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A TRANSMEMBRANE pH GRADIENT IN *STREPTOCOCCUS FAECALIS*: ORIGIN, AND DISSIPATION BY PROTON CONDUCTORS AND *N,N'*-DICYCLOHEXYLCARBODIIMIDE

F. M. HAROLD, EMILIE PAVLASOVÁ* AND J. R. BAARDA

Division of Research, National Jewish Hospital, Denver, Colo. and Department of Microbiology, University of Colorado School of Medicine, Denver, Colo. (U.S.A.)

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SUMMARY

The distribution of [^{14}C]dimethyloxazolidinedione was employed to measure the internal pH of *Streptococcus faecalis*. Glycolyzing cells maintained an internal pH more alkaline than that of the medium by 0.5–1 unit. The pH difference disappeared upon exhaustion of glucose. Arginine metabolism did not establish a pH gradient.

The pH gradient was abolished by metabolic inhibitors of two kinds: inhibitors of the membrane-bound ATPase including *N,N'*-dicyclohexylcarbodiimide and chlorhexidine; and proton conductors including tetrachlorosalicylanilide, carbonylcyanide *m*-chlorophenylhydrazone and the antibiotics nigericin and monensin. The antibiotics valinomycin and monactin which facilitate K^+ diffusion did not affect the pH gradient so long as the external K^+ concentration was relatively high; at low K^+ concentrations they lowered the internal pH.

To study the relationship of internal pH to K^+ accumulation we employed cells in which K^+ was completely replaced by Na^+. Such Na^+-loaded cells also maintained a pH gradient during glycolysis, albeit a small one (0.3 unit), which was again abolished by dicyclohexylcarbodiimide and by proton conductors. Addition of K^+ raised the internal pH to the level characteristic of K^+-loaded cells. The results suggest that the pH gradient is formed by energy-dependent extrusion of protons from the cell; this renders the interior alkaline and generates a membrane potential (interior negative). K^+ accumulation apparently results from movement of K^+ down its electrochemical gradient.

INTRODUCTION

Compounds which uncouple oxidative phosphorylation generally inhibit active transport. Surprisingly, this is true even under anaerobic conditions, even though uncouplers do not interfere with ATP generation at the substrate level[1–5]. A striking example of this unexpected phenomenon was described in a previous paper[3]. A series

Abbreviations: TCS, 3,5,3',4'-tetrachlorosalicylanilide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DMO, dimethyloxazolidinedione.

* Permanent address: Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia.

of uncouplers including 3,5,3′,4′-tetrachlorosalicylanilide (TCS), carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and others inhibit energy-dependent transport of K^+, orthophosphate and amino acids by a strain of *Streptococcus faecalis*. This organism does not carry out oxidative phosphorylation and generates ATP *via* glycolysis and arginine degradation. The uncouplers do not inhibit glycolysis, nor the synthesis or turnover of ATP, but prevent the utilization of metabolic energy for membrane transport. We further noted that TCS, CCCP and other uncouplers markedly accelerate the diffusion of protons (and protons only) across the cytoplasmic membrane of *S. faecalis*, and proposed that the inhibition of transport is due to the increased proton flux[3,4].

A second class of transport inhibitors was subsequently found. *N,N′*-Dicyclohexylcarbodiimide (DCCD) and the antibiotic Dio 9 are known to inhibit energy-transfer reactions in mitochondria and chloroplasts[6,7] (for review see ref. 5). In *S. faecalis*, DCCD, Dio 9 and the synthetic guanidine derivative chlorhexidine[8] inhibit the ATPase which is associated with the plasma membrane, and also block the accumulation of K^+ (refs. 9, 10). The ATPase appears to be involved in ion transport and presumably mediates utilization of ATP.

The experiments described below were guided by a modified version of Mitchell's chemiosmotic hypothesis[11,12]. We postulate that the ATPase participates in the electrogenic extrusion of protons from the cells, generating a gradient of pH and of electrical potential across the membrane. We further assume that a gradient of H^+ activity or of electrical potential is required for active transport of various metabolites. Uncouplers dissipate the proton gradient by accelerating diffusion of protons back across the membrane; DCCD and other inhibitors of the ATPase prevent establishment and maintenance of the gradient.

