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METABOLIC MODULATION OF STOICHIOMETRY IN A PROTON PUMP

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Summary

The current-voltage characteristics of the ATP-dependent proton pump in the plasma membrane of *Neurospora* have been explored under varied metabolic conditions imposed by mutation and by differential respiratory inhibition. The reversal potential, or presumed equilibrium potential, for the pump was observed at about —400 mV under energy-replete conditions, and at about —200 mV during a stable metabolic downshift of 55 percent. Steady-state levels of adenine nucleotides and inorganic phosphate, however, were not affected by this partial energy restriction, so that under both normal and restricted conditions the apparent free energy of ATP hydrolysis remained near —500 mV. The results suggest that a normal pump stoichiometry of 1 H^+ extruded/1 ATP split is modified to 2 H^+/1 ATP, by chronic energy restriction.

Introduction

It has been known for more than 15 years that ion transport systems can function with variable stoichiometry. Thus, Cross et al. [1] noted a variability in current generated (per unit sodium transported), by the sodium pump in frog sartorius muscle, dependent on calcium status and seasonal changes. The finding was taken to represent variability in the stoichiometry of potassium influx coupled to sodium efflux. Variable coupling (K^+/Na^+) was also inferred for the sodium pump from a comparison of rubidium and potassium uptake [2] in frog muscle and from a study of ouabain-sensitive sodium and potassium fluxes in dialyzed squid nerve [3]. A variable ratio of potassium taken up to

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ATP split was reported by Cockrell et al. [4], for isolated non-respirating mitochondria, dependent on the transport gradient for potassium. Because potassium in those experiments could be assumed to exchange for pumped protons, a variable H^+/ATP stoichiometry could be inferred for the mitochondrial ATPase; and although proton stoichiometries are especially difficult to determine, genuine variability in the H^+ : ATP stoichiometry would reconcile a number of other conflicting reports on mitochondrial H^+ pumps [5—10]. Additional evidence for variable stoichiometry in proton pumps has come from quantum-yield and deprotonation experiments with suspensions of purple membrane from halobacteria [11,12], where the controlling variable seems to be overall ionic strength.

Proton-dependent substrate cotransport systems apparently can also change their stoichiometry depending on external conditions. Thus, Tanner and his collaborators [13,14] have reported H^+ : sugar stoichiometries ranging from 1 : 1 to 1 : 6, depending on the particular sugar, for the derepressible hexose uptake system in the alga *Chlorella vulgaris*. In the fungus *Neurospora crassa*, membrane depolarization resulting from coupled H^+/glucose influx was found to be the same, at saturation, for glucose and for the non-metabolized analogue 3-*O*-methyl glucose. The measured 3-*O*-methyl glucose influx, however, is half that of glucose, so that the apparent stoichiometry H^+/glucose must be half that for H^+/3-*O*-methyl glucose [15]. Variable stoichiometry (K^+/H^+) in an artificial counter transport system, nigericin-doped black lipid membranes, can also be inferred from electrical data [16]. In that case the critical system variable seems to be the K^+/H^+ concentration ratio, which governs carrier dimerization, thereby altering the levels of several charged species in the membrane.

Although acidic carriers like nigericin may provide plausible models for variable stoichiometry in non-enzymatic reactions, no correspondingly attractive explanation has been offered for the inconstancy of reactant : product ratios in the enzyme-dependent (ATPase) active transport systems. Furthermore, no functional significance has yet been assigned to the ability of transport systems to shift stoichiometry. While functional significance is likely to differ from system to system, an interesting general interpretation has newly emerged from studies on the electrogenic proton pump in the plasma membrane of *Neurospora*: the stoichiometry of that pump (H^+ transported/ATP split) seems to be regulated for greater or lesser efficiency commensurate with the rate of energy production in the cells. The data which have led to this interpretation are outlined below.

