Effect of Basolateral CO$_2$/HCO$_3^-$ on Intracellular pH Regulation in the Rabbit S3 Proximal Tubule

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ABSTRACT We used the absorbance spectrum of the pH-sensitive dye dimethyl-carboxyfluorescein to monitor intracellular pH (pHi) in the isolated perfused S3 segment of the rabbit proximal tubule, and examined the effect on pHi of switching from a HEPES to a CO$_2$/HCO$_3^-$ buffer in the lumen and/or the bath (i.e., basolateral solution). Solutions were titrated to pH 7.40 at 37°C. With 10 mM acetate present bilaterally (lumen and bath), this causing steady-state pHi to be rather high (~7.45), bilaterally switching the buffer from 32 mM HEPES to 5% CO$_2$/25 mM HCO$_3^-$ caused a sustained fall in pHi of ~0.26. However, with acetate absent bilaterally, this causing steady-state pH$_i$ to be substantially lower (~6.9), bilaterally switching to CO$_2$/HCO$_3^-$ caused a transient pH$_i$ fall (due to the influx of CO$_2$), followed by a sustained rise to a level ~0.18 higher than the initial one. The remainder of the experiments was devoted to examining this alkalinization in the absence of acetate. Switching to CO$_2$/HCO$_3^-$ only in the lumen caused a sustained pH$_i$ fall of ~0.15, whereas switching to CO$_2$/HCO$_3^-$ only in the bath caused a transient fall followed by a sustained pH$_i$ increase to ~0.26 above the initial value. This basolateral CO$_2$/HCO$_3^-$-induced alkalinization was not inhibited by 50 μM DIDS applied shortly after CO$_2$/HCO$_3^-$ washout, but was slowed ~73% by DIDS applied more than 30 min after CO$_2$/HCO$_3^-$ washout. The rate was unaffected by 100 μM bilateral acetazolamide, although this drug greatly reduced CO$_2$-induced pH$_i$ transients. The alkalinization was not blocked by bilateral removal of Na$^+$ per se, but was abolished at pH$_i$ values below ~6.5. The alkalinization was also unaffected by short-term bilateral removal of Cl$^-$ or SO$_4^2-$ Basolateral CO$_2$/HCO$_3^-$ elicited the usual pH$_i$ increase even when all solutes were replaced, short or long-term (>45 min), by N-methyl-D-glucammonium/glucuronate (NMDG$^+$/Gl$^-$). Luminal CO$_2$/HCO$_3^-$ did not elicit a pH$_i$ increase in NMDG$^+$/Gl$^-$.
sustained pH increase elicited by basolateral CO₂/HCO₃⁻ could be due to a basolateral HCO₃⁻ uptake mechanism, net reabsorption of HCO₃⁻ by the S3 segment, as well as our ACZ data, suggest instead that basolateral CO₂/HCO₃⁻ elicits the sustained pH increase either by inhibiting an acid-loading process or stimulating acid extrusion across the luminal membrane (e.g., via an H⁺ pump).

INTRODUCTION

The reabsorption of HCO₃⁻ by the mammalian proximal tubule is the result of acid-base transport processes at both the luminal and basolateral membrane (for review, see Hamm and Alpern, 1992). The luminal step of HCO₃⁻ reabsorption is the neutralization of luminal HCO₃⁻ by H⁺ that is extruded by the proximal-tubule cell into the lumen. As many as three transporters may contribute to luminal acidification. The first is the Na-H exchanger, which is generally regarded as the dominant mechanism, and was originally proposed by Berliner (Berliner, 1952). This exchanger has been identified both in brush-border membrane vesicles (Murer, Hopfer, and Kinne, 1976; Kinsella and Aronson, 1980) and in intact proximal tubules (Alpern and Chambers, 1986; Sasaki, Shiigai, and Takeuchi, 1985; Schwartz, 1981; Kurtz, 1987; Nakhoul, Lopes, Chaillet, and Boron, 1988). The second mechanism of luminal acidification, demonstrated thus far only in the absence of HCO₃⁻, is the luminal uptake of Na⁺ plus acetate via a Na/acetate cotransporter, followed by the exit of acetic acid across both luminal and basolateral membranes (Nakhoul and Boron, 1988). In the absence of HCO₃⁻, this mechanism is about twice as powerful as the Na-H exchanger for intracellular pH (pHi) regulation, and also makes a substantial contribution to luminal acidification (Geibel, Giebisch, and Boron, 1989). Acetate paradoxically produces a slight inhibition of acid secretion in the presence of HCO₃⁻ (Geibel et al., 1989). A third mechanism that could contribute to luminal acidification is an H⁺ pump. Its existence is supported by two observations. First, HCO₃⁻ reabsorption continues in the absence of Na⁺ (Chan and Giebisch, 1981). Second, Na⁺ removal from both the luminal and basolateral cell surfaces induces a rapid pH decrease followed by a slower pH increase to a value higher than the initial one (Nakhoul and Boron, 1985; Nakhoul et al., 1988). However, it should be pointed out that it is yet to be demonstrated that an H⁺ pump does indeed function at the luminal membrane, or that it plays a role in HCO₃⁻ reabsorption in the presence of Na⁺.

The basolateral step of HCO₃⁻ reabsorption is brought about by the efflux of HCO₃⁻ across the basolateral membrane. The major mechanism of this HCO₃⁻ exit in proximal tubules of both the rat (Alpern, 1985; Yoshitomi, Burckhardt, and Frompter, 1985) and rabbit (Sasaki et al., 1985; Biagi, and Sohtell, 1986; Kurtz, 1989a; Nakhoul, Chen, and Boron, 1990) is believed to be the electrogenic Na/HCO₃ cotransporter (Boron and Boulpaep, 1983). Work on vesicles derived from basolateral membranes of mammalian proximal tubules also provided evidence for Cl/HCO₃ exchange (Low, Friedrich, and Burckhardt, 1984; Grassl, Karniski, and Aronson, 1985), as did work on the LLC-PK₁ cell line (Chaillet, Amsler, and Boron, 1986). Studies of pH regulation have also provided evidence for Cl-HCO₃ exchange at the basolateral membrane of the rabbit S3 proximal tubule (Kurtz, 1989b; Nakhoul et al., 1990). Both the Na/HCO₃ cotransporter and the Cl-HCO₃ exchanger would
mediate the net efflux of HCO₃⁻, and thereby produce a sustained intracellular acid load. Thus, we were surprised at an incidental finding made in an earlier study (Nakhoul et al., 1990): in the absence of acetate, switching the luminal and basolateral buffer from HEPES to CO₂/HCO₃⁻ (at a constant external pH of 7.4) caused a sustained increase in pHᵢ. This last result implies that the net effect of CO₂/HCO₃⁻ is to promote acid extrusion to a greater extent than it promotes acid loading via Na/HCO₃ cotransport and Cl⁻-HCO₃ exchange.

In the present study, we investigated the mechanism of the CO₂/HCO₃⁻-induced alkalinization in isolated perfused S3 segments of the rabbit proximal tubule. Computing pHᵢ from the absorbance spectrum of a pH-sensitive dye, we found that the alkalinization can be elicited by adding CO₂/HCO₃⁻ to the bath (i.e., basolateral solution) only, but not to the lumen only. The basolateral CO₂/HCO₃⁻-induced alkalinization did not require Na⁺ or Cl⁻ and, in fact, occurred when bath HCO₃⁻ was added to tubules bathed in and perfused with a solution in which all solutes were replaced with N-methyl-D-glucammonium/glucuronate. The rate of alkalinization was not reduced by 50 μM DIDS applied shortly after CO₂/HCO₃⁻ washout, but was inhibited ~73% by 100–1,000 μM DIDS applied at least 30 min after CO₂/HCO₃⁻ washout. The alkalinization was blocked by reducing pHᵢ below ~6.5. Because the alkalinization was still extremely fast when carbonic anhydrase was blocked with acetazolamide, it is unlikely that the alkalinization can be accounted for by a HCO₃⁻-influx mechanism. Instead, it is most likely that the pHᵢ increase elicited by basolateral CO₂/HCO₃⁻ is mediated at least in part by a luminal H⁺ pump.

Portions of this work have been published previously in abstract form (Nakhoul and Boron, 1987).

MATERIALS AND METHODS

Biological Preparation

The experiments were performed on the isolated perfused S3 segment of the rabbit proximal tubule, as detailed previously (Nakhoul et al., 1988; Nakhoul and Boron, 1988; Nakhoul et al., 1990). Briefly, we used female, New Zealand white rabbits, weighing 3–5 lbs. Tubules were isolated and perfused at 37°C in a manner similar to that of Burg and co-workers (Burg, Grantham, Abramow, and Orloff, 1966). We isolated the most distal ~1 mm of the proximal tubule, extending to the junction with the thin descending limb. The isolated tubule was transferred to a chamber and then perfused at 37°C. The time constant for bath solution changes was ~2 s. Movement of the tubule in the optical path was minimized by limiting the length of exposed tubule between holding pipettes to 200–400 μm. The tubules can be assumed to have been S3 segments, inasmuch as we isolated the terminal ~1 mm, and perfused considerably less than this (see Kaissling and Kriz, 1979).

Solutions

The compositions of solutions are given in Table 1. All were titrated to 7.40 at 37°C. Nominally HCO₃⁻-free solutions were buffered with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and HCO₃⁻-containing solutions were continuously bubbled with 5% CO₂ at 37°C. Solutions were delivered by gravity to either bath or lumen through CO₂-impermeable Saran tubing (Clarkson Equipment and Controls, Detroit, MI). The osmolality of all solutions was measured before the experiment, and verified to be between 295 and 305 mOsm/kg.
| COMPONENT | HEPS | HEPES | HEPES | HEPES+ | SO4- + | SO4- + | SO4- + | SO4- + | SO4- + | GLUC | GLUC |
|-----------|------|-------|-------|--------|--------|--------|--------|--------|--------|------|------|
| Na+       | 146.4| 153.6 | 153.6 | 146.4  | 153.6  | 153.6  | 153.6  | 153.6  | 153.6  | 153.6 | 153.6 |
| K+        | 5.0  | 5.0   | 5.0   | 5.0    | 5.0    | 5.0    | 5.0    | 5.0    | 5.0    | 5.0  | 5.0  |
| NMDG+     | 0.0  | 0.0   | 0.0   | 0.0    | 0.0    | 0.0    | 0.0    | 0.0    | 0.0    | 0.0  | 0.0  |
| Mg2+      | 1.2  | 1.2   | 1.2   | 1.2    | 1.2    | 1.2    | 1.2    | 1.2    | 1.2    | 1.2  | 1.2  |
| Ca2+      | 1.0  | 1.0   | 1.0   | 1.0    | 1.0    | 1.0    | 1.0    | 1.0    | 1.0    | 1.0  | 1.0  |
| mEq(+)    | 155.8| 163.0 | 155.8 | 163.0  | 157.3  | 164.5  | 163.0  | 163.0  | 162.6  | 163.0 | 155.2 | 163.0 |
| Cl-       | 122.0| 122.0 | 132.0 | 132.0  | 0.0    | 0.0    | 134.4  | 134.4  | 155.4  | 130.8 | 131.4 | 132.0 |
| H2PO4-    | 0.4  | 0.4   | 0.4   | 0.4    | 0.4    | 0.4    | 0.4    | 0.4    | 0.4    | 0.4  | 0.4  |
| HPO4-     | 1.6  | 1.6   | 1.6   | 1.6    | 1.6    | 1.6    | 1.6    | 1.6    | 1.6    | 1.6  | 1.6  |
| Glucuronate| 0.0 | 0.0   | 0.0   | 0.0    | 133.5  | 133.5  | 0.0    | 0.0    | 0.0    | 0.0  | 160.2 |
| HEPES     | 17.8 | 0.0   | 17.8  | 0.0    | 17.8   | 0.0    | 17.8   | 0.0    | 17.8   | 0.0  | 17.8 |
| pH        | 7.4  | 7.4   | 7.4   | 7.4    | 7.4    | 7.4    | 7.4    | 7.4    | 7.4    | 7.4  | 7.4  |

All concentrations are given in mM. NMDG+ is N-methyl-o-glucamine. All HCO3- containing solutions were continuously bubbled with 5% CO2. SITS and DIDS were added directly in powder form to solutions with alanine absent (replaced isosmotically with NaCl).
Na⁺-free solutions, Na⁺ was replaced with N-methyl-D-glucamine (NMDG). In Cl⁻-free solutions, Cl⁻ was replaced with either glucuronate or cyclamate. We did not compensate for the chelation of Ca²⁺ by the Cl⁻ substitute in early experiments with Cl⁻-free/Ca²⁺-containing solutions. However, in later experiments we compensated by raising total Ca²⁺ four-fold, as indicated in Table I. HEPES, NMDG, glucuronate and cyclamate were obtained from Sigma Chemical Co. (St. Louis, MO). 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS; Pfaltz and Bauer, Waterbury, CT) and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS; Sigma Chemical Co.) were added as powders to the appropriate solutions immediately before they were used. The colorless dye precursor 4',5'-dimethyl-5 (and-6)-carboxyfluorescein diacetate (Molecular Probes, Inc., Eugene, OR) was added to solution 1 from a 100 mM stock solution in dimethyl sulfoxide (DMSO; Sigma Chemical Co.), to a final concentration of 100 μM.

