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 dimethylcarboxyfluorescein to monitor intracellular pH (pH_i) in the isolated perfused S3 segment of the rabbit proximal tubule, and examined the effect on pH_i 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 pH_i 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 pH_i 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 $SO_4^=$. 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 ($NMDG^+/Glr^-$). Luminal CO_2/HCO_3^- did not elicit a pH_i increase in $NMDG^+/Glr^-$. Although the

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sustained pH_i increase elicited by basolateral CO_2/HCO_3^- could be due to a basolateral HCO_3^- uptake mechanism, net reabsorption of HCO_3^- by the S3 segment, as well as our ACZ data, suggest instead that basolateral CO_2/HCO_3^- elicits the sustained pH_i 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 HCO_3^- 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 HCO_3^- reabsorption is the neutralization of luminal HCO_3^- 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 HCO_3^-, 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 HCO_3^-, this mechanism is about twice as powerful as the Na-H exchanger for intracellular pH (pH_i) 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 HCO_3^- (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, HCO_3^- 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_i decrease followed by a slower pH_i 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 HCO_3^- reabsorption in the presence of Na^+.

The basolateral step of HCO_3^- reabsorption is brought about by the efflux of HCO_3^- across the basolateral membrane. The major mechanism of this HCO_3^- 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, 1989*a*; Nakhoul, Chen, and Boron, 1990) is believed to be the electrogenic Na/HCO_3 cotransporter (Boron and Boulpaep, 1983*b*). Work on vesicles derived from basolateral membranes of mammalian proximal tubules also provided evidence for $Cl\text{-}HCO_3$ exchange (Low, Friedrich, and Burckhardt, 1984; Grassl, Karniski, and Aronson, 1985), as did work on the LLC-PK_1 cell line (Chaillet, Amsler, and Boron, 1986). Studies of pH_i regulation have also provided evidence for $Cl\text{-}HCO_3$ exchange at the basolateral membrane of the rabbit S3 proximal tubule (Kurtz, 1989*b*; Nakhoul et al., 1990). Both the Na/HCO_3 cotransporter and the $Cl\text{-}HCO_3$ exchanger would

mediate the net efflux of HCO_3^-, 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 CO_2/HCO_3^- (at a constant external pH of 7.4) caused a sustained increase in pH_i. This last result implies that the net effect of CO_2/HCO_3^- is to promote acid extrusion to a greater extent than it promotes acid loading via Na/HCO_3 cotransport and $Cl\text{-}HCO_3$ exchange.

In the present study, we investigated the mechanism of the CO_2/HCO_3^--induced alkalinization in isolated perfused S3 segments of the rabbit proximal tubule. Computing pH_i from the absorbance spectrum of a pH-sensitive dye, we found that the alkalinization can be elicited by adding CO_2/HCO_3^- to the bath (i.e., basolateral solution) only, but not to the lumen only. The basolateral CO_2/HCO_3^--induced alkalinization did not require Na^+ or Cl^- and, in fact, occurred when bath HCO_3^- 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 CO_2/HCO_3^- washout, but was inhibited ~73% by 100–1,000 μM DIDS applied at least 30 min after CO_2/HCO_3^- washout. The alkalinization was blocked by reducing pH_i 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 HCO_3^--influx mechanism. Instead, it is most likely that the pH_i increase elicited by basolateral CO_2/HCO_3^- 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 I. All were titrated to 7.40 at 37°C. Nominally HCO_3^--free solutions were buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and HCO_3^--containing solutions were continuously bubbled with 5% CO_2 at 37°C. Solutions were delivered by gravity to either bath or lumen through CO_2-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. For

TABLE I

*Solutions**

COMPONENT	1 HEPES +Ac	2 HCO_3^- +Ac	3 Std HEPES	4 Std HCO_3^-	5 0 Cl^- HEPES	6 0Cl^-	7 0 $SO_4^=$	8 0 $SO_4^=$ HCO_3^-	9 2 HEPES	10 HCO_3^- + 2 HEPES	11 0 Na^+	12 0 Na^+ HCO_3^-	13 NMDG/ GLUC	14 NMDG/ GLUC HCO_3^-
Na^+	146.4	153.6	146.4	153.6	141.9	149.1	146.4	153.6	153.2	153.6	0.	0.	0.	0.
K^+	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	0.	0.
$NMDG^+$	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	145.8	153.6	160.2	168.0
Mg^{++}	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.	0.
Ca^{++}	1.0	1.0	1.0	1.0	4.0	4.0	1.0	1.0	1.0	1.0	1.0	1.0	0.	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	160.2	168.0
Cl^-	122.0	122.0	132.0	132.0	0.	0.	134.4	134.4	155.4	130.8	131.4	132.0	0.	0.
$H_2PO_4^-$	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
$HPO_4^=$	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	0.	0.
Glucuronate$^-$	0.	0.	0.	0.	133.5	133.5	0.	0.	0.	0.	0.	0.	160.2	143.0
HEPES$^-$	17.8	0.	17.8	0.	17.8	0.	17.8	0.	1.2	1.2	17.8	0.	0.	0.
$SO_4^=$	1.2	1.2	1.2	1.2	1.2	1.2	0.	0.	1.2	1.2	1.2	1.2	0.	0.
HCO_3^-	0.	25.0	0.	25.0	0.	25.0	0.	25.0	0.	25.0	0.	25.0	0.	25.0
Ac^-	10.0	10.0	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.
mEq (−)	155.8	163.0	155.8	163.0	157.3	164.5	155.8	163.0	162.6	163.0	155.2	163.0	160.2	168.0
Glucose	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	0.	0.
Alanine	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	0.	0.
HEPES	14.4	0.	14.4	0.	14.4	0.	14.4	0.	0.8	0.8	14.4	0.	0.	0.
pH	7.4	7.4	7.4	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, $NMDG^+$ in *N*-methyl-D-glucamine. All HCO_3^--containing solutions were continuously bubbled with 5% CO_2. 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 Ca^{++} by the Cl^- substitute in early experiments with Cl^--free/Ca^{++}-containing solutions. However, in later experiments we compensated by raising total Ca^{++} 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_i 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_i to predetermined values using the nigericin/high-K^+ method (Thomas, Buchsbaum, Zimniak, and Racker, 1979). The calibration curve relating the ratio A_{510}/A_{470} to pH_i 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_i change were determined by using a computer to fit a line to the pH_i vs time data.

RESULTS

Effect on pH_i of Bilateral CO_2/HCO_3^- 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

CO_2/HCO_3^-, and two acid-loading mechanisms that require CO_2/HCO_3^-. 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, 1983*a*). At least a portion of the pH_i fall is due to the influx of CO_2, its hydration to form H_2CO_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 $Cl\text{-}HCO_3$ 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

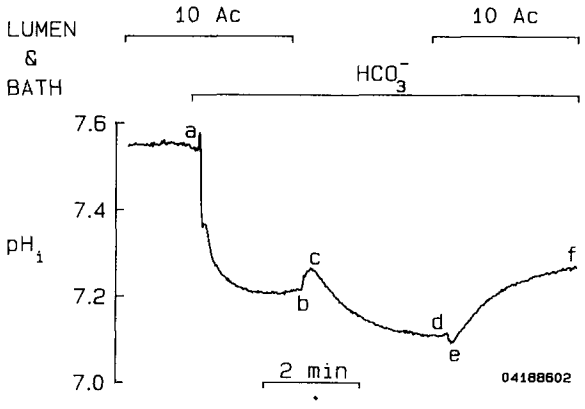

FIGURE 1. Effect of bilateral CO_2/HCO_3^- in the presence of bilateral 10 mM acetate. At point *a*, the buffer in both the lumen and bath was switched from 32 mM HEPES (solution 1) to 5% CO_2/25 mM HCO_3^- (solution 2) in the continuous presence of 10 mM acetate, causing a sustained pH_i fall. The subsequent bilateral removal of acetate (solution 4) caused a transient pH_i increase (*bc*), followed by a fall (*cd*). This series of pH_i changes was reversed by returning the acetate to the bath and lumen (*def*). Four similar experiments were performed.

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_i increase in segment-*bc* is caused by the efflux of acetic acid, whereas the pH_i decrease in segment-*cd* reflects the cessation of an acetate-dependent acid-extrusion process. Quantitatively, however, both the transient pH_i rise (*bc*) and the sustained pH_i fall (*b* vs *d*) elicited by acetate removal in the presence of CO_2/HCO_3^- were substantially smaller than those previously observed in the absence of CO_2/HCO_3^-. For example, the net pH_i 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 CO_2/HCO_3^-. The smaller magnitude of the transient pH_i rise elicited by acetate removal probably reflects, at least in part, the higher buffering power expected to prevail in the presence of CO_2/HCO_3^- (Nakhoul and Boron, 1988), as well as the lower initial pH_i in the presence of CO_2/HCO_3^-. The smaller magnitude of the sustained pH_i fall caused by acetate removal in CO_2/HCO_3^- suggests that the loss of an acetate-dependent acid-extrusion process is partially compensated by a CO_2/HCO_3^--dependent acid-extrusion process that is stimulated (and/or an acid-loading process that is inhibited) by the intracellular acidification evoked by acetate removal.

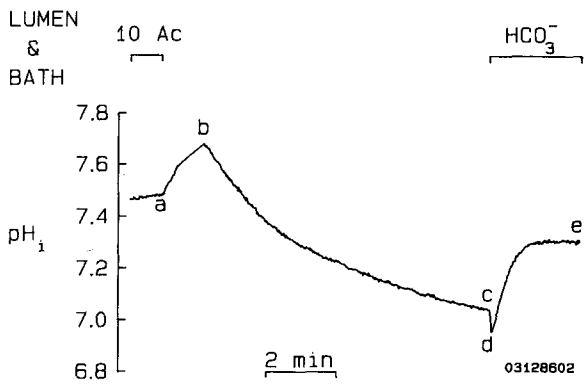

FIGURE 2. Effect of bilateral CO_2/HCO_3^- in the absence of acetate. At point *a*, 10 mM acetate (solution 1) was removed bilaterally (solution 3), causing a transient alkalinization, followed by a sustained pH_i decrease. Subsequently, switching the buffer in both the lumen and bath at *c* from 32 mM HEPES (solution 3) to 5% CO_2/25 mM HCO_3^- (solution 4), caused a transient pH_i fall, followed by a sustained increase. We performed 17 similar experiments.

