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H^+ Transport and the Regulation of Intracellular pH in Ehrlich Ascites Tumor Cells

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Summary. The intracellular pH (pH_i) of Ehrlich ascites tumor cells, both in the steady state and under conditions of acid loading or recovery from acid loading, was investigated by measuring the transmembrane flux of H^+ equivalents and correlating this with changes in the distribution ratio of dimethyloxazolidine-2,4-dione (DMO). The pH_i of cells placed in an acidic medium (pH_o below 7.15) decreases and reaches a steady-state value that is more alkaline than the outside. For example when pH_o is acutely reduced to 5.5, pH_i falls exponentially from 7.20 ± 0.06 to 6.29 ± 0.04 with a halftime of 5.92 ± 1.37 min, suggesting a rapid influx of H^+. The unidirectional influx of H^+ exhibits saturation kinetics with respect to extracellular $[H^+]$; the maximal flux is 15.8 ± 0.05 mmol/(kg dry wt · min) and K_m is 0.74 ± 0.09 × 10^{-6} M.

Steady-state cells with pH_i above 6.8 continuously extrude H^+ by a process that is not dependent on ATP but is inhibited by anaerobiosis. Acid-loaded cells (pH_i 6.3) when returned to pH_o 7.3 medium respond by transporting H^+, resulting in a rapid rise in pH_i. The halftime for this process is 1.09 ± 0.22 min. The H^+ efflux measured under similar conditions increases as the intracellular acid load increases. An ATP-independent as well as an ATP-dependent efflux contributes to the restoration of pH_i to its steady-state value.

Key Words Ehrlich cells · intracellular pH · pH regulation · H^+ transport

Introduction

The recognition that many cellular processes are sensitive to the intracellular pH (pH_i) has in recent years stimulated the study of pH regulatory mechanisms, primarily transport processes, which operate to remove H^+ (acid equivalents) produced as a result of metabolism as well as those which passively enter the cell. Regulation of pH_i has been most intensively studied in three excitable invertebrate cell types: snail neurons (Thomas, 1976, 1977, 1982), barnacle muscle (Boron, McCormick & Roos, 1979, 1981), and squid giant axon (Russell & Boron, 1976; Boron & Russell, 1983). The pH regulatory systems of these tissues share a number of features, which include: (1) stimulation when pH_i is reduced below normal, (2) a requirement for extracellular Na^+ and HCO_3^- as well as intracellular Cl^-, and (3) sensitivity to anion transport inhibitors. ATP is required for pH regulation in the squid axon but is apparently not needed in the snail neuron (Boron, 1983).

In contrast to the invertebrate systems, little is known about the mechanisms underlying the regulation of pH_i of nonexcitable mammalian cells including the Ehrlich cell or indeed whether such cells possess a pH regulatory system. In previous studies the transmembrane movements of H^+ have been inferred from changes in the steady-state pH_i in response to imposed pH gradients. Poole, Butler and Waddell (1964) reported that at constant P_{CO_2} pH_i decreased from 7.2 to 7.1 in Ehrlich cells incubated in HCO_3^- buffered media ranging from pH 7.60 to 6.80. However, in P_i-buffered media (pH 6.3 to 7.8) free of added HCO_3^-, the relationship between pH_i and extracellular pH (pH_o) was linear with a slope of 0.50, indicating that the cell interior was maintained alkaline with respect to the medium. In a recent ^{31}P-NMR study Gillies, Ogino, Shulman and Ward (1982) investigated the response of pH_i to changes in extracellular medium (20 mM HCO_3^-, pH 6–8). Their results are similar to those of Poole et al. (1964) and suggest that H^+ is not at electrochemical equilibrium but rather is regulated when pH_o is in the range of 6.5 to 7.2.

The mechanism by which the intracellular H^+ is regulated is not known. Both Gillies et al. (1982) and Geck, Pietrzyk, Heinz and Pfeiffer (1978) suggest that, since normal metabolism is necessary for maintaining even a small pH gradient across the membrane, active transport of H^+ or H^+ equivalents must be involved. This is consistent with the work of Heinz, Sachs and Schafer (1981) who presented evidence for activation by glycolysis of an electrogenic H^+ efflux.

The purpose of the present study is to extend

these observations in an attempt to gain additional insights into those factors responsible for the development, maintenance, and dissipation of a pH gradient across the Ehrlich cell membrane. To accomplish this we have investigated the time course of changes in pH_i in response to an external acid load and have correlated this with transmembrane fluxes of H^+ measured directly with a pH-stat. Since extrusion of acid is a hallmark of pH regulation, the ability of these cells to recover from an acid load and the energy dependence of this process have also been studied.

Materials and Methods

General

Experiments were conducted with nonglycolyzing Ehrlich-Lettré mouse ascites tumor cells maintained and prepared as previously described (Levinson, 1972). The initial wash medium contained (mM): 154, NaCl; 5, P_i; 6, KCl; and 10, HEPES-NaOH buffer (pH 7.4, 285–300 mOsm).

Cells were initially resuspended to a density of 19 mg dry wt/ml suspension and maintained at 25°C prior to use. To alter the pH of the extracellular medium, cell suspension was centrifuged, resuspended to a density of 9.1 mg dry wt/ml in a medium of the desired pH, and incubated at 37°C for 30 min in the presence of 100% O_2. This medium had a composition similar to that of the initial wash medium, except that the HEPES-NaOH buffer was replaced, for pH 5.5 to 6.7, with 2 (N-morpholino) ethanesulfonic acid (MES), and for pH 7.5 to 8.0 with N,N-bis (2-hydroxyethyl) glycine (Bicine). All solutions were free of added HCO_3^- and glucose. Before use, the cells were centrifuged and resuspended with fresh medium. Unless otherwise noted, all experiments were conducted at 37°C, and 100% O_2 was supplied at the surface of the cell suspension.

In some experiments, cells were resuspended immediately prior to use with a weakly buffered medium, prepared from the media previously described by tenfold dilution with an unbuffered, isosmotic solution containing (mM): 148, Na^+; 6, K^+; and 154, Cl^-. The pH of this unbuffered solution was adjusted to 7.0 with 1.0 N NaOH.

Intracellular pH

Intracellular pH was estimated from the distribution (cell/medium) of dimethyloxazolidine-2,4-dione, 5,5-[2-^{14}C] (DMO). [^{14}C]-DMO (0.025 μCi/ml cell suspension; New England Nuclear) was added in a solution containing unlabeled DMO such that the final DMO concentration was approximately 10^{-5} M. In most experiments, a minimum of 2 min was allowed for equilibration of the DMO. Portions of the cell suspension (1.0 ml) were then removed, placed in 1.5 ml microcentrifuge tubes, and centrifuged at 15,000 × g for 30 sec. The supernatant fluid was sampled and the remainder was aspirated. Cells were lysed and proteins precipitated by mixing 1.0 ml of ice-cold 1% (vol/vol) perchloric acid with the cell pellet. Samples were kept in an ice bath at least 30 min then centrifuged at 15,000 × g to remove the perchloric acid-insoluble residue.

Aliquots of the perchloric acid extract of the cells and the extracellular medium were assayed for [^{14}C]-DMO radioactivity by liquid scintillation counting. Intracellular water was determined from the wet and dry weights of cell pellets sampled in parallel as previously described (Bowen & Levinson, 1982). The ratio of wet wt/dry wt was 4.16 ± 0.01 (SE, n = 400). The extracellular space containing trapped [^{14}C]-DMO was estimated to be 21% of the total volume of the centrifuged cell pellet. This value was obtained from measurements using [^{32}P] P_i or [^{3}H]-methoxyinulin as extracellular space markers (Bowen & Levinson, 1983). The appropriate correction was applied to the intracellular [^{14}C]-DMO radioactivity and the ratio (cpm/ml cell water)/(cpm/ml medium) was calculated. The pK'_a of DMO at 37°C was taken as 6.13 and at 25°C was 6.22 (Boron & Roos, 1976). The extracellular pH was measured with a Corning semimicro combination electrode, or was obtained from the end point of the pH-stat if the samples were taken from the pH-stat chamber. Intracellular pH was then calculated by the method of Waddell and Butler (1959). Variations in the extracellular space had a relatively small effect on this calculation; for example, a 30% change in the trapped volume caused at most a 0.03 pH unit difference in the calculated intracellular pH.

