Electrical Properties of *Neurospora crassa*

Effects of external cations on the intracellular potential

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ABSTRACT Glass micropipette electrodes have been employed to study the transsurface potential difference of *Neurospora crassa*. For mature hyphae grown in agar cultures, the internal potential is large and negative, often exceeding −200 mv. The potential is sensitive to the concentrations of extracellular potassium, sodium, hydrogen, and calcium ions, but does not vary in a manner which is readily explained by ionic diffusion potentials. With extracellular solutions containing only potassium chloride (or sulfate) and sucrose, the internal potential shifts toward zero (becomes less negative) at 45 mv per tenfold increase of potassium, over the range 0.1 to 10 mM. A similar result has been found with sodium, though the slope is only 33 mv/log unit. Calcium (1 mM) diminishes the influence of potassium and sodium by 60 to 70 per cent. As potassium or sodium is raised above 20 mM, the slope of the internal potential increases sharply to 85 to 90 mv/log unit, both in the presence and absence of calcium. With increasing hydrogen ion concentration, too, the internal potential shifts toward zero; in this case the slope is about 12 mv/pH unit at pH 9 and rises smoothly to 33 mv/pH unit at pH 3. All these phenomena are probably properties of the plasma membrane. The polysaccharide cell wall contains few fixed negative charges, has a low transverse resistance, and supports very little potential difference when separated from the plasma membrane.

INTRODUCTION

Neurospora was selected for these investigations because of the possibility that its electrical properties could be studied genetically. Highly efficient techniques have been developed for isolating specific biochemical mutants of *Neurospora* (18). Among existing mutants are a lipid-defective strain, whose membranes are known to be anomalous chemically (12, 20), and a number of salt-sensitive strains, which grow poorly in the presence of high concentrations of sodium chloride (8), and whose membranes may be abnormally permeable to sodium. Recently, also, a mutant (36, 39) has been isolated

which has a lowered internal potassium concentration and which takes up potassium less efficiently than the wild type. *Neurospora* can be prepared in large quantities for standard biochemical procedures, and it can be grown under a wide variety of conditions to control the intracellular ionic composition (37). In addition, the cells of *Neurospora* are sufficiently large and rugged to be punctured and studied with microcapillary electrodes (34).

The present experiments have been limited to wild-type *Neurospora* and form the groundwork for projected studies with mutants. The main purpose of the experiments has been to explore the transsurface potential difference as a function of the concentrations of potassium, sodium, calcium, and hydrogen in the external medium. *Neurospora* has not displayed either electrical excitability or rectification under any conditions tested thus far, and the experiments have been concerned only with establishing the nature of the resting potential. Two preliminary accounts of this work have appeared elsewhere (31, 34), and a detailed study of potassium transport in *Neurospora* has been made by C. W. Slayman (37, 38).

MATERIALS AND METHODS

The Cells For electrical measurements, Petri plate cultures of wild-type *Neurospora crassa*, strain RL21a, were grown for 20 to 35 hours at 25°C on sucrose-minimal agar (composition below). The inoculum, taken from a conidiated slant culture, was placed at the center of a cellophane disk supported by 7 ml of agar medium in a 9 cm Petri plate. Sterile technique was employed in the preparation of all cultures. Since *Neurospora* attaches more firmly to a rough surface than to a smooth one, the cellophane was usually scratched with fine emery cloth.

Cultures grown in this manner consist of numerous branching filaments, called hyphae, which radiate several centimeters from the point of inoculation. Individual hyphae appear as in Fig. 1A; they reach a maximal diameter of 20 μ, and are limited by a chitinous wall (*W*) several tenths of a micron in thickness. The plasmalemma (*P*, Fig. 1B), a 75 A unit membrane, is slightly convoluted and is pressed tightly against the wall. Hyphae are divided into segments, which will be called "cells," by incomplete cross-walls or septa (*S*), each having a central pore about 1 μ across. A septum and pore (*P*) are shown in the electron micrograph of Fig. 1B. In that case, a crystal of stored ergosterol (*E*; reference 43) has lodged in the pore, but in healthy, growing cells the pores are open; cytoplasm streams continuously from older cells to newer ones. Under the light microscope the cytoplasm appears relatively homogeneous, lacking the prominent vacuoles found in many higher plants. The electron microscope reveals a normal complement of organelles: nuclei (not shown in Fig. 1B, but usually many in each cell), mitochondria (*M*), ribonucleoprotein particles (*R*), small vesicles and other membranous elements, and carbohydrate granules (*C*).

Most cells studied were 10 to 20 μ in diameter and were located 7 to 9 mm behind the growing margin of the colony. It has already been demonstrated (34) that such cells display maximal membrane potentials.

The Recording Chamber The chamber consisted of two lucite plates (75 mm × 25 mm × 4 mm) bolted together. A hole 25 mm × 15 mm was cut through the