Effect on pH_i of Bilateral CO_2/HCO_3^- in the Absence of Acetate

In a second series of experiments, we examined the effect of exposing the tubule bilaterally to CO_2/HCO_3^- 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 HCO_3^- free. The bilateral removal of acetate (solution 3) caused a transient pH_i increase (*ab*), followed by a slower but sustained decrease (*bc*), as described previously (Nakhoul and Boron, 1988). The subsequent bilateral addition of CO_2/HCO_3^- caused a transient acidification (*cd*), due to the influx of CO_2, followed by a rapid and sustained alkalinization (*de*) to a level substantially higher than that prevailing in the absence of CO_2/HCO_3^- (*c* vs *e*). In 17 similar experiments, pH_i averaged 6.92 ± 0.05 after removal of acetate, and the mean net pH_i increase caused by bilateral CO_2/HCO_3^- 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

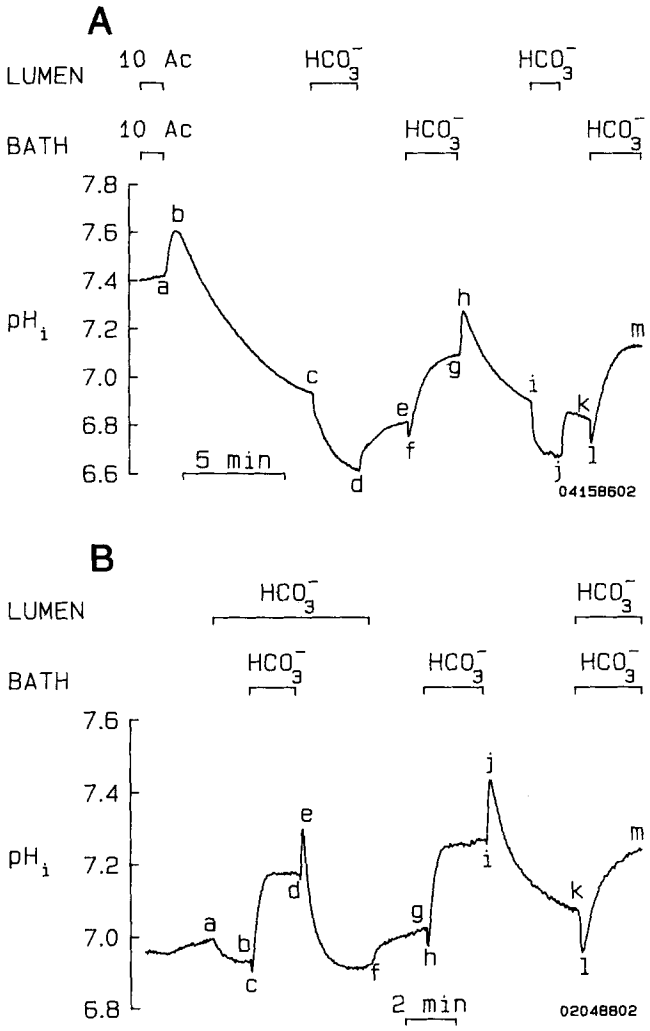

FIGURE 3. Effect of uni- or bilateral CO_2/HCO_3^-. (*A*) Effect of unilateral CO_2/HCO_3^- in the absence of acetate. At point *a*, 10 mM acetate (solution 1) was removed bilaterally (solution 3), causing a transient alkalinization, followed by a sustained pH_i decrease. Subsequently switching the buffer in the lumen from 32 mM HEPES (solution 3) to 5% CO_2/25 mM HCO_3^- (solution 4), caused a sustained pH_i decrease (*cd*) that was reversible (*de*). Switching the buffer to CO_2/HCO_3^- in the bath caused a transient pH_i fall followed by a sustained increase (*efg*), and these changes were also reversible (*ghi*). The responses to a second period of CO_2/HCO_3^- from lumen (*ijk*) or bath (*klm*) were similar to the first. Three such experiments were performed. (*B*) Effect of adding basolateral CO_2/HCO_3^- in the presence of luminal CO_2/HCO_3^-, in the absence of luminal CO_2/HCO_3^-, and together with luminal CO_2/HCO_3^-. The protocol was similar to that in part (*A*). Luminal CO_2/HCO_3^- was present during the first bath CO_2/HCO_3^- pulse (*bcd*) and absent during the second (*ghi*). In the third pulse, CO_2/HCO_3^- 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 CO_2/HCO_3^- buffer was followed by a transient pH_i fall and a sustained rise (*klm*). These results indicate that the addition of CO_2/HCO_3^- to the bath causes a sustained intracellular alkalinization, independent of the status of CO_2/HCO_3^- in the lumen.

Effect of Potential Inhibitors

Effect of applying DIDS shortly after removal of CO_2/HCO_3^-. In the next set of experiments, we examined the effect of DIDS, an inhibitor of anion exchange, on the pH_i increase caused by basolateral CO_2/HCO_3^-. As shown in Fig. 4, adding CO_2/HCO_3^- to only the bath caused the usual pH_i changes (*abc*), which were reversed upon removing CO_2/HCO_3^- (*cde*). Addition of 50 μM DIDS to the bath (*e*) caused pH_i to increase slowly (*ef*), after which we applied CO_2/HCO_3^- to the bath a second time

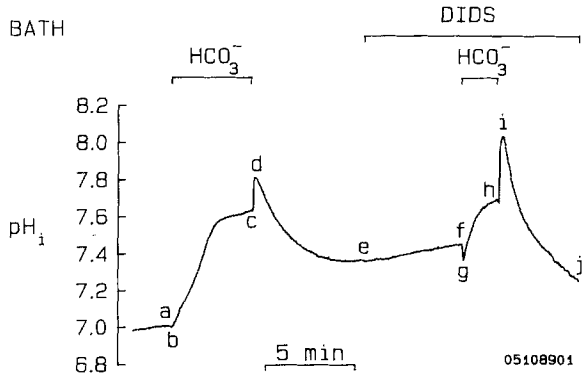

FIGURE 4. Effect of DIDs on the basolateral CO_2/HCO_3^--induced alkalinization. Switching the basolateral buffer from 32 mM HEPES (solution 3) to 5% CO_2/25 mM HCO_3^- (solution 4) caused a sustained pH_i increase, with only a hint of a CO_2-induced acidification (*abc*). CO_2/HCO_3^- removal caused a partial reversal of the effect (*cde*). Application of CO_2/HCO_3^- a second time, but in the presence of 50 μM DIDS, caused a transient CO_2-induced acidification (*fg*) followed by an alkalinization (*gh*) that was even faster than that observed in the absence of DIDS at a comparable pH_i (e.g., ~7.4). Throughout the experiment, 5% CO_2/25 mM HCO_3^- 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 CO_2/HCO_3^--induced alkalinization, the drug actually increased the rate of alkalinization (*gh*) at comparable pH_i values. Removal of bath CO_2/HCO_3^- once again caused the usual series of pH_i changes (*hij*). In a series of eight similar paired experiments, we found that the rate of the CO_2/HCO_3^--induced alkalinization was $30 \pm 3 \times 10^{-4}$ pH/s at an average pH_i of 7.31 ± 0.02 in the absence of DIDS, and $60 \pm 9 \times 10^{-4}$ pH/s at the same pH_i in the presence of DIDS. Thus, on average, the second exposure to CO_2/HCO_3^-, made in the presence of DIDS, elicited a pH_i 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_i increase in the experiment shown in Fig. 4 is that the second exposure to CO_2/HCO_3^- always evokes a faster pH_i 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_i for the initial portion of the alkalinization (points *b* and *g* in Fig. 4) was higher for second CO_2/HCO_3^- pulse (pH_i = 7.17) than for the first (pH_i = 7.01). This is consistent with the notion that some of the effects of the CO_2/HCO_3^- exposure (i.e., stimulating acid extrusion or inhibiting acid loading) persist even after removal of the CO_2/HCO_3^-. When measured at comparable pH_i values, the rate of pH_i increase was substantially greater during the second ($dpH_i/dt = 53.3 \times 10^{-4}$ s^{-1}) than during the first CO_2/HCO_3^- pulse ($dpH_i/dt = 29.8 \times 10^{-4}$ 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 CO_2/HCO_3^--induced alkalinization in this twin-pulse protocol. Earlier work (Nakhoul et al., 1990) showed that DIDS applied in the presence of CO_2/HCO_3^- substantially inhibits the two HCO_3^- transporters that act as intracellular acid loaders, the basolateral Cl-HCO_3 exchanger and Na/HCO_3 cotransporter. Thus, our data are consistent with the hypothesis that, shortly after the removal of CO_2/HCO_3^-, DIDS had no effect on the CO_2/HCO_3^--induced alkalinization per se, but inhibited the two acid loaders that otherwise would have opposed the pH_i increase.

TABLE II

*Rates of Basolateral CO_2/HCO_3^--Induced Alkalinization in Back-to-Back CO_2/HCO_3^- Pulses**

First CO_2/HCO_3^- *pulse:*	
Initial pH_i	7.01 ± 0.03
dpH_i/dt at initial pH_i of first pulse	25.7 ± 3.3 × 10^{-4} s^{-1}
dpH_i/dt at a pH_i comparable to that of the second pulse	29.8 ± 5.4 × 10^{-4} s^{-1}
Second CO_2/HCO_3^- *pulse:*	
Initial pH_i	7.17 ± 0.07‡
dpH_i/dt at initial pH_i of second pulse	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 HCO_3^- (first CO_2/HCO_3^- pulse; solution 4) → standard HEPES (solution 1) → standard HCO_3^- (second CO_2/HCO_3^- 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.

‡$p < 0.005$, compared to the initial pH_i of the first pulse.

§$p < 0.01$, compared to the dpH_i/dt of the first pulse, measured at the initial pH_i of the first pulse. $p < 0.002$ compared to the dpH_i/dt of the first CO_2/HCO_3^- pulse, measured at the initial pH_i of the second pulse.

Effect of applying DIDS long after removal of CO_2/HCO_3^-. In a second series of experiments with DIDS, we examined the effect of the drug on the CO_2/HCO_3^--induced alkalinization in tubules that had not been exposed to CO_2/HCO_3^- for at least 30 min. We used a single-CO_2/HCO_3^--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 42.3×10^{-4} s^{-1}; this value was between

TABLE III

*Effect of DIDS on the Basolateral CO_2/HCO_3^--Induced Alkalinization when the DIDS is Applied After a Long Absence of CO_2/HCO_3^-**

Controls:	
Initial pH_i	7.07 ± 0.02
dpH_i/dt at initial pH_i	$42.3 \pm 6.1 \times 10^{-4}$ s^{-1}
n	18
100–1,000 μM DIDS:	
Initial pH_i	7.05 ± 0.02‡
dpH_i/dt at initial pH_i	$11.6 \pm 1.5 \times 10^{-4}$ s^{-1}§
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.

‡Not significantly different from the initial pH_i of controls ($p > 0.6$).