We shall demonstrate here that glycolyzing cells of *S. faecalis* do indeed maintain an interior pH considerably more alkaline than the exterior and that this pH gradient is abolished by uncouplers and by DCCD as predicted by the hypothesis. Our results suggest that proton extrusion is a primary translocation and not a secondary consequence of K^+ accumulation.

MATERIALS AND METHODS

Growth media

S. faecalis ATCC 9790* was grown on complex medium KTY containing 1 % dextrose, 1 % tryptone, 0.5 % yeast extract and 1 % K_2HPO_4; K^+ is thus for all practical purposes the only monovalent cation. The pH of overnight cultures was adjusted to 7 to permit exchange of any intracellular H^+ for K^+. The cells were then harvested by centrifugation, washed with 2 mM $MgCl_2$ and resuspended at 2 mg/ml (dry weight) in either water or 50 mM KCl. Arginine-adapted cells were grown on medium supplemented with 0.6 % arginine·HCl.

Na^+-loaded cells were prepared by use of the antibiotic monactin, as described earlier[13]. Cells grown on medium KTY were incubated at 37° in 0.1 M sodium maleate (pH 8) with 2 μg/ml monactin to increase the cation permeability of the membrane.

* Strictly speaking, the characteristics of this strain are closer to those of *S. faecium* than of *S. faecalis*. We retain here the designation used by the American Type Culture Collection.

After 20 min the antibiotic was removed by repeated washing with water and the membrane recovered its impermeability to cations.

General experimental conditions

All experiments were conducted at room temperature. In most experiments glucose (4 mg/ml) served as energy source. Glycolysis was monitored at constant pH by automatic titration of the lactic acid produced, by means of a Radiometer pH-Stat fitted with an electrode containing NaCl in place of KCl. Production of H^+ and of lactate are equivalent under the conditions of the present experiments[14,15]. Arginine metabolism was likewise followed by automatic addition of HCl. Samples of the cells were collected by filtration through Millipore filters. In a few experiments K^+ in the medium was measured by flame photometry.

Determination of the internal pH by distribution of dimethyloxazolidinedione (DMO)

DMO is a weak acid, metabolically inert, which diffuses passively across many biological membranes. The permeability coefficient of the uncharged acid is generally much greater than that of the anion. The distribution of DMO is therefore a function of the pH; conversely, the internal pH of cells and organelles can be calculated from the DMO distribution by means of the Henderson–Hasselbach equation[16–18].

To a cell suspension (2 mg dry weight per ml) maintained at a known external pH we added both [^{14}C]DMO (0.1 mM) and [^{3}H]methoxyinulin (4 μg/ml). Samples (2 ml) were taken by filtration, without washing; the filters were dried and counted in a Nuclear Chicago scintillation counter. The tritium content of each filter was taken as a measure of the extracellular water. The amount of extracellular DMO was calculated from the ratio of [^{14}C]DMO to [^{3}H]inulin in the whole suspension; this was subtracted from the total [^{14}C]DMO to yield the intracellular [^{14}C]DMO. The pK of DMO was taken as 6.30 under our conditions[17]. The internal water space of *S. faecalis*, measured by use of [^{3}H]inulin, was 2.43 ml per g (dry weight) of cells.

A series of control experiments was performed to validate the calculation of internal pH from the DMO distribution. DMO appears to be metabolically inert at concentrations below 1 mM (higher levels inhibit phosphate uptake, perhaps by proton conduction). There was no measurable adsorption of DMO to the cells. The ratio of intracellular to extracellular DMO was constant over the range from 0.01 to 1 mM [^{14}C]DMO (in cells glycolyzing at external pH 7.5, the following ratios were obtained: 0.01 mM DMO, 3.6; 0.1 mM DMO, 3.6; 1 mM DMO, 3.3). This is strong evidence against active transport of DMO, which would be expected to obey saturation kinetics[16–18]. The steady state was established within 2 min after addition of DMO.

