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Depolarization of the Plasma Membrane of *Neurospora* During Active Transport of Glucose: Evidence for a Proton-Dependent Cotransport System

(chemiosmotic hypothesis/membrane potential/electrogenic pumps/microelectrodes)

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ABSTRACT **Intracellular microelectrodes were used to measure the effects of glucose transport on membrane voltage and membrane resistance in *Neurospora crassa*. Sudden activation of glucose uptake, via the high-affinity, derepressible system II, results in a very large depolarization of the plasma membrane. At saturating concentrations of glucose, the depolarization averages 120 mV; it is diphasic in time, with an initial shift at rates of 100–200 mV/sec followed by a slow, spontaneous, partial repolarization. Changes in intracellular ATP concentration are small and could account for only 10 mV of the initial depolarization, while the rest appears to depend upon the transport process itself. A plot of peak depolarization against the extracellular glucose concentration gives a saturation curve which is half-maximal at 42 μM, in good agreement with the $K_{1/2}$ reported for glucose transport via system II. The nonmetabolized analogue 3-*O*-methyl-D-glucose also causes depolarization, and in addition leads to a pulsed alkalinization of the medium occurring at approximately the same rate as 3-*O*-methyl-D-glucose uptake. The membrane resistance falls only slightly during glucose depolarization, a fact which requires the transport system itself to have a high internal resistance, or the membrane current-voltage relationship in glucose-starved cells to be quite nonlinear. All of the data support Mitchell's notion that sugar and hydrogen ions are cotransported under the influence of the membrane potential, and lead to values for H^+:glucose stoichiometry of 0.8 to 1.4.**

An important outgrowth of the chemiosmotic hypothesis of photo- and oxidative phosphorylation (1) has been the notion that—in bacteria (2–7) and very likely in eukaryotic microorganisms and plant cells (8–11)—uptake of a variety of metabolic substrates may occur as cotransport with hydrogen ions, driven by a membrane potential. Though the model can be drawn in a number of forms, one of the most useful is a cyclic carrier scheme similar to that postulated for sodium-dependent cotransport of sugars and amino acids in animal epithelial membranes and cultured cells (12). A simple version of this scheme, and one that places emphasis on the charge-carrying properties of the system, is shown in Fig. 1 (*upper half*). A neutral substrate (S) and a hydrogen ion are assumed to react in sequence with a carrier (X) at the membrane outer surface to form a charged, doubly loaded carrier (XSH^+). The carrier then crosses the membrane under the influence of the existing membrane potential, dissociates at the inner surface, and recrosses the membrane in the neutral, unloaded form.

Considerable support for this general notion has come from transport studies on bacteria and yeast, where stoichiometric influxes of sugars or amino acids and H^+ ions have been measured (3–9), and where in one special situation—in the presence of valinomycin, with the membrane potential presumably defined by the potassium gradient—a quantitative relationship has been demonstrated between substrate accumulation and the electrochemical gradient for H^+ ions (6, 7).

The model has much broader implications for the electrical properties of the membrane, however, and can be understood most clearly in terms of a specific equivalent circuit such as that shown in the lower half of Fig. 1. Since the cotransport scheme postulates a net movement of charge (which can be driven by gradients of H^+ and S as well as by the membrane potential), one convenient representation is an electromotive force (EMF) (E_C) and resistance (R_C) in series. The other two limbs in the circuit represent the membrane diffusion regime (L, leakage pathways) and any electrogenic ion pumps (P) that may be present in the membrane; and the interaction of the cotransport system with these leaks and pumps can be formulated in terms of its effects on the EMF and total resistance of the membrane. In particular, it is clear that the net inflow of charges carried by the cotransport system should depolarize the membrane; the extent and time-course of the depolarization will depend upon the magnitudes of E_C and R_C in relation to the other circuit parameters.