§$p < 0.00002$, compared to the dpH_i/dt of controls.

the first- and second-pulse values for control CO_2/HCO_3^- pulses summarized in Table II. In the DIDS experiments, we noted no substantial difference between dpH_i/dt values for DIDS concentrations of 100, 200, 400, and 1,000 μM. Therefore we lumped these DIDS data, obtaining a mean dpH_i/dt was 11.6×10^{-4} s^{-1}. This corresponds to an inhibition of ~73%. We found an inhibitions of ~42% ($n = 5$) at 25 μM DIDS, and ~63% ($n = 5$) at 25 μM DIDS (not shown). Thus, when DIDS is applied long after (typically 90–150 min) the removal of CO_2/HCO_3^-, the drug produces a sizable, though not complete, inhibition of the CO_2/HCO_3^--induced alkalinization.

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 CO_2/HCO_3^- produced a slight pH_i decline (*ab*). In the continued presence of ACZ, switching the bath solution to one buffered with CO_2/HCO_3^- caused no observable transient acidification, but a large and rapid alkalinization (*bc*). The absence of the transient pH_i decrease was probably due to the combination of the low initial pH_i and the inhibition of carbonic anhydrase by ACZ, both of which are expected to blunt formation of H^+ and HCO_3^- from CO_2. The absence of a CO_2-induced pH_i transient is even more striking upon removal of bath CO_2/HCO_3^-, 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 CO_2/HCO_3^- was repeated, with similar results (*ef* and *fgh*). After ACZ was removed from bath and lumen at *h*, the addition and removal of bath CO_2/HCO_3^- again produced the usual series of pH_i changes: a transient pH_i decrease (*ij*) followed by a rapid

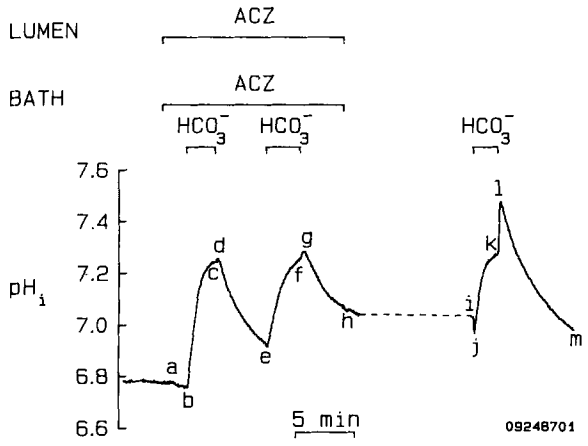

FIGURE 5. Effect of acetazolamide (*ACZ*) on the basolateral CO_2/HCO_3^--induced alkalinization. At point *a*, 100 μM ACZ was added to solution 3 in the lumen and bath. In the continued presence of ACZ, 5% CO_2/25 mM HCO_3^- (solution 4) was added to the bath on two separate occasions (*bc* and *ef*). ACZ was removed at *h*, after which 5% CO_2/25 mM HCO_3^- was added to the bath a third time. An air bubble was removed from the chamber during the period indicated by the broken line. Acetate was absent throughout. Six similar experiments were performed.

increase (*jk*) upon addition of CO_2/HCO_3^-, and a transient pH_i increase (*kl*) followed by a large decrease (*lm*) upon removal of CO_2/HCO_3^-. 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 CO_2/HCO_3^- from 70 ± 13 × 10^{-4} pH/s (mean pH_i: 7.10) to 58 ± 8 × 10^{-4} pH/s (mean pH_i: 7.15; difference in pH_i/dt values: N.S.). Thus, although ACZ substantially reduced, as expected, the CO_2-induced pH_i transients, it had little effect on the basolateral CO_2/HCO_3^--induced pH_i increase. As discussed in the Appendix, this failure of ACZ to produce a substantial inhibition of the CO_2/HCO_3^--induced alkalinization makes it very unlikely that the CO_2/HCO_3^--induced alkalinization is due to an influx of HCO_3^-.

Effect of Removing Single Ions

Effect of chloride removal. The Cl-HCO_3 exchanger identified at the basolateral membrane of the S3 segment (Kurtz, 1989*b;* Nakhoul et al., 1990) appears normally to mediate a net efflux of HCO_3^-, rather than the influx necessary to account for the alkalinization elicited by the simultaneous addition of CO_2/HCO_3^- to lumen and bath. Moreover, the DIDS data from Fig. 4 indicate that, even when CO_2/HCO_3^- is added to only the bath, the two DIDS-sensitive basolateral HCO_3^- transporters (i.e., the Cl-HCO_3 exchanger and the Na/HCO_3 cotransporter), taken as a pair, fail to mediate a net influx of HCO_3^-. Nevertheless, to determine if Cl-HCO_3 exchange makes a contribution to the CO_2/HCO_3^--induced alkalinization, we examined the effect of adding basolateral CO_2/HCO_3^- in the bilateral absence of Cl^-. As shown in Fig. 6, bilateral removal of acetate caused the usual series of pH_i changes (*abc*).

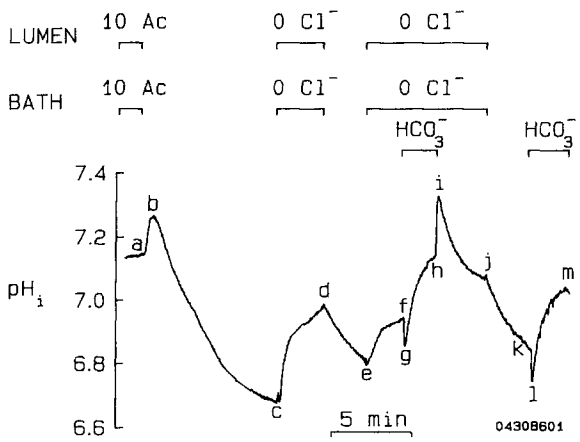

FIGURE 6. Effect of short-term Cl^- removal on the basolateral CO_2/HCO_3^--induced alkalinization. At point *a*, a solution containing 10 mM acetate (solution 1) was switch to one that was acetate free (solution 3) in both lumen and bath. Cl^- was removed bilaterally (replaced with glucuronate; solution 5) twice (*c* and *e*). During the second Cl^--free period, 5% CO_2/25 mM HCO_3^- (solution 6) was added to the bath (*fgh*). After the bilateral readdition of Cl^- (*j*), 5% CO_2/25 mM HCO_3^- (solution 4) was added to the bath one more time. Five similar experiments were performed.

Bilateral removal and return of Cl^- (solution 5) resulted in a reversible increase in pH_i (*cde*), due presumably to Cl-base exchange as described previously (Nakhoul et al., 1990). After pH_i had peaked during a second period of bilateral Cl^- removal (*ef*), the addition of CO_2/HCO_3^- (solution 6) to the bath still caused a transient acidification (*fg*) followed by a sustained alkalinization (*gh*). Removal of bath CO_2/HCO_3^- produced the usual series of pH_i changes (*hij*). pH_i did not fully recover until the bilateral return of Cl^- (*jk*), presumably because Cl-base exchange plays a role in the recovery of pH_i from alkali loads. Finally, the addition of CO_2/HCO_3^- to the bath in the continued presence of Cl^- caused a series of pH_i changes (*klm*) indistinguishable from those observed in the absence of Cl^-. In a total of five similar paired experiments, the mean initial rate of pH_i increase elicited by bath CO_2/HCO_3^- in the absence of Cl^- (e.g., *gh*) was $123 \pm 26 \times 10^{-4}$ pH/s at an average pH_i of 7.24 ± 0.13.

The difference between this rate and that observed in the presence of Cl^-, 140 ± 26 × 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, $[Cl^-]_i$ 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-HCO_3 exchange is responsible for proximal-tubule $SO_4^=$ reabsorption (Prichard and Renfro, 1983). Inasmuch as our solutions normally contained 1.2 mM SO_4, we examined whether $SO_4^=$ is required for the basolateral CO_2/HCO_3^--induced alkalinization. In the experiment illustrated in Fig. 7, $SO_4^=$ was

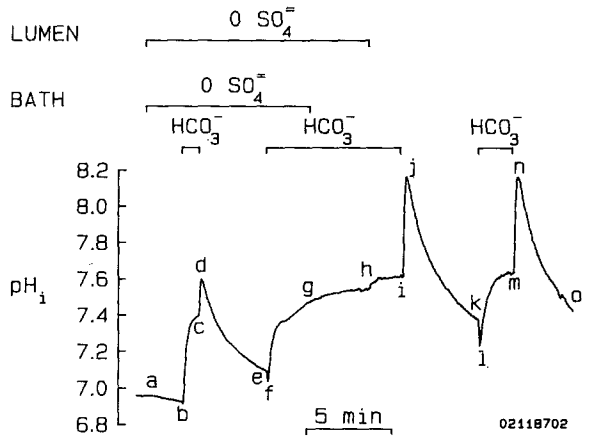

FIGURE 7. Effect of short-term $SO_4^=$ removal on the basolateral CO_2/HCO_3^--induced alkalinization. At point *a* (solution 3), 1.2 mM $SO_4^=$ was removed from both lumen and bath (solution 7). In the continued absence of $SO_4^=$, the basolateral buffer was twice switched (*b* and *e*) to 5% CO_2/25 mM HCO_3^- (solution 8). During the second period of CO_2/HCO_3^-, $SO_4^=$ was sequentially returned to the bath (*g*) and lumen (*h*). After removal of bath CO_2/HCO_3^- (*ijk*), basolateral CO_2/HCO_3^- was added (*klm*) and removed (*mno*) a third time, in the continued presence of $SO_4^=$. Acetate was absent during the entire experiment. Four similar experiments were performed.

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 $SO_4^=$, basolateral addition and then removal of CO_2/HCO_3^- (solution 8) caused the usual pH_i changes (*bcde*). Even a second application of bath CO_2/HCO_3^- caused the usual sustained pH_i increase (*efg*). After $SO_4^=$ 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^-

[1] We assume that intrinsic (i.e., non-CO_2 buffering) power averages 33 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. 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.

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 H_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 $[HEPES]_i$ 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 $CO_2/$

[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 $SO_4^=$.