The cardinal assumption on which calculation of the internal pH is based is that the concentration of undissociated DMO (HA) is the same on both sides of the membrane. As discussed by Roos[19], the validity of this assumption depends on the ratio of the permeability coefficients, P, of the undissociated acid and of the anion: if P_{HA}/P_{A^-} is greater than 500, the distribution of DMO will be essentially unaffected by a membrane potential. Evidence that undissociated DMO passes across the membrane whereas the anion does not is shown in Fig. 1. At alkaline pH DMO stabilized lysozyme protoplasts[20] to osmotic lysis but below pH 6.3 rapid lysis occurred. The ratio of the permeability coefficients was estimated by assuming that the rate of protoplast lysis is directly proportional to the permeability coefficient of the osmotic

stabilizer. Protoplasts were suspended in 0.4 M DMO, brought to pH 5.3 or 7.5 with NaOH. Gramicidin (0.5 μg/ml) was added to render the cytoplasmic membrane freely permeable to Na^+ (ref. 21); lysis would thus be determined only by the diffusion of DMO. At room temperature, the time required for 50 % decrease in turbidity was less than 2 sec at pH 5.3, about 360 sec at pH 7.5, from which we estimate that P_{HA}/P_{A^-} is greater than 180. Thus our estimate of the internal pH should be virtually independent of any membrane potential.

Two other sources of error may be mentioned. If [^{3}H]inulin is excluded by the cell wall, our estimate of the internal water space will be too high, and the calculated internal pH too low. Moreover, packing of cells on the filter during sampling may interfere with glycolysis which is required to maintain an elevated internal pH; this will also tend to make the estimated pH too low. We have chosen to neglect these errors and thus report minimal values for the internal pH*.

Chemicals

[^{14}C]DMO and [^{3}H]inulin were purchased from New England Nuclear Co., DCCD and CCCP from Calbiochem. Gifts of various antibiotics are acknowledged below.

O-Methyl-TCS was prepared by M. B. Goren by methylation of TCS with a 20-fold excess of diazomethane in methanol–ether for 18 h. It crystallized as needles from methanol, melting point 150.5–152°. The infrared spectrum (KBr pellet) showed

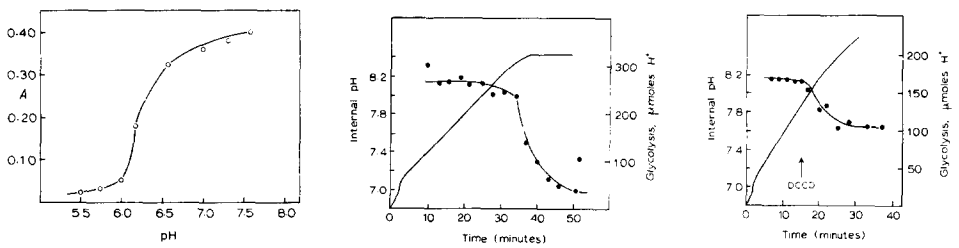

Fig. 1. Stabilization of protoplasts by DMO. Protoplasts were prepared by incubation with lysozyme in 0.5 M sucrose–0.05 M sodium phosphate, centrifuged and resuspended in a small volume of 0.5 M sucrose. Small aliquots were pipetted into 0.4 M DMO at various pH values and incubated at 37°. Absorbance was followed at 600 nm; experimental points shown are after 10 min incubation.

Fig. 2. Role of glycolysis in maintaining the pH gradient. Cells were suspended in 40 mM KCl at a density of 2 mg/ml. 160 μmoles glucose were added at 0 min and glycolysis (corrected for sampling) was monitored continuously on the pH-stat at pH 7.5. [^{3}H]Inulin and [^{14}C]DMO were added at 5 min and sampling commenced at 8 min. Solid symbols designate the calculated internal pH, the continuous line is the trace of the pH-stat.

Fig. 3. Abolition of the pH gradient by DCCD. 4 mg/ml glucose were added to a suspension of cells in KCl at 0 min, and glycolysis was monitored at pH 7.5. 0.1 mM DCCD was added at 15 min. For other details and symbols see legend to Fig. 2.

