^{31}P Nuclear Magnetic Resonance Evidence for the Regulation of Intracellular pH by Ehrlich Ascites Tumor Cells

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ABSTRACT The phenomenon of intracellular pH (pH^{in}) regulation in cultured Ehrlich ascites cells was investigated using ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Measurements were made with a Bruker WH 360 wide bore NMR spectrometer at a ^{31}P frequency of 145.78 MHz. Samples at a density of 10^8 cells ml^{-1} were suspended in a final volume of 2 ml of growth medium in 10 mm diameter NMR tubes.

Intracellular pH was calculated from the chemical shifts of either intracellular inorganic phosphate (P_i^{in}) or intracellular 2-deoxyglucose-6-phosphate ($2dG6P^{in}$). The sugar phosphate was used as a pH probe to supplement the P_i^{in} measurements, which could not always be observed. When available, the pH^{in} calculated from the P_i^{in} peak was identical within experimental error to the pH^{in} calculated from the $2dG6P^{in}$ peak.

Intracellular pH was measured to be more alkaline than the medium at an external pH (pH^{ex}) below 7.1. Typical values were $pH^{in} = 7.00$ for $pH^{ex} = 6.50$. These measurements were constant for times up to 165 min using well-energized, respiring cells. This pH gradient was seen to collapse immediately upon onset of anaerobic shock. Above a pH^{ex} of 7.2 there was no significant difference between pH^{in} and pH^{ex}. These results unequivocably demonstrate the steady state nature of the pH regulation and its dependence upon energization.

In the past few years, it has become increasingly apparent that intracellular pH (pH^{in}) plays a vital role in the regulation of cellular metabolism and proliferation (1). For instance, pH^{in} has been shown to be important in the metabolic derepression following fertilization of sea urchin eggs (2, 3), in gap junction conductance changes in blastomeres (4), and in glycolytic regulation in erythrocytes (5) and yeast (6). Changes in pH^{in} have been correlated with cellular proliferation in *Tetrahymena* (7), *Physarum* (8), yeast (9), and lymphocytes (10).

For the most part, however, studies describing the control of cellular functions by pH^{in} are not complete. In contrast, the regulation of pH^{in} itself has been extensively studied in a variety of systems and is the subject of a recent, comprehensive review (11). From these studies it is clear that both procaryotic and eucaryotic unicellular organisms, such as *Escherichia coli* (12), *Tetrahymena* (7), and yeast (9) have a great capacity for pH^{in} regulation over a wide range of pH^{ex}. In addition, quite a few metazoan cells have also been shown to have the capacity to regulate pH^{in} in the face of induced acid loads. For the most part, however, the systems used are either excitable, such as sheep heart Purkinje fibers (13), or are specialized for pH regulation, such as salamander proximal tubule (14).

It is apparent from some of these studies that mammalian cells have the genetic capacity to generate a pH^{in} regulatory mechanism. However, it has been a subject of some controversy whether or not nonexcitable mammalian cells express this capability (see references 1, 15). It has been pointed out that, given a high permeability of protons through the plasma membrane (16) and a negative membrane potential, one would expect pH^{in} to be more acidic than the pH^{ex} and that, for no pH gradient to exist, a proton-equivalent extruding mechanism must be employed.

In the present study we have asked whether or not the phenomenon of pH^{in} regulation exists in cultured mammalian cells. To measure pH^{in}, we have used ^{31}P NMR spectroscopy (see reference 17 for review). Ehrlich ascites tumor cells were chosen for this particular investigation because they have been extensively studied with regard to their pH^{in} and the data have been fragmentary and inconclusive (15, 18–25).

MATERIALS AND METHODS

Cells

Ehrlich ascites tumor cells were obtained from frozen stocks derived from those of van Venrooij et al. (26). Recovered cells were subsequently confirmed as Ehrlich ascites cells by karyotype analysis. Cells were cultured in minimum

