

Mechanism of Pyrithione-Induced Membrane Depolarization in *Neurospora crassa*

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Pyrithione is a general inhibitor of membrane transport in fungi and is widely used in antidandruff shampoos as an antifungal agent. An electrophysiological approach has been used to determine the mode of action of pyrithione on the plasma membrane of the model ascomycete, *Neurospora crassa*. At pH 5.8, pyrithione induces a dramatic dose-dependent electrical depolarization of the membrane which is complete within 4 min, amounts to 110 mV at saturating pyrithione concentrations, and is half maximal between 0.6 and 0.8 mM pyrithione. Zinc pyrithione induces a similar response but exerts a half-maximal effect at around 0.3 mM. The depolarization is strongly dependent on external pH, being almost absent at pH 8.2, at which the concentration of the uncharged form of pyrithione—which might be expected to permeate the membrane freely—is markedly lowered. However, quantitative considerations based on cytosolic buffer capacity, the pK_a of pyrithione, and the submillimolar concentration at which it is active appear to preclude significant cytosolic acidification on dissociation of the thiol proton from the uncharged form of pyrithione. Current-voltage analysis demonstrates that the depolarization is accompanied by a decrease in membrane electrical conductance in a manner consistent with inhibition of the primary proton pump and inconsistent with a mode of action of pyrithione on plasma membrane ion channels. We conclude that pyrithione inhibits membrane transport via a direct or indirect effect on the primary proton pump which energizes transport and that the site of action of pyrithione is likely to be intra- rather than extracellular.

Pyrithione (2-mercaptopyridine-*N*-oxide) is a fungistatic and antimicrobial agent (8), and its zinc salt enjoys widespread use in antidandruff shampoos (22). However, the mode of action of pyrithione remains uncertain. Albert et al. (1) suggested that pyrithione acts by entering the cell in its chelate form (see Fig. 2 for the structure) and then dissociates in the cytoplasm. In the cell, pyrithione might act by chelating metal cofactors of enzymes through its sulfhydryl group (8). Alternatively, pyrithione has been shown to interfere with thymidine uptake (9) and also has been suggested to act as an antimetabolite for nicotinic acid or vitamin B₆ (4). Previous work by Chandler and Segel (3) has demonstrated that low concentrations of pyrithione (0.1 to 1.0 mM) inhibit plasma membrane transport of a wide variety of unrelated solutes in *Penicillium chrysogenum*. Nevertheless, the primary mode of action of pyrithione on membrane transport remains to be elucidated.

Chandler and Segel (3) have hypothesized that pyrithione might act as a general transport inhibitor through weak-acid-induced cytosolic acidification and hence partial dissipation of the transmembrane proton motive force (PMF) on which the transport of many organic and inorganic solutes is reliant in fungi (16). Thus, according to their hypothesis, the uncharged form of pyrithione (pyrithione-SH), which is hydrophobic, would diffuse across the membrane, dissociate in the relatively alkaline cytosol to pyrithione-S⁻, and hence lower the cytosolic pH (pH_c). The dissociation reaction has a pK_a of 4.7 and would itself tend to sustain the concentration gradient favoring further uptake of the uncharged form.

Direct measurements of cytosolic pH in *Neurospora crassa* with pH-sensitive microelectrodes have revealed that weak acids such as butyric acid can indeed acidify the cytosol when

applied externally in the millimolar concentration range (18). However, a priori considerations based on the cytosolic buffer capacity of *N. crassa* militate against a weak acid/acidification mechanism as a mode of action of pyrithione on transport: the buffer capacity is sufficiently high to limit acidification of cytosolic pH to within about 0.1 to 0.2 unit when pyrithione is applied at inhibitory submillimolar concentrations at pH 6.

An alternative manner in which pyrithione might have such far-reaching effects on transport could be through membrane electrical depolarization. Since most H⁺-coupled transport systems in fungi carry inward current (16), the membrane potential (V_m) forms part of the driving force. Inhibition of transport might then arise either directly through reduction of this driving force or indirectly as a result of changed cytosolic conditions which result from membrane depolarization.

This study was initiated to characterize the effects of pyrithione on the transplasma membrane PMF in *N. crassa*. We show that pyrithione depolarizes the membrane in a dose- and pH-dependent manner and, furthermore, demonstrate through current-voltage analysis that this depolarization has its origins in an inhibition of the primary electrogenic H⁺ pump rather than via changes in ion channel activity or a more general nonspecific leakiness induced by pyrithione.