Properties of the organism

The fungus *Neurospora* is an obligate aerobe [17]. While the biochemical basis for this absolute oxygen-dependence is not fully understood, a reasonable view is that pyridine nucleotides reduced in glycolysis cannot be reoxidized adequately in the absence of respiration. During periods of respiratory restriction (but not blockade!) — whether produced by extrinsic inhibitors or by mutational defect — the cells derepress synthesis of an alternate terminal oxidase [18,19]. This enzyme, which is linked to the mitochondrial iron-sulfur proteins [20], runs at high velocity without being coupled to phosphorylation [21]. Its function, therefore, appears to be simply to augment

substrate-level phosphorylation, by reoxidizing pyridine nucleotides. The alternate oxidase is insensitive to cyanide and other conventional respiratory inhibitors, but is blocked by hydroxamic acids.

The so-called *poky f* strain of *Neurospora*, which has a primary defect in mitoribosomes [22], is depleted of mitochondrial cytochromes of types *a* and *b* but contains a high level of the alternate oxidase [23]. Under normal circumstances both growth and total energy turnover in *poky f* occur at half the rate for wild-type *Neurospora*. Cyanide inhibition of *poky f*, however, represents only a 55% energetic reduction, rather than total blockade as in the wild type. At the onset of cyanide treatment in *poky f*, intracellular ATP drops to 45% of the control level; but over the course of about 2 min the ATP recovers to approx. 80% of control, and by 15—30 min it has recovered completely, at which point growth has stabilized at 45% of the control rate [21] *. These observations have led to postulation of a positive feed-forward control mechanism which adjusts ATP consumption and growth, to the rate at which ATP is being produced, thereby stabilizing that overall composition of the cells. Since all major ATP-consuming pathways must be included in this control process, and since membrane transport can account for 20—40% of ATP consumption [24,25], transport in particular must be regulated in response to the metabolic downshift.

Current-voltage curve analysis

Since the major transport systems in the *Neurospora* plasma membrane are current-carrying systems, we have explored the question of transport regulation by examining the current-voltage (*I-V*) relationships — for the whole membrane and for the principal electrogenic proton pump. Complete details of the *I-V* method used have already been presented [25]. Briefly, a computer-generated current-pulse scan is driven through one intracellular microelectrode in order to force the membrane potential over the range —300 mV to 0 mV (the normal resting potential is near —180 mV), and the actual voltage displacements are measured at two other microelectrodes: one close to the current electrode, and one placed farther away, to accommodate the cable decay. The input current and voltage data are then used to calculate the membrane *I-V* curve via an algorithm somewhat similar to that of Adrian and Marshall [26]. A detailed study of wild-type *Neurospora* showed that rapid depletion of ATP, as by maximal cyanide treatment, inhibited the proton pump along a single exponential time-course [24], and the difference in membrane *I-V* curves before cyanide and after 30—60 s in cyanide was taken to be the *I-V* curve for the pump [25]. (Current leakage around the intracellular electrodes was evaluated from serial punctures, and was found to contribute about 5 percent of the normal apparent membrane conductance. It tends slightly to linearize the individual membrane *I-V* curves, but has a negligible effect on pump *I-V* curves, because those are always calculated as differences between membrane curves.)

For reference, Fig. 1 gives three records of membrane potential, to show the

* Simultaneous treatment of *poky f* with cyanide and salicylhydroxamic acid blocks both respiratory pathways, and therefore abolishes essentially all ATP synthesis. That is equivalent to cyanide-treatment of wild-type *Neurospora*, except for a somewhat slower course of depletion [21].

