

# Basolateral Membrane Na/Base Cotransport Is Dependent on CO<sub>2</sub>/HCO<sub>3</sub> in the Proximal Convoluted Tubule

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**ABSTRACT** The mechanism of basolateral membrane base transport was examined in the *in vitro* microperfused rabbit proximal convoluted tubule (PCT) in the absence and presence of ambient CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> by means of the microfluorometric measurement of cell pH. The buffer capacity of the cells measured using rapid NH<sub>3</sub> washout was 42.8 ± 5.6 mmol·liter<sup>-1</sup>·pH unit<sup>-1</sup> in the absence and 84.6 ± 7.3 mmol·liter<sup>-1</sup>·pH unit<sup>-1</sup> in the presence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. In the presence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, lowering peritubular pH from 7.4 to 6.8 acidified the cell by 0.30 pH units and lowering peritubular Na from 147 to 0 mM acidified the cell by 0.25 pH units. Both effects were inhibited by peritubular 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS). In the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, lowering peritubular pH from 7.4 to 6.8 acidified the cell by 0.25 pH units and lowering peritubular Na from 147 to 0 mM decreased cell pH by 0.20 pH units. Lowering bath pH from 7.4 to 6.8 induced a proton flux of 643 ± 51 pmol·mm<sup>-1</sup>·min<sup>-1</sup> in the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> and 223 ± 27 pmol·mm<sup>-1</sup>·min<sup>-1</sup> in its absence. Lowering bath Na from 147 to 0 mM induced proton fluxes of 596 ± 77 pmol·mm<sup>-1</sup>·min<sup>-1</sup> in the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> and 147 ± 13 pmol·mm<sup>-1</sup>·min<sup>-1</sup> in its absence. The cell acidification induced by lowering bath pH or bath Na in the absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> was inhibited by peritubular SITS or by acetazolamide, whereas peritubular amiloride had no effect. In the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, cyanide blocked the cell acidification induced by bath Na removal, but was without effect in the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. We reached the following conclusions. (a) The basolateral Na/base<sub>n>1</sub> cotransporter in the rabbit PCT has an absolute requirement for CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. (b) In spite of this CO<sub>2</sub> dependence, in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, metabolically produced CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> is sufficient to keep the transporter running at 30% of its control rate in the presence of ambient CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. (c) There is no apparent amiloride-sensitive Na/H antiporter on the basolateral membrane of the rabbit PCT.

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## INTRODUCTION

The mammalian proximal convoluted tubule (PCT) reabsorbs 80% of the filtered bicarbonate. Most of this bicarbonate reabsorption depends on two Na-dependent mechanisms, one on each side of the proximal tubule cell: an amiloride-sensitive Na/H antiporter on the apical membrane, and a recently described, stilbene-inhibitable, electrogenic Na/base cotransporter on the basolateral membrane. This latter transport system, first described in the salamander (Boron and Boulpaep, 1983), has now been found in the rat (Alpern, 1985; Yoshitomi et al., 1985) and rabbit PCT (Sasaki et al., 1985; Biagi and Sothell, 1986), in basolateral membrane vesicles from the rabbit renal cortex (Akiba et al., 1986a; Grassl and Aronson, 1986), in bovine corneal endothelial cells (Jentsch et al., 1984), and in a kidney epithelial cell line from the monkey (BSC-1; Jentsch et al., 1985). It has not been resolved whether the mechanism of Na/base exit is Na/HCO<sub>3</sub><sup>-</sup> or Na/OH cotransport (or, equivalently, Na/H antiport). Because it is not possible to eliminate CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> in vivo, in vitro studies are needed to differentiate among these possibilities.

A basolateral membrane amiloride-sensitive Na/H antiporter has been described in the salamander PCT (Boron and Boulpaep, 1983), but could not be found in rabbit cortical basolateral membrane vesicles (Ives et al., 1983) and in in vitro perfused S<sub>3</sub> segments of the rabbit PCT (Nakhoul and Boron, 1985). In rabbit basolateral membrane vesicles, <sup>22</sup>Na uptake was stimulated by pH gradients in the absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, but at a much slower rate than in its presence (5–20%), which raises the possibility that the Na/base cotransporter can transport HCO<sub>3</sub><sup>-</sup> and/or OH<sup>-</sup> (Grassl and Aronson, 1986).

The purposes of our studies were therefore (a) to examine the mechanisms of basolateral Na-coupled base exit in the rabbit PCT, (b) to determine the CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> dependence of the base exit step, and (c) to confirm the presence or absence of a basolateral membrane Na/H antiporter in the rabbit PCT. To examine these questions, we have adapted the technique of measuring intracellular pH using the pH-sensitive dye (2',7')-bis-(carboxyethyl)-(5,6)-carboxyfluorescein (BCECF) to the in vitro microperfused rabbit PCT. The results demonstrate that the basolateral membrane of the rabbit PCT contains an Na/HCO<sub>3</sub><sup>-</sup> cotransporter with an absolute requirement for CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. In spite of this CO<sub>2</sub> dependence, the cotransporter is able to run at approximately one-third of its control rate in the absence of exogenous CO<sub>2</sub> (utilizing metabolic CO<sub>2</sub>). There is no detectable amiloride-sensitive Na/H antiporter on this membrane.

Portions of this work have been presented previously and have appeared in abstract form (Krapf et al., 1987).

## METHODS

In this study, the technique of in vitro microperfusion of isolated rabbit PCT, as previously described (Burg et al., 1966), was used. Kidneys from New Zealand white rabbits, killed by decapitation, were quickly removed and cut into thin (~1 mm) coronal slices. Cortical PCT (S<sub>1</sub> and S<sub>2</sub> segments) were dissected in the cooled (4°C) solution of the respective experiment (Table I). Late PCT, as identified by their attachment to straight tubules, were not used. The tubules were transferred to a bath chamber with a volume of ~150 μl. The bath fluid was continuously exchanged at ~10 ml/min by hydrostatic pressure.

With this setup, a complete bath fluid exchange could be achieved within ~1 s. This was confirmed when solutions were changed from a control solution to one containing a fluorescent dye (BCECF salt; see below). The bath pH was continuously monitored by placing a flexible commercial pH electrode (MI 21960, Microelectrodes, Inc., Londonderry, NH) into the bath. The bath solutions were preheated to 38°C and equilibrated with appropriate gasses (see Table I). Another water bath, placed just before the bath chamber, permitted adjustment of the bath temperature to a constant  $38 \pm 0.5^\circ\text{C}$ .

The perfusion solutions used in this study are listed in Table I. CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>-free solutions were bubbled with 100% O<sub>2</sub> passed through a 3-N KOH CO<sub>2</sub> trap. The protocol that excluded exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> was always performed first, when the effects of the absence or presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> were compared to ensure the absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. SITS was obtained from ICN Pharmaceuticals (Cleveland, OH). Amiloride, nigericin, and acetazolamide were purchased from Sigma Chemical Co. (St. Louis, MO).

After the tubules were allowed to equilibrate at 38°C for 15 min, they were loaded with the acetoxymethyl derivative of BCECF (BCECF-AM; Molecular Probes, Inc., Eugene, OR). This compound does not fluoresce and is lipid soluble. It therefore diffuses rapidly into the cells, where cytoplasmic esterases cleave off the acetoxymethyl group, forming the fluorescent BCECF, which leaves the cells only slowly owing to its anionic charges. The tubules were loaded for 5–8 min. Since the intracellular cleavage of ester bonds constitutes an acid load to the cell, the first fluorescence measurements were made no earlier than 5 min after loading the tubules. During the performance of these studies, we found that the tubules could be loaded from the lumen (dye concentration, 100 μM) as well as from the bath (dye concentration, 4 μM). Loading from the bath yielded, on average, a higher signal-to-background ratio than loading from the lumen. The intracellular calibration curve of the dye (see below) was similar in tubules loaded from the lumen ( $n = 8$ ) and from the bath ( $n = 4$ ).

