Intracellular pH Regulation in the S3 Segment of the Rabbit Proximal Tubule in HCO$_3^-$-free Solutions

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ABSTRACT We used the absorbance spectrum of 4',5'-dimethyl-5-(and 6) carboxyfluorescein to measure intracellular pH (pHi) in the isolated, perfused S3 segment of the rabbit proximal tubule. Experiments were conducted in HCO$_3^-$-free solutions. pH$_i$ recovered from an acid load imposed by an NH$_4^+$ prepulse, indicating the presence of one or more active acid-extrusion mechanisms. Removal of Na$^+$ from bath and lumen caused pH$_i$ to decrease by ~0.6, whereas Na$^+$ readdition caused complete pH$_i$ recovery. Removal of Na$^+$ from the bath caused only a slow pH$_i$ decrease that was enhanced about fourfold when Na$^+$ was subsequently removed from the lumen also. Similarly, the pH$_i$ recovery produced by the readdition of Na$^+$ to the bath and lumen was about ninefold faster than when Na$^+$ was returned to the bath only. Amiloride (1–2 mM) inhibited the pH$_i$ recovery that was elicited by returning 15 or 29 mM Na$^+$ to lumen by only ~30%. However, in the absence of external acetate (Ac$^-$), 1 mM amiloride inhibited ~66% of the pH$_i$ recovery induced by the readdition of 29 mM Na$^+$ to the lumen only. The removal of external Ac$^-$ reduced the pH$_i$ recovery rate from an NH$_4^+$-induced acid load by ~47%, and that elicited by Na$^+$ readministration, by ~67%. Finally, when bilateral removal of Na$^+$ was maintained for several minutes, pH$_i$ recovered from the initial acidification, slowly at first, and then more rapidly, eventually reaching a pH$_i$ ~0.1 higher than the initial one. This Na$^+$-independent pH$_i$ recovery was not significantly affected by lowering [HEPES]$_i$ from 32 to 3 mM or by adding N'N'-dicyclohexylcarbodiimide (10$^{-4}$ M) to the lumen, but it was reduced ~57% by iodoacetate (0.5 mM) plus cyanide (1 mM). We conclude that in the nominal absence of HCO$_3^-$, three transport systems contribute to acid extrusion by S3 cells: (a) a Na$^+$-independent mechanism, possibly an H$^+$ pump; (b) a Na-H exchanger, confined primarily to the luminal membrane; and (c) an Ac$^-$ and luminal Na$^+$-dependent mechanism. The contribution of these three mechanisms to total acid extrusion, assessed by the rapid readdition of Na$^+$, was ~13, ~30, and ~57%, respectively.

INTRODUCTION

The mammalian proximal tubule is a major site of transepithelial acid secretion. This process, which results in the acidification of the luminal fluid and the net reab-
sorption of HCO₃⁻, involves two discrete steps: H⁺ efflux across the luminal membrane, and HCO₃⁻ efflux across the basolateral membrane (for review see Koeppen et al., 1985). It is to be expected that the acid-base transport mechanisms that mediate these fluxes affect and are affected by cytoplasmic pH (pHi). Thus, changes in pHi could serve as a link among the individual acid-base transporters at the luminal and basolateral membranes. Furthermore, it is likely that the mechanisms involved in pHi regulation also participate in transepithelial acid secretion.

The luminal step of acid secretion is believed to involve the extrusion of H⁺ in exchange for luminal Na⁺, as proposed for the mammalian proximal tubule (Bailariner, 1952). The existence of Na-H exchange has been confirmed in brush border membrane vesicles derived from the renal cortex (Murer et al., 1976; Kinsella and Aronson, 1980), and shown to be inhibited by the diuretic amiloride (Kinsella and Aronson, 1981). Two lines of evidence are consistent with luminal Na-H exchange in intact mammalian proximal tubules. Schwartz (1981) has described a Na⁺-dependent, amiloride-sensitive change in luminal pH in isolated perfused rabbit proximal convoluted tubules. In addition, Sasaki et al. (1985), through measuring pHi in proximal straight tubules of the rabbit, found that lowering luminal [Na⁺] causes an amiloride-sensitive fall in pHi. However, there have been no studies of the role of Na-H exchange in pHi regulation in mammalian proximal tubules. Furthermore, it remains to be established to what extent other HCO₃⁻-independent transport mechanisms contribute to pHi regulation in the intact tubule.

There are two major approaches available for monitoring rapid pHi changes in renal tubules, pH-sensitive microelectrodes and dyes. Although the small size of mammalian proximal-tubule cells makes their impalement with microelectrodes very difficult, successful pHi measurements have been reported using ion-selective microelectrodes in rat kidney (Yoshitomi et al., 1985; Henderson et al., 1986) and isolated perfused rabbit tubules (Sasaki et al., 1985). Dye techniques, on the other hand, are relatively easy to use and are capable of monitoring very rapid pHi changes. Earlier work from this laboratory exploited the pH-sensitivity of the absorbance spectrum of a fluorescein derivative (Chaillet and Boron, 1985) to measure pHi in isolated perfused rabbit cortical collecting tubules (Chaillet et al., 1985). Another alternative is to employ the pH sensitivity of the fluorescence excitation spectrum, as was done in monitoring pHi in vivo rat proximal convoluted tubules (Alpern, 1985).

In this study we examine the influence of HCO₃⁻-independent acid-base transport systems on pHi regulation in the S3 segment of the rabbit proximal straight tubule (i.e., the most distal 1 mm of the proximal tubule). The tubules were isolated and perfused in nominally HCO₃⁻-free solutions to minimize the contribution of HCO₃⁻-transporting mechanisms to pHi changes. pHi was determined from the absorbance spectrum of the pH-sensitive dye 4',5'-dimethyl-5-(and 6)-carboxyfluorescein (Me₂CF), a technique validated in our laboratory by comparing it with measurements made with microelectrodes in salamander proximal tubules (Chaillet and Boron, 1985). We found that (a) as anticipated, a Na-H exchanger, confined primarily to the luminal membrane, contributes to pHi regulation; (b) there is evidence for a Na⁺-independent acid-extruding mechanism, possibly a H⁺ pump, that also contributes to the recovery of pHi from intracellular acid loads, and finally, (c) that these cells possess an acid-extruding mechanism that requires acetate (Ac⁻) and
luminal Na+. At least in nominally HCO₃⁻-free solutions, this Na⁺- and Ac⁻-dependent mechanism is substantially more potent than the Na-H exchanger.

Portions of this work have been published in preliminary form (Chaillet and Boron, 1984; Nakhoul and Boron, 1985, 1986).