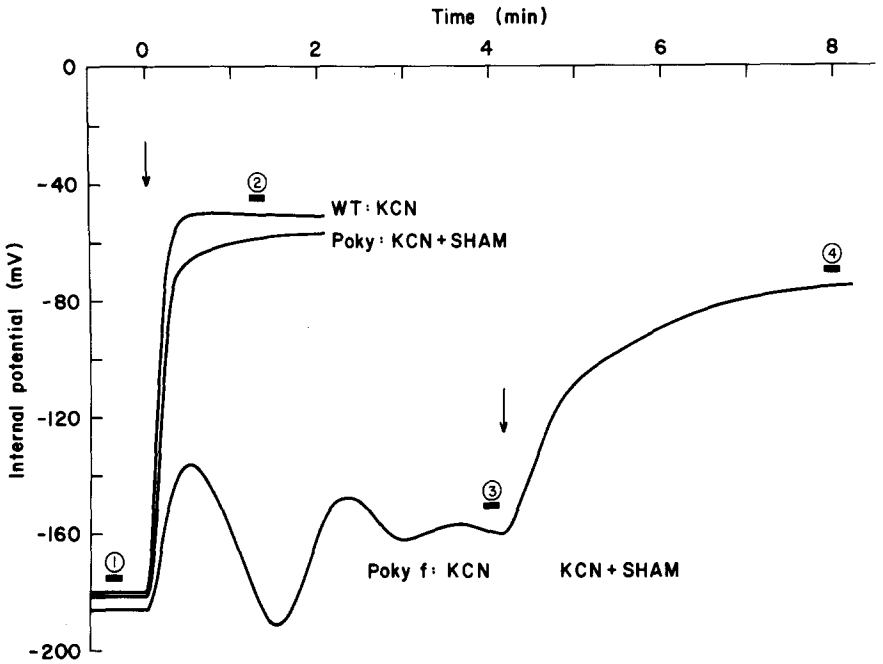

Fig. 1. Effect of cyanide and salicylhydroxamic acid (SHAM) on membrane potential in *Neurospora*. Averaged curves for 4 or more separate trials. Cyanide concentrations: 25 mM for the wild type; 1 mM for *poky f*; SHAM concentration: 1 mM. Records redrawn from Ref. 24 Fig. 4 (WT), Ref. 27 Fig. 12 (poky: KCN + SHAM), Ref 27, Fig. 4 (Poky f: KCN). All experiments carried out in a standard buffered salt solution consisting of 20 mM dimethylglutaric acid, 25 mM KOH (pH 5.8), 1 mM $CaCl_2$, and 1% glucose. Strains: Wild-type = RL21a; *poky f* = NSX *f a*. Numbered bars designate approximate times for current-voltage curve scans, used in subsequent figures.

gross effects of various combinations of inhibitors (cyanide, salicylhydroxamic acid) on the wild-type and *poky f* strains of *Neurospora*. As is seen in the upper curve, KCN depolarizes the wild type quickly and with barely an indication of a second (repolarizing) effect. The combination of KCN plus salicylhydroxamic acid similarly depolarizes *poky f* (middle curve), but with a clear slow component amounting to perhaps 10 mV. Cyanide alone produces a train of damped oscillations in *poky f* [27], with the membrane potential stabilizing only slightly depolarized after 3—5 min — a time which is comparable to that required for the major portion of ATP recovery (see above, and Ref. 21). Once the membrane potential has stabilized in cyanide, addition of salicylhydroxamic acid produces the full depolarization roughly synchronously with ATP decline, but more slowly than when the two inhibitors are introduced simultaneously.

Current-voltage curves for the plasma membrane of wild-type *Neurospora* are shown in Fig. 2, with the plotted points representing average curves for nine separate hyphae. (The circled numbers refer to the approximate times over which the pulse data were accumulated, as designated in Fig. 1.) The membrane *I-V* curves, both before cyanide treatment (curve 1) and in the presence of cyanide (curve 2) are best described as segments of inverted parabolas [25],