#### *Cell pH Measurement*

Measurements were made with an inverted fluorescent microscope (Fluovert, E. Leitz, Inc., Rockleigh, NJ) using a 25× objective. An adjustable measuring diaphragm was placed over the tubule and opened to ~40–70 μm<sup>2</sup>. Within this range, no difference in the reliability of the data was observed. The average tubule length exposed to the bath fluid was 300–400 μm. Background fluorescence was measured before loading the tubule with the dye. After this measurement, the measuring diaphragm was left in the same place for the entire experiment. The signal-to-background ratio at the end of the experiments varied from ~25 to 200 at 500 nm excitation and from ~15 to 120 at 450 nm excitation (see analysis below).

#### *Analysis*

BCECF has a peak excitation at 504 nm that is pH sensitive, and an isosbestic point at 436 nm, where fluorescence is independent of pH. Peak emission is at 526 nm (Alpern, 1985). Fluorescence was measured, as previously described (Alpern, 1985), alternately at 500 and 450 nm excitation and at an emission wavelength of 530 nm (interference filters, Corion Corp., Holliston, MA). After correcting all measurements for background, the mean of two 500-nm excitation measurements was divided by the 450-nm excitation measurement between them, thereby yielding the fluorescence excitation ratio ( $F_{500}/F_{450}$ ). For each determination, the measurements were performed twice and their mean was used to estimate cell pH. The use of the ratio provides a measurement that is unaffected by changes in the dye concentration (Thomas et al., 1979). After a solution change, the steady state cell pH values were determined when the 500-nm excitation fluorescence had stabilized.

TABLE I  
Perfusion Solutions

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	7.4 + HCO <sub>3</sub>	6.8 + HCO <sub>3</sub>	7.4 - HCO <sub>3</sub>	6.8 - HCO <sub>3</sub>	147 Na + HCO <sub>3</sub>	0 Na + HCO <sub>3</sub>	147 Na - HCO <sub>3</sub>	0 Na - HCO <sub>3</sub>	50 Na + HCO <sub>3</sub>	NH <sub>4</sub> Cl + HCO <sub>3</sub>	50 Na - HCO <sub>3</sub>	NH <sub>4</sub> Cl - HCO <sub>3</sub>	141 Na, 0 sub- strate	0 Na, 0 sub- strate
Na <sup>+</sup>	147	143.5	147	143.5	147	147	147	147	50	50	50	50	141.2	141.2
K <sup>+</sup>	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Ca <sup>++</sup>	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Mg <sup>++</sup>	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Choline <sup>+</sup>						147		147	104	84	104	84		141.2
NH <sub>4</sub> <sup>+</sup>										20		20		
Cl <sup>-</sup>	114	138.4	139	143.4	114	114	139	139	121	121	146	146	133.2	133.2
HCO <sub>3</sub> <sup>-</sup>	25	5	1	1	25	25	1	1	25	25	1	1	1	1
HPO <sub>4</sub> <sup>-</sup>	1	1	1	1	1	1	1	1	1	1	1	1	1	1
SO <sub>4</sub> <sup>-</sup>	1	1	1	1	1	1	1	1	1	1	1	1	1	1
HEPES <sup>-</sup>	14.6	6.7	14.6	6.7	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6
Glucose	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Alanine	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Urea	5	5	5	5	5	5	5	5	5	5	5	5	5	5
HEPES	10.4	18.3	10.4	18.3	10.4	10.4	10.4	10.4	10.4	10.4	10.4	10.4	10.4	10.4
CO <sub>2</sub> (%)	7	7	0	0	7	7	0	0	7	7	0	0	0	0
O <sub>2</sub> (%)	93	93	100	100	93	93	100	100	93	93	100	100	100	100
pH	7.4	6.8	7.4	6.8	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4

All concentrations are in millimoles per liter.

### Dye Calibration

In order to correlate the fluorescence excitation ratios with cell pH, the dye was calibrated intracellularly using the method of Thomas et al. (1979). Tubules were perfused with well-buffered solutions (25 mM HEPES, 33 mM phosphate) containing 7  $\mu$ M nigericin (a K/H antiporter) and 66 mM K. Since there are no data on the intracellular K activity for the rabbit PCT, we used the values as reported for the proximal straight tubule (PST) of the rabbit (48 mM; activity coefficient, 0.73; Biagi et al., 1981). In the above setting, cell pH is predicted to approximately equal extracellular pH. In order to test whether a difference in the K concentration would affect the calibration, three tubules were calibrated at pH 7.3 and external K concentrations of 66 or 132 mM. The ratios obtained were similar in the same tubules at these two external K concentrations. (The lack of an effect of a twofold increase in external K on the calibration is probably due to the high K conductance in PCT cells, which, together with the K/H antiporter, would be expected

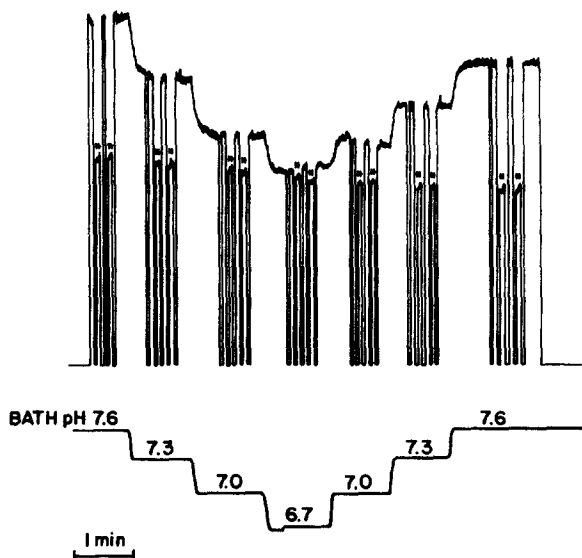


FIGURE 1. Intracellular dye calibration: typical study. Bath and luminal pH are simultaneously varied in the presence of nigericin (K/H antiporter) and a high K concentration (66 mM) in luminal and bath perfusates. Changes in cell pH are followed at 500 nm excitation. In the steady state, measurements at 500 and 450 (\*) nm excitation yield the fluorescence ratio  $F_{500}/F_{450}$ .

to equilibrate internal and external K, even if they were initially unequal.) Before exposure to nigericin, the tubules were loaded with BCECF and were then perfused in the lumen and the bath with the above solutions at different pH values. A typical tracing is shown in Fig. 1. The results of this calibration in a total of 12 tubules are shown in Fig. 2. As noted by others (Alpern, 1985; Chaillet and Boron, 1985), the effect of cell pH on the intracellular excitation ratios is shifted toward higher pH values as compared with the results of the extracellular calibration.

### Acidification Rate

To measure  $dpH/dt$ , fluorescence was followed at 500 nm while a fluid exchange was performed, and recorded on a chart recorder (LS 52, Linseis, Inc., Princeton Junction, NJ). The slope of a line drawn tangent to the initial deflection ( $dF_{500}/dt$ ) defined the initial rate of change in 500 nm fluorescence. Because fluorescence with 450 nm excitation is pH insensitive (Alpern, 1985), it can be considered constant. By measuring fluorescence at 450 nm before and after the fluid exchange, the actual value of the 450-nm excitation

at the time of the initial deflection of 500 nm could be interpolated. The rate of change in the fluorescence ratio was then calculated using the formula:

$$\frac{d(F_{500}/F_{450})}{dt} = \frac{(dF_{500}/dt)}{F_{450}} \quad (1)$$

Because the slope of the line (intracellular calibration) relating fluorescence ratio to pH in Fig. 2 is  $1.13 \cdot \text{pH unit}^{-1}$ :

$$\frac{d\text{pH}_i}{dt} = \frac{(dF_{500}/dt)}{F_{450} \times 1.13} \quad (2)$$

### Buffer Capacity

The buffer capacity ( $B$ ) was determined using the technique of rapid  $\text{NH}_3$  washout (Roos and Boron, 1981). Tubules were perfused at pH 7.4 in the control period. The bath solution was then changed to a similar solution with 20 mM  $\text{NH}_3/\text{NH}_4^+$  added (solutions 9–12, Table I). The  $\text{NH}_3$  in these solutions rapidly enters the cells and combines with intracellular protons to form  $\text{NH}_4^+$ . When external  $\text{NH}_3/\text{NH}_4^+$  are rapidly removed,