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essential medium supplemented with 5% fetal bovine serum (MEM) obtained from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY. Cultures were grown to a density of 6–8 × 10^5 cells ml^{-1} for each experiment. 250 ml of these cultures were placed in an ice bath for 1 h, after which they were collected at 500 *g* in a 250 ml conical polypropylene centrifuge tube (Corning Glass Works, Science Products Div., Corning, NY). The pellet, containing ~250 μl of packed cells, was resuspended with 1.2–1.5 ml MEM (pH 7.2) containing 25 mM HEPES buffer, 20 mM $NaHCO_3^-$ and 0.5% sodium succinate to give a final density of 2 × 10^8 cells in 2 ml. This suspension was transferred to a 10-mm diameter nuclear magnetic resonance (NMR) tube (Wilmad Glass Co., Inc., Buena, NJ) containing 25 μl of Antifoam B emulsion (Sigma Chemical Co., St. Louis, MO) and used immediately in the spectrometer. Although Antifoam B contributes to cell lysis, the half-time for this to occur was on the order of 8 h, as determined by hemocytometer. Since the present experiments were concluded within 3 h of harvesting, Antifoam-associated cytolysis was not considered to be a significant concern.

NMR Spectroscopy

The spectrometer used was a Bruker WH-360-WB equipped with a 10-mm broadband probe operating at 145.78 MHz. Spectra were obtained every 5 min by Fourier-transforming the sum of 2750 free induction decays arising from 45° tipping pulses with repetition times of 0.1024 s. These pulsing conditions were selected in order to optimize signals arising from intracellular metabolites and they cause a partial saturation (~60%) of the extracellular orthophosphate (P_i^{ex}) signals. All spectral shifts are expressed as ppm relative to 85% phosphoric acid. The in vivo spectra are initially referenced to the α-phosphate of ATP at −9.97 ppm at pH^{ex} = 7.2. Acid extract spectra are referenced to creatine phosphate at −2.35 ppm.

Experimental

The cell suspension as described above was outfitted with a 3 mm O.D. glass tube through which a mixture of 95% O_2:5% CO_2 was introduced at a rate of ~2 ml/min. The bubbling rate was adjusted to maintain O_2 tensions at 50% of saturation as measured by a Clark-type electrode. This value has been determined to be at least 10-fold higher than the K_m for O_2 consumption in this system. The tip of the aeration tube was typically drawn out to 20–30 μm and the relatively small bubbles were shown not to contribute to cell lysis.

At the beginning of each experiment, the cold cell suspension containing the aeration tube was placed in the NMR probe at 37°C and allowed to equilibrate for 20 min. After this time, an aliquot (25–35 μl) of 2% 2-deoxyglucose (2dG) in MEM was added to the suspension, to give 2.5 mM 2dG final concentration. The 2dG was phosphorylated by cellular hexokinase and the chemical shift of the resultant 2-deoxyglucose-6-phosphate (2dG6P) was used to estimate the intracellular pH, as previously described (17). In the pH titration experiments, the suspension was infused with either 300 mM HEPES (pH 4.0) or TAPS (pH 9.4) at a rate of 0.34 ml/h. Although addition of these buffers did lead to some cell lysis, the remaining cells were physiologically sound, as evidenced by both NMR spectra (indicating ATP levels) and dye exclusion. In the absence of titration, the cell suspensions have been seen to remain viable for at least 4.5 h, the longest time tested.

RESULTS

Fig. 1 *B* presents a 5-min spectrum of Ehrlich ascites cells in the absence of 2dG. Note the virtual absence of a P_i^{in} peak separate from the P_i^{ex} peak. In some spectra, a P_i^{in} peak could be seen as a shoulder downfield from the P_i^{ex} peak (Fig. 1 *D*). The shoulder has been assigned to P_i^{in} first because of the absence of a second peak at this chemical shift in spectra of perchloric acid extracts of these cells. Second, the P_i^{in} peak has been reproducibly observed in spectra of cells suspended in buffer with low (0.33 mM) P_i (data not shown). Given its small size relative to the large P_i^{ex} peak, the P_i^{in} peak could not always be used to estimate pH^{in}. As an alternative indicator of pH^{in}, we have decided on the use of 2dG6P as originally proposed by Navon et al. (15) and subsequently employed by Bailey et al. (27). 2dG6P has the advantages of being localized in the cytoplasm and of remaining in relatively stable, high concentrations throughout the course of experiments. The main drawback of this method is that it precludes investigation of pH^{in} under glycolytic conditions.

