Net Uptake of Potassium in *Neurospora*

Exchange for sodium and hydrogen ions

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ABSTRACT Net uptake of potassium by low K, high Na cells of *Neurospora* at pH 5.8 is accompanied by net extrusion of sodium and hydrogen ions. The amount of potassium taken up by the cells is matched by the sum of sodium and hydrogen ions lost, under a variety of conditions: prolonged preincubation, partial respiratory inhibition (DNP), and lowered $[K]_o$. All three fluxes are exponential with time and obey Michaelis kinetics as functions of $[K]_o$. The V_{max} for net potassium uptake, 22.7 mmoles/kg cell water/min, is very close to that for K/K exchange reported previously (20 mmoles/kg cell water/min). However, the apparent K_m for net potassium uptake, 11.8 mM $[K]_o$, is an order of magnitude larger than the value (1 mM) for K/K exchange. It is suggested that a single transport system handles both net K uptake and K/K exchange, but that the affinity of the external site for potassium is influenced by the species of ion being extruded.

INTRODUCTION

We have selected the fungus *Neurospora crassa* for a comprehensive study of ion transport, because genetic techniques are available to isolate mutants with defective transport systems (39), and because such mutants can be compared with the normal wild-type strain by means of electrophysiological (36) as well as standard flux measurements. Background studies on the wild-type have revealed that (*a*) during logarithmic growth, the intracellular potassium concentration of *Neurospora* (180 mmoles/kg cell water) greatly exceeds that in the growth medium (down to 0.3 mM; reference 37); (*b*) under steady-state conditions, the cells carry out an exchange of internal potassium for external potassium at a maximal rate of 20 mmoles/kg cell water/min, or about 13 pmoles/cm²/sec. The rate of K/K exchange is a saturable function of extracellular potassium, with an apparent Michaelis constant of 1 mM (38); and (*c*) this steady-state exchange is under genetic

control. Mutant strain R2449, isolated by virtue of its abnormally high potassium requirement for growth, was found to have an elevated K_m for K/K exchange (39).

The significance of the steady-state potassium exchange is not yet clear. It appears to be carrier-mediated, as evidenced by saturation at external potassium concentrations around 10 mM. Furthermore, influx and efflux are tightly coupled, since both are inhibited in parallel at low external potassium concentrations or in the presence of metabolic inhibitors (sodium azide, dinitrophenol; reference 38). These results stand in sharp contrast to the classical picture of an inwardly directed "pump" for potassium balanced by an outwardly directed "leak." It seems unlikely that an energy-requiring carrier system would have potassium turnover as its principal function, and the present experiments were undertaken to explore the possibility that the same system might also be responsible for net uptake of potassium.

METHODS

Preparation of Low K Cells Wild-type strain RL21a of *Neurospora crassa* was used throughout this work; the general methods of handling the cells have been described previously (37). Log-phase cells were grown in liquid medium at 25°C, from an inoculum of 10^6 conidia/ml. The cultures were aerated either by constant shaking or by a steady stream of air bubbles. Previous work had shown that such cells, grown in the standard minimal medium (42), maintain a constant, high intracellular potassium concentration of 180 ± 3 mmoles/kg cell water (mean ± SE), but when the initial potassium content of the medium is reduced below 0.3 mM, the cells lose potassium (and gain sodium) slowly (37). By adjusting the medium to 0.2 mM potassium, it is possible to prepare reasonable quantities of midlog phase cells (16 hr) which are partially depleted of potassium (56 ± 1 mmoles/kg cell water) and loaded with sodium (107 ± 4 mmoles/kg cell water; see Fig. 1). These cells are still capable of growing at an appreciable rate.

Flux Experiments For measurements of ion fluxes, cells were harvested, rinsed several times in distilled water (37), and resuspended in a K-free buffer solution (see below). The suspension was split into two parts (about 150 ml/500 ml flask) for simultaneous duplicate experiments. Cell density was about 2 mg dry weight/ml of solution, and the suspension was kept aerated by continuous shaking. All experiments were run at 25°C ± 0.2°C. In most cases the cells were preincubated in the buffer for 20 min before potassium was added (the effect of the length of this incubation upon the magnitude of the measured fluxes is discussed later; see Fig. 6). At the end of the 20 min preincubation, intracellular potassium had fallen to a steady value of 37 ± 2 mmoles/kg cell water, while sodium had risen to 152 ± 5 mmoles/kg cell water (see Table I). The cells gain more sodium than they lose potassium during preincubation, but other ion movements that may occur during this period have not been investigated.

At zero time, potassium was added to the suspension in a small volume of 1 N solution. 10 ml samples were removed at intervals (1, 2, 4, 6, 8, 10, 15, 20, 30, and

40 min) and harvested on Millipore filters; fluxes were stopped by three quick rinses with 10 ml distilled water. The cell mats were dried overnight at 90°C, weighed, and extracted into 1 N HCl at 100°C for 1 hr. Flame analyses for sodium and potassium were carried out on the acid extracts, diluted 1:10 with distilled water.

Intracellular cation concentrations were calculated from the total amount of sodium or potassium in each cell pellet, the dry weight of that pellet, and the pre-