MATERIALS AND METHODS

Fungal culture. *N. crassa* (wild-type strain RL21a) was grown at 26°C on scratched cellophane discs which overlaid a minimal medium (27) containing 2% (wt/vol) glucose in 2% (wt/vol) agar. After about 30 h, a portion of the hyphal mat and cellophane was excised and soaked in standard buffer (SB) (see below) for 30 min before impalement. This pretreatment allowed the cells to come to a steady state in which they were actively respiring but no longer growing.

Electrophysiological measurements. The preparation was mounted in a Plexiglas chamber and viewed under bright-field illumination with an M2 micromanipulation microscope (Oxford Microinstruments, Oxford, United Kingdom). Hyphae were impaled with glass microelectrodes (tip diameter, about 0.3 μm) filled with 100 mM KCl (electrode resistance, 30 to 40 MΩ) and connected with Ag|AgCl half-cells. Micromanipulation was performed with Huxley-Goodfellow micromanipulators (Goodfellow Metals, Cambridge, United Kingdom). For volt-

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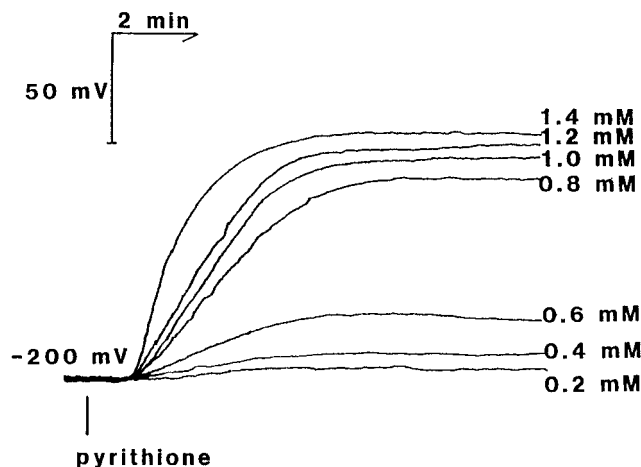


FIG. 1. Time dependence of plasma membrane depolarization in *N. crassa* on application of different concentrations of pyrithione. The lag period between the arrow indicating the time of application is accounted for by dead space between the supply tap and the recording chamber. Each trace is from a different hypha, and resting membrane potentials before the application of pyrithione were in the range of -198 to -205 mV.

age recordings, output from a single microelectrode was passed via a high-input impedance differential amplifier (FD-223; WPI, Sarasota, Fla.) and recorded on a chart recorder. Current-voltage measurements were made essentially as described previously (5). Briefly, a single cell was impaled with voltage-reporting and current-injecting microelectrodes connected respectively with the S7071A electrometer and S7050A voltage clamp modules of a S7000A microelectrode system (WPI). The voltage clamp routine took the form of a bipolar staircase of 100-ms pulses either side of the resting potential. A third electrode was used to record voltage deflections via the FD-223 amplifier in an adjacent cell; this enabled correction for current leakage along the hypha by using the algorithm of a lumped cable, described previously (5). Output from the two voltage-reporting electrodes was recorded continuously on a chart recorder and also sampled and recorded at the end of each pulse on a microcomputer via a Labmaster data acquisition card with programmable gain (model PCL-818; Semaphore Systems Ltd., London, United Kingdom). For all recordings, the circuit within the chamber was completed with a KCl-filled agar bridge positioned in the external medium.

All experiments were performed at room temperature (typically 23°C) with the preparation superfused continuously with the recording medium.

Solutions. Standard buffer (SB) comprised 20 mM dimethylglutarate, 25 mM KOH, 1 mM CaCl_2 , and 56 mM glucose (pH 5.8). At pH 4.2, the KOH concentration was reduced to 16 mM, while at pH 8.2, dimethylglutarate was replaced by *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). Inhibitors were added in an SB background: pyrithione was added from a 20 mM aqueous stock solution, while zinc pyrithione was added from stock dissolved in dimethyl sulfoxide. All standard chemicals were of reagent grade and were obtained from Sigma Chemical Co., Poole, England. Pyrithione was obtained from Aldrich Chemical Co., Gillingham, England, and zinc pyrithione was the kind gift of Zeneca Specialties.

