Sodium Transport and Phosphorus Metabolism in Sodium-Loaded Yeast: Simultaneous Observation with Sodium-23 and Phosphorus-31 NMR Spectroscopy in Vivo[†]

Herbert Höfeler,[‡] Dye Jensen,[‡] Martin M. Pike,[‡,§] Jean L. Delayre,[‡] Vincent P. Cirillo,[‖] Charles S. Springer, Jr.,[*,§] Eric T. Fossel,[‡] and James A. Balschi[‡,§]

NMR Laboratory, Harvard Medical School, Boston, Massachusetts 02115, and Departments of Chemistry and Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

Received January 29, 1986; Revised Manuscript Received March 27, 1987

ABSTRACT: Simultaneous ^{23}Na and ^{31}P NMR spectra were obtained from a number of yeast suspensions. Prior to NMR spectroscopy, the yeast cells were Na-loaded: this replaced some of the intracellular K$^+$ with Na$^+$. These cells were also somewhat P-deficient in that they had no polyphosphate species visible in the ^{31}P NMR spectrum. In the NMR experiments, the Na-loaded cells were suspended in media which contained inorganic phosphate, very low Na$^+$, and a shift reagent for the Na$^+$ NMR signal. The media differed as to whether dioxygen, glucose, or K$^+$ was present individually or in combinations and as to whether the medium was buffered or not. The NMR spectra revealed that the cells always lost Na$^+$ and gained phosphorus. However, the nature of the Na$^+$ efflux time course and the P metabolism differed depending on the medium. The Na$^+$ efflux usually proceeded linearly until the amount of Na$^+$ extruded roughly equalled the amount of NH$_4$$^+$ and orthophosphate initially present in the medium (external phosphate was added as NH$_4$H$_2$PO$_4$). Thus, we presume this first phase reflects a Na$^+$ for NH$_4$$^+$ exchange. The Na$^+$ efflux then entered a transition phase, either slowing, ceasing, or transiently reversing, before resuming at about the same value as that of the first phase. We presume that this last phase involves the simultaneous extrusion of intracellular anions as reported in the literature. The phosphorus metabolism was much more varied. In the absence of exogenous glucose, the P taken up accumulated first as intracellular inorganic phosphate; otherwise, it accumulated first in the "sugar phosphate" pool. In most cases, at least some of the P left the sugar phosphate pool and entered the polyphosphate reservoir in the vacuole. However, this never happened until the phase probably representing Na$^+$ for NH$_4$$^+$ exchange was completed, and the P in the polyphosphate pool never remained there permanently but always eventually reverted back to the sugar phosphate pool. These changes are interpreted in terms of hierarchical energy demands on the cells under the different conditions. In particular, the energy for the Na$^+$ for NH$_4$$^+$ exchange takes precedence over that required to produce and store polyphosphate. This conclusion is supported by the fact that when the cells are "forced" to exchange K$^+$, as well as NH$_4$$^+$, for Na$^+$ (by the addition of 5 times as much K$^+$ to the NH$_4$$^+$-containing medium), polyphosphates are never significantly formed, and the initial linear Na$^+$ efflux phase persists possibly 6 times as long. The ultimate consumption of polyphosphate species probably occurs because (inorganic) phosphate is required for the metabolic processes which produce the anions for the Na$^+$ anion coextrusion phase.

Phosphorus-31 NMR spectroscopy has been widely used to study yeast metabolism [see Alger et al. (1982) and references cited therein and Nicolay et al. (1983) and references cited therein]. In a more recent development, the introduction of aqueous shift reagents for cation NMR (Pike & Springer, 1982; Gupta & Gupta, 1982) has made possible the discrimination of the extra- and intracellular ^{23}Na and ^{7}Li resonances

in suspensions of yeast cells (Balschi et al., 1982). This allowed the rather continuous monitoring of Na$^+$ efflux from Na$^+$-rich yeast in the latter study. Since ion transport often puts significant demands on metabolic processes, Shulman and co-workers have discussed the advantages of monitoring the ^{23}Na and/or ^{39}K NMR spectrum of a yeast suspension (containing shift reagent) along with the ^{31}P NMR spectrum (Ogino et al., 1983).

One of the more interesting aspects of yeast metabolism is the formation of vacuolar polyphosphate species, P$_n$, in response to a number of different conditions [see Nicolay et al. (1983) and references cited therein and Dürr et al. (1979) and references cited therein]. In particular, the question of whether the P$_n$ serve an energy storage function as well as that of phosphorus storage is fascinating but still unresolved. In the present study, we have again used yeasts (*Saccharomyces cerevisiae*) which are Na$^+$ rich and, in addition, also P$_n$ depleted. We have monitored Na$^+$ efflux from these yeasts under a number of different external conditions, all including added orthophosphate, P$_i$. By simultaneously observing the ^{31}P NMR

Table I: Protocols for the Na$^+$ Efflux Experiments

external conditions	wet wt of yeast (g)	cell suspension [(w/v) %]	additions at time zero	external medium at time zero
aerobic (only)	4.60	22.3	none	standard medium[a]
anaerobic (only)	4.39	21.5	none	standard medium
aerobic, potassium	4.80	23.1	1 mL of 300 mM KCl	standard medium plus 18.8 mM K$^+$
anaerobic, potassium	4.65	22.5	1 mL of 300 mM KCl	standard medium plus 18.8 mM K$^+$
aerobic, glucose	4.55	22.1	2 mL of 2.0 M glucose	standard medium plus 250 mM glucose
anaerobic, glucose	4.74	22.9	2 mL of 2.0 M glucose	standard medium plus 250 mM glucose
aerobic, glucose (buffered)	4.73	22.8	2 mL of 2.0 M glucose	standard medium plus 250 mM glucose plus 68 mM MES[b]
anaerobic, glucose (buffered)	4.63	22.4	2 mL of 2.0 M glucose	standard medium plus 250 mM glucose plus 62 mM MES[b]

[a]Standard medium = 6.3 mM SR, 11.3 mM choline bicarbonate, and 3.8 mM NH$_4$H$_2$PO$_4$. [b]Titrated with choline bicarbonate to pH 6.8.

spectrum, we have been able to assay some of the metabolic manifestations of this process. In particular, we find the temporal correlations of a number of phenomena quite interesting.

EXPERIMENTAL PROCEDURES

Reagents. The shift reagent (SR) chol$_3$DyTTHA·3cholCl where chol is choline was prepared as described by Chu et al. (1984). Other reagents used were as follows: NH$_4$H$_2$PO$_4$ (Fisher ACS certified grade) and choline bicarbonate (45%) (K & K Laboratories); 2-(N-morpholino)ethanesulfonic acid (MES), triethylenetetraminehexaacetic acid (H$_6$TTHA), and N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid (HEPES) (Sigma); and DyCl$_3$·6H$_2$O (Alfa).

Preparation of Sodium-Rich Polyphosphate-Depleted Yeast. Red Star bakers' yeast (Standard Brands; Feb 1983; Long Island, NY) was washed 3 times with sterile distilled water and made up to a 50% (w/v) stock suspension. A portion of commercial yeast cake (not from the surface) was taken to assess cell viability. A volumetric aliquot of a suspension, whose cell density was known by hemocytometry, was incubated for 2–3 days on agar permeated with 1% yeast extract, 2% peptone, and 2% glucose. The colonies were counted, and 100 ± 2% of the cells were found to be viable.

(A) Sodium Loading. Typically, 16 mL of stock suspension (8 g of cells) was added to 400 mL of a 0.2 M trisodium citrate/5% (w/v) (278 mM) glucose solution and bubbled with 95% O$_2$/5% CO$_2$ while being slowly stirred at room temperature (Rothstein, 1974). Loading lasted about 2 h on the average.

(B) Harvesting. In order to minimize Na$^+$ efflux during harvesting, the Na$^+$ loading suspension containing the yeasts was cooled on ice prior to spin down. Equal portions of suspension were added to each of two tubes which were then centrifuged at 4000 rpm for 4 min at 20 °C. The supernatants were discarded. The remainder of the loading suspension was then divided equally between the two tubes already containing pelleted yeast, and 100 mL of ice-cold distilled water was added to each. These were centifuged as above, and the supernatants were discarded. Each portion of the yeast was resuspended in 150 mL of cold distilled water and centrifuged as above. The supernatants were again discarded. The yeast pellets thus washed were weighed and resuspended in cold standard medium. The standard medium consisted of 6.3 mM SR, 11.3 mM choline bicarbonate, and 3.8 mM NH$_4$H$_2$PO$_4$. An aliquot of a suspension of Na$^+$-loaded yeast cells was also taken for a viability determination as described above. It was found that 94% of these cells were alive.

