^{31}P NMR Measurements of the ADP Concentration in Yeast Cells Genetically Modified To Express Creatine Kinase†

Kevin Brindle,* Peta Braddock, and Sandra Fulton
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, England
Received March 13, 1989; Revised Manuscript Received November 8, 1989

ABSTRACT: Rabbit muscle creatine kinase has been introduced into the yeast *Saccharomyces cerevisiae* by transforming cells with a multicopy plasmid containing the coding sequence for the enzyme under the control of the yeast phosphoglycerate kinase promoter. The transformed cells showed creatine kinase activities similar to those found in mammalian heart muscle. ^{31}P NMR measurements of the near-equilibrium concentrations of phosphocreatine and cellular pH together with measurements of the total extractable concentrations of phosphocreatine and creatine allowed calculation of the free ADP/ATP ratio in the cell. The calculated ratio of approximately 2 was considerably higher than the ratio of between 0.06 and 0.1 measured directly in cell extracts.

The free ADP in the cell is an important effector and substrate of a number of enzymes on the pathways of ATP production and utilization, and its concentration is a determinant of the cellular energy charge (Atkinson, 1977). Methods for determining the free ADP concentration in vivo are, therefore, of great value in the study of cellular bioenergetics. A powerful feature of the ^{31}P NMR experiment is its facility, in those systems possessing creatine kinase, to measure the free ADP concentration (Lawson & Veech, 1979). Creatine kinase (EC 2.7.3.2) catalyzes the reaction:

$$PCr^{2-} + MgADP^{-} + H^{+} \rightleftharpoons Cr + MgATP^{2-}$$

^{31}P NMR measurements of the phosphocreatine (PCr)[1] and ATP concentrations, the pH [from the chemical shift of the P_i resonance (Moon & Richards, 1973)], and the free Mg^{2+} concentration [from the chemical shifts of the ATP resonances (Gupta et al., 1983)], together with measurements of the total extractable concentrations of creatine (Cr) plus phosphocreatine, allow calculation of the near-equilibrium free ADP concentration in the cell. These measurements have been made, for example, in mammalian heart and skeletal muscle (Matthews et al., 1982; Meyer et al., 1982; Shoubridge et al., 1984).

The introduction of creatine kinase into cells which do not normally express the enzyme should allow noninvasive ^{31}P NMR measurements of the free ADP concentration in these cells as well. We describe here the introduction of creatine kinase into the yeast *Saccharomyces cerevisiae*. Transformation of the cells with a multicopy plasmid containing the coding sequence for the rabbit muscle enzyme, under the control of the yeast PGK promoter, resulted in intracellular creatine kinase concentrations similar to those found in mammalian heart muscle. Incubation of the cells with creatine resulted in creatine uptake and phosphorylation to produce phosphocreatine. ^{31}P NMR measurements of the phosphocreatine and H^{+} concentrations in vivo allowed calculation of the free ADP/ATP ratio in the cell. However this ratio was considerably higher than the ratio measured directly in cell extracts and the ratio calculated from the near-equilibrium concentrations of the substrates of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (Brindle, 1988a). The possible reasons for this marked discrepancy, which implies some form of unexpected compartmentation of the enzyme's substrates in the cell, are discussed.

The introduction of rat brain creatine kinase into *Escherichia coli*, as a fusion protein with β-galactosidase, has recently been described (Koretsky & Traxler, 1989). Incubation of the cells with creatine resulted in the production of phosphocreatine, the concentration of which was shown to be sensitive to the energy status of the cells. However, no correlation was made between the intracellular phosphocreatine concentration and the ADP/ATP ratio.

Materials and Methods

Restriction enzymes and oligonucleotide linkers were purchased from Gibco BRL and used as directed by the manufacturer. NAD^{+}, $NADP^{+}$, ATP, ADP, AMP, phosphocreatine, phosphoenolpyruvate, lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.10), hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), creatine kinase, and triethanolamine hydrochloride were obtained from Boehringer Mannheim. Bovine hemoglobin, creatine, acetyl-CoA, oxaloacetate, low gelling temperature agarose, Mes, Mops, Hepes, amino acids, NADH, diadenosine pentaphosphate, and the other nucleoside phosphates used to assign resonances in cell extracts were obtained from Sigma. Yeast nitrogen base was from Difco. Glass beads (40 mesh) were from BDH. All other reagents were of analytical grade.

Plasmid Construction. The coding sequence for rabbit muscle creatine kinase was obtained as two overlapping cDNAs, in plasmids pCKM19 and pCKM15 (Putney et al., 1984). The cDNAs were a gift from Prof. Paul Schimmel, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. The two segments of the coding sequence were joined at the unique *Bgl*II site (Putney et al., 1984) and the 5′ noncoding DNA removed by Bal 31 deletion to leave five bases in front of the start ATG codon. *Bam*HI oligo-

† This work was supported by the Medical Research Council of Great Britain and by the Royal Society. K.B. was the recipient of a 1983 University Research Fellowship from the Royal Society, and P.B. received financial support from the Science and Engineering Research Council.

[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); PGK, phosphoglycerate kinase (EC 2.7.2.3); NTP, nucleoside triphosphate(s); NDP, nucleoside diphosphate(s); PCr, phosphocreatine; Cr, creatine; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; T_1, spin–lattice relaxation time; HPLC, high-performance liquid chromatography.

0006-2960/90/0429-3295$02.50/0

nucleotide linkers (CCGGATCCGG) were introduced at the 5′ end and at the *Sma*I site in the 3′ nontranslated region. Plasmid pBF9 contains the modified coding sequence inserted into the *Bgl*II site of the yeast expression vector pMA91 (Mellor et al., 1983), where it is under the control of the yeast PGK promoter and terminator sequences. Plasmid DNA was prepared as described by Chinault and Carbon (1979). The plasmid was used to transform the yeast strains described below to leucine prototrophy (Hinnen et al., 1978).

Plasmid Rescue. Plasmids were rescued from large-scale yeast culture by the method of Nasmyth and Reed (1980; Ferguson et al., 1981) and transformed into *E. coli* strain MC1061 (F′, *araD139*, Δ(*lacIPOZYA*) *X74* Δ(*araABOIC -leu*) *7697*, *galk*, *hsdR*, *hsdM*, *rpsL*) (Maniatis et al., 1982). DNA from 12 transformants was digested with *Hin*dIII/*Bgl*II and *Pst*I. Agarose gel electrophoresis of the digests gave banding patterns identical with those of the original plasmid, showing that there had been no gross plasmid rearrangement.

