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Extrusion of Sodium and Hydrogen Ions as the Primary Process in Potassium Ion Accumulation by *Streptococcus faecalis*

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Glycolyzing cells of *Streptococcus faecalis* accumulate K^+ with concurrent extrusion of equivalent amounts of H^+ and Na^+. An attempt was made to clarify the retionship between the movements of Na^+ and K^+. Sodium was displaced from cells glycolyzing in the presence of ammonia, diethylamine, tris(hydroxymethyl)aminomethane, and other nitrogenous cations; by contrast, K^+ was completely retained. Accumulation of K^+ by heterologous exchange for Na^+ was not inhibited by antibiotics which facilitate diffusion of K^+ across the membrane, but was blocked by proton conductors. The results indicate that extrusion of Na^+ and H^+ from the cells is a primary, energy-linked process which generates an electrical potential (interior negative); K^+ accumulation occurs in response to this potential. Two mutants deficient in K^+ accumulation and retention were examined in terms of this model. One mutant is apparently defective in exchange of K^+ for H^+. In the other mutant, exchange of K^+ for Na^+ is impaired.

Streptococcus faecalis, like other microorganisms, accumulates K^+ even when growing in a medium rich in Na^+, and largely excludes Na^+. Net accumulation of K^+ has been studied chiefly in suspensions of nongrowing cells. Bacteria harvested after overnight growth in certain media are relatively depleted of K^+ but contain large amounts of Na^+ and H^+. When provided with an energy source such cells accumulate K^+ with extrusion of equivalent amounts of Na^+ and H^+ (7, 20-22, 26, 30). In *S. faecalis*, at least, the immediate energy donor is probably adenosine triphosphate (ATP), since both glycolysis and arginine degradation support cation exchange (7, 30). Cation exchange is blocked by *N,N'*-dicyclohexylcarbodiimide (DCCD) and other agents which inhibit the membrane-bound adenosine triphosphatase, thus implicating this enzyme in the vectorial exchange of cations across the membrane (9, 10).

In principle, one can envisage a number of mechanisms by which the net flux of K^+ inward and that of Na^+ and H^+ outward can be coupled to each other and to the source of metabolic energy. At one extreme, there may be obligatory coupling at the level of an enzymatic process. The Na^+, K^+-dependent adenosine triphosphatase of mammalian cell membranes appears to catalyze a translocation of this kind, as both Na^+ and K^+ are required for the hydrolysis of ATP (for reviews, *see* 16, 23). At the other extreme, coupling of the cation fluxes may be purely electrical. Pressman and his associates (12, 19) have proposed that K^+ accumulation by mitochondria is mediated by an electrogenic pump which drives K^+ inward, against the electrochemical gradient; the positive potential generated thereby would tend to displace Na^+ and H^+. The chemiosmotic hypothesis (1, 15, 16) also envisages electrical coupling, but in the opposite sense: the *primary* process would be the electrogenic extrusion of H^+ and Na^+; this would generate a negative potential, drawing K^+ into the cell down the electrochemical gradient.

Our studies on the internal *p*H of *S. faecalis* and the effect of various inhibitors thereon (Harold, Pavlasova, and Baarda, Biochim. Biophys. Acta, *in press*) led to the conclusion that glycolyzing cells maintain an internal *p*H considerably more alkaline than that of the medium

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by energy-dependent extrusion of protons. The focus of the present paper is on the movement of Na^+. The evidence now available, particularly the pattern of inhibition by ion-conducting antibiotics, argues in favor of the generalized model shown in Fig. 1. It appears that *S. faecalis* extrudes both Na^+ and H^+ by an energy-dependent process which generates a potential difference across the membrane, inside negative. K^+ accumulation occurs in response to this electrical potential. Certain mutants, which require high concentrations of K^+ for growth yet retain the capacity to transport K^+ across the membrane (11) are probably deficient in the extrusion of either H^+ or Na^+.

MATERIALS AND METHODS

Organisms and growth media. *Streptococcus faecalis* strain 9790 and mutants derived from it were used. (We recognize that the characteristics of this strain are closer to those of *S. faecium*, but retain the designation by which the organism is generally known.) The organisms were grown on the following complex media: medium KTY, containing 150 mM K^+ and traces of Na^+; and medium NaTY, containing 5 mM K^+ and 150 mM Na^+ (5–11, 30).