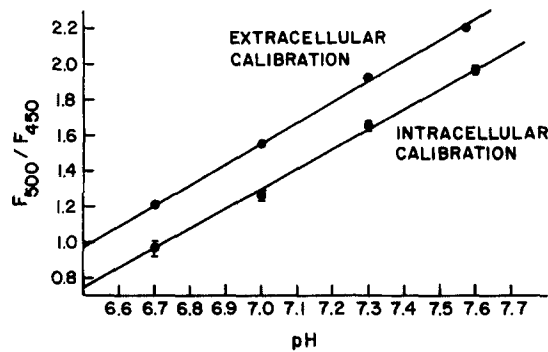


FIGURE 2. Dye calibration by fluorescence microscopy. The results of the extracellular and intracellular calibrations ( $n = 12$  tubules) are shown. The intracellular calibration curve is shifted upward by  $\sim 0.24$  pH units at pH 7.4.

intracellular  $\text{NH}_4$  dissociates into  $\text{NH}_3$  and protons. Because of its high permeability ( $6 \times 10^{-2}$  cm/s in the rabbit PCT; Hamm et al., 1985),  $\text{NH}_3$  rapidly diffuses out of the cell, while the protons are left behind and constitute the intracellular acid load. Since for each  $\text{NH}_4^+$  molecule dissociated, one intracellular proton is produced, the acid load per liter cell is  $\Delta[\text{NH}_4^+]_i$ . The buffer capacity (in millimoles per liter times pH units) is given by the formula

$$B = \frac{\Delta[\text{NH}_4^+]_i}{\Delta\text{pH}_i} \quad (3)$$

where  $[\text{NH}_4^+]_i$  is the intracellular  $\text{NH}_4^+$  concentration just before removal of external  $\text{NH}_3/\text{NH}_4$ . This is calculated as

$$[\text{NH}_4^+]_i = [\text{NH}_3]_i \times 10^{(\text{pK}_a - \text{pH}_i)} \quad (4)$$

where  $[\text{NH}_3]_i$  is the intracellular  $\text{NH}_3$  concentration (assumed to equal the extracellular  $\text{NH}_3$  concentration).  $\text{pH}_i$  was calculated from the fluorescence excitation ratios described above. A  $\text{pK}_a$  of 9.4 was used.

### Calculation of Proton Fluxes

The proton fluxes ( $J_H$ , in picomoles per liter times millimeters times minutes) induced by the maneuvers in the different protocols were calculated using the formula

$$J_H = dpH_i/dt \cdot V \cdot \text{mm}^{-1} \cdot B, \quad (5)$$

where  $dpH_i/dt$  is the initial rate of cell acidification (in pH units per minute),  $V \cdot \text{mm}^{-1}$  is the approximate cellular volume of the tubules per millimeter, and  $B$  is the buffer capacity (in millimoles per liter times pH units). For an outer tubular diameter of 60  $\mu\text{m}$  and an inner diameter of 25  $\mu\text{m}$ ,  $V = 23.4 \times 10^{-10}$  liter  $\cdot \text{mm}^{-1}$ . Reported  $J_H$  values represent the means of the  $J_H$  values for the acidification induced by the experimental solution and the alkalization induced by the control solution in the recovery period. In the steady state, cell pH is constant and there are proton fluxes from bath to cell and from cell to lumen. The proton flux ( $J_H$ ) referred to here is actually the change in the proton flux induced by the experimental maneuver.

### *Statistics*

All studies were paired, comparing two protocols within the same tubule. After the first protocol, the tubules were left to equilibrate in the control solution of the second protocol for 5 min. The data were analyzed using the paired  $t$  test. The calibration data were fitted using linear regression. Results are reported as means  $\pm$  standard error.

## RESULTS

### *Determination of Buffer Capacity*

Buffer capacity was determined in the absence and presence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. An accurate determination of the buffer capacity requires that acid-extrusion processes be blocked. We attempted to meet this requirement by perfusing the tubules with 1 mM amiloride in the lumen (to block the apical Na/H antiporter) and 1 mM SITS in the bath (to block the Na/base cotransporter and Cl/base exchanger). To prevent competition of Na ions to the amiloride-binding site on the antiporter (Kinsella and Aronson, 1981), the tubules were perfused symmetrically with 50 mM Na (solutions 9–12, Table I). That the acid extrusion processes were effectively blocked in this setting is illustrated by the fact that cell pH defense against the acid load induced by NH<sub>3</sub> washout was very slow (e.g., 0.05–0.08 pH units  $\cdot \text{min}^{-1}$  in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>). As illustrated by Table II, in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, the buffer capacity of the cells was  $42.8 \pm 5.6$  mmol  $\cdot \text{liter}^{-1} \cdot \text{pH unit}^{-1}$  ( $B_1$ ), and in its presence, the buffer capacity was  $84.6 \pm 7.3$  mmol  $\cdot \text{liter}^{-1} \cdot \text{pH unit}^{-1}$  ( $B_T$ ,  $n = 10$ ). The mean resting cell pH was  $7.26 \pm 0.02$  in the presence and  $7.24 \pm 0.03$  in the absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> (NS).

### *Na/Base Cotransport in Rabbit PCT*

To determine whether an Na/base cotransporter is present in the rabbit PCT, we examined the effects of changes in peritubular pH and Na concentration on cell pH in the absence and presence of 1 mM bath SITS. As shown in Fig. 3, lowering the bath HCO<sub>3</sub><sup>-</sup> concentration from 25 to 5 mM (solutions 1 and 2, Table I) decreased cell pH by  $0.30 \pm 0.02$  pH units in the absence of SITS as compared with  $0.09 \pm 0.02$  in the presence of SITS ( $p < 0.001$ ,  $n = 6$ ). The proton flux,  $J_H$ , was  $592 \pm 71$  pmol  $\cdot \text{mm}^{-1} \cdot \text{min}^{-1}$  in the control period and was inhibited 94% to  $37 \pm 14$  pmol  $\cdot \text{mm}^{-1} \cdot \text{min}^{-1}$  by bath SITS. When peritubular Na was lowered from 147 to 0 mM (Fig. 4; solutions 5 and 6, Table I), the cells acidified by a mean of  $0.25 \pm 0.03$  pH units. In the presence of SITS, the pH

TABLE II  
*Intracellular Buffer Capacity of Rabbit PCT  
 in the Presence and Absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>*

	Millimoles per liter per pH unit	pH <sub>i</sub>
$B_T$	84.6±7.3	7.26±0.02
$B_1$ (- CO <sub>2</sub> /HCO <sub>3</sub> <sup>-</sup> )	42.8±5.6	7.24±0.03
Measured $B_{CO_2}$	42.2±6.1	
Calculated $B_{CO_2}$	39.0±3.2	

Comparison of the total buffer capacity ( $B_T$ ) with the buffer capacity in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> ( $B_1$ ). The difference between these two values is the buffer capacity of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> ( $B_{CO_2}$  measured). Calculated  $B_{CO_2}$  represents the buffer capacity calculated from the estimated intracellular HCO<sub>3</sub><sup>-</sup> concentration (see text).

decrease was reduced to  $0.03 \pm 0.02$  ( $p < 0.001$ ,  $n = 5$ ). The  $J_H$  was  $332 \pm 51$  pmol·mm<sup>-1</sup>·min<sup>-1</sup> in the control period and was reduced to  $10 \pm 5$  pmol·mm<sup>-1</sup>·min<sup>-1</sup> by SITS.

These studies show the presence of a stilbene-inhibitable, Na-coupled base exit mechanism on the basolateral membrane of the rabbit PCT and confirm studies that found this transporter in the salamander (Boron and Boulpaep, 1983), rat (Alpern, 1985; Yoshitomi et al., 1985), and rabbit (Biagi, 1985; Sasaki et al., 1985; Biagi and Sothell, 1986) PCT.

#### CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> Dependence of Na/Base Cotransport

Since this Na-coupled transporter could possibly transport either HCO<sub>3</sub><sup>-</sup> and/or H<sup>+</sup>/OH<sup>-</sup> coupled to Na, the next studies were designed to determine the CO<sub>2</sub> requirements of this transport system. Tubules were symmetrically perfused at pH 7.4 and the bath fluid was changed to pH 6.8, first in the absence (solutions 3 and 4, Table I) and then in the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> (solutions 1 and 2, Table I). As illustrated in Fig. 5 (left), lowering bath pH in the absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> decreased cell pH from  $7.38 \pm 0.04$  to  $7.14 \pm 0.04$  ( $p < 0.001$ ,  $n = 10$ ). Cell pH returned to  $7.40 \pm 0.04$  ( $p < 0.001$ ) when bath pH was returned to 7.4. In the presence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> (Fig. 5, right), lowering bath pH acidified the cells from  $7.35 \pm 0.04$  to  $6.97 \pm 0.04$  ( $p < 0.001$ ), with a recovery

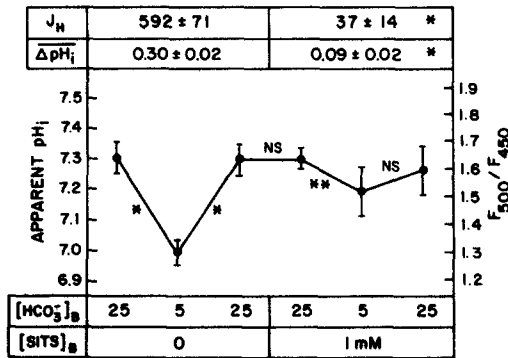


FIGURE 3. Effect of lowering bath pH in the presence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> on cell pH and  $J_H$ . Bath HCO<sub>3</sub><sup>-</sup> was lowered from 25 to 5 mM in the absence and presence of 1 mM bath SITS. \*  $p < 0.001$ , \*\*  $p < 0.05$ ,  $n = 6$ .



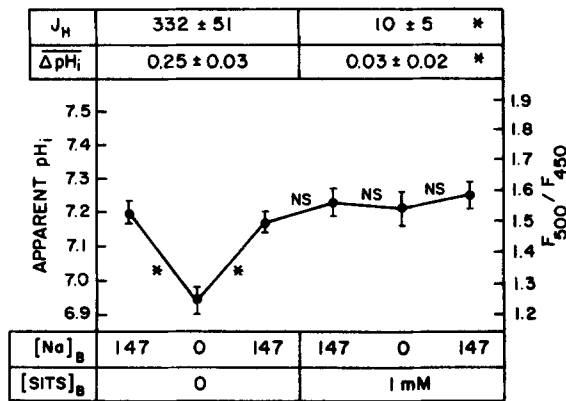


FIGURE 4. Effect of bath Na removal in the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub> on cell pH and  $J_H$ . Bath Na was replaced by choline in the presence and absence of 1 mM bath SITS. \*  $p < 0.001$ ,  $n = 5$ .

to  $7.36 \pm 0.05$  ( $p < 0.001$ ). The mean decrease of cell pH was  $0.25 \pm 0.04$  in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub> and  $0.38 \pm 0.04$  in its presence ( $p < 0.005$ ). The associated  $J_H$  values were  $223 \pm 27$  pmol·mm<sup>-1</sup>·min<sup>-1</sup> in the absence of CO<sub>2</sub>/HCO<sub>3</sub> and  $643 \pm 51$  pmol·mm<sup>-1</sup>·min<sup>-1</sup> in its presence ( $p < 0.001$ ). Thus, the  $J_H$  in response to a given change in bath pH is reduced by only 65% upon removal of ambient CO<sub>2</sub>/HCO<sub>3</sub>.

The effect of lowering the bath Na concentration from 147 to 0 mM in the absence and presence of CO<sub>2</sub>/HCO<sub>3</sub> (solutions 5–8, Table I) is shown in Fig. 6. In the absence of CO<sub>2</sub>/HCO<sub>3</sub>, cell pH decreased from  $7.25 \pm 0.03$  to  $7.08 \pm 0.02$  ( $p < 0.001$ ) and recovered to  $7.21 \pm 0.04$  ( $p < 0.001$ ,  $n = 7$ ). In the presence of CO<sub>2</sub>/HCO<sub>3</sub>, cell pH decreased from  $7.28 \pm 0.03$  to  $6.93 \pm 0.05$  ( $p < 0.001$ ) and returned to  $7.30 \pm 0.05$  ( $p < 0.001$ ). The mean change of cell pH was  $0.17 \pm 0.03$  in the absence of CO<sub>2</sub>/HCO<sub>3</sub> and  $0.35 \pm 0.07$  in its presence ( $p < 0.005$ ), and  $J_H$  values were  $147 \pm 13$  and  $596 \pm 77$  pmol·mm<sup>-1</sup>·min<sup>-1</sup>, respectively ( $p < 0.005$ ). Again, a significant  $J_H$  value in response to lowering the bath Na concentration was observed in the absence of ambient CO<sub>2</sub>/HCO<sub>3</sub>.

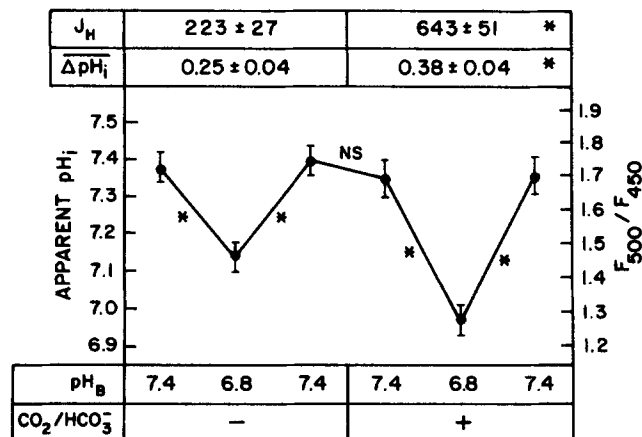


FIGURE 5. Effect of lowering bath pH in the absence and presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub> on cell pH and  $J_H$ . Bath pH was lowered from 7.4 to 6.8. \*  $p < 0.001$ ,  $n = 10$ .

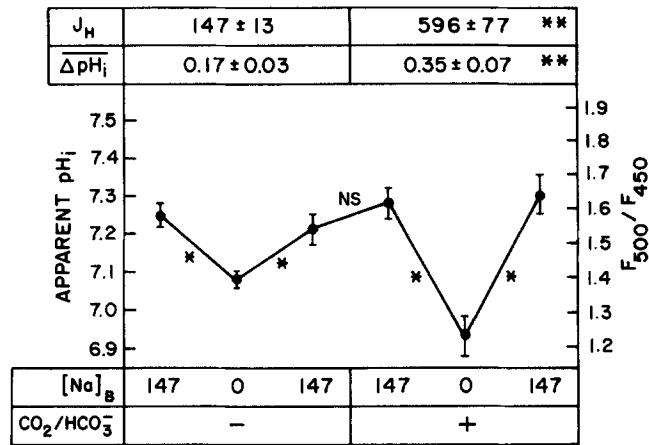


FIGURE 6. Effect of removing bath Na in the absence and presence of exogenous  $CO_2/HCO_3^-$  on cell pH and  $J_H$ . Na was removed from the bath and replaced by choline. \*  $p < 0.001$ ,  $n = 8$ .

Thus, these studies show the persistence of Na/base cotransport in the absence of  $CO_2/HCO_3^-$ . In theory, the above results could be explained by the presence of an Na/OH or an Na/ $HCO_3^-$  cotransporter and/or an Na/H antiporter. To differentiate among these mechanisms, we studied the effects of SITS (an inhibitor of Na/OH and Na/ $HCO_3^-$  cotransporters) and amiloride (an inhibitor of Na/H antiporters) in the absence of exogenous  $CO_2/HCO_3^-$ .

