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^{39}K, ^{23}Na, and ^{31}P NMR studies of ion transport in *Saccharomyces cerevisiae*

(stoichiometry/cation exchange/proton pump/intracellular pH/dysprosium tripolyphosphate)

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ABSTRACT **The relationship between efflux and influx of K^+, Na^+, and intracellular pH (pH_{in}) in yeast cells upon energizing by oxygenation was studied by using the noninvasive technique of ^{39}K, ^{23}Na, and ^{31}P NMR spectroscopy. By introducing an anionic paramagnetic shift reagent, $Dy^{3+}(P_3O_{10}^{5-})_2$, into the medium, NMR signals of intra- and extracellular K^+ and Na^+ could be resolved, enabling us to study ion transport processes by NMR. Measurements showed that 40% of the intracellular K^+ and Na^+ in yeast cells contributed to the NMR intensities. By applying this correction factor, the intracellular ion concentrations were determined to be 130–170 mM K^+ and 2.5 mM Na^+ for fresh yeast cells. With the aid of a home-built solenoidal coil probe for ^{39}K and a double-tuned probe for ^{23}Na and ^{31}P, we could follow time courses of K^+ and Na^+ transport and of pH_{in} with a time resolution of 1 min. It was shown that H^+ extrusion is correlated with K^+ uptake and not with Na^+ uptake upon energizing yeast cells by oxygenation. When the cells were deenergized after the aerobic period, K^+ efflux, H^+ influx, and Na^+ influx were calculated to be 1.6, 1.5, and 0.15 μmol/min per ml of cell water, respectively. Therefore, under the present conditions, K^+ efflux is balanced by exchange for H^+ with an approximate stoichiometry of 1:1.**

^{31}P, ^{13}C, and ^{1}H NMR have been applied to the study of bioenergetics and of metabolism in intact cells, organelles, and perfused organs and to whole animals (1, 2). In particular, ^{31}P NMR has been used to measure intracellular pH (pH_{in}) together with levels of phosphate metabolites. Therefore, ^{31}P NMR gives information about transport of protons across the cell membrane (refs. 3 and 4; A. Ballarin-Denti, personal communication). Because transport of protons is intimately connected with the transport of cations, NMR studies of transport of ions such as K^+ and Na^+ would complement the ^{31}P NMR studies. Until recently, however, direct observation by NMR of physiologically relevant ion transport was not possible because separate resonances from intra- and extracellular ions could not be observed. Balschi *et al.* (5) and Gupta and Gupta (6) have introduced anionic paramagnetic shift reagents, which allowed separation of resonances from intra- and extracellular $^{23}Na^+$ (Na^+_{in} and Na^+_{out}) in human erythrocytes, frog skeletal muscle, and yeast. This approach has opened the possibility of applying ^{39}K and ^{23}Na NMR to the study of ion transport in intact cells.

When yeast cells oxidize ethanol in the presence of K^+, K^+ is taken up by the cells and H^+ is excreted into the medium (7). The kinetics of influx and efflux pathways for cations in yeast cells have been extensively studied, but relationships and mechanisms of cation transports have not yet been clearly established (ref. 7; ref. 8 and references cited therein). For example, Conway *et al.* (9) and Ryan and Ryan (10) suggested that separate carriers were involved in the influx of K^+ and in the efflux of Na^+. However, Rothstein (11) has proposed a single cation transport system with different cation specificities and kinetics on the inside and outside of the membrane. More recently, Rodrigues-Navarro and Sancho (12) have shown that Mg^{2+} in the medium inhibits cation exchanges in yeast and that, in the absence of Mg^{2+}, a two-transport-system hypothesis gives a better explanation of their observed data. Yeast cells possess a proton translocating ATPase in the plasma membrane; however, the precise role of K^+ in proton pumping is still the subject of controversy (ref. 13 and references cited therein; ref. 14).

In this work we studied by the noninvasive technique of NMR spectroscopy the relationship between efflux and influx of Na^+, K^+, and pH_{in} when energizing yeast cells by oxygenation. With the aid of a home-built solenoidal coil probe and an anionic shift reagent, we now can follow time courses of K^+ with a time resolution of 1 min in yeast suspensions. In separate experiments under the same conditions, Na^+ transport and pH_{in} could be followed on one sample by using a probe double-tuned for ^{23}Na and ^{31}P and switching back and forth every 1 min.