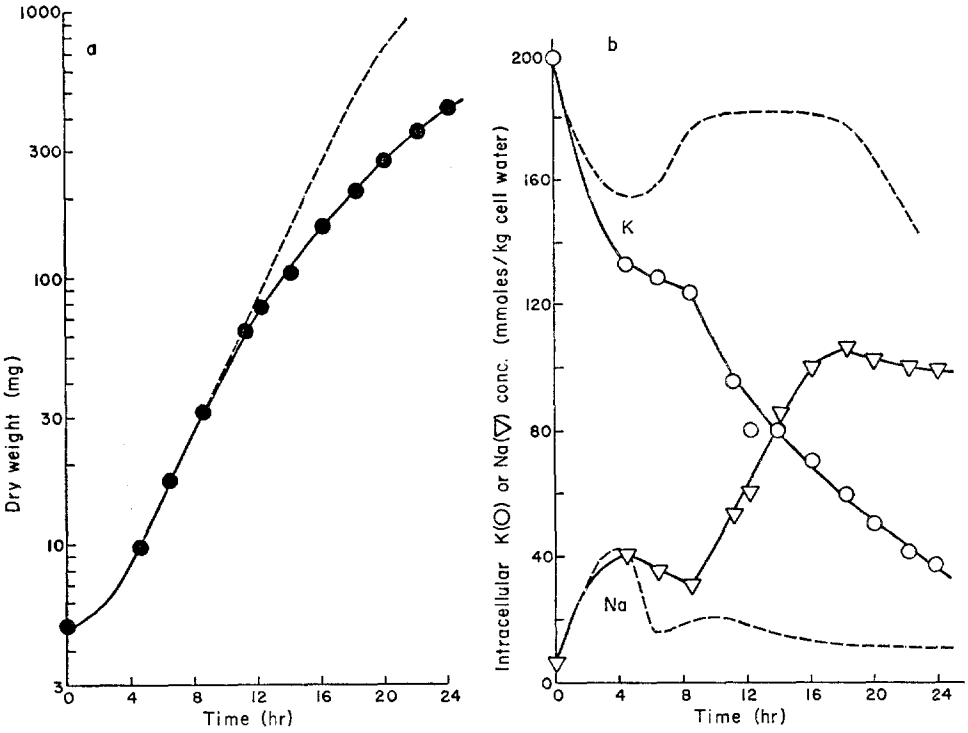

FIGURE 1. Growth of *Neurospora* on 0.2 mM potassium; 25°C. (*a*) Dry weight, on a logarithmic scale, plotted against time after inoculation. The dashed curve shows growth of the cells in normal K, 37 mM (37). (*b*) Intracellular K and Na concentrations. Again, the dashed curves represent cells grown in normal K. With $[K]_o$ = 0.2 mM, growth was normal for 8 hr, but during this period $[K]_o$ declined below 0.09 mM and $[K]_i$ fell to approximately 120 mmoles/kg cell water. After 8 hr, growth slowed out of log phase, $[K]_i$ fell, and $[Na]_i$ rose in an approximately compensatory fashion.

viously determined value (2.54) for the ratio intracellular water/dry weight (37). The factor 2.54 is based on inulin estimates of the extracellular space and may be 20% too large, if inulin does not penetrate the cell wall (41). Intracellular cation concentrations and ion fluxes, then, may be 20% too small, but this is a constant error in all calculations.

Net fluxes were calculated from semilog plots of intracellular concentration vs. time. In each case the least-squares line was fitted to the data for times 1 min–15 min and the computed slope and intercept of this line were used to calculate the flux at zero time. Data at 20 min and beyond were not used in the calculations because of

scatter, and the data at zero time were not included because of a rapid, though small (ca. 6 mM) shift of sodium and potassium within the cell wall.[1] This shift made it impossible to calculate initial net fluxes simply from concentration differences. In order to convert the fluxes into rates per unit membrane area, the cells were assumed to be uniform, long cylinders 2 μ in diameter. The resultant conversion factor is 2.5 $\times$ 10^7 cm^2/kg cell water, so that 1 mmole/kg cell water/min = 0.66 pmole/cm^2/sec. The calculation neglects slight convolutions of the plasma membrane, which would make the actual membrane area about 20% larger (35). Values throughout the paper are stated as mean ± 1 SEM.

Buffers All experiments were carried out at pH 5.8, which is the pH of the growth medium. The standard buffer solution contained 20 mM 3,3-dimethylglutaric acid (DMG; pK's of 3.66 and 6.20) brought to pH 5.8 with NaOH (final concentration, 25 mM), and 1% glucose. DMG proved to be metabolically neutral, neither supporting growth of *Neurospora* in the absence of an energy source, nor inhibiting

TABLE I

CATION CONCENTRATIONS IN NORMAL AND POTASSIUM-LIMITED *Neurospora*

	Average intracellular ion concentrations		No. of experiments
	K	Na	
	(*mmoles/kg cell water*)		
Normal cells, freshly harvested (16 hr)	180±3	14±1	44
Low K cells, freshly harvested (16 hr)	56±1	107±4	10
Low K cells, preincubated 20 min in K-free buffer	37±2	152±5	13
Low K cells, preincubated then incubated 40 min in 30 mM K	181±5	28±3	9

growth in the presence of sucrose or glucose. A search was made for an organic base to replace the NaOH, but all substances tested—including imidazole, histidine, Tris, choline, triethylamine, ethanolamine, and ammonium hydroxide—produced a rapid loss (10–50 mmoles/kg cell water/min) of sodium and potassium from the cells.

H^+ Measurements In order to determine the amount of hydrogen ion released from *Neurospora* during the net uptake of potassium, a pH electrode-reference electrode (Ag-AgCl) combination unit (A. H. Thomas, Philadelphia, Pa., No. 4858-L15) was mounted in a sidearm of each incubation flask. Loss of KCl from the reference electrode through the porous plug was less than 100 μmoles/hr and did not interfere with the potassium flux measurements. The pH of the cell suspension was monitored continuously, with a precision of 0.002 pH unit. The rate of H release was then calculated from the measured change of pH over a given time interval, and a standard titration curve for the DMG buffer. During any single run, the total fall of pH was 0.05–0.3 unit. Control experiments showed that weak-acid anions released by the

[1] Slayman, C. W., and C. L. Slayman. Net uptake of potassium in *Neurospora*: pH dependence of K-Na coupling. Data to be published.

cells have an average pH between 5.5 and 6.5; but the amount released is small enough to produce no more than 5% error in the buffer capacity of the DMG solution.