**Optical Measurements of Intracellular pH**

Intracellular pH was calculated from absorbance spectra (obtained once per second) of an intracellular pH-sensitive dye, as detailed previously (Chaillet and Boron, 1985). The dye 4',5'-dimethyl-5 (and-6)-carboxyfluorescein was introduced into tubule cells by perfusing the lumen with the dye's colorless and relatively permeant diacetate precursor. The chamber in which the isolated tubule was perfused was located on the stage of an inverted microscope. The tubule rested on a cover slip that constituted the bottom of the chamber. A second cover slip that formed the ceiling of the chamber was fixed to a 32× objective serving as a condenser, so that the tubule and ~1 mm of solution were sandwiched between the two cover slips. A 25-μm diam spot of white light was focused on the tubule, and the transmitted light collected by a 10× objective and focused onto a diffraction grating. The resulting spectrum was then projected on a linear array of 1,024 photodiodes (Princeton Applied Research, Princeton, NJ), and the measured light intensities digitized with 14-bit precision. Adjacent photodiodes were grouped, so that the spectra were made up of 512 data points. An LSI 11/73 computer stored these intensity spectra on a hard disk, from which they were later retrieved for the computation of absorbance spectra. The total measured absorbance was corrected for the absorbance due to the tubule cells (Chaillet and Boron, 1985), yielding the absorbance of the intracellular dye alone. This is done by subtracting the absorbance data at wavelengths >600 nm, a region at which the cells, but not the dye, absorb light. pH, was calculated from the ratio of dye absorbance at the wavelength of peak absorbance (510 nm) to the absorbance near the in-vitro isosbestic wavelength (at 470 nm). We used the same intracellular dye calibration coefficients described previously for the S3 segment (Nakhoul et al., 1988), obtained by clamping pH, to predetermined values using the nigericin/high-K⁺ method (Thomas, Buchsbaum, Zimniak, and Racker, 1979). The calibration curve relating the ratio A₅₁₀/A₄₇₀ to pH, is described by a standard pH titration curve with a pKₐ of 7.26, and lower and upper asymptotes (i.e., ratios) of 0.75 and 2.61, respectively.

**Statistics and Data Analysis**

Means are reported ± the standard error. Statistical significance was judged from paired and unpaired t tests, as indicated in the text. Initial rates of pH, change were determined by using a computer to fit a line to the pH, vs time data.

**RESULTS**

**Effect on pH, of Bilateral CO₂/HCO₃⁻ in the Presence of Acetate**

As summarized in the Introduction, the S3 segment of the rabbit proximal tubule has at least three acid-extrusion mechanisms that function in the nominal absence of
Fig. 1 illustrates the result of an experiment on an isolated perfused S3 proximal tubule, in which the pH buffer was switched bilaterally from HEPES at pH 7.40 (solution 1) to CO$_2$/HCO$_3^-$ at pH 7.4 (solution 2), in the continued presence of 10 mM acetate. This switch caused a rapid and sustained decrease in pH$_i$ (segment $ab$), similar to that observed previously in the salamander proximal tubule (Boron and Boulpaep, 1983a). At least a portion of the pH$_i$ fall is due to the influx of CO$_2$, its hydration to form H$_2$CO$_3$, and the subsequent dissociation into H$^+$ and HCO$_3^-$. The activity of the two HCO$_3^-$-dependent acid loaders, the Na/HCO$_3$ cotransporter and Cl/HCO$_3$ exchanger, may have contributed to the initial pH$_i$ decline, and probably prevented acid-extrusion mechanisms from returning pH$_i$ to its initial value (i.e., at point $a$). In six similar experiments conducted in the presence of acetate, pH$_i$ averaged 7.45 ± 0.07 in HEPES, and 7.18 ± 0.08 in CO$_2$/HCO$_3^-$ buffered solutions; the pH$_i$ decrease averaged 0.26 ± 0.02. The sustained decrease in pH$_i$ implies that, in the presence of acetate, CO$_2$/HCO$_3^-$ promotes intracellular acid loading to a greater extent than acid extrusion.

As noted above, our previous work on the S3 segment has shown that acetate transport is a major mechanism of acid extrusion in the absence of CO$_2$/HCO$_3^-$, and that acetate transport is largely responsible for the high steady-state pH$_i$ maintained in the absence of CO$_2$/HCO$_3^-$ (Nakhoul et al., 1988; Nakhoul and Boron, 1988). To determine if acetate transport contributes to pH$_i$ regulation in the presence of CO$_2$/HCO$_3^-$, we removed acetate bilaterally in the experiment shown in Fig. 1. Acetate removal (solution 3) in the continued presence of CO$_2$/HCO$_3^-$ caused a transient pH$_i$ increase ($bc$), followed by a slower but sustained pH$_i$ decrease ($cd$). When Ac was returned to bath and lumen, the pH$_i$ transients ($def$) were similar but opposite in direction to those elicited by acetate removal. This series of pH$_i$ changes is very similar qualitatively to that caused by removal of acetate in the absence of CO$_2$/HCO$_3^-$ (Nakhoul and Boron, 1988). Thus, by analogy to the previous data, we...
presume that the pH increase in segment-bc is caused by the efflux of acetic acid, whereas the pH decrease in segment-cd reflects the cessation of an acetate-dependent acid-extrusion process. Quantitatively, however, both the transient pH rise (bc) and the sustained pH fall (b vs d) elicited by acetate removal in the presence of CO₂/HCO₃⁻ were substantially smaller than those previously observed in the absence of CO₂/HCO₃⁻. For example, the net pH decrease (b vs d) averaged only (0.06 ± 0.02; n = 4) in the present study, compared to 0.35 previously observed in the absence of CO₂/HCO₃⁻. The smaller magnitude of the transient pH rise elicited by acetate removal probably reflects, at least in part, the higher buffering power expected to prevail in the presence of CO₂/HCO₃⁻ (Nakhoul and Boron, 1988), as well as the lower initial pH in the presence of CO₂/HCO₃⁻. The smaller magnitude of the sustained pH fall caused by acetate removal in CO₂/HCO₃⁻ suggests that the loss of an acetate-dependent acid-extrusion process is partially compensated by a CO₂/HCO₃⁻-dependent acid-extrusion process that is stimulated (and/or an acid-loading process that is inhibited) by the intracellular acidification evoked by acetate removal.

**Figure 2.** Effect of bilateral CO₂/HCO₃⁻ in the absence of acetate. At point a, 10 mM acetate (solution 1) was removed bilaterally (solution 3), causing a transient alkalinization, followed by a sustained decrease. Subsequently, switching the buffer in both the lumen and bath at c from 32 mM HEPES (solution 3) to 5% CO₂/25 mM HCO₃⁻ (solution 4), caused a transient acidification (cd), followed by a sustained pH fall, followed by a sustained increase. We performed 17 similar experiments.

**Effect on pH of Bilateral CO₂/HCO₃⁻ in the Absence of Acetate**

In a second series of experiments, we examined the effect of exposing the tubule bilaterally to CO₂/HCO₃⁻ in the absence of acetate. At the outset of the experiment illustrated in Fig. 2, the luminal and basolateral solutions contained 10 mM acetate and were nominally HCO₃⁻ free. The bilateral removal of acetate (solution 3) caused a transient pH increase (ab), followed by a slower but sustained decrease (bc), as described previously (Nakhoul and Boron, 1988). The subsequent bilateral addition of CO₂/HCO₃⁻ caused a transient acidification (cd), due to the influx of CO₂, followed by a rapid and sustained alkalinization (de) to a level substantially higher than that prevailing in the absence of CO₂/HCO₃⁻ (c vs e). In 17 similar experiments, pH averaged 6.92 ± 0.05 after removal of acetate, and the mean net pH increase caused by bilateral CO₂/HCO₃⁻ was 0.18 ± 0.03. This net alkalinization implies that, in the
absence of acetate, the bilateral addition of CO\(_2\)/HCO\(_3^-\) promotes acid extrusion to a greater extent than acid loading.

The data presented thus far demonstrate that CO\(_2\)/HCO\(_3^-\) has markedly different effects when applied in the presence of acetate, when pH\(_i\) is high (Fig. 1), as opposed to the absence of acetate, when pH\(_i\) is low (Fig. 2). The remainder of our experiments is devoted to characterizing the net pH\(_i\) increase elicited by the addition of CO\(_2\)/HCO\(_3^-\) in the absence of acetate.

Effect on pH\(_i\) of Unilateral CO\(_2\)/HCO\(_3^-\) in the Absence of Acetate

We investigated the sidedness of the CO\(_2\)/HCO\(_3^-\)-induced alkalinization by examining the effect of adding CO\(_2\)/HCO\(_3^-\) to only the lumen or to only the bath. As shown in Fig. 3 A, the bilateral removal of acetate in the absence of CO\(_2\)/HCO\(_3^-\) caused the usual series of pH\(_i\) changes (abc). In the continued absence of acetate, the addition of CO\(_2\)/HCO\(_3^-\) to only the lumen caused a rapid and sustained pH\(_i\) decrease (cd) that was partially reversed by removing the CO\(_2\)/HCO\(_3^-\) (de). The rapid pH\(_i\) decrease was due to the influx of CO\(_2\), possibly augmented by the efflux of HCO\(_3^-\) (mediated by Cl/HCO\(_3^-\) exchange and Na/HCO\(_3^-\) cotransport) across the basolateral membrane. This continuing HCO\(_3^-\) efflux is expected to represent a sustained intracellular acid load and prevent the recovery of pH\(_i\). On the other hand, the addition of CO\(_2\)/HCO\(_3^-\) to only the bath caused only a transient acidification (ef), followed by a sustained alkalinization (fg). Removing the CO\(_2\)/HCO\(_3^-\) from the bath caused the opposite series of pH\(_i\) changes (ghi). Second additions of CO\(_2\)/HCO\(_3^-\) to only the lumen (ij) and only the bath (klm) had the same effects as the first. In three similar paired experiments, the average pH\(_i\) decrease caused by luminal CO\(_2\)/HCO\(_3^-\) was 0.15 ± 0.03, whereas the average pH\(_i\) increase upon exposure to basolateral CO\(_2\)/HCO\(_3^-\) was 0.26 ± 0.01. Thus, the intracellular alkalinization elicited by bilateral CO\(_2\)/HCO\(_3^-\) can be reproduced by addition of CO\(_2\)/HCO\(_3^-\) only to the basolateral surface of the tubule. These results imply that, regardless of whether CO\(_2\)/HCO\(_3^-\) is added bilaterally or only to the bath, the net effect is for CO\(_2\)/HCO\(_3^-\) to promote acid extrusion more than intracellular acid loading.