Direct Measurement of H^+ Transport

Transmembrane movements of H^+ (i.e., acid equivalents) were measured with a Radiometer-Copenhagen pH-stat, consisting of a TTT2 Titrator set to the expanded pH scale, Titrigraph module PHA 943, Autoburette ABU13, and Titrigraph SBR3 chart recorder. The autoburette was modified by replacing the burette assembly with a 2-ml glass syringe in a plexiglass holder. The syringe was fitted with an 18-gauge needle. PE tubing (1 mm OD) attached to the needle served as the delivery tube and was threaded through an electrode holder then introduced into the chamber containing the cell suspension. This modification allowed accurate delivery of small titrant volumes and minimized leakage from the delivery tube. The cell suspension (8 to 9 ml) was maintained at 37°C in a double walled glass chamber (about 12 ml total volume) by a Haake FJ circulating water bath. The surface of the cell suspension was flooded with 100% O_2. The suspension was constantly stirred by a 14-mm Teflon-coated magnetic stir bar.

To measure net H^+ influx, cells equilibrated at pH 7.4 in HEPES-P_i buffered medium, were resuspended in weakly buffered medium. Cell suspension (8 ml, 9 mg dry wt/ml) was added to the pH-stat chamber and the cells were incubated for 2 min to permit temperature equilibration. A small volume (20 to 200 μl) of an isosmotic solution of MES (328 mM, 288 mOsm, pH 4) was then added to rapidly decrease the pH of the cell suspension. The end point of the titrator, roughly preset to the target pH on the basis of trial runs, was adjusted to the new pH of the cell suspension within 10 sec, and titration was initiated. The titrant was 0.005 N HCl, prepared by dilution of standardized 1.0 N HCl with the isosmotic, unbuffered Na/K/Cl solution described above. The rate of titration during H^+ influx measurements was linear for at least 4 min after the extracellular pH was changed. Following the titration period (6 to 12 min), duplicate 1.0-ml samples were removed from the chamber for determination of wet weight, dry weight, and intracellular water. In some cases, the cell suspension was also sampled to determine the intracellular pH from the distribution of [^{14}C]-DMO.

The procedures for the measurement of the net efflux of H^+ varied, depending on the nature of the experiment and on the rate of H^+ efflux expected. The titrant was either 0.005 N or 0.010 N NaOH in an isosmotic Na/K/Cl solution. For measurements of H^+ efflux from cells equilibrated at pH_o 7.3, a volume of cell suspension was centrifuged, resuspended with weakly buffered medium (pH 6.7 or 7.3, depending on the end point), and incubated for 3 min at 37°C with [^{14}C]-DMO. Samples were then removed for the measurement of the DMO distribution ratio and for cell water content. An 8-ml aliquot of cell suspension (9 mg dry wt/ml) was placed into the pH-stat chamber, and the pH of the cell suspension was altered by the titrator to the desired end point between pH 6.7 and 8.0.

To study the effect of intracellular pH on H^+ efflux, cells were equilibrated for 30 min at 37°C at a medium pH between 5.5 and 7.3. The cell suspension was centrifuged and resuspended with weakly buffered medium of the same pH. [^{14}C]-DMO was added, and after 3 min samples were removed to assess the initial intracellular pH. An 8-ml aliquot of cell suspension (9 mg dry wt/ml) was transferred to the pH stat chamber and a small volume of isosmotic HEPES-NaOH (155 mM, 295 mOsm, pH 8.5) was added to change the medium pH to 7.3. In some cases H^+ efflux was so rapid that, following the pH change, any delay in adjusting the end point caused a significantly shorter initial linear phase of titration and thus prevented a reliable measurement of the H^+ efflux. As an alternate method, cells were incubated and centrifuged as before, but the resulting cell pellet (about 0.6 ml) was resuspended with 0.5 ml of weakly buffered medium. The pH-stat chamber contained 8.0 ml of medium previously adjusted to the pH end point. The pH-stat was turned on and 0.8 ml of the concentrated cell suspension was added directly to the chamber to initiate the titration.

H^+ influx or efflux was calculated from the rate of acid or base addition (nmoles/min) necessary to maintain a constant pH. This rate was normalized to the total dry weight of cells in the chamber (obtained from the dry weight of 1.0 ml cell suspension samples and the final volume of the cell suspension in the chamber). Net H^+ influx or efflux was then expressed as mmol H^+/(kg dry wt · min) ± SEM.

Calculation of Net H^+ Transport from Changes in pH_i

If changes in pH_i result only from the transmembrane movement of H^+ equivalents then net H^+ transport can be determined from the product of the rate constant describing the rate of change of pH_i, the intracellular buffering capacity, and the difference between the initial and final pH_i (Boron, 1983). The ability of the Ehrlich cell to buffer H^+ was estimated in the following way. Tumor cells (19 mg dry wt/ml), equilibrated at pH_o 7.3–7.35, were rapidly washed once and resuspended at the same density in unbuffered Na/K/Cl solution. Four ml of cell suspension were added to a small chamber maintained at 37°C. The chamber contained 4 ml of 150 mM KCl and purified digitonin (Murphy, Coll, Rich & Williamson, 1980) such that the final digitonin concentration was 0.25 mg/ml cell suspension. In preliminary experiments we determined that this concentration of digitonin was adequate to permeabilize the cell membrane to solutes ranging in size from the nonmetabolized amino acid, alpha-aminoisobutyric acid, to the cytosolic enzyme lactic acid dehydrogenase. Neither cytochrome oxidase nor diaphorase, enzymes associated with mitochondria and endoplasmic reticulum, respectively, were released from the cells.

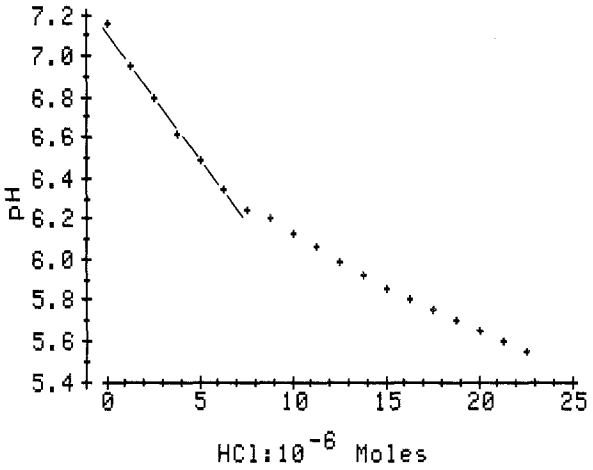

Fig. 1. Determination of cellular buffering capacity. Cells (76 mg dry wt) were permeabilized with digitonin (0.25 mg/ml) and the pH of the cell suspension was recorded following the addition of 0.25 N HCl

Figure 1 shows the results of a typical experiment in which the change in pH was measured as a function of acid addition. Upon addition of HCl to the cell suspension the pH initially decreases linearly from 7.15 to 6.35 then curves and decreases at a lower rate. In eight other experiments the initial linear change in pH ranged from 7.2 to 6.10. Since in our experiments the intracellular pH was never lower than 6.30, we used this portion of the curve to calculate the buffering capacity of the cells. The buffering capacity was taken as the reciprocal of the slope, dpH/dA, where dA is the amount of HCl that must be added to the intracellular phase to lower pH_i by dpH. In nine experiments the buffering capacity over the pH range of 7.10 to 6.10 was 100.3 ± 5.2 mmol H^+/(kg dry wt) per pH unit or 27.7 ± 1.6 mmol H^+/(kg cell water) per pH unit. In two other experiments the cell membrane was made permeable to H^+ by the addition of the ionophore monensin (10^{-5} M). The response of these cells to the addition of acid was virtually identical to that described above.