RESULTS

Net K and Na Transport When 30 mM potassium is added to a suspension of the low K cells in standard Na-DMG buffer, there is a rapid net influx of potassium which restores the intracellular concentration to the normal level,

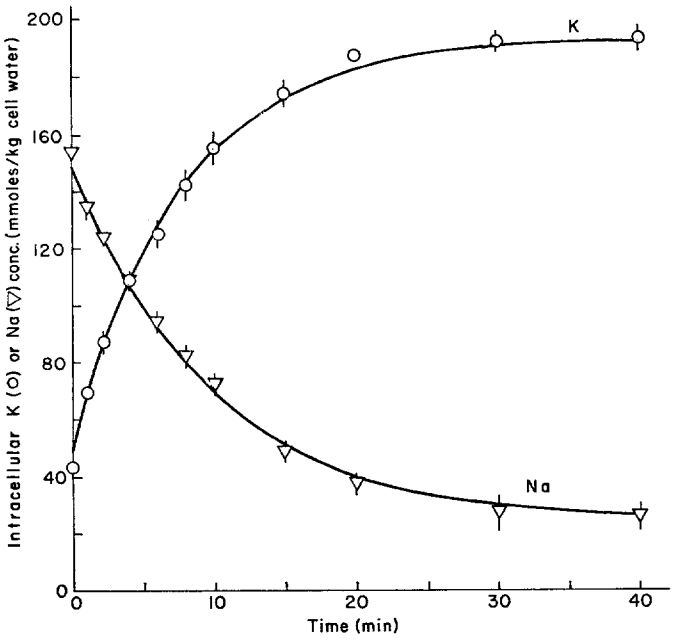

FIGURE 2. Net cation movements in low K cells. Cells were grown in 0.2 mM K for 16 hr, and preincubated in K-free buffer for 20 min at 25°C. 30 mM KCl was introduced at 0 time. Net uptake of potassium: 144 ± 4 mmoles/kg cell water; net loss of sodium: 126 ± 5 mmoles/kg cell water. All points are averages for five experiments. Vertical bars indicate ±1 SE. The curves are redrawn from the least squares lines in Fig. 3. For potassium, $[K]_t = 49 + 144\,(1 - e^{-t/7.5})$; for sodium, $[Na] = 23 + 126e^{-t/9.9}$; 49 + 144 = 193 mM K, and 23 mM Na are the end points for K uptake and Na release estimated directly from the above data plots.

181 ± 5 mmoles/kg cell water (see Table I), in 20–40 min (Fig. 2). The initial rate of potassium uptake (calculated from semilog plots; Fig. 3) is 19.1 ± 0.7 mmoles/kg cell water/min. A concomitant net loss of sodium takes place with an initial rate of 12.7 ± 0.7 mmoles/kg cell water/min. Potassium uptake and sodium release both appear to be simple exponential functions of time (Fig. 3), having time constants of 7.5 min and 9.9 min, respectively, in these experiments. [During the first minute of potassium uptake, the net fluxes apparent in Fig. 2 are about 6 mmoles/kg cell water/min larger than

the values quoted above and calculated from the semilog plots of Fig. 3. As has already been mentioned, these small and brief shifts in sodium and potassium probably represent cation binding in the cell wall.[1] Throughout recovery, potassium taken up exceeds sodium lost, and after 40 min the net discrepancy is about 25 mmoles/kg cell water.

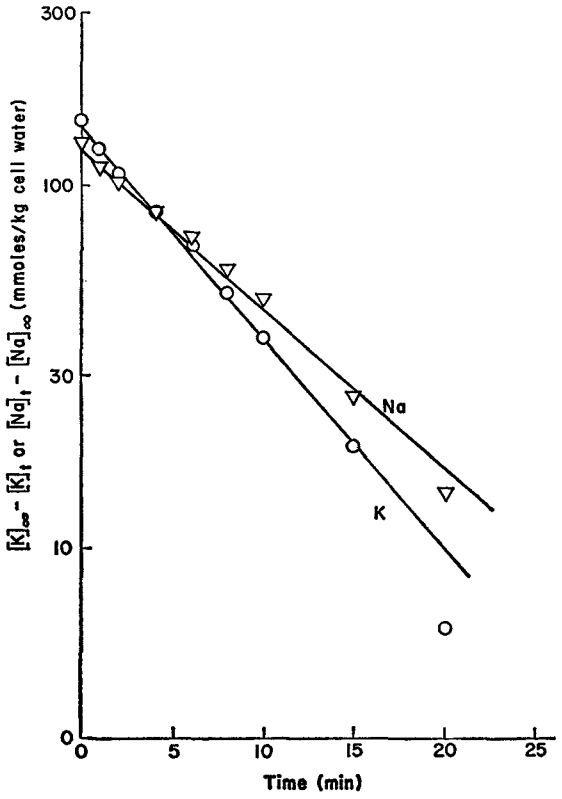

FIGURE 3. Semilog plots of intracellular cation concentrations; the same data as in Fig. 2. Computed intercepts and time constants for the least squares line: potassium, 144 ± 4 mM and 7.5 ± 0.2 min; sodium, 126 ± 5 mM and 9.9 ± 0.4 min (see Table IV).

Uptake of Anions Since the movement of sodium does not balance that of potassium, electroneutrality requires the simultaneous uptake of an anion, the simultaneous release of another cation, or some combination of the two processes. The only anions present in the standard medium are chloride and DMG. But, as is shown in Table II, the nature of these anions does not strongly influence either sodium or potassium fluxes. Chloride can be replaced by sulfate, or phosphate and DMG by phosphate; and the discrepancy between potassium and sodium fluxes remains. This lack of specificity suggests that anions are probably not involved in the charge balance.

More conclusive evidence has been obtained from direct measurements of anion fluxes. In one set of experiments $^{36}Cl^-$, $^{35}SO_4^{--}$, or ^{32}P-phosphate (pH 5.8) was added, as the potassium salt, to the DMG-buffered cell suspension to give a final potassium concentration of 30 mM. In a separate experiment the cells were preincubated in phosphate buffer (pH 5.8) and 0.5 mM

TABLE II

CATION FLUXES IN THE PRESENCE OF SEVERAL DIFFERENT ANION SPECIES

Anions in the medium	Cation fluxes	
	K	Na
	(mmoles/kg cell water/min)	
20 mM DMG 30 mM Cl	19.1±0.7	−12.7±0.7
20 mM DMG 15 mM SO_4	20.2±1.3	−12.5±0.2
20 mM PO_4 33 mM Cl	20.8±1.2	−12.4±1.7

In addition to the anions listed, all media contained 25 mM Na, 30 mM K, and 1% glucose; pH 5.8. All results are averages for at least three experiments.