Preparation of Cells for NMR Experiments. Cells of the *Saccharomyces cerevisiae* strains DBY745 (α, *ura* 3-52, *ade* 1-100, *leu* 2-3, *leu* 2-112), AH22 (a, *his* 3-52, *leu* 2-3, *leu* 2-112), and BJ4581 (a, *leu* 2, *vph* 1-1) were grown aerobically at 30 °C on a synthetic medium containing 6.7 g/L yeast nitrogen base, 2% glucose, and an amino acid mixture lacking leucine. The main cultures were inoculated from precultures at 4×10^5 cells/mL and harvested 24 h later. The cells were transformed with plasmid pBF9, which complemented the *leu* 2 mutations. The plasmid construction is described above. The vacuolar mutant, BJ4581, was a gift from Dr. Rob Preston and Dr. Beth Jones, Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA. *vph* 1-1 denotes a vacuolar mutation which was detected by vacuolar staining with carboxyfluorescein (Preston et al., 1989). Cells were analyzed for plasmid stability as described above. Cell harvest, immobilization in agarose, and perifusion in the magnet were carried out as described previously (Brindle & Krikler, 1985). The cells, a total of 4 g wet weight (unless otherwise stated), were perifused at 30 °C in a total sample volume of 17 mL with a buffer containing 2 mM $MgSO_4$, 1.7 mM NaCl, 2 mM KCl, 50 mM glucose, and 50 mM Mes. The pH was adjusted to 6.0 with NaOH.

NMR Measurements on Cells. The experiments were performed on a Bruker AM300 spectrometer, operating at a ^{31}P NMR frequency of 121.5 MHz using a home-built probe made to accommodate 20-mm-diameter NMR tubes. All measurements were made at 30 °C. Spectra were acquired into 2048 data points with a sweep width of 8 kHz, a 90° pulse, and a recycle delay of 5.127 s, unless otherwise indicated. The free induction decays were zero-filled to 4096 data points prior to Fourier transformation. Intracellular metabolite concentrations were determined by comparing their resonance intensities with that of a methylenediphosphonate (MDP) standard contained in a coaxial capillary suspended in the center of the NMR tube, as described previously (Brindle & Krikler, 1985; Brindle, 1988a). Resonance assignments in the cell spectra were made by comparing them with previously published spectra (den Hollander et al., 1981, 1986; Navon et al., 1979) and by assigning the resonances in cell extracts by adding the compound to the extract. Resonance intensities were determined by cutting the peaks out of the plotted spectra and weighing them or by use of the integration routine in the computer software. In some cases (where specified), the peaks were first fitted to Lorenztian line shapes and then integrals taken from the fitted lines. Intracellular concentrations were calculated by assuming that 1.67 g of wet yeast contains 1 mL of cell water (Gancedo & Gancedo, 1973). Intracellular pH was estimated from the chemical shift difference between the P_i and MDP resonances. Intracellular free Mg^{2+} concentration was estimated from the chemical shift difference between the α- and β-phosphate resonances of the nucleoside triphosphates, as described by Gupta et al. (1983). The predominant nucleoside triphosphate in the yeast strains used here is ATP (see Table III). Magnetization transfer measurements were carried out as described previously (Brindle, 1988a; Brindle & Radda, 1985) and in the legend to Figure 3.

Measurements of Metabolite Concentrations in Cell Extracts. Cell extracts were prepared by emptying the contents of the NMR tube into a mortar cooled with liquid nitrogen. The frozen cell/agarose matrix was then ground to a powder, and 5 mL of 30% ice-cold perchloric acid was added. The mixture was then reground and freeze-thawed 3 times in liquid nitrogen. The resulting precipitate was removed by centrifugation at 4 °C. EDTA was then added to give a concentration in the final neutralized extract (total volume 45 mL) of 10 mM. The extract was then adjusted to pH 6.0 with K_2CO_3 and the precipitate removed by centrifugation at 4 °C. This procedure is similar to methods described previously (den Hollander et al., 1986; Sáez & Lagunas, 1976). Inclusion of EDTA prior to neutralization was found to give higher ATP/ADP ratios in the final extract. The neutralized extract was poured down a Chelex column and lyophilized for NMR and HPLC measurements. For both sets of measurements, the lyophilized material was redissolved in 5 mL of a buffer containing 50 mM triethanolamine buffer, pH 8.0, and 1 mM EDTA. The pH was adjusted to 8.0 with dilute NaOH.

NMR cell extract spectra were acquired into 8192 data points with a sweep width of 8 kHz, a 60° pulse, and a recycle delay of 4.127 s. Increasing the recycle delay to 8.127 s had no significant effect on the resonance intensities from the extract. The spectra were acquired with composite pulse decoupling during the acquisition period with a power level of 1 W. The free induction decays were zero-filled to 16 384 data points prior to Fourier transformation. A line broadening of 1 Hz was applied. The samples were not spun. The concentrations of the metabolites were determined either by adding a known concentration of the compound to the extract and recording a second spectrum or by comparing the intensities of the compound's resonances with the resonance intensity of an MDP standard contained in a coaxial capillary. Both methods of determining concentration gave similar values.

HPLC measurements were made by using a 25-cm Whatman Partisil 10-SAX column. The nucleoside phosphates were eluted from the column by using a two-step linear phosphate gradient (Harmsen et al., 1982) and were detected by measuring their absorbance at 254 nm. The assignment of peaks and the determination of concentrations were made by running interleaved standards.

Creatine Uptake Measurements. Creatine uptake was measured by incubating 0.5-g aliquots of cells in 50 mL of Mes buffer containing 100 mM creatine. The other components of the buffer are described above. The cells were spun down at various time intervals, and the cell pellet was extracted with 0.5 mL of 30% perchloric acid. The resulting precipitate was removed by centrifugation and the supernatant adjusted to pH 6.0 with K_2CO_3. The precipitate was removed by centrifugation and the supernatant assayed for creatine. The creatine concentration was determined enzymatically as described by Bernt et al. (1974). The creatine measured in the cell pellet at $t = 0$ was assumed to represent creatine trapped in the cell pellet and was subtracted from all subsequent measurements

at longer incubation times. In a single transport measurement with the yeast strain AH22, the cells were separated from the incubation medium by rapid filtration through a 0.45-μm pore diameter Millipore filter, as described by Grenson et al. (1986). Rapid washing of the cells with cold water on the filter removed all creatine trapped extracellularly. The kinetics of creatine uptake measured by this method were identical with those determined by the centrifugation method described above.