To confirm that basolateral CO\(_2\)/HCO\(_3^-\) causes a pH\(_i\) increase, regardless of whether or not CO\(_2\)/HCO\(_3^-\) is present in the lumen, we added CO\(_2\)/HCO\(_3^-\) to the bath three times in the same experiment: first in the presence of luminal CO\(_2\)/HCO\(_3^-\), then in the absence of luminal CO\(_2\)/HCO\(_3^-\) and, finally, simultaneously with luminal CO\(_2\)/HCO\(_3^-\). As illustrated in Fig. 3 B, switching from a HEPES to a CO\(_2\)/HCO\(_3^-\) buffer in only the lumen caused a sustained fall in pH\(_i\), (ab), as noted above regarding Fig. 3 A. In the continued presence of luminal CO\(_2\)/HCO\(_3^-\), switching from HEPES to CO\(_2\)/HCO\(_3^-\) in the bath produced a transient pH\(_i\) decrease, followed by a rapid increase to a level substantially higher than the original one (bcd). Sequentially removing the CO\(_2\)/HCO\(_3^-\), first from the bath and then from the lumen, produced a series of pH\(_i\) changes that were the reverse of the ones elicited by adding CO\(_2\)/HCO\(_3^-\) (def and fg). Later in the same experiment, CO\(_2\)/HCO\(_3^-\) was added to the bath in the absence of luminal CO\(_2\)/HCO\(_3^-\), producing pH\(_i\) changes (ghi) that were nearly identical to those observed when CO\(_2\)/HCO\(_3^-\) was present in the lumen (compare with bcd). Finally, after removing CO\(_2\)/HCO\(_3^-\) from the bath caused the usual pH\(_i\) changes (ijk), CO\(_2\)/HCO\(_3^-\) was added to the bath at the same time it was added to the
Figure 3. Effect of uni- or bilateral CO₂/HCO₃⁻. (A) Effect of unilateral CO₂/HCO₃⁻ in the absence of acetate. At point a, 10 mM acetate (solution 1) was removed bilaterally (solution 3), causing a transient alkalinization, followed by a sustained pH decrease. Subsequently switching the buffer in the lumen from 32 mM HEPES (solution 3) to 5% CO₂/25 mM HCO₃⁻ (solution 4), caused a sustained pH decrease (cd) that was reversible (de). Switching the buffer to CO₂/HCO₃⁻ in the bath caused a transient pH fall followed by a sustained increase (efg), and these changes were also reversible (ghi). The responses to a second period of CO₂/HCO₃⁻ from lumen (ghi) or bath (klm) were similar to the first. Three such experiments were performed. (B) Effect of adding basolateral CO₂/HCO₃⁻ in the presence of luminal CO₂/HCO₃⁻, in the absence of luminal CO₂/HCO₃⁻, and together with luminal CO₂/HCO₃⁻. The protocol was similar to that in part (A). Luminal CO₂/HCO₃⁻ was present during the first bath CO₂/HCO₃⁻ pulse (bcd) and absent during the second (ghi). In the third pulse, CO₂/HCO₃⁻ was added to lumen and bath together (klm). Acetate was absent throughout (solutions 3 and 4). Six similar experiments were performed.
lumen. For a third time, the basolateral switch from a HEPES to a CO₂/HCO₃⁻ buffer was followed by a transient pH fall and a sustained rise (klm). These results indicate that the addition of CO₂/HCO₃⁻ to the bath causes a sustained intracellular alkalinization, independent of the status of CO₂/HCO₃⁻ in the lumen.

Effect of Potential Inhibitors

Effect of applying DIDS shortly after removal of CO₂/HCO₃⁻. In the next set of experiments, we examined the effect of DIDS, an inhibitor of anion exchange, on the pH increase caused by basolateral CO₂/HCO₃⁻. As shown in Fig. 4, adding CO₂/HCO₃⁻ to only the bath caused the usual pH changes (abc), which were reversed upon removing CO₂/HCO₃⁻ (cde). Addition of 50 μM DIDS to the bath (e) caused pH to increase slowly (ef), after which we applied CO₂/HCO₃⁻ to the bath a second time.

Figure 4. Effect of DIDS on the basolateral CO₂/HCO₃⁻-induced alkalinization. Switching the basolateral buffer from 32 mM HEPES (solution 3) to 5% CO₂/25 mM HCO₃⁻ (solution 4) caused a sustained pH increase, with only a hint of a CO₂-induced acidification (abc). CO₂/HCO₃⁻ removal caused a partial reversal of the effect (cde). Application of CO₂/HCO₃⁻ a second time, but in the presence of 50 μM DIDS, caused a transient CO₂-induced acidification (fg) followed by an alkalinization (gh) that was even faster than that observed in the absence of DIDS at a comparable pH (e.g., ~7.4). Throughout the experiment, 5% CO₂/25 mM HCO₃⁻ was present in the lumen, and acetate was absent bilaterally. Eight similar experiments were performed. In addition to these experiments with DIDS, we performed six similar experiments in which we found that the alkalinization is unaffected by 0.5 mM SITS.

(f). Not only did basolateral DIDS at this concentration fail to slow the CO₂/HCO₃⁻-induced alkalinization, the drug actually increased the rate of alkalinization (gh) at comparable pH values. Removal of bath CO₂/HCO₃⁻ once again caused the usual series of pH changes (hij). In a series of eight similar paired experiments, we found that the rate of the CO₂/HCO₃⁻-induced alkalinization was 30 ± 3 × 10⁻⁴ pH/s at an average pH of 7.31 ± 0.02 in the absence of DIDS, and 60 ± 9 × 10⁻⁴ pH/s at the same pH in the presence of DIDS. Thus, on average, the second exposure to CO₂/HCO₃⁻, made in the presence of DIDS, elicited a pH increase that was about twice as fast as the first, made in the absence of DIDS.

One explanation for the difference in rates of pH increase in the experiment shown in Fig. 4 is that the second exposure to CO₂/HCO₃⁻ always evokes a faster pH increase, regardless of whether DIDS is present. To explore this possibility, we
followed a protocol similar to that for Fig. 4, except that the DIDS was omitted. The results are summarized in Table II. As suggested by the experiment shown in Fig. 4, we found that the average pH for the initial portion of the alkalinization (points b and g in Fig. 4) was higher for second CO₂/HCO₃⁻ pulse (pHᵢ = 7.17) than for the first (pHᵢ = 7.01). This is consistent with the notion that some of the effects of the CO₂/HCO₃⁻ exposure (i.e., stimulating acid extrusion or inhibiting acid loading) persist even after removal of the CO₂/HCO₃⁻. When measured at comparable pH values, the rate of pHᵢ increase was substantially greater during the second (dpHᵢ/ dt = 53.3 × 10⁻⁴ s⁻¹) than during the first CO₂/HCO₃⁻ pulse (dpHᵢ/ dt = 29.8 × 10⁻⁴ s⁻¹). The ratio of these mean rates in the absence of DIDS was 1.79, somewhat less than the ratio of 2.00 observed when DIDS was present during the second pulse. Thus, we can conclude that, if anything, DIDS accelerated the CO₂/

### Table II

**Rates of Basolateral CO₂/HCO₃⁻-Induced Alkalinization in Back-to-Back CO₂/HCO₃⁻ Pulses***

|                  | First CO₂/HCO₃⁻ pulse: | Second CO₂/HCO₃⁻ pulse: |
|------------------|------------------------|-------------------------|
| **Initial pHᵢ**  |                        |                         |
| dpHᵢ/dt at initial pHᵢ of first pulse | 7.01 ± 0.03            | 7.17 ± 0.07¹           |
| dpHᵢ/dt at a pHᵢ comparable to that of the second pulse | 25.7 ± 3.3 × 10⁻⁴ s⁻¹ | 53.3 ± 8.6 × 10⁻⁴ s⁻¹  |

*The data were obtained on seven tubules that were subjected to the following series of basolateral solution changes: standard HEPES (solution 1) → standard HCO₃⁻ (first CO₂/HCO₃⁻ pulse; solution 4) → standard HEPES (solution 1) → standard HCO₃⁻ (second CO₂/HCO₃⁻ pulse; solution 4). The luminal solutions were standard HEPES (solution 1) throughout. The values presented are means ± SEM. The statistical analyses were the result of paired, single-tailed t tests.

¹p < 0.005, compared to the initial pHᵢ of the first pulse.

²p < 0.01, compared to the dpHᵢ/dt of the first pulse, measured at the initial pHᵢ of the first pulse. p < 0.002 compared to the dpHᵢ/dt of the first CO₂/HCO₃⁻ pulse, measured at the initial pHᵢ of the second pulse.

HCO₃⁻-induced alkalinization in this twin-pulse protocol. Earlier work (Nakhoul et al., 1990) showed that DIDS applied in the presence of CO₂/HCO₃⁻ substantially inhibits the two HCO₃⁻ transporters that act as intracellular acid loaders, the basolateral Cl⁻-HCO₃⁻ exchanger and Na⁺/HCO₃⁻ cotransporter. Thus, our data are consistent with the hypothesis that, shortly after the removal of CO₂/HCO₃⁻, DIDS had no effect on the CO₂/HCO₃⁻-induced alkalinization per se, but inhibited the two acid loaders that otherwise would have opposed the pHᵢ increase.

**Effect of applying DIDS long after removal of CO₂/HCO₃⁻.** In a second series of experiments with DIDS, we examined the effect of the drug on the CO₂/HCO₃⁻-induced alkalinization in tubules that had not been exposed to CO₂/HCO₃⁻ for at least 30 min. We used a single-CO₂/HCO₃⁻-pulse protocol, similar to that followed in the first part of Fig. 4 (abc). In some experiments, 100–1,000 μM DIDS
was added ~5 min before the basolateral CO$_2$/HCO$_3^-$ pulse. In controls performed on the same day, DIDS was absent throughout. We studied up to five tubules per day, all from the same animal. The control (i.e., DIDS-free) experiments usually were the first and last experiments of the day. For the first experiment, the tubule had been incubated in a nominally CO$_2$/HCO$_3^-$-free medium for ~30 min by the time of the basolateral CO$_2$/HCO$_3^-$ pulse (abc in Fig. 4). The second experiment (i.e., the first with DIDS) was begun ~60 min after the first, so that CO$_2$/HCO$_3^-$ had been absent for ~90 min; for the third, the time was ~150 min, and so on. The results of these experiments are summarized in Table III. The pH$_i$ at which the initial pH$_i$ was measured was not significant different between the control and DIDS-treated tubules. However, DIDS substantially slowed the CO$_2$/HCO$_3^-$-induced alkalinization. In the day-matched controls, the mean dpH$_i$/dt was 42.3 $\times$ 10$^{-4}$ s$^{-1}$; this value was between

| Controls: |  |
|-----------|
| Initial pH$_i$ | 7.07 ± 0.02 |
| dpH$_i$/dt at initial pH$_i$ | 42.3 ± 6.1 $\times$ 10$^{-4}$ s$^{-1}$ |
| n | 18 |

| 100–1,000 µM DIDS: |  |
|-------------------|
| Initial pH$_i$ | 7.05 ± 0.02$^\dagger$ |
| dpH$_i$/dt at initial pH$_i$ | 11.6 ± 1.5 $\times$ 10$^{-4}$ s$^{-18}$ |
| n | 19 |

*We measured the initial rate of pH$_i$ increase (dpH$_i$/dt) after switching the bath solution from standard HEPES (solution 1) to standard HCO$_3^-$ (solution 4). The luminal solutions were standard HEPES (solution 1) throughout. Before the CO$_2$/HCO$_3^-$ pulse, the tubules had been bathed in a nominally CO$_2$/HCO$_3^-$-free solution for at least 30 min. The values presented are means ± SEM. The statistical analyses were the result of paired, two-tailed t tests.

$^\dagger$Not significantly different from the initial pH$_i$ of controls (p > 0.6).

