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# $pH_i$ Regulation in Ehrlich Mouse Ascites Tumor Cells: Role of Sodium-dependent and Sodium-independent Chloride-Bicarbonate Exchange

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**Abstract:** $pH_i$ recovery in acid-loaded Ehrlich ascites tumor cells and $pH_i$ maintenance at steady-state were studied using the fluorescent probe BCECF.

Both in nominally $HCO_3^-$-free media and at 25 mM $HCO_3^-$, the measured $pH_i$ (7.26 and 7.82, respectively) was significantly more alkaline than the $pH_i$ value calculated assuming the transmembrane $HCO_3^-$ gradient to be equal to the $Cl^-$ gradient. Thus, $pH_i$ in these cells is not determined by the $Cl^-$ gradient and by $Cl^-/HCO_3^-$ exchange.

$pH_i$ recovery following acid loading by propionate exposure, $NH_4^+$ withdrawal, or $CO_2$ exposure is mediated by amiloride-sensitive $Na^+/H^+$ exchange in $HCO_3^-$-free media, and in the presence of $HCO_3^-$ (25 mM) by DIDS-sensitive, $Na^+$-dependent $Cl^-/HCO_3^-$ exchange. A significant residual $pH_i$ recovery in the presence of both amiloride and DIDS suggests an additional role for a primary active $H^+$ pump in $pH_i$ regulation. $pH_i$ maintenance at steady-state involves both $Na^+/H^+$ exchange and $Na^+$-dependent $Cl^-/HCO_3^-$ exchange.

Acute removal of external $Cl^-$ induces a DIDS-sensitive, $Na^+$-dependent alkalinization, taken to represent $HCO_3^-$ influx in exchange for cellular $Cl^-$. Measurements of $^{36}Cl^-$ efflux into $Cl^-$-free gluconate media with and without $Na^+$ and/or $HCO_3^-$ (10 mM) directly demonstrate a DIDS-sensitive, $Na^+$-*dependent* $Cl^-/HCO_3^-$ exchange operating at slightly acidic $pH_i$ ($pH_o$ 6.8), and a DIDS-sensitive, $Na^+$-*independent* $Cl^-/HCO_3^-$ exchange operating at alkaline $pH_i$ ($pH_o$ 8.2).

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**Key words:** pH regulation — $pH_i$ maintenance — $Na^+/H^+$ exchange — $Na^+$-dependent $Cl^-/HCO_3^-$ exchange — $Na^+$-independent $Cl^-/HCO_3^-$ exchange — $^{36}Cl^-$ Efflux

## Introduction

Cytoplasmic pH ($pH_i$) regulation is of fundamental significance for almost any cell function since virtually all cellular processes show a marked pH sensitivity. In recent years, therefore, $pH_i$ regulation has been studied in several cells and tissues. The results indicate that the $pH_i$ regulatory mechanisms vary considerably between different cells, but most eucaryotic cells appear to have two or more concurrent pH regulatory systems. For recent reviews on intracellular pH and its regulation, *see e.g.,* Frelin et al. (1988); Grinstein, Rotin & Mason (1989); Hoffmann & Simonsen (1989); Boron (1992). The Ehrlich ascites tumor cell is an unspecialized mammalian cell which is rather well characterized with respect to its membrane transport systems and their regulation (*see* Hoffmann, Simonsen & Lambert, 1993). Cellular pH in this cell is affected, e.g., during volume regulation (Livne & Hoffmann, 1990), and on the other hand, cellular pH will influence the activation of ion transport systems in a complex way. Mechanisms involved in $pH_i$ regulation in this undifferentiated cell are, therefore, of relevance.

The maintenance of $pH_i$ at steady-state under physiological conditions in some cell types, e.g., rat thymic lymphocytes, involves the transmembrane $Cl^-$ gradient and the operation of $Cl^-/HCO_3^-$ exchange (Grinstein, Garcia-Soto & Mason, 1988). In these cells, the trans-

membrane $Cl^-$ gradient equals the $HCO_3^-$ gradient, implying the operation of a fast $Cl^-/HCO_3^-$ exchanger at physiological $pH_i$. In Ehrlich cells, $pH_i$ maintenance at steady-state has not previously been studied. One purpose of the present work, therefore, is to explore the role of the transmembrane $Cl^-$ gradient in the control of cellular pH.

After cytoplasmic acidification in Ehrlich cells, it has previously been demonstrated that, similar to the findings in most other cell types, an amiloride-sensitive $Na^+/H^+$ exchanger represents a principal $pH_i$ recovery system (Kramhøft, Lambert & Hoffmann, 1988; Livne & Hoffmann, 1990). This system is in most cell types almost silent at physiological $pH_i$. Another purpose of the present study is to address the possible role after an acid load in Ehrlich cells of $pH_i$ regulatory systems other than the $Na^+/H^+$ exchanger. The putative involvement of such systems in steady-state $pH_i$ maintenance is also studied.

Three different systems, where the $Na^+$ gradient directly drives an influx of $HCO_3^-$, have been described in recent years in other cell types: (i) $Na^+$-dependent $Cl^-/HCO_3^-$ exchange has been reported in various invertebrate cells (Boron, McCormick & Roos, 1981; Moody, 1981; Thomas, 1984; Thomas & Schlue, 1986) as well as in some mammalian cells (for references, *see* Grinstein et al., 1989). (ii) Sodium carbonate ion-pair formation and $NaCO_3^-/Cl^-$ exchange via the inorganic anion exchanger has been demonstrated to account for $pH_i$ regulation in the squid axon (Boron, 1985; Boron & Knakal, 1989). $Na^+$ uptake mediated by this system has been demonstrated in human red blood cells (Becker & Duhm, 1978; Funder, 1980). In Vero cells (a monkey kidney cell line) $NaCO_3^-/Cl^-$ exchange has been proposed to play a role in $pH_i$ regulation (Olsnes et al., 1987; Tønnesen et al., 1987). (iii) Electrogenic as well as electroneutral, DIDS-sensitive $Na^+$,$HCO_3^-$ cotransport systems have been reported in cells from various epithelia, cells of neuronal origin, and in smooth muscle cells (*see* Boron & Boulpaep, 1989; Soleimani & Aronson, 1989; La Cour, 1989, 1991; Aalkjær & Hughes, 1991; Stahl et al., 1992).

The present report demonstrates the existence in the Ehrlich ascites tumor cell of a DIDS-sensitive, $Na^+$-*dependent* $Cl^-/HCO_3^-$ exchange system operating as an acid-extruding system in acid-loaded cells, in addition to the amiloride-sensitive $Na^+/H^+$ exchanger. This system also seems to participate in cellular $pH_i$ maintenance at steady-state. At alkaline $pH_i$, the operation of a DIDS-sensitive, $Na^+$-*independent* $Cl^-/HCO_3^-$ exchange system is also demonstrated. Finally, a residual $pH_i$ recovery in acid-loaded cells is demonstrated under conditions where the $Na^+/H^+$ exchanger as well as the $Na^+$-dependent $Cl^-/HCO_3^-$ exchanger is inhibited, suggesting a role for a primary active $H^+$ pump in $pH_i$ regulation in Ehrlich cells. A preliminary report has previously been published (Kramhøft & Hoffmann, 1989).

## Materials and Methods

### Cell Suspensions and Incubation Media

Ehrlich ascites tumor cells were obtained from white Theiler mice seven days after intraperitoneal implantation. The mice were sacrificed by cervical dislocation, and the tumor cells harvested in standard incubation medium containing heparin (2.5 IU/ml). The cells were washed by centrifugation (700 × *g*, 45 sec), once with standard incubation medium and twice in the appropriate experimental solution. The composition of the standard incubation medium was (mM): 150 $Na^+$, 5 $K^+$, 150 $Cl^-$, 1 $Mg^{2+}$, 1 $Ca^{2+}$, 1 $SO_4^{2-}$, 1 $PO_4^{3-}$, 3.3 MOPS, 3.3 TES, and 3.3 HEPES (*see* Eagle, 1971); pH was adjusted to 7.4 with NaOH or KOH, except where otherwise indicated. For experiments performed at $pH_o$ 8.2, MOPS, TES, and HEPES were replaced by 5 mM BICINE and 5 mM TRICINE. Control experiments confirmed that extracellular pH did not change significantly during the incubation of the cell suspensions. In $Li^+$ and $K^+$ media, LiCl and KCl, respectively, were substituted for NaCl in equimolar amounts. In propionate medium, 150 mM $Na^+$ propionate was substituted for NaCl, and pH adjusted to 7.2 (*see* Kramhøft et al., 1988). In NMDG medium, NaCl was replaced by NMDG chloride, added from a 1 M NMDG stock solution titrated with HCl to pH 7.4. In nitrate or gluconate medium, all chloride salts were replaced by the corresponding nitrate or gluconate salts, respectively. No attempt was made to compensate for $Ca^{2+}$ chelation in the gluconate media. All media were nominally bicarbonate-free, unless otherwise indicated. When required, $NaHCO_3$ or $KHCO_3$ was added from 0.5 M stock solutions adjusted to pH 7.4 by equilibration with 100% $CO_2$. The cell suspensions were incubated at 37°C, except during measurements of intracellular pH, which were generally performed at 25°C (*see below*).