Mutant 687A is of the class designated Cn^-_{K6} in a previous paper (11) and requires increased levels of K^+ for growth at *p*H 6. (The properties of this strain are identical with those of mutant 325 B, which has been lost.)

Mutant 7683 is representative of a class of mutants which has thus far been described only in a brief abstract (F. M. Harold, Bacteriol. Proc. p. 111, 1968). The wild type was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (in KTY medium; 25 μg/ml, 4 hr). Mutants which grew on medium KTY but not on NaTY were selected with penicillin; the growth requirement was most pronounced at *p*H above 7. All mutants were identified as derivatives of strain 9790 by means of nutritional markers (11), and all reverted readily to the wild phenotype.

General experimental conditions. Cells harvested after overnight growth were washed with 2 mM $MgCl_2$ and resuspended in water or in buffer at a density of 1 to 2 mg of cells (dry weight) per ml. Glycolysis (4 mg of glucose per ml) was monitored at constant *p*H by automatic titration of the lactic acid produced, by the use of a Radiometer *p*H-Stat (7–11, 30). Samples were filtered at intervals, washed with 2 mM $MgCl_2$, and analyzed.

Analytical methods. Procedures for the determination of K^+, Na^+, ^{42}K, and ^{22}Na have been described (5–11). The internal *p*H was calculated from the distribution of ^{14}C-dimethyloxazolidinedione (DMO) as reported elsewhere (Harold et al., Biochim. Biophys. Acta, *in press*).

The H^+ content of the cells was determined by a modification of the method of Gear et al. (3). A sample of the cell suspension containing 5 mg of cells (dry weight) was filtered, washed once, and immediately transferred to a tube containing 2.0 ml of boiling water. After 5 min, the suspension was cooled; 0.20 ml of 0.5 N Na_2SO^6 was added, and the *p*H was determined. Differences in the *p*H of two cell suspensions were converted into changes of H^+ content by use of a calibrated titration curve. The method is not entirely satisfactory, and has a precision of ±10% at best. One serious source of error arises from the continued production of lactic acid by the cells during sampling: so long as the rate of glycolysis remains constant throughout the experiment, this does not matter, but any change in the rate of glycolysis introduces an error into the estimated H^+ content.

Preparation of cells loaded with Na^+ or H^+. Cells in which K^+ has been fully replaced by Na^+ were prepared by the monactin procedure as described earlier (7). Cells grown on medium KTY were incubated at 37 C in 0.1 M sodium maleate, *p*H 8.0, with 2 μg of monactin per ml to accelerate exchange of cations across the membrane. After 20 min, the antibiotic was removed by repeated washing with water, and the membrane recovered its impermeability to cations.

Partial replacement of K^+ by H^+ was carried out by a similar procedure. Cells suspended in water were incubated at room temperature with 2 μg of monactin per ml; the *p*H was maintained at 6.0 by periodic addition of HCl. When equilibrium was reached, the cells were filtered and washed with water to remove the antibiotic.

RESULTS

Dissociation of sodium and potassium movements. Accumulation of K^+ by nongrowing cells is accompanied by concurrent extrusion of equivalent amounts of sodium and hydrogen ions. The coupling between the ion fluxes is not, however, an obligatory one. When cells loaded with Na^+ were allowed to glycolyze in the presence of NH_3, diethylamine, tris(hydroxymethyl)aminomethane (Tris), or triethanolamine, Na^+ was rapidly displaced; by contrast, K^+ was completely re-

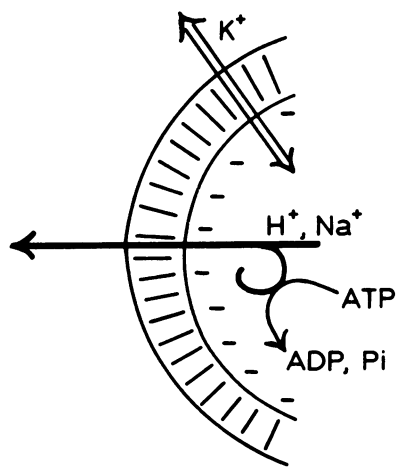

FIG. 1. *Cation transport in S. faecalis. A schematic representation to show energy-linked extrusion of H^+ and Na^+ as the primary process. K^+ accumulates secondarily in response to the electrical potential.*

tained by the cells (Fig. 2A). The extrusion of Na^+ required concurrent glycolysis and was blocked by 0.1 mM DCCD, suggesting the participation of the adenosine triphosphatase. We shall see later that mutant 7683 is deficient in Na^+ extrusion (Fig. 2B).