Effect of acetazolamide. To determine whether activity of carbonic anhydrase (CA) is required for the development of the CO$_2$/HCO$_3^-$-induced alkalinization, we added CO$_2$/HCO$_3^-$ to the bath with the CA inhibitor acetazolamide (ACZ) present bilater-
ally. As shown in Fig. 5, the bilateral addition of 100 μM ACZ in the nominal absence of CO₂/HCO₃⁻ produced a slight pH decline (ab). In the continued presence of ACZ, switching the bath solution to one buffered with CO₂/HCO₃⁻ caused no observable transient acidification, but a large and rapid alkalinization (bc). The absence of the transient pH decrease was probably due to the combination of the low initial pH and the inhibition of carbonic anhydrase by ACZ, both of which are expected to blunt formation of H⁺ and HCO₃⁻ from CO₂. The absence of a CO₂-induced pH transient is even more striking upon removal of bath CO₂/HCO₃⁻, which elicited a transient alkalinization that was barely perceptible (cd), followed by the large acidification normally observed (de). The same protocol of adding and then removing bath CO₂/HCO₃⁻ was repeated, with similar results (ef and fgh). After ACZ was removed from bath and lumen at h, the addition and removal of bath CO₂/HCO₃⁻ again produced the usual series of pH changes: a transient pH decrease (ij) followed by a rapid increase (jk) upon addition of CO₂/HCO₃⁻, and a transient pH increase (kl) followed by a large decrease (lm) upon removal of CO₂/HCO₃⁻. In a total of six similar paired experiments, ACZ reduced the magnitudes of the transient acidifications and alkalinizations by 76 ± 12% and 67 ± 6%, respectively, but only reduced the initial rate of alkalinization elicited by bath CO₂/HCO₃⁻ from 70 ± 13 × 10⁻⁴ pH/s (mean pHj: 7.10) to 58 ± 8 × 10⁻⁴ pH/s (mean pHj: 7.15; difference in pHj/dt values: N.S.). Thus, although ACZ substantially reduced, as expected, the CO₂-induced pH transient, it had little effect on the basolateral CO₂/HCO₃⁻-induced pH increase. As discussed in the Appendix, this failure of ACZ to produce a substantial inhibition of the CO₂/HCO₃⁻-induced alkalinization makes it very unlikely that the CO₂/HCO₃⁻-induced alkalinization is due to an influx of HCO₃⁻.
Effect of Removing Single Ions

Effect of chloride removal. The Cl-HCO\textsubscript{3} exchanger identified at the basolateral membrane of the S3 segment (Kurtz, 1989b; Nakhoul et al., 1990) appears normally to mediate a net efflux of HCO\textsubscript{3}\textsuperscript{-}, rather than the influx necessary to account for the alkalinization elicited by the simultaneous addition of CO\textsubscript{2}/HCO\textsubscript{3} to lumen and bath. Moreover, the DIDS data from Fig. 4 indicate that, even when CO\textsubscript{2}/HCO\textsubscript{3} is added to only the bath, the two DIDS-sensitive basolateral HCO\textsubscript{3} transporters (i.e., the Cl-HCO\textsubscript{3} exchanger and the Na/HCO\textsubscript{3} cotransporter), taken as a pair, fail to mediate a net influx of HCO\textsubscript{3}\textsuperscript{-}. Nevertheless, to determine if Cl-HCO\textsubscript{3} exchange makes a contribution to the CO\textsubscript{2}/HCO\textsubscript{3}-induced alkalinization, we examined the effect of adding basolateral CO\textsubscript{2}/HCO\textsubscript{3} in the bilateral absence of Cl\textsuperscript{-}. As shown in Fig. 6, bilateral removal of acetate caused the usual series of pH\textsubscript{1} changes (abc).

Bilateral removal and return of Cl\textsuperscript{-} (solution 5) resulted in a reversible increase in pH\textsubscript{1} (cde), due presumably to Cl-base exchange as described previously (Nakhoul et al., 1990). After pH\textsubscript{1} had peaked during a second period of bilateral Cl\textsuperscript{-} removal (ef), the addition of CO\textsubscript{2}/HCO\textsubscript{3} (solution 6) to the bath still caused a transient acidification (fg) followed by a sustained alkalinization (gh). Removal of bath CO\textsubscript{2}/HCO\textsubscript{3} produced the usual series of pH\textsubscript{1} changes (hij). pH\textsubscript{1} did not fully recover until the bilateral return of Cl\textsuperscript{-} (jk), presumably because Cl-base exchange plays a role in the recovery of pH\textsubscript{1} from alkali loads. Finally, the addition of CO\textsubscript{2}/HCO\textsubscript{3} to the bath in the continued presence of Cl\textsuperscript{-} caused a series of pH\textsubscript{1} changes (klm) indistinguishable from those observed in the absence of Cl\textsuperscript{-}. In a total of five similar paired experiments, the mean initial rate of pH\textsubscript{1} increase elicited by bath CO\textsubscript{2}/HCO\textsubscript{3} in the absence of Cl\textsuperscript{-} (e.g., gh) was $123 \pm 26 \times 10^{-4}$ pH/s at an average pH\textsubscript{1} of 7.24 ± 0.13.
The difference between this rate and that observed in the presence of Cl\(^-\), 140 ± 26 \(\times 10^{-4}\) pH/s at an average pH\(_i\) of 7.09 ± 0.12, was not statistically significant.

One caveat in the interpretation of the above experiments is that the cytoplasm may not have been completely Cl\(^-\) free at the time CO\(_2\)/HCO\(_3^-\) was added to the bath. However, [Cl\(^-\)] at the time of CO\(_2\)/HCO\(_3^-\) addition (point g) would have had to be over 16 mM\(^1\) to account for the observed extent of pH\(_i\) increase during segment gh. Further evidence against the involvement of Cl-HCO\(_3^-\) exchange is presented below in connection with Fig. 12. The data in Fig. 6 also show that Cl\(^-\) base exchange is not necessary for at least a part of the pH\(_i\) recovery from an alkali load (ij in Fig. 6).

**Effect of sulfate removal.** Studies on basolateral membrane vesicles from rat kidney cortex indicate that SO\(_4^2^-\)-HCO\(_3^-\) exchange is responsible for proximal-tubule SO\(_4^2^-\) reabsorption (Prichard and Renfro, 1983). Inasmuch as our solutions normally contained 1.2 mM SO\(_4^2^-\), we examined whether SO\(_4^2^-\) is required for the basolateral CO\(_2\)/HCO\(_3^-\)-induced alkalinization. In the experiment illustrated in Fig. 7, SO\(_4^2^-\) was removed bilaterally in the absence of CO\(_2\)/HCO\(_3^-\) (solution 7), causing a slow and very small decrease in pH\(_i\) (ab). In the continued absence of external SO\(_4^2^-\), basolateral addition and then removal of CO\(_2\)/HCO\(_3^-\) (solution 8) caused the usual pH\(_i\) changes (bcede). Even a second application of bath CO\(_2\)/HCO\(_3^-\) caused the usual sustained pH\(_i\) increase (efg). After SO\(_4^2^-\) was returned first to the bath (g) and then to the lumen (h), the sequential removal (ijk), addition (klm) and removal (mno) of bath CO\(_2\)/HCO\(_3^-\) was required that ~16.6 mM of acid be extruded from the cell. If reversed Cl-HCO\(_3^-\) exchange were responsible for this acid extrusion, 16.6 mM of Cl\(^-\) would have had to have left the cell.

\(^1\) We assume that intrinsic (i.e., non-CO\(_2\) buffering) power averages 35 mM between pH\(_i\) 6.85 and 7.15 (Chen and Boron, unpublished observations). We also assume that [CO\(_2\)] is 1.2 mM, and the pK of CO\(_2\)/HCO\(_3^-\) is 6.1. After the initial CO\(_2\)-induced acidification (fg in Fig. 6), pH\(_i\) rose from ~6.85 to ~7.15. Analyzing this problem using a Davenport diagram indicates that this pH\(_i\) increase required that ~16.6 mM of acid be extruded from the cell.
caused pH_{i} changes that were very similar\(^2\) to those observed in the absence of SO\(_{4}^-\). In four such experiments, the initial rate of CO\(_2\)/HCO\(_3^-\)-induced alkalinization was hardly affected by the absence of SO\(_{4}^-\), averaging 114 ± 19 × 10\(^{-4}\) pH/s in the presence of SO\(_{4}^-\) (mean pH\(_i\), 7.19 ± 0.05) and 117 ± 14 × 10\(^{-4}\) pH/s in the absence of SO\(_{4}^-\) (mean pH\(_i\), 7.30 ± 0.06).

It is true that some SO\(_{4}^-\) might have remained in the cytoplasm at the time of the first basolateral addition of CO\(_2\)/HCO\(_3^-\) (b in Fig. 7). However, as outlined in footnote 1 for Cl-HCO\(_3^-\) exchange, it is highly unlikely that sufficient SO\(_{4}^-\) could have been inside the cell to support the required degree of SO\(_4^-\)/HCO\(_3^-\) exchange for two separate CO\(_2\)/HCO\(_3^-\)-induced alkalinizations (bc and fg). These results also indicate that SO\(_4^-\)/HCO\(_3^-\) exchange could not have accounted for the pH\(_i\) recovery from the alkali load (e.g., segment de) observed after CO\(_2\)/HCO\(_3^-\) removal, inasmuch as SO\(_{4}^-\) was absent from the outside of the cell.

**Addition of CO\(_2\)/HCO\(_3^-\) in the continued presence of a low level of HEPES.** The HCO\(_3^-\)-free solutions in our experiments were normally buffered with 32 mM HEPES (e.g., solution 1), whereas our CO\(_2\)/HCO\(_3^-\)-containing solutions were normally HEPES free. If HEPES could enter the cells during the exposure to CO\(_2\)/HCO\(_3^-\)-free solutions, it theoretically would be possible for the anionic form of cellular HEPES to exchange for bath HCO\(_3^-\), and thus mediate the CO\(_2\)/HCO\(_3^-\)-induced alkalinization. This would not be a very efficient means of alkalinizing the cell because the efflux of anionic HEPES would tend to lower pH\(_i\). Nevertheless, we explored the possibility that HEPES transport contributes to the CO\(_2\)/HCO\(_3^-\)-induced alkalinization in experiments in which we reduced [HEPES]\(_o\) from 32 to 2 mM, and maintained [HEPES]\(_o\) at 2 mM even during the addition of CO\(_2\)/HCO\(_3^-\). As shown in Fig. 8, lowering [HEPES]\(_o\) in both the lumen and bath from 32 to 2 mM (solution 9) caused, at most, a slight pH\(_i\) increase (ab). When we added and then removed basolateral CO\(_2\)/HCO\(_3^-\) in the continued presence of 2 mM HEPES (solution 10), so that there was no change in [HEPES]\(_o\), we observed the usual pH\(_i\) changes (bcde). The pH\(_i\) changes were not substantially affected by simultaneously removing the 2-mM basolateral HEPES from the bath at the same time we added the CO\(_2\)/HCO\(_3^-\) to the bath (efghi). At i, [HEPES]\(_o\) was returned to 32 mM in both lumen and bath, with no effect on pH\(_i\) (ij). A third period of basolateral CO\(_2\)/HCO\(_3^-\) addition and removal (with CO\(_2\)/HCO\(_3^-\) replacing the 32 mM HEPES), caused the usual pH\(_i\) changes (jklmn).

In four similar paired experiments, the initial rate of CO\(_2\)/HCO\(_3^-\)-induced alkalinization was 131 ± 17 × 10\(^{-4}\) pH/s in the continued presence of 2 mM basolateral HEPES (mean pH\(_i\); 7.01 ± 0.01), and 128 ± 14 × 10\(^{-4}\) pH/s with the simultaneous removal of 2 mM bath HEPES (mean pH\(_i\); 7.06 ± 0.07). These experiments argue against a significant HEPES-dependence of the CO\(_2\)/HCO\(_3^-\)-induced alkalinization. Moreover, unless [HEPES]\(_o\) were extraordinarily high, or unless intracellular HEPES lost via HEPES-HCO\(_3^-\) exchange could be rapidly replenished by extracellular HEPES, it would be impossible for HEPES transport to account for the CO\(_2\)/

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2 The only significant difference in this experiment was that the CO\(_2\)-induced pH\(_i\) transients in the presence of SO\(_{4}^-\) (ij, kl, and mn) were substantially larger than in the absence of SO\(_{4}^-\) (b, cd, and ef). This reflects the higher pH\(_i\) prevailing in the latter half of the experiment, in the presence of SO\(_{4}^-\).
HCO₃⁻-induced alkalinization. This issue is addressed further in the experiment illustrated in Fig. 12.

Effect of sodium removal. As many as four Na⁺-dependent acid-base transporters have been identified in mammalian proximal tubules, including a luminal Na-H exchanger (Alpern and Chambers, 1986; Nakhoul and Boron, 1985), a luminal Na/monocarboxylate cotransporter (Nakhoul et al., 1988; Nakhoul and Boron, 1988), a basolateral Na/HCO₃ cotransporter (Kurtz, 1989b; Nakhoul et al., 1990), and a basolateral Na⁺-dependent Cl-HCO₃ exchanger (Alpern and Chambers, 1987). In principle, any of these could have been stimulated by basolateral CO₂/HCO₃⁻, and thus produced an alkalinization. The involvement of acetate has been ruled out by experiments conducted in the absence of acetate. We attempted to rule out the others in a series of experiments, the first of which is shown in Fig. 9A. The simultaneous removal of acetate and Na⁺ from lumen and bath (solution 11) caused a transient pHᵢ increase (ab), followed by an extreme acidification (bc). This pattern has been observed previously (Nakhoul and Boron, 1988). The basolateral addition of CO₂/HCO₃⁻ (solution 12) and then its removal (solution 11), in the continued absence of acetate and Na⁺, elicited no discernible pHᵢ change (cd). The lack of CO₂-induced pHᵢ changes (i.e., a reversible acidification) was expected, given the low pHᵢ. When the bath CO₂/HCO₃⁻ was added a second time, there was a slow alkalinization (ef); however, this was not reversed by the removal of bath CO₂/HCO₃⁻ (fg). Thus, the slow pHᵢ increase during efg was probably due to the Na⁺-independent acid-extrusion mechanism identified previously (Nakhoul and Boron, 1985; Nakhoul et al., 1988). The bilateral readdition of Na⁺ produced a rapid pHᵢ increase (gh). During this period of rapid pHᵢ recovery in the presence of Na⁺, basolateral addition of CO₂/HCO₃⁻ produced a transient acidification (hi), followed by the usual CO₂/HCO₃⁻-induced alkalinization (ij). Removal of CO₂/HCO₃⁻ had the opposite effects (kl).