### Measurements of Intracellular pH

Intracellular pH was measured using the fluorescent probe BCECF, as described previously (Kramhøft et al., 1988). Briefly, cell suspensions were incubated with the acetoxymethylester of BCECF, final concentration 3.6 μM, for about 30 min at 37°C, and subsequently washed with isotonic medium containing bovine serum albumin (BSA, 1 mg/ml) to remove excess probe. The washed cells were subsequently incubated at 25°C (except where otherwise indicated) to minimize the leakage of BCECF from the cells. The fluorescence was recorded after 50-fold dilution of the cell suspension using a Perkin Elmer LS-5 luminescence spectrometer with excitation at 495 nm (slitwidth 5 nm) and emission recorded at 525 nm (slitwidth 5 nm). In recent experiments (including Fig. 4), $pH_i$ was measured using the ratio between the isosbestic and the pH-sensitive excitation wavelengths (440 nm/495 nm). In four independent experiments using the ratio method, $pH_i$ was measured at 7.30 ± 0.03 at $pH_o$ 7.4. This value is in good agreement with the value obtained using single wavelength excitation (7.26 ± 0.03, $n = 8$; *see* Results). Calibration was carried out after each set of experiments according to the method of Thomas et al. (1979) in KCl media (KCl substituted for NaCl) containing 5 μM nigericin. Calibration of amiloride experiments was always performed in the presence of amiloride, because amiloride (0.2 mM) was found to reduce the fluorescence signal by about 10% (*see* Kramhøft et al., 1988).

### Measurement of Cellular $Cl^-$

Samples (1 ml) of the cell suspension (cytocrit 6–8%) were transferred to preweighed Eppendorf vials for determination of $Cl^-$ content and cell water, as previously described (Hoffmann et al., 1983). Briefly, the vials were centrifuged (20,000 × *g*, 60 sec) and the pellets subsequently lysed in distilled water. The lysate was deproteinized with perchloric acid (PCA, final concentration 1.2 M), and centrifuged (20,000 × *g*, 10 min). The dry weight of the PCA precipitate was converted to cell dry weight by multiplication by the factor 0.77 (Lambert, Hoffmann & Jørgensen, 1989). The $Cl^-$ content of the suspending medium and the deproteinized cell lysate was assessed by coulometric titration (CMT10 chloride titrator, Radiometer, Denmark) and corrected for extracellular trapped medium defined as the $^3$H-inulin space in the pellet (Hoffmann et al., 1983).

### Measurement of $^{36}Cl^-$ Efflux

Cell suspensions (cytocrit 8%) were equilibrated with $^{36}$Cl (32 kBq/ml) for 30 min. Then the cell suspension was centrifuged (30 sec, 700 × *g*) and the packed cells subsequently resuspended in the appropriate $Cl^-$-free gluconate efflux medium. Samples (0.5 ml) of the cell suspension were transferred to preweighed vials and immediately centrifuged.Subsequently, the samples were treated as described in the previous section, and the $^{36}Cl^-$ activity was measured by liquid scintillation counting. Correction for extracellular trapped $^{36}Cl^-$ was made as described above.

### Reagents

All reagents were analytical grade and, unless otherwise indicated, purchased from Sigma (St. Louis, MO). $^3$H-inulin was purchased from New England Nuclear, UK, and $^{36}$Cl from Amersham, UK. BCECF-AM was obtained from Molecular Probes (Eugene, OR). Amiloride was dissolved in water and kept as frozen stock (10 mM, −20°C). DIDS was dissolved in water (10 mM), titrated with NaOH to pH about 7.5 and kept frozen. BCECF-AM was dissolved in dry DMSO (1 mg/ml) and kept frozen in small aliquots (about 30 µl). Nigericin was dissolved in ethanol (1 mg/ml) and kept at −20°C.

### Statistical Evaluations

All values are given as mean ± SEM with the number of independent experiments in parentheses. Significance was tested by a Student's *t*-test.

### Abbreviations

| | |
|---|---|
| BCECF: | free acid of BCECF-AM |
| BCECF-AM: | 2′,7′-bis-(2-carboxyethyl)-5,6 carboxyfluorescein, tetraacetoxymethyl ester |
| BICINE: | *N,N*-bis(2-hydroxyethyl)glycine |
| BSA: | bovine serum albumin, fraction V |
| DIDS: | 4,4′-diisothiocyano-2,2′stilbene-disulfonic acid |
| DMSO: | dimethylsulfoxide |
| HEPES: | *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid |
| MOPS: | 3-(*N*-morpholino)propanesulfonic acid |
| NMDG: | *N*-methyl-D-glucamine |
| TES: | *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid |
| TRICINE: | *N*-tris(hydroxymethyl)methylglycine |
| TRIS: | tris(hydroxymethyl)aminomethane |

## Results

### Steady-State $pH_i$ in Nominally Bicarbonate-free Medium and in 25 mM Bicarbonate-containing Medium

Cellular pH ($pH_i$) is measured at 7.26 ± 0.03 ($n$ = 8) for cells suspended in nominally $HCO_3^-$-free standard incubation medium at $pH_o$ 7.38 ± 0.01 ($n$ = 8) and a temperature of 25°C. The membrane potential under these conditions is −55 mV (Christensen & Hoffmann, 1992). If $H^+$ and $HCO_3^-$ were at electrochemical equilibrium across the cell membrane, the predicted value for $pH_i$ can be calculated from the Nernst equation at 6.46. The tendency of the cells to become acidified by passive influx of $H^+$ or efflux of $HCO_3^-$ must, therefore, be counteracted by regulatory acid-extruding mechanisms.

Cellular pH and the transmembrane $H^+$ and $HCO_3^-$ gradients could conceivably be influenced by $Cl^-/HCO_3^-$ exchange. This would be the case even in nominally $HCO_3^-$-free media, which inevitably contain small amounts of bicarbonate (about 0.2 mM) due to contamination with atmospheric and metabolic $CO_2$ (Ludt et al., 1991). Even low $HCO_3^-$ concentrations can mediate substantial $H^+$ fluxes via $Cl^-/HCO_3^-$ exchange because $HCO_3^-$ and $CO_2$ will recycle via the Jacobs-Stewart cycle (Jacobs & Stewart, 1942). The net driving force for an electroneutral $Cl^-/HCO_3^-$ exchange (without coupling to other ion gradients) is determined by the combined chemical gradients for $Cl^-$ and $HCO_3^-$. At zero net flux via this $Cl^-/HCO_3^-$ exchanger, the chemical gradient for $HCO_3^-$ must be equal to the chemical gradient for $Cl^-$. Moreover, the chemical gradients for $HCO_3^-$ and $H^+$ must be equal, since $CO_2$ easily permeates the cell membrane and is hydrated to carbonic acid which dissociates into $H^+$ and $HCO_3^-$ on both sides of the membrane. Therefore, under conditions of zero net flux via the $Cl^-/HCO_3^-$ exchanger, the following relation will apply:

$$\frac{[Cl^-]_i}{[Cl^-]_o} = \frac{[HCO_3^-]_i}{[HCO_3^-]_o} = \frac{[H^+]_o}{[H^+]_i}$$

where the subscripts ($i$) and ($o$) denote intracellular and extracellular concentrations, respectively. Under the present experimental conditions, the $Cl^-$ distribution ratio $r_{Cl^-} = [Cl^-]_i/[Cl^-]_o$ is measured at 0.36 ± 0.02 ($n$ = 8). From the equation above, it can be calculated that the chemical $HCO_3^-$ gradient would be equal to the in-

ward $Cl^-$ gradient at $pH_i = 6.94 \pm 0.02$ ($n = 8$), which is significantly on the acid side of the actually measured $pH_i$ value at 7.26 ($P < 0.01$). This calculation assumes the activity coefficient for $Cl^-$ to be equal on both sides of the cell membrane. The actual value for the activity coefficient is presumably lower in cell water than in the extracellular medium, and a correction for this difference would shift the calculated $pH_i$ value in the acid direction.

