Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5903–5907, September 1981
Physiological Science

Role of the plasma membrane proton pump in pH regulation in non-animal cells

(intracellular pH/electrogenic pump/current–voltage analysis/*Neurospora*)

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Communicated by Robert W. Berliner, June 15, 1981

ABSTRACT **Possible methods by which eukaryotic cells can regulate intracellular pH (pH_i) in response to experimental acid loading were investigated by using as a model cell the fungus *Neurospora*. Attention was focused on the role of membrane transport in such regulation, starting from the fact that this organism possesses a powerful electrogenic proton extrusion pump. Intracellular acidification was forced by introducing butyric acid into the recording medium, and subsequent changes in pH_i and membrane potential were determined with intracellular microelectrodes. In separate experiments, membrane current–voltage curves were obtained and resolved—by an explicit kinetic model—into distinct pump and leak components. Decreased pH_i causes increased outward pumping of H^+ ions, in a manner quantitatively consistent with their role as a substrate for the proton pump. This increased pumping is often manifest as a transient hyperpolarization at the onset of cytoplasmic acidification. With a considerably slower time course, decreased pH_i also produces a large increase in membrane leak conductance, which brings about net membrane depolarization and further stimulates the pump (by virtue of the reduced back electromotive force). Although the identity of the ion responsible for increased leak conductance is not yet known, the evident modulation of conductance seemingly plays an important role in stabilizing the intracellular pH: Stimulation of the pump alone would have little net effect on pH_i because it would result simply in enhanced backflux of H^+ (to which the membrane is most permeable in normal circumstances). An increased leak to non-protons, however, would allow the pump to accomplish net H^+ ejection.**

Normal operation of metabolism in most cells and tissues results in net production of hydrogen ions. This arises from the generation of carbonic acid by catabolism of neutral carbon substrates, as well as from synthesis of proteins which, in the whole cell, contain an excess of carboxyl over amino residues (1). The requirement that intracellular pH (pH_i) be maintained within relatively narrow limits therefore necessitates constant removal of free protons from the cytoplasm. Although protein buffering can accomplish this task for small loads or over the short term, over the long term, protons must actually be expelled across cell plasma membranes.

How this is accomplished might be expected to vary from one type of cell to another, depending on other metabolic constraints. In the case of animal cells, which organize most of their transport mechanisms around the sodium ion, stabilization of pH_i appears to be accomplished by exchange of H^+ for Na^+ and exchange of HCO_3^- for Cl^- (2, 3) which, in some cases, may involve obligatory multiple-ion coupling (4). Whether the conspicuous proton pumps resident in certain epithelial membranes [e.g., gastric mucosa and turtle bladder (5, 6)] function in cytoplasmic pH control *per se* is not clear; their primary job is to effect trans-tissue secretion.

However, in eukaryotic non-animal cells, and in prokaryotic organisms as well, transport is largely organized around hydrogen ions, and it has been postulated on theoretical grounds (7, 8) that active transport of H^+ in some of these systems functions to stabilize pH_i. Although it must be admitted that proton pumps do provide the background against which regulation of pH_i takes place, supposing them to be the primary agent of regulation raises a serious conflict. Their most solidly demonstrated function is to maintain a large stable electrochemical potential difference ($\Delta\bar{\mu}_{H^+}$) for protons, which serves as the driving force to concentrate metabolic substrates. [In certain bacteria, proton pumps—reversed by redox-supported $\Delta\bar{\mu}_{H^+}$—actually carry out ATP synthesis (9, 10).] It is easy to imagine physiological circumstances in which the demonstrated task of maintaining a large $\Delta\bar{\mu}_{H^+}$ and the postulated task of regulating pH_i could not be accomplished by the same ion pump. It is no surprise, therefore, that acute regulation of pH_i in bacteria has been found to occur *via* exchange of H^+ ions for K^+ or Na^+, mediated by membrane-bound carrier systems that are completely separate from the proton pumps (11–13).

In the present paper, we analyze the behavior of pH_i and of the plasma membrane proton pump in a model eukaryotic non-animal cell, the fungus *Neurospora crassa*, examining specifically the physiological responses to acid loading imposed by extracellular butyric acid. Decreased pH_i does enhance proton (efflux) pumping, in a manner that is quantitatively consistent with the notion that increased cytoplasmic H^+ concentration provides additional substrate for the pump. But another—and quite separate—membrane response to decreased pH_i is also apparent: a progressive increase in membrane conductance, accompanied by membrane depolarization. It seems likely that this conductance change is at least as important as the proton pump itself in stabilizing pH_i.