Fig. 1 *A* shows a 5-min spectrum of cells incubated in the presence of 3 mM 2dG. This concentration was selected to maximize the resultant 2dG6P peak without significantly reducing cellular ATP levels. Note the presence of a large intracellular 2dG6P peak. This peak was identified as being intracellular by lysing approximately half of the cells in the suspen-

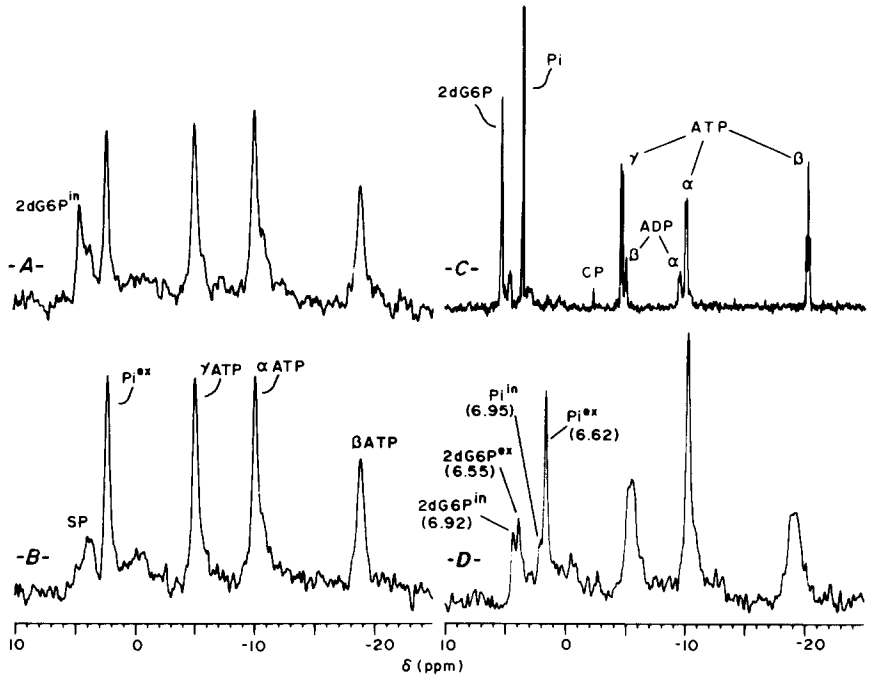

FIGURE 1 145.78 MHz ^{31}P NMR spectra of Ehrlich ascites cells either in vivo (*A–C*) or perchloric acid extract (*D*). Experimental details are given in the methods. Spectra are presented as chemical shift (δ) relative to 85% phosphoric acid referenced to either the primary phosphate of ATP at −10.05 ppm (*A–C*) or creatine phosphate at −2.35 ppm (*D*). (*A*) 5-min spectrum of 10^8 cells ml^{-1} in MEM containing 0.5% sodium succinate and 0.1% D-glucose aerobically. pH^{in} = 7.17, pH^{ex} = 6.85. (*B*) Spectrum of cells under identical conditions as in *A*, except that 2-deoxyglucose was added to final concentration of 3 mM. (*C*) Spectrum of cells under identical conditions as *B* except that approximately half of the cells had been lysed by the addition of a bolus of 0.1 N HCl. Numbers in parentheses refer to the pH estimated from the chemical shifts of the various indicators and compartments. (*D*) Spectrum of cells extracted with 5% perchloric acid after accumulation of spectrum *C*. Spectrum is the Fourier transform of 7,200 free induction decays arising from 45° tipping pulses with a repetition rate of 1.0 sec. Note the presence of a single 2-deoxyglucose-6-phosphate peak in this spectrum. Abbreviations: *SP* sugar phosphates (phosphomonoesters); *Pi* ^{in}, *Pi* ^{ex}, intra- and extracellular orthophosphate; *α, β, γ ATP*, primary, middle, and terminal phosphates of ATP; *α, β ADP*, primary and terminal phosphates of *ADP*; *2dG6P* ^{in}, *2dG6P* ^{ex}, intra and extracellular 2-deoxyglucose-6-phosphate; *CP*, creatine phosphate (δ = − 2.35 ppm).

sion with a 100-μl bolus of 0.1 N HCl. This treatment created two 2dG6P peaks, one outside at low pH and one intracellular at a higher pH (Fig. 1 *D*). In this spectrum, note the close agreement between pH values obtained using the chemical shifts of either P_i or 2dG6P in both the intra- and extracellular compartments. The 2dG6P peaks were identified by the single peak in spectra of perchloric acid extracts that titrated exactly like commercially available 2dG6P (Fig. 1 *C*).