In media containing a physiological bicarbonate concentration (25 mM), the measured $pH_i$ is 7.82 ± 0.07 ($n = 9$) at extracellular pH 7.48 ± 0.08 ($n = 9$), and also significantly more alkaline ($P < 0.01$) than the calculated $pH_i$ value at which the chemical bicarbonate gradient would be equal to the chemical $Cl^-$ gradient (measured $r_{Cl^-} = 0.41 \pm 0.03$, $n = 9$; calculated $pH_i = 7.00 \pm 0.03$, $n = 9$). Cellular pH of Ehrlich cells, therefore, is not determined by the chemical $Cl^-$ gradient and by $Cl^-/HCO_3^-$ exchange, neither in the absence of $HCO_3^-$ nor in its presence, and accordingly other mechanism(s) for $pH_i$ regulation must exist.

### $HCO_3^-$-dependent $pH_i$ Recovery after Cytoplasmic Acidification by Propionate Exposure or $NH_4^+$ Withdrawal

The cells were acid-loaded by exposure to propionic acid, a technique elegantly used by Grinstein and coworkers (Grinstein et al., 1984). The penetration of the lipid-soluble, undissociated propionic acid into the cells is followed by intracellular dissociation and cytoplasmic acidification. A typical experiment is seen in Fig. 1, upper panel, in which exposure of cells to isotonic sodium propionate (150 mM, pH 7.20) is shown to induce a rapid cellular acidification. This is followed by a $pH_i$ recovery, whose initial rate is reduced to less than one-third by 0.2 mM amiloride. It has previously been found that 0.1 mM amiloride suffices to completely block the cell swelling caused by activation of the $Na^+/H^+$ exchanger in Ehrlich cells suspended in sodium propionate medium (Kramhøft et al., 1988). Substitution of $Na^+$ propionate by $K^+$ propionate reduced the rate of $pH_i$ recovery to a similar extent (*not illustrated*). These findings are consistent with the previous demonstration that cytoplasmic acidification in these cells activates an amiloride-sensitive, $Na^+$-dependent $H^+$ extrusion, which is mediated via the $Na^+/H^+$ exchanger (Kramhøft et al., 1988). Figure 1, upper panel, also demonstrates that the $pH_i$ recovery is incomplete and apparently levels off at $pH_i$ about 7.1, in agreement with the previous finding that the "set-point" for activation of the $Na^+/H^+$ exchanger is slightly below the steady-state $pH_i$ value (Livne & Hoffmann, 1990). In Fig. 1, lower panel, the cells were exposed to isotonic $Na^+$ propionate (150 mM) in the presence of amiloride (0.2 mM), and $pH_i$ was followed with time by addition (arrow) of either 25 mM $NaHCO_3$ or 25 mM NaCl (control). It is seen that $pH_i$ recovery is much faster and also more complete in the presence of $NaHCO_3$, suggesting the existence of an amiloride-insensitive, $HCO_3^-$-dependent $pH_i$ recovery mechanism which can alkalinize the cell until the normal steady-state $pH_i$ value is reached.

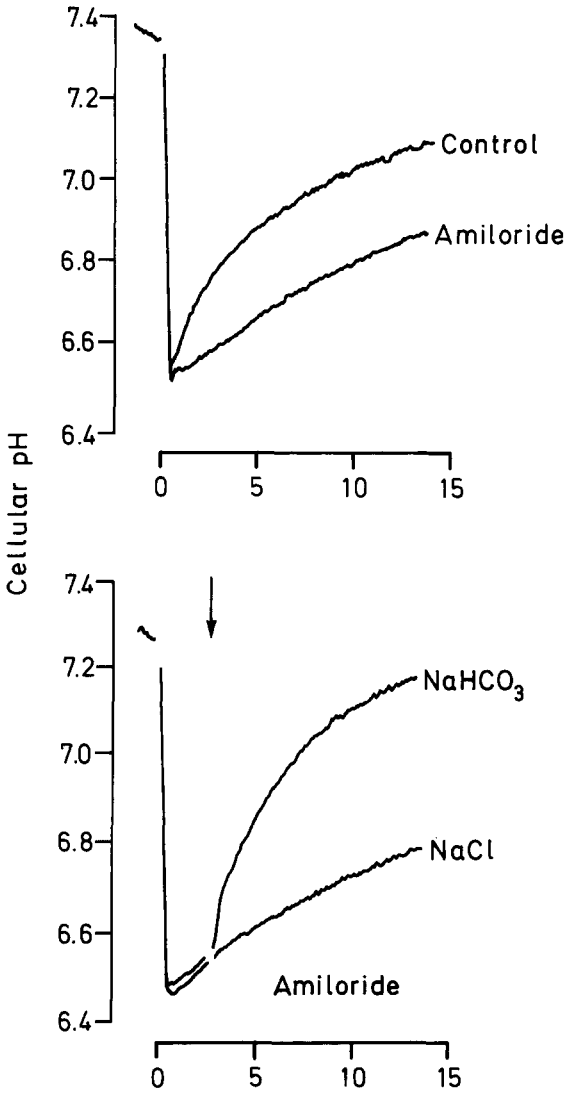


**Fig. 1.** $pH_i$ recovery in Ehrlich ascites tumor cells exposed to isotonic $Na^+$ propionate medium (pH 7.2) at zero time. The $Na^+$ propionate medium was nominally $HCO_3^-$-free. Cellular pH ($pH_i$) was measured at 25°C using the fluorescent probe BCECF with single wavelength excitation (*see* Materials and Methods). In the upper panel, the $pH_i$ recovery was monitored in the presence and absence of amiloride (0.2 mM). In the lower panel, amiloride (0.2 mM) was present in both experimental groups. At the time indicated by the arrow, $NaHCO_3$ (25 mM) was added from a 0.5 M stock solution equilibrated with 100% $CO_2$ (pH 7.4) (upper trace). The control group (lower trace) received NaCl (25 mM). The initial pH trace represents $pH_i$ of cells in standard incubation medium at $pH_o$ 7.2. Both panels are representative of four independent experiments.

Another widely used technique for acid-loading cells is based on prolonged exposure to $NH_4^+$, leading to cellular accumulation of $NH_4^+$ ions (*see* Roos & Boron, 1981). Upon a subsequent shift to a $NH_4^+$-free solution, the cellular $NH_4^+$ dissociates into $H^+$ and $NH_3$, with the latter rapidly leaving the cell by diffusion, leaving the $H^+$ behind and resulting in cytoplasmic acidification. The experiment shown in Fig. 2 confirms the result shown in Fig. 1: following cytoplasmic acidification in the nominal absence of $HCO_3^-$, $pH_i$ regulation occurs via an amiloride-sensitive mechanism. However, in the presence of amiloride, addition of $HCO_3^-$ greatly increases the cellular capacity for $pH_i$ regulation.

Furthermore, it may be noted (lower traces in both panels) that an amiloride-insensitive $pH_i$ recovery occurs also in nominally $HCO_3^-$-free media. To explore this residual $pH_i$ recovery, the experiment with $NH_4^+$ prepulse in nominally $HCO_3^-$- and $Na^+$-free NMDG-medium was repeated in the presence of both amiloride and DIDS (*not illustrated*). Under these conditions, neither $Na^+/H^+$ exchange nor any DIDS-sensitive anion exchange can contribute to $pH_i$ recovery. However, a residual $pH_i$ recovery was still observed which amounted to 0.013 pH units/min (average from two experiments). This residual $pH_i$ recovery suggests that a primary active $H^+$ pump, which has previously been reported to be present in the Ehrlich cell (Heinz, Sachs & Schafer, 1981; Bowen & Levinson, 1984) contributes to the $pH_i$ recovery in acid-loaded cells.