MATERIALS AND METHODS

Growth of Cells. Wild-type *Neurospora*, strain RL 21a, was grown on Vogel's minimal medium/2% sucrose as described (14). Cells 12–14 μm in diameter were used for measurement of intracellular pH, and slightly larger ones (15–18 μm in diameter) were used to determine the membrane current–voltage (*I–V*) characteristic.

Solutions. All experiments were carried out in 20 mM dimethylglutaric acid/1 mM $CaCl_2$/1% glucose (wt/vol) titrated to pH 5.8 with KOH (final K^+ concentration, 25 mM). Butyrate, when present, was added as the Na^+ salt from a stock solution at pH 5.8.

Measurement of Intracellular pH. Recessed-tip glass pH

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Abbreviations: pH_i, intracellular pH; *I–V*, current–voltage (relationship).

microelectrodes of the Thomas design (15, 16) were used. The time constant for response of different pH electrodes varied from 5 to 20 sec. All experiments were carried out on cells superfused with continuously flowing medium, and the time constant for exchange of solutions *at the cell surface* was 45 sec, as judged by microelectrode measurements of extracellular pH during test step-changes.

I–V Measurements and Kinetic Analysis. Membrane potential in all experiments was monitored with KCl-filled capillary microelectrodes of standard design. The *I–V* characteristic of the plasma membrane was determined by a computer-controlled three-electrode method described previously (17). The resultant curve was then decomposed into "pump" and "leak" components, assumed to function electrically in parallel. The leak conductance (G_{leak}), was taken as linear, and the H^+ pump was represented as a cyclic carrier system in which the number of conformational states was reduced to two (18, 19) by lumping together all voltage-independent reaction steps. Thus, the pump was described by four apparent reaction constants: two, k_{io} and k_{oi}, representing the (forward and backward) charge-carrying step and two, κ_{oi} and κ_{io}, representing the lumped voltage-independent steps. For the charge-carrying step, voltage dependence was introduced by means of a symmetric Eyring barrier: $k_{io} = k^0_{io}\exp(zF\Delta\psi/2RT)$ and $k_{oi} = k^0_{oi}\exp(-zF\Delta\psi/2RT)$, where k^0_{io} and k^0_{oi} are the rate constants at zero membrane potential ($\Delta\psi = 0$) and z (= 1), F, R, and T have their usual meanings. All parameters (k^0_{io}, k^0_{oi}, κ_{oi}, κ_{io}, and G_{leak}) were obtained by fitting the model equation jointly to sets of *I–V* curves generated at successive intervals following a shift of intracellular pH. The backward reaction constants (k^0_{oi} and κ_{io}) could be held in common to all *I–V* curves in any one set, but both forward reaction constants (k^0_{io} and κ_{oi}) and G_{leak} needed to be optimized for each curve to obtain consistently satisfactory fits. (A more detailed description of the pump model is given in the Appendix, along with a justification for use of a linear leak.)

RESULTS

Decreasing the Intracellular pH. A typical response of pH_i to the addition of 5 mM butyrate/butyric acid, pH 5.8, to the bathing medium is illustrated in Fig. 1. After a short delay (which represents the time taken for the new medium to reach the cell), internal pH decreases from ≈7.15 to ≈6.60 with an apparent first-order time constant of 90 sec.

[Presumably, this effect of butyrate arises from intracellular dissociation of the free butyric acid as these neutral lipophilic molecules diffuse inward across the plasma membrane (20).] This interpretation is experimentally supported by the absence of butyrate effects, on either pH_i or membrane potential, at high external pH (8.2; where little free acid is present), and by the absence of detectable metabolism of [^{14}C]butyrate, which might have produced secondary effects.]