Direct measurements of the electrical properties of the transporting membranes are not yet possible for the bacteria and yeasts, but are quite simple to make on the large mycelial fungus *Neurospora*, which has membrane potentials in the range of 200 mV (interior negative) and appears to sustain the potentials by an ATP-driven electrogenic H^+ extrusion system (13–16). Indirect arguments for the existence of voltage- and H^+-dependent cotransport systems in *Neurospora* have already been presented (11). The experiments described below provide a direct demonstration of the electrical consequences of activating one transport system in *Neurospora*: glucose system II (17), which is readily derepressed during carbon starvation. The results provide strong support for the concept of voltage- and H^+-driven cotransport systems, and raise a number of interesting questions about the model.

MATERIALS AND METHODS

Wild-type strain RL21a of *Neurospora crassa* was used throughout these studies. For microelectrode experiments, mycelial cultures were grown (24–36 hr) on cellophane supported by agar in Vogel's minimal medium (18) with 2% sucrose. The glucose transport system was derepressed by transferring strips of mycelium, affixed to the cellophane, to a large volume of liquid minimal medium without sugar for

Abbreviation: OMeGlc, 3-*O*-methyl-D-glucose.

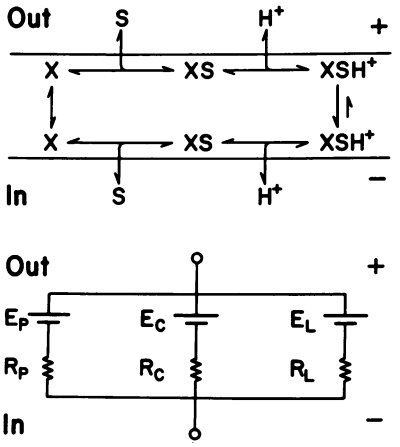

FIG. 1. (*Upper*) One possible cyclic carrier model for cotransport of hydrogen ions (H^+) along with a neutral substrate (S). The *unequal arrows* at XSH^+ indicate that the normal membrane potential would drive the doubly loaded carrier inward. (*Lower*) Electrical equivalent circuit for a membrane containing an electrogenic ion pump (P), a set of ion-dependent cotransport systems (C), and ionic diffusion pathways (L). The individual circuit elements are not meant to be fixed, but to vary with conditions.

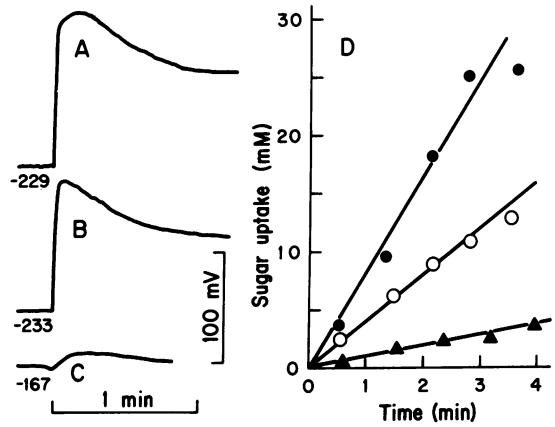

FIG. 2. (*A* and *B*) Voltage traces obtained from a hypha derepressed for glucose transport system II, showing responses to 1 mM glucose (*A*) and 2 mM 3-*O*-methyl-D-glucose (*B*). Membrane potentials (hyphal interior negative) are plotted downward, according to the usual electrophysiological convention, so that depolarization is upward. The numbers at the left below the traces are the control membrane potentials before addition of glucose or OMeGlc. Rapid flow chamber; sugar inflow commenced about 1 sec before upstroke in the traces. (*C*) Glucose effect on a repressed hypha. (*D*) Uptake of [^{14}C]glucose (●, ▲), and [^{14}C]OMeGlc (○) by derepressed (●, ○) and normally repressed (▲) shaking culture cells. mM = mmol/kg of cell water.

3–5 hr. The optimal recording solution was found to be diluted (0.3×) Vogel's medium with 2.3 mM $CaCl_2$ added in order to bring the free Ca^{++} level (buffered by citrate) to 1 mM. Methods for making the electrical measurements have been described (13, 19). Voltage recording was done at the ambient temperature, 21–23°C; the standard temperature for all other experiments was 25 ± 0.1°C.

