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# Regulation of intracellular pH in proximal tubules of avian loopless reptilian-type nephrons

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**Martinez, Christina L., Olga H. Brokl, Apichai Shuprisha, Diane E. Abbott, and William H. Dantzer.** Regulation of intracellular pH in proximal tubules of avian loopless reptilian-type nephrons. *Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42):* R1845–R1854, 1997.—In proximal tubules isolated from chicken superficial loopless reptilian-type nephrons, intracellular pH ( $pH_i$ ), measured with pH-sensitive fluorescent dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein, was  $\sim 7.1$ – $7.2$  under control conditions (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid-buffered medium with pH 7.4 at 37°C), and was reduced to  $\sim 6.9$  in response to  $NH_4Cl$  pulse. The rate of recovery of  $pH_i$  (control value  $\cong 5 \times 10^{-3}$  pH U/s) from this acid level was 1) significantly decreased by removal of  $Na^+$  or both  $Na^+$  and  $Cl^-$  from the bath or addition of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (0.25 mM) to the bath, 2) significantly increased by high bath  $K^+$  (75 mM), and 3) unchanged by removal of  $Cl^-$  alone from the bath or addition of ethylisopropylamiloride (1 mM) or  $Ba^{2+}$  (5 mM) to the bath. Resting  $pH_i$  was 1) significantly decreased by  $Na^+$  or simultaneous  $Na^+$  and  $Cl^-$  removal, 2) significantly increased by high  $K^+$ , and 3) unchanged by  $Cl^-$  removal alone or addition of  $Ba^{2+}$ . The data do not fit the concept of  $pH_i$  regulation by the most commonly suggested basolateral transporters ( $Na^+/H^+$  exchanger,  $Na^+$ -dependent and  $Na^+$ -independent  $Cl^-/HCO_3^-$  exchangers, or  $Na^+-HCO_3^-CO_3^{2-}$  cotransporter).

chickens; ammonium chloride pulse; intracellular acidification; intrinsic buffering capacity; sodium-coupled basolateral acid-base fluxes

THE AVIAN KIDNEY HAS a highly complex organization involving two major nephron populations, with gradations between the two (3, 9, 26). The most superficial cortical nephrons (often referred to as reptilian-type nephrons) (13) are small, lack loops of Henle, and empty at right angles into collecting ducts (6, 26, 27). The deepest medullary nephrons (often referred to as mammalian-type nephrons) (13) are large, with complex convoluted proximal tubules and long loops of Henle lying parallel to collecting ducts (6, 26). The gradation between the reptilian-type and the mammalian-type nephrons is made up of transitional nephrons. These have relatively straight proximal tubules and short-looped intermediate segments that do not lie parallel to collecting ducts (6, 26).

The role of the proximal tubules of these different types of nephrons in the maintenance of acid-base homeostasis for the animal as well as the factors involved in the maintenance of pH within the tubule cells themselves [intracellular pH ( $pH_i$ )] are only beginning to be explored. This is despite the fact that the proximal tubules of birds, like those of other tetrapod vertebrates, must deal with systemic dietary acid or

base loads while maintaining their own intracellular acid-base homeostasis. Some in vivo micropuncture studies on superficial loopless reptilian-type avian nephrons (19, 20) and in vitro micropuncture studies on short-looped transitional avian nephrons (7) indicate that along the proximal tubule there is little acidification of luminal fluid, that net bicarbonate reabsorption proceeds at the same rate as net fluid reabsorption, and that net fluid reabsorption is not dependent on bicarbonate reabsorption.

An initial study on proximal tubules from chicken short-looped transitional nephrons indicated that resting  $pH_i$  is higher than that generally found under similar circumstances in rabbit and snake proximal tubules and that maintenance of  $pH_i$  is dependent on both  $Na^+$ - and  $Cl^-$ -coupled acid-base fluxes at the basolateral membrane (14).  $pH_i$  studies were undertaken initially on proximal tubules from short-looped transitional nephrons because they were the only group of avian proximal tubules that we were able to tease from fresh tissue. Recently, we have learned to tease proximal tubules from superficial loopless reptilian-type avian nephrons. Because these superficial loopless reptilian-type nephrons in the avian kidney are so different structurally from the deeper short-looped transitional nephrons, we undertook to examine  $pH_i$  and its regulation in proximal tubules from this population. The results indicate that in proximal tubules from these nephrons 1) resting  $pH_i$  is lower than resting  $pH_i$  in proximal tubules from short-looped transitional nephrons and is about the same as resting  $pH_i$  in snake and rabbit proximal tubules and 2) basolateral mechanisms for acid-base fluxes involved in  $pH_i$  regulation are different from those in proximal tubules from short-looped transitional nephrons.