#### Effect of SITS

The effects of 1 mM peritubular SITS on the cell pH response to changes in bath pH or bath Na in the absence of ambient  $CO_2/HCO_3^-$  were examined. A typical tracing is shown in Fig. 7. SITS markedly inhibited the response of cell pH to changing bath pH in the absence of exogenous  $CO_2/HCO_3^-$ . Fig. 8 shows results from six tubules. In the absence of SITS, lowering bath pH from 7.4 to 6.8 caused a decrease in cell pH from  $7.52 \pm 0.04$  to  $7.23 \pm 0.03$  ( $p < 0.001$ )

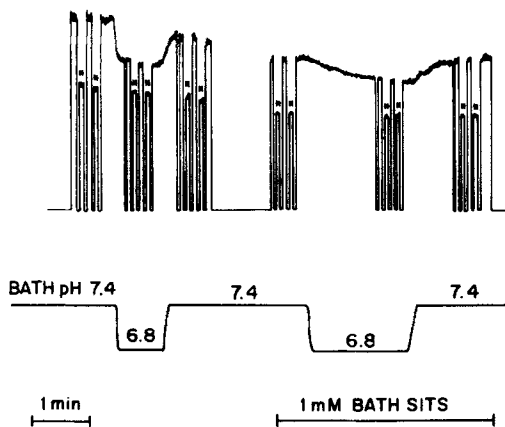


FIGURE 7. Effect of SITS on cell pH changes induced by lowering bath pH in the absence of exogenous  $CO_2/HCO_3^-$ : typical study. On the left of the figure, the effect of lowering bath pH on the fluorescence ratio is shown in the absence, on the right of the figure in the presence, of 1 mM bath SITS. The changes are followed at 500 nm excitation. The asterisk denotes measurements at 450 nm excitation.

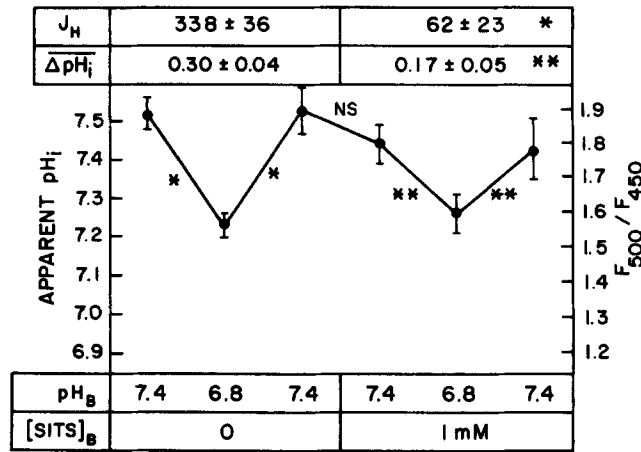


FIGURE 8. Effect of SITS on cell pH changes and  $J_H$  induced by lowering bath pH in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. Bath pH was changed from 7.4 to 6.8 in the absence and presence of 1 mM bath SITS. \*  $p < 0.001$ , \*\*  $p < 0.005$ ,  $n = 6$ .

and a return to  $7.53 \pm 0.05$  in the recovery period ( $p < 0.001$ ). In the presence of 1 mM bath SITS, cell pH decreased from  $7.44 \pm 0.05$  to  $7.26 \pm 0.05$  ( $p < 0.005$ ) and recovered to  $7.43 \pm 0.08$  ( $p < 0.005$ ). The mean changes in cell pH and  $J_H$  were  $0.30 \pm 0.04$  pH units and  $338 \pm 36$  pmol·mm<sup>-1</sup>·min<sup>-1</sup>, respectively, in the absence of SITS and  $0.17 \pm 0.05$  ( $p < 0.005$ ) pH units and  $62 \pm 23$  pmol·mm<sup>-1</sup>·min<sup>-1</sup> ( $p < 0.001$ ) in the presence of SITS.

Fig. 9 demonstrates that SITS decreased the mean change in cell pH induced by removing bath Na from  $0.16 \pm 0.03$  to  $0.05 \pm 0.02$  pH units ( $p < 0.001$ ,  $n = 8$ ) and inhibited  $J_H$  from 165 to 33 to  $13 \pm 7$  pmol·mm<sup>-1</sup>·min<sup>-1</sup> ( $p < 0.001$ ). In the period without SITS, cell pH decreased from  $7.47 \pm 0.04$  to  $7.30 \pm 0.04$  ( $p < 0.001$ ) and returned to  $7.45 \pm 0.04$  in the recovery period ( $p < 0.025$ ). In the presence of 1 mM bath SITS, cell pH changed from  $7.34 \pm 0.03$  to  $7.31 \pm 0.06$  when bath Na was removed (NS) and was unchanged ( $7.31 \pm 0.06$ ) upon readdition of Na.

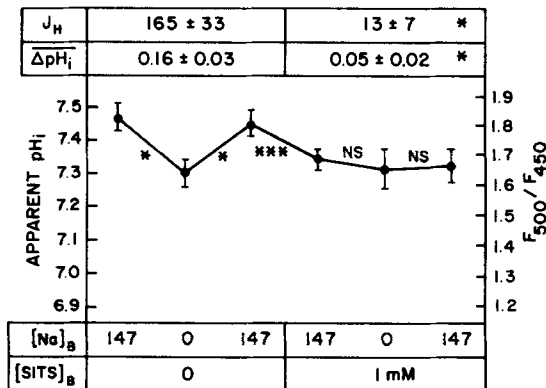


FIGURE 9. Effect of SITS on cell pH changes and  $J_H$  induced by lowering bath Na in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. Na was lowered from 147 to 0 mM in the absence and presence of 1 mM bath SITS. \*  $p < 0.001$ , \*\*\*  $p < 0.005$ ,  $n = 8$ .

These data show that cell acidification induced by lowering bath pH or Na in the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$  is SITS sensitive and is inhibited by >80% by this agent. In these protocols, SITS caused a slight decrease of cell pH (see Figs. 8 and 9). Since time controls did not show any acidification, this effect seems to be SITS related, although its mechanism is presently unclear.

#### Effect of Amiloride

Fig. 10 shows that 1 mM bath amiloride did not inhibit the cell acidification induced by removing bath Na in the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$ . Cell acidification was  $0.18 \pm 0.03$  pH units in the absence of amiloride and  $0.19 \pm 0.04$  pH units in the presence of amiloride (NS,  $n = 11$ ). The corresponding  $J_{\text{H}}$  values were  $131 \pm 23$  and  $118 \pm 21$   $\text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$  (NS). In terms of absolute

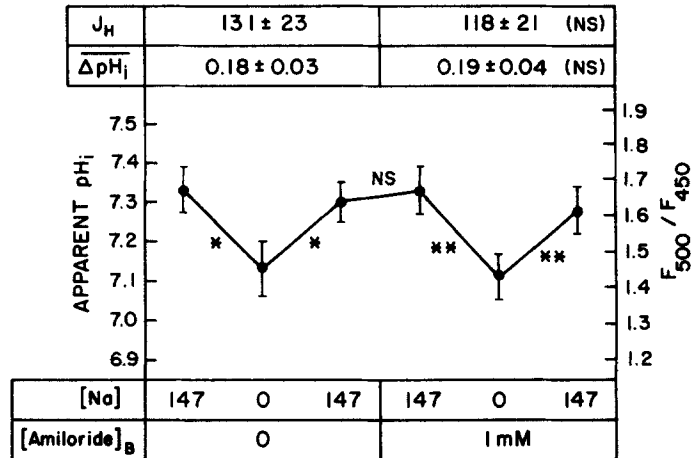


FIGURE 10. Effect of amiloride on cell pH changes and  $J_{\text{H}}$  induced by bath Na removal in the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$ . Na was removed from the bath in the absence and presence of 1 mM bath amiloride. \*  $p < 0.001$ , \*\*  $p < 0.005$ ,  $n = 11$ .

pH values, Na removal caused a decrease of cell pH from  $7.33 \pm 0.06$  to  $7.13 \pm 0.07$  ( $p < 0.001$ ), with a recovery to  $7.30 \pm 0.05$  ( $p < 0.001$ ) in the absence of amiloride. In the presence of amiloride, cell pH decreased from  $7.33 \pm 0.06$  to  $7.11 \pm 0.06$  ( $p < 0.005$ ) and returned to  $7.28 \pm 0.06$  ( $p < 0.005$ ).