Sugar and hydrogen ion fluxes and ATP levels were measured on cells from 15- to 16-hour shaking liquid cultures, grown in Vogel's medium with 2% sucrose and derepressed for 3–5 hr in the voltage-recording medium without carbon source. D-[^{14}C]glucose or 3-*O*-methyl-D-[^{14}C]glucose (uniformly labeled; New England Nuclear Corp.) was injected. Aliquots (5 ml) were withdrawn in quick succession, filtered rapidly, and rinsed with distilled water. The resultant pellets were dried at 90°C, weighed (2–5 mg), and counted on a Mark I scintillation counter (Nuclear-Chicago Corp). Net hydrogen ion fluxes were measured with a pH-glass electrode on well-stirred suspensions of cells (20) that had been derepressed and then resuspended in 0.3× Vogel's medium plus calcium. ATP levels were determined by a modification (21) of the Strehler technique (22), with firefly luciferase. All flux and ATP data were obtained in terms of amount per unit dry weight of cells and were converted to amount per kg of cell water with the factor 2.54 = intracellular water/dry weight (23).

RESULTS

Derepression of Glucose Transport System II. It seemed important, in looking for possible effects of H^+-linked cotransport on the electrical properties of the *Neurospora* membrane, to choose a transport system with as high a maximal velocity as possible. One promising candidate was glucose: Schneider and Wiley (17) had shown that wild-type cells, incubated for 1–2 hr in Vogel's medium without carbon source, derepress an energy-dependent glucose transport system (system II), with a V_{max} of about 20 mmol/kg of cell water·min–considerably higher than transport rates for other nonelectrolytes in *Neurospora*. Maximal derepression in the present experiments was obtained by incubating the cells for 2–3 hr in voltage-recording medium. As shown in Fig. 2*D*, the rate of glucose uptake at a saturating concentration (1 mM) increases about 9-fold, from 0.9 mmol/kg of cell water·min in normal cells to 8.4 mmol/kg of cell water·min in derepressed cells. In five other experiments, an average derepressed rate of 8.8 ± 1.1 mmol/kg of cell water·min was measured. The nonmetabolized glucose analogue 3-*O*-methyl-D-glucose (OMeGlc) is also transported by glucose system II, with a velocity of 3.7 mmol/kg of cell water·min in the experiment of Fig. 2*D* and 3.8 ± 0.2 mmol/kg of cell water·min in three other experiments (external concentration = 2 mM, again saturating).

Effect of Glucose Transport on the Membrane Potential. Initial electrical experiments revealed that control membrane potentials in carbon-starved hyphae are very similar to those previously described in normal hyphae (13, 16, 19). The potentials were large (generally in excess of 200 mV); they persisted for several hours, presumably drawing energy from the oxidation of endogenous reserves; and they were only slightly altered by changes in the ionic composition of the medium, but were extremely sensitive to respiratory inhibitors such as potassium cyanide. Thus, the postulated ATP-driven electrogenic H^+ pump (16) appeared to be fully active in carbon-starved hyphae.

An important difference, however, was in the response of the membrane potential to glucose. Whereas sudden addition of 1 mM glucose to the recording medium has only a minor effect on normal, repressed hyphae (Fig. 2*C*), it produced a dramatic depolarization of the derepressed hyphae, followed by a slow, spontaneous partial recovery (Fig. 2*A*). Observed peak depolarizing responses ranged from 57 to 197 mV, with an average of 120 ± 3 mV (86 trials on 20 hyphae). Initial rates of depolarization often exceeded 100 mV/sec, and in a