On the surface, these data suggest that the CO₂/HCO₃⁻-induced alkalinization is
FIGURE 9. Effect of bilateral Na⁺ removal on the basolateral CO₂/HCO₃⁻-induced alkalinization. (A) Apparent blockade by bilateral Na⁺ removal. At point a (solution 1), Na⁺ and acetate were each removed bilaterally (solution 11), causing a transient pH increase followed by a profound decrease (abc). During two subsequent exposures to basolateral CO₂/HCO₃⁻ (cd and ef; solution 12), pH₁ was not affected differently than in adjacent CO₂/HCO₃⁻-free periods (de and fg). After Na⁺ was reintroduced bilaterally and pH₁ recovered (gh), the basolateral application (hij) and removal (jkl) of CO₂/HCO₃⁻ had the usual effects. Ten similar experiments were performed. (B) Lack of blockade by bilateral Na⁺ removal. The protocol was similar to that in part (A) except that the bilateral removal of acetate (point a) and the sequential removal of Na⁺ from lumen (a) and bath (c) happened to cause pH₁ to fall only to ~6.6 (abcdef). On two occasions (ef and ij), the application of CO₂/HCO₃⁻ in the continued bilateral absence of Na⁺ elicited rapid alkalinizations. We performed 11 similar experiments.
indeed Na⁺ dependent. However, it should be noted that the removal of Na⁺ also lowered pHᵢ. Thus, it is possible that the blockade of the basolateral CO₂/HCO₃⁻-induced alkalinization was not due to the absence of Na⁺ per se, but by the extremely low pHᵢ. We found the results of several repetitions of the Fig. 9 A protocol to be inconsistent. In ten tubules, Na⁺ removal blocked the CO₂/HCO₃⁻-induced alkalinization, whereas in 11 others, Na⁺ removal seemingly had no effect. The results from one of this second group of tubules is illustrated in Fig. 9 B. The bilateral removal of acetate together with the luminal removal of Na⁺ caused a transient pHᵢ increase (ab), followed by a large decrease (bc). pHᵢ decreased further when Na⁺ was removed from the bath (cd). In the continued bilateral absence of Na⁺, there was a slow, spontaneous recovery of pHᵢ (de), due to the Na⁺-independent acid-extrusion mechanism (Nakhoul et al., 1988), possibly a luminal H⁺ pump. The subsequent addition of CO₂/HCO₃⁻ elicited the usual alkalinization (ef); the absence of the CO₂-induced pHᵢ transient at e is expected, given the low pHᵢ. Removal of bath CO₂/HCO₃⁻ elicited a small CO₂-induced pHᵢ increase (fg), which was followed by Na⁺-independent pHᵢ recovery (gh). Finally, a second application of bath CO₂/HCO₃⁻ elicited the usual transient acidification and sustained alkalinization (hi).

Three conclusions can be drawn from the experiment shown in Fig. 9, A and B. First, the CO₂/HCO₃⁻-induced alkalinization is not blocked by the bilateral removal of Na⁺ per se (Fig. 9 B). Second, the rate of CO₂/HCO₃⁻-induced alkalinization was greater at higher pHᵢ (ij in Fig. 9 B) than at low pHᵢ (ef, Fig. 9 B), suggesting that the process may be pHᵢ dependent. Third, closer examination of the experiments done according to the protocol of Fig. 9, A and B, revealed that pHᵢ was very low (<6.6, averaging 6.50 ± 0.05) in the ten experiments in which Na⁺ removal blocked the CO₂/HCO₃⁻-induced alkalinization, but that pHᵢ was relatively high (>6.6; averaging 7.01 ± 0.07) in the experiments in which Na⁺ removal failed to block the alkalinization.

pHᵢ Dependence

To determine whether pHᵢ controls the rate of the CO₂/HCO₃⁻-induced alkalinization, we performed the experiments illustrated in Fig. 10, A and B. In the experiment shown in Fig. 10 A, the bilateral removal of acetate and the sequential removal of luminal and basolateral Na⁺ caused the usual pHᵢ transients (abcd), and resulted in a pHᵢ of ~6.4. In the continued absence of Na⁺, switching from a HEPES to a CO₂/HCO₃⁻ buffer in the bath produced a small CO₂-induced acidification (de), followed by a slow alkalinization (ef). However, this slow pHᵢ increase was probably mediated by the Na⁺-independent acid-extrusion mechanism, inasmuch as pHᵢ increased at about the same rate after the removal of bath CO₂/HCO₃⁻ (gh). This result confirms the result summarized above: the CO₂/HCO₃⁻-induced alkalinization is absent when pHᵢ is below ~6.6. At h we restored the Na⁺ to only the lumen, which served two purposes. First, it caused pHᵢ to increase to ~6.88 (hi), presumably due to luminal Na-H exchange, so that we could examine the effect of bath CO₂/HCO₃⁻ at a higher pHᵢ. Second, it established a cell-to-bath concentration gradient for Na⁺, so that any Na/HCO₃⁻ cotransport would have to occur in the cell-to-bath direction. When we switched the basolateral buffer to CO₂/HCO₃⁻ after pHᵢ had reached ~6.88, an initial fall of pHᵢ (ij) was followed by a CO₂/HCO₃⁻-induced alkalinization (jk), even though Na⁺ was still absent from the bath. In six similar experiments, we found that the
Figure 10. Dependence of the basolateral CO₂/HCO₃⁻-induced alkalinization on pHᵢ. (A) pHᵢ increased by exposure to luminal Na⁺. The bilateral removal of acetate (point a) and the sequential removal of Na⁺ from lumen (a) and bath (c; solution 11) caused pHᵢ to fall (abcd) to a value sufficiently low (i.e., ~6.4) to block the CO₂/HCO₃⁻-induced alkalinization. In the continued bilateral absence of Na⁺, the basolateral application of CO₂/HCO₃⁻ (ef; solution 12) produced no more rapid a pHᵢ increase than the adjacent CO₂/HCO₃⁻-free period (gh). After readdition of Na⁺ to only the lumen caused a partial pHᵢ recovery (hi), the basolateral application of CO₂/HCO₃⁻ caused the usual transient pHᵢ decrease followed by the sustained increase (ijk). Six similar experiments were performed. (B) pHᵢ allowed to increase via the Na⁺-independent acid-extrusion mechanism. Acetate and Na⁺ had been removed bilaterally before the start of the record, causing pHᵢ to fall to nearly 6.3, and were absent thereafter. During five periods in which 5% CO₂/25 mM HCO₃⁻ was present in the bath (bed, fgh, jkl, nop, and rst), the rate of pHᵢ increase, compared to the preceding and succeeding CO₂/ HCO₃⁻-free periods, gradually rose as pHᵢ rose. Four similar experiments were performed.
CO₂/HCO₃⁻-induced alkalinization was imperceptible at an average pHᵢ of 6.50 ± 0.03 in the absence of luminal Na⁺, but occurred at a rate of 45 ± 10 × 10⁻⁴ s⁻¹ at an average pHᵢ of 6.80 ± 0.03 in the presence of luminal Na⁺. Thus, by using the luminal Na-H exchanger to raise pHᵢ, we were able to show that the CO₂/HCO₃⁻-induced alkalinization is independent of bath Na⁺, but inhibited at pHᵢ values as below ~6.5.

In Fig. 10 A, we raised pHᵢ during the experiment by exposing the tubule to luminal Na⁺. To rule out the possibility that luminal Na⁺, rather than pHᵢ, is the key factor for the basolateral CO₂/HCO₃⁻-induced alkalinization, we used a protocol similar to that of Fig. 10 A, but allowed the Na⁺-independent acid-extrusion mechanism to raise pHᵢ in the continuous bilateral absence of Na⁺. In the experiment shown in Fig. 10 B, both acetate and Na⁺ had been removed bilaterally (not shown), so that pHᵢ had fallen to ~6.33 by the time point a was reached. The first of five basolateral additions of CO₂/HCO₃⁻ caused a barely perceptible acidification (bc), followed by a slow alkalinization (cd). However, this slow pHᵢ increase was probably caused by the Na⁺-independent acid-extrusion mechanism, inasmuch as the rate of alkalinization was even greater after CO₂/HCO₃⁻ removal (ef). The rate of pHᵢ increase during a second period of CO₂/HCO₃⁻ (gh), at a pHᵢ of ~6.43, was slightly greater than during the preceding (ef) and succeeding (ij) CO₂/HCO₃⁻-free periods, indicating the presence of a small CO₂/HCO₃⁻-induced alkalinization. During the third CO₂/HCO₃⁻ pulse (kl), at a pHᵢ of ~6.6, the alkalinization rate exceeded that during the bracketing CO₂/HCO₃⁻-free periods (ij and mn) by an even greater margin. Similarly, the rates of alkalinization during the fourth (op) and fifth (st) CO₂/HCO₃⁻ pulses were substantially greater than the adjacent CO₂/HCO₃⁻-free periods. Comparable results were obtained in four other experiments. Thus, even in the bilateral absence of Na⁺, the basolateral CO₂/HCO₃⁻-induced alkalinization was absent at pHᵢ values below ~6.4, and became increasingly more prominent at higher pHᵢ values.

We performed five other experiments (not shown) similar to that shown in Fig. 10 B, but in which we removed Cl⁻ bilaterally, in addition to removing acetate and Na⁺. The results were comparable to those of Fig. 10 B: The basolateral CO₂/HCO₃⁻-induced alkalinization was minimal at low pHᵢ values, but gradually increased as pHᵢ increased.

Data on the rates of 119 CO₂/HCO₃⁻-induced alkalinizations (all protocols) are plotted as a function of pHᵢ in Fig. 11. The plot shows that the rate of CO₂/HCO₃⁻-induced alkalinization is absent at a pHᵢ of ~6.3, is minimal at a pHᵢ of ~6.5, peaks at a pHᵢ of ~7.0, and then falls off slightly at higher pHᵢ values.

**Effect of Removing all Solutes Except H⁺ and CO₂/HCO₃⁻**

As indicated in Table I, our solutions routinely contained a variety of solutes in addition to the ones whose importance for the CO₂/HCO₃⁻-induced alkalinization we had already tested individually (i.e., Na⁺, Cl⁻, SO₄²⁻, HEPES). Rather than individually assess each of these, we removed all of them simultaneously. The normal CO₂/HCO₃⁻-free saline was replaced with an isotonic solution of N-methyl-D-glucammonium/glucuronate (NMDG⁺/Glr⁻) titrated to pH 7.40 (solution 13). Although NMDG⁺ is a very weak acid and Glr⁻, a very weak base, their concentrations are so high that NMDG⁺/Glr⁻ has a measured buffering power of 3.6 mM/pH unit. As
illustrated in Fig. 12 A, the bilateral replacement of the standard CO₂/HCO₃⁻-free saline with NMDG⁺/Glr⁻ caused pHᵢ to fall rather rapidly by ~0.4, and then to continue to fall more slowly (ab). After the NMDG⁺/Glr⁻ had been present ~3 min, CO₂/HCO₃⁻ was added to the bath (HCO₃⁻ replacing Glr⁻; solution 14). This elicited the characteristic alkalinization (bc) that was reversed by the removal of the CO₂/HCO₃⁻ (cde), and reproduced by a second exposure to basolateral CO₂/HCO₃⁻ (ef).

Fig. 12 B shows the results of a similar experiment in which the NMDG⁺/Glr⁻ had been present since the time of dissection, a total of >45 min. The application of basolateral CO₂/HCO₃⁻, at an initial pHᵢ of ~6.75, produced the typical transient acidification and sustained alkalinization (bc). Thus, the basolateral CO₂/HCO₃⁻-induced alkalinization can be elicited in tubules exposed, even for prolonged periods, to solutions containing no other natural solutes other than H⁺ and CO₂/HCO₃⁻.

