Intracellular pH-regulating Mechanism of the Squid Axon

Interaction between DNDS and Extracellular Na⁺ and HCO₃⁻

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ABSTRACT Intracellular pH (pHi) of the squid axon is regulated by a stilbene-sensitive transporter that couples the influx of Na⁺ and HCO₃⁻ (or the equivalent) to the efflux of Cl⁻. According to one model, the extracellular ion pair NaCO₃⁻ exchanges for intracellular Cl⁻. In the present study, the ion-pair model was tested by examining the interaction of the reversible stilbene derivative 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) with extracellular Na⁺ and HCO₃⁻. Axons (initial pHi ~ 7.4) were internally dialyzed with a pH 6.5 solution containing 400 mM Cl⁻ but no Na⁺. After pHi, as measured with a glass microelectrode, had fallen to ~6.6, dialysis was halted. In the presence of both external Na⁺ and HCO₃⁻ (pH₀ = 8.0, 22°C), pHi increased due to the pHi-regulating mechanism. At a fixed [Na⁺]₀ of 425 mM and [HCO₃⁻]₀ of 12 mM, DNDS reversibly reduced the equivalent acid-extrusion rate (JₚH) calculated from the rate of pHi recovery. The best-fit value for maximal inhibition was 104%, and for the [DNDS]₀ at half-maximal inhibition, 0.3 mM. At a [Na⁺]₀ of 425 mM, the [HCO₃⁻]₀ dependence of JₚH was examined at 0, 0.1, and 0.25 mM DNDS. Although JₚH max was always ~20 pmol cm⁻² s⁻¹, KₚH(HCO₃⁻) was 2.6, 5.7, and 12.7 mM, respectively. Thus, DNDS is competitive with HCO₃⁻. At a [HCO₃⁻]₀ of 12 mM, the [Na⁺]₀ dependence of JₚH was examined at 0 and 0.1 mM DNDS. Although JₚH max was ~20 pmol cm⁻² s⁻¹ in both cases, KₚH(Na⁺) was 71 and 179 mM, respectively. At a [HCO₃⁻]₀ of 48 mM, JₚH max was ~20 pmol cm⁻² s⁻¹ at [DNDS]₀ levels of 0, 0.1, and 0.25 mM. However, KₚH(Na⁺) was 22, 45, and 90 mM, respectively. Thus, DNDS (an anion) is also competitive with Na⁺. The results are consistent with simple competition between DNDS and NaCO₃⁻, and place severe restrictions on other kinetic models.

INTRODUCTION

The intracellular pH (pHi) of the squid giant axon, like that of the snail neuron (Thomas, 1977) and giant barnacle muscle fiber (Boron et al., 1981), is regulated by...
a Na⁺-dependent Cl-HCO₃ exchanger. In response to intracellular acid loads, this transporter mediates the uptake of Na⁺ and HCO₃⁻ (or an equivalent species), and the efflux of Cl⁻ (for reviews, see Roos and Boron, 1981; Thomas, 1984). The stoichiometry is one mole of Na⁺ taken up for each mole of Cl⁻ lost, and for every two equivalents of acid neutralized inside the cell (Boron and Russell, 1983). However, the mechanism by which Na⁺ and the acid-base equivalents are transported across the membrane remains to be established. Among the possibilities (see Boron, 1985) are that (a) one Na⁺ and one HCO₃⁻ enter, and one H⁺ exits; (b) one Na⁺ and two HCO₃⁻ enter; (c) one Na⁺ and one CO₃²⁻ enter; and (d) one NaCO₃⁻ ion pair enters.

The likelihood of the above alternatives was examined in an earlier kinetic study of the acid-extrusion rate (i.e., equivalent H⁺ efflux, J_H) in the squid axon (Boron, 1985). It was found that when [HCO₃⁻]₀ is varied, the apparent maximal J_H (J_max) is independent of [Na⁺]₀, but that the apparent K_m for HCO₃⁻ (K_m[HCO₃⁻]) is inversely proportional to [Na⁺]₀. Conversely, J_max(Na) is independent of [HCO₃⁻]₀, but K_m(Na) is inversely proportional to [HCO₃⁻]₀. Furthermore, when the J_H data were plotted as a function of the calculated [NaCO₃⁻]₀, all the data fell along the same curve, regardless of whether the [HCO₃⁻]₀ was varied at various fixed values of [Na⁺]₀, or vice versa. These results were consistent with the NaCO₃⁻ ion-pair model, and placed restrictions on other kinetic models.

In the present work, we further test the ion-pair model by exploring the effect of 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) on the axon's pH-regulating system. Acid-extrusion rates were calculated from rates of pHᵢ recovery from acid loads in internally dialyzed squid axons. We found that DNDS, which is a divalent anion, is a reversible inhibitor of acid extrusion. In experiments in which we examined J_H as a function of [HCO₃⁻]₀ at different values of [DNDS]₀, we found that this stilbene appears to be a simple competitive inhibitor with respect to HCO₃⁻. Similarly, in experiments in which we examined J_H as a function of [Na⁺]₀, we found that DNDS appears to be competitive with respect to Na⁺. These results are consistent with the NaCO₃⁻ ion-pair model, and place severe restrictions on other kinetic models.

Portions of this work have been reported in preliminary form (Boron and Knakal, 1985).

METHODS

General

The experiments were conducted at the Marine Biological Laboratory, Woods Hole, MA, during May and June, 1984. Live specimens of the squid Loligo pealei were decapitated, and the first stellar nerve from each side was removed and placed in cold (~4°C) Woods Hole seawater. A 4-5-cm length of giant axon (generally 400-600-μm diameter) was isolated from the nerve by microdissection, and cannulated horizontally in a chamber (see Boron and Russell, 1983) designed for internal dialysis (Brinley and Mullins, 1967). A length of dialysis tubing (see below) was threaded through one cannula, down the length of the axon, and out the opposite cannula. In addition, pH- and voltage-sensitive microelectrodes were introduced through opposite cannulas, so that their tips were within 500 μm of one another at the middle of the axon. Artificial seawater continuously flowed around the axon. The temperature, controlled by a circulating water bath connected to the water jacket on the underside of the chamber, was 22°C.
Solutions

The standard extracellular fluid (i.e., artificial seawater, ASW) had the following nominal composition (in millimolar): 425.2 Na\(^+\), 12 K\(^+\), 3 Ca\(^{2+}\), 57.5 Mg\(^{2+}\), 531 Cl\(^-\), 12 HCO\(_3^-\), 0.1 EDTA\(^-\), 15 of the anionic form of [2-hydroxyethyl]-l-piperazine-propane sulfonic acid (EPPS), and 15 of the neutral form of EPPS (pK \~ 8.0). The pH was 8.00 and the osmolality was ~980 mosmol/kg. ASW was delivered to the chamber through CO\(_2\)-impermeable Saran tubing (Clarkson Equipment and Controls, Detroit, MI). When [Na\(^+\)]\(_o\) was lowered, Na\(^+\) was replaced mole for mole by N-methyl-D-glucammonium, made by titrating the free base (Sigma Chemical Co., St. Louis, MO) with HCl. When [HCO\(_3^-\)]\(_o\) was varied, HCO\(_3^-\) was exchanged for Cl\(^-\) on a mole-for-mole basis.

The HCO\(_3^-\)-containing seawaters were made as described previously (Boron, 1985). Throughout this paper, the [HCO\(_3^-\)]\(_o\) values reported are nominal values. However, because some of the added HCO\(_3^-\) went on to form CO\(_2\), CO\(_3^-\), and various ion pairs (i.e., NaCO\(_3\), MgCO\(_3\), CaCO\(_3\)), the actual [HCO\(_3^-\)]\(_o\) values were probably ~10% less than the nominal values. See Appendix 1 for a derivation of the equations describing the fate of HCO\(_3^-\) added to aqueous solutions.

In the initial and final phases of each experiment, the axons were exposed to HCO\(_3^-\)-free ASW. This was similar to the standard ASW except that it contained no HCO\(_3^-\) (Cl\(^-\) replacing HCO\(_3^-\)), only 10 mM K\(^+\), and only 10 mM total EPPS (MgCl\(_2\) replacing MgEPPS on an osmole-for-osmole basis).

The internal dialysis fluid (DF) had the following composition (in millimolar): 0 Na\(^+\), 415.3 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. The pH was adjusted to 6.5 with HCl or KOH, and osmolality was adjusted to ~980 mosmol/kg with glycine. The ATP was added to the DF on the day of the experiment from a 400-mM stock solution (pH 7.0) that was stored at ~5°C.

Internal Dialysis

Internal dialysis (Brinley and Mullins, 1967) permits control of the intracellular ionic environment. Details on our use of this technique can be found in earlier papers (Boron and Russell, 1983; Boron, 1985). The dialysis capillaries were made from cellulose acetate tubing (Fisher Research Laboratories, Inc., Dedham, MA) having an outer diameter of 140 µm. An 18-mm length of this tubing was rendered permeable to low molecular weight solutes by hydrolyzing it in 0.1 N NaOH for 18–24 h. The dialysis capillaries were perfused with DF at a rate of ~5 µl/min.

Measurement of pH

The pH-sensitive microelectrodes were of the design of Hinke (1967). They were constructed of lead glass (0120; Corning Glass Works, Corning, NY) and pH-sensitive glass (Clark Electro-medical Instruments, Pangbourne, England). Details on the construction of these microelectrodes, the electrical arrangements, and the control of the experiments by computer can be found in earlier papers (Boron and Russell, 1983; Boron, 1985).

Calculation of Acid Extrusion Rates

We define the acid extrusion rate (J\(_H\)) as the net flux of H\(^+\) out of the cell plus the flux of HCO\(_3^-\) (or other alkali equivalents) into the cell. The experimental protocol was to lower pH\(_i\) by dialyzing the axon with a low-pH solution, and then halt dialysis. The subsequent pH\(_i\) recovery rate (dpH\(_i\)/dt) was used to compute J\(_H\), which was taken as the product of dpH\(_i\)/dt,
the total intracellular buffering power (as modified by the presence of CO$_2$/HCO$_3^-$), and the
axon diameter (see Boron, 1985). For each axon, $J_{H}$ was determined for up to four external
solutions, one of which was always our standard ASW, which had the following composition:

$[Na^+]_o = 425$ mM, $[HCO_3^-]_o = 12$ mM, $pH_o = 8.00$, and $[DNDS]_o = 0$. Each of the calculated
$J_{H}$ values was divided by the $J_{H}$ value obtained under these standard conditions to yield nor-
malized $J_{H}$ values, as described previously (Boron, 1985). This normalization procedure
reduces the scatter of the data, but sometimes necessitates special curve-fitting routines, as
described below.