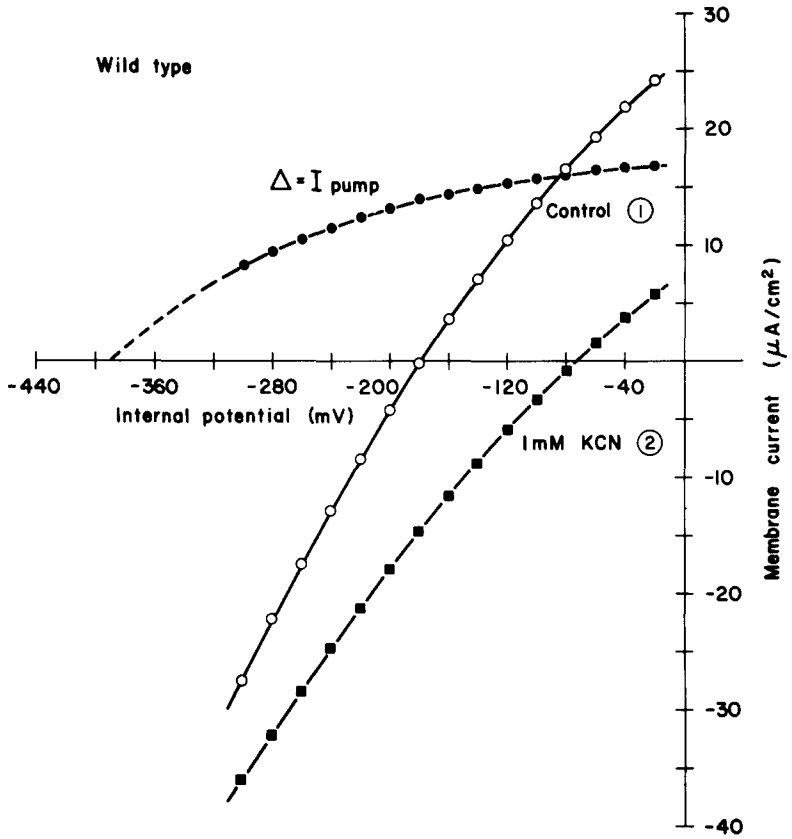

Fig. 2. Average current-voltage curves for wild-type *Neurospora*. Circled numbers refer to the *I-V* scan intervals designated in Fig. 1. Two lower curves are plots of averaged membrane *I-V* curves for nine separate experiments, with each experiment containing a scan before (1) and during (2) cyanide treatment. The upper curve is the difference between the lower two, and is taken to represent the *I-V* relationship for the electrogenic proton pump. The nine separate difference curves have been presented in Ref. 25, Fig. 8.

which would converge at strong hyperpolarization. The difference (upper plot), which is taken to be the *I-V* relationship for the electrogenic proton pump, can be fitted by a single exponential equation:

$$I_{\text{pump}} = I_{\text{max}}(1 - e^{-(V_0 - E_p)/\epsilon}), \tag{1}$$

in which I_{max} (the maximal current) = 18.6 ± 3.3 μA/cm^2, E_p (the extrapolated reversal potential for the pump) = —390 ± 29 mV, and ϵ (the voltage characteristic) = 153 ± 23 mV.

Poky f Neurospora, in the absence of cyanide, shows a membrane current-voltage curve which is essentially the same as that for the wild type, but with a somewhat expanded scale on the current axis (Fig. 3, curve 1). In the presence of cyanide (Fig. 3, curve 3), when the membrane potential has stabilized at a high value, the curve is also similar, but in most experiments shows an upward concavity in the hyperpolarizing region. We have not cal-