The rate of recovery of pH_i from an imposed acid load can be described by

$$d(pH_i)/dt = -k[pH_i(\infty) - pH_i(t)]. \quad (1)$$

A similar relationship describes the rate of decrease of pH_i when cells are challenged by an extracellular acid load. Integration of this equation yields

$$pH_i(t) = [pH_i(o) - pH_i(\infty)]\, e^{-kt} + pH_i(\infty) \quad (2)$$

where $pH_i(t)$ is the intracellular pH at time t, $pH_i(o)$ is the initial pH_i, $pH_i(\infty)$ is pH_i at the new steady state and k is the rate constant (min^{-1}). The data (pH_i as a function of time) were fit to this equation by a program written for the Apple II microcomputer. After an initial value of k is estimated the program increases k by 2% iteratively and ultimately chooses a value which maximizes the correlation coefficient of the regression line. The predicted value of $pH_i(\infty)$ and $pH_i(o)$ are provided by the intercept and the slope plus the intercept, respectively.

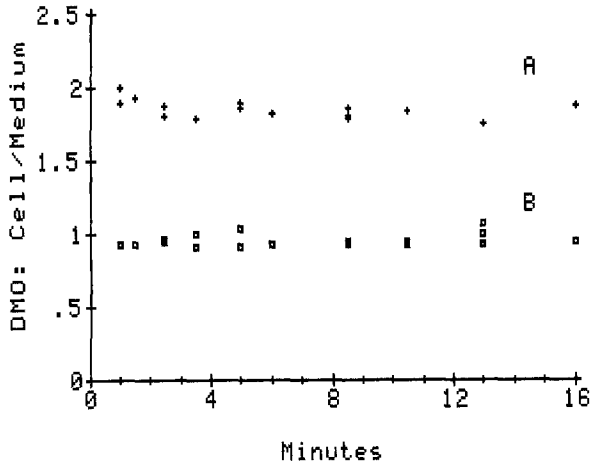

Fig. 2. The rate of equilibration of DMO across the Ehrlich cell membrane. Cells were incubated at either pH_o 5.5–5.6 (*A*) or 7.3–7.35 (*B*) for 30 min at 37°C. [^{14}C]-DMO was added and samples of the cell suspension were removed as a function of time. The distribution ratio of DMO (cell/medium) was determined from the [^{14}C]-radioactivity in perchloric acid-soluble cellular extracts and in the cellular medium. The results of three separate experiments conducted at each pH_o are shown

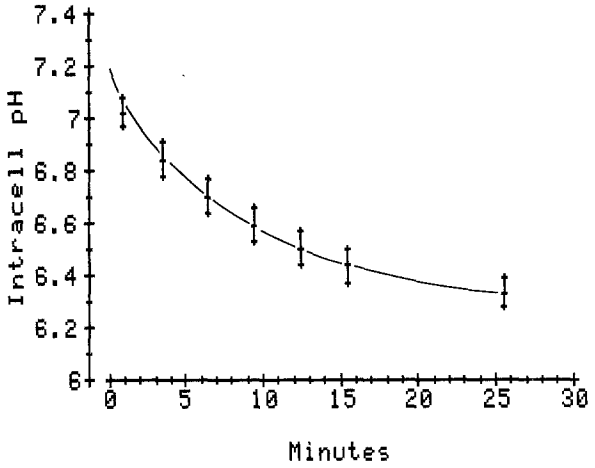

Fig. 3. The response of pH_i to an external acid load. MES buffer was added to cells incubated at pH_o 7.3 to rapidly change pH_o to 5.5–5.6. The pH_i was estimated from the distribution ratio of [^{14}C]-DMO. The means ± SE from nine experiments are shown. The decrease in pH_i in each experiment was fit to an exponential curve (*see* Methods) and for all experiments $k = 0.117 \pm 0.027$ min^{-1} ($t_{1/2} = 5.92 \pm 1.37$ min).

ATP Assay

Cellular ATP was measured in 0.05-ml samples of cell suspension that were immediately mixed with 0.05 ml ice-cold 2% (vol/vol) perchloric acid. After storing in an ice-bath for 30 min, the samples were centrifuged for 2 min at 15,000 × *g* to remove the perchloric acid-insoluble residue. The ATP in 0.05 ml of this extract was determined in a 5-ml arsenate buffer system by adding 0.05 ml of a luciferin-luciferase mixture (a threefold dilution of Sigma firefly lantern extract FLE-50). The resulting bioluminescence was quantified in a liquid scintillation spectrophotometer (Stanley & Williams, 1969). The samples were counted for 0.2 min; the interval between the addition of the reaction mixture and the start of the counting period was the same for all samples. The standards contained from 4.1 to 65.6 × 10^{-10} moles ATP.

Results

Equilibration of DMO

The early work of Poole et al. (1964) demonstrated that [^{14}C]-DMO equilibrated across the Ehrlich cell membrane in less than 5 min and that the addition of unlabeled DMO in concentrations as high as 7.7 × 10^{-4} M did not significantly affect the distribution ratio of [^{14}C]-DMO or the calculated intracellular pH. Since we were interested in measuring not only the steady-state pH_i but also changes as a function of time, we investigated the rate at which DMO equilibrated across the cell membrane. As shown in Fig. 2, when cells were incubated at an extracellular pH of 7.3–7.35 or pH 5.5–5.6 for 30 min at 37°C, DMO attained a steady-state distribution within about 90 sec. The intracellular pH was 7.27 ± 0.02 and 6.31 ± 0.04, respectively. Identical results were obtained when the unlabeled DMO concentration was increased fivefold. In a separate experiment cells were incubated at pH 7.3, DMO was added and the incubation continued for 10 min. Samples were removed for DMO analysis and the remaining cell suspension was washed free of extracellular DMO and resuspended in fresh medium. There was no detectable [^{14}C]-DMO associated with the washed cells, which demonstrated that DMO is readily removed from the intracellular phase.

Relationship between pH_i and pH_o

In order to determine the time course of the response of pH_i to an extracellular acid load, tumor cells were equilibrated at pH_o 7.30 in the presence of DMO. At time zero pH_o was suddenly decreased to pH 5.5–5.6 by the addition of MES buffer (*see* Methods) and the change in pH_i measured during the next 30 min. Figure 3, which summarizes the results of nine experiments, shows that pH_i decreases exponentially from 7.20 ± 0.06 to 6.29 ± 0.04 with a rate constant (k) of 0.117 ± 0.027 min^{-1}. If the change in pH_i is due only to the uptake of H^+ from the medium then the initial net H^+ influx is 10.7 ± 2.5 mmol/(kg dry wt · min), which was calcu-

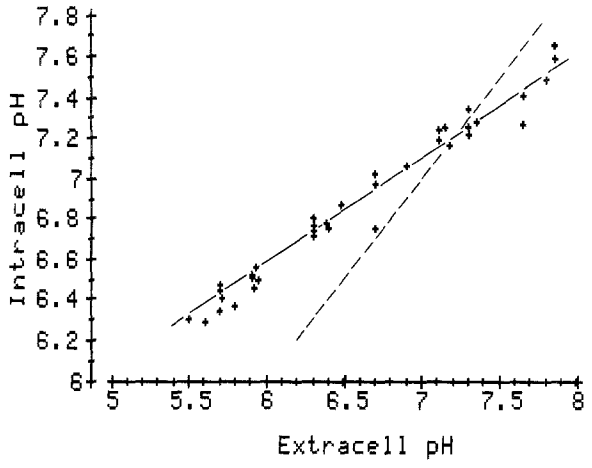

Fig. 4. The relationship between the steady-state intracellular and extracellular pH. Cells were incubated a minimum of 30 min at each pH_o. Each point represents the mean of at least two measurements. The best fit regression line has a slope of 0.53 and y-intercept of 3.38 (r = 0.987). The dashed line indicates where $pH_i = pH_o$