EXPERIMENTAL

The *Saccharomyces cerevisiae* strain NCYC 239 was grown at 30°C for 24 hr in 2% Bacto-peptone, 1% yeast extract, and 2% glucose. Before harvest, the cultures were chilled to 4°C, after which the cells were collected by low-speed centrifugation. The cells were washed twice and resuspended to a pellet/medium density of 1:2 in a resuspension medium consisting of 0.85 g of KH_2PO_4, 0.15 g of K_2HPO_4, 0.5 g of $MgSO_4$, and 0.1 g of NaCl per ml in 50 mM 2-(*N*-morpholino)ethanesulfonic acid/25 mM Tris buffer. The extracellular pH was about 6.0. The cells were kept at ice temperature until used.

A Bruker WH 360 WB NMR spectrometer was used at 25°C, operating at 145.78 MHz for ^{31}P, 95.26 MHz for ^{23}Na, and 16.81 MHz for ^{39}K. For ^{31}P, 45° pulses were used with pulse intervals of 0.3 s; for ^{23}Na and for ^{39}K, 90° pulses were used with intervals of 0.05 s. The Bruker 20-mm ^{13}C probe was tuned without modification to ^{23}Na. In addition, we used the Bruker $^{13}C/^{31}P$ double-tuned probe (15-mm OD sample tubes), which could be double-tuned to ^{23}Na and ^{31}P. ^{39}K experiments were done in a separate experiment with our home-built solenoidal coil probe and a sample volume of 25 ml. The home built coil was a 12-turn solenoid tuned to 16.81 MHz with a sample diameter of 28 mm. We obtained with this probe a signal-to-noise ratio of 63:1 for a 150 mM KCl solution in one pulse, which is >5 times better than with the Bruker 20-mm broad-band probe.

In all experiments, gas was bubbled through the suspension. In aerobic experiments, a 95% O_2/5% CO_2 was used; in an-

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Abbreviations: pH_{in}, intracellular pH; Na^+_{out} and K^+_{out}, extracellular Na^+ and K^+; Na^+_{in} and K^+_{in}, intracellular Na^+ and K^+.

aerobic experiments, 95% N_2/5% CO_2 was used (3). Calibration of intracellular concentrations was done by assuming that 1.67 g of wet yeast cells contain 1 ml of cell water.

The shift reagent used was $Dy^{3+}(P_3O_{10}^{5-})_2$ (6). It was prepared by adding a stoichiometric amount of Dy_2O_3 to a solution of $Na_5P_3O_{10}$. In most experiments, 2.5 mM shift reagent was used, leading to the addition of 25 mM Na^+ to the suspension.

RESULTS

Fig. 1 shows the 16.81-MHz ^{39}K NMR spectra of aerobic and anaerobic yeast suspensions at 25°C in the presence of the shift reagent. Two well-resolved peaks were observed, separated by about 10 ppm. The peak shifted upfield corresponds to extracellular K^+ (K^+_{out}), whereas the unshifted position corresponds to intracellular K^+ (K^+_{in}). The peak assignments were based upon the observation that the K^+_{in} chemical shift was virtually unaffected by the presence of the paramagnetic shift reagent. The linewidth of the K^+_{in} signal was about 10 ppm, and deconvolution helped to separate the two signals (Fig. 1, spectrum C). Relative peak intensities of K^+_{in} and K^+_{out} signals were quite constant for at least 3 hr, indicating that net movement of K^+ was small under these conditions (anaerobic, without exogenous substrate).

The observation of separate signals for K^+_{in} and K^+_{out} can be utilized to determine the fraction of K^+_{in} that is NMR visible.