Exchange of ^{22}Na for Na^+. *S. faecalis* does not ordinarily accumulate Na^+, but external Na^+ readily enters the cells by exchange. As shown in Fig. 3, $^{22}Na/Na^+$ exchange was strongly stimulated by glucose and reached equilibrium in about 30 min. No net change in the Na^+ content of the cells occurred in this experiment. The rate of ^{22}Na uptake appears to be a saturable function of the external ^{22}Na level, with an apparent dissociation constant of over 20 mM. [It may be recalled that the apparent K_m for $^{86}Rb/Rb^+$ exchange is 0.17 mM (5).]

$^{22}Na/Na^+$ exchange, like net Na^+ extrusion, was blocked by DCCD. It was also inhibited by proton conductors, a point to be reexamined below.

Na^+ competitively inhibits K^+ and Rb^+ uptake (5), and we therefore expected Na^+ uptake to occur via the K^+ transport system. Somewhat surprisingly, even 5 mM K^+ did not inhibit the initial uptake of ^{22}Na (Fig. 3); ^{22}Na thus appears to enter the cells via sites other than the K^+-specific ones. As K^+ accumulated in the cells, ^{22}Na which entered at first was progressively extruded.

Effect of ion-conducting antibiotics on exchange of K^+ for Na^+. Antibiotics which render membranes permeable to specific cations provide additional evidence that Na^+ extrusion is a primary, energy-dependent process and not a secondary consequence of K^+ accumulation. If K^+ accumulation by an electrogenic K^+ pump were the primary process, cation exchange should be blocked by antibiotics which facilitate K^+ movements (*see* Discussion). As shown in Fig. 4, K^+ accumulation was little affected by monactin (7), despite the presence of a 10-fold excess of Na^+. Similar results were obtained with valinomycin (6). By contrast, the proton conductor tetrachlorosalicylanilide (8) blocked cation exchange.

A synthetic, sodium-selective polyether designated cyclohexyl-15-crown-5 (18) failed to interact with *S. faecalis*.

Genetic defect in K^+/H^+ exchange: mutant 687A. A previous paper from this laboratory (11) described a class of mutants designated Cn^-_{K6} and defined this phenotype by the following criteria: (i) the mutants require high concentrations of K^+ for growth at *p*H 6, but grow normally at alkaline *p*H; (ii) under certain conditions the mutants lose K^+ to the medium by exchange for Na^+; (iii) the mutants are severely deficient in net uptake of K^+ by heterologous exchange for Na^+ and H^+ at *p*H 6, and indeed glycolyze poorly at acid *p*H; (iv) however, homologous exchange of ^{42}K for K^+ is as rapid in the mutant as in the wild type.

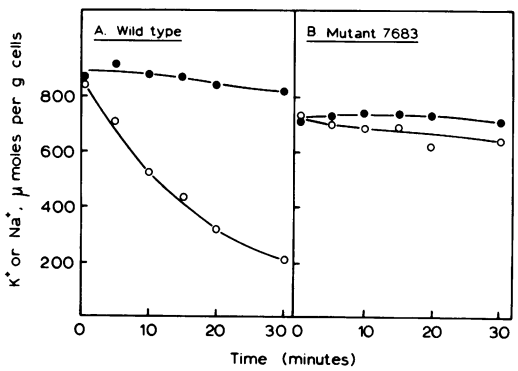

FIG. 2. *Displacement of K^+ and Na^+ by Tris. Cells were grown on KTY. To study K^+ retention, the cells were suspended in 50 mM Tris chloride, pH 7.5, at 37 C; glucose was added at 0 min. Sodium-loaded cells were prepared as described in Materials and Methods. The cells were then incubated at 37 C in Tris chloride (50 mM)-sodium sulfate (10 mM Na^+), with glucose. Symbols: ●, K^+ in K^+-loaded cells; ○, Na^+ in Na^+-loaded cells.*