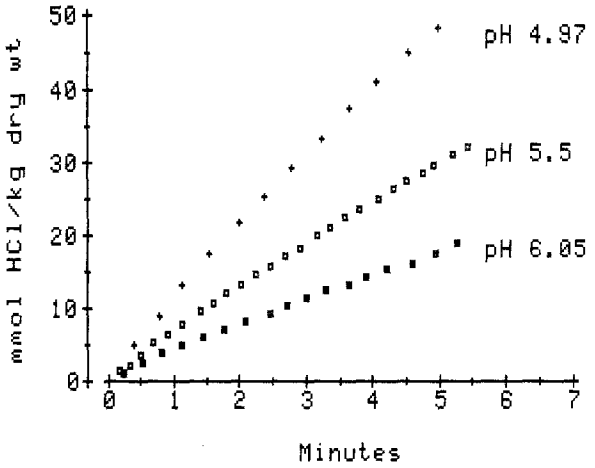

Fig. 5. Acid delivered by the pH-stat to cells preincubated at pH_o 7.3 and subsequently exposed to an acidic medium. The pH_o of the cell suspension (originally 7.3) was rapidly altered to the pH indicated in the figure for three representative experiments. The pH-stat maintained pH_o constant by the addition of HCl. The total amount of HCl delivered as a function of time is shown

lated from: $k \cdot \Delta\ pH_i \cdot$ buffering capacity. This rate was temperature dependent and in similar experiments decreased to zero when the cells were maintained at 4°C (data not shown.) We then systematically investigated the response of pH_i to changes in pH_o over the range 7.8 to 5.5. In these studies tumor cells were incubated a minimum of 30 min in each experimental medium. As illustrated in Fig. 4 intracellular and extracellular pH are equal at 7.15. However, when pH_o is decreased below 7.15 pH_i remains above pH_o. Conversely, when pH_o is greater than 7.15, pH_i is below pH_o. The slope of the regression line relating pH_i and pH_o is 0.53 and the pH gradient ($pH_o - pH_i$) linearly increases from zero at pH 7.15 to −0.79 at pH_o 5.5.

H^+ Influx

The observation that pH_i decreased with a decrease in pH_o suggested that H^+ had entered the cell. In order to study this process more directly a series of experiments was performed in which H^+ influx was measured in the pH-stat as a function of pH_o. Cells equilibrated at pH_o 7.30–7.40 (pH_i 7.15–7.22) were centrifuged and resuspended in weakly buffered medium, pH 7.40. Eight ml of cell suspension (9 mg dry wt/ml) were added to the pH-stat and incubated 2 min to permit temperature equilibration. A small volume (10–200 μl) of isosmotic MES (pH 4) was added to rapidly decrease the pH of the cell suspension. The response of the titrator to the sudden decrease in the pH_o is shown in Fig. 5. The rate of acid delivery required to maintain the extracellular pH increases as pH_o decreases and remains almost linear for about 4 min. The rate of titrant delivery estimated from the initial slope is 3.9 mmol/(kg dry wt · min) at pH_o 6.05 and increases to 10.7 at pH_o 4.97. Figure 6 summarizes the results of these studies and shows that H^+ influx increases linearly when pH_o is decreased from 6.6 to 4.8.

Since MES is a weak acid ($pKa' = 6.15$), it is possible that the increase in H^+ influx with a decrease in pH_o was due to the entry of the undissociated acid. To determine whether H^+ influx could occur in the absence of weak acids, cells were suspended in unbuffered $Na^+/K^+/Cl^-$ solution and pH_o was decreased by the addition of 0.15 N HCl. Results of these experiments (Fig. 6) were identical to those obtained when pH_o was decreased by the addition of MES.

When the pH_o is expressed in terms of $[H^+]$, H^+ influx exhibits saturation-type kinetics (Fig. 7). It is important to note that the H^+ influx measured in these experiments represents the difference between the unidirectional H^+ influx and efflux. Therefore, to evaluate the unidirectional H^+ influx it is necessary to measure the unidirectional H^+ efflux. To do this we assumed as a working hypothesis that the unidirectional efflux was dependent only on the intracellular H^+ concentration. To arrive at the H^+ efflux in the absence of any significant H^+ influx, net H^+ efflux was measured in the pH-stat when pH_o varied from 6.5 to 8.0 (or from a $[H^+]$ of 0.32 to 0.01 $\times$ 10^{-6} M). The pH_i in each of these experiments was between 7.18 and 7.23. Fig-

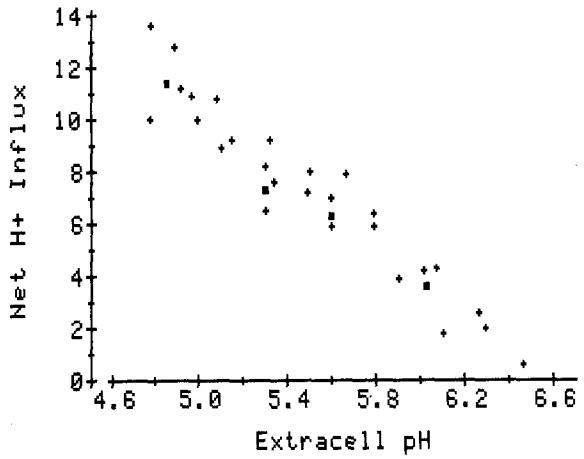

Fig. 6. Relationship between net H^+ influx and extracellular pH. Net H^+ influx [mmol/(kg dry wt · min)] determined from the initial linear uptake of H^+ was measured as a function of pH_o, which was decreased by MES (+) or by HCl (■). Extracellular pH was maintained constant by the pH-stat

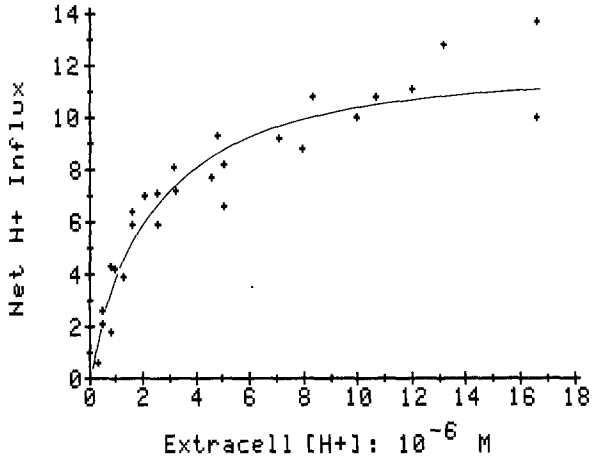

Fig. 7. Net H^+ influx as a function of extracellular $[H^+]$. The H^+ influx data of Fig. 6 were replotted with pH_o expressed in terms of extracellular $[H^+]$

ure 8 displays the results and shows that the increase in H^+ efflux is relatively linear as pH_o increases. A regression line fit to these data yields an intercept ($[H^+] = 0$) of 4.56 mmol H^+/(kg dry wt · min), which provides an estimate of the unidirectional efflux. This value was then added to each net influx value shown in Fig. 7, and these data were fit to the Michaelis-Menten equation by the weighted linear regression method of Wilkinson (1961) as previously described (Bowen & Levinson, 1982). The K_m is 0.743 ± 0.098 × 10^{-6} M H^+, which corresponds to a pH_o of 6.13 ± 0.05. The maximal unidirectional H^+ influx is 15.8 ± 0.05 mmol H^+/(kg dry wt · min).

H^+ Efflux

The results to this point are consistent with the view that a decrease in pH_o (7.30 to 5.50) results in H^+ influx which in turn is responsible for the decrease in pH_i. The degree to which pH_i decreases and eventually reaches a new steady state depends to a large extent on the capacity of the cells to buffer H^+ as well as their ability to remove H^+. This led to two questions. First, whether the unidirectional H^+ influx at the new steady state would be balanced by a corresponding H^+ efflux such that the net flux is zero. Second, since the unidirectional influx of H^+ increases with a decrease in pH_o, does the efflux of H^+ increase with a decrease in pH_i.