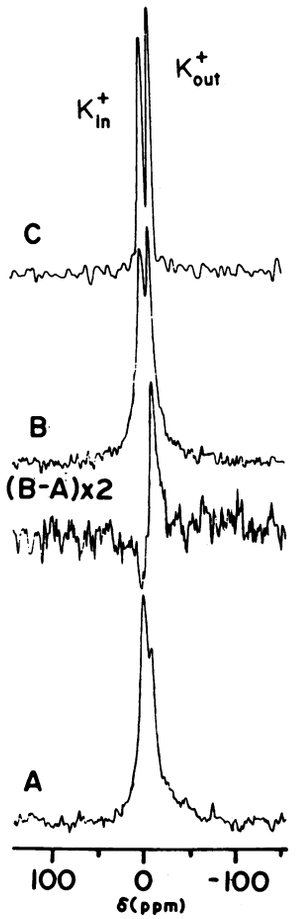

FIG. 1. ^{39}K 16.81-MHz NMR spectra of an aerobic (spectrum A) and anaerobic (spectrum B) yeast suspension at 25°C. The difference spectrum multiplied by 2 [(B − A) × 2] is shown between spectrums A and B. Exponential multiplication was used for spectrums A and B, whereas Gaussian multiplication was applied to the same free induction decay of spectrum B to obtain spectrum C.

Upon switching well-oxygenated cells to anaerobic conditions, K^+ was released by the cells into the medium, resulting in changing intensities of the K^+_{in} and K^+_{out} signals. However, the increase of K^+_{out} was greater than the decrease of the K^+_{in} signal. We calculated, based upon the change in the relative signal intensities in the difference spectrum of Fig. 1, that only 30–40% of the total K^+ present in the yeast cells is NMR visible. By addition of known amounts of K^+ to the suspension and comparison of the ^{39}K NMR intensities of the K^+_{out} signal to a control of a known amount of K^+ in buffer, we ascertained that all K^+_{out} is NMR visible. By using this correction factor, the K^+_{in} concentration was determined to be 130–170 mM for fresh yeast cells.

In a similar way we determined the NMR visibility of the Na^+_{in} signal in these yeast cells. Fig. 2 shows a representative 95.26-MHz ^{23}Na NMR spectrum of an anaerobic yeast suspension. The Na^+_{out} signal, somewhat broadened by the shift reagent, shifted upfield by 8 ppm, while the Na^+_{in} signal remained unshifted. After permeabilization of the yeast cells by the addition of 5% of a 1:4 toluene/ethanol mixture and 5 min of vigorous mixing of the cell suspension (15), the Na^+_{out} signal shifted back to the same position as the Na^+_{in} signal. This collapse of the chemical shift difference between Na^+_{in} and Na^+_{out} signals is probably due to hydrolysis of tripolyphosphate used in the shift reagent by cellular phosphatases. Note, however, that the total integrated intensity increased upon permeabilization. On the basis of the observation that all Na^+ is NMR visible after permeabilization, we calculated that only 40% of Na^+_{in} (before permeabilization) is NMR visible. Using this correction factor, we determined that the concentration of Na^+_{in} is about 2.5 mM in these fresh yeast cells.

Fig. 3 shows the time course of K^+ and Na^+ transport as well as changes in pH_{in} obtained at 25°C upon alternating between oxygen and nitrogen bubbling every 20 min. No substrate was added in this experiment. Under anaerobic conditions, before the first oxygenation, little change in Na^+ and K^+ was observed, but pH_{in} gradually decreased from the initial value of 6.5. Upon oxygenation, pH_{in} increased in a few minutes to a steady-state value of 7.1. At the same time, there was a very small change in the level of Na^+_{in}. On the other hand, K^+ was taken up rapidly by the cells to a new steady-state level, which was reached within a few minutes of oxygenation. After oxygenation ceased, pH_{in} started to decrease from 7.1 to 6.5 over a period of 20 min. In this period, Na^+_{in} started to increase by

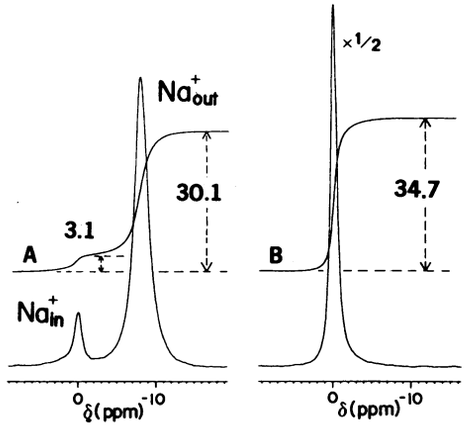

FIG. 2. ^{23}Na 95.26-MHz NMR spectra of an anaerobic yeast suspension at 25°C in the presence of 1 mM shift reagent before (*A*) and after (*B*) permeabilization of the cells. Numbers are the integral intensity for each peak.