RESULTS

Membrane depolarization by pyrithione. When measured in SB at pH 5.8, the resting potential (V_m) of *N. crassa* is in the region of -200 mV (23, 25). Figure 1 shows that addition of pyrithione to SB results in a time-dependent membrane depolarization which is complete within 4 min. Allowing for the delay introduced by unstirred-layer effects at the lower concentrations, the depolarization appears to be approximately monophasic and is sustained for at least 5 min in the continued presence of pyrithione. At millimolar concentrations, the overall depolarization induced by pyrithione amounts to 100 ± 8.6 mV ($n = 10$), or around half the resting membrane potential. In the majority of trials, the effects of pyrithione on V_m were only partially reversible during a postwashout recording time of up to 40 min.

The dose dependence of the depolarization is shown for a

typical series of six trials in Fig. 2A. The rather abrupt transition in effect between 0.6 and 0.8 mM is consistent between experiments, although reasons for this form of dose dependence are obscure. The depolarization induced by pyrithione has a concentration for half-maximal effect ($K_{1/2}$) of about 0.74 ± 0.11 mM ($n = 6$).

The effects of zinc pyrithione, which is a dimer but is probably biologically active as a monomer (1), are shown in Fig. 2B. As might be anticipated, the $K_{1/2}$ for the Zn^{2+} salt, which is in the region of 0.31 ± 0.06 mM ($n = 5$), is lower by a factor of about 2 than that of the free acid. A possible effect of Zn^{2+} itself on the membrane potential was tested by addition of 1 and 5 mM ZnCl_2 to SB. At these relatively high concentrations, no effect on the membrane potential was observed, at least within 10 min after application of Zn^{2+} (data not shown).

Chandler and Segel (3) have demonstrated that the effect of pyrithione on transport is markedly dependent on external pH. This is also true of the pyrithione-induced depolarization (Fig. 3A; results from a typical series of eight experiments). Thus, when pyrithione is applied at 6 mM, its effects on membrane electrical parameters are almost eliminated at pH 8.2, while at pH 4.2 they are exacerbated compared with the control at pH 5.8.

It will be noted from Fig. 3A that before the addition of pyrithione, the membrane is more hyperpolarized at pH 8.2 than at pH 5.8. Therefore, to ensure that the absence of an effect of pyrithione at pH 8.2 is due to an effect of pH per se and not to some secondary response resulting from membrane

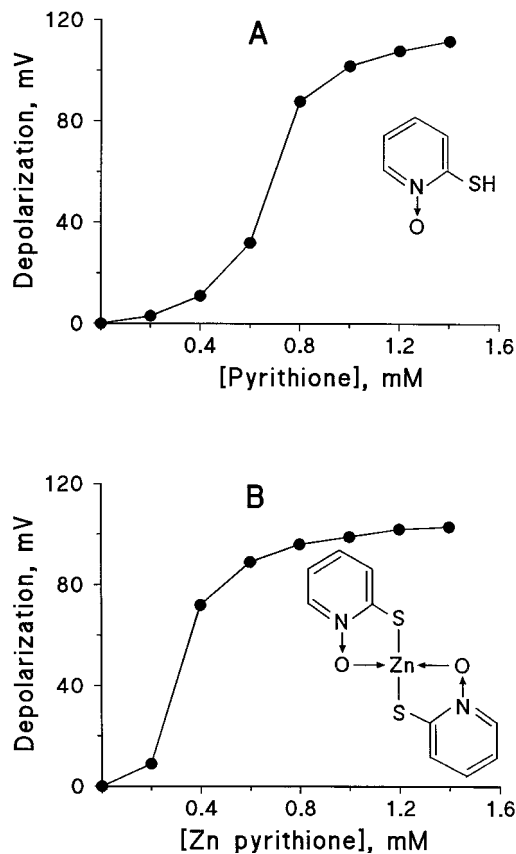


FIG. 2. (A) Dose dependence of the steady-state depolarization induced by pyrithione. Results were obtained from Fig. 1. (B) Dose dependence of steady-state depolarization by zinc pyrithione. The molecular structures of pyrithione and zinc pyrithione are given in the respective insets (10).

