# $Na^+$-dependent Cl-$HCO_3$ Exchange in the Squid Axon

## *Dependence on Extracellular pH*

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ABSTRACT Intracellular pH ($pH_i$) in squid giant axons recovers from acid loads by means of a $Na^+$-dependent Cl-$HCO_3$ exchanger, the actual mechanism of which might be exchange of: (*i*) external $Na^+$ and $HCO_3^-$ for internal $Cl^-$ and $H^+$, (*ii*) $Na^+$ plus two $HCO_3^-$ for $Cl^-$, (*iii*) $Na^+$ and $CO_3^=$ for $Cl^-$, or (*iv*) the $NaCO_3^-$ ion pair for $Cl^-$. Here we examine sensitivity of transport to changes of extracellular pH ($pH_o$) in the range 7.1–8.6. We altered $pH_o$ in four ways, using: (*i*) classical "metabolic" disturbances in which we varied $[HCO_3^-]_o$, $[NaCO_3^-]_o$, and $[CO_3^=]_o$ at a fixed $[CO_2]_o$; (*ii*) classical "respiratory" disturbances in which we varied $[CO_2]_o$, $[NaCO_3^-]_o$, and $[CO_3^=]_o$ at a fixed $[HCO_3^-]_o$; (*iii*) novel mixed-type acid–base disturbances in which we varied $[HCO_3^-]_o$ and $[CO_2]_o$ at a fixed $[CO_3^=]_o$ and $[NaCO_3^-]_o$; and (*iv*) a second series of novel mixed-type disturbances in which we varied $[CO_2]_o$, $[CO_3^=]_o$, and $[Na^+]_o$ at a fixed $[HCO_3^-]_o$ and $[NaCO_3^-]_o$. Axons (initial $pH_i$ ~7.4) were internally dialyzed with a pH 6.5 solution containing 400 mM $Cl^-$ but no $Na^+$. After $pH_i$, measured with a glass microelectrode, had fallen to ~6.6, dialysis was halted. The equivalent acid extrusion rate ($J_H$) was computed from the rate of $pH_i$ recovery (i.e., increase) in the presence of $Na^+$ and $HCO_3^-$. When $pH_o$ was varied by method (*i*), which produced the greatest range of $[CO_3^=]_o$ and $[NaCO_3^-]_o$ values, $J_H$ increased with $pH_o$ in a sigmoidal fashion; the relation was fitted by a pH titration curve with a pK of ~7.7 and a Hill coefficient of ~3.0. With method (*ii*), which produced smaller changes in $[CO_3^=]_o$ and $[NaCO_3^-]_o$, $J_H$ also increased with $pH_o$, though less steeply. With method (*iii*), which involved changes in neither $[CO_3^=]_o$ nor $[NaCO_3^-]_o$, $J_H$ was insensitive to $pH_o$ changes. Finally, with method (*iv*), which involved changes in neither $[HCO_3^-]_o$ nor $[NaCO_3^-]_o$, but reciprocal changes in $[CO_3^=]_o$ and $[Na^+]_o$, $J_H$ also was insensitive to $pH_o$ changes. We found that decreasing $pH_o$ from 8.6 to 7.1 caused the apparent $K_m$ for external $HCO_3^-$ ($[Na^+]_o$ = 425 mM) to increase from 1.0 to 26.7 mM, whereas $J_{max}$ was relatively stable. Decreasing $pH_o$ from 8.6 to 7.4 caused the apparent $K_m$ values for external $Na^+$ ($[HCO_3^-]_o$ = 48 mM) to increase from 8.6 to 81 mM, whereas $J_{max}$ was relatively stable. These data are consistent with the ion pair model, and severely restrict kinetic parameters for other models.

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## INTRODUCTION

In squid giant axons, the transporter responsible for recovery of intracellular pH ($pH_i$) from acid loads is the $Na^+$-dependent Cl-$HCO_3$ exchanger. Characterized in squid axons (Boron and De Weer, 1976; Russell and Boron, 1976) and snail neurons (Thomas, 1976, 1977), this acid extrusion mechanism was later described in a variety of other cells, both invertebrate (Moody, 1981; Boron, McCormick, and Roos, 1981) and vertebrate (Rothenberg, Glaser, Schlesinger, and Cassel, 1983; L'Allemain, Paris, and Pouyssegur, 1985; Tonnessen, Ludt, Sandvig, and Olsnes, 1987; Boyarsky, Ganz, Sterzel, and Boron, 1988; Cassel, Scharf, Rotman, Cragoe, and Katz, 1988). The transporter seems to exchange extracellular $Na^+$ and $HCO_3^-$ for intracellular $Cl^-$ and $H^+$, though the precise roster of transported ions has not been established. Three other possibilities (Boron, 1985) are that intracellular $Cl^-$ is exchanged for: (*i*) extracellular $Na^+$ and two $HCO_3^-$ ions, (*ii*) extracellular $Na^+$ and one $CO_3^=$ ion, and (*iii*) the extracellular $NaCO_3^-$ ion pair.

Kinetic studies are one approach for understanding how the transporter may respond to physiologically relevant perturbations, as well as for probing the potential ionic mechanisms of transport. Previous kinetic work has focused on how the squid axon's $Na^+$-dependent Cl-$HCO_3$ exchanger is affected by changes in $[Na^+]_o$ and/or $[HCO_3^-]_o$ at the single $pH_o$ of 8.0 (Boron and Russell, 1983; Boron, 1985). The results indicate that the equivalent acid extrusion rate ($J_H$) is approximately governed by the product $[Na^+]_o \times [HCO_3^-]_o$. These data are most easily explained by the $NaCO_3^-$ ion pair hypothesis, but do not exclude highly restricted versions of the other models. Another squid axon study, which focused on how the transporter's $[Na^+]_o$ and $[HCO_3^-]_o$ dependencies are affected by the reversible inhibitor 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), showed that the divalent anion DNDS appears to be a competitive inhibitor with respect to both extracellular $Na^+$ and $HCO_3^-$ (Boron and Knakal, 1989). These results, too, are explained most easily by the $NaCO_3^-$ ion pair hypothesis. Although the DNDS data do not rule out other models, these results place restrictions on these other models that are even more severe than those required by the earlier work. These data provide a framework for understanding the interaction among the $Na^+$-dependent Cl-$HCO_3$ exchanger and extracellular $Na^+$ and $HCO_3^-$, though only at the single extracellular pH ($pH_o$) of 8.00. In spite of the importance of the $Na^+$-dependent Cl-$HCO_3$ exchanger, we know relatively little about how it is affected by $pH_o$ changes that may be important both physiologically and pathophysiologically. Work on the $Na^+$-dependent Cl-$HCO_3$ exchanger in barnacle muscle fibers has shown that $J_H$ is inhibited by metabolic acidosis (i.e., a reduction in $[HCO_3^-]_o$ at a fixed $pCO_2$). However, because $HCO_3^-$ (or a $[HCO_3^-]$-dependent solute such as $CO_3^=$ or $NaCO_3^-$) is a substrate of the transporter, the effects of metabolic acidosis provide little insight into the effect of acidosis per se.

The purpose of the present work is to analyze how changes in $pH_o$ influence the $Na^+$-dependent Cl-$HCO_3$ exchanger. We examine the effects of the two classical acid–base disturbances: (*i*) metabolic (in which $[HCO_3^-]_o$, $[CO_3^=]_o$ and $[NaCO_3^-]_o$ vary, but $[CO_2]_o$ is fixed), and (*ii*) respiratory (in which $[CO_2]_o$, $[CO_3^=]_o$, and $[NaCO_3^-]_o$ vary, but $[HCO_3^-]_o$ is fixed). However, a study of how $J_H$ is affected by only these two disturbances, both of which involve sizable changes in $[CO_3^=]_o$ and $[NaCO_3^-]_o$,

provides little insight into a mechanism that may involve $CO_3^=$ or $NaCO_3^-$. Even if $HCO_3^-$ were the true substrate of the transporter, a complete characterization of the transporter would require a response to the "mixed-type" acid–base disturbances that arise spontaneously or during compensation of classical metabolic and respiratory disturbances. Therefore, in this study we introduce two novel acid–base disturbances and examine how they affect $Na^+$-dependent Cl-$HCO_3$ exchange: (*iii*) mixed-type 1 (in which $[CO_2]_o$ and $[HCO_3^-]_o$ vary, but $[CO_3^=]_o$ and $[NaCO_3^-]_o$ are fixed), and (*iv*) mixed-type 2 (in which $[CO_2]_o$, $[CO_3^=]_o$ and $[Na^+]_o$ vary, but $[HCO_3^-]_o$ and $[NaCO_3^-]_o$ are fixed).

Our approach was to internally dialyze a squid axon to a low $pH_i$ and use a pH-sensitive microelectrode to monitor the rate of $pH_i$ recovery, from which we computed $J_H$. We examined how $J_H$ is affected by $pH_o$ changes in the range 7.1–8.6, altering $pH_o$ according to the four approaches outlined above. In addition, we examined how changes in $pH_o$ affect the dependence of $J_H$ on $[Na^+]_o$, $[HCO_3^-]_o$, $[CO_3^=]_o$, and $[NaCO_3^-]_o$. We found that reducing $pH_o$ by a classical metabolic acidosis (method *i*) caused a steep decline in $J_H$, whereas reducing $pH_o$ by a classical respiratory acidosis (method *ii*) caused a less steep decline in $J_H$. However, $J_H$ was unaffected by reducing $pH_o$ at a fixed $[CO_3^=]_o$ and $[NaCO_3^-]_o$ (method *iii*), or by reducing $pH_o$ at a fixed $[HCO_3^-]_o$ and $[NaCO_3^-]_o$ (method *iv*). We found that $pH_o$ changes do not significantly affect the apparent maximal $J_H$. However, external acidosis causes steep increases in apparent values for $K_m(Na^+)$ and $K_m(HCO_3^-)$, without substantially affecting either $K_m(CO_3^=)$ or $K_m(NaCO_3^-)$. The most straightforward explanation for the data is the $NaCO_3^-$ ion pair model. However, with appropriate ad hoc assumptions, and with extreme values for certain kinetic parameters, the models involving $HCO_3^-$ and $CO_3^=$ also fit the data. Models aside, a useful guideline is that, regardless of $pH_o$, the transport rate is approximately governed by the product $[Na^+]_o \times [CO_3^=]_o$.

Portions of this work have been published in abstract form (Boron and Knakal, 1986).