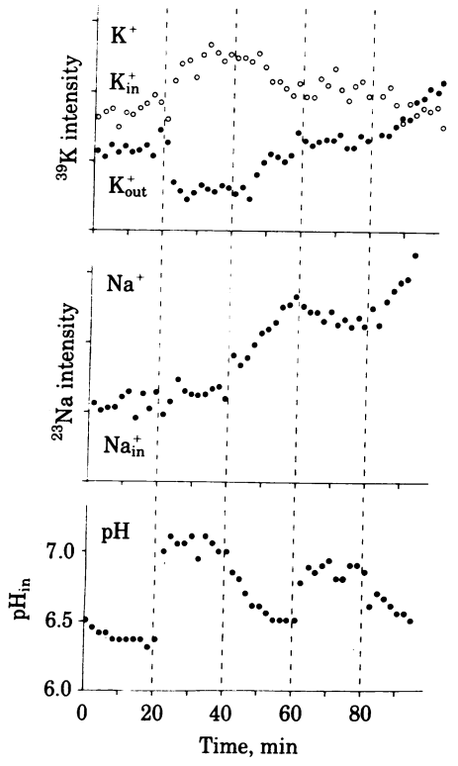

FIG. 3. Time courses of pH_{in} and concentrations of Na^+_{in} and K^+_{in} upon alternating between oxygen and nitrogen bubbling every 20 min. No exogenous substrate was used. pH_{in} and Na^+_{in} of the same sample were obtained by using a probe double-tuned for ^{31}P and ^{23}Na.

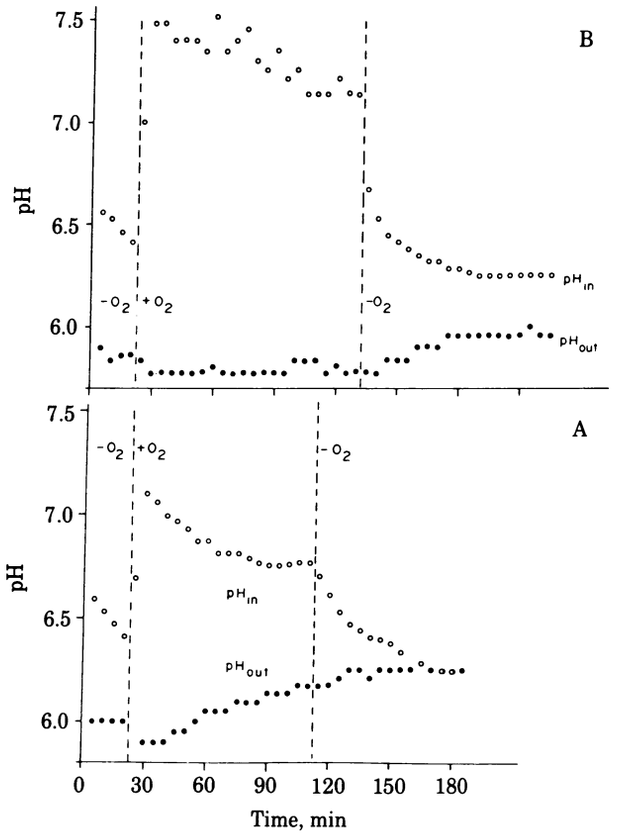

FIG. 4. Time courses of pH_{in} (○) and pH_{out} (●) obtained from the ^{31}P NMR chemical shift of the intracellular and extracellular P_i signals in the absence (*A*) and presence (*B*) of 5% ethanol in the medium.

influx of Na^+ from the medium, while at the same time an efflux of K^+ was observed.

Upon reoxygenation of the cell suspension, pH_{in} increased only to 6.9, while no uptake of K^+ was observed. This difference from the first aerobic period was thought to be due to exhaustion of endogenous substrates. To study this, we followed pH_{in}, Na^+_{in}, and K^+_{in} upon oxygenation of yeast suspensions in the absence and presence of ethanol, an exogenous substrate.