* We have also attempted to measure H^+ movements by a modification of the method of GEAR *et al.*[22] and of MITCHELL AND MOYLE[23]. These investigators lysed mitochondria with a nonionic detergent; the pH of the lysate is a function of the intramitochondrial pH and differences in the pH of lysates can be converted into changes of H^+ content by use of appropriate calibration curves. *S. faecalis* is not sensitive to detergents and was disrupted by boiling in water. Use of this method was abandoned when we recognized that the pH of boiled suspension is determined, in part, by lactic acid produced during sampling; thus conditions which alter the rate of glycolysis introduce serious errors into the estimated H^+ content.

no OH^- adsorption at 3500 cm^{-1}, but did have the NH band at 3360 cm^{-1}. Analysis: calculated for monomethyl-TCS, $C_{14}H_9NCl_4O_2$: 46.06 % C, 2.48 % H; found: 45.86 % C, 2.52 % H.

RESULTS

Energy-dependence of the pH gradient

A suspension of cells in 40 mM KCl was allowed to metabolize a limited amount of glucose at an external pH of 7.5. Radioactive DMO and inulin were added, and samples were taken at intervals for determination of the internal pH. Results are shown in Fig. 2. As measured by DMO distribution, the internal pH was about 8.1 so long as glucose was present, and fell to about 7 when the substrate was exhausted.

The absolute internal pH of glycolyzing cells and the magnitude of the gradient across the membrane both depend upon the external pH. At external pH 7.5, the internal pH was 8.0–8.2; at pH 7.0, it was around 7.7; while at an external pH of 6.0 the internal pH was 7.2.

Arginine serves as an alternative energy source for *S. faecalis*; it generates ATP and supports K^+ accumulation, but is a poor energy source for phosphate uptake[15,24]. Arginine metabolism did not produce a measurable pH gradient; at external pH 6.5, the internal pH was near 6.3; possible reasons will be considered below.

Role of the ATPase in maintenance of the gradient

Addition of 100 μM DCCD to cells glycolyzing in 40 mM KCl at pH 7.5 progressively slowed glycolysis to about half the control rate. The pH gradient as judged by DMO distribution was abolished within 5 min (Fig. 3). The guanidine derivative, chlorhexidine (1,6-di-4′-chlorophenyldiguanidohexane) had a parallel effect: 20 μM chlorhexidine reduced the internal pH of glycolyzing cells from 8.1 to 7.8. Both DCCD and chlorhexidine inhibit the ATPase associated with the protoplast membrane of *S. faecalis*[9,10] and the present results therefore suggest that the ATPase participates in maintaining the pH gradient.

Dissipation of the pH gradient by proton conductors

The effect of TCS (6 μM) on glycolysis and on the internal pH is shown in Fig. 4A: the rate of glycolysis approximately doubled while the internal pH dropped

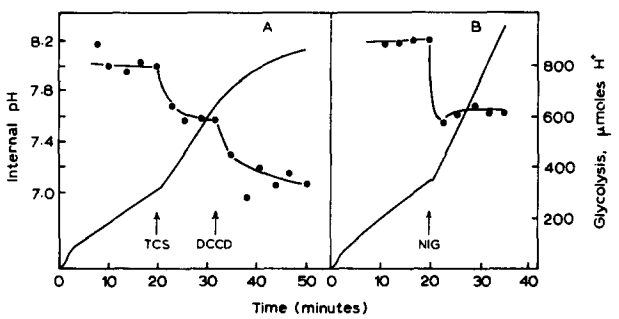

Fig. 4. Dissipation of the pH gradient by proton conductors. Cells were suspended in 40 mM KCl. 4 mg/ml glucose were added at 0 min and glycolysis was monitored at pH 7.5. Inulin and DMO were added at 5 min. A. 6 μM TCS was added at 20 min, followed by 0.1 mM DCCD at 32 min. B. Nigericin (NIG, 1 μM) was added at 20 min.

abruptly. Subsequent addition of DCCD sharply inhibited glycolysis together with a further lowering of the internal pH. The effects of TCS are evidently related to its capacity to conduct protons across the membrane: *O*-methyl-TCS, in which the phenolic hydroxyl group is blocked, does not catalyze proton translocations[4] and failed to lower the internal pH of glycolyzing cells even at a concentration of 50 μM. Similar effects were produced by other uncouplers, including CCCP and also by the antibiotics nigericin (Fig. 4B) and monensin.