Curve-fitting Procedures
We used various nonlinear least-squares methods to curve fit our $J_{H}$ data. Data pertaining to
the reduction of $J_{H}$ by extracellular DNDS (i.e., percent inhibition vs. $[DNDS]_o$) was fitted
with a standard Michaelis-Menten curve using an iterative procedure (Scarborough, 1966).
The other original $J_{H}$ data reported in this paper can be grouped into eight series: (1) $J_{H}$ vs.
$[HCO_3^-]_o$ at 425 mM Na$^+$/0 mM $[DNDS]_o$, (2) the same at 0.1 mM DNDS, (3) the same at
0.25 mM DNDS, (4) $J_{H}$ vs. $[Na^+]_o$ at 12 mM $[HCO_3^-]_o$/0 mM DNDS, (5) the same at 12
$HCO_3^-$/0.1 DNDS, (6) the same at 48 $HCO_3^-$/0 DNDS, (7) the same at 48 $HCO_3^-$/0.1 DNDS,
and (8) the same at 48 $HCO_3^-$/0.25 DNDS. Data from each of these eight series of experi-
ments were individually fitted to a standard Michaelis-Menten equation (see Segel, 1975), or
to a modified form in which the curve was forced through an arbitrary point (Boron, 1985).
The curve-fitting approach noted above (Scarborough, 1966).

The eight fitting procedures described above yielded best-fit values for the apparent maxi-
mal $J_{H}$ ($J_{max}$) and apparent $K_m$ values for either Na$^+$ or HCO$_3^-$, but only at a single $[DNDS]_o$. Because apparent $J_{max}$ and $K_m$ ought to be independent of $[DNDS]_o$, one could simultaneously fit data from experiments at two or three values of $[DNDS]_o$ to an equation of the form:

$$J_{H} = J_{max} \left[ \frac{[S]_o}{[S]_o + K_m^S (1 + [DNDS]_o/K_{DNDS})} \right]_m,$$

where $[S]_o$ is the concentration of the varied external substrate (i.e., either Na$^+$ or HCO$_3^-$),
$J_{max}$ is the apparent maximal flux when $[S]_o$ is varied, and $K_m^S$ is the apparent Michaelis con-
stant for $S$. The advantage of this approach is that single best-fit values are obtained for $J_{max}$,
$K_m^S$ and $K_{DNDS}$. Because the $J_{H}$ data for each experiment were normalized to the mean $J_{H}$ (i.e.,
16.8 ± 0.5 pmol cm$^{-2}$ s$^{-1}$, n = 169) obtained under “standard” conditions (i.e.,
$[HCO_3^-]_o = 12$ mM, $[Na^+]_o = 425$ mM, and $[DNDS]_o = 0$) in this study, the fitted curve must
be forced through this standard point ($[HCO_3^-]_o = 12$ mM, $[Na^+]_o = 425$ mM, and
$[DNDS]_o = 0, J_{H} = 16.8$ pmol cm$^{-2}$ s$^{-1}$). This was accomplished by fitting the data to the fol-
lowing variant of Eq. 1:

$$J_{H} = \frac{[S]_o}{[S]_o + K_m^S (1 + [DNDS]_o/K_{DNDS})} \cdot \frac{J_{H}(S' + K_{DNDS})}{S'},$$

where $J_{H}$ is the value of $J_{H}$ that prevails under standard conditions (i.e., 16.8 pmol cm$^{-2}$ s$^{-1}$),
and $S'$ is $[S]_o$ under standard conditions (i.e., 425 mM when $S$ is Na$^+$, and 12 mM when $S$ is
$HCO_3^-$). In deriving Eq. 2, we assumed that $[DNDS]_o = 0$ under standard conditions. When
data are fitted to Eq. 2, the result is best-fit values for the apparent $K_m^S$ and $K_{DNDS}$. $J_{max}$ is calcu-
lated from the relation:

$$J_{max} = \frac{J_{H}(S' + K_{DNDS})}{S'}.$$
Simultaneous fittings of data were performed for three groups of results: (a) experiments in which [HCO₃⁻]o was varied at 425 mM [Na⁺]o at three [DNDS]o levels (series 1–3); (b) experiments in which [Na⁺]o was varied at 12 mM [HCO₃⁻]o at two [DNDS]o levels (series 4 and 5); and (c) experiments in which [Na⁺]o was varied at 48 mM [HCO₃⁻]o at three [DNDS]o levels (series 6–8).

Finally, data from all eight series of experiments (348 points at various values of [HCO₃⁻]o, [Na⁺]o, and [DNDS]o were simultaneously fitted to each of four rapid-equilibrium kinetic models (see Discussion). We found that, with the approach described by Scarborough (1966), the fits did not converge for the more complex of these models. Therefore, we employed an iterative approach in which we systematically varied each of the fitted parameters, minimizing the sum of squares of residuals. Although this least-squares fitting approach is time consuming and does not yield the standard deviations of the fitted parameters, it always converges.

Previously Reported Data

Two series of data used in the present analysis were previously reported (Boron, 1985). These are the data for series 1 (Jn vs. [HCO₃⁻]o at 425 mM Na⁺/0 mM [DNDS]o) and the data for series 4 (JH vs. [Na⁺]o at 12 mM [HCO₃⁻]o/0 mM DNDS). Because the data from each axon were normalized to the same standard condition, both in the previous study and in the present one, the fact that the data from series 1 and 4 were obtained in a different year from those of the other series should not affect our analysis. In addition, our analysis of rapid-equilibrium kinetic models (see Discussion) includes four other series of data, all obtained in the absence of DNDS, from the earlier study (Boron, 1985): (A) Jn vs. [HCO₃⁻]o at 212 mM Na⁺, (B) JH vs. [HCO₃⁻]o at 106 mM Na⁺, (C) Jn vs. [Na⁺]o at 6 mM HCO₃⁻, and (D) Jn vs. [Na⁺]o at 3 mM HCO₃⁻.

Statistics

The apparent $K_m$ and $J_{\text{max}}$ values, derived from iterative least-squares curve fits (see above), are given ± the standard deviation. Mean values of acid extrusion rates and pHᵢ are given ± the standard error.

RESULTS

Effect of DNDS on pHᵢ Recovery from an Acid Load

Fig. 1 illustrates the results of an experiment in which the internal dialysis technique was used to impose an intracellular acid load on a squid axon. Throughout the experiment, pHᵢ was 8.00 and [Na⁺]o was 425 mM. Initially, the ASW was HCO₃⁻ free. At the time indicated by point a, dialysis is begun with a dialysis fluid (DF) buffered to pH 6.5 that contained no Na⁺ but 400 mM Cl⁻. The absence of intracellular Na⁺ should prevent reversal of the pHᵢ-regulating mechanism. The presence of 400 mM intracellular Cl⁻ should nearly saturate the transporter, which has an apparent $K_m$ for Cl⁻ of ~84 mM (Boron and Russell, 1983). Finally, the low pH of the DF causes pHᵢ to progressively fall (segment ab), thereby stimulating the pHᵢ-regulating mechanism. When dialysis is halted at point b, pHᵢ drifts upwards very slowly and then stabilizes (bc). Switching the ASW to one containing 12 mM HCO₃⁻/0.5% CO₂ produces an immediate fall in pHᵢ (cd) due to the influx of the CO₂ and the resultant production of H⁺ and HCO₃⁻. This is followed by a sustained pHᵢ recovery (de) that reflects the activity of the axon’s pHᵢ-regulating system. The rate of pHᵢ recovery corresponds to a $J_{\text{H}}$ of 10.8 pmol cm⁻² s⁻¹, after correction (see...
Boron, 1985) for the slight pH drifts during the initial HCO₃⁻-free baseline period (bc) and a final baseline period in SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulfate) (gh).

The addition of 1 mM DNDS to the ASW (ef) reduces the rate of pHᵢ recovery (predicted \( j_H = 1.7 \text{ pmol cm}^{-2} \text{s}^{-1} \)). This inhibitory effect of DNDS is fully reversible, as evidenced by the increase in the pHᵢ recovery rate when the DNDS is removed (fg). The rate of alkalinization during this period corresponds to a \( j_H \) of 12.8 pmol cm\(^{-2} \) s\(^{-1} \), very similar to the value during de, before the addition of DNDS. The observation that \( j_H \) in squid axons is relatively insensitive to pHᵢ changes in the range 6.6–6.9 (e.g., compare de and fg) has been made previously (Boron, 1985), and is consistent with the observation that \( j_H \) increases only about twofold as pHᵢ is increased from ~6.7 to ~7.3. If the \( j_H \) values in the initial (de) and final (fg) control periods are averaged, the inhibition by DNDS in this experiment amounts to ~86%. Finally, application of 0.5 mM SITS, known to block the axon's pHᵢ-regulating system, blocks further recovery of pHᵢ (gh). The promptness of the blockade by
SITS indicates that delays caused by mixing of the ASWs in the chamber are minimal.

In a group of similar experiments, the inhibitory effect of DNDS on acid extrusion was determined for drug concentrations between 0.1 and 2 mM under the "standard" conditions of our experiments (i.e., \([Na^+]_o = 425\) mM, \([HCO_3^-]_o = 12\) mM, \(pH_o = 8.00\)). A total of 40 data points were obtained, with up to three values of \([DNDS]_o\) tested in each axon. In most experiments, the blockade of acid extrusion during the final baseline period (corresponding to \(g_h\) in Fig. 1) was achieved by nominal removal of \(HCO_3^-/CO_2\). The data from this study are summarized in Fig. 2. The results of a nonlinear least-squares curve fit (see Methods) indicate that half-maximal inhibition was achieved at \(0.31 \pm 0.06\) mM DNDS, with an apparent maximal inhibition of \(104 \pm 7\%\). Because the maximal inhibition by the drug was indistinguishable from 100\%, these data rule out substantial contribution from rapid-equilibrium kinetic schemes (e.g., partial competitive inhibition, partial non-

![Graph showing inhibition of acid extrusion by DNDS](image)

**Figure 2.** Inhibition of acid extrusion by DNDS. All data were obtained at \([Na^+]_o = 425\) mM, nominal \([HCO_3^-]_o = 12\) mM, and \(pH_o = 8.00\).