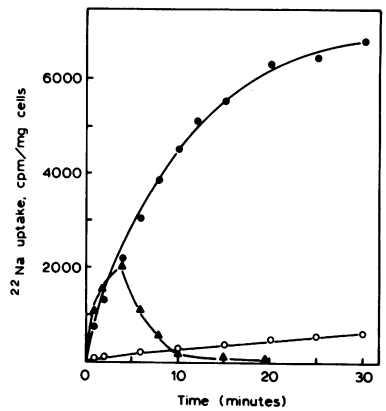

FIG. 3. *Exchange of ^{22}Na for Na^+. Sodium-loaded cells, prepared as described in Materials and Methods, were suspended in water and kept at pH 7.5 by means of the pH-stat. ^{22}Na, specific activity 11,500 counts per min per μmole. Symbols: ●, glycolyzing cells; 10 mM ^{22}Na (sulfate) was added at 0 min. At 30 min the specific radioactivity of the internal $^{22}Na^+$ had reached 91% of the external one, and the sodium content of the cells was unchanged; ○, as above but without glucose; ▲ glycolyzing cells; at 0 min, 10 mM $^{22}Na^+$ and 5 mM K^+ were added simultaneously.*

It was originally thought that the primary defect in the mutant involved the retention of K^+ (11). Experiments in which K^+/H^+ and K^+/Na^+ exchanges were examined separately prove that this suggestion was incorrect. As shown in Fig. 5, cells of mutant 687A fully loaded with Na^+ glycolyzed fairly well at *p*H 6.2 and readily exchanged K^+ for Na^+. However, cells preloaded with H^+ did not glycolyze at *p*H 6.2 and did not accumulate K^+, until the level of external K^+ was raised to 50 mM.

The genetic defect in mutant 687A thus is not in the retention of K^+, but in the exchange of K^+ for H^+. We have previously observed that *S. faecalis* maintains an alkaline interior *p*H by extrusion of H^+ in exchange for K^+ (Harold et al., Biochim. Biophys. Acta, *in press*). As would be expected, mutants of phenotype Cn^-_{K6} are deficient in this, even in 50 mM K^+: at external *p*H 6.0, the internal *p*H of the mutant was 6.8, compared with 7.3 for the wild type. The defect in proton extrusion presumably accounts for the observation that uptake of phosphate and of amino acids by the mutant was also reduced (8; Harold et al., *in preparation*).

Genetic defect in K^+-Na^+ exchange. The meta-

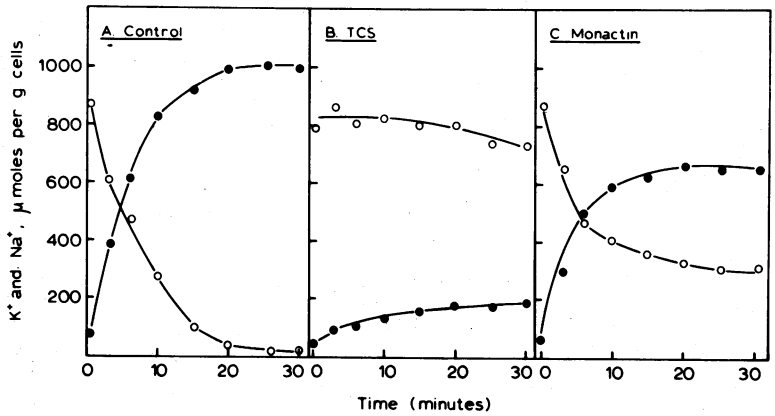

FIG. 4. *Effect of ion-conducting antibiotics on exchange of Na^+ for K^+. Sodium-loaded cells were prepared as described in Materials and Methods and were resuspended in 20 mM Na^+ (sulfate). The cells were allowed to glycolyze on the pH-stat, pH 7.5, at room temperature with or without antibiotics. K^+ (2 mM) was added at 0 min. Symbols: ●, K^+; ○, Na^+. (A) Control, no additions. (B) Tetrachlorosalicylanilide, TCS, 6 × 10^{-6} M. (C) Monactin, 2.5 × 10^{-6} M.*