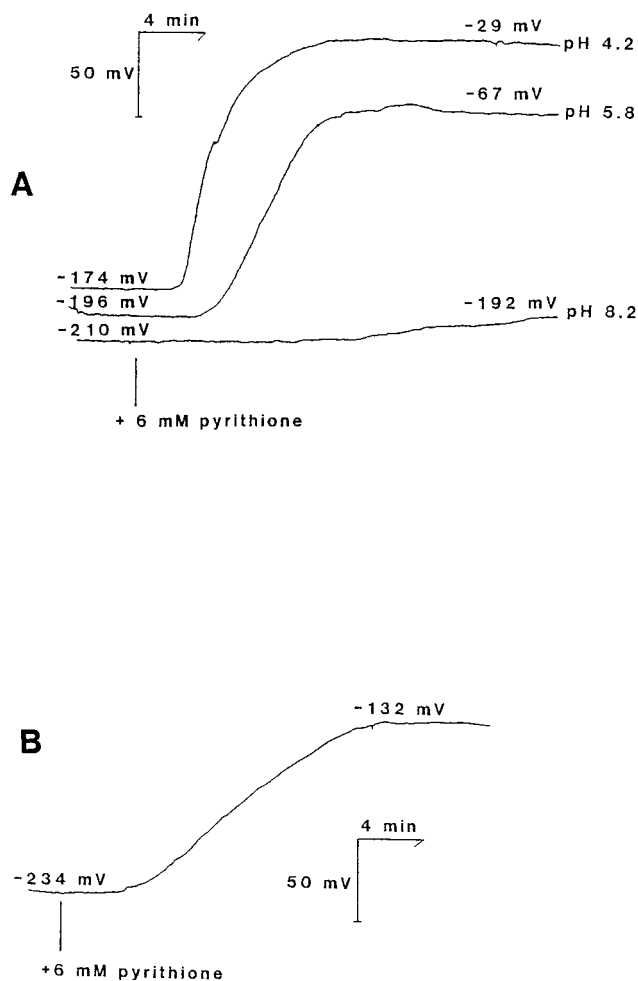


FIG. 3. (A) pH dependence of pyrithione-induced depolarization. At the time indicated by the vertical line below the curves, pyrithione was applied at 6 mM in a background of SB with respective medium pHs adjusted as described in Materials and Methods. (B) Pyrithione-induced depolarization at pH 5.8 in K⁺-free SB. Pyrithione (6 mM) was added at the time indicated by the vertical line below the curve in a background solution in which all KOH in SB was replaced by Tris.

hyperpolarization, the membrane was hyperpolarized at pH 5.8 by replacing external K⁺ with 25 mM Tris. Figure 3B shows that the capacity of pyrithione to depolarize the membrane at pH 5.8 remains undiminished, even starting from a resting potential of -230 mV. Thus, the attenuation of the pyrithione-induced depolarization at high pH appears to be a direct effect of pH.

Origins of the membrane depolarization. In principle, pyrithione might depolarize the plasma membrane in one or more of four general ways: (i) nonspecific permeability breakdown analogous to that induced, for example, by nystatin; (ii) opening of a channel passing an ion current with an equilibrium potential more positive than the resting membrane potential (17); (iii) conversely, closing of a channel passing an ion current with an equilibrium potential more negative than the resting membrane potential; or (iv) inhibition of the primary H⁺-pumping ATPase, which normally serves to hyperpolarize the membrane (6).

Preliminary evidence that pyrithione inhibits the H⁺-ATPase was obtained by using cyanide to deplete intracellular ATP

levels and hence restrict H⁺-ATPase activity. In the presence of cyanide, the hyperpolarizing activity of the H⁺-ATPase is largely abolished (5, 6, 25) and the membrane potential is close to the equilibrium potential for K⁺ (23). This effect is shown in Fig. 4 (results from a typical series of six experiments). Addition of 3 mM pyrithione in the presence of cyanide has a very limited effect on the membrane potential, causing depolarization (at most) by 5 mV (cf. Fig. 2A). However, although the nonadditivity of the cyanide and pyrithione effects on V_m might be taken to indicate a common mode of action, these data are subject to wider interpretation. In particular, it could be argued that any treatment causing depolarization will attenuate the response to pyrithione because of the more restricted range of values at which V_m might reside in the presence of pyrithione. Current-voltage (I-V) analysis offers an alternative and more definitive approach to the problem.