Effect of DNDS on the \(HCO_3^-\) Dependence of Acid Extrusion

To determine the nature of the inhibition of acid extrusion by DNDS, we studied the effect of the drug on the external-\(HCO_3^-\) dependence of acid extrusion. The experiments were similar in design to that of Fig. 1, and were always carried out at the \(pH_o\) of 8.00 and a \([Na^+]_o\) of 425 mM. A nonlinear least-squares curve fit of the data obtained in the absence of DNDS yielded a \(J_{max}\) of 20.5 pmol cm\(^{-2}\) s\(^{-1}\), and a \(K_m(HCO_3^-)\) of 2.6 mM, as previously reported (Boron, 1985). Similar individual curve fits (see Methods) were also performed on data obtained at \([DNDS]_o\) levels of 0.1 and 0.25 mM. As is evident from Fig. 3 and Table I, \(J_{max}\) was not significantly
FIGURE 3. Effect of DNDS on the external HCO₃⁻ dependence of acid extrusion. All data were obtained at [Na⁺]ₒ = 425 mM and pH₀ = 8.00. [DNDS]ₐ was either 0, 0.1, or 0.25 mM. The inset is a replot of the data in double reciprocal form.

affected by raising [DNDS]₀, whereas \( K_m(HCO_3^-) \) increased substantially, rising to 5.7 and 12.7 mM at [DNDS]₀ levels of 0.1 and 0.25 mM, respectively. Thus, DNDS behaves as a competitive inhibitor with respect to extracellular HCO₃⁻.

The apparent inhibitory constant for DNDS (\( K_i \)) could be determined by simultaneously fitting the 106 normalized data points of Fig. 3 (also summarized in Table I) to an equation of the form of Eq. 2 (see Methods), which forces the fitted curve through the point describing our “standard” conditions (i.e., [HCO₃⁻]₀ = 12 mM, [Na⁺]₀ = 425 mM, [DNDS]₀ = 0 mM, and \( J_u = 16.8 \) pmol cm⁻² s⁻¹). The fitting procedure independently determines only \( K_m(HCO_3^-) \) and \( K_i(DNDS) \), with \( J_{max} \) computed from the best-fit values of the apparent \( K_m \) and \( K_i \) using Eq. 3 (see Methods).

| [Na⁺] | [DNDS]₀ | n | Fit* | Apparent \( K_m(HCO_3^-) \) | Apparent \( J_{max} \) |
|-------|--------|---|------|------------------|-----------------|
| mM    | mM     |   |      | mM               | pmol cm⁻² s⁻¹  |
| 425   | 0      | 31| 2    | 2.6 ± 0.3        | 20.5 ± 2.2      |
| 425   | 0.1    | 40| 1    | 5.7 ± 1.3        | 19.5 ± 1.3      |
| 425   | 0.25   | 35| 1    | 12.7 ± 4.3       | 19.3 ± 2.5      |

*Nonlinear least-squares curve fits, as described in methods, were individually performed on each of the three sets of data points. Type 1 fit: standard Michaelis-Menten equation. Type 2 fit: forced through a standard point ([Na⁺]₀ = 425 mM, [HCO₃⁻]₀ = 12 mM, pH₀ = 8.00, and [DNDS]₀ = 0 mM). n is the number of points fitted. The data at [DNDS]₀ = 0 mM were previously reported (Boron, 1985).
TABLE II

Kinetic Parameters Obtained from Simultaneous Fits of Data*

| Substrate varied | [HCO₃]₀ (mM) | [Na⁺]₀ (mM) | n | Apparent Kₘ(HCO₃) (µM) | Apparent Kᵢ(DNDS) (µM) | Apparent Jₘ (pmol cm⁻² s⁻¹) |
|------------------|--------------|-------------|---|------------------------|-------------------------|-----------------------------|
| HCO₃             | —            | 425         | 106 | 2.4 ± 0.4              | —                       | 55 ± 9                      |
| Na⁺              | 12           | —           | 59  | 70.1 ± 10.5            | 53 ± 13                 | 19.5                        |
| Na⁺              | 48           | —           | 118 | 19.5 ± 4.0             | 64 ± 16                 | 19.9                        |

*The fits in line 1 were performed on the data of Table I, those in line 2, on the data of Table III (top), and those in line 3, on the data of Table III (bottom).

The results of this fit, summarized in Table II, are a Kₘ(HCO₃) of 2.4 ± 0.4 mM, and a Kᵢ(DNDS) of 55 ± 9 µM. The Jₘ computed from these values is 20.1 pmol cm⁻² s⁻¹.

Effect of DNDS on the Na⁺ Dependence of Acid Extrusion

The effect of DNDS on the extracellular Na⁺ dependence of acid extrusion was determined at two levels of [HCO₃]₀, 12 and 48 mM, always at a pHe₀ of 8.00. Data obtained at a [HCO₃]₀ of 12 mM are summarized in Fig. 4 and in the top portion of Table III. The results of an individual curve fit indicate that in the absence of DNDS, Jₘ is 19.7 pmol cm⁻² s⁻¹ and Kₘ(Na⁺) is 71 mM, as reported in a previous study (Boron, 1985). Raising [DNDS]₀ to 0.1 mM has no substantial effect on Jₘ.

\[ \text{ACID EXTRUSION RATE} (\text{pmol cm}^{-2} \text{s}^{-1}) \]

**Figure 4.** Effect of DNDS on the external Na⁺ dependence of acid extrusion. All data were obtained at nominal [HCO₃]₀ = 12 mM and pHe₀ = 8.00. [DNDS]₀ was either 0 or 0.1 mM. The inset is a replot of the data in double reciprocal form.
but increases $K_m(Na^+)$ to 179 mM. This is consistent with simple competitive inhibition of DNDS with respect to $Na^+$. It would have been desirable to extend this study to a $[DNDS]_o$ of 0.25 mM. However, at a $[HCO_3^-]_o$ of only 12 mM, the likely $K_m(Na^+)$ in 0.25 mM DNDS would have approached the maximal $[Na^+]_o$ we could have achieved under isotonic conditions (i.e., 425 mM). Because it would have been difficult to obtain $J_m$ and $K_m(Na^+)$ values from curve fits of data obtained under these conditions, we decided to forgo further experiments at a $[HCO_3^-]_o$ of 12 mM.

The two series of data obtained at a $[HCO_3^-]_o$ of 12 mM (i.e., $[DNDS]_o = 0$ or 0.1 mM) can be used to obtain an apparent inhibitory constant for DNDS. Our approach was to simultaneously fit all 59 data points obtained with a $[HCO_3^-]_o$ of 12 mM (see Fig. 4 and the top of Table III) to an equation of the form of Eq. 2 (see Methods), which forces the fitted curve through the “standard” point ($[HCO_3^-]_o = 12$ mM, $[Na^+]_o = 425$ mM, $[DNDS]_o = 0$, and $J_m = 16.8$ pmol cm$^{-2}$ s$^{-1}$). The results of this calculation, summarized in line 2 of Table II, are a $K_m(Na^+)$ of 70 ± 10 mM, and a $K(DNDS)$ of 53 ± 13 μM. The $J_m$ computed from these values is 19.5 pmol cm$^{-2}$ s$^{-1}$.

Because it was not feasible to examine the effect of 0.25 mM DNDS on the $[Na^+]_o$ dependence of $J_m$ when $[HCO_3^-]_o$ was only 12 mM, (see above) we performed additional experiments at a $[HCO_3^-]_o$ of 48 mM. Inasmuch as increasing $[HCO_3^-]_o$ in the range of 3–12 mM causes a reciprocal change in the apparent $K_m$ for $Na^+$ (Boron, 1985), we anticipated that increasing $[HCO_3^-]_o$ to 48 mM would cause $K_m(Na^+)$ to fall. The ion-pair model predicts that $K_m(Na^+)$ should decline to ~20 mM in the absence of DNDS. This would have the advantage of allowing us to examine the effect of relatively high doses of DNDS (i.e., 0.25 mM), while $K_m(Na^+)$ is kept substantially below the maximal obtainable $[Na^+]_o$. The results of this series of experiments are summarized in Fig. 5 and the lower portion of Table III. In the absence of DNDS, $J_m$ was 20.8 pmol cm$^{-2}$ s$^{-1}$, and the $K_m(Na^+)$ was 22 mM. Comparing these values to those obtained in the absence of DNDS in 12 mM HCO$_3^-$, we see that raising $[HCO_3^-]_o$ to 48 mM has a minimal effect on $J_m$, but causes a substantial reduction in $K_m(Na^+)$. Thus, this result agrees with the prediction of the ion-pair

### Table III

| $[HCO_3^-]_o$ | $[DNDS]_o$ | $n$ | Fit* | $K_m(Na^+)$ | $J_m$ |
|---------------|-------------|-----|------|-------------|------|
| mM            | mM          |     |      | mM          | pmol cm$^{-2}$ s$^{-1}$ |
| 12            | 0           | 35  | 2    | 71 ± 12     | 19.7 |
| 12            | 0.1         | 24  | 1    | 179 ± 54    | 18.5 ± 2.4 |
| 48            | 0           | 42  | 1    | 22 ± 6      | 20.8 ± 1.8 |
| 48            | 0.1         | 43  | 1    | 45 ± 12     | 20.3 ± 1.8 |
| 48            | 0.25        | 33  | 1    | 90 ± 31     | 18.6 ± 2.4 |

*Nonlinear least-squares curve fits, as described in Methods, were individually performed on each of the three sets of data points. Type 1 fit: standard Michaelis-Menten equation. Type 2 fit: forced through a standard point ($[HCO_3^-]_o = 12$ mM, $[Na^+]_o = 425$ mM, $[DNDS]_o = 0$, and $J_m = 16.8$ pmol cm$^{-2}$ s$^{-1}$). $n$ is the number of points fitted. The data at $[HCO_3^-]_o = 12$ mM/$[DNDS]_o = 0$ mM were previously reported (Boron, 1985).
model. With \([\text{HCO}_3^-]_o\) at 48 mM, raising \([\text{DNDS}]_o\) to 0.1 and 0.25 mM has a minimal effect on \(J_m\), but causes \(K_m(\text{Na}^+)\) to increase to 45 and 90 mM, respectively.

The 118 data points obtained at a \([\text{HCO}_3^-]_o\) of 48 mM can be simultaneously fitted to an equation of the form of Eq. 2, as described above. The results of such a calculation, summarized on line 3 of Table II, are a \(K_m(\text{Na}^+)\) of 19.5 ± 4.0 mM, and apparent \(K(d)(\text{DNDS})\) of 64 ± 16 \(\mu\)M.