## METHODS

### *General*

The experiments were conducted at the Marine Biological Laboratory, Woods Hole, MA. Live squid (*Loligo pealei*) were decapitated, and the first stellar nerve of each side was removed and placed in cold (~4°C) natural seawater. From this nerve we microdissected a 3–4-cm length of giant axon, 400–700 μm in diameter. This was cannulated horizontally in a chamber designed for internal dialysis (Brinley and Mullins, 1967); our use of the method is detailed in earlier papers (Boron and Russell, 1983; Boron, 1985). A length of cellulose acetate dialysis tubing (Fisher Research Laboratories, Dedham, MA), with an outer diameter of 140 μm, was inserted through one cannula and threaded down the axon and out the opposite cannula. This tubing was arranged so that an 18-mm length of tubing, previously permeabilized by hydrolyzing 18–24 h in 0.1 N NaOH, was centered in the axon. The dialysis capillary was perfused with dialysis fluid (DF) at a rate of ~5 μl/min. Also inserted into the axon through opposite cannulas were a voltage-sensitive and a pH-sensitive microelectrode, arranged so that their tips were centered in the axon within ~500 μm of each other. The open-tipped voltage electrode was filled with 3 M KCl. The pH-sensitive microelectrodes were of the design of Hinke (Hinke,

1967), and constructed of pH-sensitive glass (Clark Electromedical Instruments, Pangbourne, UK) and lead glass (0120; Corning Glass Works, Corning, NY). Details on the construction of the microelectrodes, the use of high-impedance electrometers and other devices to handle the electrode signals, the acquisition of data by computer, and the computer control of the experiments have been provided previously (Boron and Russell, 1983; Boron, 1985). The axon was superfused continuously with artificial seawater (ASW). The temperature was maintained at 22°C by using a circulating bath to pump water through the jacketed chamber.

### *Solutions*

*Artificial seawaters.* The standard extracellular fluid was an ASW buffered to pH 8.00 and having the following composition (mM): 425.2 $Na^+$, 12 $K^+$, 3 $Ca^{2+}$, 57.5 $Mg^{2+}$, 531 $Cl^-$, nominally 12 $HCO_3^-$, 0.1 $EDTA^=$, 15 of the anionic form of [2-hydroxyethyl]-1-piperazine-propane sulfonic acid (EPPS), and 15 of the neutral form of EPPS (pK ~8.0). When $HCO_3^-$ is added to a solution that is initially $CO_2/HCO_3^-$ free, some of the added $HCO_3^-$ undergoes the reaction $HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O$, and some undergoes the reaction $HCO_3^- \rightarrow H^+ + CO_3^=$; the $CO_3^=$ can go on to form ion pairs with $Ca^{2+}$, $Mg^{2+}$, and $Na^+$. Thus, the actual $[HCO_3^-]_o$ is less than the nominal $[HCO_3^-]_o$. We made our standard ASW as previously described (Boron and Knakal, 1989), titrating a $CO_2/HCO_3^-$-free EPPS-buffered stock solution to pH 8.00, adding $NaHCO_3$ (which causes a paradoxical pH decrease), and then returning $pH_o$ to 8.00 by gassing with 0.5% $CO_2$/balance $O_2$ (thereby evolving $CO_2$). As described in a previous analysis (Boron and Knakal, 1989), when $pH_o$ is 8.00, [EPPS] is 30 mM, and the nominal $[HCO_3^-]$ is 12 mM, the actual $[HCO_3^-]$ is computed to be ~10% less (i.e., ~10.8 mM). The predicted magnitude of this discrepancy increases at higher $[HCO_3^-]$ values, and increases substantially at higher pH values (Boron and Knakal, 1989). Therefore, in designing ASWs for this study, we increased the added $HCO_3^-$ as necessary to keep the computed $[HCO_3^-]$ within ~10% of the nominal $[HCO_3^-]$. The calculation of actual $[HCO_3^-]_o$ was done by computer, using the approach outlined previously (Boron and Knakal, 1989). This addition of extra $HCO_3^-$ was necessary only for $pH_o$ values of 8 or greater. Table I summarizes values for nominal (i.e., desired) $[HCO_3^-]_o$, added $[HCO_3^-]_o$, and computed $[HCO_3^-]$ for each of the 54 combinations of pH, $[HCO_3^-]$, and $[Na^+]$ used in this study. The table also identifies which of the 13 series of experiments each solution was used in, the mean observed flux (i.e., $J_H$) ± SEM, and the number of observations from which this mean was computed. In computations of kinetic parameters, we always used computed $[HCO_3^-]_o$ values rather than nominal or added values.

For an extracellular buffer we chose 30 mM EPPS (pK ~8.0), and we established $pH_o$ values between 7.1 and 8.6 by titrating 30 mM EPPS free acid with different amounts of MgO at 22°C. We chose to use a single buffer (rather than different buffers for different $pH_o$ values) in order to avoid the criticism that observed changes might be buffer- rather than $pH_o$-dependent. On the other hand, we recognize that $pH_o$ changes are necessarily accompanied by changes in the ratio $[EPPS{\cdot}H^+]_o/[EPPS^o]_o$. We chose EPPS as the buffer because it was used in previous studies, and because its pK is approximately in the middle of the desired $pH_o$ range. We compensated for changes in osmolality by varying the amount of $MgCl_2$ added to the solutions. The osmolality of the final ASWs was adjusted as necessary to ~975 mosm/kg by the addition of $H_2O$ or $MgCl_2$. The ASW was delivered to the chamber through $CO_2$-impermeable Saran tubing (Clarkson Equipment and Controls, Detroit, MI). When $[HCO_3^-]_o$ was varied, $HCO_3^-$ was exchanged mole-for-mole with $Cl^-$. When $[Na^+]_o$ was lowered, $Na^+$ was replaced mole-for-mole with $K^+$. Previous work (Boron and Russell, 1983) showed that the depolarization associated with increased $[K^+]_o$ does not affect the rate of the $Na^+$-dependent Cl-$HCO_3$ exchanger.

*Dialysis fluids.* The internal dialysis fluid (DF) had the following composition (mM): 0 $Na^+$, 413.3 $K^+$, 7 $Mg^{2+}$, 8 $Tris^+$, 400 $Cl^-$, 14 glutamate, 4 $ATP^=$, 1 $EGTA^=$, 13.3 of the anionic form of 2-[*N*-morpholino]-ethanesulfonic acid (MES), 6.7 of the neutral form of MES, 215 glycine, and 0.5 phenol red. KOH or HCl was used to titrate the pH to 6.5 at 22°C. The

TABLE I

*Composition of $HCO_3^-$-containing Seawaters*

| Solution No. | Used in series No. | pH | Nominal [$Na^+$] | Nominal [$CO_2$] | Nominal [$HCO_3$] | Added [$HCO_3$] | Computed [$HCO_3$] | Computed [$CO_3$] | Computed [$NaCO_3$] | Flux | *n* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mM* | % | *mM* | *mM* | *mM* | *μM* | *μM* | *pmol* $cm^{-2}\ s^{-1}$ | |
| 1 | 1,10 | 8.6 | 425 | 0.5 | 48 | 57.8 | 48.0 | 1,911 | 4,779 | 21.6 ± 2.3 | 7 |
| 2 | 2,10 | 8.6 | 425 | 0.125 | 12 | 16.3 | 12.0 | 478 | 1,194 | 22.4 ± 1.2 | 7 |
| 3 | 10 | 8.6 | 425 | 0.0625 | 6 | 8.6 | 6.0 | 238 | 595 | 21.6 ± 2.3 | 7 |
| 4 | 3,10 | 8.6 | 425 | 0.0313 | 3 | 4.5 | 3.0 | 119 | 298 | 17.3 ± 0.8 | 18 |
| 5 | 10 | 8.6 | 425 | 0.0156 | 1.5 | 2.3 | 1.5 | 59 | 148 | 13.7 ± 1.4 | 9 |
| 6 | 10 | 8.6 | 425 | 0.00781 | 0.75 | 1.15 | 0.73 | 29.0 | 72.5 | 8.8 ± 0.5 | 15 |
| 7 | 10 | 8.6 | 425 | 0.00391 | 0.375 | 0.58 | 0.364 | 14.5 | 36.2 | 6.4 ± 0.6 | 15 |
| 8 | 10 | 8.6 | 425 | 0.00195 | 0.1875 | 0.30 | 0.1873 | 7.5 | 18.6 | 4.1 ± 0.4 | 13 |
| 9 | 13 | 8.6 | 26.5 | 0.5 | 48 | 55.4 | 48.1 | 1,914 | 298 | 15.0 ± 2.3 | 4 |
| 10 | 4,13 | 8.6 | 13.25 | 0.5 | 48 | 55.4 | 48.2 | 1,917 | 149 | 16.0 ± 2.3 | 6 |
| 11 | 13 | 8.6 | 6.625 | 0.5 | 48 | 55.4 | 48.2 | 1,919 | 74.8 | 9.0 ± 1.6 | 10 |
| 12 | 13 | 8.6 | 3.313 | 0.5 | 48 | 55.4 | 48.3 | 1,920 | 37.4 | 4.9 ± 0.6 | 6 |
| 13 | 13 | 8.6 | 1.656 | 0.5 | 48 | 55.4 | 48.3 | 1,921 | 18.7 | 4.0 ± 0.5 | 5 |
| 14 | 1,9 | 8.3 | 425 | 0.5 | 24 | 24.0 | 20.0 | 400 | 1,000 | 18.6 ± 0.5 | 9 |
| 15 | 2,9 | 8.3 | 425 | 0.25 | 12 | 14.6 | 12.0 | 239 | 598 | 19.3 ± 0.7 | 13 |
| 16 | 3,9 | 8.3 | 425 | 0.125 | 6 | 7.4 | 6.0 | 119 | 298 | 19.0 ± 0.6 | 18 |
| 17 | 9 | 8.3 | 425 | 0.0625 | 3 | 3.7 | 3.0 | 59.0 | 148 | 14.7 ± 0.9 | 9 |
| 18 | 9 | 8.3 | 425 | 0.0313 | 1.5 | 1.85 | 1.5 | 29.3 | 73.4 | 9.6 ± 0.4 | 12 |
| 19 | 9 | 8.3 | 425 | 0.0156 | 0.75 | 0.93 | 0.74 | 14.7 | 36.8 | 6.5 ± 0.5 | 11 |
| 20 | 9 | 8.3 | 425 | 0.00781 | 0.375 | 0.47 | 0.372 | 7.4 | 18.6 | 4.9 ± 0.3 | 10 |
| 21 | 4 | 8.3 | 26.5 | 1.0 | 48 | 53.2 | 48.0 | 958 | 149 | 15.1 ± 2.1 | 9 |
| 22 | 11 | 8.0 | 106 | 2.0 | 48 | 48.0 | 44.8 | 447 | 279 | 17.5 ± 0.3 | 364 |
| 23 | 1,2,3,8 | 8.0 | 425 | 0.5 | 12 | 12.0 | 10.8 | 107 | 269 | 15.4 ± 0.5 | 7 |
| 24 | 8 | 8.0 | 425 | 0.25 | 6 | 6 | 5.4 | 53.6 | 134 | 11.3 ± 1.3 | 6 |
| 25 | 8 | 8.0 | 425 | 0.125 | 3 | 3 | 2.7 | 26.7 | 66.8 | 7.1 ± 0.2 | 6 |
| 26 | 8 | 8.0 | 425 | 0.0625 | 1.5 | 1.5 | 1.34 | 13.4 | 33.4 | 5.3 ± 0.7 | 8 |
| 27 | 8 | 8.0 | 425 | 0.0313 | 0.75 | 0.75 | 0.67 | 6.7 | 16.7 | −0.4 ± 0.5 | 4 |
| 28 | 8 | 8.0 | 425 | 0.0156 | 0 | 0 | 0 | 0 | 0 | 17.7 ± 0.5 | 6 |
| 29 | 4,12 | 8.0 | 53 | 2.0 | 48 | 48.0 | 45.0 | 449 | 140 | 15.9 ± 1.6 | 10 |
| 30 | 12 | 8.0 | 26.5 | 2.0 | 48 | 48.0 | 45.1 | 450 | 70.0 | 11.2 ± 1.1 | 7 |
| 31 | 12 | 8.0 | 13.25 | 2.0 | 48 | 48.0 | 45.1 | 451 | 35.1 | 8.3 ± 0.4 | 7 |
| 32 | 12 | 8.0 | 6.625 | 2.0 | 48 | 48.0 | 45.2 | 451 | 17.6 | 5.6 ± 1.2 | 7 |
| 33 | 12 | 8.0 | 0 | 2.0 | 48 | 48.0 | 45.2 | 451 | 0 | 0.5 ± 1.1 | 4 |
| 34 | 7 | 7.7 | 425 | 4.0 | 48 | 48.0 | 45.4 | 227 | 568 | 19.0 ± 2.0 | 7 |
| 35 | 3,7 | 7.7 | 425 | 2.0 | 24 | 24.0 | 22.7 | 113 | 284 | 16.7 ± 0.7 | 19 |
| 36 | 2,7 | 7.7 | 425 | 1.0 | 12 | 12.0 | 11.3 | 56.7 | 142 | 13.1 ± 0.9 | 11 |
| 37 | 1,7 | 7.7 | 425 | 0.5 | 6 | 6.0 | 5.7 | 28.4 | 70.9 | 9.4 ± 0.8 | 12 |
| 38 | 7 | 7.7 | 425 | 0.25 | 3 | 3.0 | 2.8 | 14.2 | 35.5 | 7.1 ± 0.7 | 14 |
| 39 | 7 | 7.7 | 425 | 0.125 | 1.5 | 1.5 | 1.4 | 7.1 | 17.7 | 4.5 ± 0.7 | 7 |
| 40 | 4 | 7.7 | 106 | 4.0 | 48 | 48.0 | 46.1 | 231 | 144 | 15.7 ± 1.8 | 8 |
| 41 | 3,6,11 | 7.4 | 425 | 8.0 | 48 | 48.0 | 46.3 | 116 | 291 | 18.2 ± 1.3 | 14 |
| 42 | 6 | 7.4 | 425 | 4.0 | 24 | 24.0 | 23.2 | 58.3 | 146 | 13.1 ± 0.8 | 7 |
| 43 | 2,6 | 7.4 | 425 | 2.0 | 12 | 12.0 | 11.6 | 29.2 | 73.0 | 10.6 ± 1.1 | 9 |
| 44 | 6 | 7.4 | 425 | 1.0 | 6 | 6.0 | 5.8 | 14.6 | 36.5 | 8.8 ± 0.8 | 7 |
| 45 | 1,6 | 7.4 | 425 | 0.5 | 3 | 3.0 | 2.9 | 7.3 | 18.3 | 3.6 ± 0.7 | 6 |
| 46 | 4,11 | 7.4 | 212 | 8.0 | 48 | 48.0 | 46.7 | 117 | 146 | 15.5 ± 1.2 | 7 |
| 47 | 11 | 7.4 | 106 | 8.0 | 48 | 48.0 | 46.8 | 117 | 73.2 | 11.2 ± 0.8 | 6 |
| 48 | 11 | 7.4 | 53 | 8.0 | 48 | 48.0 | 46.9 | 118 | 36.7 | 8.7 ± 1.4 | 8 |
| 49 | 11 | 7.4 | 26.5 | 8.0 | 48 | 48.0 | 46.9 | 118 | 18.4 | 6.0 ± 0.6 | 6 |
| 50 | 11 | 7.4 | 0 | 8.0 | 48 | 48.0 | 47.0 | 118 | 0. | 4.0 | 1 |
| 51 | 3,5 | 7.1 | 425 | 32.0 | 96 | 96.0 | 92.9 | 117 | 292 | 19.7 ± 1.7 | 13 |
| 52 | 4,5 | 7.1 | 425 | 16.0 | 48 | 48.0 | 46.8 | 58.8 | 147 | 19.2 ± 1.7 | 9 |
| 53 | 2,5 | 7.1 | 425 | 4.0 | 12 | 12.0 | 11.8 | 14.8 | 37.0 | 6.8 ± 1.1 | 10 |
| 54 | 1,5 | 7.1 | 425 | 0.5 | 1.5 | 1.5 | 1.5 | 1.9 | 4.6 | 1.3 ± 1.5 | 6 |