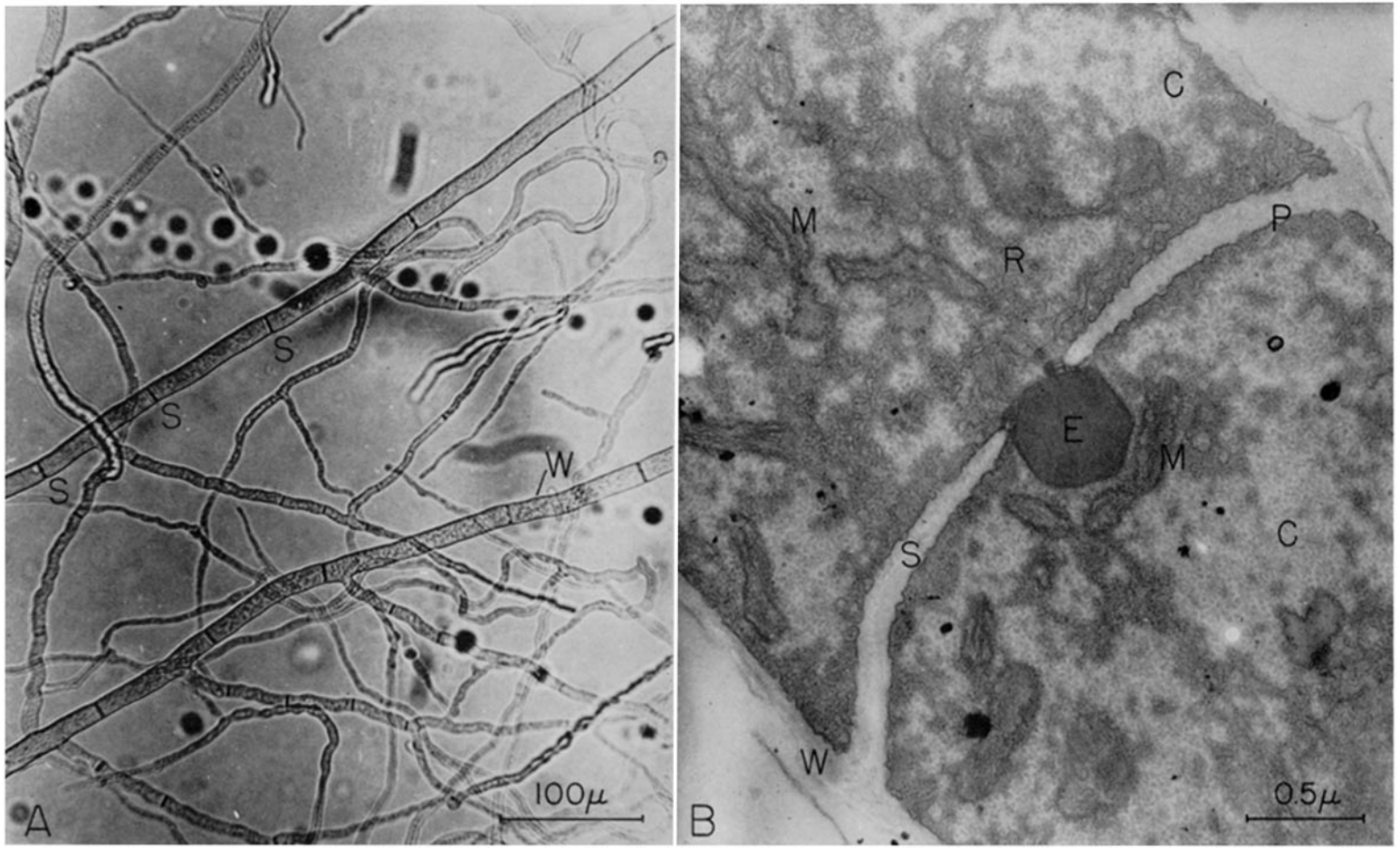

FIGURE 1. *A*, a light micrograph of wild-type *Neurospora* grown on agar medium. Two large hyphae appear in the middle. *B*, an electron micrograph of a mature, agar-cultured hypha, showing the region near a septum. *W*, the cell wall; *S*, septum; *E*, a crystal of ergosterol occluding the septal pore; *M*, mitochondrion; *R*, ribonucleoprotein particles; *P*, the plasmalemma; *C*, carbohydrate granules. Micrographs were kindly provided by Dr. J. F. Wilson (A) and Dr. D. J. L. Luck (B). A, × 200; B, × 34,000.

upper plate to form the body of the recording chamber, and a square of cellophane cut slightly wider than 15 mm could be clamped between the plates, with the attached *Neurospora* exposed. Fluids were added and removed (by aspiration) through channels bored in the lucite plates. Microelectrodes were inserted obliquely through the air–water interface, and the recording chamber was fitted to the stage of a compound microscope, permitting direct visual observation of the micropunctures.

Solutions The sucrose–minimal agar on which the cultures were grown contained 8.4 mM sodium citrate, 36.8 mM potassium dihydrogen phosphate, 25.0 mM ammonium nitrate, 0.81 mM magnesium sulfate, 0.68 mM calcium chloride, trace elements, 1 per cent sucrose, and 2 per cent agar (44); it was buffered at pH 5.9. However, the cells could maintain large and steady internal potentials in much simpler media—consisting of a single salt and sucrose, and the recording solutions were generally kept as simple as possible. The solutions were prepared from reagent grade chemicals; actual concentrations of sodium and potassium were determined by flame photometry. Sucrose was added in reciprocal relation to the salts to give a total osmotic strength of 156 mOsM, slightly hypoosmotic to the nutrient medium. (156 mM sucrose is 3½ per cent sucrose by volume, so that the actual molal salt concentrations were not more than 3½ per cent greater than the molar concentrations.) The pH of these solutions and also of the distilled water was 5.8–6.0. Oxygen diffusion from the air was calculated to be adequate for normal respiration of the cells. All experiments were conducted at the ambient temperature of 23 ± 2°C.

The following notation will be adopted to introduce specific solutions in the text: 10 mM KCl/1 mM $CaCl_2$/suc., for example, will designate a solution containing 10 mM potassium chloride, 1 mM calcium chloride, and sucrose (added to give a total osmolarity of 156 mOsM).

Buffered solutions, for a study of pH effects upon the transsurface potential difference, were made up with dimethylglutaric acid (DMGA), tris(hydroxymethyl) aminomethane (tris), or phosphate, as shown in Table I. The solutions contained 10 mM potassium, 20 mM sodium, 1 mM calcium, 85 mM sucrose, and chloride, along with buffer anions.

Electronic Apparatus The amplifiers (21) were direct-coupled and single-sided, with grid currents less than 4×10^{-12} A, time constants less than 100 μsec., and input impedances of 10^{10} ohms. The recording electrode was led to the first stage grid, while the extracellular solution was connected to the circuit ground through a low impedance potentiometer. The output from each amplifier was direct-coupled to an oscilloscope and a chart recorder.

All leads from the amplifier, both to the "indifferent" electrode and to the microelectrodes, terminated in stable silver-silver chloride half-cells embedded in 3 M KCl–3 per cent agar. The half-cells were matched to within 1 mv. The junction between the indifferent electrode and the extracellular solution was made with a 3 M KCl-agar bridge. This minimized junction potential shifts occurring with solution changes, and introduced no complications from salt leak as long as the extracellular solution was replaced frequently.

Microelectrodes Glass micropipettes with tip diameters less than 1 μ were filled (vacuum boiling, 90–95°C) with 3 M potassium chloride and had resistances greater than 5 megohms, when measured with their tips in 3 M KCl. Since the extracellular solutions were ionically dilute, the tip junction potentials (tip potentials, TP) of the microelectrodes were generally rather large (Fig. 2). Selection of electrodes for small tip potentials, a procedure that has proved satisfactory with concentrated extracellular solutions (1), was numerically impractical. It was therefore necessary to estimate the change occurring in the tip potential during each penetration.

When their tips were broken, all microelectrodes gave zero deflection of the amplifier, no matter what extracellular solution was used. Hence, the tip potential

TABLE I

COMPOSITIONS OF BUFFER SOLUTIONS
(CONCENTRATIONS IN mM)

Buffer						
DMGA	NaOH	NaCl	KCl	$CaCl_2$	Sucrose	Measured pH
10.0	0.4	19.6	10	1	85	2.9
10.0	6.6	13.4	10	1	85	4.0
10.0	9.2*	10.8	10	1	85	5.0
10.0	13.0	7.0	10	1	85	5.9
10.0	18.1	1.9	10	1	85	6.8
Na_2HPO_4	NaH_2PO_4					
1.2	8.8	8.8	10	1	85	6.1
6.1	3.9	3.9	10	1	85	7.1
9.5	0.5	0.5	10	1	85	8.0
Tris	HCl					
10.0	9.6	20	10	1	85	6.9
10.0	5.3	20	10	1	85	8.0
10.0	1.5	20	10	1	85	8.8

* In the case of DMGA at pH 5, the original pH was 4.6, and a slight amount of NaOH was added above the designed 9.2 mM.

of any electrode could be determined by simply noting the deflection of the amplifier before a puncture. In order to estimate the *change* of TP which occurred as an electrode entered a cell, the following procedure was adopted. A new batch of microelectrodes was prepared immediately prior to each experiment, and after each experiment a graph like that of Fig. 2 was constructed from measurements on 5 to 10 unused electrodes. The TP of each experimental electrode was determined immediately before each puncture (TP's after withdrawal averaged about 5 mv different, $\pm$, from those before puncture), and that value was assumed to lie on a curve scaled to the average curve. The scaling factor was simply the ratio of the TP measured immediately before puncture to the average curve TP for the relevant extracellular solution. The TP of each electrode inside each cell was then estimated as the product of the average curve TP for imitation cytoplasm (see below) times the scaling factor. The difference between the TP actually measured before puncture and that estimated for

the tip in imitation cytoplasm was then added to the potential difference recorded across the cell boundary, to give an approximation to the true transsurface potential difference.