METHODS

Biological Preparation

We used 'pathogen-free', female, New Zealand white rabbits (Dutchland, Inc., Reston, VA), weighing 3-5 lb. After the animal was killed, the kidney was rapidly removed and cut in transverse sections ~1 mm thick. The slices were placed in cold HEPES Ringer that was similar to solution 1 (see Table I), except that it was titrated to pH 7.4 at 4°C. Under microscopic observation, forces were used to tease apart the slice, yielding a fine bundle of tubules running from the inner medulla into the cortex. From the outer medullary region of this bundle, we obtained a proximal straight tubule still connected to its thin descending limb of Henle's loop. We isolated the most distal ~1 mm of this proximal tubule that extended to the junction of the thin descending limb. The isolated tubule was transferred to a chamber designed to permit relatively fast changes of bath solutions (~2 s). It was perfused at 38°C in a manner similar to that described by Burg and co-workers (1966). To minimize movement of the tubule in the optical path, the exposed length of the tubule between the holding pipette was kept short, 200-400 μm.

The S3 segment of the proximal tubule (Kaissling and Kriz, 1979) consists of the terminal ~1 mm of the proximal straight tubule in a juxtamedullary nephron (Woodhall et al., 1978) and is slightly longer in a superficial nephron (Schafer and Barfuss, 1982). Inasmuch as we isolated the terminal ~1 mm, and perfused considerably less than this, our proximal straight tubules can be assumed to be S3 segments of the proximal tubule.

Solutions

The composition of the bath and perfusion solutions are given in Table I. All solutions were nominally HCO₃⁻-free and were buffered with HEPES at 38°C. The osmolality of all solutions was measured before the experiment, and was verified to be within a range of 295 to 305 mosm/kg. Solutions with varied Na⁺ concentrations were made by mixing either solutions 1 and 3 (in the case of Ac⁻-containing solutions), or solutions 5 and 6 (Ac⁻-free solutions). Amiloride (a gift of Merck, Sharp and Dohme, West Point, PA) was added as a powder to solutions, to a final concentration of 1 or 2 mM. N,N'-dicyclo-hexylcarbodiimide (DCCD) was obtained from Merck Chemical Co. (St. Louis, MO) and used at a concentration of 10⁻⁴ M. Potassium cyanide (Allied Chemicals, Morristown, NJ) was used at a concentration of 1 mM. Iodoacetic acid (Matheson, Coleman & Bell, Norwood, OH) was used at a concentration of 0.5 mM. The colorless dye precursor Me₂CF diacetate (Molecular Probes, Inc., Junction City, OR) was added to solution 1 from a 100-mM stock solution in dimethyl sulfoxide to a final concentration of 100 μM. Nigericin (Calbiochem-Behring, La Jolla, CA) was added to solution 10 from a stock solution of 10 mM in ethanol, to a final nigericin concentration of 10 μM. The nigericin calibrating solutions were titrated to different pH values at 38°C with either HCl or N-methyl-d-glucamine (NMDG). NMDG and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO).

Optical Measurements of pH_i

We calculated pH_i values from absorbance spectra (obtained once per second) of a pH-sensitive dye incorporated intracellularly. Because the details of the method have been described
previously (Chaillet and Boron, 1985), we will only summarize them here. The pH-sensitive
dye Me$_2$CF was introduced into the tubule cells by perfusing the lumen with the dye’s color-
less diacetate precursor (Me$_2$CFAc$_2$), to which the cells are relatively permeant. Once the
precursor is inside the cell, native esterases cleave the acetate esters, releasing Me$_2$CF. 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
spot of white light (10 μm in diameter) was focused on the tubule with 32x objective that
acted as a condenser. A second cover slip that formed the ceiling of the chamber was fixed to
this condenser, so that the tubule and an ~1-mm-thick section of solution were sandwiched
between the two cover slips. The light leaving the tubule was collected by a 10x objective and
focused onto a diffraction grating using a field/relay-lens combination. The resulting spec-

### Table I

| Component | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-----------|---|---|---|---|---|---|---|---|---|----|
| Standard | Na$^+$ | 146.4 | 126.4 | 0 | 152.9 | 0 | 146.4 | 126.4 | 145.8 | 0 |
| HEPES    | K$^+$ | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 0 | 105.0 |
| HEPES    | NH$_4^+$ | 0 | 20.0 | 0 | 0 | 0 | 20.0 | 0 | 0 | 0 |
| HEPES    | NMDG$^+$ | 0 | 0 | 146.4 | 0 | 145.8 | 0 | 0 | 145.8 | 46.4 |
| HEPES    | Mg$^{2+}$ | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 |
| HEPES    | Ca$^{2+}$ | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| HEPES    | meq (+) | 155.8 | 155.8 | 155.8 | 162.3 | 155.2 | 155.8 | 155.8 | 155.2 | 155.8 |
| HEPES    | Cl$^-$ | 120.0 | 122.0 | 122.0 | 144.5 | 131.4 | 132.0 | 122.0 | 135.0 | 135.0 |
| HEPES    | H$_2$PO$_4^-$ | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0 | 0 | 0 |
| HEPES    | HPO$_4^{2-}$ | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 0 | 0 | 1.6 |
| HEPES    | Ac$^-$ | 10.0 | 10.0 | 10.0 | 10.0 | 0 | 0 | 0 | 0 | 10.0 |
| HEPES    | HEPES$^-$ | 17.8 | 17.8 | 17.8 | 17.8 | 17.8 | 17.8 | 17.8 | 17.8 | 17.8 |
| HEPES    | SO$_4^{2-}$ | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 |
| HEPES    | meq (-) | 155.8 | 155.8 | 155.8 | 162.3 | 155.2 | 155.8 | 155.8 | 155.2 | 155.8 |
| Glucose  | Glucose | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 |
| Alanine  | Alanine | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| HEPES    | HEPES | 14.4 | 14.4 | 14.4 | 14.4 | 14.4 | 14.4 | 14.4 | 14.4 | 14.4 |
| pH       | pH | 7.4 | 7.4 | 7.4 | 7.4 | 7.4 | 7.4 | 7.4 | 7.4 | 7.4 |

All concentrations are given in millimolar.

trum was then projected on a linear array of 1024 photodiodes (Princeton Allied Research,
Princeton, NJ), and the measured light intensities were 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. Absorbance (A) at a given wavelength is
calculated from the equation:

\[
A = \log \left( \frac{I_o - I_{\text{dark}}}{I - I_{\text{dark}}} \right)
\]

where \(I_o\) is the light intensity in the absence of a tubule, \(I_{\text{dark}}\) is the measured intensity in the
absence of incident light, and \(I\) is the intensity in the presence of a tubule.

The total measured absorbance was corrected for the absorbance due to the tubule cells
(Chaillet and Boron, 1985), so that the absorbance of the intracellular dye alone was derived.
This correction relies on the absorbance data at wavelengths at which the cells, but not the dye, absorb light (i.e., >600 nm). pH\textsubscript{i} was calculated from this corrected intracellular dye spectrum by comparing it with a series of intracellular calibration spectra (see next section). The aforementioned method for computing pH\textsubscript{i} from 512-point is so time consuming that it cannot be performed on-line. We were able to obtain rough estimates of pH\textsubscript{i} on-line by using only 20 points of the spectrum, the wavelength at the dye’s absorbance peak, the isosbestic wavelength, and 18 discrete wavelengths between 600 and 750 nm.