osmolality was adjusted to ~975 mosmol/kg by addition of $H_2O$ or glycine. On the day of the experiment, ATP was added to the DF from a 400-mM Tris/ATP stock (pH 7.0) stored at −5°C.

### *Calculation of Acid Extrusion Rates*

As described previously (Boron and Knakal, 1989), $pH_i$ data were acquired by computer, and rates of $pH_i$ recovery from acid loads ($dpH_i/dt$) were determined from a linear curve fit to the data. Acid extrusion rate ($J_H$) is defined as the net efflux of $H^+$ (or other acid) plus the net influx of $HCO_3^-$ (or other base), and computed as the product of $dpH_i/dt$, total intracellular buffering power, and volume-to-surface ratio. In each experiment, we computed fluxes from data obtained under our standard conditions,[1] as well as with up to three additional ASWs. All fluxes were corrected for initial and final drifts in the $pH_i$ baseline, as previously described (Boron, 1985). Each corrected flux was normalized by dividing it by the flux computed for that experiment under standard conditions. Finally, these ratios of fluxes then converted to normalized absolute fluxes by multiplying by 17.5 pmol $cm^{-2}$ $s^{-1}$, the average acid extrusion rate observed under standard conditions in 364 axons in this and previous studies (see below). Fluxes were computed from rates of $pH_i$ recovery obtained between $pH_i$ values of ~6.6 and ~6.9. We randomized the order in which solutions were presented to axons, as described previously (Boron, 1985).

### *Curve Fitting*

In plots of the $J_H$ vs. $pH_o$ data, the choice of which function to use in a curve fit is somewhat arbitrary. In the case of the metabolic acid–base disturbances (series 1, Fig. 2 *A*), the plot of $J_H$ vs. $pH_o$ resembled a pH titration curve with an unusually steep slope. Therefore, we chose to fit the data with a variant of a pH titration function that included a Hill coefficient. Because our data were normalized, we forced the fitted curve through a point described by our standard conditions. The unforced function has the form:

$$J = a + \frac{b}{1 + 10^{h(pK-pH)}} \quad (1)$$

where $h$ is the Hill coefficient. If the function is constrained to the normalization value $J_N$ when the pH is the normalization pH ($pH_N$), then the function becomes:

$$J = J_N - \frac{b}{1 + 10^{h(pK-pH_N)}} + \frac{b}{1 + 10^{h(pK-pH)}} \quad (2)$$

In our experiments, the $pH_N$ was 8.00 and $J_N$ was 17.5 pmol $cm^{-2}$ $s^{-1}$, the average acid extrusion rate observed under standard conditions in 364 axons. As a control, we not only fitted normalized series 1 data with Eq. 2, we also fitted unnormalized series 1 data with Eq. 1. The results were very similar, except that the standard deviations were larger with the unnormalized data.

Because the plot of $J_H$ vs. $pH_o$ for the respiratory acid–base disturbances (series 2, Fig. 2 *B*) did not clearly resemble a pH titration curve, and because there was no theoretical reason to expect the data to be fitted by any other function, we chose to fit these data with a variant of a second-order polynomial that forced the data through a point that described our standard conditions. Because the plots of $J_H$ vs. $pH_o$ for the mixed-type acid–base disturbances (series 3

[1] Standard conditions: $[Na^+]_o$ = 425 mM, $pH_o$ 8.00, nominal $[HCO_3^-]_o$ = 12 mM (computed $[HCO_3^-]_o$ = 10.8 mM), nominal $[CO_2]_o$ = 0.5%, $[Cl^-]_i$ = 400 mM, nominal $[ATP]_i$ = 4 mM).

and 4, Fig. 2 *C*) appeared linear, we chose to fit these data with a variant of a linear fit that forced the data through a point that described our standard conditions.

Values for apparent $K_m$ and $J_{max}$ were derived from iterative least-squares curve fits, and are presented ± the standard deviation. In all fits of $J_H$ vs. $[HCO_3^-]_o$, $[CO_3^=]_o$, or $[NaCO_3^-]_o$, the value of the independent variable was taken as the "computed" value listed in Table I. All fits of $J_H$ vs. $[Na^+]_o$ were nonlinear least-squares fits of the Michaelis-Menten equation. In the fit of $J_H$ vs. $[HCO_3^-]_o$ at a $pH_o$ of 8.0, the Michaelis-Menten curve was forced through the normalization point described by $[HCO_3^-]_o$ = 10.8 mM (the computed $[HCO_3^-]_o$ under standard conditions) and $J_N$ = 17.5 pmol $cm^{-2}$ $s^{-1}$, as described previously for a "type 2 fit" (Boron, 1985). For the remainder of the fits of $J_H$ vs. $[HCO_3^-]_o$, we used the unnormalized Michaelis-Menten equation. In the fit of $J_H$ vs. $[NaCO_3^-]_o$ at the single $pH_o$ of 8.0, as well as in the two fits of $J_H$ vs. $[NaCO_3^-]_o$ that encompassed data obtained at all $pH_o$ values, the curves were forced through the normalization point described by $[NaCO_3^-]_o$ = 269 μM (the computed $[NaCO_3^-]_o$ under standard conditions) and $J_N$ = 17.5 pmol $cm^{-2}$ $s^{-1}$, as described previously for a type 2 fit (Boron, 1985).

### *Previously Reported Data*

The present work is part of a larger study of the kinetics of the $Na^+$-dependent Cl-$HCO_3$ exchanger in the squid giant axon (see Boron, 1985; Boron and Knakal, 1989). The average acid extrusion rate observed under standard conditions ($J_N$ in Eq. 2) was taken as the mean value obtained in 63 (Boron, 1985) and 106 (Boron and Knakal, 1989) axons reported previously, as well as 195 axons described for the first time in the present study. Thus, the average $J_H$ in 364 axons was 17.5 ± 0.3 pmol $cm^{-2}$ $s^{-1}$. Because $J_H$ data in each experiment were normalized so that $J_H$ had a value of 17.5 pmol $cm^{-2}$ $s^{-1}$ under standard conditions, the present data should be directly comparable to data obtained previously. The $J_H$ vs. $[HCO_3^-]_o$ data obtained at a $pH_o$ of 8.0 and $[Na^+]_o$ of 425 mM were reported previously (Boron, 1985), as were some of the $J_H$ vs. $[Na^+]_o$ data obtained at a $pH_o$ of 8.0 and a $[HCO_3^-]_o$ of 12 mM. Similarly, the $J_H$ vs. $[Na^+]_o$ data obtained at a $pH_o$ of 8.0 and a $[HCO_3^-]_o$ of 48 mM were reported previously (Boron and Knakal, 1989).

### *Statistics*

Apparent values of $K_m$ and $J_{max}$ (see above) are expressed ± the standard deviation. Other data (e.g., mean $J_H$ values) are expressed ± SEM.