The lower curve in Fig. 1 shows the simultaneous response of membrane potential to butyrate treatment. A small (10–15 mV) transient hyperpolarization, peaking within 30–40 sec, coincides with the initial change in pH_i. The hyperpolarization is followed by sustained depolarization, in this case occurring with a time constant of ≈110 sec (i.e., slower than the change in pH_i) and giving a steady membrane potential near −155 mV. The amplitude and duration of the hyperpolarizing phase are rather variable from preparation to preparation, but the depolarizing phase is more nearly constant, both in amplitude and speed. Depolarization always requires a longer time to reach a steady state than pH_i.

Membrane *I–V* Curves. At first sight, the depolarization shown in Fig. 1 is surprising, as decreased pH_i should accelerate a true H^+-extrusion pump, thus driving current through the membrane, which ought to produce *hyperpolarization*. [The

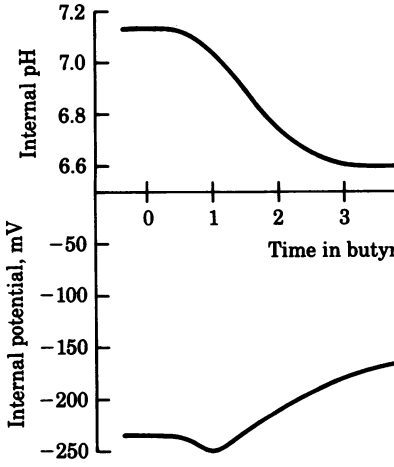

FIG. 1. Time course of intracellular pH and membrane potential of a hypha after addition of 5 mM butyrate, pH 5.8.

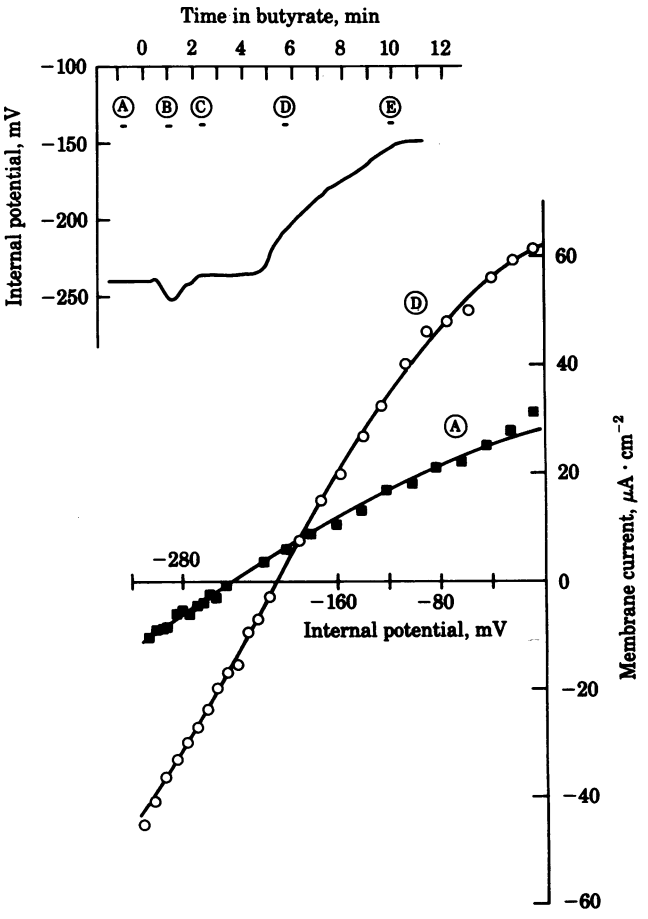

FIG. 2. *I–V* curves of the plasma membrane before (A) and during (D) treatment of a hypha with 5 mM butyrate, pH 5.8. Membrane currents calculated from the input *I–V* data are shown as the plotted points, and the solid curves are least squares fits of the pump-leak model to the plotted points. (*Inset*) Trace of membrane potential over period of butyrate-induced depolarization. *I–V* curves were obtained at the five times indicated, and the pump-leak model was jointly fitted to all five curves. For the sake of clarity, only two of the five curves are displayed.

pump current normally generates ≈80% of the membrane potential in *Neurospora* (21, 22).] Indeed, the existence of an abbreviated hyperpolarizing phase, along with the fact that depolarization lags behind the decrease in pH_i, indicates that the observed change in membrane potential must reflect at least two separate processes. Two relatively simple interpretations are (*i*) that the pump is initially activated but subsequently inhibited by cytoplasmic acidification or (*ii*) that activation of the pump is overridden by other processes, such as increased membrane leak conductance. To discriminate between these possibilities, we have determined the *I–V* relationship of the membrane under the same conditions as those used to measure the simple voltage and pH_i responses to butyrate.