The effect of pyrithione on the I-V relationship of the membrane—whose slope defines the membrane electrical conductance—can be assessed by clamping the membrane at a series of discrete potentials both before and after pyrithione treatment. Thus, possibilities i and ii, above, will generate an increase in membrane conductance, possibility iii will generate a decrease, and possibility iv will generate a decrease in accord with the well-established effects of ATP depletion on the current passed by the H⁺-ATPase (5, 6). In practice, possibility iii can be eliminated for experiments performed in SB because the electrogenic pump serves to shift the resting potential of the membrane to values more negative than those of the equilibrium potentials for any of the ions (the most negative being that for K⁺ at about -35 mV in SB).

Figure 5 shows I-V relationships measured before and after the application of 3 mM pyrithione in SB (pH 5.8) (result from typical series of 10 experiments). Clearly, the effect of pyrithione is to decrease membrane conductance—a result incompatible with membrane depolarization resulting from a general membrane permeability breakdown or the opening of ion channels. It appears, then, that the origins of the membrane depolarization reside in inhibition of the electrogenic H⁺-ATPase. Two further observations reinforce this conclusion. First, the

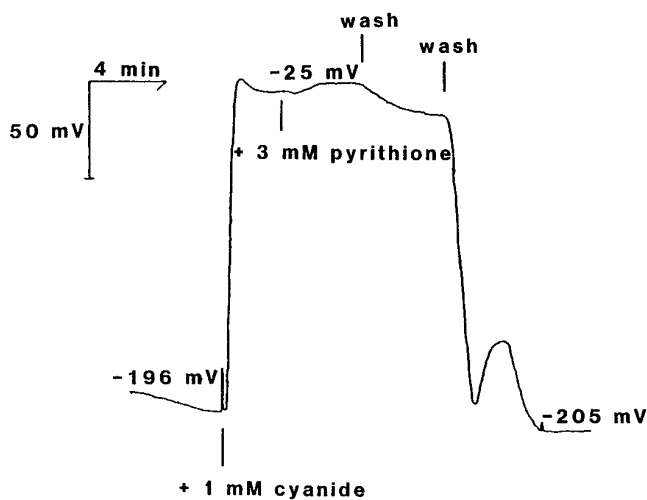


FIG. 4. Limited capacity of pyrithione to induce membrane depolarization in the near absence of electrogenic pump activity. The pump was inhibited by the application of 1 mM NaCN at the time shown, and 3 mM pyrithione was then added in the presence of CN⁻. Return to pyrithione-free solution resulted in a small repolarization (first wash), while removal of CN⁻ resulted in recovery of the original resting potential (second wash).

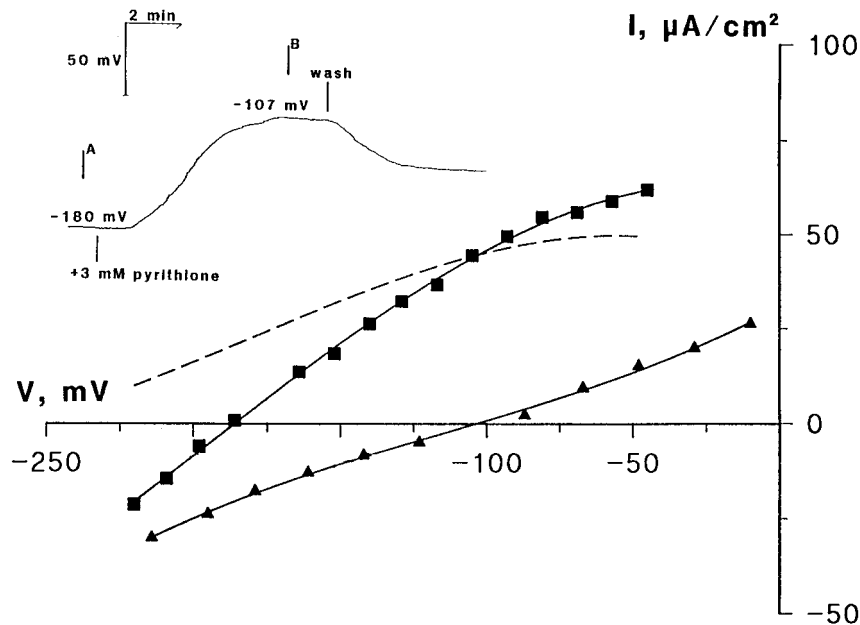


FIG. 5. I-V relationships of the *N. crassa* plasma membrane in the absence (■) and presence (▲) of 3 mM pyrithione. The dashed line is the pyrithione-dependent I-V difference relationship. (Inset) Membrane potential recording from the same cell showing the time of measurement of the two I-V relationships (A and B, respectively).