To determine whether upon the establishment of a steady-state distribution of H^+ across the cell membrane the net flux of H^+ fell to zero, tumor cells were equilibrated in media (pH 7.80 to 5.50) containing DMO. The corresponding pH_i ranged from 7.52 to 6.31. Aliquots of cell suspension were centrifuged, resuspended in weakly buffered media at the same pH, and transferred to the pH-stat, which was set up to measure either H^+ influx or efflux. The data shown in Fig. 9 demonstrate that at pH_i 6.80 (pH_o 6.5) and below (not shown) there was zero net flux, indicating that H^+ influx equalled efflux. However, in cells equilibrated in media greater than pH_o 6.50 there was a net acid efflux which increased to about 4.1 mmol/(kg dry wt · min) at pH_i 7.52 (pH_o 7.80). Although the cells extruded H^+ at pH_i greater than 6.80 the pH_i did not change. This steady-state rate of H^+ removal was unaffected when cellular ATP was depleted by the addition of 5 mM 2-deoxyglucose. However, in control cells (pH_i 7.20; pH_o 7.3–7.4) it was completely inhibited at 4°C or when the cells were incubated in a N_2-equilibrated, anaerobic medium.

The second question, that is, whether H^+ efflux increases with a decrease in pH_i, was investigated in the following way. Tumor cells with different pH_i values (6.30 to 7.20) were prepared by incubation in media of pH_o 5.50 to 7.30. Aliquots of the cell suspension were centrifuged and resuspended in a small volume (0.8 ml) of weakly buffered medium at the same pH_o. The cell suspension was then transferred to the pH-stat (adjusted to maintain pH_o 7.30), which contained in addition to DMO 8.0 ml of medium weakly buffered to pH 7.30. At this extracellular pH the unidirectional H^+ influx is only about 1 mmol/(kg dry wt · min) so that the initial net H^+ efflux provides a reasonable estimate of the unidirectional efflux. The response of the pH-stat to

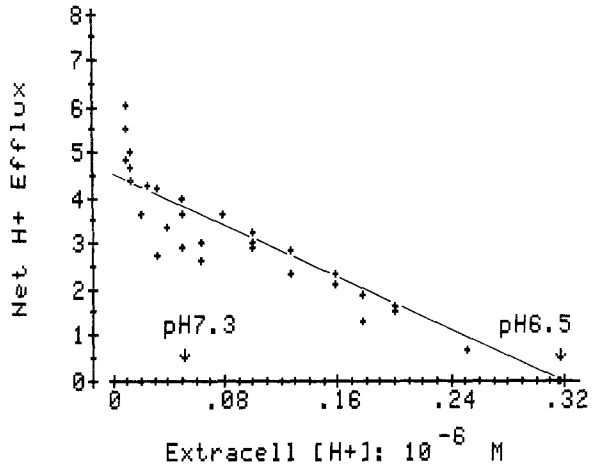

Fig. 8. Net H^+ efflux when pH_o is between 6.5 and 8.0. Net H^+ efflux [mmol/(kg dry wt · min)] is shown as a function of extracellular $[H^+]$. Cells were incubated at pH_o 7.3 prior to the efflux measurement; pH_i was between 7.18 and 7.23. The *y*-intercept determined from the regression line is 4.56 mmol/(kg dry wt · min) and $r = 0.91$

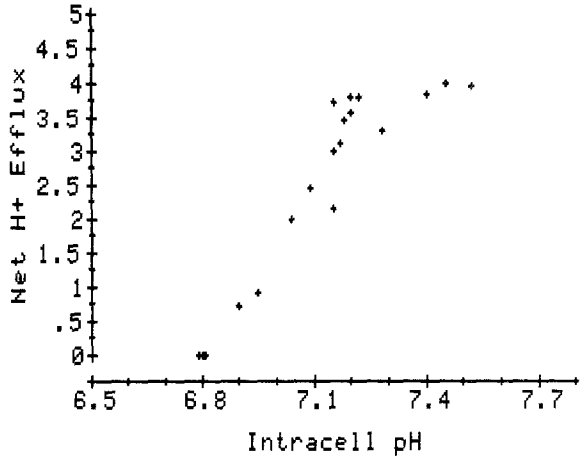

Fig. 9. Relationship between net H^+ extrusion and the steady-state pH_i. Cells were incubated at pH_o 6.5 to 7.8 until a steady-state pH_i (6.80 to 7.52) was attained. A continuous net production of H^+ [mmol/(kg dry wt · min)] was measured in the pH-stat even though pH_i did not change

the addition of cells at three representative pH_i is shown in Fig. 10. The rate of delivery of NaOH required to maintain the extracellular pH at 7.30 is clearly a function of the initial pH_i. For example, at pH_i 6.31 the rate of titration is approximately 25 times that for control cells (pH_i 7.28). The initial rapid rate of OH^- addition decreases within about 20 sec, and by 3 min the rate is constant and identical to that of the control cells. At this point the intracellular pH determined from the distribution ratio of DMO was in each case between 7.18 and 7.22 and remained constant even though the cells continued to extrude H^+ at the rate of 3.5 mmol/(kg dry wt · min). The relationship between the initial rate of H^+ efflux and pH_i expressed as $[H^+]$ is shown in Fig. 11. At pH_i 7.20 the initial as well as the steady-state rate of H^+ efflux is about 3 mmol/(kg dry wt · min) but increases to over 100 mmol/(kg dry wt · min) at pH_i 6.30. These data suggest that the initial rapid phase of base addition by the pH-stat corresponds to a rapid efflux of H^+ with a concomitant rise in pH_i. Figure 12 shows that this is indeed the case. Tumor cells were incubated in pH 5.8 medium containing DMO for 30 min after which the pH_i had decreased to 6.48 ± 0.13. When the extracellular pH was rapidly increased to 7.28–7.35 by the addition of Hepes buffer there was a rapid exponential rise in pH_i, which asymptotically approached pH 7.22 ± 0.01. In eight experiments the rate constant (k) describing the rate of change of pH_i is 0.634 ± 0.13 min^{-1}. If it is assumed that the extrusion of H^+ is the only process responsible for the change in pH_i then net H^+ efflux is 49.6 ± 9.9 mmol/(kg dry wt · min), which was calculated from: $k \cdot \Delta\ pH_i \cdot$ buffering capacity. This value, although within the same range as that found by the direct measurement of H^+ efflux (Fig. 11), is lower. The most likely reason for this is the inability of DMO to redistribute across the membrane at a sufficiently high rate in response to very rapid rates of H^+ efflux. However, the number of acid equivalents removed from the cells, calculated from the change in pH_i (6.5–7.22; Fig. 12) averaged 72 mmol/kg dry wt. This value is virtually identical with that calculated from acid efflux measured in the pH-stat after correction for a base line flux of 3.5 mmol/kg dry wt · min (Fig. 10).

Role of ATP in H^+ Efflux

The results of the H^+ efflux experiments (Figs. 11 and 12) indicate that the Ehrlich cell responds to acid loading by removing H^+ at a rapid rate, which results in a rise in pH_i. When pH_o is within the physiological range (7.25–7.35) pH_i returns to 7.15–7.22. Since it has been suggested that the pH_i of this cell may be regulated by an ATP-dependent process (Gillies et al., 1982), we were interested in establishing whether the change in pH_i associated with H^+ efflux was dependent on ATP.