Effect of K^+-conducting antibiotics on the pH gradient

The antibiotics valinomycin and monactin are known to catalyze specifically the diffusion of K^+ across biological membranes (for reviews see refs. 5 and 25), including the cytoplasmic membrane of *S. faecalis*[21]. The effect of these antibiotics on the internal pH of glycolyzing cells was complex, and dependent upon the K^+ concentration in the medium. Neither antibiotic measurably altered the internal pH of cells glycolyzing in 40 mM K^+ (Fig. 5A). However, when the external K^+ concentration was decreased to 1 mM, both monactin and valinomycin caused some lowering of the internal pH together with partial release of K^+ from the cells; subsequent addition of TCS lowered the internal pH still further, with loss of additional K^+ from the cells (Fig. 5B).

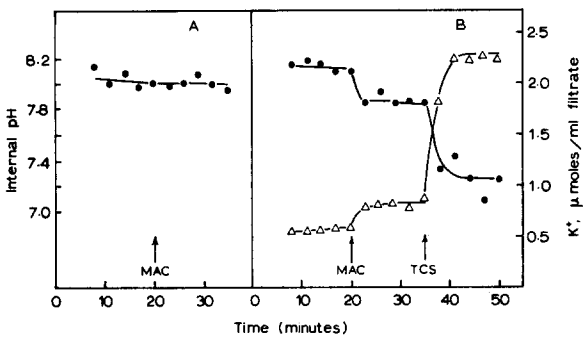

Fig. 5. Effect of monactin on the pH gradient. A. Cells were suspended in 40 mM KCl. 4 mg/ml glucose were added at 0 min and glycolysis allowed to proceed at pH 7.5. 2.5 μM monactin (MAC) was added at 20 min. B. Cells were suspended in 1 mM KCl and allowed to glycolyze at pH 7.5 as above. 2.5 μM monactin (MAC) was added at 20 min, 6 μM TCS at 35 min. Samples were filtered at intervals and the K^+ content of the filtrate was determined. The total K^+ content of cells *plus* medium was 2.60 μmoles/ml suspension. ●, internal pH; △, K^+.

The antibiotic gramicidin also facilitates cation movements; it is quite non-specific, conducting Na^+ and H^+ as well as K^+ (refs. 21, 26) (for review see ref. 5). At concentrations as low as 0.2 μM, gramicidin D significantly reduced the internal pH of glycolyzing cells, presumably by permitting the entry of protons.

Formation of a pH gradient in Na^+-loaded cells

S. faecalis, like other microorganisms, accumulates K^+ during glycolysis and extrudes both Na^+ and H^+ (ref. 15). In order to determine whether H^+ extrusion or K^+ accumulation is the primary process, formation of the pH gradient in cells fully loaded with Na^+ was investigated.

Na^+-loaded cells were prepared as described in the experimental section,

suspended in water and allowed to glycolyze at pH 7.5. As shown in Fig. 6A, the internal pH of the cells was somewhat elevated. This small pH gradient, about 0.3 unit, was abolished by DCCD and also by TCS (not shown). The trace amount of K^+ remaining in the Na^+-loaded cells did not change in the course of the experiment; limited extrusion of protons can thus occur in the absence of K^+. However, formation of a larger pH gradient required K^+. Addition of 2 mM K^+ caused a prompt increase in the internal pH, to the level characteristic of K^+-loaded cells (Fig. 6B). By contrast, addition of 10 mM or even 50 mM NaCl did not permit the internal pH of Na^+-loaded cells to rise further.

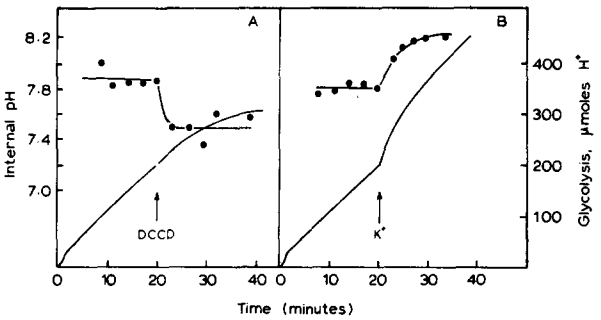

Fig. 6. Formation and dissipation of a pH gradient in Na^+-loaded cells. Cells were loaded with Na^+ by the monactin procedure, washed and resuspended in water at 2 mg/ml. A. 4 mg/ml glucose were added at 0 min, DMO and inulin at 5 min, and glycolysis was monitored continuously at pH 7.5. 0.1 mM DCCD was added at 20 min. B. Like A, except that 2 mM KCl was added at 20 min.