This procedure depends upon the nature of the solution chosen to imitate cytoplasm. The potassium and sodium concentrations of *Neurospora* cytoplasm have been found to be approximately 180 mmoles/kg cell water and 20 mmoles/kg cell water, respectively, and the osmotic strength of cytoplasm (590 mOsM, reference 3) requires those ions to be unbound. Therefore, a solution of 180 mM KCl/20 mM NaCl/200 mM suc. was used to represent the cytoplasm. Tests with other salts of potassium—phthal-

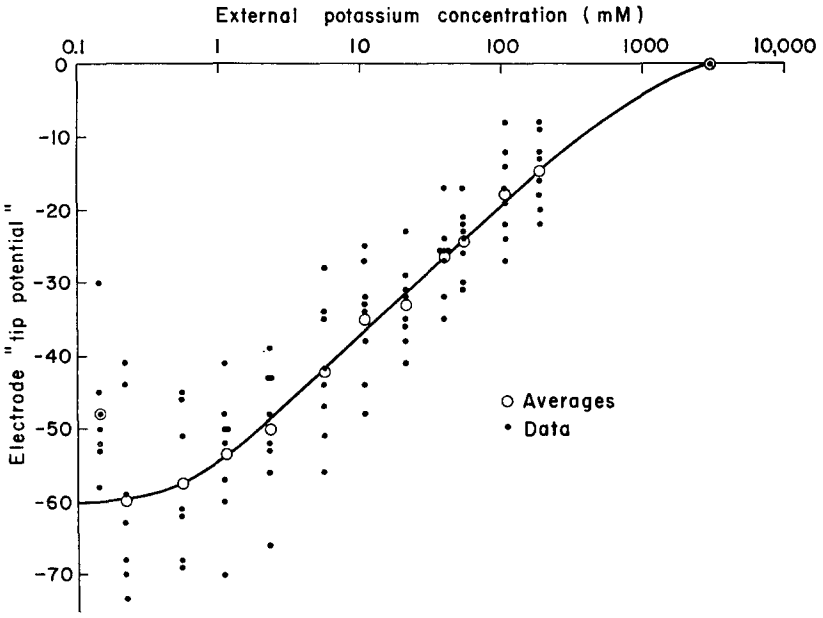

FIGURE 2. Effect of the external potassium chloride concentration upon the tip potential of micropipette electrodes. The 8 pipettes were filled with 3 M KCl and were dipped into solutions of KCl/suc. Slope of the linear portion of the curve, 18 mv/log unit. Similar curves have been presented by other authors (1, 17).

ate, tartrate, albuminate (pH 6)—revealed no significant error arising from the different anion mobilities.

There were two principal consequences of this correction procedure: (*a*) The adjusted transsurface potential differences were larger than those recorded, since the computed corrections usually had the same electrical sign as the measured potential differences. Corrections averaged about 25 mv for extracellular solutions with 0.1 to 0.2 mM salts, 8 mv for 20 mM salts, and 0 to 2 mv for 100 mM salts. Thus, TP corrections account for approximately 8 mv/log unit, in the slopes calculated for Figs. 4 to 6, 9. (*b*) Corrected potentials were less scattered than the measured ones. For 75 per cent of the cell groups studied, standard deviations of the average corrected

potentials were smaller (by as much as 50 per cent) than the standard deviations of the measured potential differences. Furthermore, with two independent pipettes recording from the same cell, the corrected potentials were always more nearly alike than the measured potentials. Some credence is given to the correction procedure by this decrease of scatter, because TP's represent a highly variable source of error which must sum with the normal biological variability.

In no case did the corrections qualitatively alter either the experimental results or their interpretations.

Recording Procedures For the present experiments a cell-sampling technique was adopted. A section of hyphal mat was transferred from the growing colony to an open recording chamber. The cells were washed three times, 1 to 3 minutes each, in the test solution. After the washings, the cells were covered with 1 to 2 mm of test solution, which was replaced at 5 to 10 minute intervals. Individual cells were observed long enough for the recorded potential to reach a steady level, and that level was read from a balancing potentiometer or from the chart record. Five to 20 cells were studied for each test condition. If a number of suitable cells still remained in the preparation, a new test solution was introduced, and the washing and sampling steps were repeated. No cell was impaled twice. When no suitable cells remained, a new population was taken from the Petri plate.

Difficulties in Recording The tough, chitinous cell wall of *Neurospora* can be a serious obstacle to micropuncture studies, particularly when the cells are flaccid or the pipettes are relatively large (45). In the present experiments the normal turgidity of the cells held the wall sufficiently rigid to permit easy puncture with the fine microelectrodes. A visible dimpling of the wall preceded penetration, but the recorded potential difference usually jumped abruptly to its stable value (Fig. 3A) or to within a few millivolts of that value. However, in about 30 per cent of the punctures the potential jumped to only 60 to 90 per cent of the stable level; it then drifted smoothly more negative for 1 to 3 minutes (Fig. 3B). Because the drift was identical at two independent electrodes in any one cell, and because it was accompanied by an apparent increase of the surface resistance, it was interpreted as the progressive formation of an electrical seal around the electrode.

At least four types of systematic noise appeared occasionally. (*a*) Sporadic depolarizing deflections 0.05 to 200 seconds in duration and 5 to 20 mv in amplitude (Fig. 3E). These were never coincident at two electrodes simultaneously in the same cell; during each episode the electrode resistance increased by as much as 100 megohms. Apparently, the cytoplasm of *Neurospora* can reversibly plug pipette tips, raising their resistances and altering their tip potentials. (*b*) Depolarizing DC shifts up to a few seconds in duration and 1 to 10 mv in amplitude (Fig. 3C). These were identical at independent electrodes and may have represented transient leaks around the microelectrodes. (*c*) Drifts, 1 to 10 minutes in duration and up to 20 mv in amplitude (Fig. 3D). Most frequently, they were hyperpolarizing in direction and were terminated by a sudden step in the recorded potential difference, back to the control value. These were never seen simultaneously at independent electrodes, and were presumed to be electrical artifacts arising near the electrode tips. (*d*) Finally, a

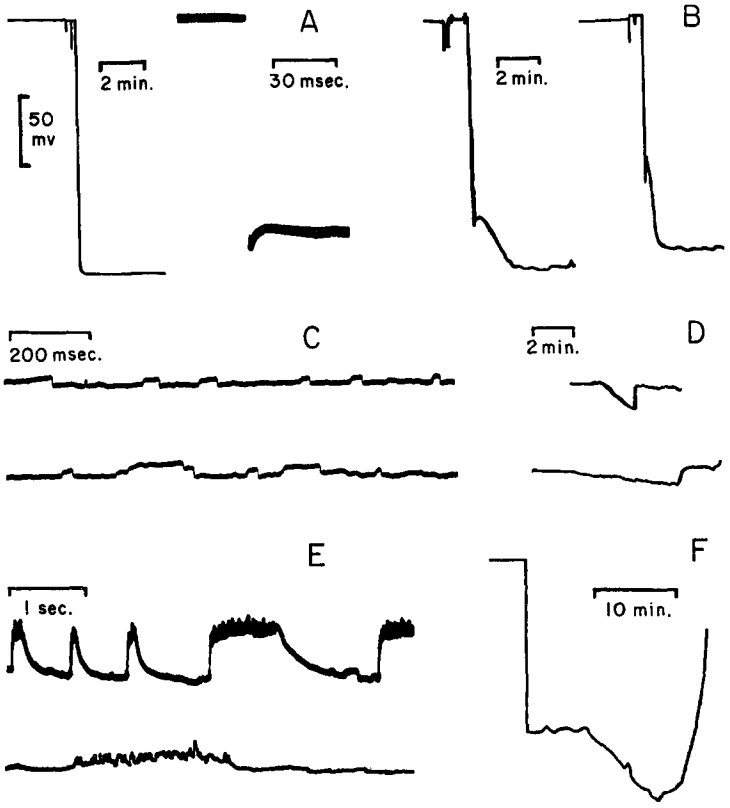

FIGURE 3. *A*, chart (left) and oscilloscope (right) records from two punctures in which the full electrical potential difference was measured at the moment of penetration. *B*, chart records from two punctures in which the last 40 to 60 mv was achieved gradually, probably because the cell surface sealed only slowly around the microelectrode. *C*, oscilloscope traces from a cell which displayed small depolarizing shifts (type *b*, in the text). *D*, chart record, showing two examples of slow drift (type *c*) in the recorded potential. *E*, oscilloscope traces illustrating the electrical artifacts caused by sporadic plugging of the micropipette electrodes. The upper record was made with an amplifier having an input resistance (10^8 ohms) of the same order of magnitude as the fluctuating resistance; hence, the disturbance was exaggerated by voltage division. A larger input resistance (10^{10}ohms) was used in making the lower record. *F*, a graph of apparent intracellular potential, showing the effect of mechanical drift which bent the micropipette electrode against the cell wall. The same voltage calibration (upper left) applies to all records.

monotonic, self-terminating rise of the recorded potential difference through 50 to 100 mv (Fig. 3F) sometimes developed. This was associated with drift in the micromanipulator and probably arose as the electrode tip was bent against the cell wall. Inevitably, the microelectrode was pulled out of the cell.