In these experiments, the NMR spectra were accumulated over periods of 5 min. Fig. 4*A* shows that pH_{in} increased as before from 6.4 to 7.1 upon oxygenation of the yeast suspension. However, during the longer period (90 min), pH_{in} was not stable in the absence of exogenous substrate. On the other hand, when ethanol was added, pH_{in} increased to 7.4 upon oxygenation, and this higher pH_{in} was rather stable for almost 2 hr (Fig. 4*B*). This observation supports the idea that the value and stability of pH_{in} depends upon the availability of carbon sources. In view of this, it is interesting to note that after the exhaustion of exogenous substrate, the amino acid pools (in particular glutamate) decreased on the same time scale in aerobic yeast (16) as pH_{in} did here during endogenous respiration. The presence of ethanol has an important effect upon the transport of K^+ (Fig. 5). Upon oxygenation K^+ was taken up rapidly by the cells, both with and without ethanol. However, in the absence of ethanol, subsequent efflux of K^+ was observed, starting 10 min after the onset of oxygenation. On the other hand, when ethanol was added, no efflux of K^+ was observed over a period of 1.5 hr (Fig. 5*B*).

Transport of Na^+ was also observed to be different in the presence and absence of ethanol. Fig. 6 shows the time courses of Na^+_{in} upon oxygenation under conditions similar to those of Figs. 4 and 5. In the absence of ethanol, the level of Na^+_{in} increased slowly upon oxygenation with a rate of 0.017 μmol/min per ml of cell water. Subsequent deenergization of the cells increased the rate of Na^+ uptake of the cells. On the other hand, when ethanol was added, Na^+_{in} increased with an initial rate of 0.42 μmol/min per ml of cell water upon oxygenation and reached a steady-state value of 19.7 μmol/ml of cell water after about 30 min. This result indicates that Na^+ was taken up by the cells in the presence of $\approx$3 mM K^+ in the medium [which is high compared with the K_m for K^+ uptake by yeast cells (8)] and that the Na^+ uptake was stimulated by the presence of ethanol. As is shown in Fig. 6, in the presence of 25 mM KCl, the rate of Na^+ accumulation was reduced by a factor of 2 compared with the control experiment.

DISCUSSION

Because ^{39}K is a nucleus of low NMR sensitivity, few ^{39}K NMR studies of biological systems have been reported (ref. 17 and references cited therein). The improvement in sensitivity of a home-built solenoidal coil probe allowed us to follow time courses of K^+ transport with a time resolution of 1 min.

As shown in Figs. 1 and 2, Na^+_{in} in yeast has a linewidth of 60 Hz, and K^+_{in} has one of 160 Hz. The linewidths of the extracellular signals increase with the concentration of shift reagent used in the experiment. The increased linewidth observed for the $^{39}K_{in}$ NMR signal compared with the $^{23}Na_{in}$ NMR signal can be explained by the greater value of the Sternheimer antishielding factor of ^{39}K because of its greater number of electrons (18).

After the first observation of $^{23}Na_{in}$ NMR signals by Cope (19), several determinations of NMR visibility of Na^+_{in} were reported, with the fractions detected ranging from 25% to 50% (17). Part of these variations may arise from the difficulties in