**pH\textsubscript{i} Calibration of the Dye**

Previous work from this laboratory on salamander proximal tubules (Chaillet and Boron, 1985) showed that the absorbance spectrum of the intracellular Me\textsubscript{2}CF differed from that obtained for the dye in a cuvette. For this reason, we performed an intracellular dye calibration by clamping pH\textsubscript{o} to predetermined values using the nigericin method of Thomas et al. (1979). The tubule was exposed from both the lumen and bath to a solution containing 10 \mu M nigericin, 105 mM K\textsuperscript{+}, and no Na\textsuperscript{+} (solution 10). The K-H exchanger nigericin approximately clamps pH\textsubscript{i} to extracellular pH (pH\textsubscript{o}). Intracellular dye absorbance spectra were then obtained over a range of predetermined pH\textsubscript{o} values. The extracellular [K\textsuperscript{+}] used in these experiments is the same reported by Chaillet et al. (1985) in calibrating the cortical collecting tubule of the rabbit.

Fig. 1 A illustrates one experiment on a rabbit S3 proximal tubule in which pH\textsubscript{o} was varied from 6.15 to 7.65 in steps of ~0.3 (solution 10). The ratio of peak absorbance (at a wavelength of 510 nm) to the absorbance near the in vitro isosbestic wavelength (470 nm) is plotted against time. The absorbance ratios (A\textsubscript{510}/A\textsubscript{470}) from experiments on 14 different tubules are plotted as a function of pH in Fig. 1 B. The curve drawn through the points was obtained from a nonlinear least-squares fit of the data to a standard pH titration curve. The result of this fit was a pK\textsubscript{o} of 7.26 \pm 0.01, a lower asymptote of 0.75 \pm 0.01, and an upper asymptote of 2.61 \pm 0.01.

In a total of 44 tubules, the average initial absorbance at 470 nm was 0.207 \pm 0.007. Assuming an average total path length of 25 \mu m through the tubule cells, and an extinction coefficient of 1.9 \times 10\textsuperscript{4} M\textsuperscript{-1} cm\textsuperscript{-1}, we calculated an average intracellular dye concentration of 4.3 \pm 0.14 mM. This is very similar to the value of 4.0 \pm 0.2 mM reported by Chaillet et al. (1985) for the rabbit cortical collecting tubule.

**Statistics and Data Analysis**

Means are reported \pm SE. Statistical significance was judged from paired and unpaired Student’s t test, as indicated in the text. Rates of pH\textsubscript{i} change were determined by using a computer to fit the pH\textsubscript{i} vs. time data to a straight line.

**RESULTS**

**Initial value of pH\textsubscript{i}**

The pH\textsubscript{i} of cells in the S3 segment of the rabbit proximal tubule was determined from the absorbance spectra of Me\textsubscript{2}CF as outlined in the Methods. The average initial pH\textsubscript{i} was 7.49 \pm 0.02 for 122 tubules bathed and perfused with solution 1 (pH\textsubscript{i} = 7.4) at 37°C. We point out that this solution contained 10 mM Ac\textsuperscript{−} and was nominally HCO\textsubscript{3}−-free. The latter should minimize the effect of HCO\textsubscript{3}−-dependent transporters on pH\textsubscript{i}. 
Intracellular Acid Loading and pH\textsubscript{i} Recovery

Acid loading with a NH\textsubscript{4}\textsuperscript{+} pulse. The acid-base transport mechanisms that regulate pH\textsubscript{i} respond to sudden intracellular acid loads by extruding acid from the cell, thereby returning pH\textsubscript{i} toward its initial value (Roos and Boron, 1981). To determine how

![Figure 1. Calibration of intracellular Me\textsubscript{2}CF. (A) Time course of changes in absorbance ratio, A(510)/A(470), while pH\textsubscript{i} was varied by altering simultaneously the pH of the bath and the lumen. (B) In vivo dye calibration curve. The data points were derived from experiments similar to that of A on 14 tubules. The curve drawn through the points is a nonlinear least-squares fit of the data to a pH titration curve. The best-fit values were a pK\textsubscript{a} of 7.26 ± 0.01, and the upper and lower asymptotes of 2.61 ± 0.01 and 0.75 ± 0.01, respectively.](image-url)
cells of the S3 segment of the rabbit proximal tubule respond to acute acid loads, we exposed the tubule bilaterally for 2 min to a solution containing 20 mM NH₄⁺, solution 2 (Boron and De Weer, 1976). As shown in Fig. 2, exposure to NH₄⁺ leads to a rapid pHᵢ increase (ab), due to the influx of the weak base NH₃, followed by a slow decline (bc), presumably due to uptake of the weak acid NH₄⁺. Upon removal of the external NH₄⁺, pHᵢ rapidly decreases (cd) as intracellular NH₄⁺ dissociates into NH₃ (which diffuses from the cell) and H⁺ (which remains trapped inside). pHᵢ spontaneously recovers from this acid load (de) as a result of one or more acid-extrusion mechanisms located in the luminal and/or basolateral cell membranes. In the absence of HCO₃⁻, one such pHᵢ-regulating mechanism that could produce such a recovery is the Na-H exchanger.

Effect of external Na⁺ removal and readdition on pHᵢ. Having established that the cells of the S3 segment of the rabbit proximal tubule regulate their pHᵢ, we next examined the Na⁺-dependence and location of the acid-extrusion mechanisms responsible for this pHᵢ regulation. If the pHᵢ recovery of Fig. 2 (de) is at least partially due to a Na-H exchanger or other Na⁺-linked acid-extruders, then removing Na⁺ from the external solution(s) should cause the transporter(s) to stop or even reverse, thereby acidifying the cell. In the experiment of Fig. 3, we first removed Na⁺ (solution 3) from the bath only. This leads to a slow and small decrease in pHᵢ (ab). When Na⁺ is also removed from the lumen, there is a further decrease in pHᵢ (bc) that is considerably faster and larger than when Na⁺ was removed from the bath only. In five such experiments, the rate of pHᵢ decrease with Na⁺ removed from
both bath and lumen was more than fourfold greater than that observed when Na\(^+\) was removed from the bath only (see Table II). Note that there is a slow pH\(_i\) recovery even in the absence of Na\(^+\) (cd). Returning Na\(^+\) bilaterally elicited a rapid pH\(_i\) recovery (de).