The \(\text{Na}^+\) dependence of \(J_{\text{n}}\), both 12 mM and 48 mM \(\text{HCO}_3^-\), indicates that DNDS has almost no effect on the apparent \(J_{\text{n}}\), but greatly increases the apparent \(K_m(\text{Na}^+)\) for \(\text{Na}^+\). These observations are consistent with simple competitive inhibition between DNDS and \(\text{Na}^+\). It should be noted that the \(K(d)(\text{DNDS})\) and \(J_{\text{n}}\) values generated by

![Figure 5](https://g8p.rupress.org/jgp/article-pdf/133/5/133/16227165/133-05-133.pdf)

FIGURE 5. Effect of DNDS on the external \(\text{Na}^+\) dependence of acid extrusion. All data were obtained at nominal \([\text{HCO}_3^-]_o\) = 48 mM and \(\text{pH}_o\) = 8.00. \([\text{DNDS}]_o\) was either 0, 0.1, or 0.25 mM. The inset is a replot of the data in double reciprocal form.

the three simultaneous curve fits were very similar (see Table II); whether (a) \([\text{HCO}_3^-]_o\) was varied at a \([\text{Na}^+]_o\) of 425 mM, or (b) \([\text{Na}^+]_o\) was varied at a \([\text{HCO}_3^-]_o\) of 12 mM, or whether (c) \([\text{Na}^+]_o\) was varied at a \([\text{HCO}_3^-]_o\) of 48 mM, the apparent \(J_{\text{n}}\) differed only slightly from 20 pmol cm\(^{-2}\) s\(^{-1}\), and the apparent \(K(d)(\text{DNDS})\) ranged from 53 to 64 \(\mu\)M.

**DISCUSSION**

**Inhibition of Acid Extrusion by DNDS**

Although disulfonic stilbene derivatives such as SITS and DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate) are known to inhibit several anion transport systems,
their effects were studied first on the erythrocyte band 3 protein, which mediates Cl-HCO₃ exchange (Cabantchik et al., 1978). The interaction of SITS and DIDS with the erythrocyte Cl-HCO₃ exchanger occurs in two steps (Cabantchik and Rothstein, 1972). The first is a rapid and reversible binding, and the second is a slower covalent reaction between the isothiocyanate group on the stilbene and an amino group on the transporter. Inasmuch as DNDS lacks the isothiocyanate group, it can only participate in the first step, which is probably an ionic interaction between the negatively charged sulfonate groups on the DNDS and positively charged regions of the Cl-HCO₃ exchanger. DNDS competitively inhibits the sulfate equilibrium exchange mediated by the Cl-HCO₃ exchanger, with an inhibitory constant of ~0.45 mM, and a Hill coefficient of unity (Barzilay and Cabantchik, 1979). Fröhlich (1982) has shown that the DNDS binding capacity of erythrocytes is about one million/cell, approximately the same as for disulfonic stilbenes possessing isothiocyanate groups. This binding of DNDS, which occurs with a binding constant of ~84 nM, is competitively inhibited by external Cl⁻ with a Cl⁻ inhibitory constant of ~6 mM. Furthermore, DNDS competitively inhibits external Cl⁻-stimulated Cl⁻ efflux with an inhibitory constant of 90 nM. Thus, the data point to an interaction between DNDS and a single class of sites on the erythrocyte, and simple competitive inhibition by DNDS of anion binding.

It is known that, qualitatively at least, the disulfonic stilbene derivatives and certain other compounds containing isothiocyanate groups behave toward the Na⁺-dependent Cl-HCO₃ exchanger in much the same way as they do toward the erythrocyte Cl-HCO₃ exchanger. Thus, DIDS and SITS block the acid extrusion (Russell and Boron, 1976; Thomas, 1976), Cl⁻ fluxes (Russell and Boron, 1976), intracellular Na⁺ activity changes (Thomas, 1977), and Na⁺ fluxes (Boron and Russell, 1983) mediated by the Na⁺-dependent Cl-HCO₃ exchangers of squid axons, snail neurons, and barnacle muscle. Moreover, DNDS reversibly inhibits acid extrusion mediated by the Na⁺-dependent Cl-HCO₃ exchanger of barnacle muscle (Boron, 1977) and the squid axon (Boron and Russell, 1983).

In light of the similar effects of the disulfonic stilbenes on erythrocyte Cl-HCO₃ exchangers and invertebrate pHₐ-regulating mechanisms, we might anticipate that the inhibition by DNDS of acid extrusion in the squid axon might be competitive with respect to anions and have a Hill coefficient of unity. These expectations have been confirmed by the present study. We found that at pHₐ 8.00 and in the presence of 12 mM HCO₃⁻ and 425 mM Na⁺, DNDS reversibly and progressively inhibited acid extrusion. The apparent Kᵢ(DNDS) under these conditions was ~0.3 mM, the best-fit maximal inhibition was 104%, and the best-fit Hill coefficient for multiple inhibitors was 1.08. Furthermore, in studies in which we examined the effect of DNDS on the [HCO₃⁻]₀ dependence of acid extrusion, we found that DNDS behaved as a simple competitor, with an inhibitory constant of ~55 μM. The three-order-of-magnitude discrepancy between the Kᵢ(DNDS) values in our squid-axon study and Fröhlich's erythrocyte study could simply reflect fundamental differences between Na⁺-dependent and Na⁺-independent Cl-HCO₃ exchangers. It should be pointed out, however, that Fröhlich (1982) found that the apparent Kᵢ(DNDS) is linearly related to [Cl⁻]₀, at least in the range 0–12 mM. Although the Kᵢ(DNDS) for the erythrocyte Cl-HCO₃ exchanger was 84 nM at [Cl⁻]₀ = 0, it rose to ~240 nM at [Cl⁻]₀ = 12 mM. Inasmuch as our experiments were conducted at a [Cl⁻]₀ of
~535 mM, it is possible that a large portion of the apparent discrepancy reflects the high levels of a potentially competing anion (i.e., Cl\(^-\)) in our seawaters.

Although the evidence we obtained for apparent competition between DNDS and HCO\(_3\) is not surprising, that for competition between DNDS and Na\(^+\) may not have been anticipated. In the classical model of competitive inhibition, the substrate and inhibitor bind to the same site, and are structurally similar. Although it is easy to interpret the competition between DNDS (a divalent anion) and HCO\(_3\) in terms of such a model, the apparent competition between DNDS and Na\(^+\) cannot be accounted for. Thus, if DNDS does behave as a classic competitive inhibitor, the most straightforward explanation for the competition between DNDS and Na\(^+\) is the NaCO\(_3\) ion-pair model (see below).

However, DNDS need not be structurally similar to Na\(^+\) in order to appear to be a competitive inhibitor. Other possibilities (see Segel, 1975) are that (a) the binding of DNDS and Na\(^+\) are mutually exclusive for steric reasons, (b) the binding sites for DNDS and Na\(^+\) are distinct but overlapping, and (c) the binding of DNDS induces a conformational change in the transporter that prevents binding of Na\(^+\).

### Analysis of Kinetic Models

Inasmuch as our observation that DNDS is apparently competitive with respect to both HCO\(_3\) and Na\(^+\) does not allow us to rule out, a priori, models in which Na\(^+\) and HCO\(_3\) (or CO\(_2\)) separately bind to the transporter, we examined the predictions of four rapid-equilibrium models: (a) binding of the NaCO\(_3\) ion pair, (b) random binding of Na\(^+\) and HCO\(_3\) (or CO\(_2\)), (c) ordered binding of Na\(^+\) and then HCO\(_3\) (or CO\(_2\)), and (d) ordered binding of HCO\(_3\) (or CO\(_2\)) and then Na\(^+\). The predictions for the binding of a single HCO\(_3\) and a single CO\(_2\) are the same, provided the data are all gathered at the same external pH. Furthermore, these models are applicable to schemes involving the binding of two HCO\(_3\), provided the affinities of the two HCO\(_3\)-binding sites are sufficiently different from one another.

A kinetic analysis of these models is detailed in Appendix 2, and summarized below and in Tables IV and VI. The goal of this analysis, and indeed the entire study, was to attempt to rule out the ion-pair model. This is straightforward if the data conflict with the kinetic predictions of the ion-pair model. However, as shown below, the present DNDS data, as well as earlier data dealing with the interaction between Na\(^+\) and HCO\(_3\) in the absence of DNDS (Boron, 1985), are nicely fitted by the ion-pair model. This raises an unavoidable dilemma that flows from the mathematical descriptions of the other kinetic models (see Appendix 2): the predictions of the other kinetic models reduce to those of the ion-pair model as their kinetic parameters approach extreme values. Thus, insofar as any data fit the ion-pair model, they can also be fitted by the other models. Although we cannot rule out these other models, we can place severe restrictions on their permissible kinetic parameters.

### Ion-Pair Model

We have analyzed the ion-pair model (see Appendix 2 for details) for each of the eight groups of data described in Results (series 1–8), as well as the four additional groups of data (series A–D) described previously (Boron, 1985). On line 1 of Table
### TABLE IV
Comparison of Observed Kinetic Parameters and Those Predicted by Rapid-Equilibrium Kinetic Models*

| Series | Na | HCO₃⁻ | DNDS | A | B |
|--------|----|-------|------|---|---|
| 1      | 425| 425   | 0    | 122| 106|
| 2      | 212| 212   | 0.1  | 5.2| 5.4|
| 3      | 106| 106   | 0    | 1.0| 1.0|

#### A. $K_a$ (HCO₃⁻), mM

| Series | 1  | 2  | 3  | A  | B  |
|--------|----|----|----|----|----|
| [Na]   | 425| 425| 425| 212| 106|
| [HCO₃⁻]|  — |  — |  — |  — |  — |
| [DNDS] |  0 |  0.1| 0.25|  0 |  0 |

1) Observed: 2.6 ± 0.3  5.7 ± 1.3  12.7 ± 4.3  5.4 ± 1.0  9.7 ± 2.1
2) Ion-pair: 2.2  6.0  11.7  4.5  8.9
3) Random: 3.2  7.3  15.5  4.7  7.3
4) Na⁺, HCO₃⁻: 3.4  7.8  14.4  5.4  9.5
5) HCO₃⁻, Na⁺: 1.6  5.1  10.2  3.2  6.0

#### B. $f_{max}$ (HCO₃⁻), pmol cm⁻² s⁻¹

| Series | 1  | 2  | 3  | A  | B  |
|--------|----|----|----|----|----|
| [Na]   | 425| 425| 425| 212| 106|
| [HCO₃⁻]|  — |  — |  — |  — |  — |
| [DNDS] |  0 |  0.1| 0.25|  0 |  0 |