### $Na^+$-Dependent, DIDS-sensitive $pH_i$ Recovery after an Acid Load by $CO_2$ Exposure

Exposure to $CO_2$, at constant external pH, can also be used to acid-load cells. The passive entry of the membrane permeant $CO_2$, with subsequent hydration catalyzed by carbonic anhydrase to form carbonic acid, causes an initial decline in $pH_i$ (*see* Roos & Boron, 1981). In Fig. 3, $CO_2$-equilibrated $HCO_3^-$ was added at the time indicated by the arrow. This causes a rapid reduction of $pH_i$ (0.3–0.4 pH units), whereafter $pH_i$ increases rapidly again until it reaches a value close to the initial one. In $Na^+$-free $K^+$ medium, the $pH_i$ recovery was strongly reduced (Fig. 3, upper panel, trace labeled $K^+$). In the experiment in Fig. 3, lower panel, DIDS (0.3 mM) was found to inhibit the $pH_i$ recovery in the presence of $Na^+$. Amiloride (0.2 mM) was present in the experimental solutions to block the $Na^+/H^+$ exchanger. Thus, the amiloride-resistant $pH_i$ recovery is $Na^+$ dependent and DIDS sensitive.

As mentioned in Materials and Methods, $pH_i$ measurements were generally carried out at 25°C due to a rapid leakage of BCECF at 37°C. To assess the possible effect of the reduced temperature on the physiological response to acid loading, the experiment shown in

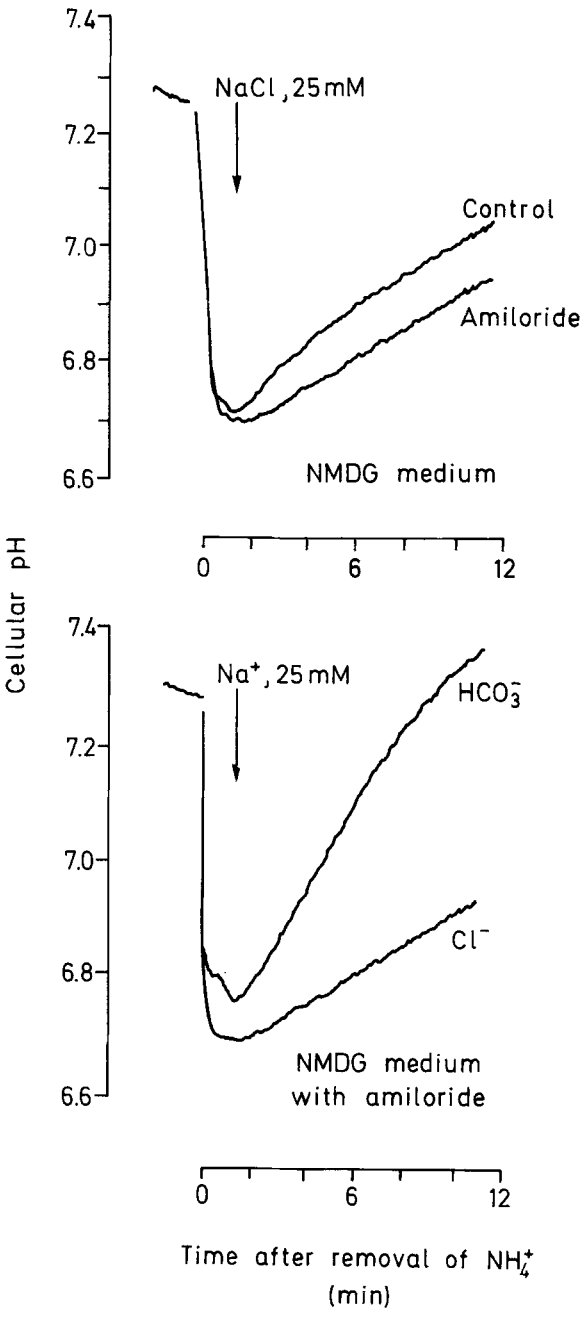


**Fig. 2.** $pH_i$ recovery after $NH_4^+$ prepulse. The cells were preincubated for about 10 min in standard incubation medium with addition of 15 mM $NH_4Cl$. At time zero, a sample of the cell suspension was diluted in $NH_4^+$-free NMDG medium. In the upper panel, NaCl (25 mM) was added at the time indicated by the arrow. The $pH_i$ recovery was monitored in the absence (control) and in the presence of amiloride (0.2 mM). In the lower panel, amiloride (0.2 mM) was present, and 25 mM NaCl (lower trace) or $CO_2$-equilibrated $NaHCO_3$ (upper trace) was added at the time indicated by the arrow. The initial trace represents $pH_i$ of cells in standard medium at $pH_o$ 7.4. Both panels are representative of two independent experiments.

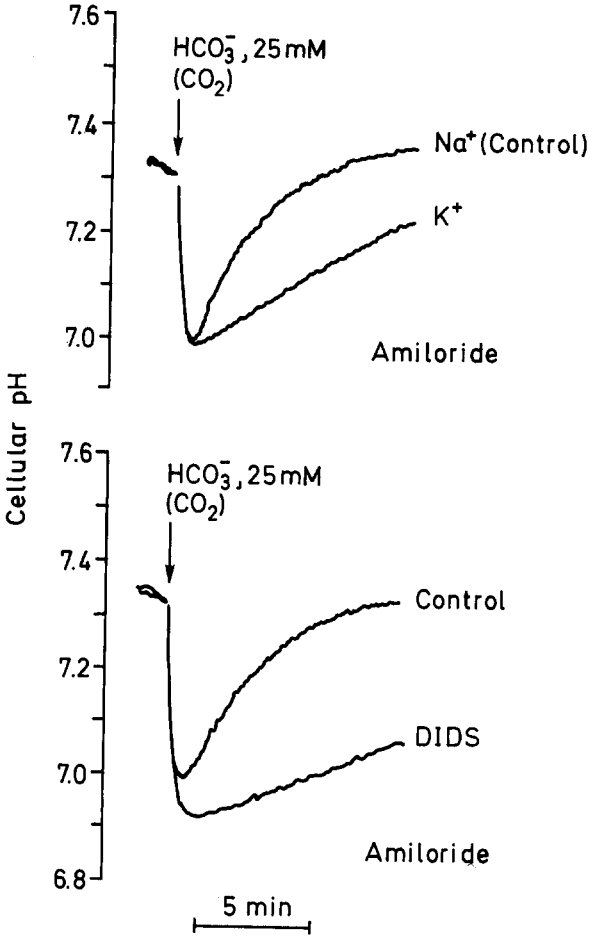


**Fig. 3.** $pH_i$ recovery after exposure to $CO_2$-equilibrated $HCO_3^-$ solution. The cells were preincubated in standard incubation medium, and a sample of the cell suspension diluted in nominally $HCO_3^-$-free, experimental medium (standard incubation medium or $K^+$ medium) containing amiloride (0.2 mM). At the time indicated by the arrow, $NaHCO_3$ or $KHCO_3$ (25 mM) was added from a 0.5 M stock solution equilibrated with 100% $CO_2$ (pH 7.4). In the upper panel, the cells were suspended in standard incubation medium (upper trace) with addition (arrow) of $NaHCO_3$, or suspended in $K^+$ medium (lower trace) with addition (arrow) of $KHCO_3$. In the lower panel, the cells were suspended in standard incubation medium in the presence or absence of DIDS (0.3 mM). $NaHCO_3$ was added at the time indicated by the arrow. The initial trace represents $pH_i$ of cells suspended in $HCO_3^-$-free standard incubation medium at pH 7.4. The traces represent five experiments in standard medium, three in $K^+$ medium, and four experiments in standard medium with DIDS.

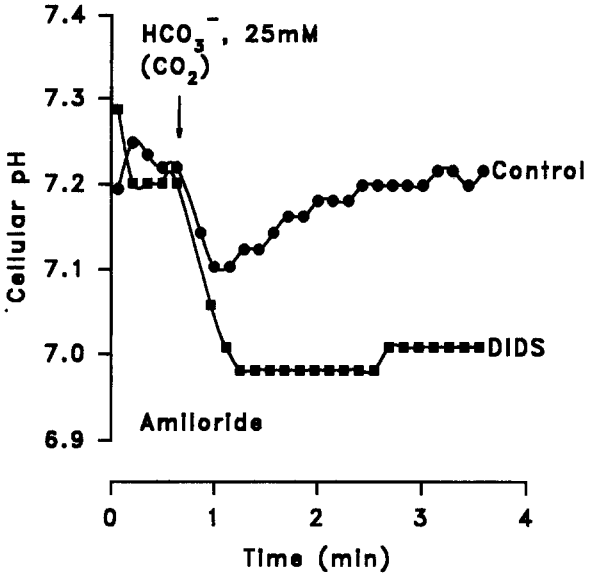


**Fig. 4.** $pH_i$ recovery at 37°C after exposure to $CO_2$-equilibrated $HCO_3^-$ solution. The experimental protocol was essentially as described in the legend to Fig. 3, lower panel, except that the $pH_i$ measurements were carried out at 37°C, and $pH_i$ was recorded using the fluorescence ratio method (*see* Materials and Methods). Amiloride (0.2 mM) was present during the experiment. To minimize errors arising from rapid leakage of BCECF at 37°C, $pH_i$ was recorded only during the initial 4 min after addition of $HCO_3^-$, and calibration was performed after each experimental condition. The figure shows one out of two individual experiments. Note that steady-state $pH_i$ measured at 25°C using the fluorescence ratio method is 7.30 ± 0.03 ($n$ = 4) and thus similar to the initial $pH_i$ value read from Fig. 4.