The data presented in this paper consistently showed that the CO₂/HCO₃⁻-induced alkalinization is produced by a basolateral, but not a luminal, addition of CO₂/HCO₃⁻. However, one might posit another explanation for this asymmetry: The alkalinizing effect of CO₂/HCO₃⁻ actually can be produced by either luminal or basolateral CO₂/HCO₃⁻, but luminal CO₂/HCO₃⁻ has an additional acidifying effect that exactly balances the alkalinizing effect. This additional acidifying effect could be produced by basolateral Cl⁻-HCO₃ exchange and Na⁺/HCO₃ co-transport, supported by the large cell-to-bath HCO₃⁻ gradient expected to prevail when CO₂/HCO₃⁻ is present only in the lumen. Although the results of the DIDS experiments (see Fig. 4) make this hypothesis unlikely, it might be argued that the DIDS may not have fully inhibited the aforementioned HCO₃⁻ transporters. Therefore, we tested the hypothesis in the experiment shown in Fig. 12 C. The protocol was similar to that in Fig. 12 A, except that the CO₂/HCO₃⁻ was twice added and then removed from the lumen before being added to the bath. NMDG⁺/Glr⁻ solutions, applied to the lumen and bath several minutes before the start of the record in Fig. 12 C, caused a gradual acidification (not shown). The first application of luminal CO₂/HCO₃⁻ (ab) had little effect on this acidification, which continued after removal of the CO₂/HCO₃⁻ (ad). The second luminal application of CO₂/HCO₃⁻ (de) also did not seem to affect pHᵢ. However, when the CO₂/HCO₃⁻ was applied to the bath, pHᵢ rose rapidly (fg). The results of twin luminal CO₂/HCO₃⁻ exposures in a total of five similar experiments are summarized in Table IV. They show that, even when
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FIGURE 12. Effect of replacing all solutes with N-methyl-D-glucammonium/glucuronate. 
(A) Short-term removal. All luminal and basolateral solutes were replaced by N-methyl-D-glucammonium/glucuronate (solution 13) at point a, causing a rapid and then slower fall in pH_i (ab). In the continued absence of other solutes, 5% CO_2/25 mM HCO_3^- (solution 14) was twice added to the bath (bc and ef), each time causing a rapid increase in pH_i. Acetate was absent throughout the experiment. Three similar experiments were performed. (B) Long-term removal. All solutes were replaced by N-methyl-D-glucammonium/glucuronate (solution 13) from the time of dissection, more than 45 min before the beginning of this record. The addition of CO_2/HCO_3^- to the bath (solution 14) caused a very small, transient acidification, followed by rapid alkalinization (bc). A similar CO_2/HCO_3^- pulse was applied several minutes before the one shown here. Three similar experiments were performed. (C) Short-term removal, with CO_2/HCO_3^- added to the lumen. All solutes were replaced by N-methyl-D-glucammonium/glucuronate (solution 13) ~ 6 min before the start of this record. During the two additions of CO_2/HCO_3^- (solution 14) to the lumen (ab and de), the rate of pH_i change was little different from the succeeding CO_2/HCO_3^- free periods (cd and ef). However, addition of CO_2/HCO_3^- to the bath caused a rapid pH_i increase (fg). Five similar experiments were performed.
basolateral Cl-HCO₃ exchange and Na/HCO₃ cotransport are blocked by removal of key ions (i.e., Cl⁻ and Na⁺, respectively), luminal CO₂/HCO₃⁻ fails to elicit the pHᵢ increase produced by basolateral CO₂/HCO₃⁻.

**DISCUSSION**

**General**

Previous work has demonstrated that the basolateral membrane of the S3 segment of the rabbit proximal tubule possesses at least two HCO₃⁻ transporters, a Cl-HCO₃ exchanger and a Na/HCO₃ cotransporter (Kurtz, 1989b; Nakhoul et al., 1990).

| Table IV |
| --- |
| Effect of Twin Luminal CO₂/HCO₃ Pulses in Tubules Exposed to NMDG⁺/Glu⁻ Solutions in Lumen and Bath* |
| First luminal CO₂/HCO₃ pulse: |  |
| Initial pHᵢ | 6.78 ± 0.09 |
| dpHᵢ/dt at initial pHᵢ | -2.7 ± 3.1 x 10⁻⁴ s⁻¹ |
| control dpHᵢ/dt | -0.5 ± 2.7 x 10⁻⁴ s⁻¹ |
| n | 4 |
| Second luminal CO₂/HCO₃ pulse: |  |
| Initial pHᵢ | 6.77 ± 0.10 |
| dpHᵢ/dt at initial pHᵢ | -1.0 ± 1.3 x 10⁻⁴ s⁻¹ |
| control dpHᵢ/dt | -2.8 ± 1.8 x 10⁻⁴ s⁻¹ |
| n | 5 |

*We measured the initial rate of pHᵢ change (dpHᵢ/dt) after switching the luminal solution from NMDG⁺/Glu⁻ (solution 13) to NMDG⁺/Glu⁻ plus CO₂/HCO₃⁻ (solution 14). The bath solutions were NMDG⁺/Glu⁻ (solution 13) throughout. The "control dpHᵢ/dt" is the rate of pHᵢ change immediately before CO₂/HCO₃⁻ was added to the lumen. Negative values for dpHᵢ/dt indicate that pHᵢ was decreasing with time. The values presented are means ± SEM. The statistical analyses were the result of paired, one-tailed t tests.

1After addition of luminal CO₂/HCO₃⁻ the rate of pHᵢ decrease was significantly greater than the paired control (p < 0.02).

*Not significantly different from the paired control (p > 0.2).

Moreover, the data indicate that both of these transporters function as intracellular acid loaders, contributing to the net efflux of HCO₃⁻ across the basolateral membrane. Direct measurements confirm that transepithelial HCO₃⁻ reabsorption occurs in the rabbit S3 proximal tubule, and is blocked by basolateral SITS (Geibel et al., 1989). Considering the role played by the Cl-HCO₃ exchanger and Na/HCO₃ cotransporter in HCO₃⁻ reabsorption, we expected that the bilateral transition from HEPES- to CO₂/HCO₃⁻-buffered solutions would have activated these two potent intracellular acid loaders, and thereby produced a sustained decrease in pHᵢ. Indeed, with the high initial pHᵢ prevailing in the presence of acetate, the addition of CO₂/ HCO₃⁻ did cause a sustained pHᵢ decrease. However, with the low initial pHᵢ prevailing in the absence of acetate, bilateral application of CO₂/HCO₃⁻ caused a substantial increase in steady-state pHᵢ. Even more surprising, the alkalinization can be attributed specifically to the presence of CO₂/HCO₃⁻ at the basolateral membrane.
Evidence Against HCO$_3^-$ Influx Across the Basolateral Membrane

The increase in steady state pH produced by bilateral or basolateral CO$_2$/HCO$_3^-$ in the absence of acetate implies that CO$_2$/HCO$_3^-$ somehow stimulates acid-extruding processes more than it stimulates acid-loading processes. In this and the following two sections, we will consider three general mechanisms by which basolateral CO$_2$/HCO$_3^-$ could lead to an increase in steady-state pH. The first, and perhaps most obvious, is that basolateral application of HCO$_3^-$ leads to a net uptake of HCO$_3^-$ across the basolateral membrane. However, four lines of evidence indicate that basolateral acid extrusion does not occur.

First, the S3 segment of the rabbit proximal tubule is a net reabsorber of HCO$_3^-$ (Geibel et al., 1989). This reabsorbed HCO$_3^-$ appears to cross the basolateral membrane via the Na/HCO$_3^-$ cotransporter and the Cl-HCO$_3^-$ exchanger. In the presence of CO$_2$/HCO$_3^-$, blockade of these transporters by DIDS produces both an increase in steady-state pH (Nakhoul et al., 1990) and a substantial inhibition of HCO$_3^-$ reabsorption (Geibel et al., 1989). Thus, because HCO$_3^-$ reabsorption requires that there be a net efflux of HCO$_3^-$ across the basolateral membrane, the bilateral addition of CO$_2$/HCO$_3^-$ cannot produce an intracellular alkalinization by instituting a net influx of HCO$_3^-$ across the basolateral membrane.

The second line of evidence against basolateral HCO$_3^-$ influx as an explanation for the CO$_2$/HCO$_3^-$-induced alkalinization is our observation that blocking known HCO$_3^-$ transporters failed to block the alkalinization. Four HCO$_3^-$ transporters have been described in animal cells: the Na$^+$-dependent Cl-HCO$_3^-$ exchanger, the Na/HCO$_3^-$ cotransporter, the Cl-HCO$_3^-$ exchanger, and a SO$_4$-HCO$_3^-$ exchanger. All four are blocked by stilbene derivatives such as DIDS, and we have previously shown that a brief exposure to this agent blocks Cl-HCO$_3^-$ exchange and substantially inhibits Na/HCO$_3^-$ cotransport in the S3 segment. Nevertheless, DIDS applied shortly after the removal of CO$_2$/HCO$_3^-$ failed to inhibit the alkalinization produced by a later application of basolateral CO$_2$/HCO$_3^-$.

An alkalinization mediated by either a Cl-HCO$_3^-$ exchanger or a Na$^+$-dependent Cl-HCO$_3^-$ exchanger would require intracellular Cl$. Yet even prolonged Cl$^-$ washouts failed to slow the pH increase. An alkalinization mediated by either a Na/HCO$_3^-$ cotransporter or a Na$^+$-dependent Cl-HCO$_3^-$ exchanger would require extracellular Na$. Yet, bilateral Na$^+$ removal had no effect on the pH increase. An alkalinization mediated by a SO$_4$-HCO$_3^-$ exchanger would require intracellular SO$_4$$. Yet bilateral SO$_4$$^2-$ removal did not slow the pH increase, even when two CO$_2$/HCO$_3^-$ pulses were delivered to the same tubule.

Based on the above results, it is not clear how the CO$_2$/HCO$_3^-$-induced alkalinization could be mediated by a known HCO$_3^-$ transporter. The alkalinization also was not blocked by maneuvers (e.g., Na$^+$ removal, acetate removal) that should have blocked acid-extrusion mechanisms independent of HCO$_3^-$ (e.g., Na-H exchange and Na/monocarboxylate cotransport, respectively). Finally, our observation that the CO$_2$/HCO$_3^-$-induced alkalinization was not blocked by a lengthy preincubation in NMDG$^+$/Glu$^-$ suggests that the alkalinization was not mediated by a transporter requiring any of the ions (other than H$^+$ or HCO$_3^-$) present in our standard saline.

The third line of evidence against basolateral HCO$_3^-$ efflux as an explanation for the CO$_2$/HCO$_3^-$-induced alkalinization is that the passive movement of HCO$_3^-$ would be in the outward direction, and thereby tend to acidify the cell. Microelectrode data
(Biagi and Vance, 1989) indicate that the basolateral membrane potential ($V_{bl}$) of the rabbit S3 segment is $-69$ mV in the bilateral presence of CO$_2$/HCO$_3^-$. Given a basolateral pH of 7.40, this $V_{bl}$ predicts an equilibrium pH$_i$ of 6.25, considerably less than the measured value of 7.18. Thus, the gradient for HCO$_3^-$ diffusion is outward, not inward as required to explain the alkalinization produced by the bilateral application of CO$_2$/HCO$_3^-$. 

The fourth line of evidence, presented in the Appendix, is that it is highly unlikely that, in the presence of ACZ, any mechanism involving the influx of HCO$_3^-$ could produce the rapid pH$_i$ increase that begins <5 s after the application of CO$_2$/HCO$_3^-$. The salient feature of this analysis is that, with minimal CA activity, pH$_i$ could rise (i.e., H$^+$ could be consumed) as rapidly as observed only if [H$_2$CO$_3$], and therefore [HCO$_3^-$], were extremely high. Our calculations show that [H$_2$CO$_3$] would have to rise from practically zero to ~6 $\mu$M within the 5-s delay that separates the application of CO$_2$/HCO$_3^-$ from the initiation of the alkalinization. At a pH$_i$ of ~7.1, this would require that [HCO$_3^-$], rise from practically zero to ~25 mM in this same ~5 s. The necessary HCO$_3^-$ flux, averaged over this initial 5 s, would thus be ~5 mM/s. This seems unreasonably high. By comparison, if the CO$_2$/HCO$_3^-$-induced alkalinization were produced by an H$^+$ efflux, the flux would have to be only ~0.17 mM/min. A further complication of the HCO$_3^-$-influx model is that after "priming" [HCO$_3^-$], to 25 mM in the first 5 s, the hypothetical HCO$_3^-$-influx mechanism would have to slow to a sustaining flux of only ~0.17 mM/min. This slowing would have to occur over a very short time, and with very little change in pH$_i$. Thus, it is difficult to account for the basolateral CO$_2$/HCO$_3^-$-induced alkalinization on the basis of an influx of HCO$_3^-$.