The absence of an amiloride effect in these studies could be due to the high Na concentration used, since amiloride competes with Na on the Na/H antiporter (Kinsella and Aronson, 1981). When the same protocol was repeated in six additional tubules, but with Na decreased from 50 to 0 mM, there was still no detectable inhibition of cell acidification. The mean changes in cell pH were  $0.11 \pm 0.01$  without and  $0.14 \pm 0.03$  with 1 mM bath amiloride (NS). The  $J_{\text{H}}$  was  $148 \pm 12$   $\text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$  and was unaltered by amiloride ( $152 \pm 16$   $\text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$ ; NS).

Thus, these studies rule out an important role for an amiloride-sensitive Na/H antiporter in the Na-coupled basolateral base exit mechanism.

*Effect of Acetazolamide and Cyanide*

The significant rate of basolateral Na-coupled base exit in the absence of ambient CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, although it is slower than in the presence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, raises the possibility that the cotransport system might operate as an Na/OH cotransporter. Alternatively, there might be sufficient metabolic CO<sub>2</sub> produced in the cell to drive an Na/HCO<sub>3</sub> cotransporter in the absence of external CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>.

The effect of acetazolamide on the cell acidification induced by lowering bath pH or bath Na might aid in distinguishing between these two possibilities. Acetazolamide would be expected to inhibit the rate of Na/HCO<sub>3</sub> cotransport, because carbonic anhydrase would be required to hydroxylate CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup>. In contrast, acetazolamide would not be expected to inhibit the rate of Na/OH

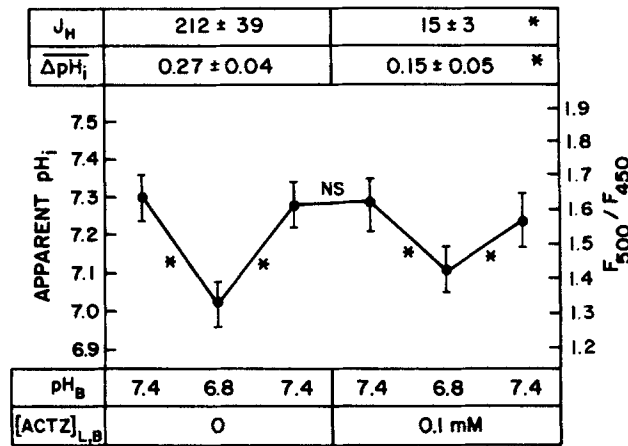


FIGURE 11. Effect of acetazolamide on cell pH changes and  $J_H$  induced by lowering bath pH in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. Bath pH was changed from 7.4 to 6.8 in the absence and presence of 0.1 mM acetazolamide in luminal and bath perfusates. \*  $p < 0.001$ ,  $n = 7$ .

cotransport, unless carbonic anhydrase were an integral part of the Na/OH symporter protein. Such a function for carbonic anhydrase has not been previously described (Dobyan and Bulger, 1982; Silverman and Vincent, 1983).

The effect of 0.1 mM acetazolamide added to bath and lumen when bath pH was lowered is shown in Fig. 11. When bath pH was lowered from 7.4 to 6.8 in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, the cell pH decreased by  $0.27 \pm 0.04$  in the absence of acetazolamide, but only by  $0.15 \pm 0.05$  ( $p < 0.001$ ,  $n = 7$ ) in the presence of acetazolamide. Acetazolamide substantially inhibited  $J_H$  from  $212 \pm 39$  to  $15 \pm 3$   $\mu\text{mol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$  ( $p < 0.001$ ). When bath Na was removed (Fig. 12), acetazolamide prevented (100% inhibition) cell acidification. The mean cell pH change was  $0.12 \pm 0.02$  in the absence and  $0.01 \pm 0.02$  in the presence of acetazolamide ( $p < 0.001$ ,  $n = 8$ ).  $J_H$  was inhibited from  $125 \pm 13$  to  $1 \pm 3$   $\mu\text{mol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$  ( $p < 0.001$ ).

As discussed above, acetazolamide sensitivity of the cell pH change is compat-

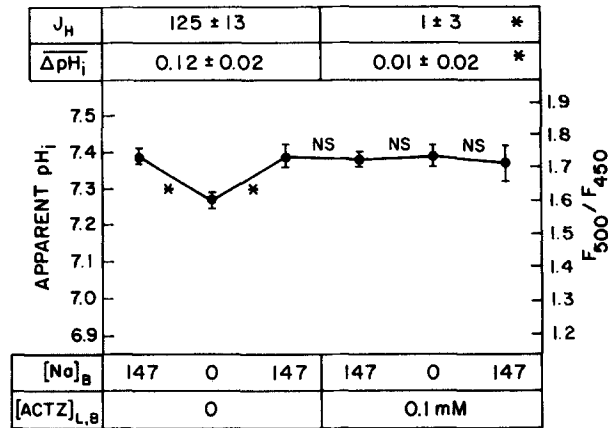


FIGURE 12. Effect of acetazolamide on cell pH changes and  $J_H$  induced by removing bath Na in the absence of exogenous  $CO_2/HCO_3^-$ . Bath Na was changed from 147 to 0 mM in the absence and presence of 0.1 mM acetazolamide in the luminal and bath perfusates. \*  $p < 0.001$ ,  $n = 8$ .

ible with the transport of  $HCO_3^-$ . To test whether a known  $H^+/OH^-$  transporter would be inhibited by acetazolamide, we examined the effect of acetazolamide on the rate of the apical Na/H antiport. This transporter was assayed as previously described (Alpern and Chambers, 1986). Glucose and alanine were eliminated from the luminal perfusate to prevent cell membrane potential changes upon luminal Na removal (solutions 13 and 14, Table I). SITS was added to the bath to prevent secondary effects of the basolateral membrane Na/ $HCO_3^-$  cotransporter. In this setting (Fig. 13), removal of luminal Na acidified the cells by  $0.41 \pm 0.08$  pH units in the absence of 0.1 mM acetazolamide and by  $0.43 \pm 0.08$  pH units in its presence (NS,  $n = 4$ ).  $J_H$  was not significantly altered and was 159

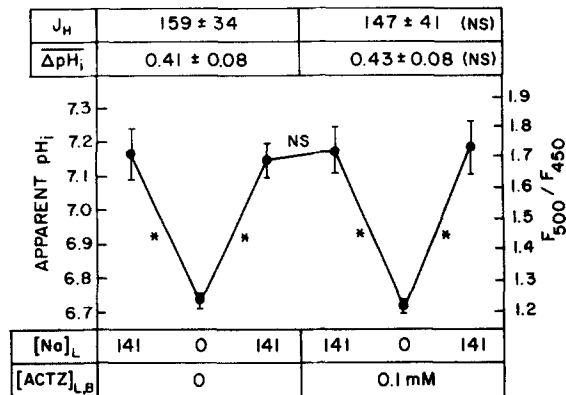


FIGURE 13. Effect of acetazolamide on cell pH changes and  $J_H$  induced by removing luminal Na in the absence of exogenous  $CO_2/HCO_3^-$ . Glucose and alanine were eliminated from the luminal perfusate and 1 mM SITS was added to the bath. Luminal Na was changed from 141 to 0 mM in the absence and presence of 0.1 mM acetazolamide in luminal and bath perfusates. \*  $p < 0.001$ ,  $n = 4$ .