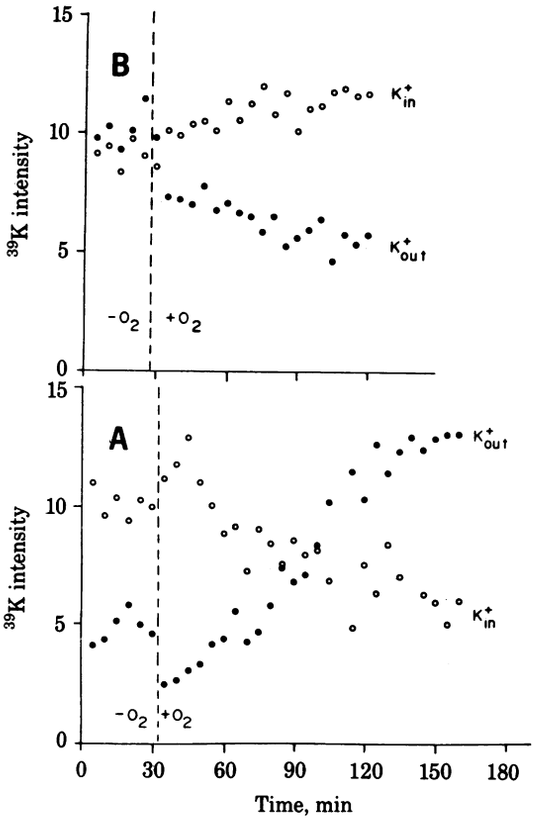

FIG. 5. Time courses of K^+_{in} (○) and K^+_{out} (●) in the absence (*A*) and presence (*B*) of 5% ethanol in the medium. Experimental conditions were as in Fig. 4.

separating signals of intracellular ions from extracellular ions, which inevitably are present. As shown in Figs. 1 and 2, direct observation of well-resolved peaks from Na^+_{in}, Na^+_{out}, K^+_{in}, and K^+_{out} in the yeast suspension can be used now to determine relative signal intensities, thereby avoiding possible artifacts of isolation procedures. In this way we determined that the NMR visibility of both Na^+_{in} and K^+_{in} in yeast cells is 40% of total ion concentrations.

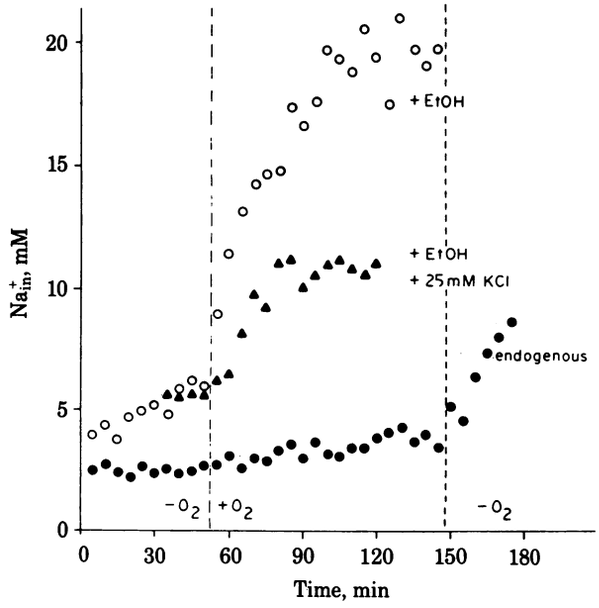

FIG. 6. Time courses of Na^+_{in} in the absence of ethanol (●), in the presence of 5% ethanol (○), and in the presence of 5% ethanol/25 mM KCl (▲) in the medium. Experimental conditions were as in Fig. 4.

The observed value of NMR visibility (40%) was explained by Berendsen and Edzes (20) on the basis of a first-order quadrupole interaction with each nucleus. This could arise either from rapid exchange between a small fraction (<1%) of immobilized cations and the free cations in the bulk or from diffusion of free cations between domains of ordered polyelectrolytes. It should be pointed out that this interpretation means that virtually all of the intracellular cations present are free in solution. Therefore, to obtain the free intracellular ion concentration, the observed NMR intensities need to be multiplied by a correction factor of 2.5.

The present experiments show that the NMR properties of cations inside yeast cells are different from those in the external medium. Our experiments show that the NMR visibility (40%) is similar for ^{23}Na and for ^{39}K, in agreement with the interpretation given by Berendsen and Edzes. Therefore, it appears that there is no significant difference in the NMR properties of $^{23}Na^+_{in}$ and of $^{39}K^+_{in}$ in yeast cells (17). This conclusion is not necessarily in disagreement with the reported difference in the apparent electrochemical activity coefficient for Na^+_{in} and K^+_{in} (21).