Creatine Kinase Assay. The enzyme was assayed spectrophotometrically at 25 °C, essentially as described by Oliver (1955). The assay system contained 50 mM Mops buffer, pH 6.7, 0.75 mM NADP, 1 mM dithiothreitol, 2 mM glucose, 10 mM $MgCl_2$, approximately 1 unit/mL hexokinase and glucose-6-phosphate dehydrogenase, 2 mM ADP, 20 mM phosphocreatine, and between 5 and 10 μL of cell extract. The reaction was initiated by the addition of phosphocreatine. Diadenosine pentaphosphate (50 μM) was added in order to inhibit adenylate kinase activity. Yeast cell extracts were prepared by freeze-thawing the cells in liquid nitrogen at a cell density of 0.2 g/mL in a buffer containing 25 mM phosphate, pH 7.0, 1 mM EDTA, and 2 mM dithiothreitol. The cells were then vortexed for 2 min with 40-mesh siliconized glass beads and then freeze-thawed again. Cell debris was removed by centrifugation on an Eppendorf microcentrifuge for 10 min at 4 °C. The rat heart extract was prepared by first briefly perfusing the heart in the Langendorf mode in order to remove blood. Following perfusion, the heart was quickly blotted to remove excess buffer and weighed. The heart was then placed in 10 mL of an ice-cold solution containing 50 mM triethanolamine hydrochloride buffer (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, and 20 mM 2-mercaptoethanol. The heart was homogenized with a Polytron homogenizer, and the resulting homogenate was centrifuged on an Eppendorf microcentrifuge for 2 min at 4 °C. The supernatant was assayed for creatine kinase activity.

Determination of the Cellular Location of Creatine Kinase. The cellular location of the enzyme was determined as described by Kovâc et al. (1986). Briefly, spheroplasts were treated with DEAE-dextran at a concentration of 1.2 pg/spheroplast. The spheroplasts were then spun down, and the supernatants and detergent-solubilized pellets were assayed for creatine kinase, pyruvate kinase, citrate synthase, and protease A activities. Protease A, which is a marker for the vacuole, was assayed as described by Lenney (1975); citrate synthase, which is a marker for the mitochondrial matrix, was assayed as described in Coore et al. (1971); and pyruvate kinase, which is a marker for the cytoplasm, was assayed as described in Barwell and Hess (1972).

Results and Discussion

Transformation of the three different yeast strains used in this study with the creatine kinase expressing vector, pBF9, which is described under Materials and Methods, produced intracellular creatine kinase activities similar to those found in the rat heart (Table I). All the cells in the cell population will have a relatively high plasmid copy number as this 2μ-based plasmid has a high copy number (Mellor et al., 1985) and in strains containing endogenous 2μ circles there is no segregation bias during cell division (Murray & Szostak, 1983). That all the cells retain the plasmid was shown by comparing the number of colonies on leucine-minus and leucine-plus plates. This experiment showed that plasmid retention was effectively 100%. Furthermore, plasmid rescue and subsequent restriction enzyme digest showed that the plasmid was retained without significant rearrangement.

Table I: Creatine Kinase Activities Measured in the Rat Heart and in the Three Plasmid-Transformed Yeast Strains Used in This Study[a]

	creatine kinase act. (units/mL of cell water)
rat heart	1506
DBY745	1042
AH22	2460
BJ4581	1615

[a] Extraction of the enzyme from the rat heart and the yeast cells is described under Materials and Methods. The cellular activities were calculated by assuming a cytosolic water content of 0.44 mL/g wet heart tissue (Kauppinen et al., 1980) and that 1.67 g of wet yeast contains 1 mL of cell water (Gancedo & Gancedo, 1973). The values shown are the mean of two determinations.

Therefore, all the cells in the cell population can be expected to contain relatively high concentrations of creatine kinase.

The enzyme appears to have a predominantly cytoplasmic location. Treatment of spheroplasts with DEAE-dextran, a procedure which has been shown to disrupt the plasma membrane but not the membranes of intracellular organelles such as mitochondria and vacuoles (Kovâc et al., 1986), resulted in coelution of pyruvate kinase, which is a marker for the cell cytoplasm, and creatine kinase. At a DEAE-dextran concentration of 1.2 pg/spheroplast, there was up to 70% release of pyruvate kinase and creatine kinase from the spheroplasts while only 10% of the mitochondrial enzyme citrate synthase and 19% of the vacuolar enzyme protease A were released. These values are similar to those found previously for cytoplasmic, mitochondrial, and vacuolar enzymes (Kovâc et al., 1986).

Perifusion of the immobilized cells with creatine, in the presence or absence of 1 mM P_i, resulted in the appearance of a phosphocreatine resonance in the ^{31}P NMR spectrum of the cells (Figure 1). The resonance was assigned on the basis of its chemical shift and by the addition of phosphocreatine to cell extracts. The appearance of the phosphocreatine resonance paralleled the measured uptake of creatine into the cells (Figure 2). Creatine uptake was measured in dilute cell suspensions incubated with creatine (see Materials and Methods and the legend to Figure 2). Measurements of the creatine content of the immobilized cells used for the NMR experiments was difficult due to the rapid loss of creatine from the cells on cell washing. Washing the immobilized cells in the NMR sample tube, total volume of sample 17 mL (see Materials and Methods), with a single volume of ice-cold buffer lacking creatine gave a creatine concentration in the wash fluid of 18 mM and a calculated intracellular creatine concentration of 65 mM, which is similar to the concentration measured in cell suspensions (see Figure 2). However, washing the immobilized cells with 4 volumes of buffer resulted in a creatine concentration in the final wash of 2.2 mM and a calculated intracellular concentration of 32 mM. Perifusing cells with creatine-free or glucose-free buffer also resulted in the rapid disappearance of the phosphocreatine resonance from the ^{31}P NMR spectrum of the cells (data not shown). The creatine uptake data in Figure 2 indicate that the final creatine concentration attained in the cells is approximately equal to the extracellular creatine concentration, although the concentration in the vacuolar mutant BJ4581 appears to be slightly lower than in the other two strains. The nature of the transport system responsible for creatine uptake is not clear, although it does not appear to involve the arginine transporter. Strain AH22 is canavanine resistant due to a defective arginine transporter (Grenson et al., 1966) but is still capable of taking up creatine (see Figure 2). This is also consistent with arginine