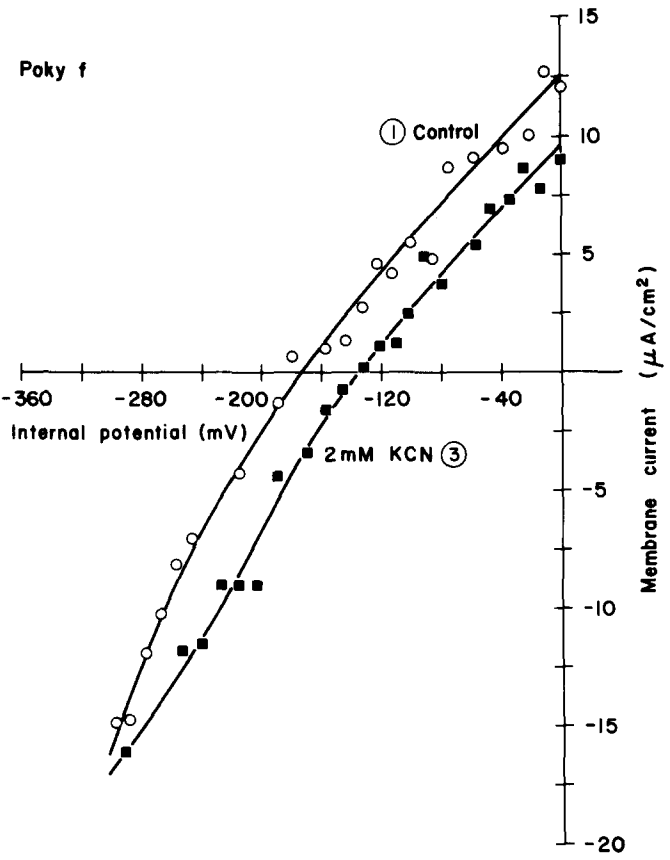

Fig. 3. Membrane current-voltage relationships for *poky f* before cyanide treatment (1) and in the presence of cyanide with stabilized high membrane potential (3). Data averaged from two widely spaced, paired trials on a single hypha. The inflection in the lower curve has been observed in very many experiments. Circled numbers refer to scans in Fig. 1.

culated the difference *I-V* curve between these two, because that difference is very small, and also probably composite (see below).

However, when salicylhydroxamic acid is added on top of cyanide (Fig. 4) after the oscillations have damped out, the *I-V* curve (curve 4) near maximal depolarization is conspicuously changed. Fig. 4 shows it to be essentially a straight line, with very little curvature in the hyperpolarizing region. This *I-V* curve intersects that for KCN alone (curve 3) within the actual range of the data; the *I-V* difference curve (Δ, upper curve) is sigmoid, and has a zero-value near −200 mV. We take this difference curve to approximate the *I-V* relationship for the ATP-dependent proton pump just before addition of salicylhydroxamic acid. Although the type of mathematical function which should be chosen to fit this curve is not so obvious as in the case of Fig. 2, the value of one parameter can be stated very clearly: the reversal potential for the pump (E_p) is −196 mV, instead of −390 mV. (The possibility that the effect of salicylhydroxamic acid might be directly on the membrane, rather than via the change in ATP level is made unlikely because the inhibitor has no effect on the