Fig. 3, lower panel, was repeated at 37°C (using the fluorescence ratio technique rather than single wavelength excitation). Figure 4 shows that the degree of acidification (control) under these conditions amounts to only about 0.15 pH units compared to 0.3–0.4 pH units at 25°C, and that the $pH_i$ recovery takes less than 3 min for completion, compared to more than 5 min at 25°C. In the presence of DIDS, however, the cells acidify by about 0.3 pH units at 37°C, i.e., close to the value of about 0.4 units at 25°C. Thus, the DIDS-sensitive $pH_i$ regulatory system seems, not unexpectedly, to operate at a much faster rate at 37°C than at 25°C. Otherwise, however, working at 25°C instead of 37°C has apparently no fundamental effect on the $pH_i$ response as such.

An interesting possibility is that the $Na^+$- and $HCO_3^-$-dependent, DIDS-sensitive $pH_i$ recovery is mediated by sodium carbonate ion-pair formation and $NaCO_3^-$ uptake via the inorganic anion exchanger. Carbonate forms soluble complexes with $Na^+$ as well as with $Li^+$, but not with $K^+$ (Becker & Duhm, 1978), and $Li^+$, but not $K^+$, has previously been found to be able to replace $Na^+$ in $pH_i$ recovery in some cell types. Table 1 shows the initial rates of $pH_i$ recovery in the presence of amiloride calculated from experiments similar to those shown in Fig. 3, including the results of substitution of NaCl by LiCl. The rate of $pH_i$ recovery is reduced to about one-third by the presence of DIDS, and also by substitution of $Na^+$ by $K^+$. The rate of $pH_i$ recovery is slightly, but not significantly reduced, when $Na^+$ is replaced by $Li^+$. The possibility of sodium (and lithium) carbonate ion-pair formation, therefore, remains an open question.

**Table 1.** Rates of $pH_i$ recovery in Ehrlich ascites tumor cells after exposure to $CO_2$-equilibrated $HCO_3^-$ solutions in the presence of amiloride

| External medium | $pH_i$ recovery pH units/min |
|---|---|
| 150 mM NaCl (control) | 0.10 ± 0.01 ($n$ = 5) |
| 150 mM NaCl, 0.3 mM DIDS | 0.03 ± 0.01 ($n$ = 4)* |
| 150 mM KCl | 0.03 ± 0.01 ($n$ = 3)* |
| 150 mM LiCl | 0.06 ± 0.01 ($n$ = 6)** |
| 150 mM LiCl, 0.3 mM DIDS | 0.03 ($n$ = 1) |

The cells were acidified by addition of 25 mM $CO_2$-equilibrated $NaHCO_3$ (NaCl medium), or $KHCO_3$ (KCl and LiCl medium) (*cf.* Fig. 3). All media contained 0.2 mM amiloride. The rate of $pH_i$ recovery was estimated from the slope (fitted by eye) of the $pH_i$ recovery curve during the first 2 min after maximal acidification. The values are given as mean ± SEM. The number of experiments are given in parentheses.

* The value is significantly different from the control ($0.01 < P < 0.05$).

** The value is not significantly different from the control ($0.05 < P < 0.10$).

## $pH_i$ Maintenance at Steady-State

A series of experiments was performed in which $pH_i$ was followed with time without prior perturbation of the cells by acid loading. In Fig. 5 (upper panel) the effect of DIDS and/or amiloride on $pH_i$ maintenance was studied in nominally $HCO_3^-$-free standard incubation medium. It is seen that amiloride and DIDS when added separately both cause a slow cytoplasmic acidification, and when added in combination cause a considerable rate of acidification, indicating that a DIDS-sensitive as well as an amiloride-sensitive system contributes to the maintenance of $pH_i$ at steady-state. In this context, it should be noted that nominally $HCO_3^-$-free media contain about 0.2 mM $HCO_3^-$ (Ludt et al., 1991). In Fig. 5 (lower panel), the cells were incubated in $Na^+$-free medium or in $Cl^-$-free $NO_3^-$ medium. Removal of $Na^+$ or substitution of $NO_3^-$ for $Cl^-$ results in a significant cellular acidification, suggesting that maintenance of steady-state $pH_i$ is achieved by $Na^+$- and $Cl^-$-dependent transport systems. In $NO_3^-$ medium, intracellular $Cl^-$ will rapidly be replaced by $NO_3^-$ via the inorganic anion exchanger (Hoffmann et al., 1979).

## $pH_i$ Changes Induced by Substituting External $Cl^-$ by Gluconate in the Presence of $HCO_3^-$

Figure 6 shows an experiment in which cells at time zero were transferred from standard incubation medium to a medium in which all $Cl^-$ salts were replaced by the corresponding salts of the nonpermeant anion gluconate (*see* Materials and Methods). The experiments were performed in the presence of 1.5 mM $HCO_3^-$ and 0.2 mM amiloride. It can be seen that the outward chloride gradient results in an alkalinization of the cells. The alkalinization was inhibited in the presence of DIDS and, moreover, in $Na^+$-free $NMDG^+$-medium (NMDG gluconate substituting for NaCl). The alkalinization was essentially unaffected by the presence or absence of amiloride (*not illustrated*). The initial acidification in the $HCO_3^-$-containing $Na^+$ gluconate medium (about 0.07 pH units) is variable and probably due to the influx of $CO_2$ from the medium.

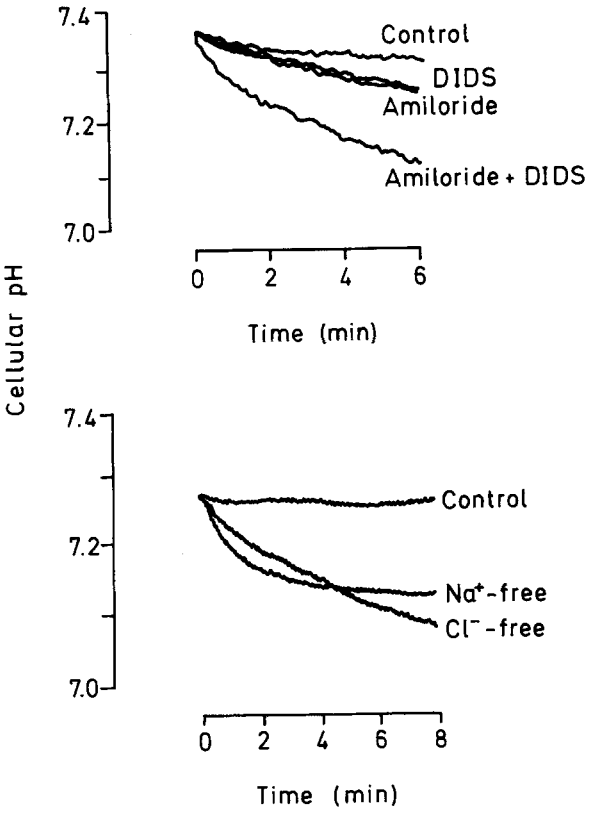


**Fig. 5.** Cellular pH followed with time at 25°C in standard incubation medium in the absence (control) or in the presence of DIDS and/or amiloride, in $Na^+$-free medium, and in $Cl^-$-free $NO_3^-$ medium. All media were nominally $HCO_3^-$-free. The cells were preincubated in standard incubation medium, and at zero time a sample of the cell suspension was diluted in the experimental media: standard medium with or without DIDS (0.3 mM) and/or amiloride (0.2 mM) (upper panel), in $Na^+$-free NMDG medium, or $Cl^-$-free $NO_3^-$ medium (lower panel). The panels are representative of five (upper panel), three (lower panel, $Na^+$-free), and two (lower panel, $Cl^-$ free) independent experiments, respectively.