1) Observed: 20.5 ± 2.2  19.5 ± 1.3  19.3 ± 2.5  18.7 ± 1.2  19.2 ± 1.5
2) Ion-pair: 19.8  19.8  19.8  19.8  19.8
3) Random: 21.3  21.3  21.3  20.7  19.6
4) Na⁺, HCO₃⁻: 21.4  21.4  21.4  21.4  21.4
5) HCO₃⁻, Na⁺: 19.1  19.1  19.1  18.5  17.5
| Series: | 4 | 5 | 6 | 7 | 8 | C |
|---------|---|---|---|---|---|---|
| [Na]:   | — | — | — | — | — | — |
| [HCO₃⁻]: 12 | 12 | 48 | 48 | 48 | 6 |
| [DNDS]: | 0 | 0.1 | 0 | 0.1 | 0.25 | 0 |

1) Observed

| 2) Ion-pair |
| 3) Random |
| 4) Na⁺, HCO₃⁻ |
| 5) HCO₃⁻, Na⁺ |

| Series: | 4 | 5 | 6 | 7 | 8 | C |
|---------|---|---|---|---|---|---|
| [Na]:   | — | — | — | — | — | — |
| [HCO₃⁻]: 12 | 12 | 48 | 48 | 48 | 6 |
| [DNDS]: | 0 | 0.1 | 0 | 0.1 | 0.25 | 0 |

1) Observed

| D. $J_{\text{m}}$(Na⁺), pmol cm⁻² s⁻¹ |

| 2) Ion-pair |
| 3) Random |
| 4) Na⁺, HCO₃⁻ |
| 5) HCO₃⁻, Na⁺ |

*For each of four kinetic parameters, observed values are presented for several experimental conditions. Also presented are values predicted for four models: ion pair, random binding, ordered binding of Na⁺ then HCO₃⁻, and ordered binding of HCO₃⁻ then Na⁺. These predicted values were computed from the best-fit values in Table VI.
IV are the observed values for each of the experimentally determined kinetic parameters: (A) $K_m$ (HCO$_3^-$), (B) $J_{max}$ (HCO$_3^-$), (C) $K_m$ (Na$^+$) and (D) $J_{max}$ (Na$^+$). On line 2, are the corresponding values predicted from a least-squares fit of the ion-pair model to our data. (See line 1 of Table VI for the best-fit parameters.) As can be seen, the fit is excellent, with predicted values always falling well within two standard deviations of the observed $K_m$'s and $J_{max}$'s. Based on the sum of squares of residuals, the ion-pair model fits the data marginally better than any of the others.

![Image of Figure 6](image_url)

**Figure 6.** Dependence of acid-extrusion rate on the calculated [NaCO$_3$]$_o$ at DNDS levels of 0, 0.1, and 0.25 mM. All 348 $J_H$ vs. [Na$^+$], or $J_H$ vs. [HCO$_3^-$], data points reported in this paper are replotted as $J_H$ vs. [NaCO$_3$]$_o$. The [NaCO$_3$]$_o$ values are nominal, and were calculated as described in footnote 1. The curves drawn through the points are the result of a simultaneous fit of all of the data to an equation of the form of Eq. 1.

For the ion-pair model to be viable, all data obtained at a single [DNDS]$_o$ must fall along the same $J_H$ vs. [NaCO$_3$]$_o$ curve, regardless of [HCO$_3^-$]$_o$ or [Na$^+$]$_o$. In Fig. 6 we have plotted $J_H$ as a function of the calculated [NaCO$_3$]$_o$ for [DNDS]$_o$ levels of 0, 0.1, and 0.25 mM. The curves drawn through the points are the result of a simultaneous fit of all 348 data points to an equation of the form of Eq. 1 (Table V, line 1). As can be seen from the three curves, as well as the individual fits of the data to the Michaelis-Menten equation (Table V, lines 2–4), $J_{max}$ is not significantly affected by changes in [DNDS]$_o$, whereas $K_m$ (NaCO$_3$) increases with increasing levels of the

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1 As discussed in Appendix 1, [NaCO$_3$]$_o$ = ([Na$^+$]$_o$[HCO$_3^-$]$_o$)/(γ $10^{-pH_o}$). We computed [NaCO$_3$]$_o$ from the nominal [HCO$_3^-$]$_o$ and [Na$^+$]$_o$, assuming that γ = $1.7 \times 10^6$ M (Garreils et al., 1961), and that pH$_o$ = 8.00.
Intracellular pH Regulation in the Squid Axon

TABLE V

Summary of Data* in Terms of Calculated [NaCO₃]

| [DNDS]₀ | n    | Fit* | Apparent Kᵦ(NaCO₃) | Apparent Kᵦ(DNDS) | Apparent Jᵦₘₙₜ |
|---------|------|------|---------------------|-------------------|-----------------|
| mM      | µM   | µM   | pmol cm⁻² s⁻¹       |
| All     | 348  | 1    | 56 ± 5              | 59 ± 6            | 19.9 ± 0.6      |
| 0       | 133  | 2    | 59 ± 6              | —                 | 20.3 ± 0.8      |
| 0.1     | 93   | 1    | 132 ± 22            | —                 | 19.4 ± 1.2      |
| 0.25    | 62   | 1    | 270 ± 67            | —                 | 18.4 ± 1.8      |

*Nonlinear least-squares curve fits, as described in Methods, were individually performed on each of the three sets of data points. Type 1 fit: standard Michaelis-Menten equation. Type 2 fit: forced through a standard point ([HCO₃]₀ = 12 mM, [Na⁺]₀ = 425 mM, [DNDS]₀ = 0 mM, and Jᵦₘₙₜ = 16.8 pmol cm⁻² s⁻¹). n is the number of points fitted. The data at [DNDS]₀ = 0 mM were previously reported (Boron, 1985).

inhibitor. Thus, this analysis is also consistent with the hypothesis that DNDS competes with the NaCO₃ ion pair.

**Random Binding of Na⁺ and HCO₃**

An analysis of the rapid-equilibrium model in which Na⁺ and HCO₃ bind randomly to the carrier, each competing with DNDS, is provided in Appendix 2. The predicted apparent Kᵦ and Jᵦₘₙₜ values are summarized in line 3 of Table IV; these were computed from the best-fit parameters (see Table VI, line 2). As noted in Appendix 2, the predictions of the random-binding model reduce to those of the ion-pair model at extreme values for the kinetic parameters. Indeed, the overall fit of the random-binding model to the 348 data points produced a sum of squares of residuals that was only ~2.6% higher than that of the ion-pair model.

**Ordered Binding of Na⁺ and HCO₃**

Appendix 2 also contains an analysis of a rapid-equilibrium model in which first Na⁺ and then HCO₃ bind to the carrier, with DNDS competing with Na⁺. The predicted apparent Kᵦ and Jᵦₘₜ values, computed from these best-fit parameters (see Table VI, line 3), are summarized in line 4 of Table IV. As for the predictions of the random-binding model, those of the ordered Na⁺-then-HCO₃ model reduce to those of the ion-pair model for extreme values of the kinetic parameters. Indeed, the overall fit

TABLE VI

Summary of Best-Fit Values for Rapid-Equilibrium Kinetic Models*

| Model            | α   | Kᵦ(NaCO₃) µM | Kᵦ mM | Kᵦ(HCO₃) µM | Kᵦ(HCO₃, Na⁺) 12.8 µM | Jᵦₘₜ pmol cm⁻² s⁻¹ | SSR  |
|------------------|-----|--------------|-------|-------------|------------------------|-------------------|-----|
| Ion pair         |     |              |       |             |                        |                   | 658 |
| Random           | 0.0321 | 385         | 4.4   | 37.4        | 21.9                   | 9.38              |     |
| Na⁺, HCO₃       |     |              |       |             |                        |                   | 21.4|
| HCO₃, Na⁺       |     |              |       |             |                        |                   | 21.4|

*The results of nonlinear least-squares curve fits are presented for four kinetic models: ion pair, random binding, ordered binding of Na⁺ then HCO₃, and ordered binding of HCO₃ then Na⁺. SSR is the sum of squares of residuals. See Appendix 2 for details.
of this random-binding model to the 348 data points produced a sum of squares of residuals that was only ~2.2% higher than that of the ion-pair model.

Ordered Binding of $\text{HCO}_3^-$ then Na$^+$

The final kinetic analysis in Appendix 2 is of a rapid-equilibrium model in which first $\text{HCO}_3^-$ and then Na$^+$ bind to the carrier, with DNDS competing with $\text{HCO}_3^-$. The predicted apparent $K_m$ and $J_{\text{max}}$ values, computed from these best-fit parameters (see Table VI, line 4), are summarized in line 5 of Table IV. As for the predictions of the random-binding and ordered Na$^+$-then-$\text{HCO}_3^-$ models, those of the ordered $\text{HCO}_3^-$-then-Na$^+$ model reduce to those of the ion-pair model for extreme values of the kinetic parameters. The overall fit of this random-binding model to the 348 data points produced a sum of squares of residuals that was only ~10% higher than that of the ion-pair model.

Conclusions

The disulfonic stilbene derivative DNDS appears to be a competitive inhibitor of acid extrusion, with respect to both $\text{HCO}_3^-$ and Na$^+$. The data are entirely consistent with the Na$^+$-HCO$_3^-$ ion-pair model. Indeed, this model provides the best fit to the data, and is probably the most straightforward. However, three other rapid-equilibrium kinetic models (random binding, ordered binding of Na$^+$ then $\text{HCO}_3^-$, and ordered binding of $\text{HCO}_3^-$ then Na$^+$) also produce reasonable fits of the data, and therefore cannot be ruled out.

Two points deserve emphasis. First, given the excellent fit provided by the ion-pair model, it was unavoidable that the other models should also fit the data. As shown in Appendix 2, the predictions of the other models reduce to those of the ion-pair model for extreme values of the kinetic parameters. This result can be reached intuitively: The ion-pair model states that Na$^+$ and CO$_3^-$ first complex with one another before binding to the carrier. However, this should be indistinguishable from the case in which the binding of Na$^+$ makes the carrier's affinity for CO$_3^-$ infinitely high, and vice versa.

Second, given the excellent fit of the ion-pair model, we would not have been able to distinguish among the models even if we had employed a broader range of values of $[\text{HCO}_3^-]_o$, $[\text{Na}^+]_o$, and $[\text{DNDS}]_o$. This is because the predictions of all of the models reduce to those of the ion-pair model, regardless of substrate or inhibitor concentration. In fact, the range of values examined in this and the preceding study was as broad as practicable. Had we used lower values of $[\text{Na}^+]_o$, we would have had to have used higher values of $[\text{HCO}_3^-]_o$ to achieve saturation. However, raising $[\text{HCO}_3^-]_o$ would require raising $[\text{CO}_3^-]_o$ to keep pH$_o$ constant. This, in turn, would have raised $[\text{HCO}_3^-]_o$, and, thus, the total intracellular buffering power. Thus, rates of pH$_i$ change would have been extremely low. Had we used lower values of $[\text{HCO}_3^-]_o$, we would have had to have used higher values of [Na$^+$]$_o$ to achieve saturation. However, we could not raise [Na$^+$]$_o$ above ~500 mM without making the solutions hypertonic.