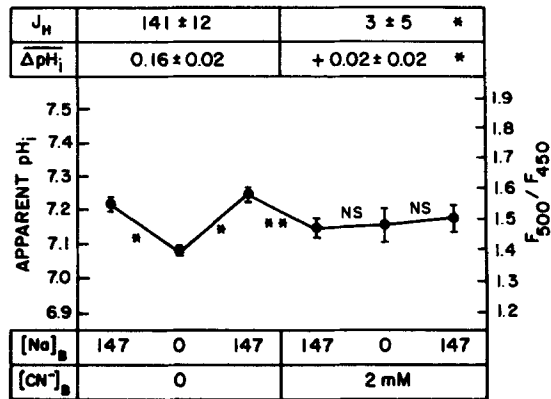


FIGURE 14. Effect of 2 mM bath KCN on cell pH changes and  $J_H$  induced by removing bath Na in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. \*  $p < 0.001$ , \*\*  $p < 0.005$ ,  $n = 6$ .

± 34 pmol · mm<sup>-1</sup> · min<sup>-1</sup> without acetazolamide and 147 ± 41 pmol · mm<sup>-1</sup> · min<sup>-1</sup> with acetazolamide (NS).

Thus, the acetazolamide sensitivity suggests that in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, HCO<sub>3</sub><sup>-</sup> provided by metabolism is the transported base species. If this is the case, metabolic inhibitors would be expected to inhibit the transporter by restriction of substrate availability. On the other hand, they should have no effect in the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. The effect of 2 mM bath KCN (2 mM KCN replaced 2 mM KCl in solutions 7 and 8, Table I) when bath Na was removed in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> is shown in Fig. 14. Na removal lowered cell pH by 0.16 ± 0.02 pH units in the absence of KCN. In its presence, however, cell acidification was completely abolished (mean cell pH change plus 0.02 ± 0.02 pH units;  $p < 0.001$ ,  $n = 6$ ). KCN inhibited  $J_H$  from 141 ± 12 to 3 ± 5 pmol · mm<sup>-1</sup> · min<sup>-1</sup> ( $p < 0.001$ ). Cyanide inhibition of the transporter could be due to an ATP dependence rather than a CO<sub>2</sub> dependence. To rule this out, additional experiments were performed on four tubules (Fig. 15) in the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> (2 mM KCN replaced 2 mM KCl in solutions 5 and 6, Table I). In this setting, KCN had no significant effect on

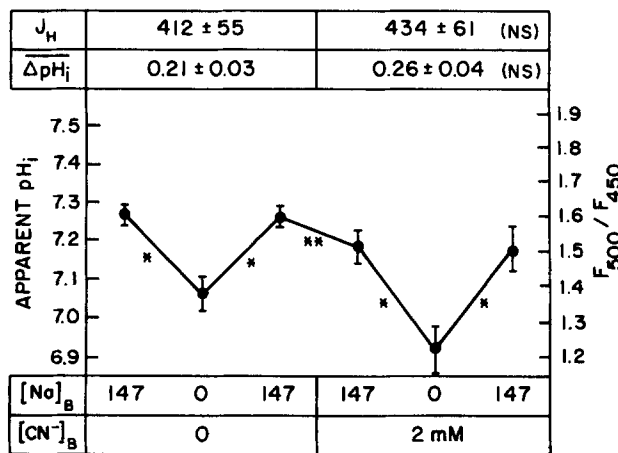


FIGURE 15. Effect of 2 mM bath KCN on cell pH changes and  $J_H$  induced by removing bath Na in the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. \*  $p < 0.001$ , \*\*  $p < 0.025$ ,  $n = 4$ .

the cell acidification induced by bath Na removal: the cells acidified by  $0.21 \pm 0.03$  pH units in the absence and by  $0.26 \pm 0.04$  in the presence of KCN.  $J_H$  was  $412 \pm 55$   $\text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$  in the absence of KCN and  $434 \pm 61$   $\text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$  (NS) in its presence.

The fact that restriction of substrate ( $\text{HCO}_3^-$ ) availability by acetazolamide and KCN inhibits cell pH changes induced by bath Na removal also provides evidence that the transporter is Na coupled. We could not prove this formally when we attempted to show an inhibitory effect of symmetrical Na removal on cell pH change induced by lowering bath pH from 7.4 to 6.8, because Na removal caused a massive, stable, and fully reversible cell acidification from  $7.28 \pm 0.04$  to  $6.84 \pm 0.05$  ( $p < 0.001$ ,  $n = 5$ ; solutions 3 and 8, Table I). The decrease in the intracellular base concentration associated with this degree of cell acidification would by itself decrease the driving force across the transporter.

#### DISCUSSION

Since the main purposes of these studies were to determine the  $\text{CO}_2/\text{HCO}_3^-$  dependence of the Na/base cotransporter and to analyze the nature of the base exit, we used the technique of in vitro microperfusion of rabbit PCT, which permits vigorous exclusion of exogenous  $\text{CO}_2/\text{HCO}_3^-$ . Microcalorimetric analysis of bath fluid samples confirmed that the total  $\text{CO}_2$  concentration was not different from zero. In addition, the special setup for these studies permitted rapid, serial fluid exchanges for the bath and the luminal perfusates independently. Therefore, we were able to assess the kinetic properties of the Na-coupled base transporter under a variety of experimental conditions. Cell pH was measured using fluorescence ratios of BCECF at two different wavelengths (500 and 450 nm), as previously described (Alpern, 1985).

In order to correlate the cell pH to the fluorescent ratios, the dye was calibrated using nigericin (a K/H antiporter) and a high external K concentration that approximated intracellular K activity (Thomas et al., 1979). In this setting, external pH approximately equals cell pH. The intracellular calibration curve was shifted to higher pH values when compared with the extracellular calibration, as also noted for the rat (Alpern, 1985) and the salamander PCT (dimethyl-6-carboxyfluorescein; Chaillet and Boron, 1985). In this study, the shift was equivalent to 0.24 pH units at pH 7.4 (Fig. 2). When tubules were perfused symmetrically with  $\text{HCO}_3^-$ -containing solutions (solution 1, Table I), the cell pH was  $7.29 \pm 0.02$  ( $n = 42$ ). This value is comparable to cell pH determinations with microelectrodes in the rabbit PST (Sasaki et al., 1985), but is lower than microfluorometrically obtained cell pH values in the rabbit PST (pH 7.46; Nakhoul and Boron, 1985). However, in the latter study, the pH data were obtained in the nominal absence of  $\text{CO}_2$  and in the presence of monocarboxylate. We observed some intertubular variability in the resting cell pH, which probably relates to the fact that the tubules were not calibrated individually. However, as all our studies were paired, comparing a control with an experimental procedure, and since the interpretation of our results depends on the changes of the cell pH rather than their absolute values, this variability is probably of minor importance.



*Determination of Buffer Capacity*

Buffer capacity was measured in the presence and absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> and was 84.6 ± 7.3 mmol·liter<sup>-1</sup>·pH unit<sup>-1</sup> in the presence and 42.8 ± 5.6 mmol·liter<sup>-1</sup>·pH unit<sup>-1</sup> in the absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. The difference between total buffer capacity and the buffer capacity in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> is equal to the buffer capacity of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> ( $B_{\text{CO}_2}$ ). According to the formula

$$B_{\text{CO}_2} = 2.3 \times [\text{HCO}_3^-]_i \quad (6)$$

(Roos and Boron, 1981), the CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> buffer capacity can be directly calculated. Since the mean pH in this set of studies was 7.26, [HCO<sub>3</sub><sup>-</sup>]<sub>i</sub> would be 17 mM and  $B_{\text{CO}_2}$  would therefore be 39 mmol·liter<sup>-1</sup>·pH unit<sup>-1</sup>. As can be seen, this value is in excellent agreement with our measured difference of 42.2 mmol·liter<sup>-1</sup>·pH unit<sup>-1</sup> (see Table II). The value for the total buffer capacity is comparable to that in the rat PCT of 102.7 ± 11.2 mmol·liter<sup>-1</sup>, when cells were exposed to a pCO<sub>2</sub> of 40 kPa or 0.5 kPa, respectively (Yoshitomi and Froemter, 1984), and the value of 65.3 ± 8.7 mmol·liter<sup>-1</sup> when pCO<sub>2</sub> was varied from 5 to 10 kPa only (Yoshitomi et al., 1985).