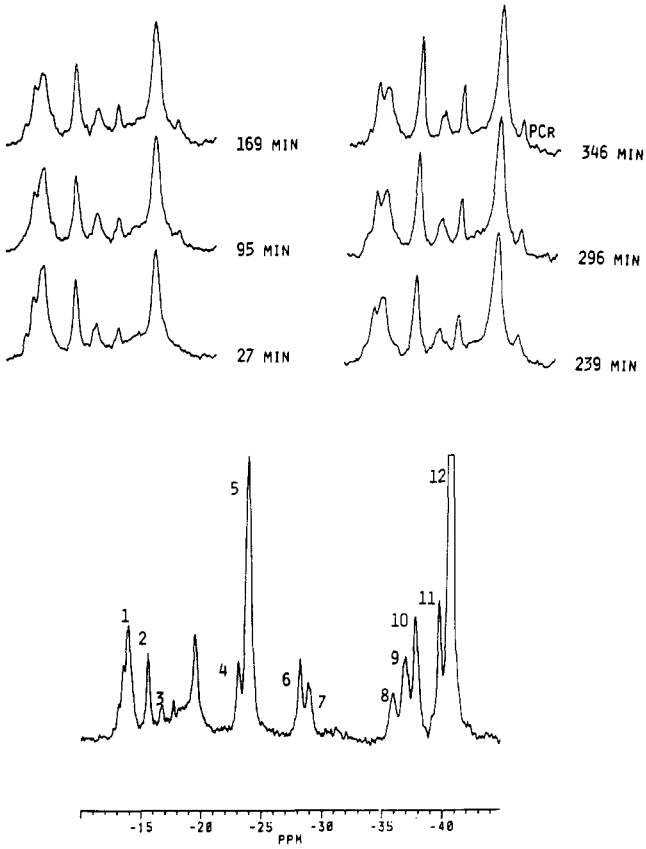

FIGURE 1: ^{31}P NMR spectra of immobilized cells showing the appearance of a phosphocreatine resonance following perifusion of the cells with 100 mM creatine. The bottom spectrum shows the complete ^{31}P NMR spectrum of the cells (strain DBY745). The full height of the resonance from the inner phosphates of long-chain polyphosphates at 41 ppm is not shown in this plot. The upper spectra show the expanded region between −12.2 and −22.6 ppm at sequential times following the start of cell perifusion with 100 mM creatine at $t = 0$ min. The phosphocreatine resonance, which appears at −20.7 ppm, is marked in the spectrum obtained at $t = 346$ min. The spectra are each the sum of 1280 scans collected in 2048 data points with an interpulse delay of 2.344 s and a sweep width of 8 kHz (see Materials and Methods). The chemical shift scale is referenced to the resonance of an MDP standard contained in a coaxial capillary. An exponential line broadening of 10 Hz was applied. The resonances were assigned by identifying the resonances in cell extract spectra (see Table II) and by comparing the cell spectra with previously published spectra (den Hollander et al., 1981, 1986; Navon et al., 1979). The resonance assignments are as follows: 1, sugar phosphates; 2, cytoplasmic P_i; 3, tentatively assigned to vacuolar P_i on the basis of its chemical shift with respect to the cytoplasmic P_i (Alger et al., 1982; Nicolay et al., 1982); 4, γ-phosphates of nucleoside triphosphates and the β-phosphates of nucleoside diphosphates; 5, terminal phosphates of polyphosphates including those from $P_3O_{10}^{5-}$ and $P_2O_7^{4-}$; 6, α-phosphates of nucleoside di- and triphosphates; 7, NAD(H); 8, 10, and 11, penultimate phosphates of polyphosphate chains including the middle phosphate of $P_3O_{10}^{5-}$; 9, β-phosphate of nucleoside triphosphates; 12, inner phosphates of long-chain polyphosphates.

transport studies which have shown that creatine has no inhibitory effect on arginine transport (Grenson et al., 1966). The transport of creatine into the cells may be non-carrier-mediated since a nonsaturable component for creatine transport has been demonstrated in mammalian cells (Loike et al., 1986).

The relatively high activity of creatine kinase measured in cell extracts indicates that the enzyme may be catalyzing a reaction that is near equilibrium. This is a necessary prerequisite if the enzyme-catalyzed reaction is to be used as an intracellular "indicator" for the free ADP/ATP ratio. The activity expressed by the enzyme in the cell was shown to be high by measuring the flux between phosphocreatine and ATP

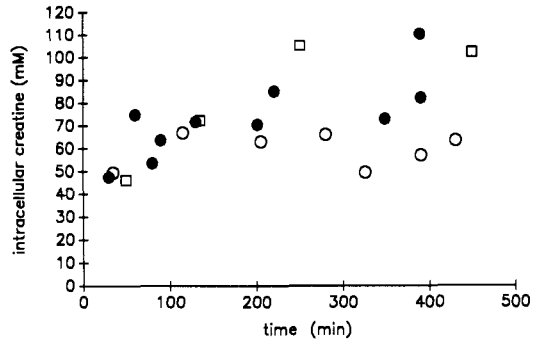

FIGURE 2: Creatine uptake in the three different plasmid-transformed yeast strains used in this study. The plot shows intracellular creatine concentration in DBY745 (●), AH22 (□), and BJ4581 (○), versus time of incubation at 30 °C. The uptake measurements are described in detail under Materials and Methods. Briefly, 0.5-g aliquots of cells were incubated in 50 mL of buffer containing 100 mM creatine. After increasing time periods of incubation, the cells were spun down, and the creatine concentration was measured in the cell pellet. The concentration of creatine in the extracellular space was determined by spinning the cells down immediately after addition to the creatine-containing medium, assuming that during the time taken for centrifugation there had been negligible transport. The concentration of extracellular creatine determined in this way was subtracted from all subsequent measurements at longer incubation times. This measurement was repeated for each series of transport measurements and was found to be similar in all experiments. Therefore, for each series of transport measurements, there is a zero point at $t = 0$ on the graph (data point not shown). The concentrations in the cell water were calculated by assuming that 1.67 g wet yeast contains 1 mL of cell water (Gancedo & Gancedo, 1973).

in the cell using ^{31}P NMR magnetization transfer measurements. Saturation of the γ-phosphate resonance of nucleoside triphosphates results in a marked decrease in the intensity of the phosphocreatine resonance (Figure 3). The magnitude of this decrease can be used to estimate a lower limit for flux in the creatine kinase reaction [see, for example, Brindle and Radda (1985)]. Assuming an intrinsic T_1 for phosphocreatine of 5 s (Shoubridge et al., 1984) and that the noise in Figure 3B represents residual phosphocreatine signal, then the data shown in Figure 3 indicate that the flux between phosphocreatine and ATP is at least 2.3 mM s^{-1}. Magnetization transfer and isotope exchange measurements on the isolated enzyme in vitro (Brindle & Radda, 1985) have shown that flux in this partial exchange reaction can be equated with the net flux through the overall enzyme-catalyzed reaction. This flux can be compared, therefore, with ^{31}P NMR and oxygen consumption measurements of net ATP turnover in yeast of between 1 and 3 mM s^{-1} (Brindle & Krikler, 1985; Campbell-Burk et al., 1987; Brindle, 1988a). The decrease in the P_i resonance intensity is due to exchange between ATP and P_i (Brindle & Krikler, 1985; Campbell-Burk et al., 1987; Brindle, 1988a). The magnitude of this decrease (see Figure 3) indicates a $P_i \rightleftarrows$ ATP exchange flux of 2.7 mM s^{-1}, assuming an intrinsic T_1 for the P_i resonance of 0.8 s (Campbell-Burk et al., 1987). Even if the PCr $\rightleftarrows$ ATP exchange flux is not in considerable excess of the $P_i \rightleftarrows$ ATP flux, the phosphocreatine/creatine ratio may still be near-to-equilibrium with the ATP/ADP ratio in the reaction catalyzed by creatine kinase, if the ATP/ADP ratio is maintained at a constant steady-state level.