The spectra of Ehrlich ascites cells presented here are somewhat different than those reported elsewhere (15, 25) in that the present spectra lack peaks corresponding to phosphorylcholine (PCh) and phosphocreatine (PCr). The absence of PCh is probably due to culture differences, since it is present in these cells if they are cultured in vivo as opposed to in vitro (unpublished results). The absence of PCr in the present study is a consequence of strain differences, because we have identified PCr in another strain of EA cells under identical circumstances. It should also be mentioned that the present cells were cultured in the absence of exogenous creatine, which could also lead to lower PCr levels (25).

The definitive presence of $2dG6P^{in}$ allows for the calculation of pH^{in} from its chemical shift, as described previously (17, 27, 28). Occasionally, as indicated in Fig. 1 *D*, we were able to observe enough P_i^{in} to allow us to also use this peak to calculate pH^{in}. These data, presented together in Fig. 2, indicate excellent agreement between pH^{in} values calculated from the chemical shifts of either $2dG6P^{in}$ or P_i^{in}. In addition, these data show that, at a $pH^{ex} < 7.1$, the pH^{in} remains more alkaline than the pH^{ex}. It is also interesting to note that the $pH^{in} = pH^{ex} > 7.1$.

The experiments outlined above were designed so that we induced a slow, but constant rate of change of extracellular pH. Consequently, it remained a question as to whether the pH^{in} values observed were steady state or simply transients in response to lowering pH^{ex}. Steady state regulation of pH^{in} was determined in several separate experiments by monitoring pH^{in} as a function of time at constant pH^{ex}, at different values between 6.4 and 6.9. A typical experiment is illustrated in Fig. 3, in which pH^{ex} was constant at ~6.8 and pH^{in} remained close to 7.05 for 2.5 h. After 165 min of incubation, N_2 was substituted for the O_2 in the gas mixture. As indicated in Fig. 3, the pH gradient promptly collapsed simultaneously with a loss of ATP and an increase in ADP and AMP. This decrease in the degree of energization was presumably due to the absence of glucose in the medium, because the changes in adenine nucleotides seen here do not occur anaerobically in the presence of glucose. Resupplying the suspension with O_2 at 180 min reversed the changes in the adenine nucleotide levels and led to a slow increase in pH^{in} (see Fig. 3).

DISCUSSION

This study illustrates the complementariness of P_i^{in} and $2dG6P^{in}$ as indicators of intracellular pH in ^{31}P NMR experiments. The 2dG6P was found to be more useful in our study because its peak was generally well resolved and intense enough to give a good signal-to-noise ratio. The $2dG6P^{in}$ peak measured in the present study presumably arises from cytoplasmic 2dG6P despite reports of hexokinase activity in mitochondria (29). The agreement between the pH^{in} estimated from the 2dG6P and P_i peaks indicates that they are in the same compartment. The P_i^{in} can be assigned as cytoplasmic and not mitochondrial due to the large volume difference between these two compartments.

The P_i^{in} peak could only rarely be observed, because of interference from the P_i^{ex} peak. With a line-width of 60 Hz and a P_i titration of 200 Hz/pH unit, it can be calculated that pH differences <0.3 pH units cannot be well resolved. From control studies in low P_i buffer, we have calculated the intracellular concentration of free P_i to be 1.5 mM, which would give a signal-to-noise ratio for the P_i^{in} peak of ~1.3 at present cell densities. This relatively low S/N predicts that the P_i^{in} peak will be only rarely visible and that the uncertainty of measuring the chemical shift will be ~50 Hz. These two predictions are substantiated in Fig. 2 and 3, in which the P_i^{in} peak (indicated by X) is observed intermittently and only when the pH gradient is >0.27 pH units. Note also that the scatter of pH values estimated from P_i^{in} is more than two times greater than those from $2dG6P^{in}$. Given the differences is S/N and sensitivity to pH, it can be calculated that 2dG6P should be four times as accurate as P_i in determining pH^{in}.