## METHODS

**Preparation of isolated renal tubules.** Male and female White Leghorn chickens, 1–3 mo old, were decapitated. Their kidneys were flushed in situ via the aorta with chilled (4°C) avian Ringer solution, quickly removed, and placed in the same solution on ice (see below for composition of solutions). Tubules were dissected from thin, vertical slices of kidney without the aid of enzymatic agents, as described previously (7). We normally used only one tubule segment from each bird (total of 51) in these experiments. The dissections were performed in chilled, oxygenated medium in a dissection dish maintained on ice. As noted above, avian nephrons vary from simple, superficial cortical nephrons without loops of Henle to deep medullary nephrons with long loops of Henle. Proximal segments ( $\sim 500$   $\mu m$  in length) were dissected from superficial loopless reptilian-type nephrons. In our previous work (7, 14), we used only proximal tubules from short-looped transitional nephrons because they were the only proximal seg-

ments that we were able to tease from avian tissue without the aid of enzymatic agents. However, as pointed out above, we have now learned to tease out proximal tubules from loopless reptilian-type nephrons to use in the nonperfused state for measurements of  $pH_i$ . The lumina of proximal tubules from these nephrons, like the lumina of proximal tubules from short-looped transitional nephrons (14), collapse rapidly so that no fluid is detectable in them. Dissections were performed in chilled medium (4°C), but all experiments were performed at 37°C.

**Ringer composition.** The components of the avian Ringer solutions used in these studies are shown in Table 1. All solutions were buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). *Solution 1* is the basic solution established previously for flushing the kidneys and for dissection of avian renal tubules (7). As noted in our previous work (7, 14), although the osmolality of this solution containing sucrose (Table 1) is substantially above the normal plasma osmolality (24), we found it to be the best solution for dissecting these tubules *in vitro*. There was no apparent change in cell volume of tubules maintained in this solution. *Solution 2*, which was identical to *solution 1* except for the removal of the sucrose (Table 1), was used for the incubation period with the pH-sensitive fluorescent dye (see below). The sucrose was removed to avoid possible interference with the dye uptake or calibration. *Solution 3* was the standard solution used for the control  $pH_i$  measurements and for studies with the addition of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and ethylisopropylamiloride (EIPA). This solution was identical to *solution 2* except that all extra organic substrates (except glucose) were replaced with NaCl to prevent any possible effect of these substrates on  $pH_i$ . *Solutions 4-8* involved modifications in *solution 3* designed to examine the effects of  $Na^+$ ,  $Cl^-$ ,  $K^+$ , and  $Ba^{2+}$  on  $pH_i$  (see RESULTS). The pH of each solution was adjusted to 7.4 at room temperature with 1 N NaOH, 1 N KOH, or tris(hydroxymethyl)aminomethane base, as appropriate. When 20 mM  $NH_4Cl$  was present in the medium, the concentration of NaCl was reduced by an equimolar amount to maintain the osmolality

and ionic strength approximately constant. The osmolality of all solutions except *solution 1* was ~290 mosmol/kgH<sub>2</sub>O (Table 1) and was checked regularly with a vapor pressure osmometer. The solutions were continuously bubbled with 100% O<sub>2</sub> and were assumed to be nominally HCO<sub>3</sub><sup>-</sup> free in the absence of tissue.

**Measurement of  $pH_i$  in single renal tubules.** We used the pH-sensitive fluorescent dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to measure  $pH_i$  in a manner similar to that described by others and used previously by us (14, 15, 17). For these measurements, we used a dual-wavelength spectrofluorimeter built around an Olympus IMT-2 inverted epifluorescence microscope. A 100-W mercury arc lamp was used as an excitation source, and specific excitation wavelengths were selected by a filter wheel mounted to the shaft of a high-speed motor. Wavelength-specific filters for measurement of pH using BCECF were centered at 445 (isobestic wavelength) and 495 nm. The selected excitation light was directed to the sample by a matched dichroic mirror. To prevent photo damage to the dye-loaded cells from the excitation light, two neutral density filters (ND 2, Oriel) were placed in front of the illumination site. The emitted fluorescent light passed through the dichroic mirror and a wavelength-specific emission filter (530 nm for BCECF). The fluorescent light emitted from a selected region of the sample was collected by a Hamamatsu HC120-03 photomultiplier tube operated in photon-counting mode. The dark current of the photomultiplier tube was zeroed out with a discriminator. Collection of fluorescent light was synchronized to the wheel rotation and excitation filter position. Synchronization, speed selection, and data collection were controlled by a microcomputer running custom software. The integrated average of 30 measurements/s was collected at 1-s intervals.

Individual tubules were held in an appropriate bathing chamber on the stage of the microscope and incubated in *solution 2* containing the acetoxymethyl ester (AM) form of BCECF (6 mM) (first dissolved in dimethyl sulfoxide) for ~45 min at 25°C. The AM form of BCECF readily enters the cells where the ester is cleaved by nonspecific esterases yielding