The intracellular concentration of P_i, phosphocreatine, and ATP and the pHs measured by ^{31}P NMR in intact cells of the three different yeast strains used in this study are summarized in Table II. The intracellular concentrations determined by high-resolution ^{31}P NMR and HPLC measurements on cell extracts are given in Table III. The HPLC and ^{31}P NMR measurements on the cell extracts show reasonable agreement.

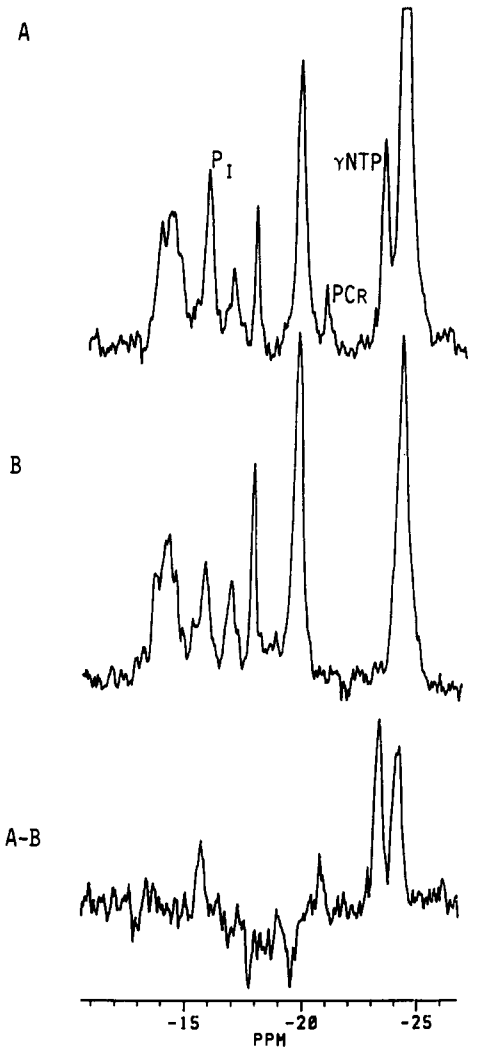

FIGURE 3: Steady-state saturation transfer measurements showing the effects of exchange between phosphocreatine and the γ-phosphate of ATP and between P_i and the γ-phosphate of ATP. An expanded region of the ^{31}P NMR spectrum of DBY745, between −11 and −26 ppm, is shown (see Figure 1). The chemical shift scale is referenced to the resonance of an MDP standard contained in coaxial capillary. The spectra are each the sum of 256 scans, collected in interleaved blocks of 16 scans into 2048 data points with a 90° pulse, an interpulse delay of 5.127 s, and a sweep width of 8 kHz. An exponential line broadening of 10 Hz was applied. Spectrum B was acquired with selective saturation of the γ-phosphate resonance of ATP (see Materials and Methods). Spectrum A was a control in which the saturating field was applied at a frequency downfield of the phosphocreatine resonance equal to the frequency difference between the resonances of phosphocreatine and the γ-phosphate of ATP. Spectrum A − B is the difference spectrum. The negative peaks in this spectrum are due to the saturating effects of the control irradiation.

For example, the total purine nucleoside triphosphate concentration determined by ^{31}P NMR in DBY745 is 2.7 mM compared with 2.6 mM determined by HPLC. The HPLC measurements show that 0.5 mM is GTP and 2.1 mM is ATP. The ^{31}P NMR resonances of these two nucleoside phosphates were not resolved. The total concentration of the nucleoside phosphates, excluding the monophosphates, measured in a cell extract from DBY745 showed good agreement with the concentration determined by ^{31}P NMR in vivo. The concentration determined by ^{31}P NMR in the cell extract was 4.7 mM compared with 4.6 mM determined by ^{31}P NMR in vivo at the time of cell extraction (see Tables II and III). The total concentration of the nucleoside phosphates was estimated in vivo and in vitro from the intensity of their unresolved α-phosphate resonances (Figure 1). The agreement between the concentrations determined in vitro and in vivo is not as good, however, in the case of AH22 and the vacuolar mutant, BJ4581, although this may reflect the fact that there were fewer experiments. The agreement between the concentrations of phosphocreatine measured in vivo and in cell extracts is poor in all three strains, and this probably reflects the relatively low concentration of phosphocreatine observed in vivo and the consequently poor signal-to-noise ratio obtained on the phosphocreatine resonance. For example, a phosphocreatine resonance could not be detected in cells of the vacuolar mutant BJ4581 (Table II), although it could be detected in cell extracts (Table III). The lower concentration of phosphocreatine in the vacuolar mutant may, in part, be due to the slightly lower concentration of creatine in these cells (Figure 2) and in part due to a higher ADP/ATP ratio (see below). The ADP/ATP ratio determined by HPLC in cell extracts from DBY745 and AH22 is between 0.06 and 0.1. This is in good agreement with the ratio of approximately 0.05 calculated from measurements of the near-equilibrium concentrations of the substrates of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase in AH22 (Brindle, 1988a). The relatively high concentration of ADP found in cell extracts in this previous study has been shown to be an artifact of cell extraction. Inclusion of 10 mM EDTA prior to neutralization of the perchloric acid extract (see Materials and Methods) prevents the accumulation of nucleoside mono- and diphosphates observed previously. The ADP/ATP ratio measured in cell extracts from the vacuolar mutant is significantly higher at 0.5. These cells also have a much higher nucleoside monophosphate concentration (Table III), AMP and GMP being undetectable in DBY745 and AH22 (Table III). Although an extraction artifact cannot be ruled out in the case of the mutant, this seems unlikely in view of the results obtained with the other two strains.

In some systems, for example, the kidney (Freeman et al., 1983), the ADP/ATP ratio has been estimated directly from the difference in intensity of the ^{31}P NMR resonances from the β-phosphate of ATP and the overlapping resonances of the γ-phosphate of ATP and the β-phosphate of ADP in the spectrum obtained in vivo (see Figure 1). If the ADP concentration is very low, then this is a very inaccurate measurement. This measurement is not normally possible in yeast because of the many overlapping resonances from cellular polyphosphates. However the vacuolar mutant largely lacks polyphosphate (Figure 4), although ^{31}P NMR measurements on cell extracts show that it does contain substantial amounts of pyrophosphate and tripolyphosphate. Extracts prepared from cells following 5 h of cell perifusion gave an intracellular pyrophosphate concentration of approximately 1 mM and a tripolyphosphate concentration of 2–3 mM. Measurements of the peak areas of the nucleoside phosphate resonances observed in cell spectra, obtained by fitting them to Lorentzian line shapes, yield a nucleoside diphosphate/nucleoside triphosphate ratio of approximately 0.2, which is similar to that obtained in cell extracts (Table III). Line fitting of the spectra obtained from DBY745 showed that, within the error of the measurements, the β-phosphate resonance of the nucleoside triphosphates was equal in intensity to the overlapping resonances of the γ-phosphates of nucleoside triphosphates and the β-phosphates of nucleoside diphosphates; i.e., there was no detectable nucleoside diphosphate. Although consistent with the low concentrations of nucleoside diphosphates measured in a cell extract, this estimate of the NDP/NTP ratio is expected to be error-prone in view of the overlap with the polyphosphate resonances.