The yeasts used in this study, at least after loading and harvesting, were polyphosphate depleted. Some batches of commercial yeasts contain polyphosphates. The reason for this variation is not known. However, variations in the nitrogen source, substrate, and exogenous phosphate are known to in-

fluence polyphosphate levels [den Hollander et al., 1981; see Dürr et al. (1979) and references cited therein; Salhany et al., 1975]. It should also be noted that the Na$^+$-loaded yeast cells are, formally, not starved cells.

Na$^+$-Efflux, P$_i$-Influx Experiments. The different gas phases and the different additions for the different experiments are indicated in Table I. The external concentrations of substances added to the suspensions were calculated on the basis of the volumes of the solutions added and assuming a yeast density of 1 g/mL (Cirillo, 1968).

The samples for NMR experiments typically consisted of 20 mL of the cell suspension in 20-mm o.d. NMR tubes. In the aerobic experiments, a 95% O$_2$/5% CO$_2$ gas mixture was bubbled through the suspension; in the anaerobic experiments, the gas mixture was 95% N$_2$/5% CO$_2$. In all experiments, the gas mixture was bubbled through a two-bubbler device essentially the same as that described by den Hollander et al. (1981). The probe tuning (^{23}Na and ^{31}P) was adjusted with a standard yeast sample prior to the beginning of the experiments. Each sample was placed in the probe and warmed to the probe temperature (ca. 25 °C). The beginning of acquisition of the first spectrum was usually ca. 3 min after the yeast pellet was resuspended in the cold standard medium. For the first two experiments in Table I, this served as the definition of time zero. For the other experiments, the addition of either KCl or glucose solutions (via the bubbler gas line) defined time zero, but this was still about 3 min from resuspension. After ca. 2 h of aerobic (only) efflux (Table I), another aliquot of the resulting suspension was taken for viability determination. It was found that 90% of these cells were viable. Thus, although the cells are not actually growing during the efflux, they are certainly alive and capable of growth. Also, at the end of most efflux experiments, the external pH was measured with a pH meter. The values were entirely consistent with extrapolations from the spectral data.

NMR Spectroscopy. All NMR experiments were conducted on a Nicolet NT-360 WB instrument modified for simultaneous ^{23}Na and ^{31}P observe capability by the provision of two entirely separate NMR transmitter/receiver channels (including pulse programmers and computers) (Jensen, 1983; Delayre et al., 1984). The ^{23}Na spectra (95.24 MHz) were obtained with a coil double-tuned to the ^{23}Na and ^{2}H (55.3-MHz) frequencies and with a spectral width of 4000 Hz represented by 2K data points. The pulses of 95-MHz radio frequency (pulse width = 85 μs) produced 90° spin flips. The ^{31}P spectra (145.75 MHz) were obtained with a separate coil in the same probe, simultaneously, with a spectral width of 8000 Hz represented by 2K data points. The pulses of 146-MHz radio frequency (pulse width = 12 μs) produced 30° spin flips.

The pulse recycle time was 0.34 s, similar to that used by den Hollander et al. (1981). Although this is greater than 6 times the largest T_1 value for ^{23}Na, it does not produce a fully

relaxed ^{31}P spectrum. Assuming that T_1 values do not change, temporal trends in the areas of individual ^{31}P NMR peaks reflects trends in concentrations. In any case, the total area under all the ^{31}P peaks does not change much during the course of an efflux experiment (see below). A total of 360 transients was accumulated for each (^{23}Na and ^{31}P) spectrum. The total accumulation time was equal to 2 min. The accumulated free induction decays (FID) for each nucleus were separately stored on hard disks. Before Fourier transformation, the ^{23}Na FID were multiplied by an exponential function sufficient to generate an 8-Hz line broadening, and the ^{31}P FID were multiplied by an exponential function sufficient to generate a 20-Hz line broadening.

The ^{23}Na spectra are referenced to the intracellular peak which is set equal to zero. The ^{31}P spectra are referenced to the peak of intracellular NAD which was set equal to 10.9 ppm (upfield from external 85% phosphoric acid). This was chosen because it could be observed in both the anaerobic and the aerobic experiments and because its chemical shift does not titrate with pH (Salhany et al., 1975). The pH values were determined from the chemical shifts of the P_i resonances by using the pH/chemical shift titration curve of Burt et al. (1979). The absolute values of these pH determinations may have some error associated, but the temporal trends should be reasonably error free. The latter are of most interest to us here.

Spectral integrations were accomplished by using the ID integration subroutine of the NMC spectral operation program. For certain spectral regions, the NMRCAP subroutine was used to resolve overlapping peaks and to measure their areas. This was necessary in the low-field regions of the ^{31}P spectra. The areas of the ^{23}Na resonances were calibrated by using a set of NaCl standards (which included the SR and were bubbled) of known concentration as described by Pike et al. (1984). The areas of both the ^{23}Na and ^{31}P resonances were also calibrated by measuring the increases in the areas of the appropriate peaks when 206 μmol of NaH_2PO_4 was added to a yeast suspension.

The computation of the fraction of total Na^+ which is extracellular, f_{Na}, is a bit involved. If one inspects Figure 1 and 2 carefully, one observes that the total ^{23}Na signal intensity increases as Na^+ efflux proceeds. This has been reported earlier (Balschi et al., 1982; Ogino et al., 1983), and it means that the Na^+-loaded yeast, at least, exhibits some ^{23}Na NMR invisibility.

Of all the experiments of Table I, we followed the Na^+ efflux in the presence of O_2 and K^+ (i.e., that depicted in Figure 1) to the greatest extent of intracellular Na^+ depletion. We conducted NMR and FP analyses on a sample identical with that of Figure 1 [except 4.58 g (w/w)] after 2 h (120 min) of Na^+ efflux in the presence of O_2, K^+, and SR. The NMR analysis consisted of comparing the total area of the inside and outside peaks in the spectrum of intact yeast cells with those of the calibrating standards (Pike et al., 1984). It yielded a value of 30.6 mM (±4%) Na^+ based on the total sample volume. The FP analysis of the same sample yielded a value of 30.5 mM (±3%). From this good agreement, we conclude that extracellular Na^+ is 100% NMR visible in these suspensions of yeast cells. This is in agreement with the results of Shulman and co-workers (Ogino et al., 1983). The NMR visibility of intracellular Na^+ in the Na^+-loaded yeast is another matter, being significantly less than 100% (Balschi, 1984; Ogino et al., 1983). This will be discussed more thoroughly in a subsequent paper (J. A. Balschi et al., unpublished results).
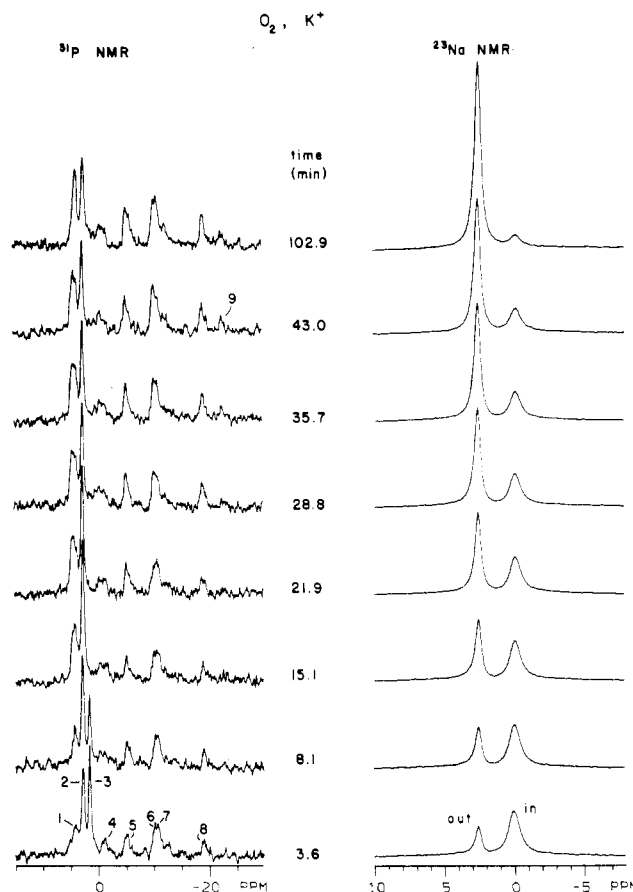

FIGURE 1: Simultaneous ^{31}P (145.75 MHz) and ^{23}Na (95.24 MHz) NMR spectra obtained at 8.5 T on a suspension of Na^+-loaded yeast. The yeast are suspended (23.1% w/v) in a medium which is low in Na^+ and which contains 3.8 mM $NH_4H_2PO_4$ and 6.3 mM $chol_3DyTTHA\cdot 3cholCl$, a downfield shift reagent for the ^{23}Na signal. The suspension was bubbled with 95% O_2/5% CO_2, and each pair of spectra is marked with the time of the midpoint of the acquisition which had elapsed since the external medium was made 18.8 mM in KCl. This is the third experiment listed in Table I. The numbered peak assignments are given in the text.