It has recently been pointed out that the chemical shifts of P_i and 2dG6P are affected by ionic strength (I) and Mg^{++} as well as by pH and it was suggested that, for these reasons, they are of limited usefulness (28, 30). However, the effect of ionic

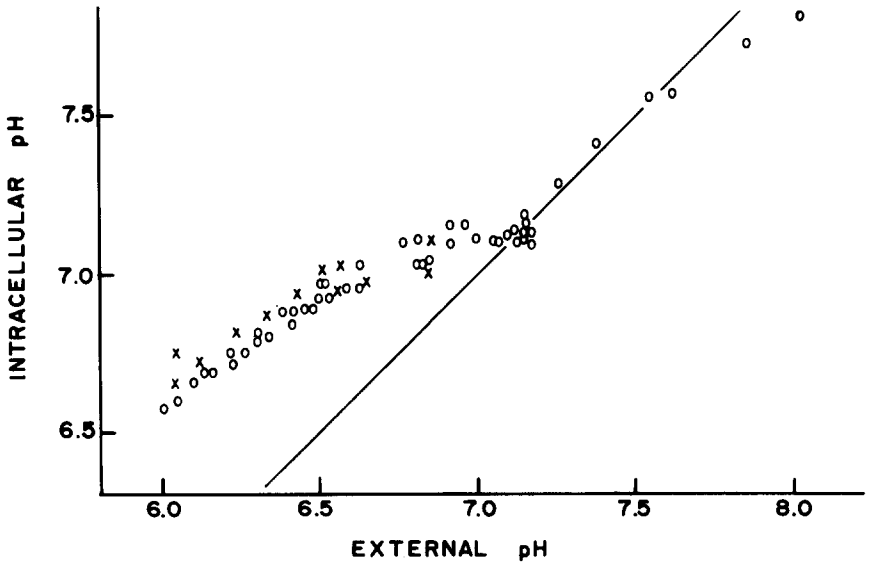

FIGURE 2 Intracellular pH of Ehrlich ascites cells as a function of extracellular pH. Intracellular pH was estimated from the chemical shift of either 2-deoxyglucose-6-phosphate (O) or orthophosphate (X), as observed in spectra identical to those shown in Fig. 1. Straight line represents condition where $pH^{in} = pH^{ex}$. Deviation from this line at an external pH greater than 8.0 is within experimental error due to the relative insensitivity of the method at high pH (20). Note the good agreement between pH^{in} estimated by either indicator.