The sidedness of this Na\(^+\)-dependent pH\(_i\) recovery was examined in the experiment of Fig. 4. Bilateral removal of Na\(^+\) causes pH\(_i\) to quickly drop (ab) as would be expected if Na\(^+\)-dependent acid-extruding mechanism(s) were reversed. Even in the absence of Na\(^+\), pH\(_i\) slowly recovers (bc). Readdition of Na\(^+\) to the bath only increases the rate of this pH\(_i\) recovery by a small amount (cd). When 146 mM Na\(^+\) is also returned to the lumen, pH\(_i\) very rapidly recovers towards its initial value (de). In four such experiments, the pH\(_i\) recovery rate with Na\(^+\) present in both the bath and lumen was about ninefold greater than with Na\(^+\) present only in the bath (see Table II). The small amount of pH\(_i\) recovery observed upon the addition of Na\(^+\) to the bath could be due to a basolateral Na\(^+\)-dependent acid-extrusion mechanism (e.g., a

### Table II

| Site of Na\(^+\) replacement | dpH\(_i\)/dt for Na\(^+\) removal | dpH\(_i\)/dt for Na\(^+\) readdition |
|-----------------------------|-----------------------------------|-----------------------------------|
| Bath only                   | \(-11 \pm 2\)                     | \(7 \pm 2\)                       |
| Bath and lumen              | \(-91 \pm 5\)                     | \(64 \pm 16\)                     |
| Bath/(bath and lumen)       | \(22 \pm 4\%)                     | \(12 \pm 3\%)                     |
| No.                         | 5                                 | 4                                 |

No. is number of paired experiments.

*Rates given in 10\(^{-4}\) pH units/s.
Na-H exchanger), or to the activity of a luminal Na⁺-dependent mechanism supported by the small amount of Na⁺ that may have leaked from bath to lumen. We have not investigated this issue further.

The data from the experiments of Figs. 3 and 4 indicate the following: (a) there are one or more Na⁺-dependent acid-extruding mechanisms that can be blocked and/or reversed by manipulating the Na⁺ gradient. (b) This Na⁺-dependent mechanism(s) is located predominantly at the luminal rather than the basolateral membrane of the S3 proximal-tubule cell.

**Na⁺-independent Acid Extrusion**

As we noted above, the bilateral removal of Na⁺ causes pHᵢ to decrease rapidly, level off, and then to spontaneously drift upward, even in the continued absence of external Na⁺ (see cd of Fig. 3 and bc of Fig. 4). In the experiment of Fig. 5A, we studied this alkaline drift over a longer Na⁺-free period. The bilateral removal of Na⁺ causes pHᵢ to rapidly decrease (ab), and then to spontaneously recover to a level higher than its initial steady-state value (bc), all in the continued absence of Na⁺. The rate of Na⁺-independent pHᵢ recovery was low at first and then more rapid after ~1 min. This could result from the summation of two processes: the waning of an acid-loading mechanism as pHᵢ falls, and the initiation of an acid-extruding mechanism. Therefore, as a measure of the rate of Na⁺-independent acid extrusion, we determined the maximal pHᵢ recovery rate (i.e., at a point after the inflection in the pHᵢ
FIGURE 5. Na⁺-independent pHᵢ recovery. (A) Spontaneous pHᵢ recovery in the bilateral absence of external Na⁺. Removal of Na⁺ from lumen and bath caused a rapid pHᵢ decrease (ab), followed by a spontaneous recovery (bc) to a value ~0.1 higher than the initial one (compare a and c). Readdition of Na⁺ to the bath caused a pHᵢ decrease (cd), and the subsequent readdition of Na⁺ to the lumen caused a transient pHᵢ decline (def). (B) Effect of reducing [HEPES] on the Na⁺-independent pHᵢ recovery. The protocol was similar to that of A, except that [HEPES] was 3.2 mM instead of 32 mM. The Na⁺-independent pHᵢ recovery (cd) was unaffected. (C) Effect of DCCD on the Na⁺-independent pHᵢ recovery. The application of
$10^{-4}$ M DCCD 3 min before the removal of external Na$^+$ did not substantially reduce the rate of Na$^+$-independent pH$_i$ recovery. (D) Effect of IAA, and of IAA plus CN$^-$ on Na$^+$-independent pH$_i$ recovery. The three records depict the Na$^+$-independent pH$_i$ recovery (i.e., bc in part A) in three experiments. In the "control" experiment, neither IAA nor CN$^-$ was present. In the second, 0.5 mM IAA was present throughout, causing a small reduction in the Na$^+$-independent pH$_i$ recovery rate. In the third, both 0.5 mM IAA and 1 mM CN$^-$ were present throughout, causing a much larger reduction in the Na$^+$-independent pH$_i$ recovery rate.
vs. time record). In eight tubules, this averaged $17.0 \times 10^{-4}$ pH units/s (see Table III). In similar experiments on five tubules, pH$_i$ overshoot its initial steady-state value by $0.10 \pm 0.02$. It is interesting to note that, once pH$_i$ has completed its Na$^+$-independent recovery, the readdition of Na$^+$ to the bath (point c), and then to the bath and lumen (point d), causes no further pH$_i$ increase, but rather a slight decrease. This failure of Na$^+$ to elicit an alkalinization implies that the Na$^+$-dependent acid-extrusion mechanism(s) that caused the pH$_i$ recoveries in the experiments of Figs. 3 and 4 is inhibited at high pH$_i$. We have no explanation for why Na$^+$ addition causes intracellular acidification under these conditions.

The Na$^+$-independent pH$_i$ recovery (bc of Fig. 5 A) could be due to permeation of a damaged cell membrane by the HEPES buffer, which is present in high concentration (i.e., 32 mM) in the external solutions. If this were true, lowering extracellular [HEPES] to 3.2 mM (solution 4) would be expected to greatly lower the rate of alkalinization. In the experiment of Fig. 5 B, bilaterally lowering [HEPES] from 32 to 3.2 mM causes a very slight decrease in pH$_i$ (ab). Subsequent removal of external Na$^+$ caused pH$_i$ to decrease rapidly (bc), and then to spontaneously recover in the continued absence of Na$^+$ to a value higher than the initial one (cd). As in the experiment of Fig. 5 A, readdition of external Na$^+$ causes a slight decrease (de) rather than an increase in pH$_i$. In three experiments, the average maximal pH$_i$ recovery rate in 3.2 mM HEPES was $21.7 \pm 5.5 \times 10^{-4}$ pH/s. Thus, the reduction in external (HEPES) had a negligible effect on the segment-cd pH$_i$ recovery.