The value of f_{Na} was calculated at any point in time by dividing the area of the external Na^+ resonance at that time by the total area expected at long times, when the Na^+ could be reasonably expected to be almost completely extracellular. This latter quantity was calculated for each experiment by multiplying the total area of the ^{23}Na NMR spectrum of the O_2, K^+ study at 102.9 min (Figure 1) by the ratio of yeast wet weight in the particular experiment over the yeast wet weight in the O_2, K^+ experiment (4.80 g, Table I). This was done in order to correct for the slightly varying amounts of Na^+ and the fact that, for most experiments, we ceased monitoring the Na^+ efflux well before complete depletion was achieved (Balschi, 1984).

Flame Emission Photometry. Flame photometric (FP) analyses for Na^+ were conducted by using a Corning flame photometer, Model 430. This instrument was calibrated and the analyses were conducted as described for erythrocytes by Pike et al. (1984).

RESULTS

Figures 1 and 2 present examples of the ^{31}P and ^{23}Na NMR spectra observed in these experiments. Figure 1 depicts representative spectra obtained during Na^+ efflux from and P_i influx into Na^+-rich, P_n-depleted yeast in the presence of both O_2 and K^+. Although only 8 ^{31}P and ^{23}Na spectra are shown, 21 pairs of simultaneous ^{31}P/^{23}Na spectra were obtained
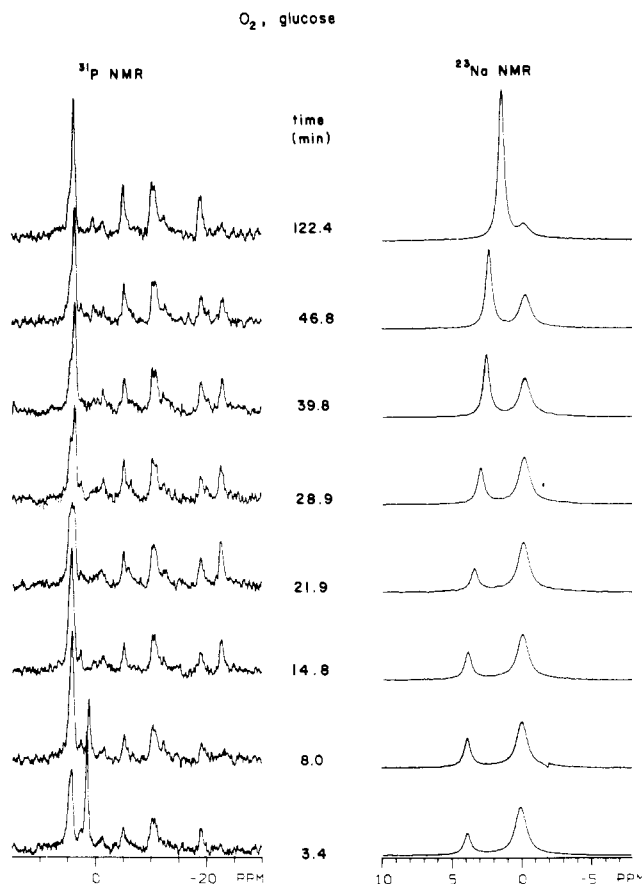
O₂, glucose

FIGURE 2: Simultaneous ^{31}P (145.75 MHz) and ^{23}Na (95.24 MHz) NMR spectra obtained at 8.5 T on a suspension of Na$^+$-loaded yeast. The yeast are suspended (22.1% w/v) in a medium which is low in Na$^+$ and which contains 3.8 mM NH$_4$H$_2$PO$_4$ and 6.3 mM chol$_3$DyTTHA·3cholCl, a downfield shift reagent for the ^{23}Na signal. The suspension was bubbled with 95% O$_2$/5% CO$_2$, and each pair of spectra is marked with the time of the midpoint of the acquisition which had elapsed since the external medium was made 250 mM in glucose. This is the fifth experiment of Table I.

during this time period. The times shown are those elapsed from the addition of KCl to the medium and are measured to the midpoints of the spectral acquisitions. The peaks in the ^{31}P spectra are enumerated. Their assignments are as follows: (1) phosphomonoesters (sugar phosphates, sP); (2) intracellular (probably cytoplasmic) orthophosphate (inorganic phosphate, P$_i$), (P$_i$)$_i$; (3) extracellular inorganic phosphate, (P$_i$)$_o$; (4) unassigned; (5) γ-P of adenosine triphosphate (ATP and β-P of adenosine diphosphate (ADP); (6) α-P of ATP and α-P of ADP; (7) P of oxidized and reduced nicotinamide adenine dinucleotide, NAD; (8) β-P of ATP; (9) nonterminal P of polyphosphate (P$_n$). This latter peak is seen more clearly in Figure 2. These assignments are based on those of Shulman and co-workers [Ogino et al., 1983; see Alger et al. (1982) and references cited therein; den Hollander et al., 1981; Salhany et al., 1975; Gillies et al., 1981; Navon et al., 1979; Ugurbil et al., 1978)] and Kaptein and co-workers [Nicolay et al., 1982; see Nicolay et al. (1983) and references cited therein]. The different resonance frequencies of the P$_i$ peaks are most likely not due to the presence of the shift reagent in the medium but to the fact that external pH (ca. 6.7) is lower than that of the intracellular milieu. The assignments of the ^{23}Na spectra are clear since Dy(TTHA)$^{3-}$ is a downfield SR for ^{23}Na$^+$ (Chu et al., 1984). They are indicated in the figure.

It is quite evident in Figure 1 that after 102.9 min, most of the Na$^+$ (89%, in fact) is extracellular. While this Na$^+$ efflux is occurring, the ^{31}P spectrum shows that P$_i$ is being

taken up by the cells. The area of the (P$_i$)$_o$ peak (3) decreases while that of the (P$_i$)$_i$ peak (2) increases.

Figure 2 shows selected spectra from a similar experiment except that glucose was substituted for the K$^+$ of Figure 1. While the Na$^+$ efflux and P influx proceed in a similar manner, the ^{31}P spectra reveal a metabolic pattern different from that of Figure 1 (see below). Also, as time progresses, the extracellular Na$^+$ peak shifts back upfield toward the intracellular peak. This is due to the fact that the yeast cells excrete protons during Na$^+$ efflux under these conditions. Although the Dy(TTHA)$^{3-}$ SR is not unduly pH sensitive, the shift it induces in the Na$^+$ resonance does decrease below a pH of 5.5 (Chu et al., 1984). The (P$_i$)$_o$ peak shifts upfield with time (as its area decreases) also indicating a decreasing external pH. If the external medium is buffered at pH 6.8 with 68 mM MES, the ^{23}Na spectral splitting remains constant with time, and the (P$_i$)$_o$ peak remains unshifted [spectra not shown here, but see Balschi (1984)].