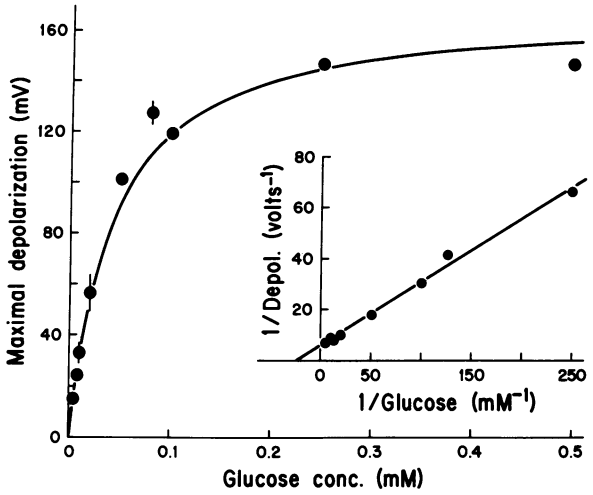

FIG. 3. Plot of the peak (maximal) depolarization of derepressed hyphae, produced by different extracellular concentrations of glucose. Each point is the average of four measurements on two hyphae; *vertical bars*, ± SEM. (*Inset*) Double-reciprocal plot of the same data. $K_{1/2}$ = 42 μM, voltage asymptote = 169 mV.

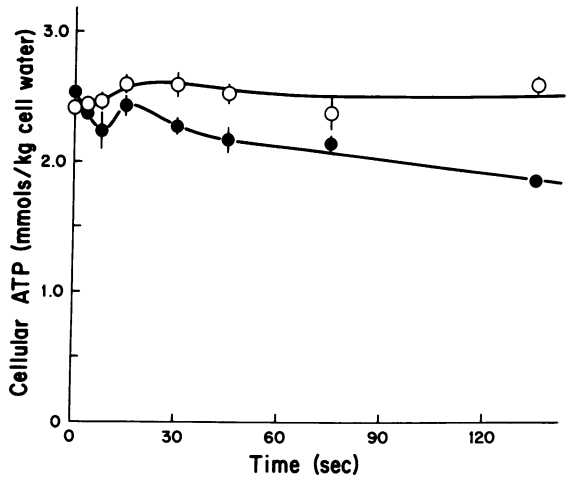

FIG. 4. ATP concentrations in derepressed cells, after addition of 1 mM glucose (●) or 2 mM OMeGlc (○). Each point is the average for three measurements; *vertical bars*, ± SEM.

number of cases exceeded 200 mV/sec. This is an order of magnitude faster than the most rapid depolarization with respiratory inhibitors (16).

In three key respects, the depolarization paralleled the behavior of glucose transport system II: (*1*) its appearance after carbon starvation (just described); (*2*) its dependence upon the extracellular glucose concentration; and (*3*) its substrate specificity.

Dependence of Depolarization on the Glucose Concentration. Fig. 3 shows a plot of the peak depolarizations observed at nine different concentrations of glucose, for a single hypha. The data clearly fall along a saturation curve which—when transformed to a double-reciprocal plot (Fig. 3, *inset*)—yields a $K_{1/2}$ of 42 μM glucose and a voltage asymptote of 169 mV. The average $K_{1/2}$ obtained by Schneider and Wiley (17) for glucose uptake by system II was 40 μM, with a range from 30 to 50 μM.

Substrate Specificity. OMeGlc (Fig. 2*B*) and 2-deoxyglucose (not shown), like glucose, produced rapid depolarization in carbon-starved hyphae. Over the range of OMeGlc concentrations from 8 to 500 μM, the peak voltage responses lay on a saturation curve similar to that with glucose, but having a $K_{1/2}$ of 75–85 μM, in accord with the value reported for OMeGlc influx (17). At saturating concentrations the voltage responses were smaller than those with glucose, a result qualitatively consistent with the lower maximal velocity of transport. Quantitative analysis has made clear that when fluxes of these different substances are compared, however, the voltage responses are not necessarily directly proportional to the rates of transport. Peak depolarization observed with saturating OMeGlc was 77 ± 8% of that with glucose (82% in the records of Fig. 2), while maximal influxes were considerably smaller (43% in these experiments; 32% reported by Schneider and Wiley, ref. 17).