Partial *I–V* data from a typical experiment are shown in Fig. 2. The control membrane *I–V* curve (A) is slightly convex upward and has a slope conductance of 170 $\mu S \cdot cm^{-2}$ (corresponding to a resistivity of 5.9 kohm·cm^2) at the resting potential. The most conspicuous *I–V* change caused by butyrate is the large increase in membrane conductance, which is shown by the steep slope of the curve (D) obtained 5.5 min after the addition of butyrate; slope conductance at the resting potential becomes 440 $\mu S \cdot cm^{-2}$ (2.3 kohm·cm^2). This result qualitatively supports alternative *ii*.

Response of the H^+ Pump to Butyrate. The component pump and leak *I–V* curves (from the data in Fig. 2) are shown in Fig. 3.

Two conspicuous changes in the pump are evident. Its apparent reversal potential moves in a negative direction, from −285 to −309 mV, which is very close to the response expected (−29 mV) for a pump transporting 1 H^+ per cycle and confronted with a sudden decrease in pH_i of 0.5 unit. More noticeably, however, pump saturation current (i_{sat^+}), which is approximated by the short-circuit current (current at 0-mV membrane potential), more than doubles in the presence of butyrate—71 $\mu A \cdot cm^{-2}$, compared with the control value (A) of 29 $\mu A \cdot cm^{-2}$. At the respective resting potentials of −206 mV (D) and −233 mV (A), the pump current increases 4-fold in butyrate (to 29 $\mu A \cdot cm^{-2}$ from 7 $\mu A \cdot cm^{-2}$), both because of the kinetic activation and because of the decrease in actual membrane potential.

The picture of the pump that emerges from Fig. 3 is strengthened by comparing the time course for internal H^+ concentration ($[H^+]_i$) with that for the product of the forward reaction constants ($\kappa_{oi}\, k^0_{io}$) of the pump (Fig. 4). This product increases in direct proportion to $[H^+]_i$, and it is especially satisfying that the major change (see legend to Fig. 3) occurs in κ_{oi}, which represents carrier recharging and contains the step of H^+ binding at the cytoplasmic surface of the membrane. In two other experiments, the change in $\kappa_{oi}\, k^0_{io}$ was 2.0- and 2.6-fold when $[H^+]_i$ had stabilized at 3 times the control values. Both $[H^+]_i$ and $\kappa_{oi}\, k^0_{io}$ have half-times of ≈2 min.

Response of Leak Conductance to Butyrate. In contrast to the close agreement between the time course of $[H^+]_i$ and the pump, the leak conductance shows a delayed and slower increase (Fig. 4), indicating that it is not simply dependent on the internal H^+ concentration. Leak conductance is stationary for ≈60 s after pH_i and the pump have begun to shift, but then increases approximately linearly with time at a rate of 29 $\mu S \cdot cm^{-2}\ min^{-1}$. (Possible causes of this increase in leak conductance are considered below.)

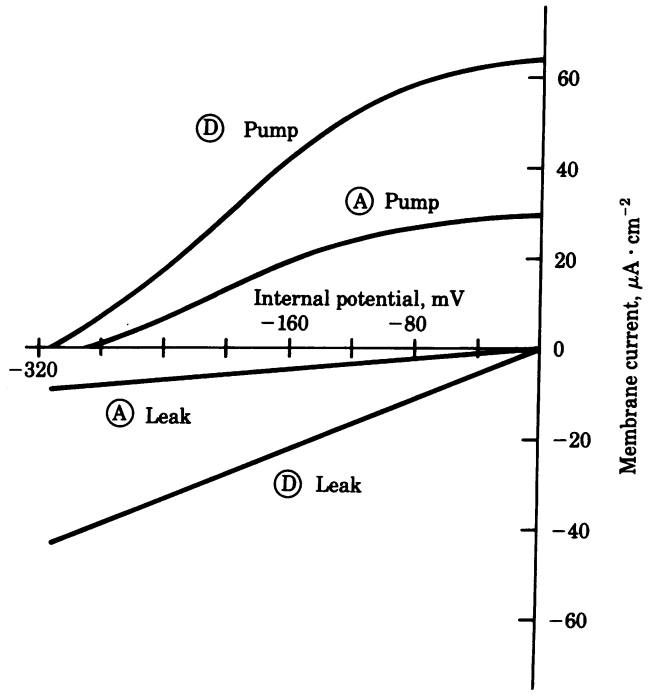

FIG. 3. Component responses of the proton pump and the leak to decreased pH_i (see Fig. 2). The parameter values are as follows:

Curve	Resting potential, mV	Reversal potential, mV	Saturating current, $\mu A/cm^2$	κ_{oi}, sec^{-1}	k^0_{io}, sec^{-1}	G_{leak}, $\mu S/cm^2$
A	−233	−285	28.5	10	$7.5 \cdot 10^4$	25
D	−206	−309	71.3	25	$8.3 \cdot 10^4$	122

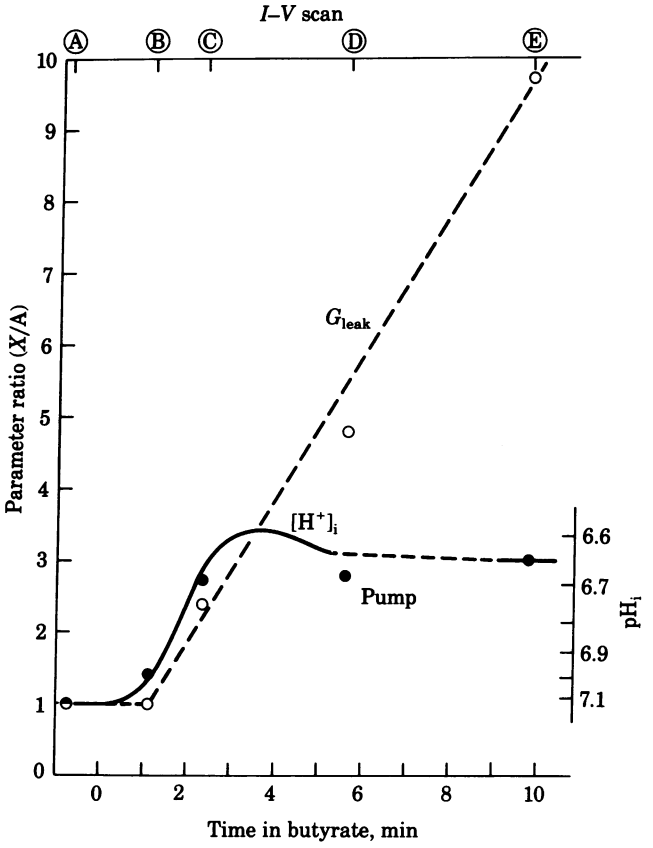

FIG. 4. Time course of pump and leak responses to cytoplasmic acidification (see Figs. 2 and 3). The ordinate scale is normalized to the value of each parameter obtained from the first *I–V* scan; *X* = A, B, C, D, or E. ——, Expected $[H^+]_i$ from parallel experiment with intracellular pH microelectrode (Fig. 1); ●, pump behavior, product of forward reaction constants ($\kappa_{oi}\, k^0_{io}$); ○, conductance of membrane leak; – –, visual guide only.

From the point of view of simple voltage measurements (Fig. 1), it is clear that the change in leak conductance overwhelms the change in pump velocity, resulting in slow depolarization. However, because the change in leak conductance is delayed, the initial increase in pump current brought about by decreased pH_i is seen as a short-lived hyperpolarization. The observed variability in this hyperpolarization is presumably a consequence of variable onset and amplitude for the pump and leak changes.

DISCUSSION

Response of the H^+ Pump to Low pH_i and Its Role in pH_i Regulation. Under physiological conditions, a proton load on the cytoplasm of a cell can arise from only two sources: the environment and metabolism. In the case of non-animal cells, which actually extract physiological work from the transmembrane electrochemical difference for H^+, the balance between metabolic and environmental sources may be crucial for survival. The central question is just how the H^+ pump can respond to these changes in such a way as to stabilize the pH_i.