One might note the relatively poor quality of the ^{31}P spectra, especially in the rich low-field region. There are two reasons for this. First, the short spectral accumulation time leads to a low signal to noise ratio. Even though the spectral parameters are set to reduce the effects of long ^{31}P relaxation (especially that of the P$_i$ resonances), the accumulated ^{23}Na signal is so strong (partly because of its short T_1) that the time resolution it allows is much better than that allowed by the ^{31}P signal. Thus, the total acquisition time used was a compromise between the good time resolution allowed by the ^{23}Na spectrum and the time required for a strong ^{31}P spectrum. This will always be an aspect of the simultaneous acquisition of spectra from different nuclei. Second, there is a broadening of the ^{31}P peaks representing intracellular molecules due to the presence of the paramagnetic SR in the extracellular medium. This has been noted for Na$_i$ before (Balschi et al., 1982; Pike et al., 1984) and is probably due to magnetic susceptibility gradient effects (Brindle et al., 1979; Fabry & San George, 1983). Since the Guptas' SR, Dy(PPP)$_2^{7-}$, produces larger shifts of the ^{23}Na resonance (Chu et al., 1984), it would be better in this regard, provided it was not harmful to the cells. If it retained its shift potency, the lower concentration of paramagnetic species required to produce the same ^{23}Na shift would produce less broadening of the intracellular resonances. We have demonstrated this with suspensions of human erythrocytes (Pike et al., 1984). Despite these problems with the simultaneous acquisition of ^{23}Na and ^{31}P NMR spectra, Figures 3–8 demonstrate the abundance of information which they contain. In addition, as we will see, there are inferences which would be difficult to reach without the absolute simultaneity of the acquisitions.

Spectra like those in Figures 1 and 2 were obtained for each of the experiments listed in Table I (Balschi, 1984). These were analyzed for the areas under the ^{31}P and ^{23}Na peaks and for the chemical shifts of the two P$_i$ peaks. Some of the results are presented in Figures 3–8. The ordinates of these plots indicate the fraction, f, of the total NMR-visible phosphorus (i.e., the total area under all the ^{31}P NMR peaks), the fraction of the total sodium, or the pH value. The unity value of f corresponds to ca. 240 μmol of P or ca. 610 μmol of Na (in a 20-mL yeast suspension). In the case of ATP, the ordinate represents 3 times the fractional area of the β resonance. The other ATP peaks are strongly overlapped with resonances of other species. The β resonance itself is strongly overlapped with terminal resonances of the polyphosphate species [Navon et al., 1979; Gillies et al., 1981; Nicolay et al., 1982; see Alger et al. (1982) and references cited therein]. However, the levels
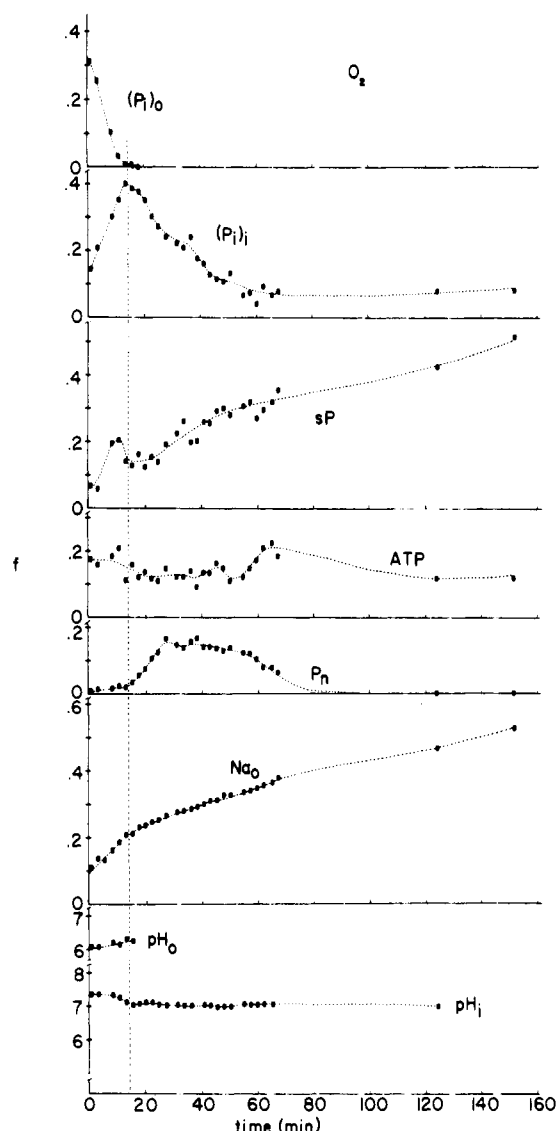
FIGURE 3: Time dependence of spectral data from the first experiment of Table I; that with aerobic (only) medium. The ordinates of the top five curves are the fractions of NMR-visible phosphorus. That of the next curve is the fraction of total Na^+ while those of the bottom two curves are pH values. The dashed lines are intended only to guide the eye. The vertical dashed line is drawn to mark the time of the end of the initial period of Na^+ efflux.
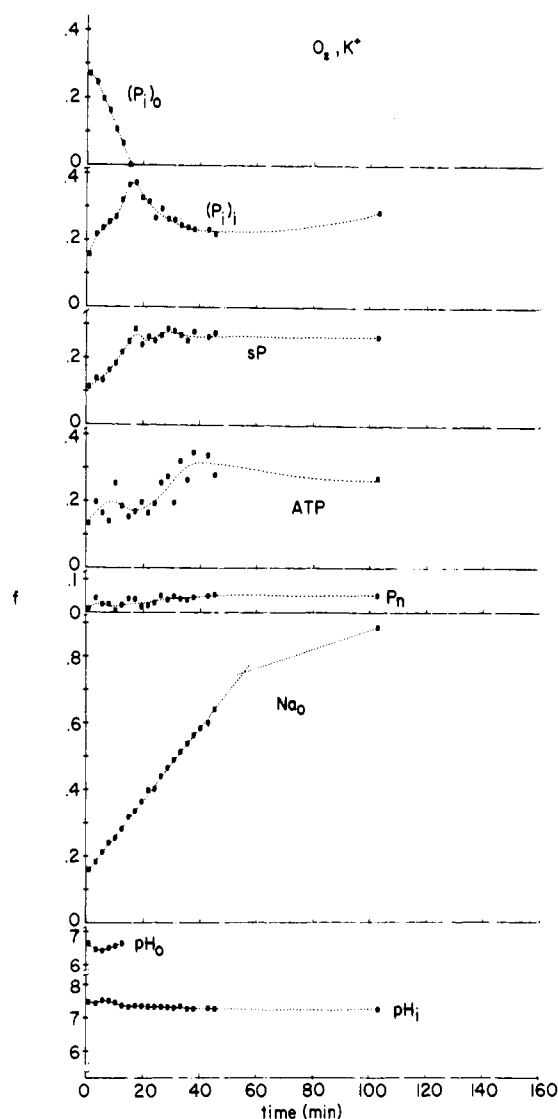
FIGURE 4: Time dependence of spectral data from the third experiment of Table I; that with aerobic, K^+-containing medium. Some spectra from this experiment are shown in Figure 1. The ordinates are the same as those of Figure 3. The dashed lines have the same significance as those of Figure 3. For the fifth curve, Na_o, the intersection of the two straight dashed lines indicates the suspected end of the initial period of Na^+ efflux.

Table II: Relative Initial Na^+ Effluxes

	N_2	O_2	K^+	glucose	glucose, buffer
N_2	1.0^a		0.98	2.0	1.6
O_2		1.6	2.2	1.7	1.7

^{a} Actual magnitude: ca. 11 μmol of Na^+ s^{-1} [kg (wet wt) of yeast]$^{-1}$.

of polyphosphate produced in our experiments are never so great that this represents a significant error (unless the average oligomeric state of the polyphosphate species is quite small). The only experiments not shown in Figures 3–8 are those under anaerobic conditions in unsupplemented standard medium (Balschi et al., 1984; Balschi, 1984) and in K^+-supplemented medium (Balschi, 1984). In both cases, the Na^+ efflux is slow (Table II), and the P_i influx is very slow.

Except for the O_2/K^+ experiment (Figure 4), the Na^+ efflux is not linear over most of the time course observed [this is also true for the two anaerobic experiments not shown (Balschi, 1984)]. An initial linear efflux averages 14.7 min and represents the extrusion of an average of 61.5 μmol of Na^+. The actual efflux values for the initial linear periods are given in

Table II. In other experiments, we have varied the initial external concentration of NH_4^+ (no K^+). The amount of Na^+ extruded during the initial linear efflux is proportional to the concentration of NH_4^+, but only if it is added as the $H_2PO_4^-$ salt. If NH_4Cl is added, in addition to $NH_4H_2PO_4$, the initial Na^+ efflux value is greater, but the amount of Na^+ extruded is still roughly equal to the amount of external P_i.