Table II: Metabolite Concentrations Measured in Vivo by ^{31}P NMR[a]

mutant	midpoint time of spectrum acquisition (min)	intracellular metabolite concn (μmol/mL of cell water)			pH (MDP − P_i)	pH (PCr − P_i)
		P_i	phosphocreatine	nucleoside di- and triphosphates		
DBY745	62	3.2 ± 0.5	0.1 ± 0.1	3.2 ± 0.3	7.20 ± 0.04	
	148	2.9 ± 0.4	0.4 ± 0.1	3.7 ± 0.4	7.16 ± 0.05	7.21 ± 0.04
	240	3.2 ± 0.8	0.4 ± 0.1	4.3 ± 0.7	7.14 ± 0.03	7.19 ± 0.04
	331	3.8 ± 0.4	0.4 ± 0.1	4.6 ± 0.4	7.16 ± 0.03	7.19 ± 0.03
AH22	66	2.5	0.1	5.1	7.11	
	153	3.2	0.2	5.4	7.04	
	242	3.6	0.3	5.5	7.01	
	329	3.5	0.2	4.7	7.01	
BJ4581	63	2.8		3.7	7.10	
	150	4.9		5.0	7.09	
	241	5.2		5.6	7.14	
	329	5.1		5.2	7.11	

[a] For DBY745, there were five separate experiments, representing five different cell batches. The errors quoted are standard deviations from the mean. The midpoint times of the spectra, from which the concentrations were determined, are with respect to the start of cell perifusion with creatine-containing buffer at $t = 0$ min. The values quoted for BJ4581 are average values from two experiments. The concentrations of metabolites were estimated by comparing their resonance intensities with that of a methylenediphosphonate standard contained in a coaxial capillary (Brindle & Krikler, 1985). The concentrations of the nucleoside di- and triphosphates were estimated from their unresolved α-phosphate resonances at −26.8 ppm (see Figure 1). The concentrations are quoted per milliliter of cell water by assuming that 1.67 g of wet yeast contains 1 mL of cell water (Gancedo & Gancedo, 1973). The cytosolic pH was estimated from the chemical shift difference between the MDP standard and the cytosolic P_i resonance (MDP − P_i) and from the chemical shift difference between the phosphocreatine resonance and the cytosolic P_i resonance (PCr − P_i).

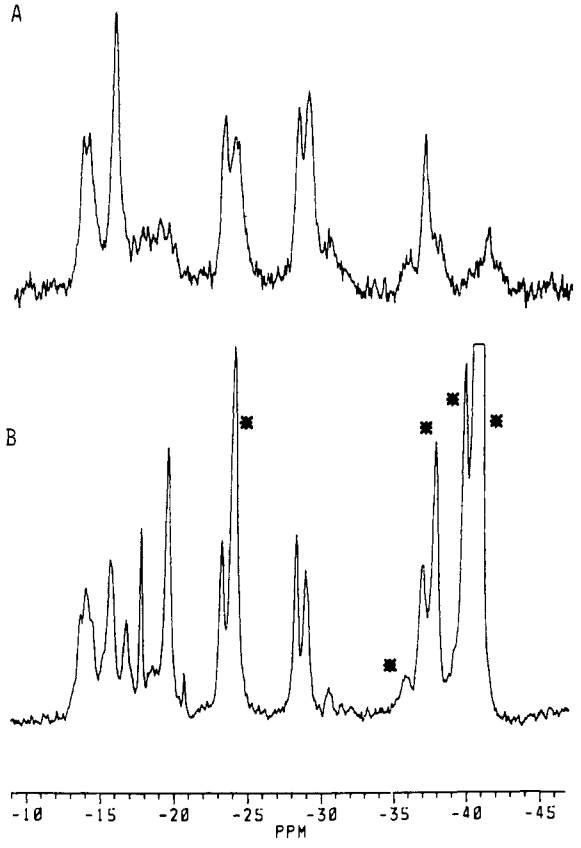

FIGURE 4: ^{31}P NMR spectra of immobilized cells of the yeast strains BJ4581 (A) and DBY745 (B). The vacuolar mutant (BJ4581) largely lacks the polyphosphate resonances observed in DBY745 (see Figure 1 for peak assignments). The polyphosphate resonances are marked with an asterisk in spectrum b. The spectra are each the sum of 1024 scans collected 330 min (DBY745) and 311 min (BJ4581) after the start of cell perifusion with 100 mM creatine. The times are the midpoint times of the spectra. The spectra were acquired under the same conditions as given in the legend to Figure 1. The NMR sample tube contained 4 g wet weight of DBY745 and 2.5 g wet weight of BJ4581.

The mean intracellular free Mg^{2+} concentration, calculated from the chemical shifts of the α- and β-phosphate resonances of nucleoside triphosphates (see Figure 1 and Materials and Methods), was 0.43 ± 0.06 mM in DBY745, 0.46 ± 0.06 mM in BJ4581, and 0.53 ± 0.04 mM in AH22. The mean intracellular pHs in the three strains were 7.17 in DBY745, 7.04 in AH22, and 7.11 in BJ4581 (Table II). A pH of 7.17 and a free Mg^{2+} concentration of 0.43 mM give a calculated equilibrium constant, K_{obs}, for the creatine kinase reaction in DBY745 of 76 (Lawson & Veech, 1979), where K_{obs} is defined as

$$K_{obs} = \frac{[ATP][Cr]}{[ADP][PCr]}$$

The calculated equilibrium constant is 112 in AH22 and 92 in BJ4581. Substitution of the measured intracellular concentrations of creatine and phosphocreatine into the above equation gives the ADP/ATP ratio. The intracellular creatine concentration at equilibrium in DBY745 is between 70 and 80 mM (Figure 2). The equilibrium phosphocreatine concentration is 0.4 mM (Table II), and therefore the calculated ADP/ATP ratio is 2.3–2.6. A similar calculation for AH22, using an intracellular creatine concentration of 100 mM (Figure 2) and a phosphocreatine concentration of 0.3 mM, gives an ADP/ATP ratio of 3.0. The intracellular creatine concentration in BJ4581 appears to be slightly lower than in the other two strains at between 50 and 70 mM. When an intracellular phosphocreatine concentration of 0.16 mM is used (Table III), this gives a calculated ADP/ATP ratio of 2.9–4.8. In all three strains, the ADP/ATP ratio calculated from the substrate concentrations of creatine kinase is appreciably higher than the ratios measured directly in cell extracts and the ratios estimated from the intensities of the nucleoside di- and triphosphate resonances observed in vivo. For example, the ADP/ATP ratio of 0.06, which was measured in the cell extract from DBY745, should give an intracellular phosphocreatine concentration of 14.4 mM, assuming an intracellular creatine plus phosphocreatine concentration of 80 mM. This is much higher than the measured value of 0.4 mM (Table II).

