31P 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\textsubscript{i}) regulation in cultured Ehrlich ascites cells was investigated using 31P nuclear magnetic resonance (NMR) spectroscopy. Measurements were made with a Bruker WH 360 wide bore NMR spectrometer at a 31P frequency of 145.78 MHz. Samples at a density of 10^6 cells ml\textsuperscript{-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\textsubscript{i}\textsuperscript{31P}) or intracellular 2-deoxyglucose-6-phosphate (2dG6p\textsubscript{31P}). The sugar phosphate was used as a pH probe to supplement the P\textsubscript{i}\textsuperscript{31P} measurements, which could not always be observed. When available, the pH\textsubscript{i} calculated from the P\textsubscript{i}\textsuperscript{31P} peak was identical within experimental error to the pH\textsubscript{i} calculated from the 2dG6p\textsubscript{31P} peak.

Intracellular pH was measured to be more alkaline than the medium at an external pH (pH\textsubscript{ex}) below 7.1. Typical values were pH\textsubscript{i} = 7.00 for pH\textsubscript{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\textsubscript{ex} of 7.2 there was no significant difference between pH\textsubscript{i} and pH\textsubscript{ex}. These results unequivocally 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\textsubscript{i}) plays a vital role in the regulation of cellular metabolism and proliferation (1). For instance, pH\textsubscript{i} 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\textsubscript{i} 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\textsubscript{i} are not complete. In contrast, the regulation of pH\textsubscript{i} 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\textsubscript{i} regulation over a wide range of pH\textsubscript{e}. In addition, quite a few metazoan cells have also been shown to have the capacity to regulate pH\textsubscript{i} 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\textsubscript{i} 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\textsubscript{i} to be more acidic than the pH\textsubscript{e} 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\textsubscript{i} regulation exists in cultured mammalian cells. To measure pH\textsubscript{i}, we have used 31P 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\textsubscript{i} 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
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 × 10^5 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') signals. All spectral shifts are expressed as ppm relative to 85% phosphoric acid.

For the sake of examining the cytoplasmic pH, 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. 1B presents a 5-min spectrum of Ehrlich ascites cells in the absence of 2dG. Note the virtual absence of a P_in peak separate from the P_i' peak. In some spectra, a P_in peak could be seen as a shoulder downfield from the P_i' peak (Fig. 1D). The shoulder has been assigned to P_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_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' peak, the P_in peak could not always be used to estimate pH_i. As an alternative indicator of pH_i, 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_i under glycolytic conditions.

Fig. 1A 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 intracellularly by lysing approximately half of the cells in the suspen-

![Figure 1](image-url)

**Figure 1** 145.78 MHz 31P 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 10^6 cells ml^(-1) in MEM containing 0.5% sodium succinate and 0.1% 2-deoxyglucose aerobically, pH_7.17, pH_i' = 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", Pi_1", 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", 2dG6P_1", intra and extracellular 2-deoxyglucose-6-phosphate; CP, creatine phosphate (δ = -2.35 ppm).
tion 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 Pi or 2dG6P in both the intracellular 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 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 Pi 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 or Pi in. In addition, these data show that, at a pH ex <7.1, the pH in remains more alkaline than the pH x. 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 in was constant at ~6.8 and pH in remained close to 7.05 for 2.5 h. After 165 min of incubation, N2 was substituted for the O2 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 O2 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 Pi in and 2dG6P as indicators of intracellular pH in 31P 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 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 Pi in peaks indicates that they are in the same compartment. The Pi in can be assigned as cytoplasmic and not mitochondrial due to the large volume difference between these two compartments.

The Pi in peak could only rarely be observed, because of interference from the Pi ex peak. With a line-width of 60 Hz and a Pi ex 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 Pi buffer, we have calculated the intracellular concentration of free Pi to be 1.5 mM, which would give a signal-to-noise ratio for the Pi in peak of ~1.3 at present cell densities. This relatively low S/N predicts that the Pi 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 Pi 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 Pi in is more than two times greater than those from 2dG6P. Given the differences in S/N and sensitivity to pH, it can be calculated that 2dG6P should be four times as accurate as Pi in determining pH in.