HCO_3^--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/HCO_3 cotransporter (Kurtz, 1989*b*; Nakhoul et al., 1990), and a basolateral Na^+-dependent $Cl\text{-}HCO_3$ exchanger (Alpern and Chambers, 1987). In principle, any of these could have been stimulated by basolateral CO_2/HCO_3^-, 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. 9*A*. The simultaneous removal of acetate and Na^+ from lumen and bath (solution 11) caused a transient pH_i increase (*ab*), followed by an extreme acidification (*bc*). This pattern

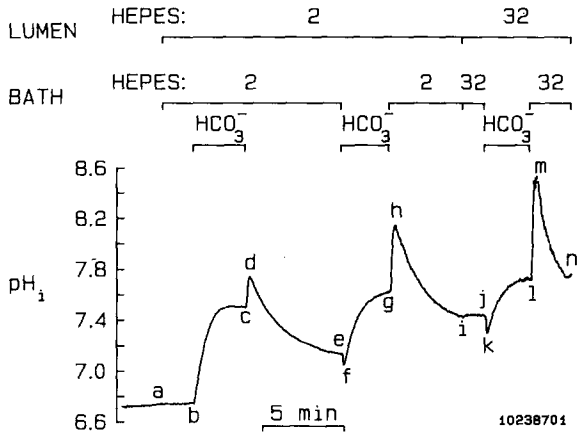

FIGURE 8. Effect of maintaining 2 mM HEPES during the exposure to basolateral CO_2/HCO_3^-. At point *a*, $[HEPES]_o$ was reduced from 32 mM (solution 3) to 2 mM (solution 9) in both lumen and bath. In the continued presence of 2 mM HEPES, 5% CO_2/25 mM HCO_3^- buffer (solution 10) was added to (*bc*) and then removed from (*cde*) the bath. At *e*, 5% CO_2/25 mM HCO_3^- was added while the HEPES was removed from the bath (solution 4). The 2 mM HEPES was replaced with 32 mM HEPES bilaterally at *i*. Finally, the basolateral buffer was switched to CO_2/HCO_3^- (*jkl*; solution 4) and back again to 32 mM HEPES (*lmn*). Acetate was absent during the entire experiment. Four similar experiments were performed.

has been observed previously (Nakhoul and Boron, 1988). The basolateral addition of CO_2/HCO_3^- (solution 12) and then its removal (solution 11), in the continued absence of acetate and Na^+, elicited no discernible pH_i change (*cde*). The lack of CO_2-induced pH_i changes (i.e., a reversible acidification) was expected, given the low pH_i. When the bath CO_2/HCO_3^- was added a second time, there was a slow alkalinization (*ef*); however, this was not reversed by the removal of bath CO_2/HCO_3^- (*fg*). Thus, the slow pH_i 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 pH_i increase (*gh*). During this period of rapid pH_i recovery in the presence of Na^+, basolateral addition of CO_2/HCO_3^- produced a transient acidification (*hi*), followed by the usual CO_2/HCO_3^--induced alkalinization (*ij*). Removal of CO_2/HCO_3^- had the opposite effects (*jkl*).

On the surface, these data suggest that the CO_2/HCO_3^--induced alkalinization is

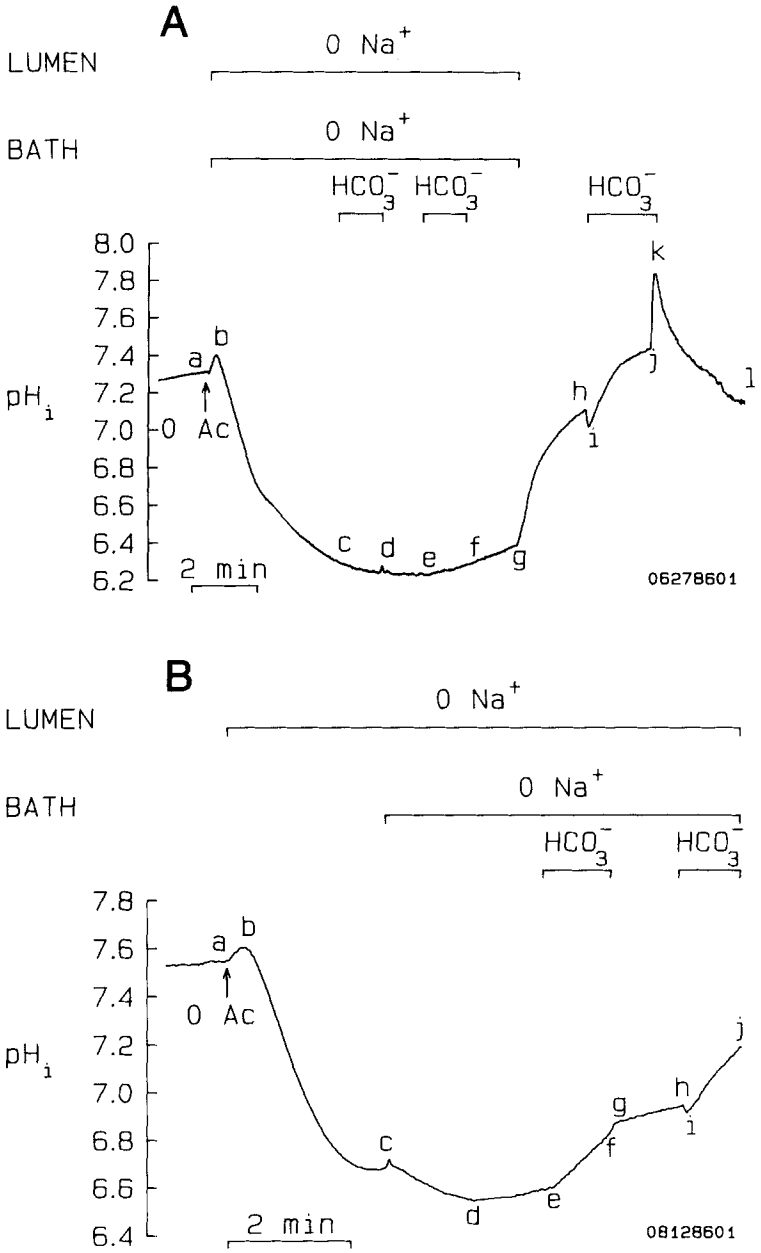

FIGURE 9. Effect of bilateral Na^+ removal on the basolateral CO_2/HCO_3^--induced alkalinization. (*A*) Apparent blockade by bilateral Na^+ removal. At point *a* (solution 1), Na^+ and acetate were each removed bilaterally (solution 11), causing a transient pH_i increase followed by a profound decrease (*abc*). During two subsequent exposures to basolateral CO_2/HCO_3^- (*cd* and *ef;* solution 12), pH_i was not affected differently than in adjacent CO_2/HCO_3^--free periods (*de* and *fg*). After Na^+ was reintroduced bilaterally and pH_i recovered (*gh*), the basolateral application (*hij*) and removal (*jkl*) of CO_2/HCO_3^- 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 pH_i to fall only to ~6.6 (*abcde*). On two occasions (*ef* and *ij*), the application of CO_2/HCO_3^- 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 pH_i. Thus, it is possible that the blockade of the basolateral CO_2/HCO_3^--induced alkalinization was not due to the absence of Na^+ per se, but by the extremely low pH_i. We found the results of several repetitions of the Fig. 9 *A* protocol to be inconsistent. In ten tubules, Na^+ removal blocked the CO_2/HCO_3^--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 pH_i increase (*ab*), followed by a large decrease (*bc*). pH_i decreased further when Na^+ was removed from the bath (*cd*). In the continued bilateral absence of Na^+, there was a slow, spontaneous recovery of pH_i (*de*), due to the Na^+-independent acid-extrusion mechanism (Nakhoul et al., 1988), possibly a luminal H^+ pump. The subsequent addition of CO_2/HCO_3^- elicited the usual alkalinization (*ef*); the absence of the CO_2-induced pH_i transient at *e* is expected, given the low pH_i. Removal of bath CO_2/HCO_3^- elicited a small CO_2-induced pH_i increase (*fg*), which was followed by Na^+-independent pH_i recovery (*gh*). Finally, a second application of bath CO_2/HCO_3^- elicited the usual transient acidification and sustained alkalinization (*hij*).

Three conclusions can be drawn from the experiment shown in Fig. 9, *A* and *B*. First, the CO_2/HCO_3^--induced alkalinization is not blocked by the bilateral removal of Na^+ per se (Fig. 9 *B*). Second, the rate of CO_2/HCO_3^--induced alkalinization was greater at higher pH_i (*ij* in Fig. 9 *B*) than at low pH_i (*ef*, Fig. 9 *B*), suggesting that the process may be pH_i dependent. Third, closer examination of the experiments done according to the protocol of Fig. 9, *A* and *B*, revealed that pH_i was very low (<6.6, averaging 6.50 ± 0.05) in the ten experiments in which Na^+ removal blocked the CO_2/HCO_3^--induced alkalinization, but that pH_i was relatively high (>6.6; averaging 7.01 ± 0.07) in the experiments in which Na^+ removal failed to block the alkalinization.

pH_i Dependence

To determine whether pH_i controls the rate of the CO_2/HCO_3^--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 pH_i transients (*abcd*), and resulted in a pH_i of ~ 6.4. In the continued absence of Na^+, switching from a HEPES to a CO_2/HCO_3^- buffer in the bath produced a small CO_2-induced acidification (*de*), followed by a slow alkalinization (*ef*). However, this slow pH_i increase was probably mediated by the Na^+-independent acid-extrusion mechanism, inasmuch as pH_i increased at about the same rate after the removal of bath CO_2/HCO_3^- (*gh*). This result confirms the result summarized above: the CO_2/HCO_3^--induced alkalinization is absent when pH_i is below ~ 6.6. At *h* we restored the Na^+ to only the lumen, which served two purposes. First, it caused pH_i to increase to ~ 6.88 (*hi*), presumably due to luminal Na-H exchange, so that we could examine the effect of bath CO_2/HCO_3^- at a higher pH_i. Second, it established a cell-to-bath concentration gradient for Na^+, so that any Na/HCO_3^- cotransport would have to occur in the cell-to-bath direction. When we switched the basolateral buffer to CO_2/HCO_3^- after pH_i had reached ~ 6.88, an initial fall of pH_i (*ij*) was followed by a CO_2/HCO_3^--induced alkalinization (*jk*), even though Na^+ was still absent from the bath. In six similar experiments, we found that the