Measurement of Intracellular ATP. An alternative explanation for the depolarizations just described might be that glucose enters the cell, is rapidly phosphorylated, and thereby causes a precipitous drop in cellular ATP. This seems an unlikely explanation, in view of the very high initial rate of depolarization and in view of the fact that OMeGlc, which is known not to be phosphorylated (17, 24), produces the same response. ATP levels were assayed directly during glucose and OMeGlc uptake to test this possibility, however, and the results are illustrated in Fig. 4. An average control value of 2.48 ± 0.04 mmol of ATP/kg of cell water was obtained, for cells starved of carbon (derepressed) for 3 hr in the voltage-recording medium; this value is practically identical with the normal ATP concentration in 15- to 16-hr shaking culture cells, removed to a standard buffer (2.56 ± 0.06 mmol/kg of cell water; ref. 21). When OMeGlc was added to carbon-starved cells (Fig. 4, *upper curve*), a slow transient rise of ATP was observed in the time interval 8–45 sec but the extent—to approximately 2.64 mmol/kg of cell water—was barely significant statistically. When glucose was added (Fig. 4, *lower curve*), there was an initial concentration drop of 0.3 mmol/kg of cell water in 8 sec, followed by a slight rebound, and then a gradual decay to the neighborhood of 1.8 mmol/kg of cell water over a 2.5-min period. A detailed analysis of the relationship between ATP levels and voltage in *Neurospora* (16) indicates that the small initial fall of ATP should itself have caused only a 9 mV depolarization, over a period of 8 sec. Clearly, the large and fast depolarizations produced by glucose and OMeGlc (Fig. 2*A* and *B*) are unrelated to these small changes in ATP levels.

Effect of Glucose Transport on Membrane Resistance. When the cotransport system is inoperative, as in the absence of transportable sugar, its electrical resistance (R_C) must be very great; and when the system is activated by supplying sugar, its resistance could be expected to fall to a finite value. Evidence for a significant decrease in total membrane resistance was therefore sought from experiments in which a single hypha was impaled with two microelectrodes: one for passing brief square pulses of current (10^{-9} A; 0.5 sec positive, then 0.5 sec negative), and the other for recording the resultant voltage displacements. Resistances were calculated by the lumped cable theory developed (13) for *Neurospora* hyphae. In nine cases, the ratio of membrane resistance at peak depolarization to that for a control period was 0.934 ± 0.021, indicating

FIG. 5. (*A*) Time-average plot of the external pH in six different suspensions of derepressed cells. OMeGlc (2 mM) was added to the suspensions at the sudden downshift. The downshift, caused by a slight alkalinity of the injected solution, indicates the direction of increasing pH and, presumably, of H^+ entry into the cells. *Dashed lines* are extrapolated parallel to the pre-OMeGlc baseline. Accumulated H^+ entry is the difference between the upper dashed line and the actual pH trace. (*B*) Derivative of the difference curve from *A*, showing net H^+ uptake for 2 min after addition of OMeGlc. mM = mmol/kg of cell water.

a 6.6% decrease of resistance with maximal glucose transport. In one case, with two spatially separated microelectrodes for recording voltage, the absolute value of membrane resistance could be calculated. During the control period the value was 18,500 ohm·cm², 4- to 5-fold greater than that seen in normal (unstarved) hyphae (13); at peak depolarization the resistance fell 9%, to 16,700 ohm·cm².

Measurement of H^+ Flux. If the model of Fig. 1 is correct, influx of glucose or OMeGlc ought to be accompanied by an equivalent influx of hydrogen ions. However, since the predominant source of the membrane potential in *Neurospora* appears to be an electrogenic H^+ efflux pump (14, 16), only a small fraction of the sugar-linked H^+ influx should be measureable as a net flux of hydrogen ions. The observation that metabolizing yeast display a brief net H^+ flux linked to sugar or amino acid influx, and of the same magnitude (8), therefore seemed worth checking in *Neurospora*. Averaged results of six experiments with OMeGlc are shown in Fig. 5*A*. Baseline hydrogen ion release in carbon-starved cells (upward slope to the left of the sudden downshift) amounted to 0.4 mmol/kg of cell water·min. When 1 mM OMeGlc was added, the pH of the medium became alkaline (right of the downshift), reaching maximal separation from the displaced baseline at 2 min, and equal to 2.4 mmol of H^+ taken up per kg of cell water. This inward pulse of H^+ was reduced at low OMeGlc concentrations and was abolished by KCN. (It could not be seen upon addition of glucose, which greatly accelerated the baseline rate of H^+ efflux.) The time derivative of the difference curve for Fig. 5*A* is plotted in Fig. 5*B*, and gives an initial H^+ influx of 4.4 mmol/kg of cell water·min, essentially equal to the value (3.8) for OMG uptake. After about 5 sec in OMeGlc the net influx of H^+ declined, and approached zero after 1–2 min. The duration of this flux is far too great for the net movement of H^+ to represent charging of membrane capacitance, so that another ionic species must simultaneously cross the membrane to maintain electroneutrality. The pathway and identity of this species are unknown.