## $Na^+$-dependent and $Na^+$-independent $Cl^-/HCO_3^-$ Exchange

The results presented above have demonstrated the existence of a $Na^+$- and $HCO_3^-$-dependent, DIDS-sensi-

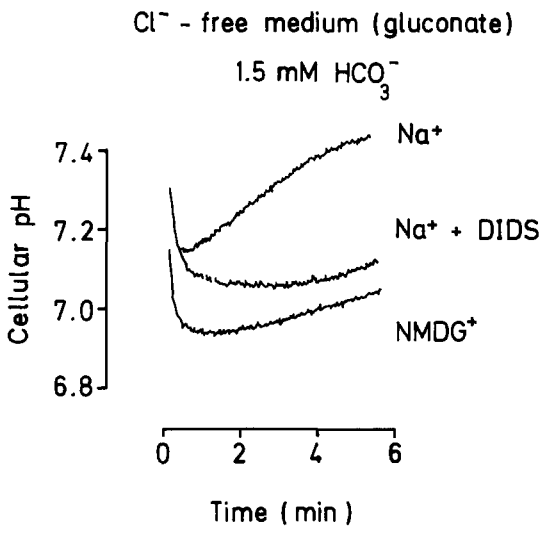


**Fig. 6.** $Na^+$-dependent, DIDS-sensitive alkalinization upon resuspension of the cells in $Cl^-$-free gluconate media containing $HCO_3^-$ (1.5 mM). All media contained amiloride (0.2 mM). The cells were preincubated in standard incubation medium. At zero time, samples of the cell suspension were diluted in $Cl^-$-free $Na^+$ gluconate medium containing 1.5 mM $NaHCO_3$, in the same medium containing 0.3 mM DIDS, and in $Na^+$-free NMDG-gluconate medium containing 1.5 mM $KHCO_3$, respectively. The figure is representative of four independent experiments in $Na^+$-gluconate medium with or without DIDS, and three experiments in NMDG-gluconate medium.

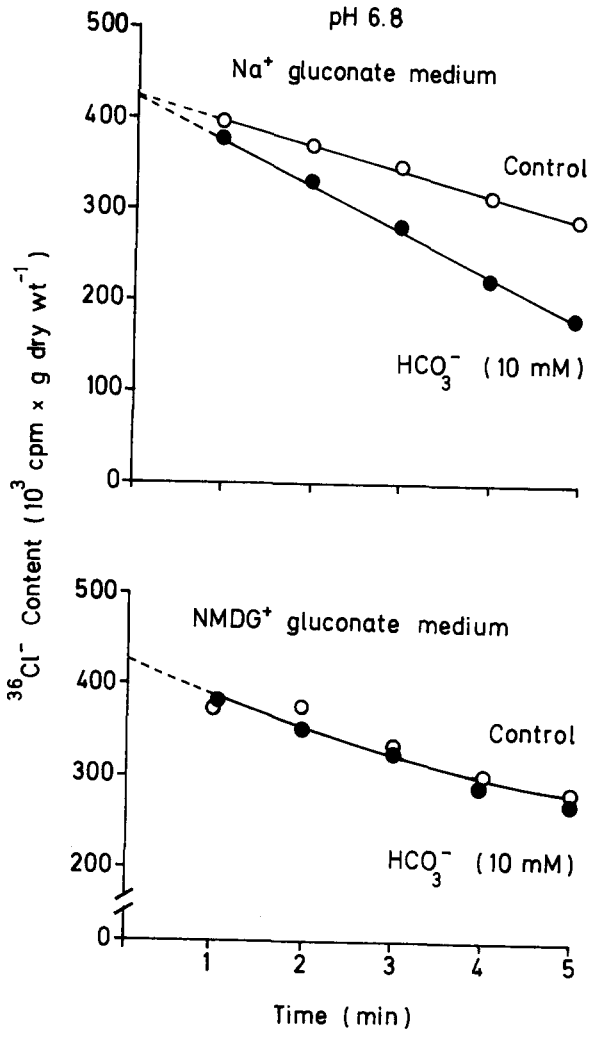


**Fig. 7.** $Na^+$- and $HCO_3^-$-dependent $^{36}Cl^-$ efflux at $pH_o$ 6.8. The cells were equilibrated with $^{36}Cl^-$ in standard incubation medium at pH 6.8 (*see* Materials and Methods), and subsequently transferred to $Cl^-$-free, gluconate efflux media at the same pH, either $Na^+$ gluconate medium (upper panel) without or with 10 mM $HCO_3^-$, or $Na^+$-free NMDG gluconate medium (lower panel) without or with 10 mM $HCO_3^-$. The cellular $^{36}Cl^-$ content was followed with time. All efflux media contained 0.2 mM amiloride and the experiments were conducted at 37°C. The figure is representative of six separate experiments.

tive system involved in $pH_i$ recovery after cytoplasmic acidification in Ehrlich cells. DIDS-sensitive inorganic anion exchange has previously been described in these cells (*see* Jessen et al., 1986). The experiments presented in Figs. 7 and 8 address the relation between $Na^+$-dependent and $Na^+$-independent $Cl^-/HCO_3^-$ exchange, assessed directly by measurements of $^{36}Cl^-$ efflux. The experiments were performed in the absence of extracellular $Cl^-$ to avoid $Cl^-/Cl^-$ self-exchange (the nonpermeant anion gluconate substituting for $Cl^-$). In the experiments at $pH_o$ 6.8 (Fig. 7), amiloride (0.2 mM) was present to avoid the effect of an activated $Na^+/H^+$ exchanger which in the absence of external $Na^+$ would drive $H^+$ into the cells in exchange for cellular $Na^+$, preventing direct comparison between the control group (Fig. 7, upper panel) and the NMDG group (Fig. 7, lower panel). Figure 7 shows that at $pH_o$ 6.8, the presence of $HCO_3^-$ (10 mM, filled circles) causes a stimulation of $^{36}Cl^-$ efflux in the presence of extracellular $Na^+$ (upper panel), but not in its absence (lower panel, $NMDG^+$ substituting for $Na^+$). Hence, Fig. 7 demonstrates the existence of a $Na^+$-dependent $Cl^-/HCO_3^-$ exchange system operating at $pH_o$ 6.8, whereas at this pH value a $Na^+$-independent $Cl^-/HCO_3^-$ exchange system does not seem to play any role. In striking contrast, Fig. 8 shows that when Ehrlich cells are suspended at an external pH value ($pH_o$ 8.2) above the physiological level, the presence of 10 mM $HCO_3^-$ (filled circles) stimulates $^{36}Cl^-$ efflux both in the presence and in the absence of $Na^+$, and almost to the same extent. This demonstrates that at $pH_o$ 8.2 a $Na^+$-independent $Cl^-/HCO_3^-$ exchange system is operating, whereas a $Na^+$-dependent $Cl^-/HCO_3^-$ exchange system does not contribute significantly at this pH. In additional experiments carried out under conditions similar to those prevailing during the efflux experiments, it was found that at $pH_o$ 6.8, $pH_i$ was in the range 7.0–7.3 in $Na^+$-containing medium, both in the presence and in