TABLE III

ANION INFLUXES IN LOW K CELLS

Tracer anion	Buffer	K or Na added as	Anion influx, initial	
			K salt	Na salt
			(mmoles/kg cell water/min)	
^{36}Cl	20 mM DMG	Cl (30 mM)	2.3, 1.8 → 2.0	2.0, 2.2 → 2.1
$^{35}SO_4$	20 mM DMG	SO_4 (15 mM)	0.21, 0.20 → 0.20	0.21, 0.20 → 0.20
$^{32}PO_4$	20 mM DMG	PO_4 (27.4 mM)	0.6, 1.6 → 1.1	0.9, 1.0 → 1.0
$^{32}PO_4$	20 mM PO_4	Cl (30 mM)		

All preincubation media contained 25 mM Na, 1% glucose; pH 5.8. Tracer was added along with 30 mM K or Na, and uptake of label was followed for 40 min.

$NaH_2{}^{32}PO_4$ was added along with the usual 30 mM KCl. In all cases both the uptake of labeled anions and the changes in cellular K and Na contents were followed as functions of time. As is shown in Table III, column 4, the initial influx of anions was never greater than 2.3 mmoles/kg cell water/min. Parallel control experiments—in which the labeled anions were added to the cell suspension as sodium salts rather than potassium salts—demonstrated that the anion influx was not dependent on rapid cation uptake (Table III,

column 5). Sodium uptake in the control experiments did not exceed 3.2 mmoles/kg cell water/min, or 17% of the normal net potassium influx.

Since small amounts of anions (1–5 mmoles/kg cell water; reference 14 and footnote 2) appear to be bound to the *Neurospora* cell wall, the initial

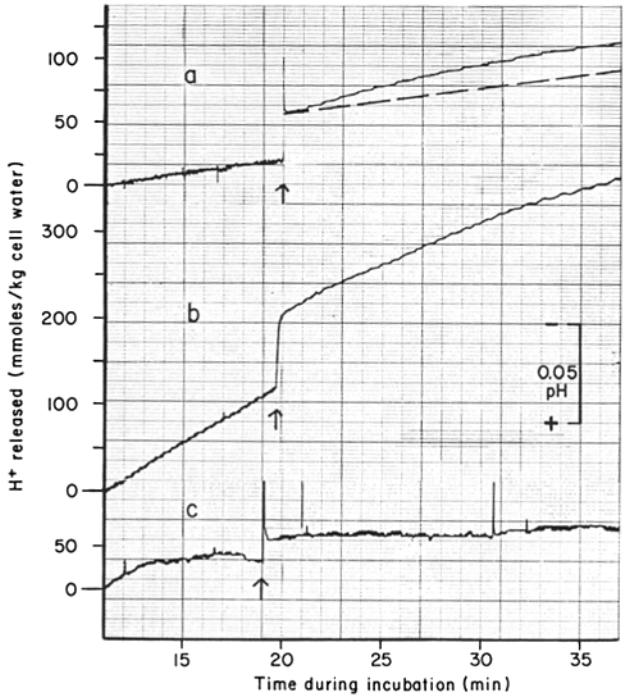

FIGURE 4. Demonstration of the extra hydrogen ion release during net uptake of potassium. All cells were preincubated in DMG buffer for 20–21 min before 30 mM KCl was added, indicated by arrows. Addition of the unbuffered KCl solution produced a variable, instantaneous, shift of pH which was followed (curve *a* only), by a prolonged acceleration of H^+ release. (*a*) Low K cells in Na-DMG buffer; (*b*) high K cells in K-DMG buffer; (*c*) low K cells, Na-DMG buffer, 10^{-4} M 2,4-DNP added at 12 min. The scale for measured pH changes is indicated to the right of curve *b*. The pH change was converted to H^+ released (ordinate scales) by calculating from the measured cell densities and the buffer capacity of the medium. Nonlinearity in the conversion was small (1.5%/0.05 pH) and was neglected.

rates listed in Table III, and estimated from the total uptake of isotope by the cells, are certainly larger than the true rates of entry into the cytoplasm. It is clear from these results that the excess potassium uptake cannot be accounted for by a simultaneous uptake of anions, and therefore that it must be accompanied by the release of cations.

[2] Lowendorf, H., and C. W. Slayman. Unpublished experiments.

Release of Hydrogen Ions Because of the results of Conway and O'Malley (7) and Rothstein and Enns (31) on yeast, Schultz, Epstein, and Solomon (33) on *Escherichia coli*, and Zarlengo and Schultz (44) on *Streptococcus fecalis*, it seemed reasonable to look for hydrogen ion secretion associated with potassium uptake in *Neurospora* as well. Our method was to monitor continuously the pH of the buffered cell suspension with a glass electrode mounted in the shaking flask (see p. 427).

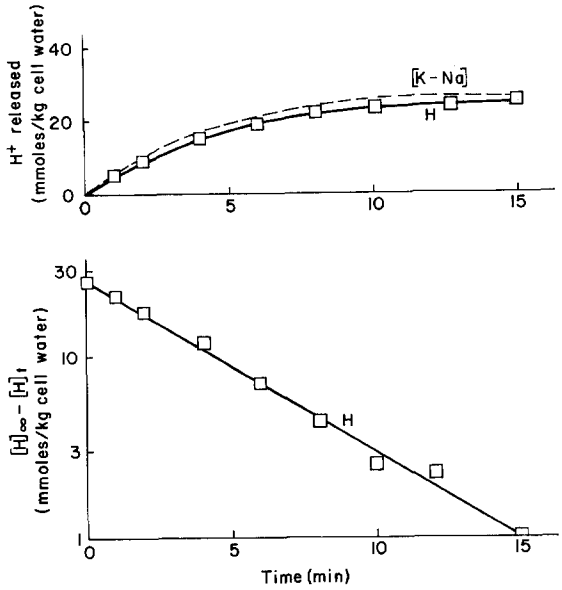

FIGURE 5. K-induced efflux of hydrogen ions from low K *Neurospora*. Lower graph semilog plot of data, with least squares line (solid); computed intercept 26 ± 1 mM time constant 4.6 ± 0.1 min. Upper graph, linear plot of data, with solid curve described by the equation, H^+ released $= 26\ (1 - e^{-t/4.6})$. All points are averages for five experiments, and in all cases ±1 SE falls within the open squares. Conditions as in Fig. 2. The dashed line shows the difference between potassium taken up and sodium lost, calculated from the curves of Fig. 2.