After the linear phase, Na^+ efflux either decreases (Figures 3 and 8), stops (Figures 6 and 7), or actually reverses (Figure 5) for only a short period which averages ca. 10 min. Subsequently, the cells enter another phase where the efflux usually resumes with a value similar to that of the initial phase. During this last phase, the cells lose much of their remaining sodium.

During the first phase of Na^+ efflux, P enters the cells from the medium. This usually results in a rapid net increase in the sP pool of P, except when glucose is not added to the medium (Figures 3 and 4). In these latter two cases, there is instead an initial net increase in the intracellular P_i pool. What sP is formed must come from the endogenous sugar reserves (glycogen and trehalose) which are mobilized initially
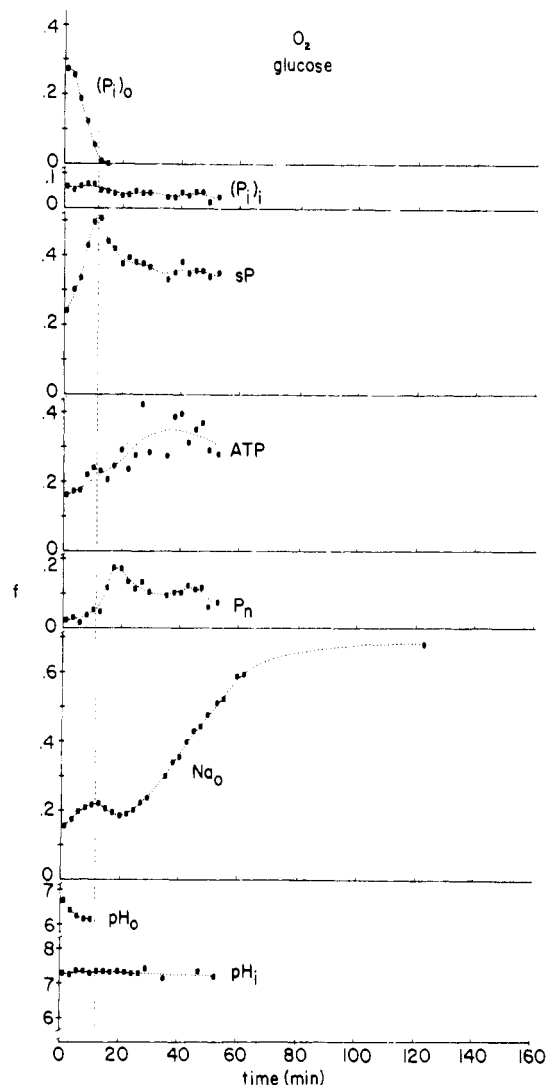
FIGURE 5: Time dependence of spectral data from the fifth experiment of Table I; that with aerobic, glucose-containing medium. Some spectra from this experiment are shown in Figure 2. The ordinates are the same as those of Figure 3. The dashed lines have the same significance as those of Figure 3.
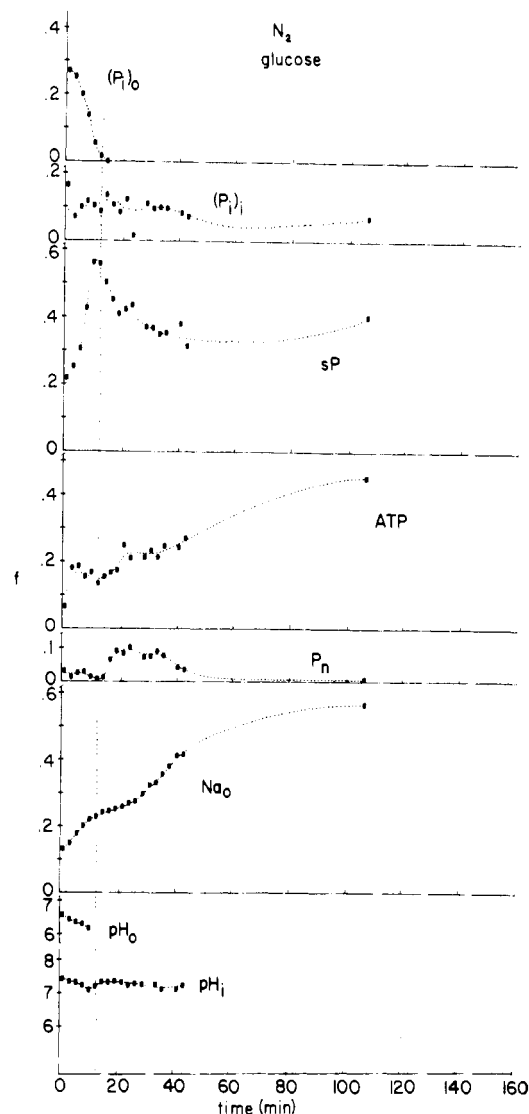

FIGURE 6: Time dependence of spectral data from the sixth experiment of Table I; that with anaerobic glucose-containing medium. The ordinates are the same as those of Figure 3. The dashed lines have the same significance as those of Figure 3.

by the availability of $(P_i)_i$ and later by P_n. In other experiments (non-NMR as well as NMR), we have found that when the yeast cells already have polyphosphate species present at the end of the Na^+-loading and washing period, the uptake of P_i is much slower.

The ATP level rises slightly during the course of these experiments [except for the O_2 (only) study (Figure 3)]. The level of ATP before commencement of gas bubbling is unknown. Certainly, in the two Na^+-efflux, P_i-influx experiments where neither O_2 nor glucose is provided, the ATP level of the yeast is barely detectable (Balschi, 1984; Balschi et al., 1984). However, in the aerobic (only) experiment, where the yeast were prepared identically, ATP is already present at an almost steady-state level when we obtain our first spectrum (Figure 3). In the other analogous experiments where either O_2 or glucose was present, the ATP level was also quite high already at the beginning (see Figures 1, 2, 4, 5, and 7). Either ATP was significant at the end of the Na^+-loading and washing period and dropped very quickly upon bubbling with N_2 or the ATP level was quite low at the end of the Na^+-loading and washing period and was quickly regenerated upon bubbling with O_2 or injection of glucose. We suspect the latter is the case because of the noticeable rise in ATP level from near zero

at the beginning of the two anaerobic experiments in glucose-containing media (Figures 6 and 8).

Perhaps the most interesting result observed in these studies is that polyphosphate species are usually formed during the course of the experiment [except in the O_2/K^+ case (Figure 4) and in the two anaerobic experiments not shown (Balschi et al., 1984; Balschi, 1984)]. There are three important generalities to be noted with regard to the formation of P_n. First, the P_n is never formed until after an initial lag period the length of which correlates rather well with that of the initial linear Na^+-efflux period [but also with the exhaustion of $(P_i)_o$]. During this time, of course, P is rapidly entering the cells. In other cases where the formation of polyphosphate species has been observed, the initiation of formation has been coincident with the beginning of plasmalemmal P influx (J. A. Balschi et al., unpublished results; Balschi, 1984; Gillies et al., 1981). Second, the P stored in the P_n species appears usually to derive mostly from the sP pool, as evidenced by very sharp drops in the level of the latter. This transformation is very rapid and has also been reported by Nicolay et al. [see Nicolay et al. (1983) and references cited therein]. Third, the formation of polyphosphate species is always transient. They are always ultimately consumed, and the final distribution of NMR-visible
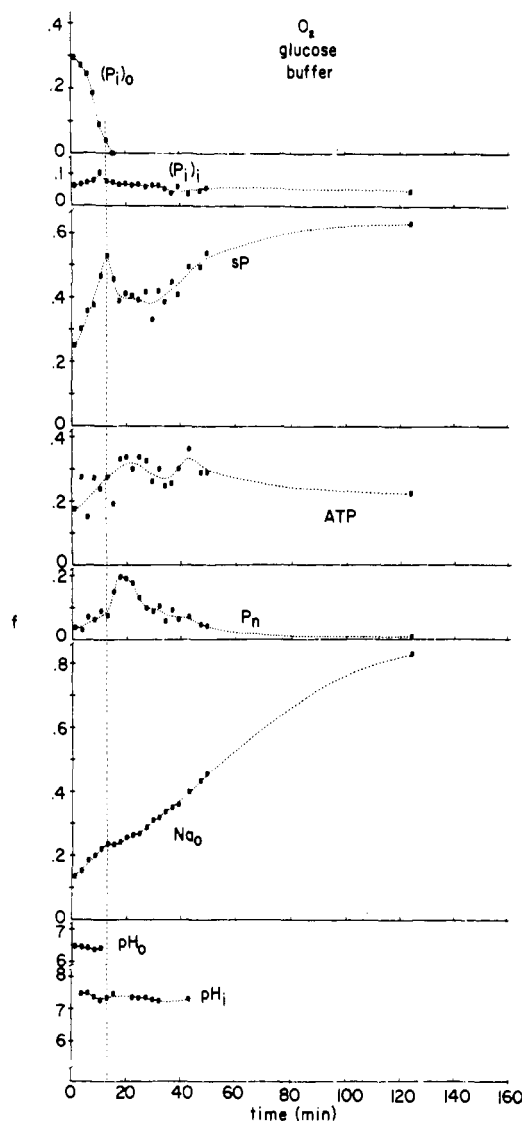
FIGURE 7: Time dependence of spectral data from the seventh experiment of Table I; that with aerobic, glucose-containing, buffered medium. The ordinates are the same as those of Figure 3. The dashed lines have the same significance as those of Figure 3.