Except for disturbances of type *c*, which could not always be distinguished by inspection from experimental changes, the various forms of noise did not interfere with determination of the intracellular potentials.

Ion Analyses. The sodium and potassium contents of *Neurospora* were determined on 24 hour, stationary cultures grown in liquid minimal medium; hyphae in such cultures are morphologically like those found on agar medium. The hyphae were removed from the medium, rinsed several times in distilled water (this causes no loss of intracellular ions, reference 37), dried to constant weight at 60°C, and extracted 1 to 2 hours in 1 N HCl at 100°C. Extracts were analyzed for sodium and potassium on a Beckman DU flame photometer. Extracellular space was estimated with inulin (using the diphenylamine color reaction, reference 2). Hyphae were soaked for 10 to 90 minutes in solutions of inulin, removed, pressed in blotting paper, weighed, and then rinsed in a fixed volume of distilled water. The concentration of inulin in the wash solution was assayed, and the hyphal mat was reweighed after drying.

RESULTS

Intracellular Ion Concentrations The most reliable estimates of the intracellular potassium and sodium concentrations in *Neurospora* are those given by C. W. Slayman (37) for 15 hour shake cultures grown in liquid minimal medium. All cells in these cultures are growing logarithmically, and they are morphologically very homogeneous (2 to 3 μ in diameter, unbranched). Stationary cultures contain a fraction of non-growing hyphae (28) which may be dying or dead, and it is likely that average ion analyses on such colonies do not accurately reflect the ionic contents of cells studied in the electrical experiments.

For the shake cultures, internal potassium and sodium were found to be 457 ± 3 mmoles/kg dry weight (mean ± SE) and 35.5 ± 0.5 mmoles/kg dry weight, respectively. The ratio of intracellular water to dry weight was 2.54 ± 0.03, giving intracellular concentrations of $[K]_i$ = 180 ± 3 mmoles/kg cell water and $[Na]_i$ = 14.0 ± 0.5 mmoles/kg cell water (37). In eight experiments with stationary cultures, internal potassium and sodium were equal to 380 ± 4 mmoles/kg dry weight and 67 ± 3 mmoles/kg dry weight, respectively. Under standard conditions the inulin extracellular space was 18.6 ± 0.3 per cent of the wet weight. This figure depended upon the blotting procedure, but it varied in parallel with the wet weight/dry weight ratio, making the more important ratio—intracellular water/dry weight—independent of the blotting procedure and equal to 2.61 ± 0.05, essentially the same as for the shake cultures. This gives concentrations of $[K]_i$ = 146 ± 4 mmoles/kg cell water and $[Na]_i$ = 26 ± 3 mmoles/kg cell water. For somewhat different conditions, Lester and Hechter (19) have given values near 160 mM K^+ and 30 mM Na^+ in germinating conidia.

The estimates of internal sodium and potassium will be too low if inulin fails to penetrate the interstices of the cell wall. From the geometry of the hyphae an error of about 20 per cent can be calculated for the shake cultures, and 10 per cent for the stationary cultures. An attempt was made to estimate the extracellular space with a smaller substance, ^{35}S-sulfate, but the result—

suggesting an inulin error of 25 per cent for stationary cultures—was spurious. Sulfate appears to be taken up and released by the cytoplasm, and it may also be bound electrostatically to the cell wall.

$[K]_i$, at least, was probably constant over the experimental period (15 to 60 minutes), since *Neurospora* loses less than 1 per cent of its internal potassium per hour into distilled water or potassium solutions (37). It loses potassium as fast as 16 per cent per hour into K-free sodium solutions, however.

In the discussions below 180 mM K^+ and 20 mM Na^+ will be taken as rounded estimates of the intracellular concentrations.

Information is available on the intracellular levels of three other inorganic ions. *Hydrogen*, no measurements have been made *in vivo*, but determinations on fresh conidia (*N. sitophila*, reference 23) gave pH's near 6. This is reasonable in view of the pH optima for extracted enzymes (41). The extent to which external pH influences internal pH is not known. *Chloride*, the total radioactivity of shake culture cells grown on $^{36}Cl^-$-labeled minimal medium indicates an intracellular concentration below 0.2 mmole/kg cell water (35), making the $[Cl]_o/[Cl]_i$ ratio greater than 6.8. *Phosphate*, from data given by Harold (13), one can compute an orthophosphate level of 15 mmoles/kg cell water. Harold's data were from shake cultures grown on a medium containing 7 mM phosphate, rather than the 36.8 mM in the growth medium of the present experiments. As far as has been determined, the major cation in *Neurospora* is potassium, and the major inorganic anion is phosphate.

Effects of Potassium and Sodium on the Intracellular Potential The resting membrane potential in many kinds of cells is potassium-sensitive in a manner which suggests that passive diffusion of potassium outward through the cell membrane gives rise to the resting potential. For this reason, the internal potential of *Neurospora* was measured as a function of the extracellular potassium concentration, $[K]_o$. Figs. 4A and 5 show that in the absence of external cations other than potassium, the internal potential of *Neurospora* did become less negative as $[K]_o$ increased. The curve broke into two portions: for $[K]_o$ values between 0.1 and about 10 mM, the change was 45 ± 2 mv/log unit concentration (regression coefficient ± SE, Fig. 4A); with $[K]_o$ between 10 and 100 mM, the slope was 85 ± 3 mv/log unit (Fig. 5, upper curve). Essentially the same results were obtained whether the external anion was chloride or sulfate, though there was more scatter in the sulfate data. Changes of potential on passing from one potassium concentration to another were reversible, except when the cells were exposed for more than a few minutes to 50 or 100 mM potassium. The smaller potentials found after long treatment with high concentrations were not studied systematically.

It is clear from the above results that the potassium gradient across the *Neurospora* cell surface influences the potential difference, but several features

of the curves in Figs. 4A and 5 indicate that there are other important factors. At all potassium concentrations tested (0.1 to 100 mM), the internal potential of *Neurospora* was more negative than the diffusion equations would predict for movement of a single cationic species through a barrier permeable only to that species. For example, at a $[K]_o$ of 10 mM, the internal potential was