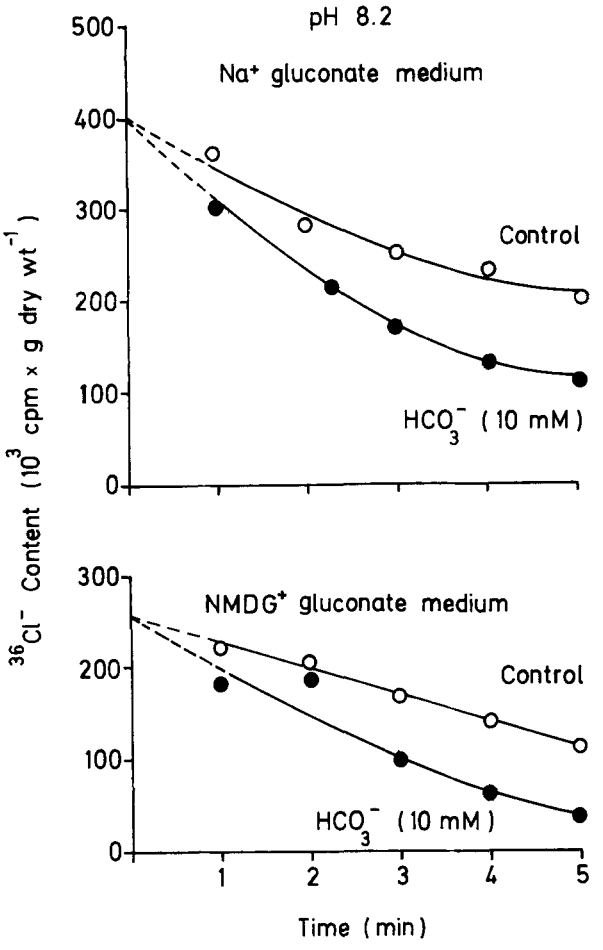


**Fig. 8.** $Na^+$-independent, $HCO_3^-$-dependent $^{36}Cl^-$ efflux at $pH_o$ 8.2. The cells were treated as described in the legend to Fig. 7, except that extracellular pH was adjusted to 8.2 in the preincubation medium as well as in the efflux medium, and that amiloride was omitted. $HCO_3^-$ (10 mM) was added to the preincubation medium 5 min prior to transfer to the $HCO_3^-$-containing efflux media to avoid an initial $CO_2$-induced acidification (*see* Fig. 4) at the start of the efflux period. The figure is representative of four separate experiments.

the absence of $HCO_3^-$, and in the range 6.8–7.0 in $Na^+$-free media. At $pH_o$ 8.2, the $pH_i$ ranges were 7.6–7.8 in the $Na^+$-containing and 7.4–7.5 in the $Na^+$-free medium, respectively. The dependence of the $Na^+$-dependent and $Na^+$-independent anion exchange systems on cellular pH was not investigated in further detail in the present study.

Table 2 shows the initial net $Cl^-$ efflux calculated from experiments similar to those shown in Figs. 7 and 8. It may be seen that in $Na^+$-containing media, addition of 10 mM $HCO_3^-$ doubles the rate of $Cl^-$ efflux both at $pH_o$ 6.8 and 8.2. Furthermore, the presence of 0.3 mM DIDS abolishes the $HCO_3^-$-induced stimulation of the $Cl^-$ efflux. In the absence of extracellular $Na^+$, $HCO_3^-$ also stimulates $Cl^-$ efflux at $pH_o$ 8.2, but fails to do so at $pH_o$ 6.8. Consequently, a $Na^+$- and $HCO_3^-$-dependent $Cl^-$ efflux can be demonstrated at $pH_o$ 6.8, whereas at $pH_o$ 8.2 the $HCO_3^-$-dependent $Cl^-$ efflux is $Na^+$ independent.

Although gluconate cannot exchange with cellular $Cl^-$ there is a significant efflux of $Cl^-$ also from cells in nominally $HCO_3^-$-free gluconate medium both at pH 6.8 and 8.2. This efflux is not inhibitable with DIDS (*not illustrated*) and is, therefore, unlikely to represent anion exchange. The nature of this residual $Cl^-$ efflux, however, was not further investigated.

## Discussion

In the Ehrlich cell, the measured $pH_i$ is higher in medium containing a physiological $HCO_3^-$ concentration than in nominally $HCO_3^-$-free medium, in agreement with observations from many other cell types (*see e.g.*, Grinstein et al., 1989; Putnam, 1990). In cells maintained under steady-state conditions at $pH_o$ 7.4, the measured $pH_i$ value is significantly more alkaline, both in the nominal absence and in the presence of bicarbonate (25 mM), than the $pH_i$ value calculated assuming the chemical gradients for $HCO_3^-$ and $Cl^-$ to be equal. Under these conditions a $Cl^-/HCO_3^-$ exchanger (without coupling to other ion gradients) would mediate inward $Cl^-$ movement in exchange for $HCO_3^-$ efflux, and hence operate to acidify the cell. Consequently, a cation-independent $Cl^-/HCO_3^-$ exchanger in Ehrlich cells must be nearly quiescent at physiological pH. Consistent with this notion, the $Cl^-$ efflux data discussed below directly demonstrate that the $Na^+$-independent $Cl^-/HCO_3^-$ exchanger operates at alkaline $pH_o$ only. These results in the case of the Ehrlich cell are at variance with results obtained with rat thymic lymphocytes in the presence of $HCO_3^-$, where it was found that the transmembrane $HCO_3^-$ gradient was equal to the $Cl^-$ gradient (Grinstein et al., 1988), compatible with the operation of a fast $Cl^-/HCO_3^-$ exchange at physiological $pH_i$ in these cells.

### $pH_i$ Recovery after Cytoplasmic Acidification: $Na^+$- and $HCO_3^-$ Dependence

In the nominal absence of $HCO_3^-$, $pH_i$ recovery following an acid load in Ehrlich cells occurs via an amiloride-sensitive mechanism (illustrated in Figs. 1 and 2, upper panels), which presumably represents the $Na^+/H^+$ exchanger previously described in these cells (Kramhøft et al., 1988; Livne & Hoffmann, 1990). Addition of $HCO_3^-$ (25 mM) strongly enhances the capacity of the cells for $pH_i$ recovery after an acid load. Moreover, the $HCO_3^-$-dependent $pH_i$ recovery is dependent on extracellular $Na^+$, and can be inhibited by the anion exchange inhibitor DIDS (Fig. 3 and Table 1).

**Table 2.** Net $Cl^-$ efflux from Ehrlich ascites tumor cells after transfer to $Cl^-$-free gluconate media with or without $HCO_3^-$ (10 mM)

| Efflux medium | Net $Cl^-$ efflux $\mu$mol × g dry wt$^{-1}$ × min$^{-1}$ | |
|---|---|---|
| | $pH_o$ 6.8 | $pH_o$ 8.2 |
| 150 mM $Na^+$, $HCO_3^-$ free | 12.6 ± 2.4 ($n$ = 4) | 25.2 ± 4.9 ($n$ = 3) |
| 150 mM $Na^+$, 10 mM $HCO_3^-$ | 27.6 ± 2.8 ($n$ = 6)* | 45.2 ± 2.2 ($n$ = 5)* |
| 150 mM $Na^+$, 10 mM $HCO_3^-$, 0.3 mM DIDS | 14.4 ± 7.3 ($n$ = 2) | 28.4 ± 8.8 ($n$ = 3) |
| 150 mM $NMDG^+$, $HCO_3^-$ free | 18.0 ± 2.3 ($n$ = 5) | 26.8 ± 0.5 ($n$ = 3) |
| 150 mM $NMDG^+$, 10 mM $HCO_3^-$ | 15.8 ± 1.4 ($n$ = 4) | 51.6 ± 7.4 ($n$ = 3)* |

After equilibration with $^{36}Cl^-$ by preincubation at $pH_o$ 6.8 or 8.2 for 30 min, the cells were transferred to $Cl^-$-free gluconate efflux medium at the same pH, and the cellular $^{36}Cl^-$ content was followed with time (*cf.* Figs. 7 and 8 ). The net $Cl^-$ efflux was calculated from the slope of the $^{36}Cl^-$ content (cpm × g dry wt$^{-1}$) plotted against time for the measurements obtained during the first 2–3 min after initiation of the efflux. A nominal $Cl^-$ content at time zero at 172 $\mu$mol × g dry wt$^{-1}$ (*see* Lambert et al., 1989) was used for the calculation of the specific activity of $Cl^-$. In the experiments performed at $pH_o$ 6.8 amiloride (0.2 mM) was present in the efflux medium. The values are given as mean ± SEM. The number of experiments are given in parentheses.
* The value is significantly different from the value measured in the parallel group in the absence of $HCO_3^-$ ($P < 0.05$).

Accordingly, a DIDS-sensitive, $Na^+$- and $HCO_3^-$-dependent system mediating $H^+$ extrusion and/or $HCO_3^-$ uptake is also involved in $pH_i$ recovery in acid-loaded cells in the presence of a physiological $HCO_3^-$ concentration. This system will presumably also be operating in nominally bicarbonate-free media since these media inevitably contain a low concentration of bicarbonate (Ludt et al., 1991).