## RESULTS

### *Dependence of Acid Extrusion Rate on $pH_o$*

We studied the effects on $J_H$ of altering $pH_o$ in four different ways. The first two were the classical "metabolic" and "respiratory" acid–base disturbances. The third was a novel mixed-type acid–base disturbance designed to vary $pH_o$ at fixed values of $[CO_3^=]_o$ and $[NaCO_3^-]_o$. The fourth was also a novel mixed-type acid–base disturbance in which $pH_o$ varied at fixed values of $[HCO_3^-]_o$ and $[NaCO_3^-]_o$. The parameters varied and fixed in each of these four series of experiments are summarized in Table II.

*Varying $[HCO_3^-]_o$ at constant $[CO_2]_o$: "metabolic" acid–base disturbances (series 1).* Fig. 1 illustrates an experiment in which we investigated the effects of varying $pH_o$ on the recovery of $pH_i$ from an acid load. The axon was initially bathed in a pH 8.00 $CO_2/HCO_3^-$-free ASW. Internal dialysis with a fluid having a pH of 6.5 caused $pH_i$ to

fall from ~7.4 to ~6.55 over a period of ~40 min (segment *ab*). After dialysis was halted, $pH_i$ slowly increased and then leveled off (*bc*). The slope of the $pH_i$ record ($dpH_i/dt$) just before point *c* is an index of the acid extrusion rate ($J_H$) during this initial background period.[2] The subsequent introduction of an ASW buffered to pH 8.3 with 24 mM $HCO_3^-$ and 0.5% $CO_2$ caused a small and transient $pH_i$ decline (*cd*), due to the influx of $CO_2$, followed by a rapid alkalinization (*de*), due to $Na^+$-dependent Cl-$HCO_3$ exchange.[3] Lowering $pH_o$ from 8.3 to 7.7, achieved by reducing $[HCO_3^-]$ to 6 mM at a fixed $[CO_2]$ of 0.5%, lowered $dpH_i/dt$ (*ef*). On the other hand, raising $pH_o$ to 8.00 by increasing $[HCO_3^-]_o$ to 12 mM at a fixed $[CO_2]$ of 0.5% (*fg*) caused $dpH_i/dt$ to increase, although not to a value as high as at $pH_o$ 8.3. When $pH_o$ was reduced to 7.4 by lowering $[HCO_3^-]_o$ to 3 mM at a fixed $[CO_2]$ of 0.5% (*gh*), $dpH_i/dt$ decreased to a very low value. Finally, returning the axon to the control, $CO_2/HCO_3^-$-free ASW caused a rapid alkalinization (*hi*), reflecting the efflux of $CO_2$, followed by a slow and steady $pH_i$ decline (*ij*) due to a background acidifying process.

The results of 40 $J_H$ determinations are summarized in Fig. 2 *A*, which describes how the $Na^+$-dependent Cl-$HCO_3$ exchanger was affected by metabolic acid–base disturbances. Of the four acid–base disturbances, this method of varying $pH_o$

TABLE II

*Parameter Values in the Four Acid–Base Disturbances*

| Acid–base disturbance | $pH_o$ | $[CO_2]_o$ | $[HCO_3^-]_o$ | $[CO_3^=]$ | $[Na^+]_o$ | $[NaCO_3^-]_o$ | $J_H$ |
|---|---|---|---|---|---|---|---|
| | | % | *mM* | μ*M* | *mM* | μ*M* | *pmol cm*$^{-2}$ s$^{-1}$ |
| Metabolic | 7.1–8.6 | Fixed: 0.5 | 1.5–48 | 1.9–1,911 | Fixed: 425 | 4.6–4,779 | 1.3–21.6 |
| Respiratory | 7.1–8.6 | 4–0.125 | Fixed: 12 | 14.8–478 | Fixed: 425 | 37.0–1,194 | 6.8–22.4 |
| Mixed-type 1 | 7.1–8.6 | 32–0.0313 | 96–3 | Fixed: ~115 | Fixed: 425 | Fixed: ~289 | 15.4–19.7 |
| Mixed-type 2 | 7.1–8.6 | 16–0.5 | Fixed: 48 | 58.8–1,917 | 425–13.25 | Fixed: ~146 | 15.1–19.2 |

produced the greatest range of $[CO_3^=]_o$ values (~1,000-fold, from 1.9 to 1,911 μM) and $[NaCO_3^-]_o$ values (also ~1,000-fold, from 4.6 to 4,779 μM). It also elicited the largest changes in $J_H$ (see Table II). The curve drawn through the points is a normalized best-fit curve for a pH titration having an apparent $pK_a$ of 7.74 and an apparent Hill coefficient of 2.98. As described in Methods, the function was forced through the point (indicated by the asterisk) describing our standard conditions ($pH_o$ = 8.00, $J_H$ = 17.5 pmol cm$^{-2}$ s$^{-1}$). It can be shown that the slope of the fitted curve is maximal when $pH_o$ = pK, so that the maximal fitted slope for our data is 31.5 pmol cm$^{-2}$ s$^{-1}$ per pH unit at a $pH_o$ of 7.74.

[2] This initial background (just before point *c* in Fig. 1), as well as the final background (segment *ij* in Fig. 1), are treated as fluxes, although they could reflect intracellular events (e.g., metabolism) in whole or in part.

[3] In computing $J_H$, we corrected for drift in the $pH_i$ baseline by subtracting an estimate of the background $J_H$ from the observed $J_H$, as described previously (Boron, 1985). In brief, we computed "initial" and "final" backgrounds as described in footnote 2, and assumed that the background flux varied linearly with time between these initial and final periods.

*Varying $[CO_2]_o$ at constant $[HCO_3^-]_o$: "respiratory" acid–base disturbances (series 2).* In 50 additional $J_H$ determinations, made according to the protocol used in Fig. 1, we varied $pH_o$ at a fixed $[HCO_3^-]_o$ of 12 mM. Compared with the metabolic disturbances, such respiratory acid–base disturbances involved smaller changes in $[CO_3^=]_o$ (~32-fold, from 14.8 to 478 μM) and $[NaCO_3^-]_o$ (also ~32-fold, from 37 to 1,194 μM). These metabolic disturbances also elicited smaller variations in $J_H$ (see Table II). The curve drawn through the data in Fig. 2 *B* is the result of a second-order polynomial curve fit that was forced through the standard point ($pH_o$ = 8.00, $J_H$ = 17.5 pmol $cm^{-2}$ $s^{-1}$). The slope of the fitted function varies linearly but inversely with $pH_o$. The maximal slope is thus at the lowest $pH_o$ studied, 7.1; at this $pH_o$, the slope is 14.8 pmol $cm^{-2}$ $s^{-1}$ per pH unit, which is slightly less than half as great as the maximal slope of the fitted curve describing the metabolic acid–base disturbances (Fig. 2 *A*).

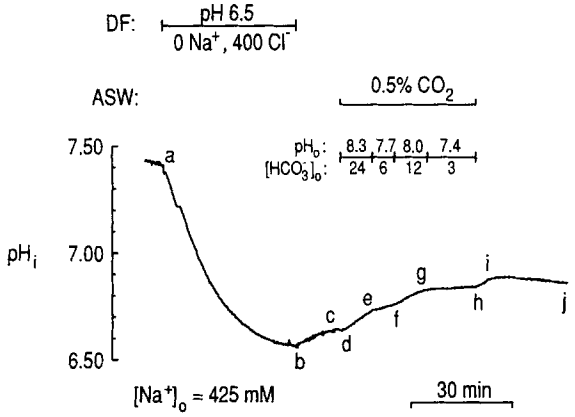


FIGURE 1. Recovery of $pH_i$ from an acid load, and the effect of varying $[HCO_3^-]_o$ at a fixed $[CO_2]_o$ of 0.5%. The axon (a050485d), which had an initial $pH_i$ of 7.42, was acid loaded by dialyzing between points *a* and *b* with a pH 6.5 solution having a $[Na^+]$ of 0 mM and a $[Cl^-]$ of 400 mM. After $pH_i$ had stabilized in the absence of $CO_2/HCO_3^-$ at *c*, we monitored the $pH_i$ recovery in the presence of $CO_2/HCO_3^-$ at $pH_o$ values of 8.3 ($[HCO_3^-]_o$ = 24 mM), 7.7 ($[HCO_3^-]_o$ = 6 mM), 8.0 ($[HCO_3^-]_o$ = 12 mM), and 7.4 ($[HCO_3^-]_o$ = 3 mM). Finally, at *h* we returned the axon to a $CO_2/HCO_3^-$-free seawater. The axon diameter was 600 μm.

*Varying $[HCO_3^-]_o$ and $[CO_2]_o$ at constant $[CO_3^=]_o$ and $[NaCO_3^-]_o$: first mixed-type acid–base disturbance (series 3).* In the first two series of experiments, variations in $pH_o$ were accompanied by changes in both $[CO_3^=]_o$ and $[NaCO_3^-]_o$. In the third series, we varied $pH_o$ by altering $[HCO_3^-]_o$ and $[CO_2]_o$ in such a way that $[CO_3^=]_o$ was held fixed at ~115 μM, $[NaCO_3^-]_o$ at ~289 μM, and $[Na^+]_o$ at ~425 mM. As summarized in Fig. 2 *C* for 82 determinations of $J_H$, this acid–base disturbance only slightly affected $J_H$, which tended to fall as $pH_o$ rose. The regression line forced through the standard point ($pH_o$ = 8.00, $J_H$ = 17.5 pmol $cm^{-2}$ $s^{-1}$) has a slope of $-0.8 \pm 0.8$ pmol $cm^{-2}$ $s^{-1}$ per pH unit. This slope is of the opposite sign and only ~2.5% of the magnitude of the maximal slope in series 1.

*Varying $[CO_2]_o$ and $[Na^+]_o$ at constant $[HCO_3^-]_o$ and $[NaCO_3^-]_o$: second mixed-type acid–base disturbance (series 4).* Although $J_H$ was not significantly affected by $pH_o$ changes in the series 3 data, the experiments did not distinguish among $[CO_3^=]_o$, $[NaCO_3^-]_o$, and $[Na^+]_o$, which were all fixed. We therefore examined the effects of a second mixed-type disturbance, one in which we varied $pH_o$ by altering $[Na^+]_o$ (~32-fold, from 425 to 13.25 mM), $[CO_2]_o$ (32-fold, from 16 to 0.5%), and $[CO_3^=]_o$