I-V difference relationship—which reflects the pyrithione-sensitive membrane transport system—exhibits a finite conductance amounting to around half of the control membrane conductance. This property agrees with that surmised already, by the independent approach of reaction kinetic analysis, for the contribution of the electrogenic pump to the overall membrane conductance (6). Second, the I-V difference relationship fails to exhibit a reversal potential (i.e., an abscissa intercept), which accords with the suggestion that the reversal potential for the H^+ -ATPase is more negative than -400 mV (5, 6).

DISCUSSION

Site of action of pyrithione. The effects of pyrithione on the membrane potential are markedly dependent on external pH, being essentially undetectable at alkaline pH. A similar pH dependence was noted for inhibition of phenylalanine transport in *P. chrysogenum* by Chandler and Segel (3). These findings suggest that inhibition by pyrithione requires that the uncharged form of the molecule (pyrithione-SH) be present in the external medium, a conclusion which is strongly supported by quantitative considerations. Thus, given a pK_a for dissociation of the thiol proton of 4.7 and a $K_{1/2}$ for pyrithione-induced membrane depolarization of 0.7 mM at pH 5.8, the effective $K_{1/2}$ for the pyrithione-SH form will be 50 μ M. At pH 8.2, at which the effects of 6 mM pyrithione are almost indiscernible, the uncharged form is present at only 1.9 μ M—a value substantially below the $K_{1/2}$ for pyrithione-SH.

In contrast with the charged form of the compound, pyrithione-SH can be expected to permeate hydrophobic bilayers freely. Our findings therefore indicate that biological activity of pyrithione involves uptake of the uncharged form of the compound and hence an internal or intramembrane site of action.

Mode of action of pyrithione on solute transport. The proposal of Chandler and Segel (3)—that uptake of pyrithione-SH acidifies the cytosol and thereby dissipates a component of the PMF which is required for energization of solute transport—

can be quantitatively evaluated with reference to cytosolic buffer capacity (β). In *N. crassa*, β has been measured as $(-40$ mM H^+ /pH unit (18), a value which coincides closely with those measured for a wide range of eukaryotic cells (15, 26). Since every H^+ ion liberated inside the cell as a result of dissociation of pyrithione-SH will be accompanied by generation of the pyrithione-S $^-$ form, we can write

$$[PS^-] = \beta(pH_{st} - pH_{fn}) \quad (1)$$

where $[PS^-]$ is the concentration of the pyrithione-S $^-$ form and pH_{st} and pH_{fn} are the respective starting and final values of cytosolic pH. Substituting equation 1 into the Henderson-Hasselbalch equation yields

$$pH_{fn} = pK_a + \log \{ \beta(pH_{st} - pH_{fn}) / [PSH] \} \quad (2)$$

where $[PSH]$ is the concentration of pyrithione-SH. For a steady-state value of pH_{fn} , we can assume that equilibration of pyrithione-SH across the plasma membrane is complete and therefore that $[PSH]$ in the cytosol is identical to that in the external medium. Numerical solution of equation 2 for $pK_a = 4.7$, $\beta = (-40$ mM/unit, and $pH_{st} = 7.20$ (18) at the $K_{1/2}$ value of $[PSH] = 50$ μ M yields a value of $pH_{fn} = 6.97$. The PMF across the plasma membrane of *N. crassa* is largely independent of external pH and of the order -300 mV (18). This projected 0.23 unit fall in cytosolic pH is equivalent to only 14 mV—a reduction of only 5% in the overall PMF.

Many plasma membrane H^+ -driven transport systems display an acute sensitivity to cytosolic pH, which is likely to reflect kinetic rather than thermodynamic control by H^+ (17, 19). Nevertheless, even this mode of inhibition of transport by pyrithione is unlikely: propionate with a pK_a almost identical to that of pyrithione is an order of magnitude less effective than pyrithione in inhibiting uptake of SO_4^{2-} and phenylalanine by *P. chrysogenum* species (3). Thus, a mechanism for pyrithione action based on cytosolic acidification through dissociation of pyrithione-SH appears not to be viable.