Fig. 4 *a* shows the actual record from such an experiment, in which low K cells were preincubated for 20 min in DMG buffer, and then exposed to 30 mM KCl. Base line hydrogen ion production by *Neurospora*—visible during the preincubation period—turns out to be a complicated phenomenon, dependent on metabolic energy and also on the pH of the incubation medium.[3] At pH 8 it is about 40 mmoles/kg cell water/min; but at pH 5.8, where potassium and sodium movements have been measured, the apparent base line H^+ release averages 5 mmoles/kg cell water/min (though quite variable, cf. Fig. 4 *a* and 4 *b*).

[3] Slayman, C. L. Unpublished experiments.

Superimposed on the base line in Fig. 4 *a* is an additional burst of hydrogen ions associated with the net uptake of potassium. The burst lasted about 15 min in this experiment and the extra H^+ produced amounted to about 25 mmoles/kg cell water.

Fig. 4 *b* and 4 *c* are controls for this experiment. In *b*, the cells were grown in medium with 37 mM potassium and therefore contained the normal, high, internal potassium concentration (180 mmoles/kg cell water). In *c*, low K cells were used, but 0.1 mM 2 ,4-dinitrophenol was added to the cell suspension 7 min before the KCl. In neither of these cases was there an accelerated H^+ release or a net uptake of potassium following addition of KCl.

Balancing Charges Like potassium uptake and sodium loss, the extra hydrogen ion release appears to be a simple exponential function of time (Fig. 5, lower). It also accounts both in magnitude (Fig. 5, upper) and in

TABLE IV
NET CATION FLUXES

	Initial rates		Time constant	Change of internal concentration in 40 min
	mmoles/kg cell water/min	*pmoles/cm²/sec*	*min*	*mmoles/kg cell water*
K (influx)	19.1±0.7	12.6±0.5	7.5±0.2	144±4
Na (efflux)	−12.7±0.7	−8.4±0.5	9.9±0.4	−126±5
H (efflux)	−5.7±0.3	−3.8±0.2	4.6±0.1	−26±1

These results are summarized from the data used in Figs. 2, 3, and 5. Because of intracellular buffering, the net loss of hydrogen ions indicated in column 4 is reflected in only a small rise, about 0.3 pH unit, of the intracellular pH (see Table VI).

rate for the discrepancy between sodium and potassium movements. In a strict sense not all the three fluxes can be exponential with time, if the sum of Na and H fluxes is equal to K flux. However, even the "ideal" K flux curve, constructed from the sum of two curves with time constants of 9.9 and 4.6 min, cannot be resolved into its components over the interval—0–15 min—in which the data are most accurate. Table IV summarizes the magnitudes, time constants, and initial net fluxes for all three ions.

Under a variety of other conditions (prolonged preincubation, partial inhibition of respiration, and variation of the extracellular potassium concentration) the difference between potassium influx and sodium efflux is made up by hydrogen ion efflux. These results, which are discussed below, are taken as further evidence for a potassium–hydrogen ion exchange process.

Effect of Preincubation on Cation Net Fluxes The initial rates of potassium uptake and sodium release (Fig. 6, upper) diminish roughly exponentially with increasing preincubation time, falling to about 50% of their 0 preincubation values in 3 hr. The difference between the K and Na rates appears

to decline linearly, however, as does the K-induced hydrogen ion efflux. For the particular experiments summarized in Fig. 6, the measured H^+ efflux was slightly larger (1 mmole/kg cell water/min) than the estimated difference between potassium and sodium fluxes; in other experiments, a slight difference in the opposite direction was seen.

Energy Dependence It has already been demonstrated (38) that the steady-state flux of potassium in *Neurospora* is dependent on respiratory metabolism. In order to verify that net potassium and sodium fluxes and the extra hydrogen ion efflux also require metabolic energy, we have examined

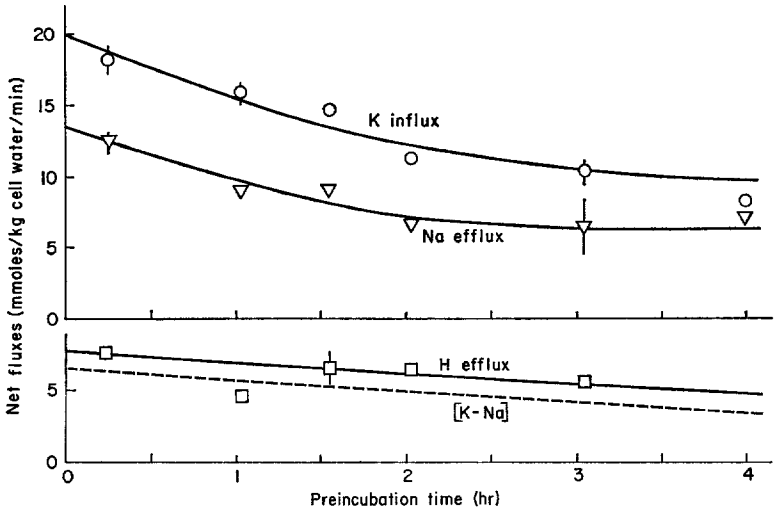

FIGURE 6. Influence of preincubation time on cation fluxes. Standard low K cells, preincubated in K-free DMG buffer for periods of 15 min to 4 hr. Fluxes were calculated from semilog plots of the data; the points represent average results for five experiments. Vertical bars, ±1 SE.

the effects of 2 ,4-dinitrophenol. *Neurospora* is a totally aerobic organism (8) whose oxygen consumption is stimulated by DNP over the range 10^{-7} to 10^{-5} M (presumably due to the uncoupling of respiration from oxidative phosphorylation), and then inhibited over the range 10^{-5} to 10^{-3} M (pH 5.8; reference 35). Fig. 7 shows that all three net fluxes—K influx, Na efflux, and the K-induced H ion efflux—are sensitive to DNP. The three curves are similar in shape, with 50% inhibition occurring at the same DNP concentration (5.2×10^{-5} M) in all of them. There is again good agreement, as shown in the lower part of Fig. 7, between the K-induced H^+ release and the difference between K and Na net fluxes.