DISCUSSION

The data presented above lend themselves to a quantitative analysis of sugar transport in relation to the electrical properties of the *Neurospora* plasma membrane. The basic observation is that a glucose flux of 8.8 mmol/kg of cell water·min (average value at 1 mM glucose) leads to a depolarization of 120 mV, from a resting membrane potential of -211 ± 3 mV to a peak of -91 ± 2 mV. There are two relatively simple limiting cases for the behavior of E_C and R_C (lower half of Fig. 1) that would generate this result:

(*1*) E_C is very large and oriented with the cell interior positive. Then the cotransport system would behave like a current source (R_C also large), depolarizing the membrane with a negligible decrease in membrane resistance. This is an attractive interpretation in view of the observed small change in membrane resistance, about 7%. The depolarizing current, flowing inward through the cotransport system, would be 120 mV/18,500 ohm·cm² = 6.5 μA/cm² = 67 pmol of charge/cm²·sec. For hyphae 15–20 μm in diameter, the glucose flux of 8.8 mmol/kg of cell water·min amounts to 48 pmol/cm²·sec (16), so that a 1.4:1 (or more likely 2:1) stoichiometry between H^+ and glucose would be required to account for the observed depolarization. Stoichiometries of 2:1 have been observed for H^+ cotransport with sugars and amino acids in yeast (8, 9), and stoichiometries of 1:1 in bacteria (3, 4).

(*2*) Alternatively, E_C could be zero, so that the activated cotransport system would behave like a shunt resistance across the membrane. In this event the total membrane resistance during cotransport would be proportional to the fraction of membrane potential remaining: for peak depolarization, $-91/-211 = 0.43$, implying a 57% decrease in membrane resistance. Such a change would be almost an order of magnitude greater than the observed decrease (7%). However, one conspicuous feature of normal *Neurospora* membranes, which has not yet been examined in carbon-starved cells, needs to be considered: the nonlinear current-voltage relationship (25). Voltage-clamp depolarization of normal hyphae from -211 mV to -91 mV causes membrane resistance to double. A similar effect in carbon-starved hyphae would yield a membrane resistance, at peak glucose depolarization, of $0.43 \times 2 \times 18{,}500$ ohm·cm² = 15,950 ohm·cm². The decrease of 14% is tolerably close to the change actually observed. An additional consequence is that the necessary peak depolarizing current would be reduced from 6.5 μA/cm² (calculated above) to 3.9 μA/cm² = 40 pmol of charge/cm²·sec. That value is less than the observed glucose influx (48 pmol/cm²·sec) and would permit an H^+:glucose stoichiometry of 0.8:1 (more likely 1:1).

Since both of these limiting cases are plausible in relation to the present data, it is evident that a detailed examination of the current-voltage relationship for carbon-starved *Neurospora*—both with and without the cotransport system operating—will be required in order to specify the behavior of the circuit elements representing the cotransport system.

Two observations that remain to be discussed are the pulsed net influx of H^+, which decays over a 1- to 2-min period (Fig. 5*B*), and the partial recovery of voltage (Fig. 2*A* and *B*)—both occurring during sustained transport of sugars. Since the time courses of these two processes are roughly the same, both might be accounted for by a time-dependent shut-down of a leakage channel, possibly for potassium ions (8). In addition, the value of E_C can be expected to become more negative as the hyphae accumulate sugar, causing the rate of net sugar influx to fall. A number of other explanations might be suggested but would be premature in the absence of more detailed experimental data relating electrical events to sugar fluxes, during the transition from initial to steady-state conditions.

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