The large discrepancy between the ADP/ATP ratio determined by a number of different methods with that calculated from measurements of the creatine/phosphocreatine ratio implies that there is compartmentation of the substrates of creatine kinase in the cell. The creatine, which was introduced into the cells, may be compartmented. One possible compartment is the vacuole, an organelle which is not found in

Table III: Metabolite Concentrations (μmol/mL of Cell Water) Measured in Cell Extracts by ^{31}P NMR and HPLC[a]

mutant	metabolite	NMR determination	metabolite	HPLC determination
DBY745	ATP + GTP	2.74	GTP	0.47
	ADP + GDP	0.30	ATP	2.08
	CTP + UTP	1.67	UTP	0.23
	phosphocreatine	0.34	CTP	0.87
			GDP	0.06
			ADP	0.13
AH22	ATP + GTP	2.31	GTP	0.42
	ADP + GDP	0.26	ATP	1.65
	CTP + UTP	1.16	UTP	0.17
	phosphocreatine	0.57	CTP	0.89
			GDP	0.01
			ADP	0.16
BJ4581[b]	ATP + GTP	1.66	GTP	0.19
	ADP + GDP	0.85	ATP	1.04
	CTP + UTP	0.95	UTP	0.14
	AMP + GMP	0.60	CTP	0.21
	phosphocreatine	0.18	GDP	0.08
			ADP	0.25
BJ4581	ATP + GTP	1.72	GTP	0.14
	ADP + GDP	0.77	ATP	1.18
	CTP + UTP	0.72	UTP	0.16
	AMP + GMP	0.58	CTP	0.34
	phosphocreatine	0.14	GDP	0.02
			ADP	0.39

[a] The NMR and HPLC determinations of metabolite concentrations were carried out on neutralized perchloric acid extracts prepared from cell preparations which had been perifused for 5 h. The lyophilized extracts, which had been Chelex-treated, were reconstituted in 5 mL of 50 mM triethanolamine buffer, pH 8.0, and 1 mM EDTA, and the pH was adjusted to 8.0 with dilute NaOH. The concentrations are quoted per milliliter of cell water by assuming that 1.67 g of wet yeast contains 1 mL of cell water (Gancedo & Gancedo, 1973). High-resolution ^{31}P NMR spectra were acquired with proton decoupling into 8192 data points with a sweep width of 8 kHz, a 60° pulse, and a recycle delay of 4.127 s. Increasing the recycle time to 8.127 s had no significant effect on the resonance intensities. The spectra were zero-filled to 16 384 data points prior to Fourier transformation. The metabolite concentrations were determined from the spectra either by adding a known concentration of the compound to the extract and recording a second spectrum or by comparing the intensities of the compound's resonances with the resonance intensity of an MDP standard contained in a coaxial capillary. Both methods of determining concentration gave similar values. The concentrations of nucleoside di- and triphosphates were determined from the intensities of their α-phosphate resonances. The α-phosphate resonances of ATP and GTP were not resolved from each other, nor were the α-phosphate resonances of ADP and GDP. The concentrations of CTP and UTP were estimated from their unresolved β-phosphate resonances, which were resolved from the unresolved β-phosphate resonances of ATP and GTP. The HPLC measurements were made with a 25-cm Whatman Partisil 10-SAX column. The nucleoside phosphates were eluted from the column by using a two-step linear phosphate gradient (Harmsen et al., 1982) and were detected by measuring their absorbance at 254 nm. The assignment of peaks and the determination of concentrations were made by running interleaved standards. [b] The results of two sets of experiments are shown for the vacuolar mutant, BJ4581.

mammalian systems. The vacuole, which can constitute up to 25% of the cytoplasmic volume of the yeast cell, can accumulate large quantities of amino acids, particularly the basic amino acids arginine and lysine (Wiemken & Dürr, 1974). The concentration of arginine is about 20 times higher in the vacuole as compared with the concentration in the cytoplasm. Retention of arginine within the vacuole has been shown to be due to binding to the negatively charged polyphosphate, which is located in the vacuole (Dürr et al., 1979). Creatine is zwitterionic at the pH of the cytoplasm and is unlikely, therefore, to be sequestered in the vacuole by this mechanism. However, transport measurements on isolated vacuoles have shown the presence of active transport systems for some amino acids, including neutral amino acids such as leucine and isoleucine. Other amino acids, however, such as valine, threonine, and alanine, are not transported (Sato et al., 1984). The active transport of amino acids into the vacuole is brought about by a substrate–H^+ antiport mechanism (Ohsumi & Anraku, 1981) and has been shown to result in 5-fold concentration gradients of some of the amino acids (Sato et al., 1984). These transport systems could, conceivably, result in sequestration of creatine within the vacuole, lowering the concentration of creatine available to creatine kinase in the cytoplasm.

There are several observations which show that this is not the case. The steady-state intracellular creatine concentration is similar to that in the perifusion buffer (Figure 2). If there were significant vacuolar accumulation of creatine, then the intracellular creatine concentration should significantly exceed the extracellular concentration. It is difficult to see how in the steady state, when the total intracellular creatine concentration is approximately constant, the cytoplasmic creatine concentration could be maintained at a concentration which is less than that in the bathing medium, given that creatine transport into the cell appears to be relatively rapid (Figure 2) and the creatine concentration in the bathing medium is effectively constant. The results obtained with the vacuolar mutant also argue strongly against creatine sequestration in the vacuole. Not only does the mutant lack substantial quantities of polyphosphate but it also appears to lack a vacuolar pH gradient, as evidenced by the lack of a vacuolar P_i resonance which is resolved from the cytoplasmic P_i resonance. The presence of residual vacuolar polyphosphate and substantial amounts of pyrophosphate, both of which break down during prolonged cell perifusion, make it likely that there is vacuolar P_i in these cells. Spectrophotometric measurements using a fluorescein pH probe indicate that a vacuolar pH gradient may still exist in these cells, although much reduced. The vacuolar pH measured in these cells using this technique was 6.9 as compared to 6.2 in the wild type (Preston et al., 1989). The cytoplasmic pH measured by ^{31}P NMR in this study was 7.1 (Table II). A reduced pH gradient would be expected to lead to lowered creatine accumulation in the vacuole. Despite this, however, the vacuolar mutant still shows an unexpectedly low phosphocreatine concentration.

Another possibility is that there is compartmentation of ATP and ADP in the yeast cell cytoplasm. How such compartmentation could be effected, however, is not clear, although there has been considerable speculation about the existence of glycolytic enzyme complexes, which may result in enzyme/substrate compartmentation [see Srere (1987) for a review].