In this series of experiments cells were prepared such that pH_i was 6.80 ± 0.04 (pH_o 6.45–6.50). The cells were concentrated to 90 mg dry wt/

ml and 4 ml were added to 35 ml of pH 6.45 DMO-containing medium which had been made O_2-free by bubbling with N_2 30 min before the start of the experiment. The cell suspension was kept anaerobic by continuously flushing the surface with N_2. Samples of cell suspension were removed periodically for the measurement of ATP and DMO. Figure 13 illustrates the results of a typical experiment. The addition of cell suspension to the anaerobic medium resulted in a rapid decrease in ATP from 13.0 to 1.0 mmol/kg dry wt. The halftime ($t_{1/2}$) for this process is 2.5 min. During this period (13 min) pH_i decreased from 6.93 to 6.55, which suggested that a net influx of H^+ had occurred. However, in parallel experiments using the pH-stat, net influx of H^+ during this period was less than 1 mmol/(kg dry wt · min), which would have decreased pH_i by no more than 0.1 pH units. A more likely reason for the decrease in pH_i is the release of H^+ concomitant with the hydrolysis of ATP (Alberty, 1968, 1969; Poole, 1967). The replacement of N_2 by 100% O_2 at 13 min resulted in the resynthesis of ATP ($t_{1/2}$ = 0.7 min). When the extracellular pH was rapidly changed to 7.28, the pH_i increased to 7.20 with $t_{1/2}$ of 2.2 min. However, when in a similar experiment the pH_o of ATP-depleted cells was changed to 7.27 (Fig. 14), pH_i increased and reached a new steady state at 6.91. After O_2 was returned to the cell suspension the pH_i increased from 6.91 to 7.29. The Table summarizes the results of three experiments performed in exactly the same manner.

Although with the introduction of O_2 pH_i increased from 6.91 to 7.24, there was a concomitant rise in cellular ATP. This raised the possibility that the change in pH_i occurred as a result of ATP resynthesis rather than H^+ efflux. In order to test this possibility the experimental conditions described in Fig. 14 were duplicated in the pH stat. When O_2 was introduced H^+ efflux increased and the total num-

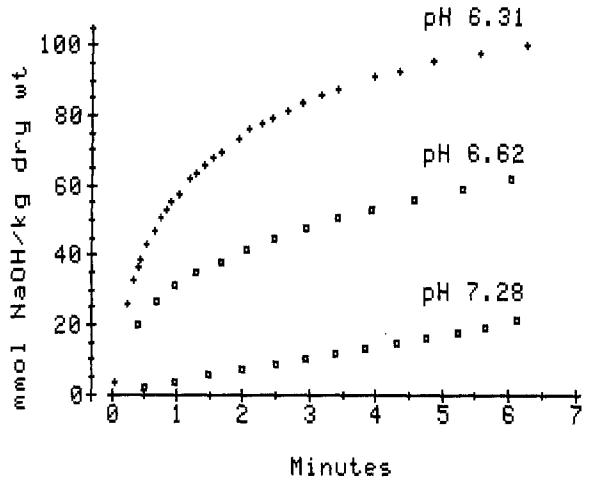

Fig. 10. Addition of base by the pH-stat when the pH_o of acid-loaded cells is raised and maintained at pH 7.3. Cells at pH_i indicated in the figure were transferred to the pH-stat set to maintain pH_o constant at 7.3. The total amount of NaOH added is shown as a function of time for three representative experiments

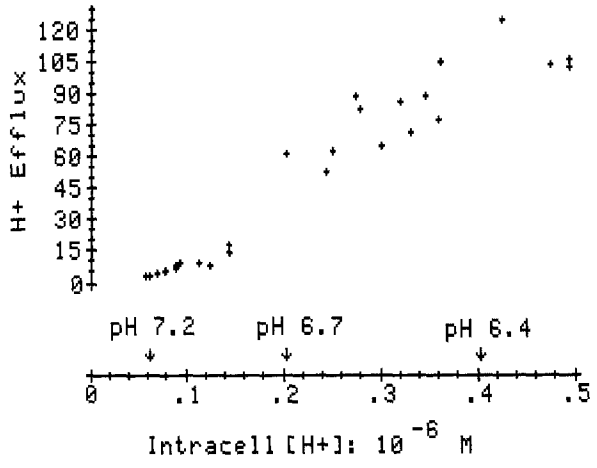

Fig. 11. Relationship between initial H^+ efflux and intracellular [H^+] when pH_o was changed to 7.3. Acid-loaded cells were added to the pH-stat containing pH_o 7.3 medium. The initial rate of H^+ efflux [mmol/(kg dry wt · min)] was calculated from the initial linear rate of addition of NaOH. H^+ efflux is displayed as a function of intracellular [H^+]; three corresponding pH_i values are indicated on the abscissa

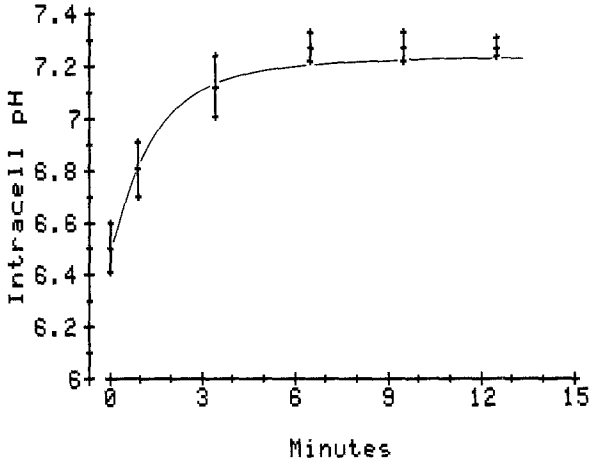

Fig. 12. Rise of pH_i of acid-loaded cells when pH_o is changed to pH 7.3. Cells were preincubated at pH_o 5.8. Hepes buffer was added at time zero to change the pH_o to between 7.28 and 7.35. The mean pH_i ± SE is shown for eight experiments. The rate constant was determined in each experiment from the best fit exponential curve. For all eight experiments, k = 0.63 ± 0.13 min^{-1} and $t_{1/2}$ = 1.09 ± 0.22 min

ber of acid equivalents lost from the cells in the following 5-min period was 34.9 ± 0.3 mmol/kg dry wt (n = 3). The data in Fig. 14 shows that pH_i increased from 6.91 to 7.24 within the same 5-min period, which represents a net acid loss of 33 mmol/kg dry wt.

Therefore, the efflux of H^+ can occur by two processes. The first which does not require ATP is capable of raising pH_i, although to a value well below that expected when pH_o is 7.30. The second is ATP-dependent and is responsible for returning pH_i to the steady-state value predicted for pH_o 7.30.

Discussion

The experiments described above were undertaken to test the hypothesis that the Ehrlich ascites tumor cell has the capacity to influence or regulate pH_i. Regulation of pH_i has often been defined as those processes which return the intracellular pH to its normal steady-state value following acidification of the cytosol (Roos & Boron, 1981; Boron, 1983). It is generally assumed that these processes are also operative in the normal physiological state to extrude H^+ or acid equivalents which originate from either cellular metabolism or from passive H^+ influx. The results of our experiments when considered in the context of this definition support the conclusion that these cells in response to a decrease in pH_i extrude H^+ and that this process involves both an energy-dependent and a passive H^+ efflux.