DISCUSSION

The merits and possible limitations of DMO as an indicator of the internal pH of cells and organelles have been discussed at length by previous investigators[16–19]. A series of control experiments was performed to warrant application of this procedure to *S. faecalis*. We shall assume in what follows that the distribution of DMO between medium and cells is a function of the pH of these two compartments and not subject to serious error due to a possible membrane potential. No attempt was made to correct our results for several other sources of error; such corrections would have had the effect of raising the calculated internal pH still further.

The internal pH of glycolyzing cells of *S. faecalis* was substantially higher than that of the medium; the absolute difference was a function of the external pH and exceeded 1 unit when the external pH was 6. The pH gradient collapsed as soon as the energy source was exhausted (Fig. 2). Curiously, the metabolism of arginine did not give rise to a pH gradient even though arginine supports ATP synthesis and net uptake of K^+ (refs. 9, 13, 15). The reason for the difference between arginine and glucose is unknown. It may be trivial, related to the fact that the rate of ATP generation from glucose is 4 times greater than that from arginine. On the other hand, the failure of arginine metabolism to generate a pH gradient may suggest that one of the enzymes of glycolysis is directly involved; experiments along this line are in progress.

We reported in previous papers[9,10] that chlorhexidine and DCCD inhibit the

ATPase of *S. faecalis* membrane and also prevent net uptake of K^+ and of certain other metabolites. Both reagents abolished the pH gradient, with little effect on the rate of glycolysis (Fig. 3). Chlorhexidine probably reacts with the ATPase protein itself[10], whereas DCCD binds to an unknown constituent of the membrane and secondarily inhibits both ATPase and NADH dehydrogenase[9]. The present results therefore specifically implicate the ATPase in the generation of the pH gradient.

The antibiotics valinomycin and monactin which facilitate K^+ diffusion across a variety of biological membranes including that of *S. faecalis*[13,21] (for reviews see refs. 5 and 25), had little effect on the internal pH. However, a series of proton conductors including TCS, CCCP and the antibiotics nigericin and monensin stimulated glycolysis as much as 2-fold and dissipated the pH gradient (Fig. 4). These compounds were earlier shown to accelerate proton diffusion across the *S. faecalis* membrane by at least 50-fold; TCS and CCCP conduct protons only[3,23,27,28] (for review see ref. 5) whereas nigericin and monensin catalyze exchange of H^+ for K^+ or Na^+ (refs. 13, 29, 30) (for reviews see refs. 5 and 25). *O*-Methyl-TCS, a derivative which does not conduct protons and does not inhibit transport[4] did not affect the internal pH. It would thus appear that the pH gradient can be maintained only if the membrane is relatively impermeable to protons.

It is recognized that in *S. faecalis*[9,15], as in other microorganisms, extrusion of protons is closely linked to the accumulation of K^+. Thus, Na^+-loaded cells could establish a maximal pH gradient only when provided with K^+ (Fig. 6). We can formulate two extreme hypotheses to account for the linkage between K^+ and H^+ movements, as shown schematically in Fig. 7. Scheme a is derived from the chemiosmotic hypothesis as formulated by MITCHELL[11,12] and by CHAPPELL AND CROFTS[26]. The diagram states that proton extrusion is the primary, energy-linked process which renders the cell interior alkaline and generates a membrane potential, inside negative. Accumulation of K^+ occurs by movement of the cation down its electrochemical gradient.

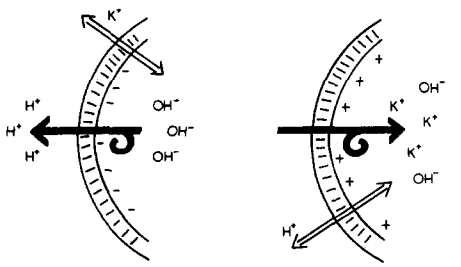

Fig. 7. Possible models for the origin of a pH gradient and its relationship to K^+ accumulation.