It has recently been pointed out that the chemical shifts of Pi 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

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**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.
strength on the pKa is ~0.30 pH units/log 10 in the case of P,
and ~0.40 pH units/log 10 for 2dG6P (17). Since the statistical
eperimental error in pH
 determinations is ± 0.05 pH units
at pH 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 observa-
tions into account, we feel that reasonable estimates of the
intracellular pH estimated by 31p NMR is accurate. Certainly
the close agreement shown here, between pH 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
 regulation measured
by microelectrodes has generally been defined as the
recovery of pH following transiently induced acid loads to the
cytosol (11). In contrast, we define regulation as an energy-
requiring process that acts to resist changes in the pH under
steady state conditions. This can best be observed by determin-
ing pH at different values of pH. The range of pH
regulation is shown in Fig. 2, in which the slope of pH vs. pH
is nonunity. Under present conditions, pH
 is regulated at pH
values between 6.5 and 7.2. Although pH
 remains more alkaline than pH
 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
 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
below 6.5. Between pH
 of 6.5 and 6.0, the absolute proton
concentration difference (and hence, the inward directed pro-
ton 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 extrud-
ing 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 mem-
brane potential (32). These observations suggest that a rela-
tionship 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 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
energy dependence and steady state pH values in

The results presented here should be compared with those of
other workers who have investigated pH of Ehrlich ascites
cells cultured in mice. An early 31P 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 col-
lapsed when the cells were de-energized. The faster collapse
in the present experiments is presumably due to the higher tem-
peratures (37°C instead of 20°C), and suggests a temperature
dependence of the proton permeability.

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

FIGURE 3 Intracellular pH (O, X), extracellular
pH (O), and adenine nucleotide levels as a
function of time in Ehrlich ascites cells. Intra-
cellular pH was estimated from the chemical
shifts of either 2-deoxyglucose-6-phosphate
(2dG6P) or orthophosphate (X), as described in
the text. Extracellular pH was estimated from
the chemical shift of extracellular orthophos-
phate. Adenine nucleotide levels are
expressed in intensity units of primary phos-
phate peaks provided by peak-picking soft-
ware supplied with the spectrometer and do
not necessarily correspond linearly with con-
centration. 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. Note the appearance of
an identifiable pH
 peak (indicated by X) when
the transmembrane pH gradient is greater
than 0.27 pH units. At T = 165 min N2 was
substituted for O2 in the aeration mixture. At
T = 180 min, the O2 was replaced. Note the
rapid recovery of ATP levels and slower recov-
y of intracellular pH, ADP, and AMP.

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earlier paper from the same laboratory compared pH\textsuperscript{in} values obtained using either 6CF or the weak acid: 5,5-dimethoxyoxazolidine-2,4-dione (DMO) (19). In general, these measurements gave good agreement between pH\textsuperscript{in} values obtained using either indicator, and the pH\textsuperscript{in} values reported are similar to ours in that, at two values of pH\textsuperscript{in} (7.4 and 6.2), they observed pH\textsuperscript{in} to be near 7.3. In the present paper, we have tried to answer the question of pH\textsuperscript{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\textsuperscript{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\textsubscript{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\textsuperscript{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\textsuperscript{in} in Ehrlich ascites cells, others have not.

We have no evidence to suggest a mechanism by which Ehrlich ascites cells regulate their pH\textsuperscript{in}. Likely candidates are Na\textsuperscript{+}/H\textsuperscript{+} exchange or an ATP-requiring Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange. However, studies using specific inhibitors of these exchanges have generally shown a close agreement between intra- and extracellular pH. Nonetheless, some of these previous measurements have been consistent with our observations. The question of pH in regulation under physiological (37°C, well-energized) and steady state conditions is the subject of a recent review by Shulman and Belt (18, 27).

Note Added in Proof: A recent publication by González-Méndez et al. (González-Méndez, R., D. Weimer, 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 31P 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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