cellular P is mostly in the sP pool (except in the experiment of Figure 4).

DISCUSSION

The Na^+-rich yeast prepared in this study were loaded with Na^+ in the absence of external phosphate (Rothstein, 1974). The cells are loaded to a level of ca. 100 mM Na^+; the intracellular K^+ concentration is reduced to about 30 mM (Rothstein, 1974).

The amount of Na^+ extruded during the initial linear efflux phase (61.5 μmol, on the average, see Results) is approximately equal to the amount of NH_4^+ and therefore also P_i (58.9 μmol, on the average) initially present in the extracellular medium (recall that NH_4^+ was added as the $H_2PO_4^-$ salt). We believe that this is significant and that the initial linear Na^+ efflux is the result of an energy-dependent one-for-one Na^+/NH_4^+ exchange process. The relative efflux values during this period are remarkably similar (1.6–2.2) for all experiments except the two where neither O_2 nor exogenous glucose is present (Table II). This latter fact is indicative of the energy requirements of this phase. The analogous Na^+/K^+ exchange has been shown to be energy dependent (Rodríguez-Navarro & Ortega, 1982), and Peña has recently shown that NH_4^+ can behave as a K^+ surrogate except that it probably enters the
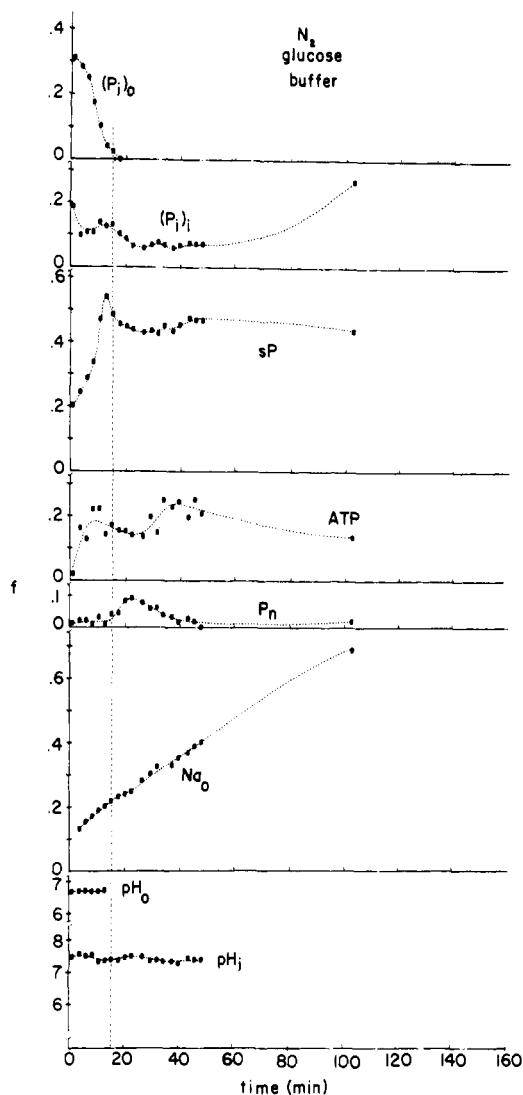

FIGURE 8: Time dependence of spectral data from the eighth experiment of Table I; that with anaerobic, glucose-containing, buffered medium. The ordinates are the same as those of Figure 3. The dashed lines have the same significance as those of Figure 3.

cell through a different pathway [Peña et al., 1987; see also Bogonez et al. (1983)].

The scheme in Figure 9 depicts many of the transport pathways known to be present in yeast (Borst-Pauwels, 1981) and which are probably employed for Na^+/NH_4^+ exchange. The influx of NH_4^+ down its electrochemical gradient, whether facilitated (process IX, Figure 9) as pictured by Peña or via some more complicated mechanism, would tend to depolarize the cell except for the nearly stoichiometric influx of $H_2PO_4^-$, most likely through the high-affinity proton–phosphate cotransporter (Borst-Pauwels, 1981) (process II, Figure 9). In any case, if intracellular NH_4^+ replaces intracellular Na^+, this would stimulate Na^+ extrusion via the Na^+/H^+ antiporter (process VII). To the extent that process VII is as (or more) electrogenic than the analogous Li^+/H^+ exchange (Rodríguez-Navarro & Ortega, 1982; Rodríguez-Navarro et al., 1981), it would tend to depolarize the cell [(Na^+/H^+) < 1). A typical yeast cytoplasmic membrane potential is ca. 80 mV (inside negative) (Boxman et al., 1982; Vacata et al., 1981). Thus, an NH_4^+ influx will cause a stimulation of the electrogenic $H^+/ATPase$ (process VIII) in an attempt not only to eliminate protons brought in by VII but possibly also to maintain the transplasmalemmal potential (Goffeau & Slayman, 1981; Borst-Pauwels, 1981).
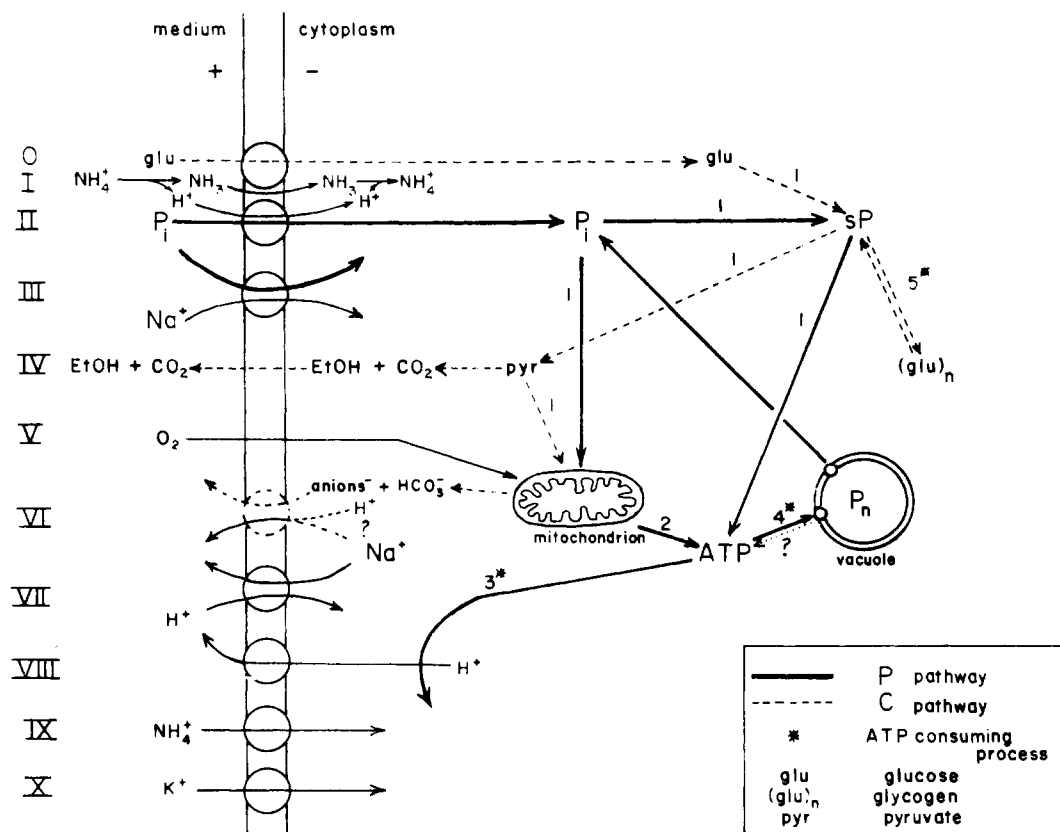
FIGURE 9: Some relevant transport and metabolic processes. Some numbers refer to metabolic processes indicated in Table III.