It is clear that, as far as the kinetics of the pump alone is concerned, the pump is activated by increased $[H^+]_i$ in a manner consistent with its role as a substrate. However, the implications of this observation for pH_i regulation must be considered in relationship to other properties of the membrane. First, as is apparent from Fig. 3, the pump velocity (current) is itself dependent on membrane potential. Thus, activation of the pump alone with no change in leak conductance tends to drive pump current to the left along the *I–V* curve until a new steady state is reached in which pump and leak currents are equal. This limits the degree to which a given change in pump reaction constants can be manifested in a proportional change in pump current. Second, in *Neurospora*, as well as in many plant cells and bacteria (23–25), the major conductance itself appears to be due to protons. Therefore, the overall consequence of an increased pump current could be simply to return an increased number of protons to the cell *via* the leak. Both of these circumstances present a fundamental dilemma to the cell if the electrogenic H^+ pump is the main mechanism of pH_i control.

The Increased Leak Conductance—a Physiological Necessity? One way in which the H^+ pump *could* exercise substantial control over pH_i is through the opening of a leak conductance to an ion (other than H^+) that has an equilibrium potential more positive than the membrane potential. This would circumvent both problems discussed above, as the ensuing depolarization would tend both to increase pump velocity (shifting to the right along the *I–V* curve) and to reduce the driving force for inward leak of H^+. Thus, it is important to establish the identity of the ion whose conductance increase at low pH is responsible for the large observed increase in overall leak conductance.

The presence of a high concentration of butyrate anion inside the cell must result in at least some increase of membrane conductance if the membrane has a finite permeability to butyrate. However, when the pH_i reaches a steady level in the presence of butyrate (the pump is then chemically short-circuited by the continuing entry of free acid), the internal concentration of butyrate anion should also be constant if the Henderson–Hasselbalch equation is to apply. (Experiments with [^{14}C]butyrate have confirmed that, during the period of stability of pH_i in the presence of butyrate, the total internal butyrate concentration is also constant.) The butyrate anion conductance would therefore be expected to follow a time course similar to that of the pH_i. However, the observed change in leak conductance occurs more slowly than the change in pH_i, so butyrate cannot be principally responsible.

It is clear, therefore, that the change in leak conductance and consequent depolarization in the presence of butyrate must arise from a time- and pH-dependent *change* of permeability to one or more ions. For a time, K^+ seemed most likely to be involved in the butyrate/pH_i effect in *Neurospora*, as that ion is normally present at an extracellular concentration of 25 mM and an equilibrium potential of about −50 mV (26). However, the butyrate-induced depolarization is still present in K^+,Na^+-free solutions (unpublished observations). As, in this circumstance, the equilibrium potential for K^+ is more negative than the control membrane potential, hyperpolarization—rather than depolarization—should result from increased K^+ conductance.

The identity of the ion(s) responsible for the large increase in leak conductance shown in Figs. 2–4 therefore remains uncertain. Organic anions are the next most likely candidates, as they are excreted in substantial quantities by *Neurospora* (unpublished experiments) and membrane permeability to certain organic anions is known to incrase with decreasing pH (extracellular) in at least one membrane system (27). The clearest point, however, is that the ion cannot be H^+, if the purpose of the conductance change is to transduce activation of the H^+ pump (by low pH_i) into a homeostatic control for cytoplasmic pH.

APPENDIX

Two-State Kinetic Model. The kinetic model used to analyze the *I–V* data shown in Fig. 2 is shown in Fig. 5. The pump is drawn in parallel with an ensemble of ionic leaks (which must be assumed to include nonspecific "channels," specialized ion uniports, and proton-coupled cotransport systems). Although the pump must, in detail, have at least five conceptually distinct molecular states (see, e.g., ref. 28), analysis of steady-state *I–V* data gives direct information about only those states that actually mediate transfer of charge across the membrane. Hence, Fig. 5 shows a two-state model with only the electrically charged forms of the "carrier", XH^+ and $P{\sim}XH^+$, explicitly included. All other forms—the unloaded carrier at the membrane outer face, the unloaded carrier at the membrane inner face, and the partially reacted carrier (energized but not H^+ charged, or *vice versa*)—are subsumed into the lumped reaction constants κ_{oi} and κ_{io}. The model also indicates that charge transfer through the membrane is coincidental with decomposition of the chemically energized state of the carrier. Though this is not thermodynamically necessary, previous *I–V* analysis of data from the H^+ pump of *Neurospora* and the Cl^- pump of *Acetabularia*