Dependence upon the Extracellular Potassium Concentration Given that *Neurospora* can carry out a net uptake of potassium balanced by the net release of

sodium plus hydrogen ions, and that these are energy-dependent processes, the question arises as to whether there are two separate transport systems, one for K/H and the other for K/Na exchange, or whether there is a single system which exchanges either sodium or hydrogen ions for potassium, depending on the relative affinities for the two cations and the intracellular concentrations of each. The fact that all three processes have the same energy dependence (as determined in the DNP experiment) is certainly consistent with the single

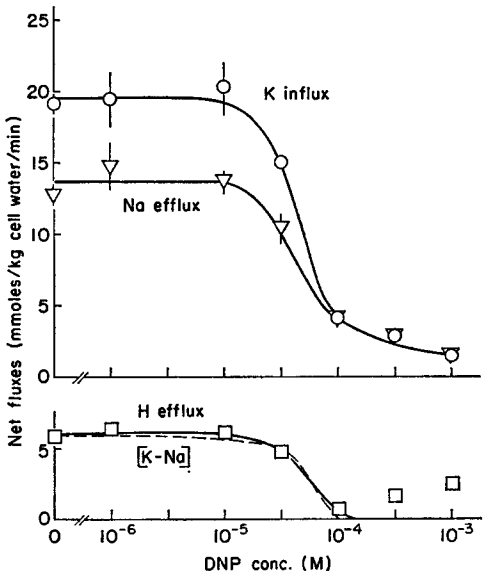

FIGURE 7. The effect of DNP on cation fluxes in *Neurospora*. Standard low K cells preincubated for 20 min in K-free buffer before addition of 30 mM KCl. 2,4-DNP was introduced 5 min ahead of the KCl. [The apparent increase of H^+ efflux produced by KCl at higher concentrations ($\geq 3 \times 10^{-4}$ M) of DNP probably is not real. At the higher DNP concentrations the H^+ base line is negative, and 30 mM KCl tends to retard this alkalinization of the medium. Whether, under these conditions, the KCl reduces H^+ uptake by the cells or suppresses the net release of base is not known.] Vertical bars, ± 1 SE.

pump hypothesis. But two pumps having similar requirements for high energy substrate could give the same result if that substrate were rate-limiting.

A further effort was made to separate the K/Na and K/H fluxes on the basis of their dependence on the extracellular potassium concentration. The results, plotted in Fig. 8, show that over the range of potassium concentrations used (5–50 mM) the difference between K and Na movements again was equal to the K-induced H^+ release. As expected for carrier-mediated processes, the fluxes all saturate at high external potassium concentrations. When a double-reciprocal plot of the same results is made (Fig. 9), it becomes evident that all three fluxes reach half-maximal saturation at essentially the same

extracellular potassium concentration, 11.7 mM (see Table V). It seems unlikely that two independent systems would have the same dependence on *both* extracellular potassium *and* high-energy substrate, and therefore more reasonable to assume that the K/Na and K/H exchanges are carried out by a single system.

The maximal velocities obtained from the intercepts of Fig. 9 are slightly larger than the values found for an external potassium concentration of 30 mM (see Fig. 3 and Table IV). The values of V_{max} and K_m listed in Table V

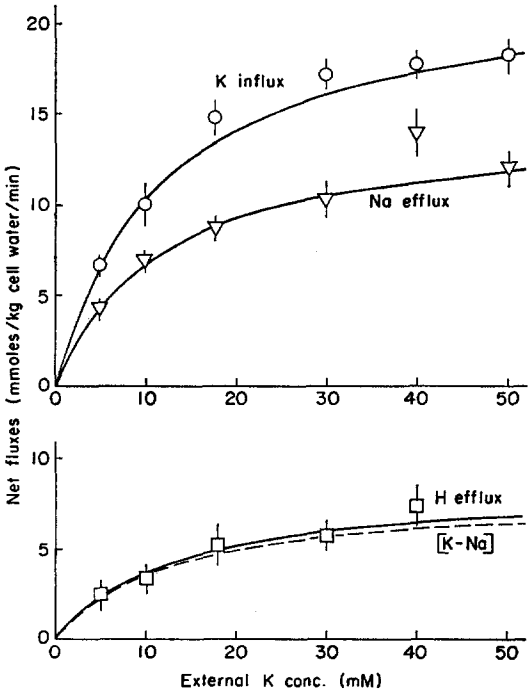

FIGURE 8. Dependence of cation fluxes on extracellular potassium. Standard low K cells preincubated 20 min; KCl added to final concentrations of 5, 10, 20, 30, 40, or 50 mM. Fluxes were calculated from the semilog plots of the data. The points represent average results for at least three experiments. Vertical bars, ±1 SE. The curves are redrawn from the least squares lines of Fig. 9, and are given by the general equation:

$$\text{Flux} = \frac{V_{max}[K]_o}{K_m + [K]_o},$$

with the values of V_{max} and K_m listed in Table V.

were computed from Fig. 9 by the method of least squares and are subject to possible weighting errors. A check of reliability was therefore made by calculation of V_{max} and K_m using two other linear transformations of the data (v vs. $v/[K]_o$; $[K]_o/v$ vs. $[K]_o$; see reference 10); and none of the recomputed values differs significantly from those listed in Table V.

Intracellular pH The fact that K/H exchange appears to be a carrier-mediated, energy-requiring process indicates that the hydrogen ions are released from within the cells (rather than, e.g., from anionic sites in the cell wall) and suggests that the intracellular pH should rise. Conway and Downey, indeed, were able to identify an increase of 0.6 unit in the intracellular pH of yeast during K/H exchange (5).