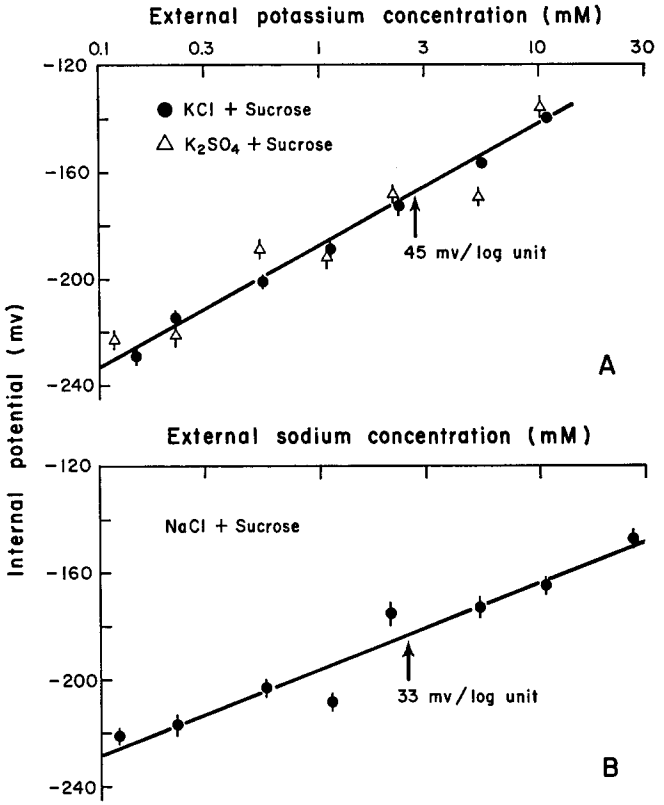

FIGURE 4. The effects of potassium (A) and sodium (B) upon the internal potential of *Neurospora*. Each point represents the average internal potential for 10 to 30 cells sampled in a given solution. Vertical bars, ±1 SE; for the slopes of both regression lines, the SE's are 2 mv/log unit.

−140 mv, while the Nernst equation ($V_i - V_o = -58 \log [K]_i/[K]_o$, in millivolts) would predict −73 mv, assuming $[K]_i$ = 180 mM, with equal activity coefficients on both sides of the cell surface. Correction of $[K]_i$ for possible cell wall volume would change the predicted internal potential only −5 mv, and would still leave a discrepancy of −62 mv between the measured and calculated potentials. Any allowance for activity coefficients would only magnify the discrepancy, since the activity coefficient for potassium at 180 mM is likely to be near 0.7, while that at 10 mM should be near 0.9.

Over both concentration ranges 10 to 100 mM, and 0.1 to 10 mM, the slopes of internal potential *versus* log $[K]_o$ differ from the theoretical value of 58 mv/log unit. The difference is significant at a level less than 0.001 in both cases. This complex dependence of the internal potential upon log $[K]_o$—with slopes both much greater and much less than the theoretical value for simple diffusion—requires some special device in order to be interpreted within the framework of ion diffusion. Unstirred layers, Donnan potentials in the cell

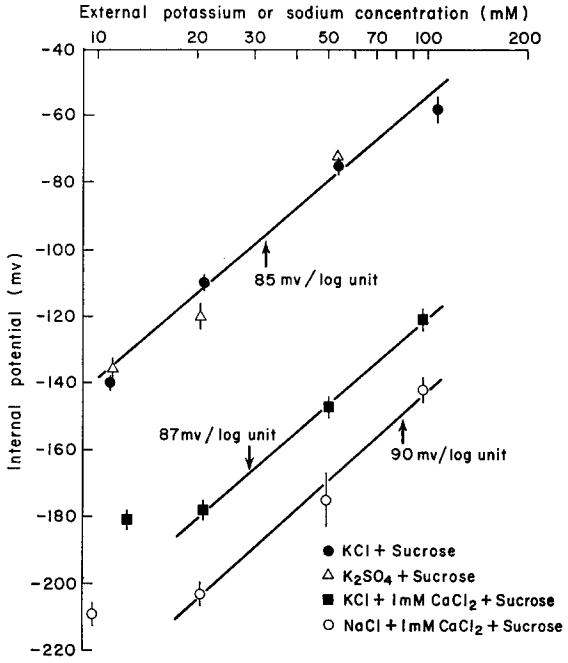

FIGURE 5. The effects of high concentrations of salts upon the internal potential of *Neurospora*. Each point represents the average internal potential for 5 to 20 cells. *Upper curve* (extended from Fig. 4A), potassium chloride or sulfate, without calcium. *Lower curves* (extended from Fig. 9), potassium chloride or sodium chloride, with 1 mM calcium. Vertical bars, ±1 SE. SE's of the slopes of the regression lines, 3, 8, and 9 mv/log unit, reading from top to bottom.

wall, and finite permeability of the cell surface to ions other than potassium might be involved, and will be treated further in the Discussion.

Direct evidence has been obtained on the question of permeability to ions other than potassium. In the absence of added potassium, the internal potential varied systematically with $[Na]_o$, having a slope of 33 ± 2 mv/log unit, over the range 0.1 to about 20 mM (Fig. 4B). With sulfate as the anion, the internal potential appeared less dependent upon sodium, but the scatter of data prevents any firm conclusion about the role of the anion. Potassium

and sodium also interact in a manner to be expected for two permeant species. Extracellular sodium "clamps" the internal potential, making it insensitive to changes of potassium at low concentrations. Thus, 5 mM sodium chloride set the internal potential at about −190 mv (Fig. 6A) for all potassium concentrations below about 1 mM. The effect was even more pronounced with

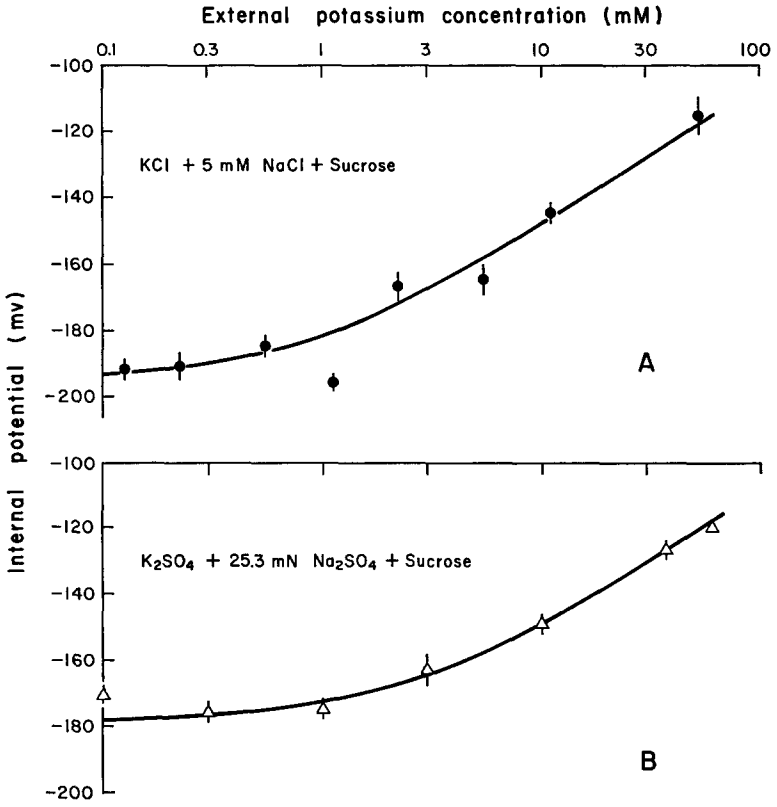

FIGURE 6. The effect of sodium upon the potassium dependence of the internal potential. Each point indicates the average internal potential for 5 to 20 cells samples in solutions with 5 mM NaCl (A) or 25.3 mN Na_2SO_4 (B) at various potassium concentrations. Vertical bars, ±1 SE.

25 mM sodium in the extracellular solution (Fig. 6B). Comparison of Fig. 6 with Fig. 4A suggests a sodium/potassium permeability ratio of about 0.1; with only one extracellular cationic species, the internal potential was the same for 0.8 mM potassium as for 5 mM sodium (−193 mv), or the same for 1.7 mM potassium as for 25 mM sodium (−178 mv). But the interaction of potassium and sodium is not completely simple; the presence of both species prevents the precipitous shift of potential which normally occurs at potassium concentrations above 10 mM (see Figs. 5 and 6B).