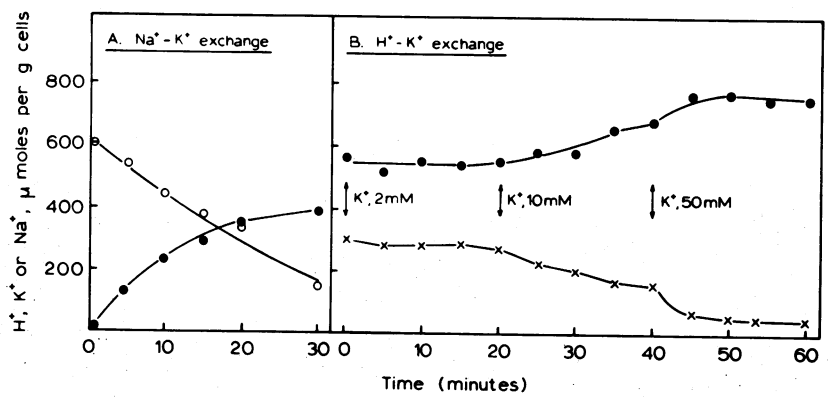

FIG. 5. *K^+ accumulation by mutant 687A at pH 6.2. (A) Cells harvested from medium KTY were loaded with Na^+ by the procedure described in Materials and Methods, resuspended in water, and allowed to glycolyze on the pH-stat at pH 6.2. K^+ (2 mM) was added at 0 min. The rate of glycolysis was 25 μmoles of lactic acid per g cells per min, about one-third of the wild-type rate. (B) Cells harvested from medium KTY were partly loaded with H^+ by the procedure described in Materials and Methods, resuspended in water (with glucose), and placed on the pH-stat at pH 6.2. K^+, 2 mM, was added at 0 min. There was no detectable glycolysis until the K^+ level was raised to 10 mM, and glycolysis was further accelerated by raising the K^+ level to 50 mM. Symbols; ●, K^+, ○, Na^+; × H^+.*

bolic pattern of the class of mutants represented by 7683 is in some respects complementary to that of Cn^-_{K6}. Since these mutants have not been previously described, some documentation is desirable. Mutant 7683 required high concentrations of K^+ for growth at alkaline *p*H: at *p*H 7.5, there was very little growth on medium NaTY (5 mM K^+) but good growth on KTY (150 mM K^+; Fig. 6). At *p*H 6, the mutant grew on medium NaTY at half the rate of the wild type.

Wild-type *S. faecalis* retains K^+ tenaciously when suspended in sodium buffers, both in the presence and in the absence of an energy source. The mutants rapidly lost K^+ by exchange for Na^+: in 50 mM sodium maleate buffer, *p*H 7.5, half the K^+ was lost in 15 min. Loss of K^+ was further stimulated by glycolysis (Fig. 7). Retention of K^+ was somewhat better at acid *p*H.

Mutant 7683 was definitely not defective in uptake of K^+ per se. Mutant cells glycolyzing at *p*H 7.5 in the presence of 0.5 mM ^{42}K carried out $^{42}K/K^+$ exchange at a rate considerably *more* rapid than that of the wild type: half the K^+ pool exchanged in 4 to 5 min, compared with 15 min for the wild type. Addition of Na^+ did not reduce the rate of ^{42}K uptake, though it did, of course, displace K^+ from the cells and thus lower the equilibrium level of ^{42}K. The mutation thus clearly did not alter the capacity of the cells to select K^+ over Na^+ for entry.

However, the genetic defect severely impairs net uptake of K^+ by exchange for Na^+. As shown in Fig. 8A, sodium-loaded cells of the mutant took up very little K^+ and failed to extrude Na^+ altogether. This should be contrasted with the rapid exchange of K^+ for Na^+ seen in the wild type (Fig. 4A). However, the internal *p*H of

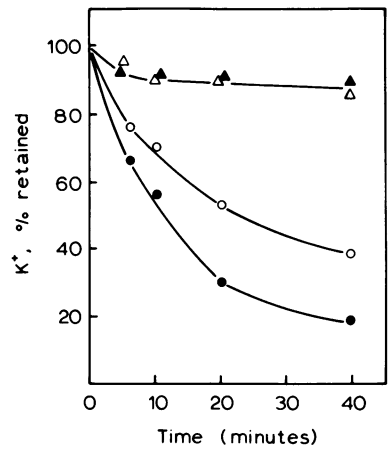

FIG. 7. *Loss of K^+ from mutant 7683. Wild type and mutant were grown on KTY containing ^{42}K. The cells were washed, resuspended in sodium maleate buffer (50 mM Na^+), pH 7.5, and incubated at room temperature. Wild type: △, buffer only; ▲, buffer with glucose. Mutant: ○, buffer only; ●, buffer with glucose.*