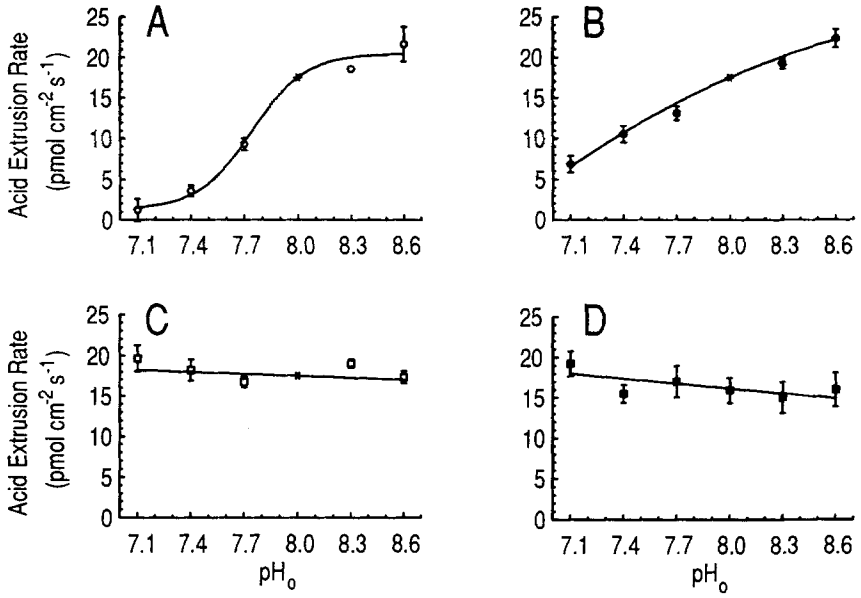


FIGURE 2. Dependence of acid extrusion rate on extracellular pH for four types of acid–base disturbances. (*A*) Metabolic acid–base disturbances (series 1). The $J_H$ data are indicated by open circles. The error bars indicate the SEM; error bars are omitted for SEMs that were less than half the vertical size of the symbol. The curve drawn through the points is the result of a nonlinear least-squares curve fit of the data by Eq. 2 (see Methods) that forces the curve through a point (*asterisk*) describing our standard conditions. Best-fit values were $b = 18.6 \pm 1.76$, pK = 7.74 ± 0.04, and $h = 2.98 \pm 0.53$ ($n = 40$). (*B*) Respiratory acid–base disturbances (series 2). The $J_H$ data are indicated by filled circles. The curve drawn through the points is the result of a least-squares curve fit of the data by a second-order polynomial (see Methods) that forces the curve through a point (*asterisk*) describing our standard conditions. Best-fit values for the polynomial $J_H = a_o + a_1 \times pH_o + a_2 \times (pH_o)^2$ were $a_o = -244.9$, $a_1 = 56.04$, and $a_2 = -2.906$ ($n = 50$). (*C*) Mixed-type acid–base disturbance 1 (series 3). The $J_H$ data are indicated by open squares. The curve drawn through the points is the result of a least-squares curve fit of the data by a line that is forced through a point (*asterisk*) describing our standard conditions. Best-fit values for the line $J_H = a_o + a_1 \times pH_o$ were $a_o = 23.97$ and $a_1 = -0.8085$ ($n = 82$). (*D*) Mixed-type acid–base disturbance 2 (series 4). The $J_H$ data are indicated by filled squares. The curve drawn through the points is the result of a least-squares curve fit of the data by the line $J_H = a_o + a_1 \times pH_o$, where $a_o = 32.5$ and $a_1 = -2.046$ ($n = 50$).

(~33-fold, from 58.8 to 1,917 μM) in such a way that $[HCO_3^-]_o$ was held fixed at 48 mM and $[NaCO_3^-]_o$ at ~146 μM. The results of 50 such $J_H$ determinations, summarized in Fig. 2 *D*, again show that the $Na^+$-dependent Cl-$HCO_3$ exchanger was not substantially affected by changes in $pH_o$, though $J_H$ showed a small tendency to decline with increasing values of $pH_o$. The regression line, not forced through a standard point, has a slope of $-2.0 \pm 1.4$ pmol cm$^{-2}$ s$^{-1}$ per pH unit. This value has the opposite sign and only ~6% of the magnitude of the maximum slope in series 1.

### *Effect of $pH_o$ Changes on the Transporter's Apparent $[NaCO_3^-]_o$, $[HCO_3^-]_o$, $[CO_3^=]_o$, and $[Na^+]_o$ Dependencies*

$[NaCO_3^-]_o$. The above data indicate that varying $pH_o$ in the range 7.1–8.6 has no substantial effect on $J_H$, provided that the product of $[CO_3^=]_o$ and $[Na^+]_o$ (and thus $[NaCO_3^-]_o$) is held constant. If the $NaCO_3^-$ ion pair were the extracellular substrate of the $Na^+$-dependent Cl-$HCO_3$ exchanger, and if the exchanger were unaffected by changes in $pH_o$ per se, then $J_H$ would depend uniquely on $[NaCO_3^-]_o$, regardless of $pH_o$. In Fig. 3 we have replotted the $J_H$ data of Fig. 2, *A–D* as a function of $[NaCO_3^-]_o$, retaining the same symbols as in Fig. 2. It is apparent that the data for all four series of experiments (i.e., the four methods for varying $pH_o$) are consistent with a single dependence of $J_H$ on $[NaCO_3^-]_o$. The curve drawn through the data in Fig. 3

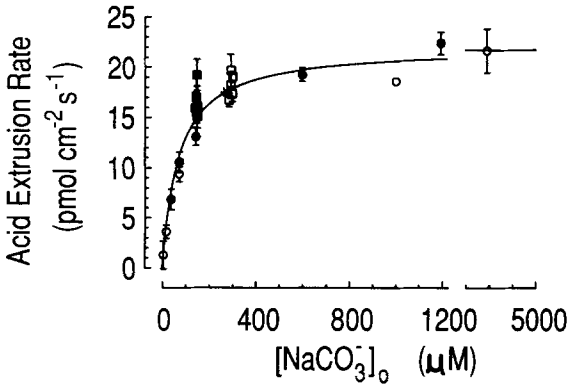


FIGURE 3. Dependence of acid extrusion rate on $[NaCO_3^-]_o$. The $J_H$ data depicted in Fig. 2, *A–D* are replotted as a function of $[NaCO_3^-]_o$. As in the previous figure, the open circles represent data from series 1 (Fig. 2 *A*); the filled circles, series 2 (Fig. 2 *B*); the open squares, series 3 (Fig. 2 *C*); and the filled squares, series 4 (Fig. 2 *D*). The curve drawn through the points is the result of a nonlinear least-squares fit that forced the curve through our standard conditions ($[NaCO_3^-]_o$ = 269 μM, $J_H$ = 17.5 pmol $cm^{-2}$ $s^{-1}$; point indicated by an asterisk near the cluster of open squares). The best-fit $K_m$ was 69 ± 9 μM, from which was computed a $J_{max}$ of 22.0 pmol $cm^{-2}$ $s^{-1}$ ($n$ = 222 $J_H$ determinations). We obtained similar kinetic constants when we fitted all of the data presented in this study (including the additional data summarized in Figs. 4 and 5): the best-fit $K_m$ was 79 ± 5 μM, from which was computed a $J_{max}$ of 22.6 pmol $cm^{-2}$ $s^{-1}$ ($n$ = 471).

is the result of using a nonlinear least-squares curve fitting procedure to fit the Michaelis-Menten equation simultaneously to all the data of Fig. 2, *A–D*. The apparent $K_m$ for $NaCO_3^-$ is 69 μM and the $J_{max}$ is 22.0 pmol $cm^{-2}$ $s^{-1}$.

We have also examined the apparent dependence of $J_H$ on $[NaCO_3^-]_o$ separately for $pH_o$ values between 7.4 and 8.6. For this analysis, we included all of the data of Fig. 2, as well as additional data (presented below) specifically gathered for a study of the effects of $pH_o$ changes on the $[HCO_3^-]_o$ and $[Na^+]_o$ dependencies of acid extrusion. The results, summarized in Table III, show that decreasing $pH_o$ from 8.3 to 7.4 has virtually no effect on the apparent $J_{max}(NaCO_3^-)$. When $pH_o$ is decreased from 8.6 to 8.3, $J_{max}(NaCO_3^-)$ increases to a value that is ~10% higher than the mean $J_{max}(NaCO_3^-)$ (i.e., 21.5 pmol $cm^{-2}$ $s^{-1}$) between $pH_o$ 7.1 and 8.3. As was the case for $J_{max}(NaCO_3^-)$, $K_m(NaCO_3^-)$ was not very sensitive to changes in $pH_o$. Decreasing $pH_o$

from 8.3 to 7.4 had no consistent effect on $K_m(NaCO_3^-)$. The $K_m(NaCO_3^-)$ at $pH_o$ 8.6 was ~58% higher than the average $K_m(NaCO_3^-)$ (i.e., ~68 mM) in the $pH_o$ range from 7.4 to 8.3.

If the $NaCO_3^-$ ion pair hypothesis were correct, then our data would imply that the $Na^+$-dependent Cl-$HCO_3$ exchanger itself is not very $pH_o$ sensitive in the range 7.1–8.6. This conclusion would lead to two kinetic predictions. First, when $J_H$ is examined as a function of $[HCO_3^-]_o$ at a fixed $[Na^+]_o$, the apparent $K_m(HCO_3^-)$ value should approximately double for each 0.3 fall in $pH_o$, whereas the apparent $J_{max}(HCO_3^-)$ values should be invariant of $pH_o$. The basis of this prediction is that $[CO_3^=]_o$ (and thus $[NaCO_3^-]_o$) should fall by half for each 0.3 fall in pH. Second, when $J_H$ is examined as a function of $[Na^+]_o$ at a fixed $[HCO_3^-]_o$, the apparent $K_m(Na^+)$ value should approximately double for each 0.3 fall in $pH_o$, whereas the apparent $J_{max}(Na^+)$ values should be stable. As noted in the Discussion, predictions experimentally indistinguishable from these are made by models calling for a separate binding

TABLE III

*Effect of Altering $pH_o$ on the $[NaCO_3^-]_o$ Dependence of Acid Extrusion*

| $pH_o$ | Range of $[NaCO_3^-]_o$ | $K_m(NaCO_3^-)$ | $J_{max}$ | $n$ |
|---|---|---|---|---|
| | $\mu M$ | $\mu M$ | *pmol cm$^{-2}$ s$^{-1}$* | |
| 7.4 | 0–291 | 64 ± 12 | 21.5 ± 1.5 | 71 |
| 7.7 | 18–568 | 78 ± 14 | 21.5 ± 1.2 | 78 |
| 8.0 | 0–279 | 54 ± 6 | 21.1 | 72 |
| 8.3 | 37–1,000 | 75 ± 9 | 21.9 ± 0.7 | 91 |
| 8.6 | 19–4,779 | 107 ± 12 | 23.7 ± 0.9 | 121 |

$K_m(NaCO_3^-)$ is the apparent Michaelis constant, and $J_{max}$ is the apparent maximal acid extrusion rate. The values of $[NaCO_3^-]_o$ were computed from the nominal $[Na^+]_o$, $[HCO_3^-]_o$, $pH_o$, and other parameters as described in Methods. The data at $pH_o$ 8.0 were fitted by a normalized Michaelis-Menten equation that forced the curve through the point dictated by our standard conditions (i.e., $[NaCO_3^-]_o$ = 269 μM, $J_H$ = 17.5 pmol cm$^{-2}$ s$^{-1}$). The data at other $pH_o$ values were fitted by the Michaelis-Menten equation. The curve-fitting procedures, the results of which are summarized in this table, were performed on a compendium of all data reported in other tables and figures in the paper. $n$ is the number of data points fitted.

of $Na^+$ and $HCO_3^-$ (or $CO_3^=$) in an ordered or random fashion. However, these other models make these predictions only when endowed with extreme kinetic parameters (Boron and Knakal, 1989), and only when certain ad hoc assumptions are made (see Discussion). We examined these predictions in the kinetic studies described below. The kinetic analyses incorporate the data summarized in Fig. 2, *A–D*, as well as additional data gathered according to the protocol of Fig. 1.