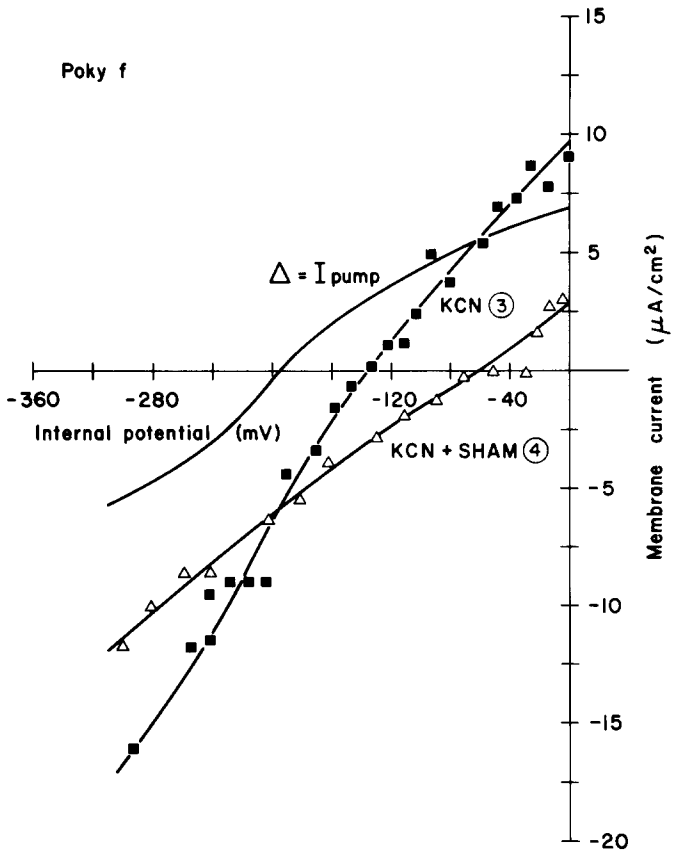

Fig. 4. Membrane current-voltage relationships for *poky f* in the presence of cyanide (3), and in the presence of cyanide + salicylhydroxamic acid (SHAM; 2 mM, in 1% ethanol; curve 4). KCN-curve replotted from Fig. 3, KCN + SHAM curve is average of 2 trials matched to those of KCN-curve. Circled numbers as in Fig. 1.

I-V relationship of wild-type *Neurospora*. Furthermore, salicylhydroxamic acid treatment alone causes membrane potential in *poky f* to oscillate, in the same manner as for cyanide [27].)

Interpretations

Before considering further the difference in reversal potentials for the electrogenic pump in these circumstances, it may be useful to explore somewhat the meaning of the shape difference between the two curves for complete inhibition (Fig. 2, curve 2, and Fig. 4, curve 4). In both cases we assume that the pump itself is off, or nearly so. The much smaller slope conductance along the hyperpolarizing limb of curve 4 probably reflects shut down of secondary charge-carrying transport systems, also as a final consequence of the mechanism producing the damped oscillations.

Now, in each case the curve for complete inhibition is used as the reference point from which to estimate the prior *I-V* relationship. For wild-type *Neuro-*

TABLE I

ADENINE NUCLEOTIDE LEVELS AND THE FREE ENERGY OF ATP HYDROLYSIS IN *NEUROSPORA*

Assume μ_0 for ATP hydrolysis = 8 kcal/mol, = $RT \ln(1/K_{ATP})$ in Eqn. 2; 100 mV = 2.3 kcal/mol. Numbers at the left refer to *I-V* curves numbered as in Fig. 1. Data from Refs. 21 and 28. SHAM, salicylhydroxamic acid.

	$[ATP]_i$ (mM)	$[ADP]_i$ (mM)	$[P_i]$ (mM)	$K = \frac{ADP \cdot P_i}{ATP}$ (mM)	$\mu_0 + RT \ln K$ (mV)
Wild type					
1 Control	2.7	0.8	10	3.0	500
2 KCN	0.6	1.1	~14	26	440
Poky f					
1 Control	3.5	0.8	10	2.3	510
3 KCN	2.6	0.8	~12	3.7	490
4 KCN + SHAM	0.5	0.8	~16	26	440

spora, a single change between curves 1 and 2 (Fig. 2) has been inferred [25]. It follows, then, that for *poky f* at least two membrane changes must have occurred between curves 1 and 4 (Fig. 4). (The fact that depolarizing currents, positive to about −70 mV, give similar results (except for a scaling factor) in curves 2 and 4, but that hyperpolarizing currents give very dissimilar results, strongly argues for a qualitative difference, rather than a simple quantitative one, between curves 2 and 4.) In *poky f*, the electrogenic pump is deprived of ATP only after the second inhibitor (salicylhydroxamic acid) has been added; so the transition from curve 3 to curve 4 corresponds to the transition from curve 1 to curve 2 in the wild type. That should be just a decrease in the magnitude of the pump component within the total membrane *I-V* relationship [25]. But a decrease in magnitude should not per se affect the measured reversal potential. Consequently, the change in reversal potential for the proton pump in *poky f* must have occurred between curves 1 and 3 (Fig. 3): during the time when membrane potential oscillates (Fig. 1) and ATP rebounds in response to the first inhibitor (cyanide).