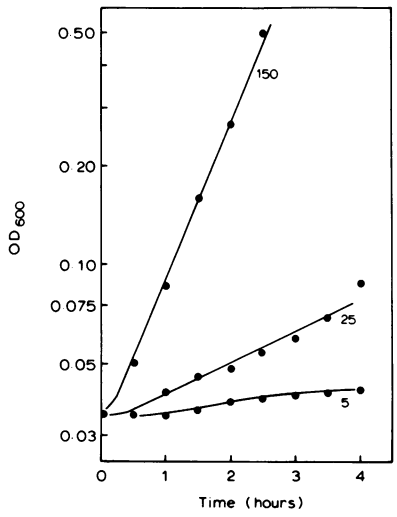

FIG. 6. *Growth of mutant 7683. Cells grown on medium KTY were washed and resuspended in the following media at pH 7.5: NaTY (5 mM K^+, 150 mM Na^+); NaTY (with added K^+, to 25 mM); and KTY (150 mM K^+). Growth was followed turbidimetrically at 37 C. The wild type, not shown, grew with a generation time of 30 min in these media.*

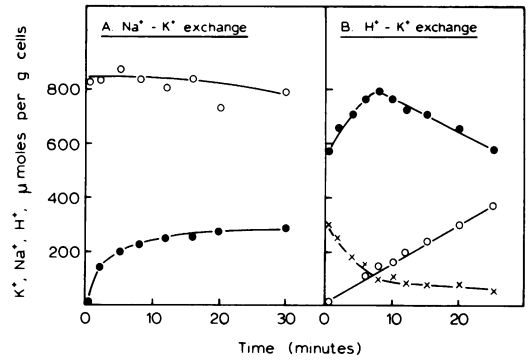

FIG. 8. *K^+ accumulation by mutant 7683 at pH 7.5. (A) Cells harvested from medium KTY were loaded with Na^+ by the procedure described in Materials and Methods, resuspended in water, and allowed to glycolyze on the pH-stat at pH 7.5. K^+, 2 mM, was added at 0 min. For uptake of K^+ by the wild type under these conditions, see Fig. 4A. (B) Cells harvested from medium KTY were partly loaded with H^+ by the procedure described in Materials and Methods, resuspended in water, and allowed to glycolyze on the pH-stat at pH 7.5. K^+, 2 mM, was added at 0 min. The metabolic pattern of the wild type (not shown) was the same, except that the Na^+ did not accumulate in the cells. Symbols: ●, K^+; ○, Na^+; ×, H^+.*

mutant cells, like those of the wild type, was 8.2 under these conditions, suggesting that the limited initial uptake of K^+ shown in Fig. 8A occurred by exchange for H^+. Indeed, as shown in Fig. 8B, H^+ was readily extruded by cells of the mutant, with concurrent uptake of both K^+ and Na^+.

The conclusion that mutant 7683 is defective in K^+/Na^+ exchange was corroborated by the experiment shown in Fig. 2B: whereas Tris readily displaced Na^+ from wild-type cells, it failed to displace Na^+ from the mutant. Similar results were obtained with NH_3. However, $^{22}Na/Na^+$ exchange in the mutant was perfectly normal.

DISCUSSION

Accumulation of K^+ by nongrowing cells of *S. faecalis* is a process of vectorial cation exchange; electroneutrality is preserved by the extrusion from the cells of stoichiometrically equivalent amounts of Na^+ or H^+, or of both. Some of the ways by which the two opposite cation fluxes could be coupled are outlined in the introduction.

The results presented here, together with those reported elsewhere (Harold et al., Biochim. Biophys. Acta, *in press*), provide considerable support for the conclusion that the ion fluxes are coupled loosely and in the manner illustrated in Fig. 1. We propose that the primary translocation, which is directly linked to the hydrolysis of ATP, is the extrusion of both Na^+ and H^+ from the cells. This generates an electrical potential, interior negative; K^+ accumulation occurs in response to this potential—against the concentration gradient, but down the electrochemical gradient.

The evidence in support of the view that K^+ and Na^+ fluxes are loosely coupled is quite convincing, and amplifies the conclusions of previous investigators (20, 21, 26). Sodium was readily displaced from glycolyzing cells, not only by K^+ but also by a variety of nitrogenous compounds such as NH_3, diethylamine, Tris, and triethanolamine; none of these displaced K^+. It might be argued that the nitrogenous cations serve as transport analogues of K^+. This suggestion is implausible on structural grounds alone, and is largely ruled out by the failure of nitrogenous cations to displace K^+ or to inhibit competitively uptake of K^+ and Rb^+. It seems much more likely that sodium extrusion is the primary event. Ammonia and substituted amines are known to diffuse passively into *S. faecalis* as the free base (29). Association with a proton, perhaps generated by glycolysis, produces a cation which can substitute for Na^+ extruded by the pump.