In general, the responses of K^+ to oxygenation are too fast to be followed accurately in time in the present experiments. By using the measured intracellular buffer capacity of yeast cells (A. Ballarin-Denti, personal communication), a net proton transport can be calculated from observed changes in pH_{in}. For example, upon oxygenation, pH_{in} increased in a few minutes to pH 7.1 or 7.4 in the absence or presence of ethanol, respectively. These pH_{in} changes correspond to a net proton consumption of 32 and 39 μmol/ml of cell water, respectively. K^+ ions were simultaneously taken up by the cells upon oxygenation—about 13–18 and 10–16 μmol/ml of cell water in the absence and in the presence, respectively, of ethanol. On the other hand, Na^+_{in} increased very slowly at a rate of 0.017 and 0.42 μmol/min per ml of cell water in the absence and in the presence, respectively, of ethanol. These results indicate that proton extrusion is correlated with K^+ uptake (7, 8, 15) and not with Na^+ uptake. However, the precise stoichiometry between proton extrusion and K^+ uptake has not been determined because it may be complicated by proton production by metabolic processes (22) and by the participation of other ions.

The efflux and influx of Na^+, K^+, and protons could be followed when the cells were deenergized after the aerobic period. On the basis of the data presented in Fig. 3, Na^+ influx, K^+ efflux, and H^+ influx were calculated to be 0.15, 1.6, and 1.5 μmol/min per ml of cell water, respectively. Thus, Na^+ influx is an order of magnitude slower than K^+ efflux, which is consistent with a report that vesicles of plasma membranes are more permeable to K^+ than Na^+ (23). Furthermore, it appears that, under the present conditions, K^+ efflux is balanced within experimental error by H^+ and Na^+ influx, in agreement with reports on Na^+-loaded cells (8). Because of the small contribution of Na^+ influx (≈10%) we conclude that the K^+ efflux is due to the exchange of K^+ for H^+ with an approximate stoichiometry of 1:1.

It has been reported that Na^+ ions enter into the yeast cells by a K^+ carrier and possibly also by Na^+/substrate symporters as described for phosphate (8). The present experiments show that the rate of Na^+ uptake was 1 order of magnitude slower during oxygenation (0.017 μmol/min per ml of cell water) than it was during deenergization (0.15 μmol/min per ml of cell water) in the absence of exogenous substrate (cf. Figs. 3 and 6). However, in the presence of exogenous ethanol, Na^+ was taken up

at a rate of 0.42 μmol/min per ml of cell water until Na^+_{in} reached a steady-state value of 19.7 mM, which was somewhat lower than the Na^+ level in the medium (25 mM). The addition of 25 mM KCl to the medium reduced the rate of Na^+ uptake to 0.21 μmol/min per ml of cell water and the steady-state level of Na^+_{in} accumulation to 10.8 mM. This suggests that, upon energization, Na^+ may enter into the cells by competing for the K^+ carrier, with a rate that also depends upon the presence of an exogenous carbon source (8, 24). A similar observation was reported for glycolyzing yeast cells by Rodrigues-Navarro and Ortega (25). The increased uptake of Na^+ in the presence of ethanol seems to be correlated with the higher pH_{in} under these conditions. This observation supports the idea that Na^+ enters into the cell by the K^+ carrier because of the correlation between K^+ uptake and H^+ extrusion.

A complication in the interpretation of the ^{23}Na NMR data arises because of the presence of vacuoles in the yeast cells. The present NMR method for measuring Na^+_{in} is not able to differentiate between different compartments and measures only the total amount of cations present in the yeast cells. If a Na^+ gradient does exist across the tonoplast, this may influence the concentration of Na^+ in the cytoplasm, and also the influx of Na^+ into the yeast cells. In fact, a vacuolar membrane-bound ATPase has been described (26), and a ΔpH can be maintained across the vacuolar membrane (4, 27). Moreover, Mg^{2+}, K^+, and P_i can be accumulated into the vacuoles of *Saccharomyces carlsbergensis* (28), although no such Na^+ accumulation has been reported.

It has been reported that in the absence of Mg^{2+} in the medium there is an enhanced exchange of K^+/Na^+ in yeast cells (12). If the tripolyphosphate used with the shift reagent causes depletion of Mg^{2+} because of complexation, this would affect the results of our experiments. However, in the absence of ethanol, no enhanced Na^+ uptake was observed, so that these effects were negligible.

In conclusion, the experiments reported here demonstrate that ^{39}K and ^{23}Na NMR can be used to study K^+ and Na^+ ion transport in intact biological samples, supplementing information on proton movement obtained by ^{31}P NMR.

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