*$[HCO_3^-]_o$.* We examined the $[HCO_3^-]_o$ dependence of acid extrusion in a total of 355 $J_H$ determinations at $pH_o$ values of 7.1 (series 5), 7.4 (series 6), 7.7 (series 7), 8.0 (series 8), 8.3 (series 9), and 8.6 (series 10). In these experiments, $[Na^+]_o$ was fixed at 425 mM and $[HCO_3^-]_o$ was varied at a constant $pH_o$ by making proportional changes in $[HCO_3^-]_o$ and $[CO_2]_o$. As discussed below, this approach necessarily produces changes in $[CO_3^=]_o$ that are proportional to those in $[HCO_3^-]_o$. The dependence of $J_H$ on $[HCO_3^-]_o$ at each of these $pH_o$ values is plotted in Fig. 4 *A*. The best-fit values for $K_m(HCO_3^-)$ and $J_{max}(HCO_3^-)$ are plotted as a function of $pH_o$ in Fig. 4 *B*, and are

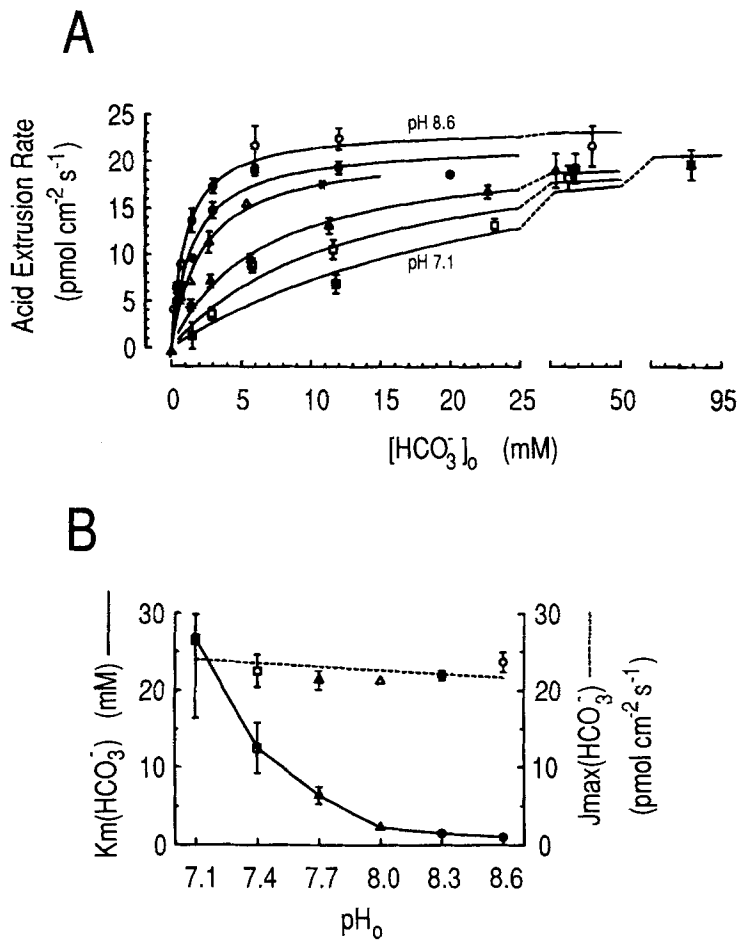


FIGURE 4. Effect of changes in $pH_o$ on the $[HCO_3^-]_o$ dependence of the $Na^+$-dependent Cl-$HCO_3$ exchanger. (*A*) Dependence of acid extrusion rate on $[HCO_3^-]_o$ at different values of $pH_o$. The top data set (*open circles*) was obtained at $pH_o$ 8.6, and the bottom set (*filled squares*) at a $pH_o$ of 7.1. The intermediated data sets were obtained at $pH_o$ values of 8.3 (*filled circles*), 8.0 (*open triangles*), 7.7 (*filled triangles*), and 7.4 (*open squares*). The curves drawn through the points are the result of nonlinear least-squares curve fits to $J_H$ vs. $[HCO_3^-]_o$, the results of which are summarized in Table IV. The fit at $pH_o$ 8.0 was to a modified form of the Michaelis-Menten equation that forced the curve through the point (*asterisk*) defined by our standard conditions ($[HCO_3^-]_o$ = 10.8 mM, $J_H$ = 17.5 pmol cm$^{-2}$ s$^{-1}$). (*B*) Dependence of $K_m(HCO_3^-)$ and $J_{max}(HCO_3^-)$ on $pH_o$. The kinetic parameters from the curve fits are plotted as a function of $pH_o$. The symbols are the same as in *A*. The solid squares representing $K_m$ ($HCO_3^-$) and $J_{max}(HCO_3^-)$ at $pH_o$ 7.1 overlay one another; the error bar extending upward from this solid square refers to the $J_{max}(HCO_3^-)$ value, the one extending downward, the $K_m(HCO_3^-)$ value. The $K_m(HCO_3^-)$ data are connected by solid line segments. The $J_{max}(HCO_3^-)$ data were fitted to a straight line (*broken curve*).

TABLE IV

*Effect of Altering $pH_o$ on the $[HCO_3^-]_o$ Dependence of Acid Extrusion in the Presence of 425 mM External $Na^+$*

| $pH_o$ | Range of $[HCO_3^-]_o$ | $K_m(HCO_3^-)$ | $J_{max}$ | $n$ |
|---|---|---|---|---|
| | *mM* | *mM* | *pmol cm$^{-2}$ s$^{-1}$* | |
| 7.1 | 1.5–92.9 | 26.7 ± 10.3 | 26.5 ± 3.4 | 38 |
| 7.4 | 2.9–46.3 | 12.5 ± 3.3 | 22.5 ± 2.1 | 43 |
| 7.7 | 1.4–45.4 | 6.4 ± 1.1 | 21.3 ± 1.2 | 70 |
| 8.0 | 0–10.8 | 2.3 ± 0.3 | 21.2 | 31 |
| 8.3 | 0.4–20 | 1.5 ± 0.2 | 21.9 ± 0.6 | 82 |
| 8.6 | 0.2–48.0 | 1.0 ± 0.1 | 23.6 ± 1.3 | 91 |

$K_m(HCO_3^-)$ is the apparent Michaelis constant and $J_{max}$ is the apparent maximal acid extrusion rate. The values of $[HCO_3^-]_o$ were computed from the nominal $[HCO_3^-]_o$, $pH_o$, and other parameters as described in Methods. At each $pH_o$, $[HCO_3^-]_o$ was varied by proportionally varying both $[HCO_3^-]_o$ and $[CO_2]_o$. The data obtained at $pH_o$ 8.00 were reported previously (Boron, 1985), and were fitted by a normalized Michaelis-Menten equation that forced the curve through the point by our standard conditions (i.e., $[HCO_3^-]_o$ = 10.8 mM, $J_H$ = 17.5 pmol cm$^{-2}$s$^{-1}$). The data at other $pH_o$ values were fitted by the Michaelis-Menten equation. $n$ is the number of data points fitted.

summarized in Table IV. Whereas lowering $pH_o$ from 8.6 to 7.1 had little effect on $J_{max}(HCO_3^-)$, the trend being to slightly increase $J_{max}(HCO_3^-)$, this decrease in $pH_o$ produced a marked increase in $K_m(HCO_3^-)$. As predicted by the ion pair model, each 0.3 reduction in $pH_o$ caused an approximate doubling of $K_m(HCO_3^-)$ between $pH_o$ values of 8.3 and 7.1. $K_m(HCO_3^-)$ fell by only 50% between $pH_o$ values of 8.6 and 8.3.

*$[CO_3^=]_o$.* We have also fitted these $J_H$ data from series 5 through series 10 as a function of $[CO_3^=]_o$ rather than as a function of $[HCO_3^-]_o$. The results of these $J_H$ vs. $[CO_3^=]_o$ fits are summarized in Table V. As was the case for $J_{max}(HCO_3^-)$, $J_{max}(CO_3^=)$ did not vary substantially with $pH_o$ in the range 8.6–7.1. However, unlike the $K_m(HCO_3^-)$ values, the $K_m(CO_3^=)$ values were relatively insensitive to changes in $pH_o$.

TABLE V

*Effect of Altering $pH_o$ on the $[CO_3^=]_o$ Dependence of Acid Extrusion in the Presence of 425 mM External Na*

| $pH_o$ | Range of $[CO_3^=]_o$ | $K_m(CO_3^=)$ | $J_{max}$ | $n$ |
|---|---|---|---|---|
| | *μM* | *μM* | *pmol cm$^{-2}$ s$^{-1}$* | |
| 7.1 | 2–117 | 34.1 ± 13.2 | 26.6 ± 3.4 | 38 |
| 7.4 | 7–116 | 31.5 ± 8.4 | 22.6 ± 2.1 | 43 |
| 7.7 | 7–227 | 31.9 ± 5.3 | 21.3 ± 1.2 | 70 |
| 8.0 | 0–54 | 23.2 ± 2.5 | 21.3 | 31 |
| 8.3 | 7–400 | 30.2 ± 3.1 | 21.9 ± 0.6 | 82 |
| 8.6 | 8–1,911 | 42.7 ± 5.1 | 23.6 ± 0.8 | 91 |

The data are the same as those summarized in Table IV, except that the curve fitting has been repeated with $[CO_3^=]_o$ as the independent variable. $K_m$ $(CO_3^=)$ is the apparent Michaelis constant and $J_{max}$ is the apparent maximal acid extrusion rate. The values of $[CO_3^=]_o$ were computed from the added $[HCO_3^-]_o$, $pH_o$, and other parameters, as described in Methods. The data obtained at $pH_o$ 8.00 were fitted by a normalized Michaelis-Menten equation that forced the curve through the point dictated by our standard conditions (i.e., $[CO_3^=]_o$ = 447, μM, $J_H$ = 17.5 pmol cm$^{-2}$ s$^{-1}$); for the other data in that fit, $[CO_3^=]_o$ ranged from 0 to 54 μM. The data at other $pH_o$ values were fitted by Michaelis-Menten equation. $n$ is the number of data points fitted.

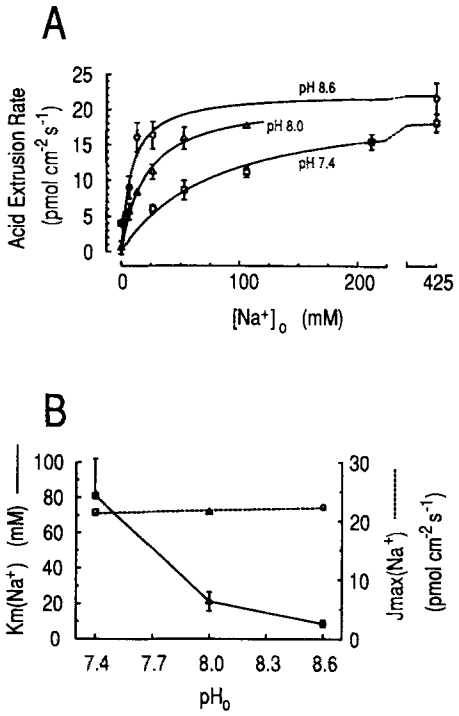


FIGURE 5. Effect of changes in $pH_o$ on the $[Na^+]_o$ dependence of the $Na^+$-dependent Cl-$HCO_3$ exchanger. (*A*) Dependence of acid extrusion rate on $[Na^+]_o$ at different values of $pH_o$. The top data set (*open circles*) was obtained at $pH_o$ 8.6, the middle set (*open triangles*) at $pH_o$ 8.0, and the bottom set (*open squares*), at $pH_o$ 7.4. The curves drawn through the points are the result of nonlinear least-squares curve fits to $J_H$ vs. $[Na^+]_o$, the results of which are summarized in Table VI. (*B*) Dependence of $K_m(Na^+)$ and $J_{max}(Na^+)$ on $pH_o$. The kinetic parameters from the curve fits are plotted as a function of $pH_o$. The symbols are the same as in *A*. The $K_m(Na^+)$ data are connected by solid line segments. The $J_{max}(Na^+)$ data were fitted to a straight line (*broken curve*).