Another possibility is that the Na$^+$-independent pH$_i$ recovery is mediated by an acid-extruding transport system, such as a H$^+$-ATPase. Evidence for such a pump has been provided in brush border membrane vesicles isolated from rat renal cortex, for which $10^{-4}$ M DCCD reduces H$^+$-ATPase activity (Krinne-Saffran et al., 1982). In the turtle urinary bladder, a 30-min pretreatment with $10^{-4}$ M DCCD reduces H$^+$ ATPase activity by ~50% (Steinmetz et al., 1981). In the experiment of Fig. 5 C, we added $10^{-4}$ M DCCD to the lumen ~2 min before the bilateral removal of Na$^+$. By itself, the addition of DCCD had no effect on pH$_i$ (not shown). Furthermore, the Na$^+$-independent pH$_i$ recovery (bc) was not affected. In five similar experiments, in which the DCCD was applied to the lumen only or to the lumen plus bath,

### Table III

| Condition | $dpH/dt$ | $pH_i$ |
|-----------|---------|-------|
| 0 mM Na$^+$ (lumen and bath) | $17.0 \pm 1.6$ (8) | $7.1 \pm 0.03$ (8) |
| 0 mM Na$^+$ (lumen and bath), $10^{-4}$ M DCCD (lumen) | $16.2 \pm 2.4$ (5) | $7.04 \pm 0.1$ (5) |
| 0 mM Na$^+$, 0.5 mM IAA (lumen and bath) | $12.7 \pm 0.3$ (5) | $6.92 \pm 0.05$ (5) |
| 0 mM Na$^+$, 0.5 mM IAA, and 1 mM CN$^-$ (lumen and bath) | $7.3 \pm 2.0$ (6) | $7.0 \pm 0.1$ (6) |

$pH_i$ is the mean pH$_i$ at which the maximal rate was determined. Number of experiments is given in parentheses.

* $dpH/dt$ is the mean maximal rate in pH units $\times 10^{-4} \cdot s^{-1}$. 

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| 0 mM Na$^+$ (lumen and bath), $10^{-4}$ M DCCD (lumen) | $16.2 \pm 2.4$ (5) | $7.04 \pm 0.1$ (5) |
| 0 mM Na$^+$, 0.5 mM IAA (lumen and bath) | $12.7 \pm 0.3$ (5) | $6.92 \pm 0.05$ (5) |
| 0 mM Na$^+$, 0.5 mM IAA, and 1 mM CN$^-$ (lumen and bath) | $7.3 \pm 2.0$ (6) | $7.0 \pm 0.1$ (6) |
the maximal rate of pH\textsubscript{i} recovery (some 7 min after DCCD application) was $16.2 \times 10^{-4}$ pH/s (see Table III), which was not significantly different from the rate in eight control tubules. Although it is possible that the allotted time was not sufficient for the inhibition by DCCD to take effect, or that $10^{-4}$ M DCCD was too small a dose, even this short exposure to $10^{-4}$ DCCD caused the proximal tubule cells to swell noticeably and to become extremely granular in appearance. Therefore, caution is advised in interpreting data from DCCD experiments because of the possibility of cell damage.

To test whether the Na\textsuperscript{+}-independent pH\textsubscript{i} recovery requires ATP, we next examined the effect of iodoacetic acid (IAA) and cyanide (CN\textsuperscript{-}). In Fig. 5 D, the record labeled 'CONTROL' is a Na\textsuperscript{+}-independent pH\textsubscript{i} recovery in the absence of inhibitors. As noted above, the average maximal pH\textsubscript{i} recovery rate was $17.0 \times 10^{-4}$ pH/s in such control tubules. The record labeled 'IAA' is from one of three experiments in which 0.5 mM IAA was added bilaterally 2-3 min before the bilateral Na\textsuperscript{+} removal. The maximal rate of Na\textsuperscript{+}-independent pH\textsubscript{i} recovery in these experiments averaged $12.7 \times 10^{-4}$ pH/s (see Table III), which represents an ~25% inhibition ($P < 0.1$). In two other experiments (data not shown), addition of 1 mM CN\textsuperscript{-} bilaterally elicited a comparable inhibition. The record labeled 'CN\textsuperscript{-} + IAA' is from one of six experiments in which both IAA and CN\textsuperscript{-} were added simultaneously. The average maximal Na\textsuperscript{+}-independent pH\textsubscript{i} recovery rate was $7.3 \times 10^{-4}$ pH/s (see Table III), which represents an inhibition of ~57% ($P < 0.001$). This inhibition is consistent with the hypothesis that at least a part of the Na\textsuperscript{+}-independent pH\textsubscript{i} recovery requires ATP. However, we cannot rule out the possibility that the inhibition of the Na\textsuperscript{+}-independent pH\textsubscript{i} recovery is an indirect consequence of the blockade of metabolism. For example, Krapf et al. (1987) found that, in the nominal absence of HCO\textsubscript{3}\textsuperscript{-}, CN\textsuperscript{-} blocked electrogenic Na/HCO\textsubscript{3} cotransport, presumably reducing the endogenous production of CO\textsubscript{2}, and thus the ambient [HCO\textsubscript{3}]\textsuperscript{-}.

Early during the pH\textsubscript{i} recovery from the acid load elicited by the bilateral removal of Na\textsuperscript{+}, the contribution made by the Na\textsuperscript{+}-independent mechanism is rather small. For example, in experiments similar to that of Fig. 3, the segment-cd pH\textsubscript{i} recovery rate was only $16.1 \pm 1.4\%$ ($n = 8$) of the sequent-de rate. However, if the reapplication of Na\textsuperscript{+} were delayed until the Na\textsuperscript{+}-independent pH\textsubscript{i} recovery rate had reached its maximal value, one would expect the contribution of the Na\textsuperscript{+}-independent mechanism to rise to ~25% of the total pH\textsubscript{i} recovery rate (i.e., Na\textsuperscript{+}-dependent plus Na\textsuperscript{+}-independent components).

Na\textsuperscript{+}-dependent Acid Extrusion

Effect of amiloride. As shown in the preceding sections, the most rapid component of pH\textsubscript{i} recovery in the nominal absence of HCO\textsubscript{3}\textsuperscript{-} is mediated by a mechanism(s) dependent upon the presence of external (presumably luminal) Na\textsuperscript{+}. The data presented thus far are consistent with the presence of a luminal Na-H exchanger. Studies on the Na-H exchanger in a variety of preparations (for review, see Boron, 1985) indicate that this transporter is sensitive to the diuretic amiloride. We therefore tested the effect of amiloride on the Na\textsuperscript{+}-dependent pH\textsubscript{i} changes observed in experiments similar to those of Figs. 3 and 4.

We first examined the effect of amiloride on the pH\textsubscript{i} decline caused by the bilat-
eral removal of external Na\(^+\). In the experiment of Fig. 6, Na\(^+\) is removed (solution 3) from the bath and lumen in the presence of 1 mM amiloride. This causes pH\(_i\) to decline rapidly at first, and then more slowly (ab). The subsequent removal of amiloride at point b, however, causes the rate of acidification to increase. When the Na\(^+\) is returned to the lumen and bath, pH\(_i\) rapidly recovers (cd). In five similar experiments, the rate of acidification just before amiloride removal (i.e., b in Fig. 6) was 74 ± 6% as large as the rate of acidification just after amiloride removal. These results suggest that a portion of the pH\(_i\) decline elicited by removal of external Na\(^+\) is mediated by an amiloride-sensitive Na-H exchanger operating in reverse.