In summary, the data are fitted well by the ion-pair model. Although we cannot rule out three other rapid-equilibrium models, we can place severe restrictions on the allowable kinetic parameters for each of them.
APPENDIX I

Calculation of Concentrations of HCO$_3^-$-derived Species after HCO$_3^-$ Is Added to a Solution

To perform the kinetic analyses presented in the Discussion, one must know the concentration of HCO$_3^-$, or of related species (e.g., NaCO$_3^-$). However, the relationship between added HCO$_3^-$ and [HCO$_3^-$] is complex. For example, we have found that adding 12 mM KHCO$_3$ to seawater, initially buffered to pH 8.00 with 30 mM EPPS, causes a paradoxical fall in pH to ~7.97. This observation implies that less of the added HCO$_3^-$ participates in the reaction HCO$_3^-$ + H$^+$ → H$_2$CO$_3$ than in the reaction HCO$_3^-$ → CO$_3^{2-}$ + H$. We suspected that the latter reaction is promoted by the complexation of CO$_3^{2-}$ with various cations to form ion pairs. Thus, the added HCO$_3^-$ ought to be distributed in several pools, including HCO$_3^-$ and several carbonates (e.g., CO$_3^{2-}$, NaCO$_3^-$, MgCO$_3$, and CaCO$_3$). To the extent that carbonate formation occurs, the assumption that [HCO$_3^-$] equals the added HCO$_3^-$ will be in error. Because this issue is crucial for the present study, as well as a forthcoming one in which we examine the effects of extracellular pH changes on acid-extrusion rates, we analyzed the chemistry of HCO$_3^-$ in solutions containing cations that complex with CO$_3^{2-}$. Our analysis shows that carbonate formation causes [HCO$_3^-$] to be only ~10% lower than the added HCO$_3^-$ at pH$_o$ 8.00 (i.e., the conditions of the present experiments), and that the error increases with increasing pH$_o$. The analysis is relevant for any HCO$_3^-$-containing solution at high pH.

HCO$_3^-$ added to an aqueous solution (in an amount [HCO$_3^-$]$^\circ$) has one of three general fates: (a) the greatest portion remains HCO$_3^-$, (b) a small amount ($x$) of the HCO$_3^-$ combines with H$^+$ to yield CO$_2$, and (c) a small amount ($y$) of the HCO$_3^-$ dissociates into H$^+$ and CO$_3^{2-}$. The flux ($x$) of HCO$_3^-$-derived carbon atoms through pathway (b) increases if the CO$_2$ formed leaves solution and enters the atmosphere, whereas the flux ($y$) through pathway (c) will be increased if the CO$_3^{2-}$ formed is consumed by the formation of CO$_3^{2-}$-containing ion pairs. The reactions can be summarized as follows:

\[
\text{HCO}_3^- \xymatrix{ y \ar[r] & \text{CO}_3^{2-} \ar[r] & \text{MCO}_3^-} \xymatrix{ x \ar[u] & \text{H}^+ \ar[u] & \text{NaCO}_3^-. \ar[u] \ar[l]^z}
\]

\text{Scheme A1}

where $x$, $y$, $z$, and $v$ are the fluxes of HCO$_3^-$-derived carbon atoms through the indicated reactions. $M$ is a divalent cation (e.g., Ca$^{++}$ or Mg$^{++}$). At equilibrium, when there is no net flux through any of the reactions,

\[
\begin{align*}
[HCO_3^-]' &= [HCO_3^-]^\circ - x - y \\
[CO_3^{2-}]' &= y - z - v \\
[MCO_3^-]' &= z \\
[NaCO_3^-]' &= v \\
[CO_2]' &= x \\
pH' &= pH^\circ + (x - y)/\beta
\end{align*}
\]

where the single primes following the parameter designations indicate the values pertaining after equilibrium is established. pH$^\circ$ is the pH prevailing before the addition of the amount
of $[\text{HCO}_3^-]$ of $\text{HCO}_3^-$, and $\beta$ is the non-$\text{CO}_2$ buffering power of the solution. The following
equations describe the four equilibria that are simultaneously established:

$$\text{pH}' = \text{pK}_1 + \log \frac{[\text{HCO}_3^-]}{[\text{CO}_2]} \quad \text{or} \quad 10^{(\text{pH}' - \text{pK}_1)} = \frac{[\text{HCO}_3^-]}{x} - y$$  \hspace{1cm} (A7)

$$\text{pH} = \text{pK}_2 + \log \frac{[\text{CO}_2]}{[\text{HCO}_3^-]} \quad \text{or} \quad 10^{(\text{pH}' - \text{pK}_2)} = \frac{y - z - v}{[\text{HCO}_3^-]} - x$$  \hspace{1cm} (A8)

$$K_M = \frac{[\text{CO}_2][\text{M}]}{[\text{MCO}_3^-]} \quad \text{or} \quad K_M = \frac{(y - z - v)[\text{M}]}{z}$$  \hspace{1cm} (A9)

$$K_{Na} = \frac{[\text{CO}_2][\text{Na}]}{[\text{NaCO}_3^-]} \quad \text{or} \quad K_{Na} = \frac{(y - z - v)[\text{Na}]}{v}$$  \hspace{1cm} (A10)

where it is assumed that $[\text{M}]$ and $[\text{Na}^+]$ are not affected significantly by the formation of $\text{CO}_2^-$
ion pairs. Algebraic manipulations of the above equations lead to the following equation,
which describes the pH obtaining at equilibrium in terms of the constants $\text{pK}_1$, $\text{pK}_2$, $K_M$, $K_{Na}$,
$\text{M}$, $\text{Na}$, $[\text{HCO}_3^-]$, and pH$^o$:

$$\beta(\text{pH}' - \text{pH}^o) + [\text{HCO}_3^-]^o \cdot \frac{P_1S[P_1 + P_1P_2(S + 1)] + P_1P_2 - (1 + P_2S)}{(1 + P_2S) \cdot [1 + P_1 + P_1P_2(S + 1)]} = 0$$  \hspace{1cm} (A11)

where $P_1 = 10^{(\text{pH}' - \text{pK}_1)}$, $P_2 = 10^{(\text{pH}' - \text{pK}_2)}$, and $S = ([\text{M}]/K_M + [\text{Na}]/K_{Na})$. This exponential
equation is solved for pH$'$ using a standard numerical technique. This value is then used to compute $x$, $y$, $z$, and $v$
using the following equations, which are also derived by algebraically manipulating Eqs. (A7) through (A10):

$$x = [\text{HCO}_3^-]^o \cdot \frac{P_2S[P_1 + P_1P_2(S + 1)] + P_1P_2}{(1 + P_2S)[1 + P_1 + P_1P_2(S + 1)]}$$  \hspace{1cm} (A12)

$$y = \frac{[\text{HCO}_3^-]^o}{1 + P_1 + P_1P_2(1 + S)}$$  \hspace{1cm} (A13)

$$z = \frac{[\text{M}]}{K_M} \cdot P_2[\text{HCO}_3^-]^o \cdot \frac{P_1 + 2P_2S + P_1P_2S}{(1 + P_2S)[1 + P_1 + P_1P_2(S + 1)]}$$  \hspace{1cm} (A14)

$$v = \frac{[\text{Na}]}{K_{Na}} \cdot P_2[\text{HCO}_3^-]^o \cdot \frac{P_1 + 2P_2S + P_1P_2S}{(1 + P_2S)[1 + P_1 + P_1P_2(S + 1)]}$$  \hspace{1cm} (A15)

These values for $x$, $y$, $z$, and $v$ are inserted into Eqs. A1–A5 to obtain values for the concentrations of
the solutes after equilibrium has been achieved after the initial addition of $\text{HCO}_3^-$: $[\text{CO}_2]^o$, $[\text{HCO}_3^-]^o$, $[\text{CO}_2^+]^o$, $[\text{MCO}_3^-]^o$, and $[\text{NaCO}_3^-]^o$.

It is instructive to examine the predictions of these equations for the standard $\text{HCO}_3^-$
containing seawater that we use in our experiments. This solution normally contains 425 mM
$\text{Na}^+$, 57 mM $\text{Mg}^{2+}$, and 3 mM $\text{Ca}^{2+}$. We used the following parameter values:

| Parameter | Value |
|-----------|-------|
| $\text{pK}_1$ | 6.1 |
| $K_M$ | 25 |
| pH$^o$ | 8.00 |
| $[\text{M}]$ | 60 mM |
| $\text{pK}_2$ | 10.0 |
| $K_{Na}$ | 170 |
| $[\text{HCO}_3^-]^o$ | 12 mM |
| [Na] | 425 mM |

The value for $\text{pK}_2$ was chosen because, when used in the calculations for a solution lacking
Na\(^+\), Ca\(^{++}\), and Mg\(^{++}\) it led to a predicted pH change (i.e., \(~+0.003\)) that was very close to that observed when 12 mM HCO\(_3\) was added to a solution containing 425 mM N-methyl-d-glucammonium-Cl and 30 mM EPPS. Note that \([M] = [Mg^{++}] + [Ca^{++}]\). The value chosen for \(K_M\) was selected because, when used in the calculations for a solution lacking Na\(^+\) but containing 57 mM Mg\(^{++}\) and 3 mM Ca\(^{++}\), it predicted a HCO\(_3\)-induced pH change very close to that observed (i.e., \(-0.014\)). Finally, the value chosen for \(K_N\) was selected because, when used in the calculations for a solution containing 425 mM Na\(^+\), 57 mM Mg\(^{++}\) and 3 mM Ca\(^{++}\) it predicted a HCO\(_3\)-induced pH change that was very close to that observed (i.e., \(~-0.03\)) for our standard 12 mM HCO\(_3\) seawater.

To further test whether our estimates of \(pK_a\), \(K_M\), and \(K_N\) were reasonable, we also computed the predicted pH changes for the addition of a wide range of amounts of HCO\(_3\) to seawaters initially at pH values ranging from 7.4 to 8.6. The observed values were always very close to those predicted by the model. For example, for pH 7.4, the model predicts that adding 12 mM HCO\(_3\) should cause pH to increase by 0.032, which is very close to the observed value. For a starting pH of 7.7, the model predicts that adding any amount of HCO\(_3\) will cause no pH change, which is our observation. At the other pH extreme, for an initial pH of 8.6, the model predicts that the addition of 12 mM HCO\(_3\) should cause pH to fall by 0.15, which is very similar to the observed value. Thus, on the basis of observed changes in pH caused by the addition of known amounts of HCO\(_3\) to defined solutions, the model appears consistent.