*$[Na^+]_o$.* We examined the $[Na^+]_o$ dependence of acid extrusion in a total of 120 $J_H$ determinations at $pH_o$ values of 7.4 (series 11), 8.0 (series 12), and 8.6 (series 13). For this study, $[HCO_3^-]_o$ was fixed at 48 mM and the different $pH_o$ values were established by varying $[CO_2]_o$. This approach necessarily involves changes in $[CO_3^=]_o$, which ranged from ~117 μM at $pH_o$ 7.1 to ~1,917 μM at $pH_o$ 8.6. The dependence of $J_H$ on $[Na^+]_o$ at each of these $pH_o$ values is plotted in Fig. 5 *A*. The best-fit values for $K_m(Na^+)$ and $J_{max}(Na^+)$ are plotted as a function of $pH_o$ in Fig. 5 *B* and summarized in Table VI. Decreasing $pH_o$ from 8.6 to 7.4 had only a slight effect on $J_{max}(Na^+)$, the trend being to slightly decrease $J_{max}(Na^+)$. However, this decrease in $pH_o$ produced a marked increase in $K_m(Na^+)$, with the 0.6 pH unit decrease from

TABLE VI

*Effect of Altering $pH_o$ on the $[Na^+]_o$ Dependence of Acid Extrusion in the Presence of 48 mM External $HCO_3^-$*

| $pH_o$ | Range of $[Na^+]_o$ | $K_m(Na^+)$ | $J_{max}$ | $n$ |
|---|---|---|---|---|
| | *mM* | *mM* | *pmol cm⁻² s⁻¹* | |
| 7.4 | 0–425 | 81 ± 21 | 21.5 ± 1.7 | 42 |
| 8.0 | 0–106 | 21.0 ± 5.3 | 21.5 ± 1.9 | 41 |
| 8.6 | 1.7–425 | 8.6 ± 1.9 | 22.4 ± 1.6 | 37 |

$K_m$ ($Na^+$) is the apparent Michaelis constant and $J_{max}$ is the apparent maximal acid extrusion rate. Some of the data (31 of 41 points) at $pH_o$ 8.00 were reported previously (Boron and Knakal, 1989); all data at this $pH_o$ were fitted by a normalized Michaelis-Menten equation that force the curve through the point dictated by our standard conditions (i.e., $HCO_3^-]_o$ = 10.8 mM, $J_H$ = 17.5 pmol cm⁻² s⁻¹). The data at other $pH_o$ values were fitted by the Michaelis-Menten equation. $n$ is the number of data points fitted.

$pH_o$ 8.0 to 7.4 quadrupling $K_m(Na^+)$ as predicted by the ion pair model. The 0.6 pH unit decrease from $pH_o$ 8.6 to 7.4 caused $K_m(Na^+)$ to increase by a factor of somewhat less than 2.5. Thus, just as was the case for the $pH_o$ dependence of $K_m(HCO_3^-)$, the $pH_o$ dependence of $K_m(Na^+)$ was somewhat smaller than expected in the highest $pH_o$ range examined.

## DISCUSSION

### *Summary*

An analysis of the effects of $pH_o$ changes on the $Na^+$-dependent Cl-$HCO_3$ exchanger is important both because $pH_o$ disturbances are common, and because the transporter is a key player in the regulation of $pH_i$ in both invertebrate and vertebrate cells. In approaching this study, we had five major goals. The first was to confirm the observation, previously made only on barnacle muscle fibers (Boron, McCormick, and Roos, 1979), that graded decreases in $pH_o$ produce graded inhibitions of $Na^+$-dependent Cl-$HCO_3$ exchange. The results, summarized in Fig. 2 *A*, indicate that metabolic acid–base disturbances have a sizable effect on the transporter, with an apparent pK of ~7.7 and a Hill coefficient of nearly 3.

Our second goal was to examine the effects of respiratory acid–base disturbances on $Na^+$-dependent Cl-$HCO_3$ exchange. To our knowledge, such an analysis had not been done. As summarized in Fig. 2 *B*, we found that graded decreases of $pH_o$ at a fixed $[HCO_3^-]_o$ produce graded inhibitions of transport. The maximal steepness of the relationship between $J_H$ and $pH_o$, however, is less than half as great as with metabolic acid–base disturbances.

Third, we wished to examine how the exchanger responds to $pH_o$ changes made at a fixed $[NaCO_3^-]_o$. Therefore, we introduced two novel methods for varying $pH_o$: (*i*) we fixed $[NaCO_3^-]_o$ (and $[CO_3^=]_o$) by making appropriate changes in both $[CO_2]_o$ and $[HCO_3^-]_o$; and (*ii*) we fixed $[NaCO_3^-]_o$ (and $[HCO_3^-]_o$) by appropriately changing $[CO_2]_o$ and $[Na^+]_o$. We were surprised to find that the transporter is minimally affected by $pH_o$ changes made by either of these two methods (see Fig. 2, *C* and *D*).

Our fourth goal was to assess how $pH_o$ changes affect the apparent $K_m$ and $J_{max}$ values obtained when varying $[Na^+]_o$, $[HCO_3^-]_o$, $[CO_3^=]_o$, or $[NaCO_3^-]_o$. As summarized in Tables IV–VI, none of the $J_{max}$ values is substantially affected by $pH_o$ changes. However, both $K_m(Na^+)$ and $K_m(HCO_3^-)$ approximately halve for each $pH_o$ increase of 0.3 in the $pH_o$ range 7.1–8.3 (Tables IV and VI). On the other hand, both $K_m(CO_3^=)$ and $K_m(NaCO_3^-)$ are virtually insensitive to these $pH_o$ changes (see Tables III and V).

Our final goal was to use the data summarized above in an attempt to determine whether it is $HCO_3^-$, $CO_3^=$ or $NaCO_3^-$ that is the base transported by the $Na^+$-dependent Cl-$HCO_3$ exchanger. The results of this analysis are summarized below.

### *Mechanisms by Which $pH_o$ Changes Are Expected to Affect $Na^+$-dependent Cl-$HCO_3$ Exchange*

$pH_o$ changes could affect the $Na^+$-dependent Cl-$HCO_3$ exchange rate in at least two ways. First, they could alter the degree of protonation of titratable groups near the transporter's extracellular surface. In principle, such direct effects could cause a $pH_o$

decrease to inhibit, stimulate, or have no influence on transport. Second, $pH_o$ changes could affect the transport rate by altering the concentration of the transported base (i.e., $HCO_3^-$, $CO_3^=$, or $NaCO_3^-$). Neglecting direct $pH_o$ effects, all four models of $Na^+$-dependent Cl-$HCO_3$ exchange (see Introduction) predict that a $pH_o$ decrease should inhibit transport only if the $pH_o$ decrease caused a fall in the concentration of the transported base. Thus, the total effect of a $pH_o$ shift on transport should have two components, the direct effect on the transporter's titratable groups, and the substrate effect. Varying $pH_o$ in different ways might provide insight into the mechanism of transport.

### *Predicted Effects of $pH_o$ Changes for Different Models of $Na^+$-dependent Cl-$HCO_3$ Exchange*

In three previous kinetic studies of the axon's $Na^+$-dependent Cl-$HCO_3$ exchanger, the approach was to examine how $J_H$ is affected by changes in $[HCO_3^-]_o$ and/or $[Na^+]_o$ in the absence or presence of DNDS. Because these earlier studies were all performed at the single $pH_o$ of 8.0, changes in $[HCO_3^-]_o$ produced proportional changes in $[CO_3^=]_o$. The results of these earlier studies were always consistent with the ion pair model, but did not rule out highly restricted versions of models involving the ordered or random binding of $Na^+$ and $HCO_3^-$ (or $CO_3^=$) (Boron and Knakal, 1989).

*$HCO_3^-$*. We will now consider if the data of series 1–series 4 can be reconciled with the hypothesis that $HCO_3^-$ itself is the base transported by the $Na^+$-dependent Cl-$HCO_3$ exchanger. We shall assume that $HCO_3^-$ interacts with the transporter only as a substrate, and that neither $CO_3^=$ nor $NaCO_3^-$ interact with the transporter. The data from series 1 show that when $pH_o$ and $[HCO_3^-]_o$ are reduced at a fixed $[CO_2]_o$, there is a steep fall in $J_H$. This could be due entirely to the decrease in substrate concentration (i.e., $[HCO_3^-]_o$), or to a combination of decreased $[HCO_3^-]_o$ and a direct inhibitory effect of low $pH_o$.

The series 2 experiments show that reducing $pH_o$ at a fixed $[HCO_3^-]_o$ causes a fall in $J_H$ that is less steep than when $pH_o$ and $[HCO_3^-]_o$ are reduced simultaneously (series 1). Thus, if $HCO_3^-$ is the substrate, the shallow $J_H$ vs. $pH_o$ profile in the series 2 experiments (Fig. 2 *B*) represents the direct effect on the transporter of varying $pH_o$, the kinetic counterpart of which would be an effect on the affinity for $HCO_3^-$. As indicated in Table IV, decreasing $pH_o$ from 8.6 to 7.1 (at a fixed $[Na^+]_o$ of 425 mM) elicits only minor changes in $J_{max}(HCO_3^-)$, but causes $K_m(HCO_3^-)$ to increase from 1 to 26.7 mM. Based on the best-fit parameters in Table IV, we predict that decreasing $pH_o$ from 8.6 to 7.1 at a fixed $[HCO_3^-]_o$ of 12 mM should cause $J_H$ to fall from 21.8 to 8.2 pmol $cm^{-2}$ $s^{-1}$, similar to the results summarized in Fig. 2 *B* and Table II. These same kinetic parameters also account for the series 1 data, predicting that decreasing $pH_o$ from 8.6 to 7.1 by decreasing $[HCO_3^-]_o$ from 48 to 1.5 mM at a fixed $[CO_2]_o$ of 0.5% should cause $J_H$ to decrease from 23.1 to 1.4 pmol $cm^{-2}$ $s^{-1}$, similar to our results (Fig. 2 *A* and Table II).

In the series 3 experiments the reduction of $pH_o$ from 8.6 to 7.1 was accomplished by increasing $[HCO_3^-]_o$ by ~32-fold but decreasing $[CO_2]_o$ by ~1,000 fold. This combination of maneuvers had no effect on $J_H$. If $HCO_3^-$ were the true substrate of the transporter, then decreasing $pH_o$ from 8.6 to 7.1 must have had offsetting effects on $K_m(HCO_3^-)$ (i.e., a direct effect) and $[HCO_3^-]_o$ (i.e., a substrate effect). Indeed,

although this $pH_o$ decrease caused $K_m(HCO_3^-)$ to increase from 1 to 26.7 mM (see Table IV), there was a corresponding increase in $[HCO_3^-]_o$, from 3 to 96 mM (see Table II). Based on the best-fit parameters in Table IV, we predict that decreasing $pH_o$ from 8.6 to 7.1 while increasing $[HCO_3^-]_o$ from 3 to 96 mM should cause $J_H$ to change from 17.7 to 20.7 pmol $cm^{-2}$ $s^{-1}$, similar to our data (Fig. 2 *C* and Table II). Thus, the series 3 data do not rule out the $HCO_3^-$ model.