We have obtained preliminary estimates of the internal pH in *Neurospora* using the distribution of DNP as an indicator, according to the method of Kotyk (21) and Neal et al. (23). DNP equilibrates rapidly—in less than 1 min—and it was assumed that only the undissociated form of the molecule crosses the cell membrane (21). A modified form of the Henderson-Hasselbach equation (43) was used to calculate the intracellular pH. A low concentration of DNP, 10^{-5} M, was chosen at which inhibition of ion fluxes (see Fig. 7)

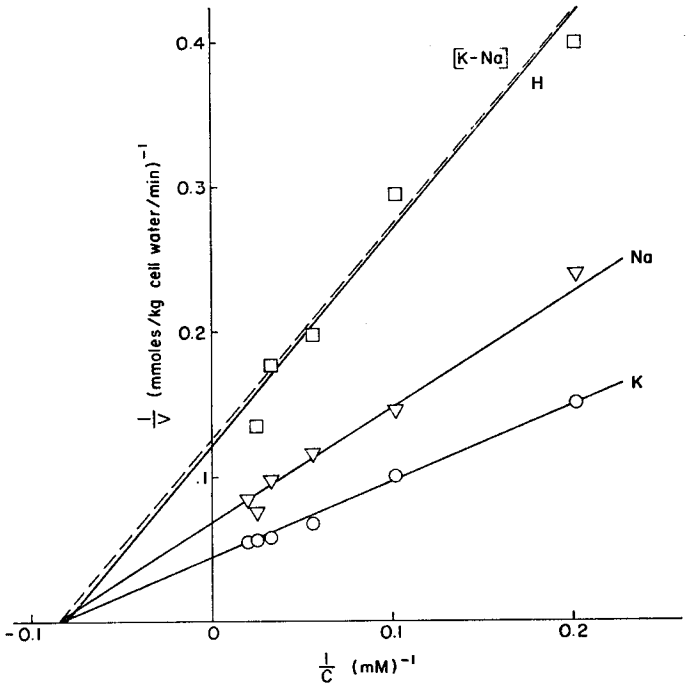

FIGURE 9. Dependence of cation fluxes on extracellular potassium. A double-reciprocal plot of the results shown in Fig. 8. The solid lines were drawn by the method of least squares; the reciprocal intercepts (V_{max}, K_m) are listed in Table V.

TABLE V

MAXIMAL VELOCITIES AND APPARENT MICHAELIS CONSTANTS FOR NET CATION FLUXES IN *Neurospora*

	Maximal velocity (V_{max})		Michaelis constant for extracellular potassium (K_m)
	mmoles/kg cell water/min	*pmoles/cm²/sec*	*mM*
K (influx)	22.7±0.5	15.0±0.3	11.8±1.1
Na (efflux)	−14.7±0.4	−9.7±0.3	11.6±0.4
H (efflux)	−8.5±1.1	−5.6±0.7	12.3±1.6

The values listed in this table were obtained from the intercepts in Fig. 9.

or of membrane potential (35) is no greater than 5%. The amount of DNP within the cells was estimated from the optical density (370 mμ; 5 cm cuvette) of sodium carbonate extracts. Cells were prepared for extraction by filtering, blotting, and air-drying to constant weight. The actual external concentration of DNP in each cell filtrate was also measured, since the cells take up a significant portion of the total DNP.

The results of six experiments are summarized in Table VI. The apparent intracellular pH rises about 0.3 unit over an interval of 3 min following addition of KCl to the low K cells. During this period the cells lose 50% of the total K-induced H^+ ions (see Fig. 5), or 12–13 mmoles/kg cell water. Thereafter, the intracellular pH falls slowly, requiring 15–20 min to stabilize at the control value.

TABLE VI

SUMMARY OF ESTIMATES OF INTRACELLULAR pH

	Control pH_i	Peak pH_i	Time of peak (min after KCl added)	Recovered pH_i
	6.44	6.69	2	6.28
	6.02	6.72	2	6.28
	6.41	6.57	3	6.38
	6.63	6.93	3	6.40
	6.75	6.87	3	6.51
	6.53	6.72	6	6.45
Average	6.46±0.10	6.75±0.05	3	6.38±0.04

10^{-5} M DNP was added to the cell suspension 5 min before the 30 mM KCl; this concentration of inhibitor reduces net K flux by less than 5%. The average pH values are arithmetic means of the numbers given in the above columns, but do not differ significantly from pH's calculated from average H^+ ion concentrations. The peak pH (6.75) is significantly different from the average control-recovered pH (6.42) with $p < 0.001$.

The absolute value of pH_i (ca. 6.4) measured in this experiment cannot be considered firm, since it is subject to an unknown error from possible intracellular binding of DNP. [The figure is a reasonable one in terms of the pH optima for enzymes (40) and the pH of cell extracts (25).] However, the change of pH—0.3 unit—accompanying the K-induced H^+ release is probably more reliable. If the normal pH_i is assumed to rest near the average pK for intracellular buffers, 0.3 pH unit/12–13 mM H^+ would require an intracellular buffer concentration of 75 mmoles/kg cell water. This figure may reflect the fact that *Neurospora* contains large amounts of phosphate. Total phosphorus has been estimated at 300 mmoles/kg cell water (15 and footnote 2), which is distributed as follows: 4% orthophosphate, 23% inorganic polyphosphate, 30% organic phosphate (small molecules), 43% nucleic acids (15).

Lack of Effect of Ouabain upon Cation Movements Because cardiac glycosides are known to be fairly specific inhibitors of the K/Na pump of the cells of higher organisms (12), it was of interest to test the effect of a representative glycoside, ouabain, on cation movements in *Neurospora*. Table VII shows that 10^{-3} M ouabain had no measurable effect, beyond the 20% depression of flux produced by 1% ethanol, in which the ouabain was dissolved. Ouabain has also been found to have little effect on K/Na exchange or membrane ATPase from other microorganisms and plant tissues (9, 13, 16, 18; except see 22, 28).

TABLE VII

CATION FLUXES IN THE PRESENCE OF 10^{-3} M OUABAIN

	Control	1% ethanol	Ethanol + ouabain
K (influx)	20.7±1.7	16.0±2.4	14.7±0.2
Na (efflux)	−13.5±0.4	−10.4±0.8	−12.5±1.3
H (efflux)	−5.7±0.5	−8.5±0.8	−7.3±0.5

All values given are initial net fluxes, in mmoles/kg cell water/min, and are averages for two separate experiments.

DISCUSSION

It is now clear that *Neurospora*—like yeast (1, 7), *Escherichia coli* (33), and *Streptococcus fecalis* (44)—is capable of rapid net potassium uptake, and in addition that there are several conspicuous differences between cation movements in microorganisms and those in the better known nerve, muscle, and red cell systems.