In our next series of experiments, we removed Na\(^+\) bilaterally and examined the effect of 1 mM amiloride on the pH\(_i\) recovery induced by the readdition of 29 mM Na\(^+\) to the lumen only. In the experiment of Fig. 7 A, Na\(^+\) is removed from bath and lumen, causing the usual fall in pH\(_i\) (ab). At point b, 29 mM Na\(^+\) is added to the lumen, causing a slow pH\(_i\) increase (bc). This low concentration of Na\(^+\) was chosen to maximize the inhibition by amiloride, which is known to be competitive with Na\(^+\) (Kinsella and Aronson, 1981). At c, 1 mM amiloride is added to the lumen and Na\(^+\) is removed, causing a slow pH\(_i\) decline (cd). When 29 mM Na\(^+\) is now returned to the lumen in the continued presence of 1 mM amiloride, pH\(_i\) recovers again (de). When the rates of pH\(_i\) recovery during the amiloride-free (bc) and amiloride (de) periods are compared, the calculated inhibition by amiloride comes to only ~20%. The average inhibition in a total of eight similar experiments was 30 ± 7%. The data of Kinsella and Aronson (1981) predict that, in the presence of 29 mM Na\(^+\), 1 mM amiloride should have inhibited Na-H exchange by 86%.
FIGURE 7. Effect of luminal amiloride on the luminal Na⁺-dependent pHᵢ recovery, in the presence of 10 mM Ac⁻. (A) Effect of 1 mM amiloride on the pHᵢ recovery caused by the readdition of 29 mM Na⁺ to the lumen. The cells were acidified by bilateral Na⁺ removal. Amiloride produced only a ~20% inhibition of the pHᵢ recovery rate (compare bc with de). (B) Effect of 2 mM amiloride on the pHᵢ recovery caused by the readdition of 15 mM Na⁺ to the lumen. This experiment is similar to that of A, except that luminal [Na⁺] was halved to 15 mM, and [amiloride] was doubled to 2 mM. The amiloride produced only a ~25% inhibition of the pHᵢ recovery rate (compare bc and fg with de).
As shown in Fig. 7 B, reducing luminal [Na\textsuperscript{+}] to 15 mM and doubling luminal [amiloride] to 2 mM did not increase the degree of inhibition any further. When the rates of pH\textsubscript{i} recovery caused by adding 15 mM Na\textsuperscript{+} to the lumen during the two amiloride-free periods (bc and fg) of Fig. 7 B are averaged and compared with the pH\textsubscript{i} recovery rate in the presence of 2 mM amiloride (de), the calculated inhibition was only ~25% (predicted inhibition, 95%). In two additional experiments performed in the presence of 146 mM Na\textsuperscript{+} (not shown), we acid loaded cells by briefly exposing the tubule bilaterally to NH\textsubscript{4}\textsuperscript{+}, and then compared the rates of pH\textsubscript{i} recovery (as in segment de of Fig. 2) in the presence and absence of 2 mM amiloride. We found that amiloride only inhibited the pH\textsubscript{i} recovery by an average of 23%, whereas the data of Kinsella and Aronson (1981) predicted an inhibition of 73%.

The relatively small inhibition produced by amiloride in our experiments suggest three possibilities: (a) the pH\textsubscript{i} recovery induced by adding Na\textsuperscript{+} to the lumen is mediated by a single transporter, namely, a Na-H exchanger that has a low apparent affinity for amiloride; (b) the pH\textsubscript{i} recovery is mediated by a single Na\textsuperscript{+}-dependent acid-extruding mechanism, other than a Na-H exchanger, that has a low apparent affinity for amiloride; and (c) the pH\textsubscript{i} recovery is due to the parallel actions of an amiloride-sensitive Na-H exchanger and an amiloride-insensitive, Na\textsuperscript{+}-dependent acid-extrusion mechanism. The observation that 1 or 2 mM amiloride produces similar degrees of inhibition at Na\textsuperscript{+} concentrations of 15 and 145 mM makes either of the first two possibilities unlikely. If the third is true, the amiloride-insensitive mechanism must be more potent than the Na-H exchanger. Moreover, blockade of the amiloride-insensitive mechanism should increase amiloride's fractional inhibition of the remaining Na\textsuperscript{+}-dependent pH\textsubscript{i} recovery.

An excellent candidate for an amiloride-insensitive, Na\textsuperscript{+}-dependent acid-extruding mechanism is the pair of monocarboxylate transporters described by Siebens and Boron (1987) for proximal tubules of the tiger salamander Ambystoma tigrinum. They described one cotransporter that mediates the uptake of Na\textsuperscript{+} and lactate across the luminal membrane, and a second that mediates the exit of H\textsuperscript{+} and lactate (or the exchange of lactate for base) across the basolateral membrane. The net effects are (a) Na\textsuperscript{+}-dependent, amiloride-insensitive extrusion of acid from the cell, and (b) presumed reabsorption of lactate. Inasmuch as our external solutions routinely contained 10 mM Ac\textsuperscript{−}, we tested the possibility that a Na\textsuperscript{+}- and Ac\textsuperscript{−}-dependent mechanism contributes to the recovery of pH\textsubscript{i} from acid loads in the S3 segment of the rabbit proximal tubule. This hypothesis makes two predictions: (a) amiloride should produce a greater inhibition of pH\textsubscript{i} recovery in the absence of Ac\textsuperscript{−} than in its presence. (b) The absolute rate of pH\textsubscript{i} recovery from an acid load should be less in the absence of Ac\textsuperscript{−} than in its presence.

Effect of amiloride in the absence of Ac\textsuperscript{−}. In the experiment of Fig. 8 A, Na\textsuperscript{+} and Ac\textsuperscript{−} are simultaneously removed from bath and lumen (solution 5), causing a rapid increase in pH\textsubscript{i} (ab) followed by a slower decrease (bc). The contribution of Ac\textsuperscript{−} to these pH\textsubscript{i} changes is described in detail in an accompanying paper (Nakhoul and Boron, 1988). The addition of 1 mM amiloride to the lumen has no effect on pH\textsubscript{i} (cd). In the continued presence of amiloride, the addition of 29 mM Na\textsuperscript{+} to the lumen causes a slow recovery of pH\textsubscript{i} (de). Washing away amiloride and Na\textsuperscript{+} causes pH\textsubscript{i} to decline (ef). When 29 mM Na\textsuperscript{+} is returned to the lumen, this time in the
FIGURE 8. Effect of luminal amiloride on the luminal Na⁺-dependent pH recovery, in the absence of Ac⁻. (A) First example. The simultaneous removal of Na⁺ and Ac⁻ from both lumen and bath produced a transient alkalinization (ab) followed by a large acidification (bc). The readDITION of 29 mM Na⁺ to the lumen in the presence of 1 mM luminal amiloride produced a slower pH recovery (de) than did the readdition of Na⁺ in the absence of amiloride (fg). Amiloride produced a 54% inhibition of the pH recovery (compare de and fg). (B) Second example. The protocol was the same as in A. In this case, amiloride produced a 90% inhibition.
absence of amiloride, pH, recovers at a somewhat greater rate (fg). Comparison of pH, recovery rates in the presence (de) and absence (fg) of amiloride indicates that amiloride inhibited the pH, recovery by ~55%. Removal of Na+ again causes a decrease in pH, (gh), whereas the luminal addition of 146 mM Na+ elicits a rapid pH, recovery (hi). A similar experiment is shown in Fig. 8 B, though in this case, 1 mM amiloride produced a 90% inhibition. In a total of five tubules, the average inhibition by amiloride, in the absence of external Ac-, was 66 ± 19% (predicted inhibition, 86%). Thus, in the absence of Ac−, the inhibition by amiloride is actually increased by a factor of 2.3. Therefore, it is reasonable to assume that, in the absence of external Ac−, the Na-H exchanger is probably the main luminal mechanism involved in the recovery of pH, from an acid load, and that this exchanger is largely blocked by amiloride.