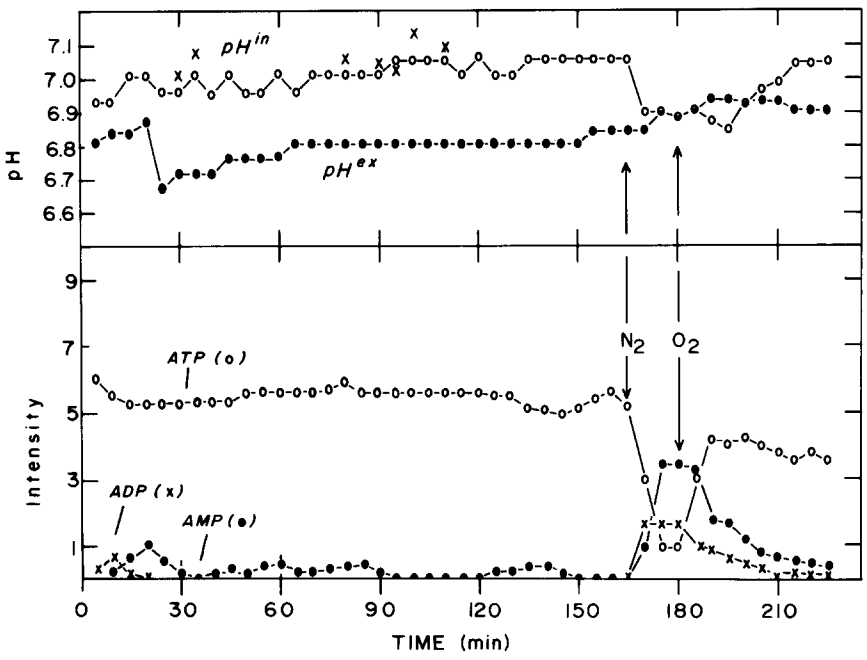

FIGURE 3 Intracellular pH (O,X), extracellular pH (●), and adenine nucleotide levels as a function of time in Ehrlich ascites cells. Intracellular pH was estimated from the chemical shifts of either 2-deoxyglucose-6-phosphate (O) or orthophosphate (X), as described in the text. Extracellular pH was estimated from the chemical shift of extracellular orthophosphate. Adenine nucleotide levels are expressed in intensity units of primary phosphate peaks provided by peak-picking software supplied with the spectrometer and do not necessarily correspond linearly with concentration. At time, T = 0 min, 2-deoxyglucose was added to a concentration of 3 mM and intracellular pH could be estimated from the 2dG6P chemical shift 5 min later. At T = 20 min, 100 μl of 300 mM HEPES (pH = 4.0) was added to lower pH^{ex}. Note the appearance of an identifiable P_i^{in} peak (indicated by X) when the transmembrane pH gradient is greater than 0.27 pH units. At T = 165 min N_2 was substituted for O_2 in the aeration mixture. At T = 180 min, the O_2 was replaced. Note the rapid recovery of ATP levels and slower recovery of intracellular pH, ADP, and AMP.

strength on the pKa' is −0.30 pH units·log I^{-1} in the case of P_i and −0.40 pH units·log I^{-1} for 2dG6P (17). Since the statistical experimental error in pH^{in} determinations is ± 0.05 pH units at pH^{ex} of 7.2, a twofold change in intracellular ionic strength would be necessary to affect our measurements significantly. It is reasonable to assume that changes of this magnitude do not occur under physiological conditions. Also, the intracellular free Mg^{++} concentrations are relatively invariable, as estimated from the chemical shift difference between the primary and middle phosphate peaks of ATP (31). Taking these observations into account, we feel that reasonable estimates of the intracellular ionic environment can be made and that the intracellular pH estimated by ^{31}P NMR is accurate. Certainly the close agreement shown here, between pH^{in} values obtained using both of these indicators, supports their use.

Our main conclusion is that Ehrlich ascites cells regulate their intracellular pH and that the basis for regulation is an active transport of proton equivalents. pH^{in} regulation measured by microelectrodes has generally been defined as the recovery of pH^{in} following transiently induced acid loads to the cytoplasm (11). In contrast, we define regulation as an energy-requiring process that acts to resist changes in the pH^{in} under steady state conditions. This can best be observed by determining pH^{in} at different values of pH^{ex}. The range of pH^{in} regulation is shown in Fig. 2, in which the slope of pH^{in} vs. pH^{ex} is nonunity. Under present conditions, pH^{in} is regulated at pH^{ex} values between 6.5 and 7.2. Although pH^{in} remains more alkaline than pH^{ex} below pH 6.5, the gradient remains constant and "regulation" cannot be said to occur, although the cells are still actively exporting proton-equivalents. These data are qualitatively similar to those seen in yeast (9) and *E. coli* (12), except that, in the case of microorganisms, the pH^{in} is more tightly "clamped" by a plasma membrane ATPase allowing the steady state pH gradient to be several pH units. In the present study, the pH gradient is never larger than 0.5 pH units and, to a first approximation, remains at 0.5 pH units at pH^{ex} below 6.5. Between pH^{ex} of 6.5 and 6.0, the absolute proton concentration difference (and hence, the inward directed proton flux across the bilayer) increases threefold. Therefore, the proton-extruding activity must also increase threefold to yield these steady state results. This suggests that the proton extruding mechanism is limited by the energy needed to overcome the pH gradient and not by the rate of the proton-extruding activity. A pH gradient of 0.5 pH units corresponds to ~30 mV, which is nearly equal and opposite to the reported membrane potential (32). These observations suggest that a relationship may exist between the membrane potential and the allowed pH gradient, and this possibility will be explored. The energy dependence and steady state nature of pH^{in} values in Ehrlich ascites cells are clearly shown in Fig. 3, in which a constant pH gradient of ~0.25 pH units is maintained for 2.5 h, until the oxygen supply is removed. These data show that energy is required for remaining pH^{in} above pH^{ex}.