In order to relate the observed *reversal potential* to the thermodynamic *equilibrium potential* for the pump, and thence to feasible stoichiometries, it is useful to consider the amount of energy that is available from hydrolysis of ATP under various conditions. Table I is a summary of measured phosphate and adenine nucleotide levels in *poky f* and wild-type *Neurospora* [21,28]. Neither ADP nor inorganic phosphate (P_i) changes much during splitting of ATP. P_i is large enough normally that the increment due to phosphate liberation is small, and ADP is stabilized by adenylate kinase. Obviously, the phosphate potentials are the same in uninhibited cells of both strains, and in KCN-treated *poky f* as well, at least if uniform distribution in the cytoplasm is assumed. The equilibrium potential for an ATP-driven pump should be:

$$E_p = \frac{RT}{nF}\left(\ln \frac{1}{K_{ATP}} + \ln \frac{ADP \cdot P_i}{ATP} + n \cdot \ln \frac{[H^+]_{out}}{[H^+]_{in}}\right) \quad (2)$$

where n is the stoichiometric ratio H^+ : ATP. Although the cytoplasmic pH in *Neurospora* is not known precisely, a crude estimate of 6.5 was obtained

with a distribution indicator [29], and the stability of pyridine nucleotides seemingly would require it to be not far from 7.0. Since the external pH in these experiments was 5.8, the proton diffusion potential must be close to 60 mV (cell interior positive), making the theoretical equilibrium potential for the pump about 440 mV if $n = 1$, and about 190 mV if $n = 2$.

Evidently, the reversal potential estimated in Fig. 2, (—)390 mV, and that measured in Fig. 4 (—)196 mV, lie close to the theoretical equilibrium values for H^+ : ATP stoichiometries of 1 and 2, respectively. This result strongly suggests that the difference in shape and position of the two pump *I-V* curves is caused by a difference in pump stoichiometry. (For technical reasons, the normal pump *I-V* curve (sudden introduction of KCN + salicylhydroxamic acid) for *poky f* itself is difficult to obtain. Qualitative data, however, indicate it to be similar in shape to that of the wild type, with a reversal potential of approx. —350 mV.) It seems likely, therefore, that the mechanism in *poky f* which stabilizes both ATP and membrane potential at high levels in the presence of cyanide also mediates a change in proton pump stoichiometry: from 1 H^+ transported per ATP split, with normal energy turnover, to 2 H^+ per ATP with restricted energy turnover. Since the stable membrane potential in both cases is about —180 mV, the change in stoichiometry represents nearly a 2-fold increase in pump efficiency * in response to the energy downshift. This effect would be synergistic with the decreased membrane conductance along the hyperpolarizing limb of curve 4 (compared with curve 2) serving to reduce the energy drain through the plasma membrane, without compromising the cells' ability to maintain normal composition at the reduced growth rate.

One interesting additional point arises from circuit considerations. In ordinary electric circuits, maximal power transfer from a source device to a load device is accomplished when the load voltage is half the source voltage. Maximal energy transfer, on the other hand, is accomplished when the load voltage equals the source voltage. Thus, *Neurospora* could be said to operate its proton pump for maximal power transfer under energy-replete conditions, but for maximal energy transfer under energy-restricted conditions. The advantage of the former, given the usual phosphate potentials in *Neurospora*, is that it leaves a surplus of 200 mV, beyond the normal membrane potential, which can be summoned for specific transport functions. (One probable case in point is ammonium uptake by NH_4-starved cells. Short-term ammonium deprivation apparently causes derepression of a high-affinity NH_4-transport system capable of conducting a current of nearly 10 $\mu A/cm^2$ (Ref. 30). With prolonged growth in the absence of ammonium, membrane resistance can rise, so that membrane potentials as high as —320 mV have been observed (Walker, N.A. and Slayman, C.L., unpublished data).

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* Efficiency here is defined as the free energy, per mol or per mol per Faraday, dissipated by the driving reaction (ATP hydrolysis under cytoplasmic conditions), divided into the free energy conserved in the driven reaction (pumping of charge against an electric field).

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