1. For one thing, microbial cell membranes have relatively low passive permeabilities to ions (11, 29), so that diffusion of potassium in these organisms amounts to only a small fraction of carrier-mediated transport. Rothstein (30) has pointed out that the low ion permeabilities seen in microorganisms may represent an adaptation to growth in dilute media. We have estimated the passive leak of potassium out of *Neurospora* hyphae in three ways: from the rate at which potassium is lost into buffer by azide-poisoned cells (38) or into distilled water by untreated cells (37), and from the unidirectional potassium flux extrapolated to the minimum extracellular concentration at which the cells remain in the steady state (0.05 mM; reference 38). Although all three methods are subject to criticism, they give values in good agreement: 0.3, 0.5, and 0.7 mmole/kg cell water/min, respectively, or less than 3% of the maximal potassium flux.

2. Essentially the entire potassium influx in microorganisms is thought to be carrier-mediated—since it saturates as a function of the extracellular K concentration and requires metabolic energy (1, 11, 33, 44). But unlike potassium transport in most higher organisms, the microbial uptake of potassium

is insensitive to ouabain—at least in *E. coli* (13) and *Neurospora*—and is not entirely coupled to the release of sodium. K/H exchange in addition to K/Na exchange has been identified in all microorganisms examined (7, 17, 31, 33, 44) as well as in mitochondria (3, 24) and the tissues of higher plants (20). On a quantitative basis, the process is less conspicuous in *Neurospora* than elsewhere, representing only 20% of the net potassium uptake, as compared with 50% or more in yeast (4), *E. coli* (33), and *S. fecalis* (44). The apparent K_m of 11.7 (extracellular potassium) in *Neurospora* compares with values of 4.5 mM in *E. coli* (33) and 0.5 mM in yeast (1). Hydrogen ions released by *Neurospora* seem to be preformed within the cells (as evidenced by the slight rise of internal pH), as has also been indicated in *S. fecalis* (44), yeast (4), and *E. coli* (33). Apparently these organisms cannot—even during maximal respiration—oxidize glucose completely to CO_2 and water, so that organic acids tend to accumulate within the cells and to leak into the medium. (One estimate of the fraction of glucose taken up by yeast which ultimately appears in the medium as weak acids gave 3% as succinate, 1% as acetic acid; reference 27.) Hydrogen ion extrusion in exchange for potassium becomes, then, an alternative to extrusion along with formate, acetate, lactate, succinate (4, 27, 32, 44) and perhaps other organic acid anions.

3. The existence of a K/H exchange process in addition to the K/Na exchange process is only one manifestation of the relative lack of specificity in ion transport by microorganisms. Both yeast (6) and *Neurospora* (37), when grown on low potassium, accumulate sodium against considerable concentration gradients (though perhaps not against electrochemical gradients), in a reaction which is blocked by respiratory inhibitors. In *Neurospora*, also, a variety of amino cations probably can exchange reversibly with either sodium or potassium (see Methods) under circumstances (normal resting potential) in which no generalized increase in membrane permeability would be expected.[3]

It is tempting to suppose, both for the sake of simplicity, and for the sake of assigning a useful function to the steady-state K/K exchange system, that all these fluxes of monovalent cations are mediated by a single carrier system. A certain amount of evidence pertinent to the three exchanges K/K, K/H, and K/Na can be extracted from the experiments presented above. The most important point is that the maximal velocity of potassium uptake is essentially the same (20 and 22.7 mmoles/kg cell water/min; see reference 38 and Table V above), whether it occurs in exchange for intracellular potassium or for intracellular sodium plus hydrogen ions. As far as the net fluxes are concerned, all the ions are equally affected by DNP and by $[K]_o$. The single-carrier hypothesis is especially attractive since a single-gene mutation affecting transport has produced a strain of *Neurospora* (39) in which K/K exchange and net K uptake are equally affected.[4] For the same

[4] Slayman, C. W. Unpublished experiments.

strain, the relative effectiveness of sodium, ammonium, or rubidium as competitive inhibitors of potassium uptake is altered[4], in comparison with the wild-type. A similar circumstance has also been identified for potassium vs. sodium efflux in the bacterial mutant *E. coli* B 525 (13).

[Many properties of microbial ion transport systems, especially the relative lack of specificity, might be accounted for by supposing that either net influxes or net effluxes were driven by an electrical gradient associated with the pump rather than by a chemical carrier. On the surface, this would seem particularly relevant in *Neurospora*, both since the resting membrane potential is highly sensitive to respiratory inhibitors (35) and since, electrically, the membrane does not discriminate strongly between alkali cations (34). But further experiments have shown this interpretation to be improbable: neither the internal concentrations of sodium and potassium nor the magnitudes of the net fluxes substantially influence the membrane potential of mature, agar-cultured hyphae.[3]]

Against the single carrier hypothesis rests the fact that the apparent potassium K_m for net transport is an order of magnitude larger than that for K/K exchange (11.7 mM instead of 1 mM, see reference 38). This could be accounted for only if the affinity of the entry site for potassium is influenced by the nature of the exciting cation. Such an effect could arise, for example, from a configuration change brought about by the exiting ion in reaction either at the entry site or at a different, allosteric, site. Numerous demonstrations of this kind of interaction are available, of which perhaps the best known is the inhibition of phosphofructokinase by ATP: at 0.5 mM and 2.3 mM ATP, respectively, the K_m values for fructose-6-phosphate are 0.2 mM and 1.2 mM (26). In a system more closely related to transport, the K-dependent acyl phosphatase prepared from (brain) microsomes has been shown to have a variable dependence upon potassium ions (19). The potassium concentration required for half-maximal activation of that enzyme was found to be 0.8 mM with carbamyl phosphate, 1.8 mM with acetyl phosphate, and 2.9 mM with *p*-nitrophenylphosphate. In the case of transport systems themselves, Armstrong and Rothstein (2) have postulated a "modifier" site to explain noncompetitive inhibition by alkali cations of potassium uptake in yeast.

Whether this kind of model will be adequate to account for the observations on *Neurospora* remains to be determined, and pertinent experiments are now in progress.

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