**Contribution of Ac− to pH, Recovery**

To determine the effect of Ac− on pH, regulation, we performed two sets of experiments in which the pH, recovery from an intracellular acid load was monitored, both in the presence and absence of Ac−. In the first, cells were acid loaded by pulsing with 20 mM NH4+. In the second, cells were acid loaded by removing Na+. In these latter experiments, the pH, recovery elicited by the addition of external Na+ also demonstrates the Na+ dependence of Ac− transport.

In the experiment shown in Fig. 9, cells were acid loaded twice by exposing the tubule bilaterally to 20 mM NH4+, first in the presence and then in the absence of 10 mM Ac− (solutions 2 and 7, respectively). The first application and removal of NH4+
causes the usual alkalinization (ab), plateau-phase acidification (bc), and undershoot (cd), as previously noted for Fig. 2. In the presence of Ac\(^{-}\), the pH\(_{i}\) recovery toward its original value (de) is very rapid. Bilateral removal of Ac\(^{-}\) (solution 6) causes pH\(_{i}\) to rapidly increase (ef), and then decrease more slowly (fg), to a value lower than the original one (compare e and g). After the tubule is again acid loaded by an NH\(_{4}\)\(^{+}\) pulse (ghij) in the absence of Ac\(^{-}\), the pH\(_{i}\) recovery is substantially slower (jk). At similar pH\(_{i}\) values (i.e., 6.9), the pH\(_{i}\) recovery rate in the absence of Ac\(^{-}\) (jk) is only 52\% as great as in the presence of Ac\(^{-}\) (de). In five paired experiments, the value was 56 ± 10\%. These results indicate that a major component of pH\(_{i}\) recovery requires Ac\(^{-}\).

In the other series of experiments, we removed external Na\(^{+}\) and then examined the effect on pH\(_{i}\) of returning the Na\(^{+}\), first in the absence and then in the presence of Ac\(^{-}\). In these experiments, external (inorganic) phosphate (Pi) was removed with the Ac\(^{-}\) (solution 9), though we now know that phosphate removal has little effect on pH\(_{i}\) (Nakhoul and Boron, 1988). As shown in Fig. 10, the bilateral removal of Ac\(^{-}\) and Pi (solution 4) results in a transient rise in pH\(_{i}\) (ab) followed by a slower decline (bc). Removal of Na\(^{+}\) from lumen and bath in the absence of Ac\(^{-}\) causes a slow, further fall in pH\(_{i}\) (cd), presumably due to a reversal of a luminal Na-H exchanger. When Na\(^{+}\) is returned to the external medium, pH\(_{i}\) slowly recovers (de). The readdition of Ac\(^{-}\) and Pi to the external medium causes a series of pH\(_{i}\) transients that are opposite to those elicited by removal of these solutes (efg). When Na\(^{+}\) is now bilaterally removed in the presence of Ac\(^{-}\), pH\(_{i}\) decreases very rapidly (gh).
and then slowly recovers (hi). The pH_i recovery is greatly accelerated by the bilateral addition of Na⁺ (ij). In three paired experiments, the maximal rate of pH_i recovery, was about threefold greater in the presence of Ac⁻ (ij) than in its absence (de).

In the experiment of Fig. 10, ionic substitutions were performed simultaneously in both lumen and bath. Because the Na⁺ dependence of acid extrusion is largely luminal (see Figs. 3 and 4), we performed two experiments similar to those of Fig. 10, but in which Na⁺ was removed from only the lumen (maintaining bath [Na⁺] at 146 mM). As shown in Fig. 11, the fall in pH_i induced by luminal Na⁺ removal is much faster in the presence of Ac⁻ (ab) than is its absence (ef). Similarly, the pH_i recovery induced by the readdition of Na⁺ to the lumen was about fourfold faster (at comparable pH_i values) in the presence of Ac⁻ (bc) than in its absence (fg). These

![Diagram](image)

**Figure 11.** Contribution of Ac⁻ to the pH_i recovery produced by luminal readdition of Na⁺. Na⁺ was removed and then returned to the lumen, first in the absence and then in the presence of 10 mM Ac⁻. The Na⁺-dependent pH_i recovery in the presence of Ac⁻ (fg) was 4.8 times faster than that in the absence of Ac⁻ (bc), with both rates measured at a pH_i of 6.85.

results are consistent with those of the experiment of Fig. 10, and indicate that the Ac⁻-dependent component of pH_i recovery is also dependent on luminal Na⁺.

The above experiments indicate that the luminal Na⁺-dependent pH_i recovery has two components, a minor one due to luminal Na-H exchange, and a major one that is Ac⁻-dependent.

**DISCUSSION**

Our results show that cells of the S3 segment of the rabbit proximal tubule possess three HCO₃⁻-independent mechanisms capable of extruding acid from the cell. The first is capable of causing the recovery of pH_i from an acid load in the total absence
of external Na\(^+\), and may be a proton pump. The second appears to be a classical Na-H exchanger at the luminal membrane. The third requires Ac\(^-\) as well as Na\(^+\).

**Na\(^+\)-independent Acid Extrusion**

In a preliminary report, we described a Na\(^+\)-independent recovery of pH\(_i\) from an acid load in the S3 segment of the rabbit proximal tubule (Nakhoul and Boron, 1985). This basic observation has been confirmed in the rat proximal tubule (Yoshitomi et al., 1985) and in the S3 segment of the rabbit proximal tubule (Kurtz, 1987). In our experiments, prolonged exposure of the tubules to Na\(^+\)-free external solutions resulted in an initial decrease in pH\(_i\), followed by a spontaneous pH\(_i\) recovery, first slowly and then more rapidly, to a value higher than the initial steady-state value in the presence of Na\(^+\) (Figs. 5, A–C). Although it is thought that the cellular production of alkali can act as a sort of biochemical buffer for acute intracellular acid loads (Folbergrova et al., 1972), we are unaware of any description of metabolism producing a de novo pH\(_i\) increase of the magnitude observed in the experiments of Fig. 5. Thus, it is most likely that an acid-extruding transport process is responsible for the pH\(_i\) recovery we observed.