*Persistence of a Stilbene-inhibitable, Na-coupled Base Exit in the Absence of Exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>*

In the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, our studies confirmed the presence of a stilbene-sensitive, Na-coupled base exit in the rabbit PCT (Figs. 3 and 4). The observed mean cell pH change of 0.30 pH units when bath bicarbonate was lowered from 25 to 5 mM is comparable to findings in the rabbit PST (microelectrode study; Sasaki et al., 1985) and the rat PCT (fluorescent studies; Alpern, 1985; Alpern and Chambers, 1986), where mean changes of ~0.31 and 0.23–0.30 pH units were recorded, respectively. Similarly, Na removal from the bath caused a mean acidification of 0.25 pH units, again quite close to values in the rabbit PST (0.21; Sasaki et al., 1985) and the rat PCT (0.20, where Na was not totally removed, but lowered to 25 mM; Alpern, 1985).

When the effects of lowering peritubular Na or pH were compared in the absence and presence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> in the same tubule (Figs. 5 and 6), it could be demonstrated that a significant acidification persisted in the total absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. The persisting proton fluxes were ~25% of the observed fluxes in the presence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> when bath Na was removed and ~35% when bath pH was lowered. The facts that (a) SITS inhibited these effects (Figs. 7–9) and (b) amiloride had no effect (Fig. 10) both support the existence of a stilbene-sensitive, Na-coupled base exit in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. The failure to find an amiloride-sensitive Na/H antiporter on the basolateral membrane agrees with studies in vesicles from the rabbit and rat renal cortex (Ives et al., 1983; Sabolic and Burckhardt, 1983) and with studies in the isolated rabbit PST (Nakhoul and Boron, 1985).

*Nature of the Na-coupled Base Exit in the Rabbit PCT*

In the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$ , an Na-coupled base exit could occur by two basic transport mechanisms: (a) an Na/OH cotransporter (equivalent to an amiloride-insensitive Na/H antiporter), or (b) an Na/ $\text{HCO}_3^-$  cotransporter.

The presence of an amiloride-insensitive Na/H antiporter has recently been described in endosomes from the rabbit renal cortex (Gurich and Warnock, 1986), but there are no data to suggest its presence in the basolateral membrane of the PCT; in addition, it would not be expected to be SITS sensitive.

The finding that acetazolamide inhibited the cell pH changes in this study could be consistent with an Na/OH cotransporter: (a) if cytoplasmic carbonic anhydrase is needed to minimize a cellular diffusion barrier to base equivalents (Gutknecht et al., 1977), (b) if basolateral carbonic anhydrase is an integral functional component of the transporter, or (c) if the transporter shares a common structural domain with carbonic anhydrase, rendering it susceptible to acetazolamide.

If carbonic anhydrase facilitated diffusion of mobile buffers from the bulk phase in the cytoplasm to the membrane transporter, acetazolamide also should have inhibited the apical Na/H antiporter in the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$ . As such an inhibition was not found, carbonic anhydrase does not seem to have an important role in facilitating cytoplasmic buffer diffusion.

Because  $\text{CO}_2$ -dependent  $^{22}\text{Na}$  uptake in rabbit cortical basolateral membrane vesicles occurs in the presence of acetazolamide (Akiba et al., 1986; Soleimani and Aronson, 1987), a direct influence of acetazolamide on the Na/base cotransporter seems improbable. The results of these studies and the effect of acetazolamide on the rate of the Na-coupled base exit observed in our study would argue against an Na/OH and favor an Na/ $\text{HCO}_3^-$  cotransport mechanism.

Even more convincing is the dependence of the basolateral membrane transporter in the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$  on metabolism. In the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$ , inhibition of metabolism by bath cyanide completely inhibited basolateral membrane Na/ $\text{HCO}_3^-$  cotransport. To prove that this was not due to a direct effect on the transporter, we showed that cyanide was without effect in the presence of exogenous  $\text{CO}_2/\text{HCO}_3^-$ . Thus, sensitivity to acetazolamide and cyanide, in the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$ , proves that the transporter is dependent on  $\text{CO}_2/\text{HCO}_3^-$ , a dependence that is probably due to the fact that  $\text{HCO}_3^-$  is the transported base species.

The observations that, in the absence of exogenous  $\text{CO}_2/\text{HCO}_3^-$ , (a) changes in bath Na concentration were able to affect changes in transport rate and (b) changes in bath pH were reversible suggest that there is a significant local  $\text{CO}_2/\text{HCO}_3^-$  concentration next to the outside of the basolateral membrane. This could be due to the effect of unstirred layers. Based on calculations of metabolic  $\text{CO}_2$  production, the intracellular  $\text{HCO}_3^-$  concentration can be estimated to be  $<0.37 \text{ mM}$ ,<sup>1</sup> while the local extracellular concentration of  $\text{HCO}_3^-$  would be even

<sup>1</sup> The  $\text{HCO}_3^-$  concentration generated from metabolic production was estimated assuming that endogenous  $\text{CO}_2$  production ( $Q_{\text{CO}_2}$ ) by metabolism is  $6.0 \mu\text{mol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$  (Burg and Orloff, 1962). The weight of a tubule with an inner and outer diameter of 25 and 60  $\mu\text{m}$ , respectively, is  $23.4 \times 10^{-6} \text{ g}\cdot\text{cm}^{-1}$ . With minutes converted to seconds,  $Q_{\text{CO}_2}$  is then  $2.34 \times 10^{-9} \text{ mmol}\cdot$

lower. In the presence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, the intracellular HCO<sub>3</sub><sup>-</sup> concentration at a pH of 7.3 is ~19 mM. However, in the absence of exogenous CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, the transporter is able to run at ~30% of its control rate in the presence of ambient CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>. This observation suggests that the transporter is sensitive to very low concentrations of bicarbonate. This might occur if one or two of the bicarbonate-binding sites had a high affinity for bicarbonate, probably in the micromolar range. However, not all of the bicarbonate-binding sites can have such a high affinity, because the transporter would become saturated at low concentrations and its rate would be insensitive to changes in cell and bath bicarbonate concentrations from 25 to 5 mM.

In conclusion, our studies demonstrate the presence of a stilbene-sensitive Na/base cotransporter on the basolateral membrane of the rabbit PCT. This cotransporter has an absolute requirement for CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, which suggests that HCO<sub>3</sub><sup>-</sup> is at least one of the transported base species.

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cm<sup>-1</sup>·s<sup>-1</sup>. In the steady state, the CO<sub>2</sub> produced must be equal to the CO<sub>2</sub> lost across the apical and basolateral membranes. Therefore,  $\Delta C = Q_{CO_2}/P = 0.0234 \text{ mmol CO}_2 \cdot \text{liter}^{-1}$ , where  $\Delta C$  is the CO<sub>2</sub> gradient across the cell membrane and  $P$  is the membrane permeability (the sum of the apical plus basolateral) to CO<sub>2</sub>. We used for  $P$  the value of 10<sup>-4</sup> cm<sup>2</sup>·s<sup>-1</sup> (Schwartz et al., 1981), the permeability of the whole epithelium. Using the Henderson-Hasselbalch equation and an intracellular pH of 7.3, the intracellular HCO<sub>3</sub><sup>-</sup> concentration would be 0.37 mM. Alternatively, the intracellular HCO<sub>3</sub><sup>-</sup> concentration can be estimated by calculation of the CO<sub>2</sub> production associated with Na transport. With a transepithelial Na flux of 250 pmol·mm<sup>-1</sup>·min<sup>-1</sup>, a ratio of Na transported to ATP consumed of 30:1 was assumed (Cohen and Kamm, 1981). With 6 ATP molecules consumed per CO<sub>2</sub> molecule produced, the  $Q_{CO_2}$  would be 0.23 × 10<sup>-9</sup> mmol·cm<sup>-1</sup>·s<sup>-1</sup>. Using the same analysis as above, the intracellular HCO<sub>3</sub><sup>-</sup> concentration would be only 0.04 mM.

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