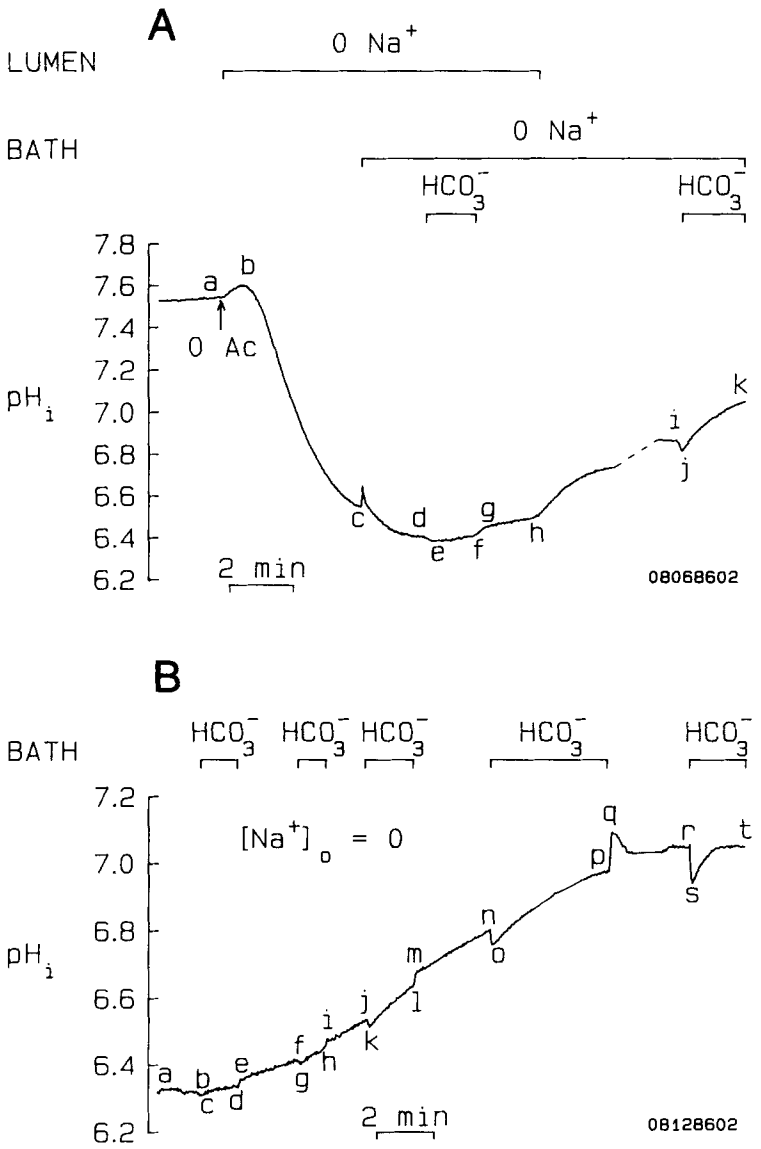

FIGURE 10. Dependence of the basolateral CO_2/HCO_3^--induced alkalinization on pH_i. (*A*) pH_i increased by exposure to luminal Na^+. The bilateral removal of acetate (point *a*) and the sequential removal of Na^+ from lumen (*a*) and bath (*c;* solution 11) caused pH_i to fall (*abcd*) to a value sufficiently low (i.e., ~6.4) to block the CO_2/HCO_3^--induced alkalinization. In the continued bilateral absence of Na^+, the basolateral application of CO_2/HCO_3^- (*ef*; solution 12) produced no more rapid a pH_i increase than the adjacent CO_2/HCO_3^--free period (*gh*). After readdition of Na^+ to only the lumen caused a partial pH_i recovery (*hi*), the basolateral application of CO_2/HCO_3^- caused the usual transient pH_i decrease followed by the sustained increase (*ijk*). Six similar experiments were performed. (*B*) pH_i allowed to increase via the Na^+-independent acid-extrusion mechanism. Acetate and Na^+ had been removed bilaterally before the start of the record, causing pH_i to fall to nearly 6.3, and were absent thereafter. During five periods in which 5% CO_2/25 mM HCO_3^- was present in the bath (*bcd, fgh, jkl, nop,* and *rst*), the rate of pH_i increase, compared to the preceding and succeeding CO_2/HCO_3^--free periods, gradually rose as pH_i rose. Four similar experiments were performed.

CO_2/HCO_3^--induced alkalinization was imperceptible at an average pH_i 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 pH_i of 6.80 ± 0.03 in the presence of luminal Na^+. Thus, by using the luminal Na-H exchanger to raise pH_i, we were able to show that the CO_2/HCO_3^--induced alkalinization is independent of bath Na^+, but inhibited at pH_i values as below ~6.5.

In Fig. 10 *A*, we raised pH_i during the experiment by exposing the tubule to luminal Na^+. To rule out the possibility that luminal Na^+, rather than pH_i, is the key factor for the basolateral CO_2/HCO_3^--induced alkalinization, we used a protocol similar to that of Fig. 10 *A*, but allowed the Na^+-independent acid-extrusion mechanism to raise pH_i 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 pH_i had fallen to ~6.33 by the time point *a* was reached. The first of five basolateral additions of CO_2/HCO_3^- caused a barely perceptible acidification (*bc*), followed by a slow alkalinization (*cd*). However, this slow pH_i increase was probably caused by the Na^+-independent acid-extrusion mechanism, inasmuch as the rate of alkalinization was even greater after CO_2/HCO_3^- removal (*ef*). The rate of pH_i increase during a second period of CO_2/HCO_3^- (*gh*), at a pH_i of ~6.43, was slightly greater than during the preceding (*ef*) and succeeding (*ij*) CO_2/HCO_3^--free periods, indicating the presence of a small CO_2/HCO_3^--induced alkalinization. During the third CO_2/HCO_3^- pulse (*kl*), at a pH_i of ~6.6, the alkalinization rate exceeded that during the bracketing CO_2/HCO_3^--free periods (*ij* and *mn*) by an even greater margin. Similarly, the rates of alkalinization during the fourth (*op*) and fifth (*st*) CO_2/HCO_3^- pulses were substantially greater than the adjacent CO_2/HCO_3^--free periods. Comparable results were obtained in four other experiments. Thus, even in the bilateral absence of Na^+, the basolateral CO_2/HCO_3^--induced alkalinization was absent at pH_i values below ~6.4, and became increasingly more prominent at higher pH_i 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 CO_2/HCO_3^--induced alkalinization was minimal at low pH_i values, but gradually increased as pH_i increased.

Data on the rates of 119 CO_2/HCO_3^--induced alkalinizations (all protocols) are plotted as a function of pH_i in Fig. 11. The plot shows that the rate of CO_2/HCO_3^--induced alkalinization is absent at a pH_i of ~6.3, is minimal at a pH_i of ~6.5, peaks at a pH_i of ~7.0, and then falls off slightly at higher pH_i values.

Effect of Removing all Solutes Except H^+ and CO_2/HCO_3^-

As indicated in Table I, our solutions routinely contained a variety of solutes in addition to the ones whose importance for the CO_2/HCO_3^--induced alkalinization we had already tested individually (i.e., Na^+, Cl^-, $SO_4^=$, HEPES). Rather than individually assess each of these, we removed all of them simultaneously. The normal CO_2/HCO_3^--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 CO_2/HCO_3^--free saline with $NMDG^+/Glr^-$ caused pH_i 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, CO_2/HCO_3^- was added to the bath (HCO_3^- replacing Glr^-; solution 14). This elicited the characteristic alkalinization (*bc*) that was reversed by the removal of the CO_2/HCO_3^- (*cde*), and reproduced by a second exposure to basolateral CO_2/HCO_3^- (*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 CO_2/HCO_3^-, at an initial pH_i of ~6.75, produced the typical transient acidification and sustained alkalinization (*bc*). Thus, the basolateral CO_2/HCO_3^--induced alkalinization can be elicited in tubules exposed, even for prolonged periods, to solutions containing no other natural solutes other than H^+ and CO_2/HCO_3^-.

The data presented in this paper consistently showed that the CO_2/HCO_3^--induced alkalinization is produced by a basolateral, but not a luminal, addition of CO_2/HCO_3^-. However, one might posit another explanation for this asymmetry: The alkalinizing effect of CO_2/HCO_3^- actually can be produced by either luminal or basolateral CO_2/HCO_3^-, but luminal CO_2/HCO_3^- has an additional

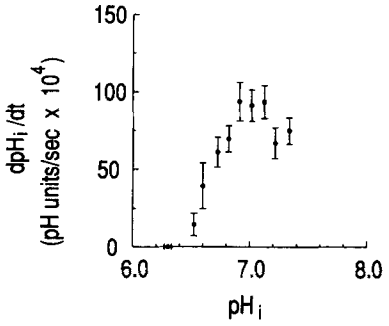

FIGURE 11. Dependence of the rate of CO_2/HCO_3^--induced alkalinization (dpH_i/dt) on pH_i. Data are summarized from 119 basolateral CO_2/HCO_3^- pulses, representing all protocols described in this study.

acidifying effect that exactly balances the alkalinizing effect. This additional acidifying effect could be produced by basolateral Cl-HCO_3 exchange and Na/HCO_3 cotransport, supported by the large cell-to-bath HCO_3^- gradient expected to prevail when CO_2/HCO_3^- 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 HCO_3^- 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 CO_2/HCO_3^- 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 CO_2/HCO_3^- (*ab*) had little effect on this acidification, which continued after removal of the CO_2/HCO_3^- (*cd*). The second luminal application of CO_2/HCO_3^- (*de*) also did not seem to affect pH_i. However, when the CO_2/HCO_3^- was applied to the bath, pH_i rose rapidly (*fg*). The results of twin luminal CO_2/HCO_3^- exposures in a total of five similar experiments are summarized in Table IV. They show that, even when

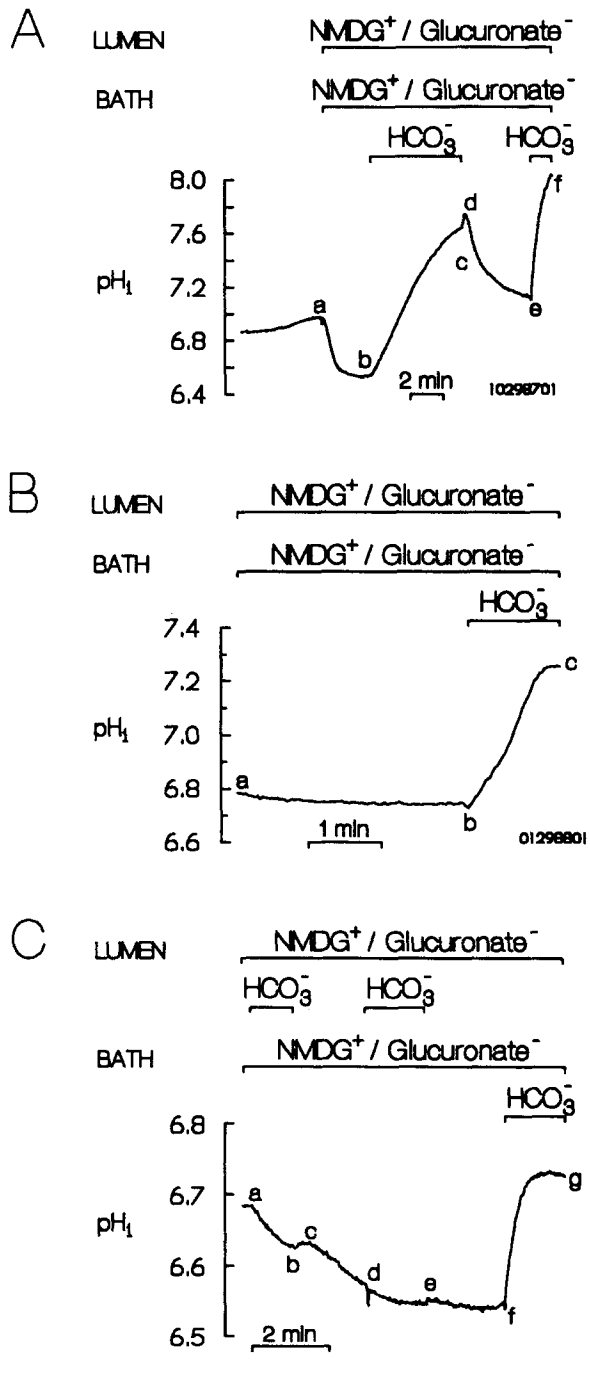

FIGURE 12. Effect of replacing all solutes with *N*-methyl-D-glucammonium/glucuronate. (*A*) Short-term removal. All luminal and basolateral solutes were replaced by *N*-methyl-D-glucammonium / glucuronate (solution 13) at point *a*, causing a rapid and then slower fall in pH_i (*ab*). In the continued 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-HCO_3 exchange and Na/HCO_3 cotransport are blocked by removal of key ions (i.e., Cl^- and Na^+, respectively), luminal CO_2/HCO_3^- fails to elicit the pH_i increase produced by basolateral CO_2/HCO_3^-.