Two aspects of our experiments are of particular interest. First, early on during the period of Na\(^+\) removal, the rate of acid extrusion in the absence of Na\(^+\) is only \(\sim 16\%\) as great as that observed when Na\(^+\) is subsequently added (paired data, measured at same pH\(_i\)). This indicates that the Na\(^+\)-independent component is relatively slow. Second, the Na\(^+\)-independent component is nevertheless capable of causing pH\(_i\) to recover to a value that is even higher than the initial steady-state pH\(_i\). According to the model of pH\(_i\) regulation presented by Roos and Boron (1981), pH\(_i\) reaches a steady state when the total rate at which the cell is loaded with acid equals the total rate of acid extrusion. Thus, one would expect that an increase in steady-state pH\(_i\) could be caused by an increase in the total acid-extrusion rate, a decrease in the total acid-loading rate, or both. Our experiments in Na\(^+\)-free solutions present an apparent contradiction; the removal of Na\(^+\) reduced the total acid extrusion rate, but increased steady-state pH\(_i\). The simplest explanation is that in the new steady state prevailing in the absence of Na\(^+\), the total acid-extrusion rate is decreased by a modest amount, whereas the acid-loading rate is reduced substantially. Some support for the notion that the acid-loading rate may be reduced in the absence of Na\(^+\) comes from the following observation. After a long-term bilateral Na\(^+\) removal, and when pH\(_i\) has recovered to a higher-than-normal value, returning Na\(^+\) to only the bath usually causes pH\(_i\) to decline, which suggests the presence of a Na\(^+\)-dependent acid-loading process.

One candidate for the Na\(^+\)-independent acid-extruding mechanism is a H\(^+\) pump. One might argue that the failure of \(10^{-4}\) M DCCD, added a few minutes before the removal of Na\(^+\), to block the Na\(^+\)-independent pH\(_i\) increase rules out the H\(^+\)-pump hypothesis. However, it is possible that significant blockade would require a more lengthy exposure to DCCD, or a higher DCCD concentration. We did not investigate either of these possibilities, given the extremely poor appearance of the tubules after our DCCD treatments. It should be pointed out that Kurtz (1987) has noted that the Na\(^+\)-independent pH\(_i\) recovery is largely blocked by 1 mM DCCD or by 0.5 mM IAA, and suggests that the mechanism is a H\(^+\) pump. In our experi-
ments, 0.5 mM IAA only moderately inhibited pH$_i$ recovery (~25%), whereas the simultaneous addition of IAA and CN$^-$ was about two times as effective (~58% inhibition). This effect of CN$^-$ and IAA could possibly indicate that metabolism is involved in the Na$^+$-independent pH$_i$ recovery. If the Na$^+$-independent pH$_i$ recovery is due to a H$^+$ pump, one question that arises is whether these pumps normally are active in the presence of Na$^+$, or whether they are activated only by the removal of external Na$^+$ or by the accompanying fall in pH$_i$. Decreases in pH$_i$ are thought to trigger the insertion of ATP-driven H$^+$ pumps into the apical membrane of the toad urinary bladder (Cannon et al., 1985), and possibly in the rabbit proximal tubule and cortical collecting tubule (Schwartz and Al-Awqati, 1985). Thus, a possible explanation for the observation that the steady-state pH$_i$ is higher in the absence of Na$^+$ than in its presence is that the acidification induced by Na$^+$ removal triggered the insertion of H$^+$ pumps whose acid-extruding activity exceeds that of the Na$^+$-dependent processes. Finally, the question of which of the epithelial membranes is involved in Na$^+$-independent pH$_i$ regulation, has yet to be resolved.

**Na-H Exchange**

The Na$^+$-dependent component of the pH$_i$ recovery from an acid load consists of luminal Na-H exchange and an Ac$^-$-dependent mechanism that also requires luminal Na$^+$. We found that the Na-H exchanger in the S3 segment is localized primarily in the luminal membrane. It is largely blocked by the diuretic amiloride and, under the conditions of our experiments, appears to account for only a modest fraction (~35%) of total Na$^+$-dependent acid extrusion. Moreover, we have provided evidence that this transporter is reversed when the Na$^+$ gradient is reversed. In experiments in which complete pH$_i$ recovery occurred in the absence of external Na$^+$, returning Na$^+$ to lumen did not cause any further pH$_i$ recovery (see Fig. 5, A and B). One explanation for this effect is that the prolonged absence of Na$^+$ produces changes in the cell that secondarily inhibit the Na-H exchanger. Alternately, the Na-H exchanger may be active only when pH$_i$ falls below a certain threshold (e.g., ~7.4). A similar pH$_i$ threshold was reported for the activation of the Na$^+$-dependent Cl/HCO$_3^-$ exchanger of the barnacle muscle fiber (Boron et al., 1978) and the Na-H exchanger of the *Ambystoma* proximal tubule (Boron and Boulpaep, 1983). At least a portion of the pH$_i$ dependence of the S3 segment's Na-H exchanger is probably due to the internal H$^+$ modifier site described for the Na-H exchanger of brush border membrane vesicles isolated from the renal cortex (Aronson et al., 1982). These vesicles were probably derived from S1 and S2 segments.

**Ac$^-$-dependent Acid Extrusion**

Of the total luminal Na$^+$-dependent acid-extruding capacity, the major component (~65%) requires the presence of Ac$^-$. Thus, this component might appear to be an amiloride-insensitive Na-H exchanger that is Ac$^-$-dependent. The properties of this Na$^+$- and Ac$^-$-dependent component of acid extrusion are examined in more detail in the accompanying paper (Nakhoul and Boron, 1988). This Ac$^-$-dependent acid-extrusion system, in addition to its importance for pH$_i$ regulation, may play a role in the transepithelial transport of Ac$^-$. 

Conclusions

Our data indicate that, in the nominal absence of HCO₃⁻, three transport systems contribute to acid extrusion in the S3 segment of the rabbit proximal tubule: a minor component that is Na⁺ independent, a minor component that is due to luminal Na-H exchange, and a major component that requires Ac⁻ and luminal Na⁺. It should be noted that all our experiments were conducted in HCO₃⁻-free solutions. Consequently, the relative contributions of these three transporters to acid extrusion and pH_{i} regulation may be different in the presence of HCO₃⁻. Furthermore, our data pertain only to the S3 segment of the rabbit proximal tubule. We might expect to find that acid-base transport in the S1 and S2 segments may be quantitatively or qualitatively different from that in the S3 segment.

We thank Andrey Yeatts for modifying the software for data acquisition, and writing the software for data analysis and plotting. We also thank Emily Tyner for plotting the figures.

N. L. Nakhoul and J. R. Chaillet were recipients of postdoctoral fellowships from the Connecticut Affiliate of the American Heart Association. W. F. Boron was a Searle Scholar and a recipient of a Research Career Development Award (DK-01022). This research was supported by a grant from the National Institutes of Health (DK-30344).

Original version received 25 January 1988 and accepted version received 5 April 1988.

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