The coupling of the ion fluxes is thus probably electrical. Either K^+ accumulation is primary and generates a positive potential which displaces Na^+ and H^+, or else Na^+ and H^+ are extruded, generating a negative potential which draws K^+ into the cells. In *Neurospora*, Slayman (24, 25) was able to demonstrate a negative potential directly by microelectrode techniques. In the absence of comparable methods for bacteria, we have used cation-conducting antibiotics as a probe to detect the potential and to determine its polarity. Mitchell (16, 17) has pointed out that, if the cell interior were electrically positive, K^+ accumulation should be blocked by antibiotics such as valinomycin or monactin. These render the membrane permeable to K^+ (6, 7, 19; Harold, Advan. Microbiol Physiol., *in press*) and should permit rapid efflux of K^+ in response to the electrochemical gradient. In fact, these antibiotics had comparatively little effect, but exchange of K^+ for Na^+ was blocked by proton conductors (Fig. 4). This observation argues that a negative potential, generated by extrusion of H^+ and of Na^+, is the driving force for K^+ accumulation by resting cells. Unfortunately, no substance is currently available which facilitates electrogenic movement of Na^+ across lipid membranes with a high degree of selectivity. Our model (Fig. 1) predicts that sodium conductors, unlike K^+ conductors, should strongly inhibit K^+/Na^+ exchange, and this experiment affords a potential test of the model.

Cells fully loaded with K^+ carry out energy-dependent uptake of ^{42}K by homologous exchange for internal K^+. The nature and role of this process are not entirely clear. Entry of K^+ by homologous and by heterologous exchange appears to involve the same specific site (5, 26) but it may be recalled that homologous $^{42}K/K^+$ exchange is unaffected by DCCD and by other inhibitors which block heterologous cation exchange (9, 10). Moreover, $^{42}K/K^+$ exchange persists in mutants defective in the extrusion of H^+ or of Na^+ (*see below*). Taken together, these observations are best accommodated by a scheme (Fig. 1) in which K^+ movements are distinct from those of H^+ and Na^+.

Finally, let us consider the physiology of mutants deficient in K^+ accumulation. It has been recognized for some time that these fall into two classes. The first class consists of mutants in which the characteristics of K^+ entry are altered (2, 5, 27). Of particular interest in the present context is the second class, which includes the original mutant of *Escherichia coli* isolated by Lubin and Ennis (4, 13, 14) as well as mutants of *Bacillus subtilis* (28) and of *S. faecalis* (11). These mutants readily carry out homologous $^{42}K/K^+$ exchange, and clearly possess the K^+-transport site, but are defective in net uptake of K^+ and in its retention (4, 11, 14). In terms of the present model, this phenotype would result from any

genetic lesion which impairs the capacity for selective extrusion of H^+ or Na^+.

Mutant 687A [and strain 325A of an earlier report (11)] appears to carry out normal exchange of K^+ for Na^+, but is unable to extrude H^+ (Fig. 5). This primary defect readily explains other aspects of the phenotype. At *p*H 6, the mutant grows poorly, glycolyzes slowly, and fails to establish a proper internal *p*H. All of these, and also a reduced capacity for uptake of phosphate and of alanine, are presumably secondary to the defect in H^+ extrusion. An important point is that mutant cells preloaded with both H^+ and Na^+ can extrude neither (11). Apparently H^+ extrusion takes precedence over Na^+ extrusion, a conclusion suggested also by the finding that proton conductors block Na^+ movements. In any event, inability of the mutant to extrude Na^+ is a sufficient explanation for the fact that high levels of Na^+ will displace K^+ (11).

The genetic defect in mutant 7683 is less clearly defined. Mutant cells readily exchange K^+ for H^+; they glycolyze rapidly, maintain a marked *p*H gradient, and exhibit normal uptake of phosphate and of alanine. However, the mutant is unable to extrude Na^+ in exchange for either K^+ or Tris (Fig. 2B and 8A). In addition, both resting and glycolyzing cells of the mutant leak K^+ by exchange for cations from the medium, and there is rapid turnover of the cellular K^+ pool. We cannot yet specify the primary genetic lesion, but its physiological consequences clearly include an impairment in the selective extrusion of Na^+. This, in itself, is a sufficient explanation for the loss of K^+ from the cells.

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