Estimation of pH_i from the distribution of the

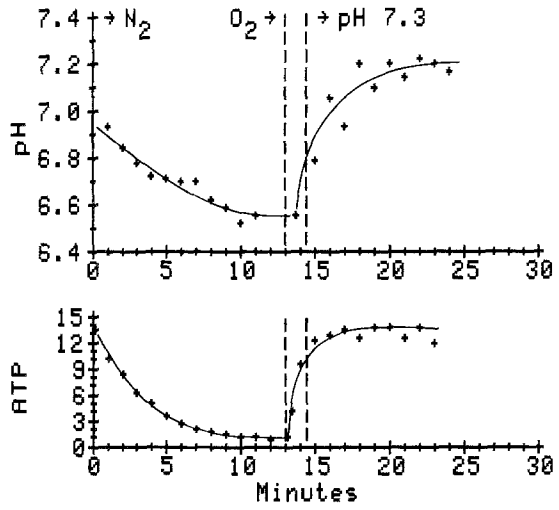

Fig. 13. The response of acid-loaded, anaerobic cells to O_2 and to a rise in pH_o. At time zero a concentrated cell suspension incubated at pH_o 6.5 (pH_i 6.91) was added to N_2-equilibrated medium at the same pH. pH_i and cell ATP (mmol/kg dry wt) were measured as a function of time. The rate of disappearance of ATP approximated an exponential decay and by a method similar to that described for the change in pH_i was fit to the equation

$$ATP(t) = [ATP(o) - ATP(\infty)]\, e^{-kt} + ATP(\infty)$$

where $ATP(t)$ is intracellular ATP (mmol/kg dry wt) at time t, $ATP(o)$ is initial ATP, $ATP(\infty)$ is ATP at the new steady state, and k is the rate constant (min^{-1}). The k describing the disappearance of ATP after the cells were placed in an anaerobic medium is 0.29 min^{-1} ($t_{1/2}$ = 2.41 min, r = 0.998). At 13 min, 100% O_2 was substituted for N_2 and ATP reappeared with k = 1.27 min^{-1} ($t_{1/2}$ = 0.55 min, r = 0.988). At 14.5 min pH_o was changed to 7.3 by the addition of a small volume of Hepes buffer

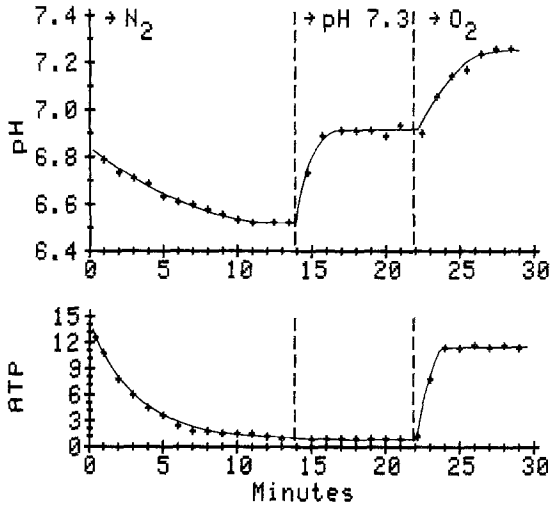

Fig. 14. Demonstration of ATP-independent and ATP-dependent H^+ efflux pathways. The experimental conditions were similar to those of Fig. 13 except that pH_o was changed to 7.3 in the presence of 100% N_2 at 14 min. ATP fell with k = 0.32 min^{-1} ($t_{1/2}$ = 2.15 min, r = 0.999). At 22 min, 100% O_2 was introduced and ATP was resynthesized with k = 1.17 min^{-1} ($t_{1/2}$ = 0.60 min, r = 0.991)

Table. Changes in cellular ATP and pH_i

Time (min)	Gas	ATP		pH_o	pH_i	
		Disappearance $t_{1/2}$ (min)	Reappearance $t_{1/2}$ (min)		Initial	Final
0–14	N_2	2.40 ± 0.20	—	6.45	6.80	6.51
14–22	N_2	—	—	7.24	6.51	6.91
22–29	O_2	—	0.76 ± 0.03	7.24	6.91	7.24

At time zero a concentrated cell suspension was added to N_2-equilibrated medium (pH_o 6.45) and samples were periodically removed for the determination of cell ATP and pH_i. At 14 min, the pH of the cell suspension was changed to 7.24 by adding a small volume of N_2-equilibrated Hepes buffer. The N_2 was replaced with O_2 at 22 min. The mean halftime ± SE for the disappearance or reappearance of ATP (*see* Fig. 13) and the mean initial and final pH_i during each period are shown for three experiments.

weak acid DMO (Waddell & Butler, 1959) assumes that the concentration of the nondissociated form is equal on both sides of the membrane while the distribution ratio of the ionized form depends on the pH of the intracellular and extracellular compartments. Since this is an equilibrium method, it can be used to follow changes in pH_i only if DMO equilibrates across the cell membrane at least as fast as pH_i changes. Our results (Fig. 2) indicate that DMO attains equilibrium distribution by 90 sec, which approaches the time resolution of the sampling technique.

The relationship between steady-state pH_i and pH_o (Fig. 4) was found to be linear over the pH_o range of 7.80–5.50. The slope of this line is 0.53, which is almost identical to that reported by Poole et al. (1964) for the nominally HCO_3^--free system. In a similar study Gillies et al. (1982) used ^{31}P-NMR to investigate pH_i as a function of pH_o in media containing HCO_3^-. Their results showed that pH_i is maintained at about 7.1 when pH_o is 6.5–7.1 and are in good agreement with those of Poole et al. (1964) for the HCO_3^--containing system. This provides an independent verification that the distribution ratio of DMO yields an accurate measure of pH_i.

As noted above, exposing the cells to an external acid load results in a decrease in pH_i. For example, at pH_o 5.50–5.60 pH_i decreases exponentially with a halftime of about 6 min by a temperature-sensitive process (Fig. 3). Direct measurement of net H^+ influx at constant pH_o showed that the rate at which H^+ entered the cell increased linearly with a decrease in pH_o and was independent of the nature of the acid. However, the relationship between net H^+ influx and the extracellular $[H^+]$ exhibits saturation-type kinetics. The observation that this process is temperature sensitive, saturable, and occurs in the absence of weak acids is suggestive of carrier-mediated H^+ influx rather than simple diffusion of weak acids. Net influx can be correlated with the rate at which H^+ accumulates within the cell and decreases pH_i. The unidirectional influx, on the other hand, provides a more accurate measure of the rate at which H^+ crosses the membrane. As noted in the Results unidirectional H^+ influx was determined from the sum of the net influx and unidirectional efflux. The latter quantity was estimated by measuring the net efflux (pH_i 7.28–7.23) as a function of increasing pH_o and extrapolating to zero extracellular $[H^+]$ (Fig. 8). Implicit in this approach is the assumption that H^+ efflux depends only on pH_i. If, however, it is influenced by pH_o then raising the extracellular pH could result in an enhanced H^+ efflux. In this case the unidirectional efflux would not be constant but would decrease as pH_o decreased, resulting in an overestimate of the correction factor and hence the unidirectional influx.

During the course of the efflux measurements we observed that cells equilibrated and maintained at the normal physiological pH (7.3–7.4) continuously exported H^+ at a rate that ranged from 3–4 mmol/(kg dry wt · min) (Figs. 8 and 9). A similar observation has been previously reported (Heinz et al., 1981; Smith & Robinson, 1981*a*). It is important to note that, although the cells continuously removed H^+, pH_i did not change. Nonglycolytic metabolism is probably the source of this H^+. Metabolic origin is suggested by our finding that the rate of net H^+ efflux decreased when the steady-state pH_i decreased from about 7.30 to 6.80 (Fig. 9). This most likely occurred because of progressive inhibition of the metabolic processes responsible for acid generation. This view is strengthened by the observation that either reducing the temperature to 4°C or incubating cells in an anaerobic medium completely inhibited net H^+ efflux.

In contrast with the steady-state case, when acid-loaded cells are returned to the normal physiological pH, efflux of H^+ greatly exceeds influx and pH_i returns to pH 7.2 (Figs. 12–14). The extrusion of an acid load by the cells is apparently accomplished by two processes, which can be separated on the basis of ATP dependence. The ATP-independent process rapidly extrudes H^+ but raises pH_i only to about 6.9. The ATP-dependent process is slower and raises pH_i from 6.9 to 7.2. The possibility exists that a fraction of the increase in pH_i results from H^+ utilization associated with ATP synthesis. However, the ATP content is 62% of its steady-state value by 1 min after the introduction of O_2 while pH_i has changed by only 0.1 pH units. Furthermore, during this period H^+ efflux, as measured in the pH-stat, increases and the total acid loss correlates well with the change in pH_i. Because of these findings we conclude that the rise in pH_i from 6.9 to 7.2 is due principally to an ATP-dependent efflux rather than to ATP synthesis. Under similar experimental conditions Gillies et al. (1982) report that addition of O_2 to an anaerobic cell suspension resulted in the synthesis of ATP as well as a rise in pH_i. However, in contrast with our results both the resynthesis of ATP and return of pH_i were very slow.