Scheme b is derived from the work of PRESSMAN[25] and HARRIS *et al.*[31] on K^+ accumulation in mitochondria and is essentially the converse. It postulates an electrogenic K^+ pump which drives K^+ into the cell, against its electrochemical gradient, at the expense of metabolic energy. In consequence a membrane potential arises, inside positive, which will tend to expel H^+ and thus render the pH of the cytoplasm alkaline.

In principle, the two hypotheses can be distinguished by measuring the membrane potential. This has been accomplished in *Neurospora*. SLAYMAN[32,33] reported

a membrane potential of as much as 200 mV, inside negative; a potential considerably in excess of the calculated K^+ diffusion potential. The membrane potential was abolished by dinitrophenol and NaN_3. Incidentally, the pH of the cytoplasm of Neurospora was also higher than that of the medium[34], but the gradient was smaller than that found here.

There is at present no way to measure the membrane potential in bacteria and we must resort to indirect evidence. Several arguments favor proton extrusion as the primary process. (i) *S. faecalis* is known to accumulate K^+ while extruding Na^+. Nevertheless as shown in Fig. 6, cells fully loaded with Na^+ established a measurable pH gradient which was discharged by DCCD and by TCS. Since *S. faecalis* does not accumulate Na^+, this pH difference is best attributed to proton extrusion. Addition of K^+ would raise the internal pH further by allowing extensive net exchange of H^+ for K^+. (ii) If K^+ were pumped inwards against its electrochemical gradient, as required by Scheme b, antibiotics which facilitate K^+ diffusion should permit K^+ to flow out in response to the positive membrane potential, and H^+ extrusion should cease. In other words, if the pH gradient were a consequence of K^+ pumping, its formation should be inhibited by monactin and valinomycin. This argument is analogous to that advanced by MITCHELL[12] and MITCHELL AND MOYLE[35] to demonstrate that H^+ extrusion by mitochondria must be a primary process. In fact, when cells were allowed to glycolyze in 40 mM K^+, the pH gradient was unaffected by K^+-conducting antibiotics but was dissipated by proton conductors (Figs. 4 and 5). While this observation favors the primacy of proton extrusion, it must be mentioned that at very low external K^+ concentrations valinomycin and monactin did partly discharge the pH gradient (Fig. 5B). The significance of this finding is uncertain in view of the evidence (for review see ref. 5) that under certain conditions valinomycin, at least, may conduct H^+ as well as K^+. (iii) By the same line of argument, it will be recalled that proton conductors block net accumulation of K^+ by depleted cells of *S. faecalis*[3] whereas valinomycin and monactin have little effect[13,21]. (iv) The observation that proton conductors such as TCS and CCCP lower the internal pH and stimulate glycolysis is consistent with the concept of a primary proton pump. These reagents induce electrogenic proton movements across artificial lipid membranes[27,28] and an electrically negative interior should favor proton entry.

We conclude that our results point to proton extrusion as a primary, energy-linked process and suggest that K^+ accumulation by exchange for H^+ occurs in response to the electrochemical gradient. This formulation leaves unspecified the mechanism of Na^+ expulsion, which will be discussed elsewhere. We recognize that more complex models can be written including obligatory exchange of K^+ for H^+ and schemes in which both K^+ and H^+ movements are energy-coupled; the effects of ion-conducting agents on such systems are not necessarily predictable, and it is not possible to rigorously exclude such models on the basis of present evidence.

It will be recalled that proton conductors inhibit not only the uptake of K^+ by *S. faecalis* but also that of phosphate and amino acids[3]. Proton conductors also block accumulation of β-galactosides in *Escherichia coli*[4]. It is therefore possible that a gradient of pH or of electrical potential is quite generally involved in coupling metabolic energy to active transport, but we have no evidence concerning the nature of this relationship.