DISCUSSION

General

Previous work has demonstrated that the basolateral membrane of the S3 segment of the rabbit proximal tubule possesses at least two HCO_3^- transporters, a Cl-HCO_3 exchanger and a Na/HCO_3 cotransporter (Kurtz, 1989*b*; Nakhoul et al., 1990).

TABLE IV

*Effect of Twin Luminal CO_2/HCO_3^- Pulses in Tubules Exposed to $NMDG^+/Glr^-$ Solutions in Lumen and Bath**

First luminal CO_2/HCO_3^- *pulse:*	
Initial pH_i	6.78 ± 0.09
dpH_i/dt at initial pH_i	$-2.7 \pm 3.1 \times 10^{-4}$ s^{-1}‡
control dpH_i/dt	$-0.5 \pm 2.7 \times 10^{-4}$ s^{-1}
n	4
Second luminal CO_2/HCO_3^- *pulse:*	
Initial pH_i	6.77 ± 0.10
dpH_i/dt at initial pH_i	$-1.0 \pm 1.3 \times 10^{-4}$ s^{-1}§
control dpH_i/dt	$-2.8 \pm 1.8 \times 10^{-4}$ s^{-1}
n	5

*We measured the initial rate of pH_i change (dpH_i/dt) after switching the luminal solution from $NMDG^+/Glr^-$ (solution 13) to $NMDG^+/Glr^-$ plus CO_2/HCO_3^- (solution 14). The bath solutions were $NMDG^+/Glr^-$ (solution 13) throughout. The "control dpH_i/dt" is the rate of pH_i change immediately before CO_2/HCO_3^- was added to the lumen. Negative values for dpH_i/dt indicate that pH_i was decreasing with time. The values presented are means ± SEM. The statistical analyses were the result of paired, one-tailed *t* tests.

‡After addition of luminal CO_2/HCO_3^- the rate of pH_i 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 HCO_3^- across the basolateral membrane. Direct measurements confirm that transepithelial HCO_3^- 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-HCO_3 exchanger and Na/HCO_3 cotransporter in HCO_3^- reabsorption, we expected that the bilateral transition from HEPES- to CO_2/HCO_3^--buffered solutions would have activated these two potent intracellular acid loaders, and thereby produced a sustained decrease in pH_i. Indeed, with the high initial pH_i prevailing in the presence of acetate, the addition of CO_2/HCO_3^- did cause a sustained pH_i decrease. However, with the low initial pH_i prevailing in the absence of acetate, bilateral application of CO_2/HCO_3^- caused a substantial increase in steady-state pH_i. Even more surprising, the alkalinization can be attributed specifically to the presence of CO_2/HCO_3^- at the basolateral membrane.

Evidence Against HCO_3^- *Influx Across the Basolateral Membrane*

The increase in steady state pH_i 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_i. 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 $Cl\text{-}HCO_3$ exchanger. In the presence of CO_2/HCO_3^-, blockade of these transporters by DIDS produces both an increase in steady-state pH_i (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 $Cl\text{-}HCO_3$ exchanger, the Na/HCO_3 cotransporter, the $Cl\text{-}HCO_3$ exchanger, and a $SO_4\text{-}HCO_3$ exchanger. All four are blocked by stilbene derivatives such as DIDS, and we have previously shown that a brief exposure to this agent blocks $Cl\text{-}HCO_3$ 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 $Cl\text{-}HCO_3$ exchanger or a Na^+-dependent $Cl\text{-}HCO_3$ exchanger would require intracellular Cl^-. Yet even prolonged Cl^- washouts failed to slow the pH_i increase. An alkalinization mediated by either a Na/HCO_3 cotransporter or a Na^+-dependent $Cl\text{-}HCO_3$ exchanger would require extracellular Na^+. Yet, bilateral Na^+ removal had no effect on the pH_i increase. An alkalinization mediated by a $SO_4\text{-}HCO_3$ exchanger would require intracellular $SO_4^=$. Yet bilateral $SO_4^=$ removal did not slow the pH_i 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 $NMDG^+/Glr^-$ 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^-.[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 $[H_2CO_3]_i$, and therefore $[HCO_3^-]_i$, were extremely high. Our calculations show that $[H_2CO_3]_i$ would have to rise from practically zero to ~6 μM 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 $[HCO_3^-]_i$ 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" $[HCO_3^-]_i$ 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

[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 CO_2/HCO_3^- 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 HCO_3^-. This model would still require a signal transduction mechanism to link basolateral CO_2/HCO_3^- to an inhibition of acid loading.

Possible Stimulation of Acid Extrusion Across the Luminal Membrane

In the third general model for the CO_2/HCO_3^--induced alkalinization, basolateral CO_2 and/or HCO_3^- stimulates the extrusion of acid equivalents across the luminal membrane. This would have the effect of not only raising pH_i, but also promoting transepithelial HCO_3^- reabsorption. The results of Fig. 12 suggest that the only ions required for such an acid-extruding process are H^+ and/or HCO_3^-. The active uptake of HCO_3^- across the luminal membrane is highly unlikely, inasmuch as the CO_2/HCO_3^--induced alkalinization was specifically not supported by luminal CO_2/HCO_3^-. This leaves as the most likely mechanism a luminal H^+ pump. Schwartz and Al-Awqati have demonstrated that bilateral CO_2/HCO_3^- 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) CO_2/HCO_3^- 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 pH_i, which leads to a rise in $[Ca^{++}]_i$. Our data are not consistent with this hypothesized role for pH_i: We found that luminal CO_2/HCO_3^- consistently caused a larger initial pH_i decrease than did basolateral CO_2/HCO_3^- (see Fig. 3 *A*). Nevertheless, it was only basolateral CO_2/HCO_3^- that caused the alkalinization.

Although our data indicate that the CO_2/HCO_3^--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 pH_i increase. In this context, it is interesting to note that recent work suggests that CO_2/HCO_3^-, specifically basolateral CO_2/HCO_3^-, stimulates luminal Na-H exchange (Chen and Boron, 1993*b*).

Is it CO_2 or HCO_3^- that Signals the Alkalinization?

We believe that the most likely explanation for the CO_2/HCO_3^--induced alkalinization is that CO_2 and/or HCO_3^- 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 CO_2 or HCO_3^- that initiates the process. Our data do not allow us to make a definitive choice between CO_2 and HCO_3^-. The observation that luminal CO_2/HCO_3^- fails to elicit the alkalinization implies that neither the CO_2 nor HCO_3^- could be acting at the luminal membrane. Moreover, because CO_2 can freely cross the luminal membrane, it is unlikely that either CO_2 or HCO_3^- could be acting at a cytoplasmic site near the apical pole of the cell. However, it is possible that CO_2 and/or HCO_3^- acts at a low-affinity cytoplasmic site near the basolateral membrane, and that local $[CO_2]_i$ and/or $[HCO_3^-]_i$ are sufficiently high to trigger the alkalinization only when CO_2 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 $CO_3^=$).

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_i, 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_i dependence would be that the acid-extruder itself has an intrinsic, biphasic pH_i dependency. Indeed, it is interesting to note that the pH_i dependence of the CO_2/HCO_3^--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 CO_2/HCO_3^--induced Alkalanization Observed in the Presence of Acetazolamide

The acid-extruding flux produced by basolateral CO_2/HCO_3^- in the presence of acetazolamide. In principle, the basolateral CO_2/HCO_3^- -induced alkalinization could be due either to stimulation of an acid-efflux mechanism (e.g., H^+ pump) or direct uptake of HCO_3^-. Examining the rate of this alkalinization when carbonic anhydrase (CA) is inhibited by acetazolamide (ACZ) should help us distinguish between the acid-efflux and HCO_3^--uptake models. In the case of the acid-efflux model, the H^+ efflux needed to account for the rate of pH_i increase observed in the presence of ACZ is simply $(dpH_i/dt) \times \beta$. Given the dpH_i/dt of 58×10^{-4} pH/s observed at a pH_i of 7.1, and the intrinsic buffering power of 30 mM/pH measured at a pH_i of 7.1 (Chen and Boron, 1993*a*), 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 HCO_3^- would be considerably greater than the required efflux of H^+.

Pathways for the removal of H_2CO_3 in the HCO_3^- uptake model. As illustrated in Fig. 13 *A*, the HCO_3^--uptake model predicts that pH_i can rise only as rapidly as the incoming HCO_3^- combines with H^+ to form H_2CO_3. Because the pK of the reaction $H_2CO_3 = H^+ + HCO_3^-$ is ~3.5, and the reaction occurs very rapidly, $[H_2CO_3]_i$ is < ~0.1% as high as $[HCO_3^-]_i$ at physiological pH_i values. Therefore, millimolar quantities of H^+ can be consumed only if H_2CO_3 can be removed more or less as rapidly as it is formed. Newly formed H_2CO_3 can be removed by three routes: (*a*) efflux of H_2CO_3, which should be proportional to the difference $[H_2CO_3]_i - [H_2CO_3]_o$; (*b*) the uncatalyzed dehydration reaction $H_2CO_3 \rightarrow H_2O + CO_2$; 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_i increase elicited by removal of basolateral CO_2/HCO_3^- 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_i increase that is produced by removal of extracellular CO_2/HCO_3^-. Fig. 13 *B* illustrates the reactions taking place. Just before the extracellular CO_2 is removed, pH_i is 7.4, $[CO_2]_i$ is 1.2 mM, $[HCO_3^-]_i$ is 24 mM, and $[H_2CO_3]_i$ is 3 μM.[4] In six experiments such as that in Fig. 5, the mean

[4] In computing $[CO_2]_i$, we assumed a pCO_2 of 40 torr, and a CO_2 solubility of 0.03 mM/torr. In computing $[HCO_3^-]_i$, we assumed that the pK governing the overall reaction $HCO_3^- + H^+ = CO_2 + H_2O$ is 6.1. In computing $[H_2CO_3]_i$, we assumed that the pK governing the reaction $H_2CO_3 = H^+ + HCO_3^-$ is 3.5.