It is useful to compare acid-loaded cells that maintain a pH gradient across the membrane to those that extrude acid. Incubation of cells in media of pH lower than 6.50 results in H^+ influx followed by a decrease in pH_i. The cells respond by transporting H^+ from the cytosol to the medium against a $[H^+]$ gradient. Since the membrane potential, at

least under physiological conditions, is in the range of 22–25 mV inside negative (Smith & Robinson, 1981*b,c*), the transport system must move H^+ against an electrical gradient as well. A steady state is established such that H^+ influx equals efflux and the cell interior is maintained alkaline with respect to the extracellular environment. The bidirectional fluxes predicted for the steady state depend on pH_o and according to our data do not exceed 16 mmol/(kg dry wt · min). In contrast with the steady-state condition, when the extracellular pH of acid-loaded cells is rapidly increased to 7.30, H^+ moves from the cell interior to the medium, resulting in a rise in pH_i. Probably both the ATP-dependent and independent processes are responsible for the rapid rise in pH_i, although the relative contribution of each remains to be determined. It is clear, however, that the initial rapid rate of H^+ efflux (Figs. 10 and 11) increases with a decrease in pH_i. At every pH_i below 7.20 this phase far exceeds the efflux predicted from the steady-state condition. This suggests that an efflux pathway(s) is either stimulated by the reduction in the extracellular $[H^+]$ or that inhibition of this pathway(s) is relieved. It is possible that this response of the efflux to the extracellular pH serves as an important component of the pH_i regulatory system of the Ehrlich cell.

In other systems, particularly squid axon, snail neuron, and barnacle muscle, considerable evidence has been presented to indicate that pH_i is regulated primarily by a cotransport system(s) that is stimulated by extracellular acidification and requires extracellular Na^+, HCO_3^- and intracellular Cl^- (Boron, 1983). Whether a similar cotransport mechanism is operative in the Ehrlich cell is unknown, although in preliminary experiments we have found that the replacement of extracellular Na^+ by choline markedly reduces the rate of acid extrusion, suggesting a role for Na^+ in this process. The H^+ extrusion mechanism of this cell is also like that of the invertebrate systems in that it is stimulated by a decrease in pH_i and is apparently inhibited by an increase in the extracellular $[H^+]$ (Boron et al., 1979). However, there is at least one significant difference between the pH_i regulatory system of the Ehrlich cell and that of invertebrate tissues. In the barnacle muscle the rate of acid extrusion falls to zero at pH_i 7.3–7.4 over a wide range of extracellular $[HCO_3^-]$ and pH_o (Boron et al., 1979), while in the Ehrlich cell the pH gradient established across the cell membrane is directly related to pH_o and may be a consequence of H^+ influx.

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References

Alberty, R.A. 1968. Effect of pH and metal ion concentration on the equilibrium hydrolysis of adenosine triphosphate to adenosine diphosphate. *J. Biol. Chem.* **243:**1337–1343

Alberty, R.A. 1969. Standard Gibbs free energy, enthalpy and entropy changes as a function of pH and pMg for several reactions involving adenosine phosphates. *J. Biol. Chem.* **244:**3290–3302

Boron, W.F. 1983. Transport of H^+ and of ionic weak acids and bases. *J. Membrane Biol.* **72:**1–16

Boron, W.F., McCormick, W.C., Roos, A. 1979. pH regulation in barnacle muscle fibers: Dependence on intracellular and extracellular pH. *Am. J. Physiol.* **237:***C*185–*C*193

Boron, W.F., McCormick, W.C., Roos, A. 1981. pH regulation in barnacle muscle fibers: Dependence on extracellular sodium and bicarbonate. *Am. J. Physiol.* **240:***C*80–*C*89

Boron, W.F., Roos, A. 1976. Comparison of microelectrode, DMO and methylamine methods for measuring intracellular pH. *Am. J. Physiol.* **231:**799–809

Boron, W.F., Russell, J.M. 1983. Stoichiometry and ion dependencies of the intracellular pH regulating mechanism in squid giant axons. *J. Gen. Physiol.* **81:**373–399

Bowen, J.W., Levinson, C. 1982. Phosphate concentration and transport in Ehrlich ascites tumor cells: Effect of sodium. *J. Cell. Physiol.* **110:**149–154

Bowen, J.W., Levinson, C. 1983. Evidence for monovalent phosphate transport in Ehrlich ascites tumor cells. *J. Cell. Physiol.* **116:**142–148

Geck, P., Pietrzyk, C., Heinz, E., Pfeiffer, B. 1978. Is there an active proton pump in Ehrlich cells? *Acta Physiol. Scand.* **Special Suppl.:** 363–372

Gillies, R.J., Ogino, T., Shulman, R.G., Ward, D.C. 1982. ^{31}P-nuclear magnetic resonance evidence for the regulation of intracellular pH by Ehrlich ascites tumor cells. *J. Cell. Biol.* **95:**24–28

Heinz, A., Sachs, G., Schafer, J.A. 1981. Evidence for activation of an active electrogenic proton pump in Ehrlich ascites tumor cells during glycolysis. *J. Membrane Biol.* **61:**143–153

Levinson, C. 1972. Phosphate transport in Ehrlich ascites tumor cells and the effect of arsenate. *J. Cell. Physiol.* **79:**73–78

Murphy, E., Coll, K., Rich, T.L., Williamson, J.R. 1980. Hormonal effects on calcium homeostasis in isolated hepatocytes. *J. Biol. Chem.* **255:**6600–6608

Poole, D.T. 1967. Intracellular pH of the Ehrlich ascites tumor cell as it is affected by sugar and sugar derivatives. *J. Biol. Chem.* **242:**3731–3736

Poole, D.T., Butler, T.C., Waddell, W.J. 1964. Intracellular pH of the Ehrlich ascites tumor cell. *J. Natl. Cancer Inst.* **32:**939–946

Roos, A., Boron, W.F. 1981. Intracellular pH. *Physiol. Rev.* **61:**296–434

Russell, J.M., Boron, W.F. 1976. Role of chloride transport in regulation of intracellular pH. *Nature (London)* **264:**73–74

Smith, T.C., Robinson, S.C. 1981*a*. Effect of H^+ on the kinetics of Na^+-dependent amino acid transport in Ehrlich ascites tumor cells: Evidence for H^+ as an alternative substrate. *J. Cell. Physiol.* **109:**507–516

Smith, T.C., Robinson, S.C. 1981*b*. The membrane potential of Ehrlich ascites tumor cells: An evaluation of the null point method. *J. Cell. Physiol.* **109:**399–406

Smith, T.C., Robinson, S.C. 1981*c*. Variable coupling of active

(Na + K)-transport in Ehrlich ascites tumor cells: Regulation by external Na^+ and K^+. *J. Cell. Physiol.* **106:**407–418

Stanley, P.E., Williams, S.G. 1969. Use of the liquid scintillation spectrometer for determining adenosine triphosphate by luciferase enzyme. *Biochem. J.* **80:**324–332

Thomas, R.C. 1976. The effect of carbon dioxide on the intracellular pH and buffering power of snail neurons. *J. Physiol. (London)* **255:**715–735

Thomas, R.C. 1977. The role of bicarbonate, chloride and sodium ions in the regulation of pH in snail neurons. *J. Physiol. (London)* **273:**317–338

Thomas, R.C. 1982. Snail neuron intracellular pH. *In:* Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions. R. Nuccitelli and D. Deamer, editors. pp. 189–204. Alan R. Liss, New York

Waddell, W.J., Butler, T.C. 1959. Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of dog. *J. Clin. Invest.* **38:**720–729

Wilkinson, G.N. 1961. Statistical estimations in enzyme kinetics. *Biochem. J.* **80:**324–332

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