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REFERENCES

1 F. WINDISCH AND W. HEUMANN, *Naturwissenschaften*, 47 (1960) 209.
2 T. GALEOTTI, L. KOVAČ AND B. HESS, *Nature*, 218 (1968) 194.
3 F. M. HAROLD AND J. R. BAARDA, *J. Bacteriol.*, 96 (1968) 2025.
4 E. PAVLASOVÁ AND F. M. HAROLD, *J. Bacteriol.*, 98 (1969) 198.
5 F. M. HAROLD, *Advan. Microbial Physiol.*, 4 (1969) in the press.
6 R. B. BEECHEY, A. M. ROBERTON, C. T. HOLLOWAY AND I. G. KNIGHT, *Biochemistry*, 6 (1967) 3867.
7 R. J. GUILLORY, *Biochim. Biophys. Acta*, 89 (1964) 197.
8 G. E. DAVIES, J. FRANCIS, A. R. MARTIN, F. L. ROSE AND G. SWAIN, *Brit. J. Pharmacol.*, 9 (1954) 192.
9 F. M. HAROLD, J. R. BAARDA, C. BARON AND A. ABRAMS, *J. Biol. Chem.*, 244 (1969) 2261.
10 F. M. HAROLD, J. R. BAARDA, C. BARON AND A. ABRAMS, *Biochim. Biophys. Acta*, 183 (1969) 129.
11 P. MITCHELL, *Biol. Rev. Cambridge Phil. Soc.*, 41 (1966) 445.
12 P. MITCHELL, *Federation Proc.*, 26 (1967) 1370.
13 F. M. HAROLD AND J. R. BAARDA, *J. Bacteriol.*, 95 (1968) 816.
14 M. ZARLENGO AND A. ABRAMS, *Biochim. Biophys. Acta*, 71 (1963) 65.
15 M. ZARLENGO AND S. G. SCHULTZ, *Biochim. Biophys. Acta*, 126 (1966) 308.
16 T. C. BUTLER, W. J. WADDELL AND D. T. POOLE, *Federation Proc.*, 26 (1967) 1327.
17 S. ADDANKI, F. D. CAHILL AND J. F. SOTOS, *J. Biol. Chem.*, 243 (1968) 2337.
18 W. J. WADDELL AND R. G. BATES, *Physiol. Rev.*, 49 (1969) 285.
19 A. ROOS, *Am. J. Physiol.*, 209 (1965) 1233.
20 A. ABRAMS, *J. Biol. Chem.*, 234 (1959) 383.
21 F. M. HAROLD AND J. R. BAARDA, *J. Bacteriol.*, 94 (1967) 53.
22 A. R. L. GEAR, C. S. ROSSI, B. REYNAFARJE AND A. L. LEHNINGER, *J. Biol. Chem.*, 242 (1967) 3403.
23 P. MITCHELL AND J. MOYLE, *Biochem. J.*, 104 (1967) 588.
24 F. M. HAROLD, R. L. HAROLD AND A. ABRAMS, *J. Biol. Chem.*, 240 (1965) 3145.
25 B. C. PRESSMAN, *Federation Proc.*, 27 (1968) 1283.
26 J. B. CHAPPELL AND A. R. CROFTS, *Biochem. J.*, 95 (1965) 393.
27 U. HOPFER, A. L. LEHNINGER AND T. E. THOMPSON, *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 484.
28 E. A. LIBERMAN AND V. P. TOPALY, *Biochim. Biophys. Acta*, 163 (1968) 125.
29 B. C. PRESSMAN, E. J. HARRIS, W. S. JAGGER AND J. H. JOHNSON, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1949.
30 P. J. F. HENDERSON, J. D. MCGIVAN AND J. B. CHAPPELL, *Biochem. J.*, 111 (1969) 521.
31 E. J. HARRIS, M. P. HÖFER AND B. C. PRESSMAN, *Biochemistry*, 6 (1967) 1348.
32 C. L. SLAYMAN, *J. Gen. Physiol.*, 49 (1965) 69.
33 C. L. SLAYMAN, *J. Gen. Physiol.*, 49 (1965) 93.
34 C. L. SLAYMAN AND C. W. SLAYMAN, *J. Gen. Physiol.*, 52 (1968) 424.
35 P. MITCHELL AND J. MOYLE, *European J. Biochem.*, 7 (1969) 471.