**Possible Inhibition of Acid Loading**

According to the second general model that we will consider for the CO$_2$/HCO$_3^-$-induced alkalinization, the primary event need not be a stimulation of acid extrusion. Instead, the CO$_2$/HCO$_3^-$ could inhibit acid loading. In the continued presence of an uninhibited acid extruder, this would lead to an increase in pH$_i$. The two known acid-loading mechanisms in this segment of the proximal tubule are the Cl-HCO$_3^-$ exchanger and the Na/HCO$_3^-$ cotransporter. Even if these transporters function as acid loaders in the nominal absence of HCO$_3^-$ (employing OH$^-$ or metabolically generated HCO$_3^-$), it is doubtful that either is inhibited by the addition of the substrate HCO$_3^-$. S3 cells probably have other acid-loading mechanisms (e.g., metabolic production of H$^+$), and it is possible that these are affected by CO$_2$/HCO$_3^-$. In experiments on suspensions of rabbit proximal tubules, Dickman and Mandel (Dickman and Mandel, 1992) have shown that CO$_2$/HCO$_3^-$ increases ouabain-sensitive and -insensitive O$_2$ consumption, and decreases lactate production. If CO$_2$/HCO$_3^-$ should promote a shift from glycolytic to oxidative metabolism, this could reduce metabolic H$^+$ production. Existing acid-extrusion mechanisms thus would be less opposed by acid loading, and pH$_i$ would rise. Our data are consistent

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3 The average delay between the beginning of the pH$_i$ decrease (due to the influx of CO$_2$) and the alkalinization was 4.2 ± 0.1 s (range: 3.4 - 4.9 s). This figure is based on 12 CO$_2$ pulses, which represent all control experiments shown in this paper, as well as other randomly selected experiments.
with a model in which CO₂/HCO₃⁻ acts primarily on metabolism. Nevertheless, even in this scenario, the alkalinization would require an intact acid-extrusion mechanism (e.g., a H⁺ pump), that is nominally independent of all ions other than H⁺ and HCO₃⁻. This model would still require a signal transduction mechanism to link basolateral CO₂/HCO₃⁻ to an inhibition of acid loading.

Possible Stimulation of Acid Extrusion Across the Luminal Membrane

In the third general model for the CO₂/HCO₃⁻-induced alkalinization, basolateral CO₂ and/or HCO₃⁻ stimulates the extrusion of acid equivalents across the luminal membrane. This would have the effect of not only raising pHᵢ, but also promoting transepithelial HCO₃⁻ reabsorption. The results of Fig. 12 suggest that the only ions required for such an acid-extruding process are H⁺ and/or HCO₃⁻. The active uptake of HCO₃⁻ across the luminal membrane is highly unlikely, inasmuch as the CO₂/HCO₃⁻-induced alkalinization was specifically not supported by luminal CO₂/HCO₃⁻. This leaves as the most likely mechanism a luminal H⁺ pump. Schwartz and Al-Awqati have demonstrated that bilateral CO₂/HCO₃⁻ stimulates the insertion of apical vesicles, which presumably contain H⁺ pumps, into the luminal membrane of the rabbit proximal straight tubule (Schwartz and Al-Awqati, 1985). Our data are consistent with this interpretation. However, it remains to be seen whether basolateral (but not luminal) CO₂/HCO₃⁻ causes such an insertion of vesicles. Al-Awqati and his colleagues (Cannon, Van Adelsberg, Kelly, and Al-Awqati, 1985; Van Adelsberg and Al-Awqati, 1986) have suggested that vesicle insertion is triggered by a decrease in pHᵢ, which leads to a rise in [Ca++]ᵢ. Our data are not consistent with this hypothesized role for pHᵢ: We found that luminal CO₂/HCO₃⁻ consistently caused a larger initial pHᵢ decrease than did basolateral CO₂/HCO₃⁻ (see Fig. 3 A). Nevertheless, it was only basolateral CO₂/HCO₃⁻ that caused the alkalinization.

Although our data indicate that the CO₂/HCO₃⁻-induced alkalinization can be produced in the absence of Na⁺, we did not specifically ask whether Na⁺ removal slows the alkalinization. Thus, it is possible that a Na⁺-dependent process contributes to the pHᵢ increase. In this context, it is interesting to note that recent work suggests that CO₂/HCO₃⁻, specifically basolateral CO₂/HCO₃⁻, stimulates luminal Na-H exchange (Chen and Boron, 1993b).

Is it CO₂ or HCO₃⁻ that Signals the Alkalinization?

We believe that the most likely explanation for the CO₂/HCO₃⁻-induced alkalinization is that CO₂ and/or HCO₃⁻ stimulates a luminal H⁺ pump, and perhaps other luminal acid-extruding mechanisms as well. Regardless of the mechanism of the alkalinization, a question that arises is whether it is CO₂ or HCO₃⁻ that initiates the process. Our data do not allow us to make a definitive choice between CO₂ and HCO₃⁻. The observation that luminal CO₂/HCO₃⁻ fails to elicit the alkalinization implies that neither the CO₂ nor HCO₃⁻ could be acting at the luminal membrane. Moreover, because CO₂ can freely cross the luminal membrane, it is unlikely that either CO₂ or HCO₃⁻ could be acting at a cytoplasmic site near the apical pole of the cell. However, it is possible that CO₂ and/or HCO₃⁻ acts at a low-affinity cytoplasmic site near the basolateral membrane, and that local [CO₂], and/or [HCO₃⁻], are sufficiently high to trigger the alkalinization only when CO₂ is added to the basolateral solution.
If this basolateral-cytoplasmic-site hypothesis is correct, then it should be possible to mimic the basolateral CO$_2$/HCO$_3^-$-induced alkalinization with a sufficiently high level of CO$_2$ added to the lumen, a line of research we have yet to pursue. Our ACZ data indicate that basolaterally applied CO$_2$/HCO$_3^-$ elicits a sustained pH$_i$ increase even when ACZ blocks the initial CO$_2$-induced pH$_i$ decrease. That is, the alkalinizing process apparently begins before significant amounts of H$^+$ and HCO$_3^-$ can be generated intracellularly from incoming CO$_2$. The ACZ data thus make it unlikely that HCO$_3^-$ acts at any intracellular site. Thus, we are left with the option that, if the site of triggering is cytoplasmic, the first messenger is most likely CO$_2$. On the other hand, if the receptor is on the extracellular side of the basolateral membrane, we presently cannot distinguish between CO$_2$ and HCO$_3^-$ (or a related species, such as CO$_2^+$.)

From the perspective of whole-animal acid-base balance, it is not clear what advantage would be gained by the evolution of a luminal acid-extrusion mechanism that is activated by elevated levels of basolateral HCO$_3^-$. According to this model, HCO$_3^-$ would be part of a positive feedback system. The ill effects of such a positive-feedback system could be avoided if the $K_m$ for HCO$_3^-$ were so low that luminal acid extrusion would be maximally activated at any physiological [HCO$_3^-$].

The alternative hypothesis is that basolateral CO$_2$ triggers luminal acid extrusion. This is attractive because it would make CO$_2$ part of a negative-feedback system in which respiratory acidosis (i.e., high [CO$_2$]) promotes H$^+$ secretion, and thus HCO$_3^-$ reabsorption. The net effect would be a metabolic compensation to the respiratory acidosis. Indeed, classic work on dogs indicates that respiratory acidosis stimulates HCO$_3^-$ reabsorption, and that this effect is correlated primarily with the increase in plasma [CO$_2$], rather than a decrease in plasma pH (Brazeau and Gilman, 1953; Dorman, Sullivan, and Pitts, 1954).

The pH$_i$ Dependence of the CO$_2$/HCO$_3^-$-induced Alkalinization

One of the more interesting features of the basolateral CO$_2$/HCO$_3^-$-induced alkalinization is its biphasic pH$_i$ dependence (see Fig. 11). The process is blocked at pH$_i$ values below ~6.4, and is apparently maximally active at pH$_i$ values of ~7. The net rate of CO$_2$/HCO$_3^-$-induced alkalinization shows a tendency to fall off as pH$_i$ approaches ~7.3, suggesting that the process might be gradually deactivated at alkaline pH$_i$ values. This pH$_i$ dependence is very different from that of the Na$^+$-dependent acid-extrusion mechanisms, which have a monotonic dependence on pH$_i$. For example, the Na$^+$-dependent Cl-HCO$_3^-$ exchange rate is zero at pH$_i$ values above a threshold pH$_0$, but increases steeply as pH$_i$ falls below this threshold (Boron, McCormick, and Roos, 1979). If the acid-extrusion mechanism responsible for the CO$_2$/HCO$_3^-$-induced alkalinization has a pH$_i$ dependence similar that of the Na$^+$-dependent acid extruders, then some other process must account for the decrease in the alkalinization rate observed at higher pH$_i$ values. One possibility is that the fall-off in the alkalinization rate is caused by a progressive stimulation of acid loading at high pH$_i$ values. Alternatively, the biphasic pH$_i$ dependence could reflect the summation of two separate pH$_i$ dependencies related to the acid-extrusion process. For example, the acid-extrusion mechanism per se could be inhibited at high pH$_i$ values, whereas the signal-transduction process responsible for activation of acid extrusion could be inhibited at low pH$_i$ values. However, the simplest explanation for
the biphasic pH dependence would be that the acid-extruder itself has an intrinsic, biphasic pH dependency. Indeed, it is interesting to note that the pH dependence of the CO₂/HCO₃⁻-induced alkalinization (see Fig. 11) is strikingly similar to that of the vacuolar-type H⁺ ATPase purified from renal brush border. The ATPase activity of this enzyme has a biphasic pH dependence, with maximal activity at a pH of ~ 7.3 (Wang and Gluck, 1990).

APPENDIX

Analysis of the CO₂/HCO₃⁻-induced Alkalinization Observed in the Presence of Acetazolamide

The acid-extruding flux produced by basolateral CO₂/HCO₃⁻ in the presence of acetazolamide. In principle, the basolateral CO₂/HCO₃⁻-induced alkalinization could be due either to stimulation of an acid-efflux mechanism (e.g., H⁺ pump) or direct uptake of HCO₃⁻. Examining the rate of this alkalinization when carbonic anhydrase (CA) is inhibited by acetazolamide (ACZ) should help us distinguish between the acid-efflux and HCO₃⁻-uptake models. In the case of the acid-efflux model, the H⁺ efflux needed to account for the rate of pH increase observed in the presence of ACZ is simply \( \frac{\Delta \text{pH}_i}{\Delta t} \). Given the \( \frac{\Delta \text{pH}_i}{\Delta t} \) of 58 \( \times \) 10⁻⁴ pH/s observed at a pH of 7.1, and the intrinsic buffering power of 30 mM/pH measured at a pH of 7.1 (Chen and Boron, 1993a), the required flux would be ~ 0.17 mM/s. That is, each second ~ 0.17 millimoles of acid would have to be removed per liter of cell water. The following analysis will show that during inhibition of CA, the required influx of HCO₃⁻ would be considerably greater than the required efflux of H⁺.

Pathways for the removal of H₂CO₃ in the HCO₃⁻ uptake model. As illustrated in Fig. 13 A, the HCO₃⁻-uptake model predicts that pH_i can rise only as rapidly as the incoming HCO₃⁻ combines with H⁺ to form H₂CO₃. Because the pK of the reaction H₂CO₃ = H⁺ + HCO₃⁻ is ~ 3.5, and the reaction occurs very rapidly, [H₂CO₃] is ~ 0.1% as high as [HCO₃⁻] at physiological pH values. Therefore, millimolar quantities of H⁺ can be consumed only if H₂CO₃ can be removed more or less as rapidly as it is formed. Newly formed H₂CO₃ can be removed by three routes: (a) efflux of H₂CO₃, which should be proportional to the difference [H₂CO₃] - [H₂CO₃]₀; (b) the uncatalyzed dehydration reaction H₂CO₃ → H₂O + CO₂; and (c) the CA-catalyzed reaction, which has the same effect as reaction (b). Although Fig. 5 indicates that 100 µM ACZ inhibits CA substantially in the S3 segment, we cannot say that the CA is blocked completely. Therefore, in our analysis we will not distinguish between the uncatalyzed dehydration reaction and the uninhibited CA reaction.