The observed $Na^+$- and $HCO_3^-$-dependent, DIDS-sensitive $pH_i$ recovery could conceivably (for references, *see* Introduction) be mediated by: (i) $Na^+$-dependent $Cl^-/HCO_3^-$ exchange, exchanging external $Na^+$ and $HCO_3^-$ for internal $Cl^-$, (ii) uptake of $Na^+$ and $CO_3^{2-}$ in the form of the ion pair $NaCO_3^-$ in exchange for intracellular $Cl^-$ via the inorganic anion exchanger, or (iii) $Na^+$,$HCO_3^-$ cotransport. The distinction between these transporters is not easy, and conclusive evidence requires analysis of the dependence of $pH_i$ recovery upon cellular $Cl^-$, or a strict kinetic analysis similar to that carried out in case of the $pH_i$ regulatory system in the squid axon (Boron & Knakal, 1989). This is beyond the scope of the present study. Figure 6 shows, however, that in the presence of a steep outwardly directed $Cl^-$ gradient (extracellular $Cl^-$ is replaced by the nonpermeant anion gluconate), $HCO_3^-$ can be driven into the cell via a $Na^+$-dependent, DIDS-sensitive transport system. This observation suggests a role for cellular $Cl^-$ in $pH_i$ maintenance, arguing against the involvement in Ehrlich cells of the $Na^+$,$HCO_3^-$ cotransporter in $pH_i$ regulation. This notion is further supported by the $Cl^-$ efflux data discussed below, which at low $pH_o$ directly demonstrate a $Na^+$- and $HCO_3^-$-dependent $Cl^-$ efflux. At low $pH_o$, moreover, the $Na^+$-independent $Cl^-/HCO_3^-$ exchanger seems to be quiescent, arguing against a role for this exchanger in $pH_i$ recovery via $NaCO_3^-$ ion-pair formation and $NaCO_3^-/Cl^-$ exchange. Although our data do not permit a definitive conclusion with respect to the transport system involved in the $Na^+$- and $HCO_3^-$-dependent $pH_i$ recovery in Ehrlich cells, the available evidence indicates that the $pH_i$ recovery is mediated by $HCO_3^-$ uptake via $Na^+$-dependent $Cl^-/HCO_3^-$ exchange.

### Steady-State $pH_i$ Maintenance in Nominally Bicarbonate-Free Media

Cells in steady-state in nominally bicarbonate-free media maintain a nearly constant $pH_i$ about 7.3 (Fig. 5). This $pH_i$-maintenance is inhibited by amiloride and DIDS (Fig. 5, upper panel) as well as by removal of $Na^+$ or $Cl^-$ (Fig. 5, lower panel). The effect of amiloride and of DIDS suggests a role for $Na^+/H^+$ exchange as well as for $Na^+$-dependent $Cl^-/HCO_3^-$ exchange in the maintenance of steady-state $pH_i$, and the additive effect of the two inhibitors indicates that the two transporters are operating in parallel in steady-state $pH_i$ maintenance. In the $Na^+$-free medium, both transporters will be prevented from operating. The evidence that the $Na^+/H^+$ exchanger is operating at a low rate at $pH_i$ about 7.3 indicates that the "set-point" of the $Na^+/H^+$ exchanger is slightly more alkaline than the value 7.0 previously reported (Livne & Hoffmann, 1990). The acidification seen in the presence of DIDS suggests a high affinity of the $Na^+$-dependent $Cl^-/HCO_3^-$ exchanger for $HCO_3^-$ since nominally $HCO_3^-$-free medi-

um contains only about 0.2 mM $HCO_3^-$ (Ludt et al., 1991). Finally, the acidification observed in $Cl^-$-free $NO_3^-$ medium, where the cellular $Cl^-$ is also replaced by $NO_3^-$ (Hoffmann et al., 1979), can presumably be ascribed to dependence upon cellular $Cl^-$ of the $Na^+$-dependent $Cl^-/HCO_3^-$ exchanger. The acidification in $NO_3^-$ medium is perhaps augmented by $NO_3^-$ inhibition of the $Na^+/H^+$ exchanger which has been reported in other systems (Parker & Castranova, 1984).

### $Na^+$-DEPENDENT AND $Na^+$-INDEPENDENT $Cl^-/HCO_3^-$ EXCHANGE

The results discussed above suggest, although indirectly, that DIDS-sensitive, $Na^+$-dependent $Cl^-/HCO_3^-$ exchange is involved in $pH_i$ regulation in Ehrlich cells. The existence of a $Na^+$-dependent $Cl^-/HCO_3^-$ exchange system under steady-state conditions in these cells was directly demonstrated in the present study by monitoring the efflux of $^{36}Cl^-$ into $Cl^-$-free gluconate medium in the absence and presence of extracellular $Na^+$ and/or $HCO_3^-$. As shown in Fig. 7 and Table 2, there is a clear $Na^+$ and $HCO_3^-$ dependence of the $^{36}Cl^-$ efflux at $pH_o$ 6.8. In contrast, at $pH_o$ 8.2 (Fig. 8 and Table 2), no $Na^+$ dependence was demonstrated, since at this $pH_o$ $HCO_3^-$ stimulates $^{36}Cl^-$ efflux both in the presence and in the absence of external $Na^+$, and almost to the same extent. Thus, Ehrlich cells possess both $Na^+$-dependent and $Na^+$-independent $Cl^-/HCO_3^-$ exchange systems.

The *$Na^+$-dependent* anion exchange system operates at acidic $pH_i$ values, consistent with a role for this system in $pH_i$ recovery in Ehrlich cells after an acid load in the presence of physiological $HCO_3^-$ concentrations. In addition, as discussed above, the system also seems to play a role in $pH_i$ maintenance at physiological steady-state even in the nominal absence of $HCO_3^-$. This implies that the *$Na^+$-dependent* $Cl^-/HCO_3^-$ exchanger is operating at physiological $pH_i$ (about 7.3). This notion is also supported by the finding (*see* Fig. 6) that the cytoplasmic alkalinization induced by the acute removal of extracellular $Cl^-$, and taken to represent $HCO_3^-$ influx in exchange for $Cl^-$ efflux driven by the outward $Cl^-$ gradient, is predominantly $Na^+$ dependent.

In contrast, the *$Na^+$-independent* anion exchange system seems to operate at more alkaline $pH_i$ values, above the physiological $pH_i$. This system is likely to play a role in the recovery from alkaline shifts in cytosolic pH. As discussed above, in the case of the Ehrlich cell, the $Cl^-$ gradient *does not* equal the transmembrane $HCO_3^-$ gradient at physiological $pH_i$ values, indicating that the *$Na^+$-independent* $Cl^-/HCO_3^-$ exchanger is quiescent at physiological $pH_i$ or operating only at a low rate. This is consistent with the finding (*see* Fig. 7 and Table 2) that no *$Na^+$-independent* $Cl^-/HCO_3^-$ exchange could be demonstrated at $pH_i$ levels just below the physiological range. The notion that the *$Na^+$-independent* anion exchanger is nearly quiescent under physiological conditions is also supported by the finding (*see* Fig. 6) that the cytoplasmic alkalinization induced by an outward $Cl^-$ gradient is nearly absent in $Na^+$-free medium.

Taken together, these findings suggest that a *$Na^+$-dependent* $Cl^-/HCO_3^-$ exchanger is involved in $pH_i$ recovery in acid-loaded cells and in the maintenance of $pH_i$ at physiological steady-state, whereas a *$Na^+$-independent* $Cl^-/HCO_3^-$ exchanger seems to be virtually quiescent at physiological $pH_i$ but operating at alkaline $pH_o$. Similar findings regarding $Na^+$-dependent and $Na^+$-independent anion exchange have recently been reported for other cell types (*see* Putnam, 1990; Tønnesen et al., 1990). The relationship between the *$Na^+$-dependent* and *$Na^+$-independent* $Cl^-/HCO_3^-$ exchange systems discussed above and the inorganic anion exchange system previously reported in Ehrlich cells (Hoffmann et al., 1979; Jessen et al., 1986) is at present unclear and under current investigation.

In conclusion, the present report demonstrates the existence in Ehrlich cells of a *$Na^+$-dependent* $Cl^-/HCO_3^-$ exchange system involved in $pH_i$ recovery after an acid load in the presence of $HCO_3^-$ and in the maintenance of physiological steady-state $pH_i$. In nominally $HCO_3^-$-free medium, $pH_i$ recovery is mediated by the $Na^+/H^+$ exchanger, which is also involved in steady-state $pH_i$ maintenance. Moreover, a primary active $H^+$ pump may also be involved in $pH_i$ regulation. Finally, the presence of a *$Na^+$-independent* anion exchange system was demonstrated, operating at $pH_i$ values above the physiological level and presumably involved in $pH_i$ recovery after an alkaline load.

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