The aforementioned calculations only pertain to the first step of our solution-making process, in which the HCO\(_3\) is added to the ASW. If the initial pH exceeds 7.7, this addition of HCO\(_3\) causes a dose-dependent acidification. Coincidentally, [CO\(_2\)]' is higher than the value that would obtain if the addition of HCO\(_3\) had not caused any pH change. The pH can be returned to its initial value by lowering [CO\(_2\)]. This can be achieved by gassing for an indefinitely long period with a gas mixture of the proper CO\(_2\) content, or by briefly gassing with a CO\(_2\)-free solution until pH has been raised from pH' to the initial value. In either case, CO\(_2\) is lost to the gas phase, causing the equilibria to be shifted as follows:

\[
\begin{align*}
\text{HCO}_3^- & \rightleftharpoons y \text{CO}_2^- + z \text{MCO}_3^- \\
x & \text{H}^+ \\
\text{CO}_2 & \xrightarrow{z} \text{NaCO}_3 \\
\end{align*}
\]

where \(x, y, z, v,\) and \(s\) are fluxes through the indicated reactions. For initial pH values below 7.7, adding HCO\(_3\) causes an alkalinization, and [CO\(_2\)]' is lower than if pH had not risen. pH can be returned from pH' to pH\(^*\) by gassing with a mixture having a CO\(_2\) content greater than the ideal. The following derivation is independent of starting pH. At the final equilibrium,

\[
\begin{align*}
[HCO_3^-]' &= [HCO_3^-] + y - x & (A16) \\
[CO_2^-]' &= [CO_2^-] + v + z - y & (A17) \\
[MCO_3^-]' &= [MCO_3^-] - z & (A18) \\
[NaCO_3^-]' &= [NaCO_3^-] - v & (A19) \\
[CO_2]' &= [CO_2]' + x - s & (A20)
\end{align*}
\]
\[
\text{pH}'' = \text{pH}' + \frac{(x - y)}{\beta}
\]

(A21)

where the double primes indicate the values prevailing after this second and final equilibrium is established. The following equations describe the four equilibria that are simultaneously established:

\[
10^{(\text{pH}' - \text{pH}'' - x)} = \frac{[\text{HCO}_3^-]' + y - x}{[\text{CO}_2]' + x - s}
\]

(A22)

\[
10^{(\text{pH}' - \text{pH}'' - y)} = \frac{[\text{CO}_2]' + v + z - y}{[\text{HCO}_3^-]' + y - x}
\]

(A23)

\[
K_M = \frac{([\text{CO}_2]' + v + z - y)[M]}{[\text{MCO}_3]'' - z}
\]

(A24)

\[
K_{Na} = \frac{([\text{CO}_2]' + v + z - y)[Na]}{[\text{NaCO}_3]'' - v}
\]

(A25)

By definition, the pH of the solution will be titrated back to pH\(^{o}\), so that pH\(^{o}\) = pH\(^{o}\). Manipulation of Eqs. A22-A25 leads to the following expressions for \(x, y, z, v, \) and \(s\):

\[
x = \frac{(P_1P_2 + 1)([\text{CO}_2]' + [\text{NaCO}_3]' + [\text{MCO}_3]') - P_2[\text{HCO}_3]'[1 - S + P_1P_2(S + 1)]}{1 + P_1P_2 - \beta(\text{pH}' - \text{pH}''[1 + P_1P_2(S + 1) + P_1P_2P_4[1 + S])}
\]

(A26)

\[
y = \frac{(P_1P_2 + 1)([\text{CO}_2]' + [\text{NaCO}_3]' + [\text{MCO}_3]') - P_2[\text{HCO}_3]'[1 - S + P_1P_2(S + 1)]}{1 + P_1P_2 - \beta(\text{pH}' - \text{pH}''[1 + P_1P_2(S + 1) + P_1P_2 + P_4P_2[1 + S])}
\]

(A27)

\[
z = [\text{MCO}_3]' - \frac{M}{K_M} \cdot ([\text{HCO}_3]' + \beta(\text{pH}' - \text{pH}''])
\]

(A28)

\[
v = [\text{NaCO}_3]' - \frac{Na}{K_{Na}} \cdot ([\text{HCO}_3]' + \beta(\text{pH}' - \text{pH}''])
\]

(A29)

\[
s = [\text{CO}_2]' - [[\text{CO}_2]' + [\text{NaCO}_3]' + [\text{MCO}_3]']
\]

(A30)

These values for \(x, y, z, v, \) and \(s\) are inserted into Eqs. A16-A21 to obtain the solute concentrations obtaining after the final equilibrium has been achieved at the desired pH: [CO\(_2\)]\(^{o}\), [HCO\(_3\)]\(^{o}\), [CO\(_3\)]\(^{o}\), [MCO\(_3\)]\(^{o}\), and [NaCO\(_3\)]\(^{o}\).

It is instructive to examine the predictions of these equations for the case of our standard HCO\(_3\) ASW. As noted above, Eq. A11 correctly predicts that pH falls from 8.00 to 7.97 upon the addition of 12 mM HCO\(_3\). Eqs. A26-A30 predict that as pH is returned to 8.00, 0.47 mM total CO\(_2\) is lost to the atmosphere as CO\(_2\). Furthermore, the final [HCO\(_3\)] is 10.8 mM, and the final [NaCO\(_3\)] is 269 \(\mu\)M. Thus, the actual [HCO\(_3\)] is about 90% as large as the nominal [HCO\(_3\)]. The predicted error is increased slightly at lower amounts of added HCO\(_3\) (e.g., 88.7% of nominal for 0.75 mM added HCO\(_3\)), and decreased slightly at higher amounts of added HCO\(_3\) (e.g., 90.8% of nominal for 48 mM added HCO\(_3\)). The discrepancy between
actual and nominal [HCO₃⁻] decreases as pH is decreased. This is expected, due to the decrease in ion-pair formation. For example, the addition of 12 mM HCO₃⁻ at pH 7.4 causes the predicted [HCO₃⁻] to be 97.1% as large as the nominal [HCO₃⁻], whereas the addition of a similar amount of HCO₃⁻ at pH 8.6 results in a predicted [HCO₃⁻] that is only 71.6% of the nominal value.

APPENDIX 2

Analysis of Kinetic Models

Ion-pair model. Consider the following reaction sequence, in which DNDS and the NaCO₂ ion pair compete for binding to the carrier X:

\[
\begin{align*}
X & \cdot \text{DNDS} \\
\text{DNDS} & + \\
X + \text{NaCO}_2 & \overset{k}{\leftrightarrow} X \cdot \text{NaCO}_2 \\
\end{align*}
\]

where \( K_i \), \( K_{\text{NaCO}_2} \), and \( \gamma \) are equilibrium constants, and \( k \) is a rate constant describing the transport of NaCO₂ across the cell membrane. \( \gamma \) is defined as \( [\text{Na}^+] [\text{HCO}_3^-] / [\text{NaCO}_2] [\text{H}^+] \), and has the value \( 1.7 \times 10^6 \text{ M} \). Rapid equilibrium kinetics predicts the following:

\[
J = \frac{[\text{Na}][\text{HCO}_3^-]/J_{\text{max}}}{\gamma[H]K_{\text{NaCO}_2}(1 + [\text{DNDS}]/K_i) + [\text{Na}][\text{HCO}_3^-]}, \quad (B1)
\]

which can in turn be rearranged to provide expressions for the experimentally determined parameters:

\[
K_m(\text{HCO}_3^-) = K_{\text{NaCO}_2} \left(1 + \frac{[\text{DNDS}]}{K_i}\right) \gamma[H] [\text{Na}], \quad J_{\text{max}}(\text{HCO}_3^-) = J_{\text{max}} \quad (B2, a \text{ and } b)
\]

\[
K_m(\text{Na}^+) = K_{\text{NaCO}_2} \left(1 + \frac{[\text{DNDS}]}{K_i}\right) \gamma[H] [\text{HCO}_3^-], \quad J_{\text{max}}(\text{Na}^+) = J_{\text{max}} \quad (B2, c \text{ and } d)
\]

We used a nonlinear least-squares method to fit the data of series 1–8 and A–D to Eq. B1, and obtained the best-fit values summarized in Table VI, line 1.

Random binding of Na⁺ and HCO₃⁻. Consider the reaction sequence in which DNDS,
HCO₃⁻, and Na⁺ all compete for binding to the carrier:

\[
\begin{align*}
X \cdot \text{DNDS} & \rightleftharpoons K_i \\
\text{DNDS} & + \\
X + \text{Na} & \rightleftharpoons X \cdot \text{Na} \\
+ & + \\
\text{HCO}_3^- & \rightleftharpoons \alpha K_{\text{HCO}_3} \\
K_{\text{HCO}_3} & \downarrow \\
X \cdot \text{HCO}_3^- + \text{Na} & \rightleftharpoons X \cdot \text{Na} \cdot \text{HCO}_3^- \\
\end{align*}
\]

Scheme B2

where \(\alpha\) describes how the binding of one substrate affects the affinity of the carrier for the other. Rapid-equilibrium kinetics predicts that:

\[
J = \frac{[\text{Na}][\text{HCO}_3^-]J_{\text{max}}}{\alpha K_{\text{HCO}_3} K_{\text{HCO}_3}(1 + \text{DNDS})/K_i + \alpha K_{\text{HCO}_3}[\text{Na}] + \alpha K_{\text{Na}}[\text{HCO}_3] + [\text{Na}][\text{HCO}_3]}.
\]  

(B3)

and the following expressions for the experimentally determined parameters:

\[
K_a(\text{HCO}_3) = \frac{\alpha K_{\text{Na}}(1 + [\text{DNDS}])}{\alpha K_{\text{Na}} + [\text{Na}]},
\]  

(B4,a)

\[
J_{\text{max}}(\text{HCO}_3) = \frac{J_{\text{max}}[\text{Na}]}{\alpha K_{\text{Na}} + [\text{Na}]},
\]  

(B4,b)

\[
K_a(\text{Na}^+) = \frac{\alpha K_{\text{HCO}_3}(1 + [\text{DNDS}])}{\alpha K_{\text{HCO}_3} + [\text{HCO}_3]}
\]  

(B4,c)

\[
J_{\text{max}}(\text{Na}^+) = \frac{J_{\text{max}}[\text{HCO}_3]}{\alpha K_{\text{HCO}_3} + [\text{HCO}_3]}
\]  

(B4,d)