The relatively small projected effects of pyrithione on cytosolic pH can be compared with the considerable impact on the other component of the PMF—the membrane potential. Thus, depolarization can amount to 100 mV, or one-third of the overall transmembrane PMF. It therefore seems very likely that the origins of the wide-ranging inhibitory effects of pyrithione on plasma membrane transport result from the dramatic effect on V_m . It is noteworthy that a variety of depolarizing treatments—including those that inhibit the electrogenic H^+ pump and those that induce ionic leaks in the membrane—are also effective in inhibiting H^+ -coupled transport in *N. crassa* (24).

It has previously been argued from I-V analysis of H^+ -driven sugar and amino acid transport systems in fungi that although these transport systems are electrophoretic, they normally operate sufficiently far from equilibrium that they are rendered insensitive to voltage changes over the span 0 to -300 mV (7, 17). At first sight, then, this argument appears to contradict the general sensitivity of H^+ -coupled transport to V_m noted above. In this context, however, a distinction must be drawn between direct and indirect effects of V_m on transport. I-V recordings are made with V_m clamped for less than 200 ms at each potential, whereas the earliest samples for radiometric solute flux measurements can be taken only after 10 s or so. We therefore conclude that the effect of sustained (i.e., seconds to minutes) membrane depolarization on transport could result from indirect, rather than direct, interaction of membrane potential with the transport systems concerned. Alternatively, the transport systems might possess some time-dependent gating mechanism which is responsible for down-regulating activity after some minutes.

Mode of action of pyrithione on the membrane potential. The conditions used for the present experiments were selected such that the equilibrium potentials of all ions in the bathing medium were positive of the resting potential. The pyrithione-induced depolarization cannot therefore result from closure of ion channels, since closure would tend to counter their normally depolarizing effect and hence hyperpolarize the membrane. Thus, the depolarization must result from ion channel opening, induction by pyrithione of a general breakdown in membrane permeability, or inhibition of the primary electrogenic pump (an H^+ -ATPase).

I-V analysis of the pyrithione-induced depolarization demonstrates convincingly that inhibition of the pump is responsible for the depolarization. Thus, (i) the membrane conductance is decreased in a manner consistent with the change observed when the pump is inhibited through ATP depletion (5) or by orthovanadate (11), and (ii) the I-V difference relationship, which reflects the I-V relationship of the inhibited element, exhibits consistently positive (outward) currents over the range of experimentally accessible V_m values. Since the reversal potential for the H^+ -ATPase is negative of -400 mV (6) and thereby experimentally inaccessible, this failure to discern a reversal potential for the I-V difference relationship also implicates inhibition of the pump.

We conclude that pyrithione is likely to inhibit transport through membrane electrical depolarization and that this depolarization results from inhibition of the primary electrogenic H^+ -ATPase at an intramembrane or internal site. The relative slowness of the response (a few minutes, even at saturating concentrations) in comparison with the rapid inhibition of the pump upon ATP depletion (a few seconds) might at first sight suggest an indirect interaction with the H^+ -ATPase. Nevertheless, the possibility remains that pyrithione interacts in a time-dependent fashion with free cysteinyl residues on the

H^+ -ATPase, analogous to inhibition of the enzyme by *N*-ethylmaleimide (2).

Possibilities for indirect control of H^+ -ATPase activity by pyrithione include phosphorylation, interaction with lysophospholipids, and other interactions with the autoinhibitory C-terminal domain (13, 21).

It is not known whether inhibition of H^+ pump activity by pyrithione is the principal fungistatic site of action of this compound or whether inhibition is merely one of a number of secondary effects of pyrithione. The observed $K_{1/2}$ of pyrithione for membrane depolarization is up to 1,000-fold higher than its MIC for growth of a number of yeast species (8). However, the disparity between the time domains over which the electrophysiological and growth experiments are conducted (a few minutes and many hours, respectively) precludes direct comparison between the two sets of data. An operational plasma membrane H^+ -ATPase is essential for growth of *Saccharomyces cerevisiae* (14, 20), and therefore inhibition of this enzyme should have far-reaching effects. Nevertheless, the H^+ -ATPase of fungi is under acute metabolic control (17, 18, 20), and a scaling down of its activity can be anticipated if other cellular functions (e.g., respiration) are interfered with. Our findings therefore accord with a recent independent assessment of the properties of the proton ATPase which concluded that this enzyme represents an excellent target for antifungal agents (12).

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