The over-all contribution of intracellular sodium to the transsurface potential difference in *Neurospora* cannot be large, however. $[Na]_i$ is only about one-tenth as large as $[K]_i$. Even if the cell surface did not discriminate between sodium and potassium, log ($[Na]_i + [K]_i$) would be only 2 per cent greater than log $[K]_i$. In fact, the cell surface discriminates against sodium, which would make the sodium contribution to the log expression much less than 2 per cent.

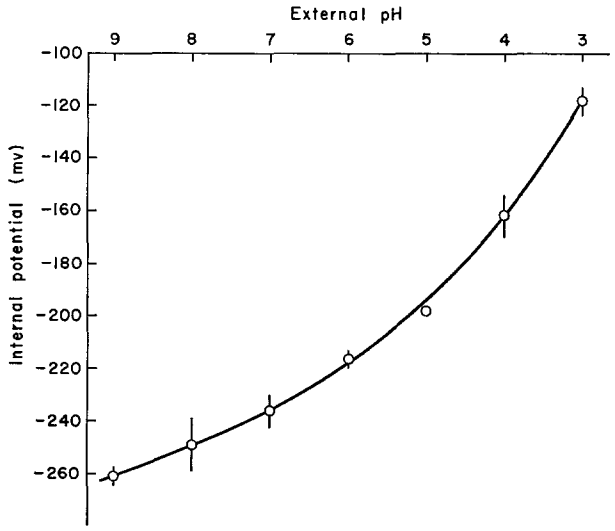

FIGURE 7. Influence of extracellular pH upon the internal potential of *Neurospora*. Four cells were studied, and the buffer solutions (Table I) were changed many times during each penetration. Vertical bars, ±1 SE. At pH 6, which is the reference condition, the average internal potential in this figure is about 30 mv negative to the average plotted in Fig. 9A (at 10 mM KCl). The reasons for this discrepancy are probably more closely related to the technique of long term recording than to the presence or absence of a specific buffer. The matter is discussed elsewhere (32). For present purposes the slope of internal potential *versus* pH is more important than the exact position of the curve along the ordinate.

The Influence of the Hydrogen Ion pH experiments were carried out not by the usual cell-sampling technique, but by a technique of long term recording from single cells during many changes of solutions (32). All buffer solutions contained 1 mM calcium chloride, since 0-calcium DMGA buffers produced an irreversible decline of transsurface potential difference. Data from four cells were averaged and plotted in Fig. 7. In all cases the internal potential moved monotonically from one level to the next with a change of pH. At pH 8–9 the internal potential shifted only about 12 mv/pH unit, whereas at pH 3–4 the shift was about 33 mv/pH unit. This indicates a substantial in-

fluence of pH, but it is not yet known whether the relevant factor is a hydrogen ion diffusion potential *per se* or a more general control by pH of the membrane permeability. Certainly, cells maintained at the acid extreme, pH 3, leak potassium more rapidly than normal cells (35).

Anions Of course, slopes substantially less than 58 mv/log unit suggest that the cell surface may be permeable to anions, particularly to chloride and sulfate. No direct and unequivocal evidence is yet available on this point, although *Neurospora* probably resembles yeast (25) in having generally low anion permeabilities. Certainly, intracellular chloride, as well as sodium and potassium, is stable to distilled water washing (37). When shake culture cells grown in the presence of $^{36}Cl^-$, $^{42}K^+$, or $^{24}Na^+$ were washed for 10 second periods in 5 successive aliquots of distilled water, the number of counts associated with the cells after each rinse remained constant within a standard error of 3 per cent for chloride and 1 per cent for potassium and sodium. Counts actually appearing in each aliquot of solution (after the first rinse) were less than 0.3 per cent of the counts associated with the cells (35). When the cells were left in warm (20–25°C) distilled water for longer periods, potassium leaked out at a rate of 1 per cent per hour. These results all suggest either that the surface of *Neurospora* is quite impermeable to anions or that the intracellular concentration of small anions is very low.

Other experiments with shake culture cells have revealed the maximal rate of uptake of ^{35}S-labeled sulfate at 27°C to be 0.1 mmole/kg cell water/minute, or 0.005-fold the maximal rate of entry of radiolabeled potassium at the same concentration (30 mM). An anion/potassium permeability ratio of 0.005 would imply that external anions could not contribute significantly to the transsurface potential difference; but the inward movement of potassium and sulfate may be regulated by active transport at least as much as by passive permeability. Flux measurements on respiration-poisoned cells—which might yield information about permeability in the absence of active transport—would be complicated by the fact that the surface resistance of *Neurospora* increases when respiration is blocked (32).

Effects of Calcium and Magnesium Calcium is well known to stabilize cells against mechanical damage and to regulate membrane permeability to other ions, particularly the alkali cations (22, 42). Its influence on *Neurospora* seems pronounced in both respects. Addition of calcium to the external medium made possible routine long term recording (10 minutes to 4 hours) from individual hyphae, and it also allowed hyphae to be punctured repeatedly without substantial deterioration of the internal potential (32).

Fig. 8 (lower curve) shows that addition of even 0.1 mM calcium to a standard medium containing 36.8 mM KCl/25.3 mM NaCl increased the recorded transsurface potential difference by more than 30 per cent, taking the average

internal potential from −131 to −173 mv. Larger amounts of calcium, 1 mM or 10 mM, had little further effect on the potential recorded under these conditions, but did further stabilize the cells mechanically.

Only one other divalent cation was tested, magnesium, which is an essential growth factor for *Neurospora* (28). Even at a concentration of 10 mM it increased the recorded potential difference by only a few millivolts (Fig. 8, upper curve). Its effect on the mechanical properties of the cells was, if any, to diminish stability.

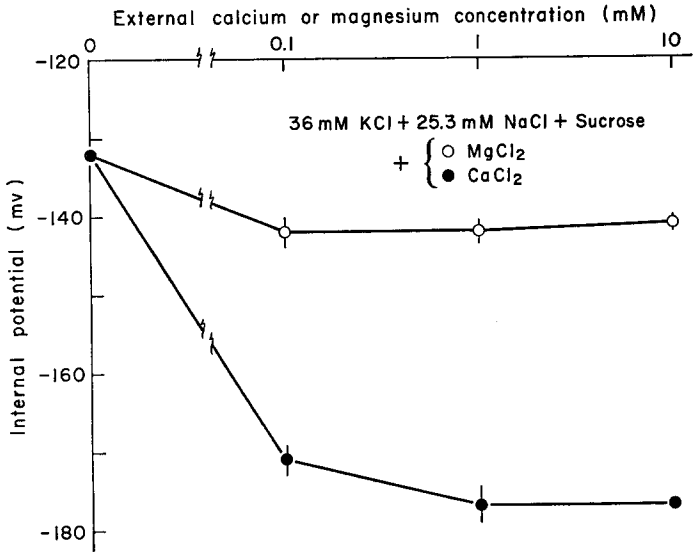

FIGURE 8. The effects of calcium (●) and magnesium (○) upon the internal potential of *Neurospora*. Each point gives the average internal potential for 5 to 15 cells tested in solutions with the same potassium and sodium concentrations as minimal medium. Vertical bars, ±1 SE.