We fitted the data of series 1–8 and series A–D to Eq. B3, and obtained the best-fit values summarized in Table VI, line 2. Given the result of the previous section, in which we showed that the data were fitted well by the ion-pair model, it is not surprising that the data are also fitted by the random-binding model. It is easily shown that the expressions for \(K_a(\text{HCO}_3)\), \(J_{\text{max}}(\text{HCO}_3)\), \(K_a(\text{Na}^+)\) and \(J_{\text{max}}(\text{Na}^+)\) of Eqs. B4, a–d (i.e., the random-binding model) reduce to the form of Eqs. B2, a–d (i.e., the ion-pair model) as \(\alpha\) approaches zero under the restricted conditions in which: (a) the product \(\alpha K_{\text{HCO}_3} K_{\text{Na}}\) is fixed and (b) the quotient \(K_{\text{Na}}/K_{\text{HCO}_3}\) also is fixed. Thus, we might expect the best fit for the random-binding model to be achieved with a very small \(\alpha\) and corresponding large values for \(K_{\text{HCO}_3}\) and \(K_{\text{Na}}\) expectations borne out by the fitting procedure.
The best-fit values for $\alpha$, $K_{\text{HCO}_3}$, and $K_N$ predict apparent $K_m$ and $J_{\text{max}}$ values (Table IV, line 3) that are similar, but not identical, to those of the ion-pair model (Table IV, line 2). However, the predictions of the random-binding model should approach those of the ion-pair model more closely if $\alpha$ is decreased and $K_{\text{HCO}_3}$ and $K_N$ compensatorily increased. For example, if (a) $\alpha$ is reduced by four orders of magnitude, (b) $K_{\text{HCO}_3}$ and $K_N$ are each raised by about two orders of magnitude, and (c) $K_i$ is raised by $\sim$55%, the predictions of the random-binding model are within 1% of those of the ion-pair model for our data. Even if one considers Na$^+$ activation curves studied at $[\text{HCO}_3]$ as high as 384 mM (eightfold higher than we used) with as much as 5 mM DNDS (20-fold higher), or HCO$_3^-$ activation curves studied at $[\text{Na}^+]$ as low as 13.25 mM (eightfold lower) with as much as 5 mM DNDS, the predictions of the random-binding model are within 2% of those of the ion-pair model. Thus, even if data obtained over a wide range of concentrations were to fit the ion-pair model perfectly, it would still be possible to fit the data nearly as well with a random-binding model.

*Ordered binding of Na$^+$ then HCO$_3^-$. Consider the ordered reaction sequence in which DNDS competes with Na$^+$ for binding to the carrier:

$$X \cdot \text{DNDS}$$

$\downarrow K_i$

$$\text{DNDS}$$

$+$

$$X + Na \rightarrow X \cdot Na$$

$+$

$$\text{HCO}_3$$

$\downarrow K_{\text{HCO}_3}$

$$X \cdot Na \cdot \text{HCO}_3 \rightarrow$$

Scheme B3

Rapid-equilibrium kinetics predicts that:

$$J = \frac{[\text{Na}][\text{HCO}_3]/J_{\text{max}}}{K_{\text{Na}}K_{\text{HCO}_3}(1 + [\text{DNDS}]/K_i) + K_{\text{HCO}_3}[\text{Na}] + [\text{Na}][\text{HCO}_3]},$$

and:

$$K_m(\text{HCO}_3) = K_{\text{HCO}_3} \frac{K_{\text{Na}}(1 + [\text{DNDS}]/K_i) + [\text{Na}]}{[\text{Na}]}$$

$$J_{\text{max}}(\text{HCO}_3) - J_{\text{max}} (B5, a \text{ and } b)$$

$$K_m(\text{Na}^+) = K_{\text{Na}} \frac{K_{\text{HCO}_3}(1 + [\text{DNDS}]/K_i)}{K_{\text{HCO}_3} + [\text{HCO}_3]}$$

$$J_{\text{max}}(\text{Na}^+) = J_{\text{max}} \frac{[\text{HCO}_3]}{K_{\text{HCO}_3} + [\text{HCO}_3]} (B6, c \text{ and } d)$$

For example, if $\alpha = 3 \times 10^{-4}$, $K_{\text{HCO}_3} = 6,990$, $K_N = 43,350$, $K_i = 0.058$, and $J_{\text{max}} = 19.8 \text{ pmol cm}^{-2} \text{ s}^{-1}$, the predicted $K_m(\text{HCO}_3)$ values are 2.2 mM for series 1, 8.9 mM for series B, and 11.8 mM for series 3. Similarly, the predicted $K_m(\text{Na}^+)$ values are 78.5, 312, and 104 mM, respectively. The apparent $J_{\text{max}}$ values range between 19.7 and 19.8 pmol cm$^{-2}$ s$^{-1}$. Thus, all predictions are within 1% of those of the ion-pair model.
When we fitted the data of series 1–8 and series A–D to Eq. B5, we obtained the best-fit values summarized in Table VI, line 3. The fit provided by the Na⁺-then-HCO₃⁻ ordered-binding model is to be expected, given the excellent fit of the ion-pair model, because it is easily shown that the predictions of this ordered-binding model (Eqs. B6, a–d) reduce to those of the ion pair model (Eqs. B2, a–d) as \( K_{\text{HCO}_3} \) approaches zero at a constant \( K_{\text{HCO}_3} K_{\text{Na}} \) product. Thus, we would expect the best fit of the Na⁺-then-HCO₃⁻ ordered-binding model to be achieved with a very low \( K_{\text{HCO}_3} \) and a correspondingly high \( K_{\text{Na}} \), as was indeed the case. The best-fit values for \( K_{\text{HCO}_3} \) and \( K_{\text{Na}} \) lead to predictions for the apparent \( K_{\text{HCO}_3} \) and \( J_{\text{app}} \) values (Table IV, line 4) that are similar, though not identical, to those of the ion-pair model (Table IV, line 2). However, if (a) \( K_{\text{HCO}_3} \) is lowered by about two orders of magnitude, (b) \( K_{\text{Na}} \) is similarly raised, and (c) \( K_{i} \) is raised by \( \sim 25\% \), the predictions of the random-binding model are within 1% of those of the ion-pair model for our data.⁵

**Ordered binding of HCO₃⁻ then Na⁺.** Consider the reaction sequence in which DNDS, HCO₃⁻, and Na⁺ all compete for binding to the carrier:

\[
\begin{align*}
X \cdot \text{DNDS} & \quad \text{1:} \quad K_i \\
\text{DNDS} & + \quad X + \text{HCO}_3^- \quad \text{1:} \quad K_{\text{HCO}_3} \\
& + \quad \text{Na}^+ \quad 1: \quad K_{\text{Na}} \\
& \quad 1: \quad K_{i} \\
X \cdot \text{Na}^+ \cdot \text{HCO}_3^- \quad 1: \quad k
\end{align*}
\]

**Scheme B4**

Rapid-equilibrium kinetics predicts that:

\[
J = \frac{[\text{Na}][\text{HCO}_3^-] J_{\text{max}}}{K_{\text{Na}} K_{\text{HCO}_3}(1 + [\text{DNDS}] / K_i) + K_{\text{Na}} [\text{HCO}_3^-] + [\text{Na}][\text{HCO}_3^-]}, \tag{B7}
\]

and:

\[
\begin{align*}
K_{\text{Na}}(\text{HCO}_3^-) &= K_{\text{HCO}_3} \frac{K_{\text{Na}} (1 + [\text{DNDS}] / K_i)}{K_{\text{Na}} + [\text{Na}]} \\
J_{\text{max}}(\text{HCO}_3^-) &= J_{\text{max}} \frac{[\text{Na}^+]}{K_{\text{Na}} + [\text{Na}^+]} \tag{B8, a and b} \\
K_{\text{Na}}(\text{Na}^+) &= K_{\text{Na}} \frac{K_{\text{HCO}_3} (1 + [\text{DNDS}] / K_i) + [\text{HCO}_3^-]}{[\text{HCO}_3^-]} \\
J_{\text{max}}(\text{Na}^+) &= J_{\text{max}} \tag{B8, c and d}
\end{align*}
\]

⁵ For example, if \( K_{\text{HCO}_3} = 0.013, K_{\text{Na}} = 72,500, K_i = 0.058, \) and \( J_{\text{max}} = 19.8 \text{ pmol cm}^{-2} \text{ s}^{-1} \), the predicted \( K_{\text{Na}}(\text{HCO}_3^-) \) values are 2.2 mM for series 1, 8.9 mM for series B, and 11.8 mM for series C. Similarly, the predicted \( K_{\text{Na}}(\text{Na}^+) \) values are 78.5, 312, and 104 mM, respectively. The apparent \( J_{\text{max}} \) values range between 19.7 and 19.8 pmol cm⁻² s⁻¹. Thus, all predictions are within 1% of those of the ion-pair model.
We fitted the data of series 1–8 and series A–D to Eq. B7, obtaining the best-fit values reported in Table VI, line 4. As was the case for the random-binding model and the Na⁺-then-HCO₃⁻ ordered-binding model, it is to be expected that the HCO₃⁻-then-Na⁺ ordered-binding model would fit the data, given the excellent fit provided by the ion-pair model. It is easily demonstrated that the predictions of the HCO₃⁻-then-Na⁺ ordered-binding model (Eqs. B8, a–d) reduce to those of the ion-pair model as Kₘₐ approaches zero at a constant (Kₐ₇₇Kₜₐ) product. As expected, the best fit of this model was achieved with a very low Kₙₐ and a correspondingly high Kₐ₇₇. Although the best-fit values for Kₐ₇₇ and Kₙₐ predict apparent Kₜ and Jₘₐ values (Table IV, line 5) that are not identical to those of the ion-pair model (Table IV, line 2), the agreement is improved if Kₙₐ is further reduced. For example, if (a) Kₐ₇₇ is increased by about two orders of magnitude, (b) Kₙₐ is decreased by about two orders of magnitude, and (c) Kₜ is raised by ~20%, the predictions of the random-binding model are within 1% of those of the ion-pair model for our data.⁴

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⁴ For example, if Kₐ₇₇ = 6,600, Kₙₐ = 0.143, Kₜ = 0.585, and Jₘₐ = 19.8 pmol cm⁻² s⁻¹, the predicted Kₜ(HCO₃⁻) values are 2.2 mM for series 1, 8.9 mM for series 3, and 11.7 mM for series 5. Similarly, the predicted Kₜ(Na⁺) values are 78.8, 315, and 104 mM, respectively. All apparent Jₘₐ values are ~19.8 pmol cm⁻² s⁻¹. Thus, all predictions are within 1% of those of the ion-pair model.
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