In the series 4 experiments, the reduction of $pH_o$ from 8.6 to 7.1 was accomplished by increasing both $[CO_2]_o$ and $[Na^+]_o$ by ~32-fold at fixed levels of $[NaCO_3^-]_o$ and $[HCO_3^-]_o$. These maneuvers had only minor effects on $J_H$. Because $[HCO_3^-]_o$ was fixed, $HCO_3^-$ could be the true substrate of the transporter only if our method of decreasing $pH_o$ from 8.6 to 7.1 did not alter $K_m(HCO_3^-)$. Although the kinetic parameters summarized in Table IV indicate that $K_m(HCO_3^-)$ approximately doubles for each 0.3 fall in $pH_o$, this analysis was based on data obtained at a fixed $[Na^+]_o$ of 425 mM. In our experiments, each 0.3 fall in $pH_o$ was accompanied by a doubling of $[Na^+]_o$. Earlier work (Boron, 1985) at the single $pH_o$ of 8.0 has established that $K_m(HCO_3^-)$ is approximately inversely proportional to $[Na^+]_o$. If this relationship between $K_m(HCO_3^-)$ and $[Na^+]_o$ holds at other $pH_o$ values between 7.1 and 8.6, then the tendency of a $pH_o$ decrease in series 4 to raise $K_m(HCO_3^-)$ would be offset exactly by the tendency of a $[Na^+]_o$ increase to lower $K_m(HCO_3^-)$. Thus, it is not unreasonable to hypothesize that the apparent $K_m(HCO_3^-)$ was stable in the series 4 experiments. If this were true, then the series 4 data, as were the data from series 1–series 3, would be consistent with the model that $HCO_3^-$ itself is the transported base.

*$CO_3^=$*. In testing the hypothesis that $CO_3^=$ is the transported base, we shall assume that neither $HCO_3^-$ nor $NaCO_3^-$ interact with the transporter. In the series 1 experiments, a $pH_o$ decrease is accompanied by a very large reduction in $[CO_3^=]_o$ (~1,000-fold when $pH_o$ falls from 8.6 to 7.1), and a steep fall in $J_H$. Although, in principle, low $pH_o$ could inhibit transporter directly, examination of Table V shows that the apparent $K_m$ and $J_{max}$ values for $CO_3^=$ at a fixed $[Na^+]_o$ of 425 mM are fairly insensitive to changes in $pH_o$ between 7.1 and 8.6. Thus, if $CO_3^=$ were the substrate, we would expect all of the inhibition in series 1 to reflect a decrease in substrate concentration. Based on the kinetic parameters in Table V, we predict that decreasing $pH_o$ from 8.6 to 7.1, accompanied by a decrease in $[CO_3^=]_o$ from 1,911 to 1.9 μM, should cause $J_H$ to fall from 23.1 to 1.4 pmol $cm^{-2}$ $s^{-1}$, similar to our data (Fig. 2 *A* and Table II).

In the series 2 experiments, decreasing $pH_o$ is accompanied by a smaller reduction in $[CO_3^=]_o$ (~32-fold when $pH_o$ falls from 8.6 to 7.1) and causes a decrease in $J_H$ that is less steep than in series 1. Because the kinetic parameters for $CO_3^=$ are relatively $pH_o$ insensitive, we expect this inhibition to reflect a decrease in $[CO_3^=]_o$ itself. Based on the parameters in Table V, we predict that decreasing $pH_o$ from 8.6 to 7.1, accompanied by a decrease in $[CO_3^=]_o$ from 478 to 14.8 μM, should cause $J_H$ to fall from 21.7 to 8.1 pmol $cm^{-2}$ $s^{-1}$, similar to our data (Fig. 2 *B* and Table II). Thus, the $J_H$ data from series 1 and series 2 are consistent with the hypothesis that $CO_3^=$ is the substrate and that the difference between Figs. 2 *A* and 2 *B* reflects the smaller changes in $[CO_3^=]_o$ in the latter case.

The series 3 experiments saw no change in either $[CO_3^=]_o$ or $J_H$ as $pH_o$ was reduced from 8.6 to 7.1. If $CO_3^=$ is indeed the transporter's substrate, these data indicate that

the transporter is virtually insensitive to $pH_o$ changes between 7.1 and 8.6, consistent with the kinetic parameters summarized in Table V, which indicate that $K_m(CO_3^=)$ and $J_{max}(CO_3^=)$ are relatively insensitive to $pH_o$ changes in this $pH_o$ range.

In the series 4 experiments, reducing $pH_o$ from 8.6 to 7.1 had no effect on $J_H$ even though $[CO_3^=]_o$ was reduced by a factor of $>32$. Although this seems inconsistent with the $CO_3^=$ model, $[CO_3^=]_o$ decrease was accompanied by a corresponding increase in $[Na^+]_o$ (Table I). As noted above, earlier work at $pH_o$ 8.0 showed that $J_H$ is unaffected by reciprocal alterations in $[Na^+]_o$ and $[HCO_3^-]_o$ (or $[CO_3^=]_o$) that leave the product $[Na^+]_o \times [HCO_3^-]_o$ (or $[Na^+]_o \times [CO_3^=]_o$) unchanged. If a similar relationship between $[Na^+]_o$ and $K_m(HCO_3^-)$ or $K_m(CO_3^=)$ holds for all $pH_o$ values between 7.1 and 8.6, then these series 4 data would be consistent with the model that $CO_3^=$ is the extracellular substrate. Thus, the series 1–series 4 data do not rule out the model that $CO_3^=$ is the transporter's substrate.

*$NaCO_3^-$.* In considering that $NaCO_3^-$ is the transporter's substrate, we shall assume that neither $HCO_3^-$, $CO_3^=$, nor $Na^+$ interact with the transporter. The analysis of the series 1 and series 2 experiments in terms of the $NaCO_3^-$ ion pair model is virtually the same as for the $CO_3^=$ model discussed above. In brief, the data summarized in Table III show that the apparent $K_m$ and $J_{max}$ values for $NaCO_3^-$ are fairly insensitive to changes in $pH_o$ between 7.1 and 8.6. The difference between the steepness of the $J_H$ vs. $pH_o$ relationships in Figs. 2 *A* and 2 *B* could be accounted for by the smaller changes in $[NaCO_3^-]_o$ in the latter case. Thus, these data are consistent with the ion pair model.

The series 3 and series 4 data are also consistent with the $NaCO_3^-$ ion pair model. In both cases, the reduction in $pH_o$ from 8.6 to 7.1 was achieved with no change in $[NaCO_3^-]_o$ and was accompanied by no change in $J_H$. In summary, if $NaCO_3^-$ is indeed the substrate, the data from series 1–series 4 would require that the transporter's kinetic parameters be virtually unaffected by changes in $pH_o$ between 7.1 and 8.6, as supported by the data of Table III.

### *Conclusions*

*Ad hoc assumptions required by the $HCO_3^-$ and $CO_3^=$ models.* Although the $NaCO_3^-$ ion pair model remains the most straightforward explanation for our data, we cannot rule out models with $HCO_3^-$ or $CO_3^=$ as the transported base. However, if either $HCO_3^-$ or $CO_3^=$ is the substrate, then our kinetic data (Tables III–VI) require that additional restrictions be placed upon allowable kinetic parameters. Moreover, at least one series of ad hoc assumptions must be imposed for either the $HCO_3^-$ or the $CO_3^=$ model to be viable.

In the case of the $HCO_3^-$ model, two ad hoc assumptions are required. First, the series 3 data can be explained only if the $\sim$32-fold increase in $[HCO_3^-]_o$ that accompanied the decrease in $pH_o$ from 8.6 to 7.1 is matched by comparable changes in $K_m(HCO_3^-)$. Also, the series 4 data can be explained only if $K_m(HCO_3^-)$ is inversely proportional to $[Na^+]_o$ over the $pH_o$ range 7.1–8.6. A question that arises is whether fulfilling these two ad hoc assumptions would be overly serendipitous. Both assumptions are predictions of the $NaCO_3^-$ ion pair model.

In the case of the $CO_3^=$ model, one ad hoc assumption is required to explain the

series 4 data: $K_m(CO_3^=)$ would have to be inversely proportional to $[Na^+]_o$ at all $pH_o$ values between 7.1 and 8.6. This is a prediction of the $NaCO_3^-$ ion pair model.

*Model-independent conclusions.* Although the preceding discussion is model dependent, our data lead to two important model-independent conclusions. First, because $J_{max}$ values are remarkably insensitive to $pH_o$ changes, inhibition of $Na^+$-dependent Cl-$HCO_3$ exchange by low $pH_o$ can be overcome by increasing the level of a substrate (i.e., $Na^+$, $HCO_3^-$, $CO_3^=$, or $NaCO_3^-$). Second, the exchanger behaves as if $J_H$ were governed uniquely by the product $[Na^+]_o \times [CO_3^=]_o$ (as predicted by the ion pair model). That is, the transporter behaves as if $[HCO_3^-]_o$ and $pH_o$ were inconsequential, and as if $[Na^+]_o$ and $[CO_3^=]_o$ were important only insofar as they determine the product $[Na^+]_o \times [CO_3^=]_o$.

*Unanswered kinetic questions.* As pointed out previously (Boron and Knakal, 1989), if kinetic data are consistent with the ion pair model, it will always be possible to fit the data with models calling for the random or ordered binding of $Na^+$ and $CO_3^=$ or $HCO_3^-$. The reason is that the binding of one substrate (e.g., $Na^+$) could sufficiently raise the transporter's affinity for the second substrate (e.g., $CO_3^=$), such that the binding of one would be tantamount to the binding of both substrates. Thus, it will be impossible to rule in the ion pair model to the exclusion of the other models. One approach for evaluating models of $Na^+$-dependent Cl-$HCO_3$ exchange would be to continue to gather data in the hope of making an observation inconsistent with the ion pair model. At least three major categories of experiments are suggested by the present work. The first involves testing the predictions made by the ion pair model about the dependence of $K_m(HCO_3^-)$ on $[Na^+]_o$ and the dependence of $K_m(Na^+)$ on $[HCO_3^-]_o$ at $pH_o$ values other than 8.0. These data on $K_m(HCO_3^-)$ and $K_m(Na^+)$ would also permit a thorough evaluation of the models involving the binding of $Na^+$ and $HCO_3^-$. Second, one could test the predictions of the ion pair model concerning the dependence of $K_m(Na^+)$ on $pH_o$ at a fixed $[CO_3^=]_o$, as well as the dependence of $K_m(Na^+)$ on $[CO_3^=]_o$ over a range of $pH_o$ values. Such data, not obtained in this study, would permit a full analysis of the model involving the binding of $Na^+$ and $CO_3^=$. Finally, one could vary $pH_o$ by means other than those used in series 1–series 4.

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