But perhaps the most important effect of calcium is to make the transsurface potential difference relatively insensitive to sodium or potassium in the extracellular solution. The internal potential of cells bathed in NaCl/1 mM $CaCl_2$/suc. shifted only 9 ± 2 mv per tenfold change of external sodium concentration in the range 0.2 to 20 mM (Fig. 9B). This slope (significantly different from 0 at a level of 0.005) must be compared with the slope of 33 mv/log unit in the absence of added external calcium (see Fig. 4B). Potassium had a somewhat larger effect than sodium on the internal potential, though the slope of 17 mv/log unit (Fig. 9A), in the range 0.5 to 20 mM, was less than one-half the value found for cells in "calcium-free" media. At concentrations of potassium or sodium above 20 mM, the presence of 1 mM calcium had little effect on the slope of internal potential *versus* log $[K]_o$ or log $[Na]_o$ (see Fig. 5, lower

two curves). Slopes of 87 ± 8 and 90 ± 9 mv/log unit were found for potassium and sodium, respectively. The combined slope for all data at high $[K]_o$ and high $[Na]_o$ is 87 mv/log unit, which is significantly different from the theoretical 58 mv/log unit at a level of 0.001. In the presence of calcium the internal potential of *Neurospora* is greater than the Nernst potential for potassium at all external potassium concentrations above 0.2 mM. For $[K]_o$ = 10

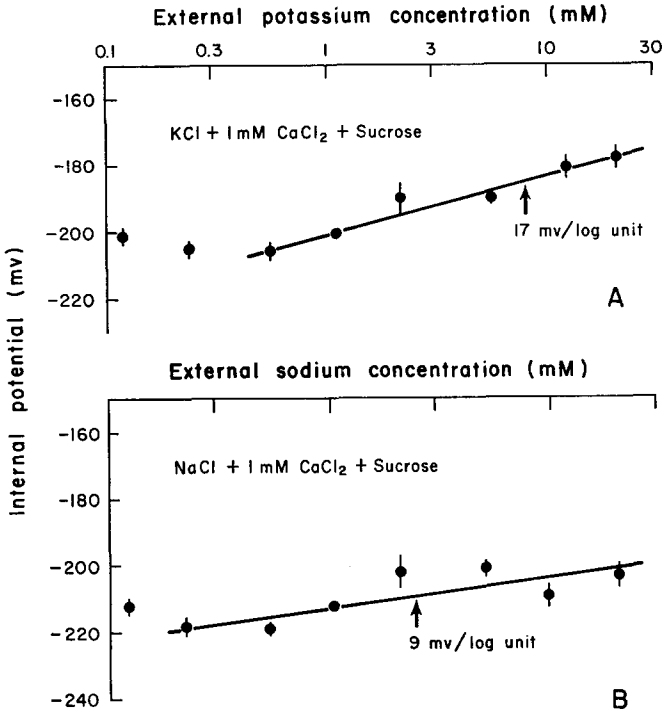

FIGURE 9. The effects of potassium (A) and sodium (B) on the internal potential of *Neurospora* in the presence of 1 mM calcium. Each point presents the average internal potential for 10 to 20 cells. Vertical bars, ±1 SE. SE's of the regression coefficients, 2 mv/log unit.

mM, the discrepancy is −105 mv. It is clear under these circumstances that the internal potential of *Neurospora* is not a simple Nernst diffusion potential for potassium, or even a combined diffusion potential for potassium and sodium.

The Role of the Cell Wall Since the surface of the *Neurospora* hypha comprises both a polysaccharide wall and the plasma membrane, it is of central importance to determine what each structure contributes to the electrical phenomena presented above. The most direct answer would come from experiments with protoplasts, which lack cell walls; such experiments are now

being undertaken. But considerable indirect information is already available which indicates that the cell wall of *Neurospora* contributes very little to the transsurface potential difference.

If the wall were to contain fixed negative charges—as in the form of phosphate, sulfate, or carboxyl residues—it could function as a cation exchange resin and, under non-steady state conditions, could contribute Donnan potentials to the total transsurface potential difference. This situation has been demonstrated in the alga *Chara*, where the internal potential does not progress monotonically from one level to another during a shift of external salt concentration, but displays a large transient overshoot (15). The magnitude and duration of the transient depend upon the tightness of the cell wall, or the relative size of the water free space which penetrates the wall and is electrically parallel to the fixed charge region.

Although chemical evidence (see Discussion) indicates that the cell wall of *Neurospora* contains few fixed negative charges, electrical evidence is somewhat equivocal. The wall alone, punctured in dead or plasmolyzed cells, was unable to support a measurable potential difference during tenfold shifts of the external KCl concentration over the entire range 0.1 to 100 mM (solutions contained KCl/1 mM $CaCl_2$/suc.). This probably means only that the parallel water free space is large, but it may also indicate that the wall does not distinguish between anions and cations. For intact cells, transient overshoots of internal potential were only sometimes observed during changes of external salt concentration. Fig. 10A (traces 4 and 5) shows the overshoots in a case when KCl was shifted from 0.1 to 10 mM (trace 4), then back to 0.1 mM (trace 5), in the presence of 1 mM calcium. But with external calcium between 0 and 0.1 mM, the internal potential usually moved monotonically from one level to the next (Fig. 10A, traces 1 to 3), regardless of the magnitude or direction of the change in salt concentration. Even with 1 mM calcium, the overshoot was not always observed. If the overshoot does reflect Donnan potentials across the cell wall, then it is a little surprising to see a more pronounced effect in the presence of calcium than in its absence. Calcium would be expected to displace univalent cations from any fixed negative charges, thus making the wall a less effective cation exchanger.

Regardless of the presence or absence of fixed charge regions, steady-state conditions are probably reached quickly. Preliminary experiments have shown the transverse resistance of the wall to be small—certainly not more than 250 ohm. cm^2 (5 per cent of the total transverse resistance of the surface), and probably much less. Also, the potential change accompanying any shift of $[K]_o$ was complete within a few minutes, whether or not an overshoot appeared. The steady-state times suggested by Fig. 10A are upper limits, since complete exchange of fluid in the recording chamber required about 2 minutes. This means that all the measurements summarized in Figs. 4 to 9 were

made after the transients had subsided. Sampling of internal potentials was not begun until the cells had been in test solution for several minutes, and sampling was completed only after 10 to 50 minutes more. Furthermore, under most of the conditions the unidirectional fluxes of potassium must have been equal inward and outward, since it has been shown that *Neurospora* does not lose net potassium into potassium-containing solutions (37).

One final piece of evidence suggests that the cell wall of *Neurospora* contributes very little to the total transsurface potential difference. The polyene

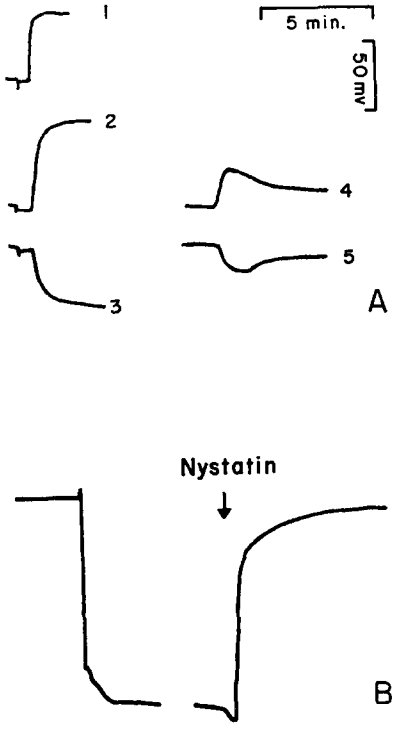

FIGURE 10. *A*, Traces of changes of internal potential during shifts of extracellular potassium concentration, showing that overshoots do not always appear. The solutions contained KCl/0.01 mM $CaCl_2$/suc. (traces 1 to 3) or KCl/1 mM $CaCl_2$/suc. (traces 4 and 5). The following steps of potassium concentration were employed. *1*, 0.3 to 3 mM; *2*, 1 to 50 mM; *3*, 1 to 0.1 mM; *4*, 0.1 to 10 mM; *5*, 10 to 0.1 mM. *B*, the effect of 10^{-6} M nystatin upon the internal potential of *Neurospora*. The break in the trace represents a period of 1 hour. The voltage and time calibrations at the upper right apply to all traces.

antibiotic nystatin (provided by the Squibb Institute for Medical Research, New Brunswick, N.J.) has been shown by Kinsky (16) to bind specifically to membrane fragments of *Neurospora*. Nystatin also abolishes the transsurface potential difference (Fig. 10B). In the record shown, the cell was punctured in 10 mM KCl/1 mM $CaCl_2$/suc., and registered a normal internal potential; but when 10^{-6} M nystatin was introduced, the internal potential collapsed, losing 140 mv in the first 2 minutes. Nystatin produces an equally rapid escape of potassium from *Neurospora* (37). Seventy-five per cent of intracellular potassium is lost into distilled water within 2 minutes; the other 25 per cent escapes more slowly, apparently limited by the rate of proteolysis. Again, the important point is that the wall is not an effective barrier to diffusion of small

ions. Any Donnan regions which might exist in the wall should be electrically shunted by the parallel water spaces.