The results presented here should be compared with those of other workers who have investigated pH^{in} of Ehrlich ascites cells cultured in mice. An early ^{31}P NMR study by Navon et al. (15) indicated the lack of pH regulation in that the pH gradient formed by an acidic pH jump collapsed with a time constant of 20 min. Those measurements were made at 20°C on de-energized cells, whereas the present study was performed at 37°C on cells energized in the presence of oxygen (Fig. 3). In agreement with the previous results, the pH gradient collapsed when the cells were de-energized. The faster collapse in the present experiments is presumably due to the higher temperatures (37°C instead of 20°C), and suggests a temperature dependence of the proton permeability.

Belt et al. (18) have investigated pH^{in} using either methylamine (MA) or 6-carboxyfluorescein (6CF) and were particularly interested in the effects of glycolysis and bioflavinoids on pH^{in}. With either indicator, they reported that pH^{in} is generally more acidic than pH^{ex}, which is in the opposite direction from our results. In the case of MA, the pH^{in} was 6.74 at a pH^{ex} of 7.3. This low value possibly arose from the sequestration of the MA in acidic compartments, as the authors suggest. The higher pH^{in} value of 7.18 observed using 6CF was seen to acidify with time, indicating that it is a non-steady-state value. A slightly

earlier paper from the same laboratory compared pH^{in} values obtained using either 6CF or the weak acid: 5,5-dimethyloxazolidine-2,4-dione (DMO) (19). In general, these measurements gave good agreement between pH^{in} values obtained using either indicator, and the pH^{in} values reported are similar to ours in that, at two values of pH^{ex} (7.4 and 6.2), they observed pH^{in} to be near 7.3. In the present paper, we have tried to answer the question of pH^{in} regulation under physiological (37°C, well-energized) and steady state conditions. In addition we attempt to distinguish these measurements from the more fragmentary and somewhat self-contradictory results measured as part of larger studies such as that of Belt et al. (18).

For years, Poole and her colleagues (20–23) have investigated pH^{in} of Ehrlich ascites cells at 37°C using DMO. Their studies have generally shown a close agreement between intra- and extracellular pH. However, the notable exception was that in the presence of bicarbonate and constant pCO_2 (30–40 mm Hg), the intracellular pH remained relatively constant around pH 7.2 at external pH between 7.2 and 6.7 (20, 21). These data are quantitatively similar to ours, although it was not demonstrated that they were obtained under steady-state conditions. In fact, in every case wherein a time course was presented, the intracellular pH was seen to acidify with time, even in the absence of glucose (21–23).

Spencer and Lehninger (24) have also used DMO at 37°C and, in some cases, have obtained results similar to ours. Their pH^{in} measurements were made after pH jumps, so that it was not possible to determine whether they were measuring a slow response to pH jumps or a steady state energy driven proton pumping. Comparing these diverse reports with the present study, it can be seen that our data are in agreement with certain parts of these earlier studies. We would like to emphasize that whereas some of these previous measurements have been consistent with steady state regulation of pH^{in} in Ehrlich ascites cells, others have not.

We have no evidence to suggest a mechanism by which Ehrlich ascites cells regulate their pH^{in}. Likely candidates are a Na^+/H^+ exchange or an ATP-requiring Cl^-/HCO_3^- exchange, which have been seen in other systems (see reference 11 for review). Either of these mechanisms could be energetically linked to the membrane potential, as described above. However, studies using specific inhibitors of these exchanges have yielded negative results (unpublished results). Recently, Heinz et al. (32) have presented evidence for electrogenic active proton extrusion in Ehrlich ascites cells from measurements of pH^{ex}. This could also be energetically linked to the membrane potential and would be consistent with our observations. The studies presented here provide sound evidence for the existence of an energy-driven proton-equivalent extruding mechanism that will, in the presence of an external acid load, maintain a relatively alkaline interior.

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Note Added in Proof: A recent publication by Gonzalez-Mendez et al. (Gonzalez-Mendez, R., D. Wemmer, G. Hahn, N. Wade-Jardetzky and O. Jardetzky. 1982. Continuous flow NMR culture system form mammalian cells. *Biochim. Biophys. Acta* 720:274–280.) has indicated that the steady state intracellular pH of Chinese hamster ovary cells as determined by ^{31}P NMR is constant at 7.15 in the external pH range from 6.0 to 8.0. There is no information at this time to explain the apparent differences between these data and those of the present study.

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