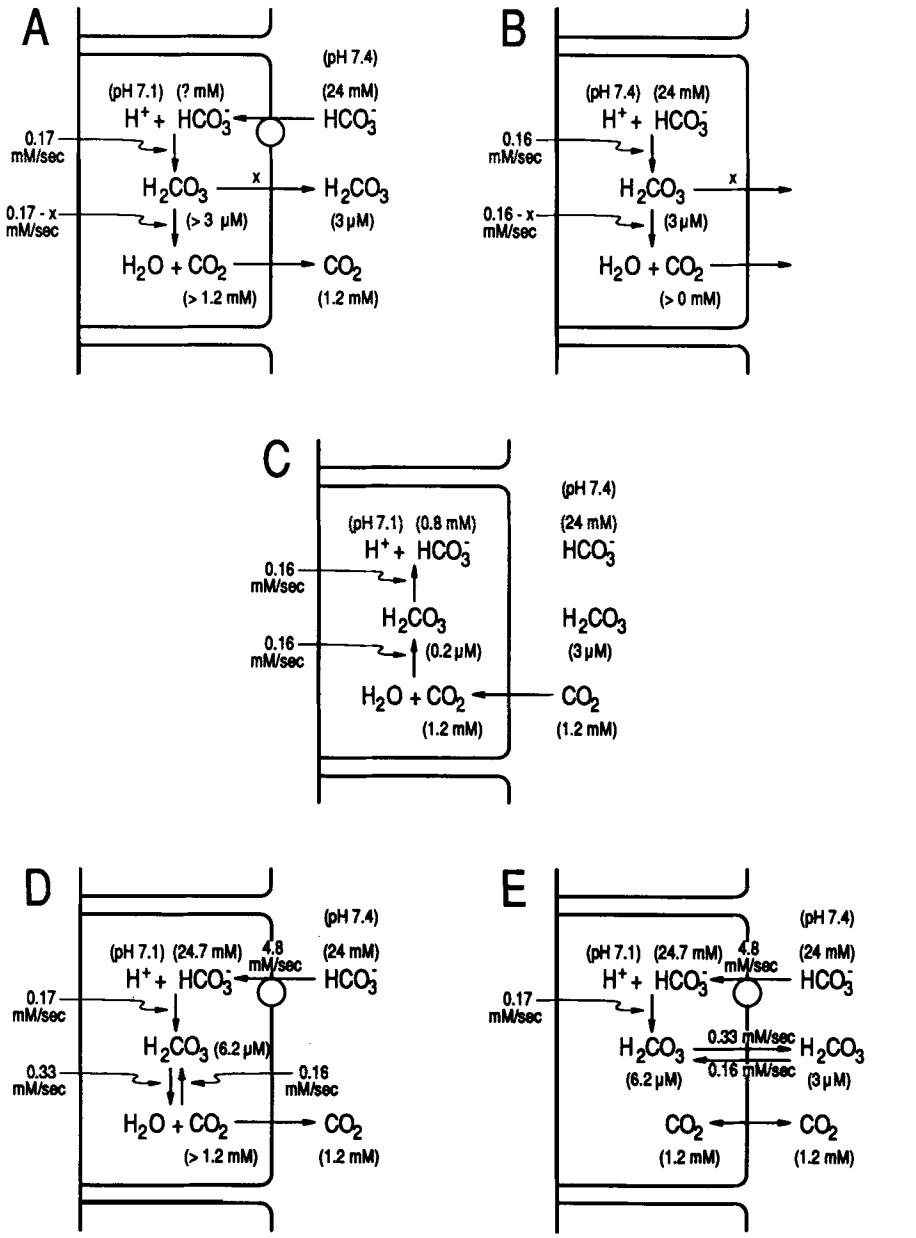

FIGURE 13. Predicted reaction rates associated with the influx of HCO_3^-, when carbonic anhydrase (CA) is blocked by acetazolamide (ACZ). (*A*) Introduction. The pH_i increase elicited by HCO_3^- uptake can proceed only as rapidly as H^+ is neutralized by incoming HCO_3^- to form H_2CO_3. However, this reaction can proceed only as rapidly as H_2CO_3 either exits the cell, or dissociates into H_2O and CO_2. From the observed rate of pH_i increase and intracellular buffering power, we calculate that H^+ disappears at the rate of 0.17 mM/s. (*B*) Washout of CO_2. When a cell previously equilibrated with CO_2/HCO_3^- is exposed to a CO_2/HCO_3^--free medium, pH_i rises due to the efflux of CO_2 and/or H_2CO_3. From the observed rate of pH_i increase at pH_i

initial (i.e., maximal) dpH_i/dt produced by CO_2 withdrawal in the presence of ACZ (see segments *cd* and *fg* in Fig. 5) was $79 \pm 16 \times 10^{-4}$ pH/s at a pH_i of ~7.4. Given a β of 20 mM/pH measured at a pH_i of 7.4 (Chen and Boron, 1993*a*), the rate of H^+ neutralization, which must have been the same as the flux of H_2CO_3 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 H_2CO_3 efflux (J_{H2CO3}), where $J_{H2CO3} = P_{H2CO3} \times ([H_2CO_3]_i - [H_2CO_3]_o)$, then the effective permeability constant P_{H2CO3} must have been $J_{H2CO3}/(([H_2CO_3]_i - [H_2CO_3]_o) = (0.16 \text{ mM/s})/(3 \mu M - 0) = \sim 53 \text{ s}^{-1}$. 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*) $[CO_2]_i$ is zero immediately after removal of external CO_2, and (*b*) that this flux ($J_{dehyd+CA}$) is proportional to $[H_2CO_3]_i$. It follows that the rate constant governing the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ would be given by $k_1 = J_{dehyd+CA}/[H_2CO_3]_i = (0.16 \text{ mM/s})/(3 \mu M) = \sim 53 \text{ s}^{-1}$. 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 H_2CO_3 consumption is governed by H_2CO_3 efflux vs conversion to CO_2 is not important for our analysis.

The delayed pH_i increase elicited by application of basolateral CO_2/HCO_3^- in the presence of ACZ. Applying basolateral CO_2/HCO_3^- in the absence of ACZ produces a transient pH_i 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_i decrease is very small; we will assume that the delay to the initiation of the pH_i increase remains 5 s. In this analysis, we will first make the simplifying assumption that, during this initial 5 s, $[CO_2]_i$ rises to 1.2 mM (the extracellular concentration) instantaneously, but that HCO_3^- cannot enter the cell. Based on the value for k_1 computed above, we estimate that 0.8 mM H_2CO_3 would be formed from the intracellular CO_2 during these initial

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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 $[H_2CO_3]_i$, we can compute the effective permeability to H_2CO_3 or the rate constant (k_1) of the inhibited dehydration reaction. (*C*) Predicted fluxes 5 s after the introduction of CO_2, allowing only an influx of CO_2. From k_1 and the equilibrium constant for the dehydration reaction, we can compute the rate of formation of H_2CO_3 from CO_2. Virtually all of this newly formed H_2CO_3 dissociates into H^+ and HCO_3^-. Over a period of 5 s, we estimate that 0.8 mM HCO_3^- would be formed. (*D*) Predicted fluxes 5 s after the introduction of CO_2/HCO_3^-, allowing the influx of HCO_3^- and the transmembrane equilibration of CO_2. In order for the net reaction $H_2CO_3 \rightarrow H_2O + CO_2$ to proceed at the required rate of 0.17 mM/s in the presence of ACZ, $[H_2CO_3]_i$ would have to be 6.2 μM. Because H_2CO_3, H^+ and HCO_3^- rapidly equilibrate, we can compute that $[HCO_3^-]_i$ must be 24.7 mM when pH_i is 7.1. This level of HCO_3^- must be achieved in within 5 s of the time CO_2/HCO_3^- is added, for an average HCO_3^- flux of 4.8 mM/s. (*E*) Predicted fluxes 5 s after the introduction of CO_2/HCO_3^-, allowing the influx of HCO_3^- and the transmembrane equilibration of H_2CO_3. The analysis here is similar to that in part *D*, except that all newly formed H_2CO_3 passively exists from the cell. The unidirectional H_2CO_3 fluxes are computed from the effective permeability constant derived from the data in part *B*.

5 s,[5] and that almost all of this H_2CO_3 would dissociate into HCO_3^- and H^+. The predicted immediate pH_i change can be predicted from this H^+ formation and the intrinsic β of 30 mM: $\Delta pH_i = -(0.8 \text{ mM})/(30 \text{ mM/pH}) = -0.027$. 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 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 H_2CO_3 is the conversion to 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_i begins to increase at the rate of 58×10^{-4} pH/s ($pH_i = 7.1$), and H^+ must disappear at the rate of ~0.17 mM/s. Thus, at t = 5 s, the net formation of CO_2 from H_2CO_3 also must proceed at ~0.17 mM/s. Inasmuch as the back-reaction ($CO_2 + H_2O \rightarrow H_2CO_3$) proceeds at 0.16 mM/s, the forward reaction ($H_2CO_3 \rightarrow CO_2 + H_2O$) would have to proceed at 0.33 mM/s. Because k_1 governing this forward reaction is 53 s^{-1}, the required $[H_2CO_3]_i$ can be calculated: $[H_2CO_3]_i = J_{uncat+CA}/k_1 = (0.33 \text{ mM } s^{-1})/(53 \text{ } s^{-1}) = 6.2 \text{ } \mu M$. Given a value of ~3.5 for the pK of the equilibrium $H_2CO_3 = H^+ + HCO_3^-$, and a pH_i of 7.1, this $[H_2CO_3]_i$ predicts a $[HCO_3^-]_i$ of ~24.7 mM. In other words, in order for a HCO_3^--uptake mechanism to account for the CO_2/HCO_3^--induced pH_i increase observed at $t = 5$ s, the conversion of the newly formed H_2CO_3 to CO_2 would require that $[HCO_3^-]_i$ rise from 0 to 24.7 mM in only 5 s. As pointed out above, CO_2 influx would cause $[HCO_3^-]_i$ to rise from 0 to 0.8 mM in 5 s. Thus, over a period of 5 s, the hypothetical HCO_3^- uptake mechanism would have to account for a $\Delta[HCO_3^-]_i$ of 24.7 − 0.8 = 23.9 mM; this corresponds to an average HCO_3^- influx of 4.8 mM/s.