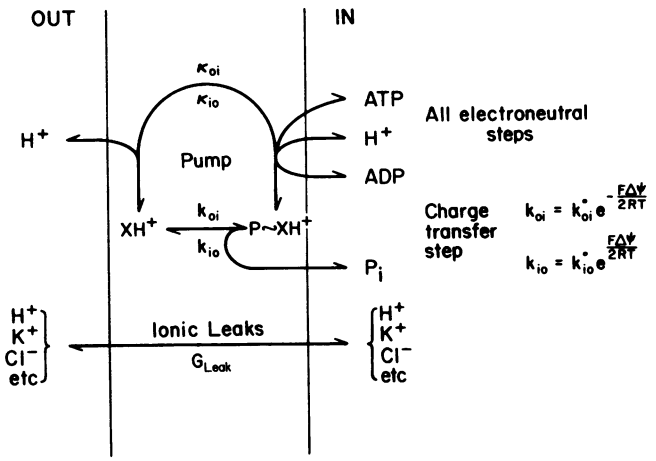

FIG. 5. Two-state carrier model for an electrogenic proton pump drawn in parallel with the membrane ionic leaks.

support it, as does the much more elaborate kinetic analysis carried out on the sodium pump of animal cell membranes (28).

At present, the form of voltage dependence for charge transfer (interconversion of XH^+ and $P{\sim}XH^+$) must be assumed rather arbitrarily. The simplest assumption is that, for transfer in either direction, charge must cross a symmetric Eyring barrier (29). Thus, the forward reaction constant k_{io} can be expanded into the product of a zero-voltage reaction constant (k_{io}^0) and a Boltzmann factor referenced to the middle of the membrane, $\exp(zF\Delta\psi/2RT)$. A corresponding relationship, with opposite sign for the membrane potential, is defined for the backward reaction constant k_{oi}. (As, in the steady-state kinetic analysis, k_{io} and k_{oi} appear only as products with $P{\sim}XH^+$ and XH^+, respectively, the above assumption is formally equivalent to considering the reaction constants as voltage independent but treating the "concentrations" of $P{\sim}XH^+$ and XH^+ as electrochemical activities.)

By means of ATPase inhibitors (30) or rapid blockade of energy metabolism (17), it is possible to separate pump behavior from leak behavior in the intact membrane of *Neurospora*. All such maneuvers thus far examined tend to linearize the membrane *I–V* relationship, suggesting that the leaks, in ensemble, are linear. In fact, in the experiments described above, quite satisfactory fits between the data and the model of Fig. 5 were obtained by assuming the total leakage conductance (G_{leak}) to be independent of the actual membrane potential.

The membrane *I–V* relationship implied by Fig. 5 is the following:

$$i = zF\bar{N}\frac{\kappa_{oi}k_{io} - \kappa_{io}k_{oi}}{k_{io} + k_{oi} + \kappa_{io} + \kappa_{oi}} + G_{leak}(\Delta\psi - E_{leak}), \qquad [1]$$

in which $\bar{N}$ is a function of the total carrier density in the membrane, E_{leak} is the net diffusion potential for the leakage pathways, and the interesting voltage dependence lies in k_{io} and k_{oi}, expanded as discussed above. The complete membrane model therefore contains seven parameters. However, preliminary curve-fitting calculations using the data in Fig. 2, as well as that from many related experiments, showed that four of the seven were uninfluenced by changes of pH_i and could be held in common for all *I–V* curves associated with any one pH shift sequence. Those four parameters were $\bar{N}$, which functions as a scaling factor and (by definition) has no effect on curve *shape*; E_{leak}, which always fell near zero and was eventually fixed at zero for fitting purposes; and the two backward reaction constants, k_{oi} and κ_{io}.

This work was supported by Grant GM-15858 from the National Institutes of Health (to C.L.S.), and Grant HA 712/6 from the Deutsche Forschungsgemeinschaft (to U.-P.H.). D.S. was a James Hudson Brown Fellow of Yale University. U.-P.H. is on leave from the Institut für Angewandte Physik, Neue Universität, Kiel, Federal Republic of Germany.

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