The immediate pH increase elicited by removal of basolateral CO₂/HCO₃⁻ in the presence of ACZ. An indication of how fast reactions (a)–(c) can proceed in the presence of ACZ is provided by the speed of the pH increase that is produced by removal of extracellular CO₂/HCO₃⁻. Fig. 13 B illustrates the reactions taking place. Just before the extracellular CO₂ is removed, pH_i is 7.4, [CO₂] is 1.2 mM, [HCO₃⁻] is 24 mM, and [H₂CO₃] is 3 µM. In six experiments such as that in Fig. 5, the mean

4 In computing [CO₂], we assumed a pCO₂ of 40 torr, and a CO₂ solubility of 0.03 mM/torr. In computing [HCO₃⁻], we assumed that the pK governing the overall reaction HCO₃⁻ + H⁺ = CO₂ + H₂O is 6.1. In computing [H₂CO₃], we assumed that the pK governing the reaction H₂CO₃ = H⁺ + HCO₃⁻ is 3.5.
Figure 13. Predicted reaction rates associated with the influx of \( \text{HCO}_3^- \), when carbonic anhydrase (CA) is blocked by acetazolamide (ACZ). (A) Introduction. The \( \text{pH} \) increase elicited by \( \text{HCO}_3^- \) uptake can proceed only as rapidly as \( \text{H}^+ \) is neutralized by incoming \( \text{HCO}_3^- \) to form \( \text{H}_2\text{CO}_3 \). However, this reaction can proceed only as rapidly as \( \text{H}_2\text{CO}_3 \) either exits the cell, or dissociates into \( \text{H}_2\text{O} \) and \( \text{CO}_2 \). From the observed rate of \( \text{pH} \) increase and intracellular buffering power, we calculate that \( \text{H}^+ \) disappears at the rate of 0.17 mM/s. (B) Washout of \( \text{CO}_2 \). When a cell previously equilibrated with \( \text{CO}_2/\text{HCO}_3^- \) is exposed to a \( \text{CO}_2/\text{HCO}_3^- \)-free medium, \( \text{pH} \) rises due to the efflux of \( \text{CO}_2 \) and/or \( \text{H}_2\text{CO}_3 \). From the observed rate of \( \text{pH} \) increase at \( \text{pH} \),
initial (i.e., maximal) dpHi/dt produced by CO2 withdrawal in the presence of ACZ (see segments cd and fg in Fig. 5) was 79 ± 16 × 10⁻⁴ pH/s at a pHi of ~7.4. Given a β of 20 mM/pH measured at a pHi of 7.4 (Chen and Boron, 1993a), the rate of H⁺ neutralization, which must have been the same as the flux of H₂CO₃ through reactions (a)–(c), was (79 × 10⁻⁴ pH/s) × (20 mM/pH) = ~0.16 mM/sec. If this flux were accounted for entirely by H₂CO₃ efflux (J_{H₂CO₃}), where J_{H₂CO₃} = P_{H₂CO₃} × ([H₂CO₃]ᵢ - [H₂CO₃]ₒ), then the effective permeability constant P_{H₂CO₃} must have been J_{H₂CO₃}/([H₂CO₃]ᵢ - [H₂CO₃]ₒ) = (0.16 mM/s)/(3 μM - 0) = ~53 s⁻¹. Similarly, this flux may have been produced entirely by the uncatalyzed dehydration reaction and the uninhibited CA reaction. We will make the simplifying assumptions that (a) [CO₂]ᵢ is zero immediately after removal of external CO₂, and (b) that this flux (J_{dehyd+CA}) is proportional to [H₂CO₃]ᵢ. It follows that the rate constant governing the reaction H₂CO₃ → CO₂ + H₂O would be given by k₁ = J_{dehyd+CA}/[H₂CO₃]ᵢ = (0.16 mM/s)/(3 μM) = ~53 s⁻¹. This figure is somewhat higher than the value of 32 s⁻¹ reported for the uncatalyzed dehydration reaction (Berliner, 1985), which is reasonable, inasmuch as the inhibition of CA by ACZ may have been incomplete. Our data do not allow us to compute J_{H₂CO₃} and J_{dehyd+CA} per se, only their sum, 0.16 mM/s. The extent to which H₂CO₃ consumption is governed by H₂CO₃ efflux vs conversion to CO₂ is not important for our analysis.

The delayed pH increase elicited by application of basolateral CO₂/HCO₃⁻ in the presence of ACZ. Applying basolateral CO₂/HCO₃⁻ in the absence of ACZ produces a transient pH decrease (ij in Fig. 5), followed within ~5 s by a rapid and sustained alkalinization (jk in Fig. 5). In the presence of ACZ, the initial pH decrease is very small; we will assume that the delay to the initiation of the pH increase remains 5 s. In this analysis, we will first make the simplifying assumption that, during this initial 5 s, [CO₂]ᵢ rises to 1.2 mM (the extracellular concentration) instantaneously, but that HCO₃⁻ cannot enter the cell. Based on the value for k₁ computed above, we estimate that 0.8 mM H₂CO₃ would be formed from the intracellular CO₂ during these initial 5 s.

7.4 in the presence of ACZ, we calculate that H⁺ must be consumed at the rate of 0.16 mM/s. From this value, and the estimated equilibrium value for [H₂CO₃]ᵢ, we can compute the effective permeability to H₂CO₃ or the rate constant (k₁) of the inhibited dehydration reaction. (C) Predicted fluxes 5 s after the introduction of CO₂, allowing only an influx of CO₂. From k₁ and the equilibrium constant for the dehydration reaction, we can compute the rate of formation of H₂CO₃ from CO₂. Virtually all of this newly formed H₂CO₃ dissociates into H⁺ and HCO₃⁻. Over a period of 5 s, we estimate that 0.8 mM HCO₃⁻ would be formed. (D) Predicted fluxes 5 s after the introduction of CO₂/HCO₃⁻, allowing the influx of HCO₃⁻ and the transmembrane equilibration of CO₂. In order for the net reaction H₂CO₃ → H₂O + CO₂ to proceed at the required rate of 0.17 mM/s in the presence of ACZ, [H₂CO₃]ᵢ must have to be 6.2 μM. Because H₂CO₃, H⁺ and HCO₃⁻ rapidly equilibrate, we can compute that [HCO₃⁻]ᵢ must be 24.7 mM when pH, is 7.1. This level of HCO₃⁻ must be achieved in within 5 s of the time CO₂/HCO₃⁻ is added, for an average HCO₃⁻ flux of 4.8 mM/s. (E) Predicted fluxes 5 s after the introduction of CO₂/HCO₃⁻, allowing the influx of HCO₃⁻ and the transmembrane equilibration of H₂CO₃. The analysis here is similar to that in part D, except that all newly formed H₂CO₃ passively exists from the cell. The unidirectional H₂CO₃ fluxes are computed from the effective permeability constant derived from the data in part B.
5 s, and that almost all of this \( \text{H}_2\text{CO}_3 \) would dissociate into \( \text{HCO}_3^- \) and \( \text{H}^+ \). The predicted immediate pH change can be predicted from this \( \text{H}^+ \) formation and the intrinsic \( \beta \) of 30 mM: \( \Delta \text{pH} = -(0.8 \text{ mM})/(30 \text{ mM}/\text{pH}) = -0.027 \). The status of the cell at the end of this 5-s initial period is indicated in Fig. 13 C.

Now let us complicate the analysis by allowing \( \text{HCO}_3^- \) to be transported into the cell during the initial 5 s. Assume for now that the only pathway for disposing of newly formed \( \text{H}_2\text{CO}_3 \) is the conversion to \( \text{CO}_2 \) via the uncatalyzed dehydration reaction and the uninhibited CA reaction (see Fig. 13 D). As noted above, by the end of this 5-s interval, pH begins to increase at the rate of \( 58 \times 10^{-4} \text{ pH/s} \) (pH = 7.1), and \( \text{H}^+ \) must disappear at the rate of \( \sim 0.17 \text{ mM/s} \). Thus, at \( t = 5 \text{ s} \), the net formation of \( \text{CO}_2 \) from \( \text{H}_2\text{CO}_3 \) also must proceed at \( \sim 0.17 \text{ mM/s} \). Inasmuch as the back-reaction \( (\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3) \) proceeds at 0.16 mM/s, the forward reaction \( (\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}) \) would have to proceed at 0.33 mM/s. Because \( k_1 \) governing this forward reaction is 53 s\(^{-1} \), the required \( [\text{H}_2\text{CO}_3] \), can be calculated: \( [\text{H}_2\text{CO}_3] = J_{\text{uncat+CA}}/k_1 = (0.33 \text{ mM s}^{-1})/(53 \text{ s}^{-1}) = 6.2 \mu\text{M} \). Given a value of \( \sim 3.5 \) for the pK of the equilibrium \( \text{H}_2\text{CO}_3 = \text{H}^+ + \text{HCO}_3^- \), and a pH of 7.1, this \( [\text{H}_2\text{CO}_3] \), predicts a \( [\text{HCO}_3^-] \), of \( \sim 24.7 \text{ mM} \). In other words, in order for a \( \text{HCO}_3^- \) uptake mechanism to account for the \( \text{CO}_2/\text{HCO}_3^- \)-induced pH increase observed at \( t = 5 \text{ s} \), the conversion of the newly formed \( \text{H}_2\text{CO}_3 \) to \( \text{CO}_2 \) would require that \( [\text{HCO}_3^-] \), rise from 0 to 24.7 mM in only 5 s. As pointed out above, \( \text{CO}_2 \) influx would cause \( [\text{HCO}_3^-] \), to rise from 0 to 0.8 mM in 5 s. Thus, over a period of 5 s, the hypothetical \( \text{HCO}_3^- \) uptake mechanism would have to account for a \( \Delta [\text{HCO}_3^-] \), of 24.7 - 0.8 = 23.9 mM; this corresponds to an average \( \text{HCO}_3^- \) influx of 4.8 mM/s.

The conclusions about the predicted \( \text{HCO}_3^- \) flux are virtually identical if we assume that all of the newly formed \( \text{H}_2\text{CO}_3 \) is dissipated by the efflux of \( \text{H}_2\text{CO}_3 \) (Fig. 13 E). Thus, regardless of whether we assume that the newly formed \( \text{H}_2\text{CO}_3 \) is converted to \( \text{CO}_2 \) or exits the cell directly as \( \text{H}_2\text{CO}_3 \), the \( \text{HCO}_3^- \) influx needed to explain the data obtained during inhibition of CA would have to be \( \sim 4.8 \text{ mM/s} \). This figure is unreasonably high when compared with the fluxes produced by \( \text{HCO}_3^- \)-dependent acid-extrusion mechanisms in other cell types. For example, renal mesangial cells exhibit a \( \text{CO}_2/\text{HCO}_3^- \)-induced alkalinization that is produced by the combination of an Na-H exchanger and a Na+-dependent Cl-HCO_3 exchanger (Boyarsky, Ganz, Sterzel, and Boron, 1988a, b). However, the computed flux at a pH of 6.8 is only \( \sim 0.16 \text{ mM/s} \) (Boyarsky et al., 1988a).

We thank Emily Tyner for preparing the figures from the computer data files, and Mrs. Eleanor Savage for expert secretarial assistance. We are also grateful to Dr. R. W. Berliner for helpful discussions related to the analysis presented in the Appendix.

This research was supported by NIH grant R01-DK30544.

Original version received 28 December 1992 and accepted version received 28 June 1993.

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5 The equilibrium constant governing the reaction \( \text{H}_2\text{CO}_3 = \text{CO}_2 + \text{H}_2\text{O} \) is \( \sim 0.0025 \). Thus, if \( k_1 \) (the rate constant for \( \text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O} \)) is \( 53 \text{ s}^{-1} \), then \( k_{-1} \) (the rate constant for \( \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3 \)) must be \( \left(53 \text{ s}^{-1}\right)/400 = 0.133 \text{ s}^{-1} \). Thus, the flux from \( \text{CO}_2 \) to \( \text{H}_2\text{CO}_3 \) must be \( k_{-1} \times [\text{CO}_2] = (0.133 \text{ s}^{-1}) \times (1.2 \text{ mM}) = 0.16 \text{ mM/s} \). During the first 5 s, the total \( \text{H}_2\text{CO}_3 \) formed comes to 0.8 mM.
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