In conclusion, the introduction of creatine kinase and its substrate creatine into a homogeneous yeast cell population has allowed an estimation of the free ADP/ATP ratio to which the enzyme is exposed in the cell cytoplasm. The large discrepancy between the ADP/ATP ratio measured in this way with that measured directly in cell extracts and that calculated from measurements of the substrate concentration of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase implies some form of unexpected compartmentation of the enzyme's substrates within the cell cytoplasm. Compartmentation of creatine has been invoked previously to explain the isotope labeling patterns observed in creatine and phosphocreatine in the perfused rabbit heart (Lee & Visscher, 1961) and, more recently, in isolated rat atria (Savabi, 1988). If creatine compartmentation were responsible for the results obtained in this study, then more than 98% or 78 mM of the 80 mM intracellular creatine pool would have to be bound or

sequestered in some way in the strain DBY745. This does not seem very likely and suggests instead compartmentation of ATP and ADP, which are present at much lower concentrations.

ACKNOWLEDGMENTS

We thank Dr. Alan Kingsman for plasmid pMA91 and Prof. George Radda for his advice and generous support.

REFERENCES

Alger, J. R., den Hollander, J. A., & Shulman, R. G. (1982) *Biochemistry 21*, 2957–2963.
Atkinson, D. E. (1977) *Cellular Energy Metabolism and Its Regulation*, Academic Press, New York.
Barwell, C. J., & Hess, B. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1178–1184.
Bernt, E., Bergmeyer, H. U., & Möllering, H. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., Ed.) Vol. 4, pp 1772–1776, Academic Press, New York.
Bessman, S. P., & Carpenter, C. L. (1985) *Annu. Rev. Biochem. 54*, 831–862.
Brindle, K. M. (1988a) *Biochemistry 27*, 6187–6196.
Brindle, K. M. (1988b) *Prog. Nucl. Magn. Reson. Spectrosc. 20*, 257–293.
Brindle, K., & Krikler, S. (1985) *Biochim. Biophys. Acta 847*, 285–292.
Brindle, K. M., & Radda, G. K. (1985) *Biochim. Biophys. Acta 829*, 188–201.
Campbell-Burk, S. L., Jones, K. A., & Shulman, R. G. (1987) *Biochemistry 26*, 7483–7492.
Chinault, A. C., & Carbon, J. A. (1979) *Gene 5*, 111–126.
Coore, H. G., Denton, R. M., Martin, B. R., & Randle, P. J. (1971) *Biochem. J. 125*, 115–127.
den Hollander, J. A., Ugurbil, K., Brown, T. R., & Shulman, R. G. (1981) *Biochemistry 20*, 5871–5880.
de Hollander, J. A., Ugurbil, K., & Shulman, R. G. (1986) *Biochemistry 25*, 212–219.
Dürr, M., Urech, K., Boller, Th., Wiemken, A., Schwenke, J., & Nagy, M. (1979) *Arch. Microbiol. 121*, 169–175.
Ferguson, J., Groppe, J. C., & Reed, S. I. (1981) *Gene 16*, 191–197.
Freeman, D., Bartlett, S., Radda, G., & Ross, B. (1983) *Biochim. Biophys. Acta 762*, 325–336.
Gancedo, J. M., & Gancedo, C. (1973) *Biochimie 55*, 205–211.
Grenson, M., Mousset, M., Wiame, J. M., & Bechet, J. (1966) *Biochim. Biophys. Acta 127*, 325–338.
Gupta, R. K., Gupta, P., Yoshok, W. D., & Rose, Z. B. (1983) *Biochem. Biophys. Res. Commun. 117*, 210–216.
Harmsen, E., de Tombe, P. Ph., & de Jong, J. W. (1982) *J. Chromatogr. 230*, 131–136.
Hinnen, A., Hicks, J. B., & Fink, G. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1929–1933.
Kauppinen, R., Hiltunen, K., & Hassinen, I. (1980) *FEBS Lett. 112*, 273–276.
Koretsky, A. P., & Traxler, B. A. (1989) *FEBS Lett. 243*, 8–12.
Kovâc, L., Nelson, B. D., & Ernster, L. (1986) *Biochem. Biophys. Res. Commun. 134*, 285–291.
Lawson, J. W. R., & Veech, R. L. (1979) *J. Biol. Chem. 254*, 6528–6537.
Lee, Y. C. P., & Visscher, M. B. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 1510–1515.
Lenney, J. F. (1975) *J. Bacteriol. 122*, 1265–1273.
Loike, J. D., Somes, M., & Silverstein, S. C. (1986) *Am. J. Physiol. 251*, C128–C135.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1986) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Matthews, P. M., Bland, J. L., Gadian, D. G., & Radda, G. K. (1982) *Biochim. Biophys. Acta 721*, 312–320.
Mellor, J., Dobson, M. J., Roberts, N. A., Tuite, M. F., Emtage, J. S., White, S., Lowe, P. A., Patel, T., Kingsman, A. J., & Kingsman, S. M. (1983) *Gene 24*, 1–14.
Mellor, J., Dobson, M. J., Roberts, N. A., Kingsman, A. J., & Kingsman, S. M. (1985) *Gene 33*, 215–226.
Meyer, R. A., Kushmerick, M. J., & Brown, T. R. (1982) *Am. J. Physiol. 242*, C1–C11.
Moon, R. B., & Richards, J. H. (1973) *J. Biol. Chem. 248*, 7276–7278.
Murray, A. W., & Szostak, J. W. (1983) *Cell 34*, 961–970.
Nasmyth, K. A., & Reed, S. I. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2119–2123.
Navon, G., Shulman, R. G., Yamane, T., Eccleshall, T. R., Lam, K.-B., Baronofsky, J. J., & Marmur, J. (1979) *Biochemistry 18*, 4487–4498.
Nicolay, K., Scheffers, W. A., Bruinenberg, P. M., & Kaptein, R. (1982) *Arch. Microbiol. 133*, 83–89.
Ohsumi, Y., & Anraku, Y. (1981) *J. Biol. Chem. 256*, 2079–2082.
Oliver, I. T. A. (1955) *Biochem. J. 61*, 116–122.
Preston, R. A., Murphy, R. F., & Jones, E. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7027–7031.
Putney, S., Herlihy, W., Royal, N., Pang, H., Aposhian, H. V., Pickering, L., Belagaje, R., Biemann, K., Page, D., Kuby, S., & Schimmel, P. (1984) *J. Biol. Chem. 259*, 14317–14320.
Sato, T., Ohsumi, Y., & Anraku, Y. (1984) *J. Biol. Chem. 259*, 11506–11508.
Savabi, F. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7476–7480.
Shoubridge, E. A., Bland, J. L., & Radda, G. K. (1984) *Biochim. Biophys. Acta 805*, 72–78.
Srere, P. A. (1987) *Annu. Rev. Biochem. 56*, 89–124.
Wiemken, A., & Dürr, M. (1974) *Arch. Microbiol. 101*, 45–57.