The above describes an energy-dependent ion-exchange process. Although the NH_4^+ transport is initially down its electrochemical potential gradient, almost all of the Na^+ extrusion is energetically uphill as long as the normal membrane potential is more or less maintained. We also detect a small amount of extracellular Na^+ at the beginning of the experiment (Figures 1 and 2; Balschi et al., 1984; Balschi, 1984), and we can calculate it to be 5 mM on the average by extrapolating the efflux curves back to time zero. This almost certainly results from insufficient washing of the cells after the Na^+-loading incubation. (Hopefully, the low temperature prevents significant Na^+ efflux until the sample is warmed in the NMR probe.) Thus, although the Na^+ efflux may not start against its electrochemical potential gradient, it must quickly became an uphill process. The combination of the electrogenic (?) NH_4^+ and $H_2PO_4^-$ transport processes, with the activities of the electrogenic $H^+/ATPase$, and the electrogenic (?) Na^+/H^+ antiporter produce an "effective Na^+/NH_4^+-ATPase". (Normally, this acts as an "effective Na^+/K^+-ATPase" employing process X.)

As mentioned under Results, recent experiments suggest that the NH_4^+ influx into Na^+-loaded yeasts is intimately tied to P_i influx. This may mean that the actual mechanism involves the transport of extra protons by the proton–phosphate cotransporter (process II, Figure 9) and the simple unfacilitated inward diffusion of NH_3, present at equilibrium (process I). Shift reagent ^{14}N NMR experiments have shown that unfacilitated diffusion of NH_3 across phospholipid bilayer membranes is extremely rapid (Balschi, 1984).

We believe that the energy dependence of the first phase provides the key to interpreting our unusual polyphosphate observations. In other cases, the formation of P_n species begins as soon as P begins entering the cell (J. A. Balschi et al., unpublished results; Balschi, 1984; Gillies et al., 1981). However, the storage of P in the form of P_n requires energy

[see Nicolay et al. (1983) and references cited therein]. This apparently cannot compete successfully with the ion transport processes described above. While the cells are using energy to exchange Na^+ for NH_4^+, they are "energy stressed" and seem not to be able to store their newly acquired P as polyphosphate. This is demonstrated most dramatically in the aerobic experiments. It is only when the extracellular NH_4^+ is exhausted that there is a low enough demand on ATP to allow P_n synthesis in these experiments. When this happens, the P comes mostly from the sP pool. When the medium is supplemented with 18.8 mM K^+, in addition to the 3.8 mM NH_4^+, the initial linear phase extrudes much more Na^+ [probably 5.9 times as much, $(18.8 + 3.8)/3.8$, Figure 4]. Both the Na^+/K^+ exchange and the Na^+/NH_4^+ exchange require that the cells expend energy. This is the only aerobic experiment where polyphosphate species are never significantly formed (Figure 4).

In addition to the kinetic considerations given above, the amounts of Na^+ pumped and P_n species transiently formed seem to qualitatively correspond to the amount of energy available to the cell. A qualitative ATP balance sheet is given in Table III. Energy is available to the cells from either endogenous or exogenous energy sources. Yeast cells use their endogenous reserves sparingly by comparison with exogenous energy sources so that the amount of energy from endogenous metabolism is much lower than from metabolism of exogenous substrates. Furthermore, although yeast cells can both ferment (process 1, Table III, Figure 9) and respire (process 2) their endogenous reserves, the energy derived from endogenous respiration is greater than that from endogenous fermentation (Sols et al., 1971). Externally added glucose can provide energy both from fermentation (glycolysis) and from respiration.

The competition between ion transport (process 3) and polyphosphate synthesis (process 4) in energy-limited cells is

Table III: Qualitative ATP Balance Sheet

experiment	relative amount of ATP production		relative amount of ATP consumption		
	fermentation (glycolysis) (1)^a	respiration (2)^a	Na$^+$/K$^+$ (NH$_4^+$) exchange (3)	P$_n$ synthesis (4)	glycogen synthesis (5)^a
N$_2$ (only)	1X^b (endogenous)		1X		
N$_2$, K$^+$	1X (endogenous)		1X		
N$_2$, glucose	7X		1X	2X	4X
O$_2$ (only)		5X (endogenous)	1X	4X	
O$_2$, K$^+$		5X (endogenous)	5X		
O$_2$, glucose		9X	1X	4X	4X

^{a} Expected, not measured. ^{b} The symbol X represents an unknown constant.

also observed in cells under anaerobic conditions (Table III). Thus, in anaerobic cells without exogenous glucose, in which ATP synthesis is low, only ion transport is observed. In anaerobic cells metabolizing glucose, the additional energy supports both ion transport and P$_n$ synthesis although the latter still only after the former is completed. While the results with cells under anaerobic conditions are consistent with this proposal, it is surprising that the amount of P$_n$ synthesized by cells metabolizing externally added glucose under anaerobic conditions (fermentation) is less than that by cells carrying out endogenous respiration (in the absence of external K$^+$) since the energy yield from such exogenous fermentation should be greater than that from endogenous respiration. The lower P$_n$ synthesis in the former case suggests that an additional energy-consuming process may be competing with P$_n$ synthesis. The most probable reaction would be glycogen synthesis (process 5); however, we have no direct evidence to support this suggestion.

In all of the experiments, there is still intracellular Na$^+$ to be eliminated when the extracellular NH$_4^+$ (and K$^+$, in the experiment of Figure 4) is exhausted. However, there is no longer any extracellular monovalent inorganic cation to be exchanged. Under these conditions, the mechanism preferred by the yeast is Na$^+$/anion coextrusion (Rothstein, 1974). We surmise that the last, mostly linear, efflux phase is the manifestation of this process (VI in Figure 9). It is curious that the value of this efflux is, in general, similar to that of the first phase. Perhaps the rate-limiting step is the same because Na$^+$ actually exists only via the same Na$^+$/H$^+$ antiporter, VII, and only the protonated anions by a separate process, VI. The anions required for extrusion, mostly succinate and bicarbonate (Goffeau & Slayman, 1981; Rothstein, 1974), must be produced by oxidative phosphorylation in the mitochondrion. Since the latter process requires the utilization of P$_i$, the P stored in P$_n$ after the end of the first phase of Na$^+$ efflux is mobilized as required during the course of the last phase. Since this latter phase is not energy requiring (Rothstein, 1974), the observed consumption of P$_n$ does not answer the important question as to whether ATP is produced upon hydrolysis of P$_n$.

During the transition between the first and last Na$^+$ efflux phases, the Na$^+$ efflux is transiently reduced. This is probably necessitated by the requirement for the buildup of sufficient levels of extrudable anions. In one curious case, the experiment with O$_2$ and glucose present in an unbuffered medium, a transient Na$^+$ influx actually occurs (Figure 5). (This has also been observed in a non-NMR experiment.) This cannot be due to the well-known Na$^+$/P$_i$ cotransporter (III) (Borst-Pauwels, 1981) since the extracellular P$_i$ is essentially exhausted by the time this occurs. This is also most likely not due to the Na$^+$/H$^+$ antiporter (VII) since the ^{23}Na spectrum indicates that the medium is quite acidic at this time. This is one of the two cases where the medium becomes considerably acidified during the first phase (the other is that of

Figure 6). Perhaps the cells become transiently hyperpolarized and the Na$^+$ is electrophoresed in through the K$^+$ (or NH$_4^+$) channels. Since this influx does not occur when buffer ions are present in the medium (Figure 7), perhaps the lower extracellular ionic strength extant in the experiment of Figure 5 allows the Na$^+$ ions to easily sense a transient hyperpolarization [possibly via surface potential effects (Borst-Pauwels, 1981)].