DISCUSSION

In trying to interpret the electrical properties of *Neurospora*, one certainly must begin with the process of ion diffusion, since that is the only unequivocally established mechanism for generating biological membrane potentials. Internal potentials near −30 mv have already been demonstrated (34) at the tips of agar-cultured *Neurospora* hyphae growing on minimal medium ($[K]_o$ = 37 mM). This value could well result from ion diffusion potentials ($[K]_i$ = 180 mM), but it is normally found only in regions where cytoplasmic volume and membrane area are expanding rapidly. By analogy with the growing tips of agar-cultured hyphae, the small and rapidly growing shake-culture cells used for ion flux measurements (37, 38) probably also have internal potentials in the neighborhood of −30 mv. As far as other microorganisms are concerned, Conway (5) has mentioned a value of −70 mv for the internal potential of yeast, obtained apparently with considerable difficulty. Seventy mv is certainly not out of reason for a cation diffusion potential.

Interpretation of the large internal potentials of mature *Neurospora* hyphae is a more difficult problem. The decrease of the transsurface potential difference with increasing extracellular salt concentration suggests cation selectivity and cation diffusion potentials. But the magnitude of the internal potential is outside the limit of any single cation diffusion potential, and probably also outside the limit of a polycation diffusion potential involving potassium, sodium, and hydrogen. The role of the hydrogen ion, however, must be explored in greater detail. With the external pH at 6, an internal pH near 3 would be required to produce a hydrogen ion diffusion potential of 180 mv. Such a pH almost certainly does not exist throughout the cytoplasm (23, 41), though conceivably it could occur in the immediate neighborhood of the plasma membrane. Conway and Downey (6) have inferred the existence of a region of low pH near the surface of yeast, but it probably lies just outside rather than just inside the plasma membrane. But even if the pH immediately inside the membrane were near 3, the membrane would need to be 100- to 1000-fold more permeable to hydrogen than to potassium for the hydrogen ion potential to be expressed—in the face of 180 mM potassium. This condition might well exist, since the slope of internal potential *versus* external pH is 33 mv/pH unit (between pH's 5 and 3; see Fig. 7) in the presence of 10 mM KCl/1 mM $CaCl_2$/20 mM Na^+ along with buffer anions. At an external pH of 6, an internal pH of 3, and an external potassium concentration of 10 mM, the hydrogen gradient might contribute −80 mv, and the potassium gradient −60 mv. The sum of −140 mv is just the level observed in the absence of extracellular calcium. With 1 mM calcium present, however, the

same argument would give little more than −100 mv, which is far from the measured internal potential of −180 mv.

The effect of calcium on the internal potential of *Neurospora* is quite unlike the effects of potassium, sodium, or hydrogen, and suggests that calcium alters the relative permeability of the surface for specific ions. Such results are known in other preparations (22, 42), but any calcium-favored ion supporting the internal potential of *Neurospora* remains to be identified. Whether calcium actually binds to the plasma membrane of *Neurospora* is unestablished. It is bound to the surface of yeast at sites which affect external enzyme activity (27), but these sites may be in the membrane, the cell wall, or both. To whatever extent calcium binds to the wall of *Neurospora*, it may also affect the electrical transients.

If the cell wall of *Neurospora* were strongly cation-selective—with fixed negative charges in its lattice—and were sufficiently tight, the measured internal potentials might be explained as sums of diffusion potentials arising across the wall and across the plasma membrane. In this case both the magnitude and the cation dependence of the internal potential would be complex functions of the extracellular cation concentrations. Evidence has already been presented to indicate that the wall is not a substantial barrier to the movement of small ions. It is probably also of low fixed charge density. The walls of bacteria, yeast, giant algae, and higher plant cells contain polyuronic acids and/or proteins (4, 7, 29), which bear fixed negative charges at densities approaching 1 equivalent/liter. But the wall of *Neurospora* comprises mainly polyglucose, chitin (poly *N*-acetylglucosamine), and polygalactosamine (14) with only traces of proteins or amino acids (29, 40). None of the polysaccharides would be expected to carry fixed negative charges, but at acid pH values chitin and polygalactosamine may be positively charged. Thus, the wall of *Neurospora* would be expected to be a weak anion exchanger but not a cation exchanger. It is known in fact to bind polyphosphate (14) and probably also binds sulfate (33). If Donnan potentials across the cell wall of *Neurospora* contribute at all to the total transsurface potential difference, their sum should be anionically directed, or should be of opposite sign to the total potential difference.

Without being a cation exchanger the wall might still create, immediately outside the cell membrane, an unstirred region whose ionic composition could be quite different from that of the external medium. Similar situations have been invoked to explain nerve and muscle after-potentials (9, 10) and post-anoxic hyperpolarization of myelinated nerves (30). For hyphae bathed in 10 mM KCl/1 mM $CaCl_2$/suc., with internal potentials near −180 mv, the Nernst equation would require potassium in the unstirred region to be about 0.2 mM, or one-fiftieth of $[K]_o$. With 1 mM potassium outside, the ratio would fall to about 1/11. The potassium transport system in the cell membrane might

help maintain such gradients, since it can move potassium into *Neurospora* at rates (15 pmoles/cm²/sec., reference 38) comparable with the maximal rates found in nerve, muscle, and other tissues. The apparent Michaelis constant for the potassium transport system of *Neurospora* is 1 mM. If the potassium concentration in the unstirred region from which potassium is extracted were actually less than 0.1 mM when $[K]_o$ equals 1 mM, then the real Michaelis constant must be less than 0.1 mM. This is ten-fold smaller than the Michaelis constant for potassium transport in wall-less cells such as erythrocytes (11). In addition, calculations based on the preliminary measurements of wall resistance indicate that the maximal rate of transport is not sufficient to reduce the potassium concentration in the unstirred region more than 1 per cent below $[K]_o$, at the steady state.

Of course, identification of the internal potential of *Neurospora* as a simple or complex ion diffusion potential depends upon the existence of passive diffusion channels through the cell surface. These certainly exist, but may be of very high resistance indeed. Both *Neurospora* and yeast (25) leak ions only very slowly into distilled water. Yeast cells deprived of oxygen and glucose also lose potassium at less than 1 mM/kg cell water/hour into triethylamine buffer solutions (24, 26); the rate of loss seems to be related as much to a carrier-mediated mechanism in the cell surface as to the requirement for electroneutrality. Interpretation of those experiments may be complicated, however, by changes of surface resistance and internal potential, such as are found in *Neurospora* (32). A suggestion to this effect has already been made by Rothstein and his associates (24, 26).

Clearly, interpretation of the large internal potentials of *Neurospora* can be clarified only by more information in at least three areas: (*a*) the relevance of extracellular and intracellular pH; (*b*) the behavior of wall-less protoplasts, along with careful measurements of transverse resistance and fixed charge density of the cell wall; and (*c*) the relationship between energy metabolism, ion transport, and the internal potential.

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