The conclusions about the predicted HCO_3^- flux are virtually identical if we assume that all of the newly formed H_2CO_3 is dissipated by the efflux of H_2CO_3 (Fig. 13 *E*). Thus, regardless of whether we assume that the newly formed H_2CO_3 is converted to CO_2, or exits the cell directly as H_2CO_3, the HCO_3^- influx needed to explain the data obtained during inhibition of CA would have to be ~4.8 mM/s. This figure is unreasonably high when compared with the fluxes produced by HCO_3^--dependent acid-extrusion mechanisms in other cell types. For example, renal mesangial cells exhibit a CO_2/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, 1988*a*,*b*). However, the computed flux at a pH_i of 6.8 is only ~0.16 mM/s (Boyarsky et al., 1988*a*).

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[5] The equilibrium constant governing the reaction $H_2CO_3 = CO_2 + H_2O$ is ~0.0025. Thus, if k_1 (the rate constant for $H_2CO_3 \rightarrow CO_2 + H_2O$) is 53 s^{-1}, then k_{-1} (the rate constant for $CO_2 + H_2O \rightarrow H_2CO_3$) is must be $(53 \text{ } s^{-1})/400 = 0.133 \text{ } s^{-1}$. Thus, the flux from CO_2 to H_2CO_3 must be $k_{-1} \times [CO_2]_i = (0.133 \text{ } s^{-1}) \times (1.2 \text{ mM}) = 0.16$ mM/s. During the first 5 s, the total H_2CO_3 formed comes to 0.8 mM.

REFERENCES

Alpern, R. J. 1985. Mechanism of basolateral membrane $H^+/OH^-/HCO_3^-$ transport in the rat proximal convoluted tubule. A sodium-coupled electrogenic process. *Journal of General Physiology.* 86:613–636.

Alpern, R. J., and M. Chambers. 1986. Cell pH in the rat proximal convoluted tubule: regulation by luminal and peritubular pH and sodium concentration. *Journal of Clinical Investigation.* 78:502–510.

Alpern, R. J., and M. Chambers. 1987. Basolateral membrane Cl/HCO_3 exchange in the rat proximal convoluted tubule. Na-dependent and independent modes. *Journal of General Physiology.* 89:581–598.

Berliner, R. W. 1952. Renal secretion of potassium and hydrogen ions. *Federation Proceedings.* 11:695–700.

Berliner, R. W. 1985. Carbon dioxide tension of alkaline urine. *In* The Kidney: Physiology and Pathophysiology. Vol 2. D. W. Seldin and G. Giebisch, editors. Raven Press, NY. 1527–1537.

Biagi, B. A., and B. A. Vance. 1989. Microelectrode Characterization of the Basolateral Membrane of Rabbit S3 Proximal Tubule. *Journal of Membrane Biology.* 108:53–60.

Biagi, B. A., and M. Sohtell. 1986. Electrophysiology of basolateral bicarbonate transport in the rabbit proximal tubule. *American Journal of Physiology.* 250:F267–F272.

Boron, W. F., and E. L. Boulpaep. 1983*a*. Intracellular pH regulation in the renal proximal tubule of the salamander. Na-H exchange. *Journal of General Physiology.* 81:29–52.

Boron, W. F., and E. L. Boulpaep. 1983*b*. Intracellular pH regulation in the renal proximal tubule of the salamander: basolateral HCO_3^- transport. *Journal of General Physiology.* 81:53–94.

Boron, W. F., W. C. McCormick, and A. Roos. 1979. pH regulation in barnacle muscle fibers: dependence on intracellular and extracellular pH. *American Journal of Physiology.* 237:C185–C193.

Boyarsky, G., M. B. Ganz, B. Sterzel, and W. F. Boron. 1988*a*. pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO_3. *American Journal of Physiology.* 255:C844–C856.

Boyarsky, G., M. B. Ganz, B. Sterzel, and W. F. Boron. 1988*b*. pH regulation in single glomerular mesangial cells. II. Na-dependent and -independent Cl^-HCO_3 exchangers. *American Journal of Physiology.* 255:C857–C869.

Brazeau, P., and A. Gilman. 1953. Effect of plasma CO_2 tension on renal tubular reabsorption of bicarbonate. *American Journal of Physiology.* 175:33–38.

Burg, M., J. Grantham, M. Abramow, and J. Orloff. 1966. Preparation and study of fragments of single rabbit nephrons. *American Journal of Physiology.* 210:1293–1298.

Cannon, C., J. Van Adelsberg, S. Kelly, and Q. Al-Awqati. 1985. Carbon-dioxide-induced exocytotic insertion of H^+ pumps in turtle-bladder luminal membrane: role of cell pH and calcium. *Nature.* 314:443–446.

Chaillet, J. R., and W. F. Boron. 1985. Intracellular calibration of a pH-sensitive dye in isolated perfused salamander proximal tubules. *Journal of General Physiology.* 86:765–794.

Chaillet, J. R., K. Amsler, and W. F. Boron. 1986. Optical measurement of intracellular pH in single LLC-PK1 cells: demonstration of Cl^-HCO_3 exchange. *Proceedings of the National Academy of Science, USA.* 83:522–526.

Chan, Y. L., and G. Giebisch. 1981. Relationship between sodium and bicarbonate transport in the rat proximal convoluted tubule. *American Journal of Physiology.* 240:F222–F230.

Chen, L. K., and W. F. Boron. 1993*a*. Intracellular pH regulation in the S3 segment of the rabbit proximal tubule: I. Effect of CO_2/HCO_3 on acid extrusion. *American Journal of Physiology.* In press.

Chen, L. K., and W. F. Boron. 1993*b*. Intracellular pH regulation in the S3 segment of the rabbit proximal tubule: II Effect of basolateral CO_2/HCO_3 on Acid Extrusion. *American Journal Physiology.* In press.

Dickman, K. G., and L. J. Mandel. 1992. Relationship between HCO_3^- transport and oxidative metabolism in rabbit proximal tubule. *American Journal of Physiology.* 263:F342–F351.

Dorman, P. J., W. J. Sullivan, and R. F. Pitts. 1954. The renal response to acute respiratory acidosis. *Journal of Clinical Investigation.* 33:82–90.

Geibel, J. P., G. Giebisch, and W. F. Boron. 1989. Effects of acetate on luminal acidification processes in the S3 segment of the rabbit proximal tubule. *American Journal of Physiology.* 257:F586–F594.

Grassl, S. M., L. P. Karniski, and P. S. Aronson. 1985. $Cl^-HCO_3^-$ exchange in rabbit renal cortical basolateral membrane vesicles (BLMV). *Kidney International.* 27:282*a*. (Abstr.)

Hamm, L. L., and R. J. Alpern. 1992. Cellular mechanisms of renal tubular acidification. *In* The Kidney: Physiology and Pathophysiology. Vol 2. D. W. Seldin and G. Giebisch, editors. Raven Press, New York. 2581–2626.

Kaissling, B., and W. Kriz. 1979. Structural analysis of the rabbit kidney. *Advances in Anatomy, Embryology and Cell Biology.* 56:1–121.

Kinsella, J. L., and P. S. Aronson. 1980. Properties of the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *American Journal of Physiology.* 238:F461–F469.

Kurtz, I. 1987. Apical Na^+/H^+ antiporter and glycolysis-dependent H^+-ATPase regulate intracellular pH in the rabbit S3 proximal tubule. *Journal of Clinical Investigation.* 80:928–935.

Kurtz, I. 1989*a*. Basolateral membrane Na^+ and Cl^--dependent H^+/base transport in the rabbit S3 proximal tubule. *Kidney International.* 35:457*a*. (Abstr.)

Kurtz, I. 1989*b*. Basolateral membrane Na^+/H^+ antiport, Na^+ cotransport, and Na^+-independent Cl^- base exchange in the rabbit S3 proximal tubule. *Journal of Clinical Investigation.* 83:616–622.

Low, I., T. Friedrich, and G. Burckhardt. 1984. Properties of an anion exchanger in rat renal basolateral membrane vesicles. *American Journal of Physiology.* 246:F334–F342.

Murer, H., U. Hopfer, and R. Kinne. 1976. Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *Biochemical Journal.* 154:597–604.

Nakhoul, N. L., and W. F. Boron. 1985. Intracellular pH regulation in rabbit proximal straight tubules: dependence on external sodium. *Federation Proceedings.* 44:1898*a*. (Abstr.)

Nakhoul, N. L., and W. F. Boron. 1987. pH_i regulation in the S3 proximal tubule: A novel HCO_3^--dependent acid-extruder of the basolateral membrane. *Kidney International.* 31:412*a*. (Abstr.)

Nakhoul, N. L., and W. F. Boron. 1988. Acetate transport in the S3 segment of the rabbit proximal tubule and its effect on intracellular pH. *Journal of General Physiology.* 92:395–412.

Nakhoul, N. L., L. K. Chen, and W. F. Boron. 1990. Intracellular pH regulation in rabbit S3 proximal tubule: basolateral Cl^-HCO_3 exchange and Na-HCO_3 cotransport. *American Journal of Physiology.* 258:F371–F381.

Nakhoul, N. L., A. G. Lopes, J. R. Chaillet, and W. F. Boron. 1988. Intracellular pH regulation in the S3 segment of the rabbit proximal tubule in HCO_3^--free solutions. *Journal of General Physiology.* 92:369–393.

Prichard, J. B., and J. L. Renfro. 1983. Renal sulfate transport at the basolateral membrane is mediated by anion exchange. *Proceedings of the National Academy of Science, USA.* 80:2603–2607.

Sasaki, S., T. Shiigai, and J. Takeuchi. 1985. Intracellular pH in the isolated perfused rabbit proximal tubule. *American Journal of Physiology.* 249:F417–F423.

Schwartz, G. J. 1981. Na^+-dependent H^+ efflux from proximal tubule: evidence for reversible Na^+-H^+ exchange. *American Journal of Physiology.* 241:F380–F385.

Schwartz, G. J., and Q. Al-Awqati. 1985. Carbon dioxide causes exocytosis of vesicles containing H^+ pumps in isolated perfused proximal and collecting tubules. *Journal of Clinical Investigation.* 75:1638–1644.

Thomas, J. A., R. N. Buchsbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 81:2210–2218.

Van Adelsberg, J., and Q. Al-Awqati. 1986. Regulation of cell pH by exocytotic insertion of H^+-ATPases. *J Cell Biology.* 102:1638–1645.

Wang, Z.-Q., and S. Gluck. 1990. Isolation and properties of bovine kidney brush border vacuolar H^+-ATPase. A proton pump with enzymatic and structural differences from kidney microsomal H^+-ATPase. *Journal of Biological Chemistry.* 265:21957–21965.

Yoshitomi, K., B.-Ch. Burckhardt, and E. Fromter. 1985. Rheogenic sodium-bicarbonate cotransport in the peritubular cell membrane of rat renal proximal tubule. *Pflugers Archiv.* 405:360–366.