The fact that the intracellular P accumulated during the courses of these experiments is ultimately mostly stored in the sP pool is interesting. It may suggest that glycolytic intermediates accumulate upstream of a block in glycolysis (Thompson & Torchia, 1984). Although our analysis of the composition of the "sugar phosphate" pool is seriously hampered by our unavoidably poor spectral resolution, the accumulated sugar may contain large amounts of fructose diphosphate (FDP). The chemical shift observed is consistent with this possibility. This suggests that the cells carry out a form of in vivo Harden–Young reaction (eq 1) by which a

$$2P_i + 2 \text{ glucose} \rightarrow FDP + 2EtOH + 2CO_2 + 2H_2O \quad (1)$$

portion of the endogenous sugar reserves are metabolized and a portion converted to sugar phosphate. However, we have no hard data to support this suggestion.

ACKNOWLEDGMENTS

REFERENCES

Alger, J. R., den Hollander, J. A., & Shulman, R. G. (1982) Biochemistry 21, 2957–2963.

Balschi, J. A. (1984) Ph.D. Dissertation, State University of New York at Stony Brook, Stony Brook, NY.

Balschi, J. A., Cirillo, V. P., & Springer, C. S. (1982) Biophys. J. 38, 323–326.

Balschi, J. A., Jensen, D., Pike, M. M., Höfeler, H., Cirillo, V. P., Delayre, J. L., Springer, C. S., & Fossel, E. T. (1984) Magn. Reson. Med. 1, 96–97.

Bogonez, E., Machado, A., & Satrustegui, J. (1983) Biochim. Biophys. Acta 733, 234–241.

Borst-Pauwels, G. W. F. H. (1981) Biochim. Biophys. Acta 650, 88–127.

Boxman, A. W., Barts, P. W. J. A., & Borst-Pauwels, G. W. F. H. (1982) Biochim. Biophys. Acta 686, 13–18.

Brindle, K. M., Brown, F. F., Campbell, I. D., Grathwohl, C., & Kuchel, P. W. (1979) Biochem. J. 180, 37–44.

Burt, C. T., Cohen, S. M., & Barany, M. (1979) Annu. Rev. Biophys. Bioeng. 8, 1–25.

Chu, S. C., Pike, M. M., Fossel, E. T., Smith, T. W., Balschi, J. A., & Springer, C. S. (1984) J. Magn. Reson. 56, 33–47.

Cirillo, V. P. (1968) J. Bacteriol. 95, 603–611.

Delayre, J. L., Jensen, D., Baldo, J., Pike, M. M., & Fossel, E. T. (1984) Magn. Reson. Med. 1, 292.

den Hollander, J. A., Ugurbil, K., Brown, T. R., & Shulman, R. G. (1981) Biochemistry 20, 5871–5880.

Dürr, M., Urech, K., Boller, Th., Wiemken, A., Schwenke, J., & Nagy, M. (1979) Arch. Microbiol. 121, 169–175.

Fabry, M. E., & San George, R. C. (1983) Biochemistry 22, 4119–4125.

Gillies, R. J., Ugurbil, K., den Hollander, J. A., & Shulman, R. G. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2125–2129.

Goffeau, A., & Slayman, C. W. (1981) Biochim. Biophys. Acta 639, 197–223.

Gupta, R. K., & Gupta, P. (1982) J. Magn. Reson. 47, 344–350.

Jensen, D. (1983) M.S. Thesis, Rheinisch-Westfalische Technische Hochschule, Achen, Federal Republic of Germany.

Navon, G., Shulman, R. G., Yamane, T., Eccleshahl, T. R., Lam, K.-B., Baronofsky, J. J., & Marmur, J. (1979) Biochemistry 18, 4487–4499.

Nicolay, K., Scheffers, W. A., Bruinenberg, P. M., & Kaptein, R. (1982) Arch. Microbiol. 133, 83–89.

Nicolay, K., Scheffers, W. A., Bruinenberg, P. M., & Kaptein, R. (1983) Arch. Microbiol. 134, 270–275.

Ogino, T., den Hollander, J. A., & Shulman, R. G. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5185–5189.

Peña, A., Pardo, J. P., & Ramirez, J. (1987) Arch. Biochem. Biophys. 253, 431–438.

Pike, M. M., & Springer, C. S. (1982) J. Magn. Reson. 46, 348–353.

Pike, M. M., Fossel, E. T., Smith, T. W., & Springer, C. S. (1984) Am. J. Physiol. 246, C528–C536.

Rodriguez-Navarro, A., & Ortega, M. D. (1982) FEBS Lett. 138, 205–208.

Rodriguez-Navarro, A., Sancho, E. D., & Perez-Lloveres, C. (1981) Biochim. Biophys. Acta 640, 352–358.

Rothstein, A. (1974) J. Gen. Physiol. 64, 608–621.

Salhany, J. M., Yamane, T., Shulman, R. G., & Ogawa, S. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4966–4970.

Sols, A., Gancedo, C., & De la Fuente, G. (1971) in The Yeasts (Rose, A. H., & Harrison, J. S., Eds.) pp 271–307, Academic Press, New York.

Thompson, J., & Torchia, D. A. (1984) J. Bacteriol. 158, 791–800.

Ugurbil, K., Rottenberg, H., Glynn, P., & Shulman, R. G. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2244–2248.

Vacata, V., Kotyk, A., & Sigler, K. (1981) Biochim. Biophys. Acta 643, 265–268.

Magnetic Resonance Studies on the Copper Site of Dopamine β-Monooxygenase in the Presence of Cyanide and Azide Anions

Atsuo Obata, Hisao Tanaka, and Hiroshi Kawazura*

Faculty of Pharmaceutical Sciences, Josai University, Keyaki-dai 1-1, Sakado, Saitama 350-02, Japan

ABSTRACT: In order to elucidate the coordination state of water molecules in the Cu(II) site of dopamine [(3,4-dihydroxyphenyl)ethylamine] β-monooxygenase, measurements of the paramagnetic ^{1}H nuclear magnetic relaxation rate of solvent water in the enzyme solution containing cyanide or azide as an exogenous ligand were carried out to obtain the values of intrinsic paramagnetic relaxation rate decrements R_p^1 and R_p^2 for the ligand–enzyme 1:1 and 2:1 complexes, respectively. R_p^1 (percent) values were 53 (pH 5.5) and 52 (pH 7.0) for cyanide and 38 (pH 5.5) and 32 (pH 7.0) for azide, while R_p^2 (percent) values were 98 (pH 5.5) and 96 (pH 7.0) for azide. Although no R_p^2 values for cyanide were obtained because of its reducing power at the Cu(II) site, the R_p^1 and R_p^2 values obtained above prove that the Cu(II) center has two coordinated water molecules that are exchangeable for exogenous ligands at either pH. Supporting evidence was provided by electron paramagnetic resonance (EPR) titration, in which the enzyme solution containing cyanide–enzyme (1:1) complex in an equal proportion to uncomplexed enzyme gave an observed paramagnetic relaxation rate decrement, R_p, of 23%. Another characteristic of the R_p^1 and R_p^2 values was their invariability with respect to pH, indicating that the three-dimensional structure of the Cu(II) site is pH-invariant within the range examined. Binding constants of ligand to enzyme K_b^1 and K_b^2 for 1:1 and 2:1 complex formation, respectively, were also determined through an analysis of the R_p values; it was found that K_b^1 was larger than K_b^2 irrespective of pH. On the basis of these results, together with the axial-symmetric EPR parameters of the 1:1 complexes, a possible coordination geometry of the two water molecules in the Cu(II) site of the enzyme is suggested.

Dopamine β-monooxygenase (EC 1.14.17.1), an indispensable copper enzyme in the human brain, regulates neurotransmitter levels by catalyzing the conversion of dopamine to norepinephrine (Rosenberg & Lovenberg, 1980). Although

numerous studies have shown that the copper moiety in the enzyme is essential for the catalysis (Villafranca, 1981), the structure of the copper site has not yet been established. Indeed, even after a recent extended X-ray absorption fine