Table 1. Avian Ringer solutions

Compounds	Solutions							
	1	2	3	4	5	6	7	8
NaCl	100	100	120				50	120
NMDG-Cl				120				
Na-gluconate					120			
NMDG-gluconate						120		
HEPES		25	25	25	25	25	25	25
Sucrose	100							
K <sub>2</sub> HPO <sub>4</sub>	3	3	3	3	3	3	3	
KH <sub>2</sub> PO <sub>4</sub>					5	5		
KCl	5	5	5	5			75	5
MgSO <sub>4</sub>	1	1	1	1	1	1	1	
MgCl <sub>2</sub>								1
CaCl <sub>2</sub>	1.2	1.2	2.7	2.7			2.7	2.7
Ca(OH) <sub>2</sub>					2.7	2.7		
BaCl <sub>2</sub>								5
D-Glucose	8	8	8	8	8	8	8	8
L-Proline	5	5						
Hemicalcium								
Lactate	1.5	1.5						
Na-pyruvate	10	10						
Glutathione	3	3						
Adenosine	5	5						
Osmolality, mosmol/kgH <sub>2</sub> O	~380	~290	~290	~290	~290	~290	~290	~290

All values except osmolality are concentrations in mM. HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NMDG, *N*-methyl-D-glucamine.

the impermeant, fluorescent form of the dye. After the loading period, the bath was replaced with dye-free solution 3. The tubule and bathing chamber were rinsed several times with this dye-free solution to remove remaining extracellular dye before an experiment was begun. For collection of fluorescent light, the microscope was equipped with a Zeiss  $\times 63$  Neofluor oil-immersion objective (1.25 numerical aperture). Wavelength-specific fluorescence was collected as described above over a 22- to 32-mm-diameter area of nonperfused tubule. The ratio of fluorescence at 495/445 nm was then used as a measurement of  $\text{pH}_i$  to eliminate the influences of changes in dye content or in cell shape or volume. Calibration of the pH sensitivity of intracellular BCECF was performed for each tubule at the end of each experiment. This involved monitoring the 495/445 nm ratio at various values of  $\text{pH}_i$  by incubating the tubule in a solution with high  $\text{K}^+$  containing the ionophore nigericin (13 mM) (which exchanges  $\text{K}^+$  for  $\text{H}^+$  and sets  $\text{pH}_i$  to approximate extracellular pH) (25). The calibration curve was linear between pH 6.5 and 8.0. Autofluorescence was insignificant compared with the fluorescence from BCECF and was taken into account by the calibration procedure.

**Chemicals.** Nigericin and DIDS were purchased from Sigma. BCECF and EIPA were purchased from Molecular Probes. All other chemicals were purchased from standard sources and were of the highest purity available.

**Statistics.** Values are summarized as means  $\pm$  SE. Significant differences between values were determined with Student's *t*-test for paired or unpaired data, as appropriate. Linear regression analyses were performed for calibrations as required. In all analyses, differences were considered statistically significant when  $P < 0.05$ .

## RESULTS

**Control measurements of  $\text{pH}_i$ .** We measured the control resting  $\text{pH}_i$  in isolated proximal tubules from chicken loopless reptilian-type nephrons before studying changes in  $\text{pH}_i$  in response to various treatments. As summarized in Table 2, the resting  $\text{pH}_i$  was  $\sim 7.2$  or below.

**Response of  $\text{pH}_i$  to exposure to  $\text{NH}_4\text{Cl}$  pulse.** To alter  $\text{pH}_i$ , we exposed tubules for 30–60 s to 20 mM  $\text{NH}_4\text{Cl}$  in the bathing medium (14, 15, 22).  $\text{NH}_3$  diffuses across cell membranes much more readily than  $\text{NH}_4^+$  (10). Therefore,  $\text{NH}_3$  should rapidly enter the tubule cells and combine with free intracellular  $\text{H}^+$  to form  $\text{NH}_4^+$  and alkalinize the cell interior. In principle,  $\text{pH}_i$  should increase until the intracellular  $\text{NH}_3$  concentration

( $[\text{NH}_3]_i$ ) is equal to the extracellular  $\text{NH}_3$  concentration.  $\text{NH}_4^+$  should enter the cells more slowly than  $\text{NH}_3$ , leading to a gradual decrease in  $\text{pH}_i$  over the exposure period. When  $\text{NH}_4\text{Cl}$  is then removed from the bathing medium, free  $\text{NH}_3$  should diffuse rapidly from the cells, leaving behind free  $\text{H}^+$  and producing rapid acidification of the cell interior. The results of this process are summarized for all individual tubules studied in Table 2. The results are also shown for individual proximal tubules from chicken loopless reptilian-type nephrons in Figs. 1–4. The expected pattern was observed, with both the maximum  $\text{pH}_i$  in the presence of  $\text{NH}_4\text{Cl}$  ( $\sim 7.7$ ) and the minimum  $\text{pH}_i$  after removal of  $\text{NH}_4\text{Cl}$  ( $\sim 6.9$ ) being significantly different from the resting  $\text{pH}_i$  (Table 2).

**Rate of  $\text{pH}_i$  change, intrinsic buffering capacity,  $\text{NH}_3$  flux across the basolateral membrane, and permeability of basolateral membrane to  $\text{NH}_3$  during  $\text{NH}_4\text{Cl}$  pulse experiments.** To examine more quantitatively the factors involved in the changes in  $\text{pH}_i$  during the  $\text{NH}_4\text{Cl}$  pulse experiments, we measured the rate of change of  $\text{pH}_i$  ( $\text{dpH}_i/\text{dt}$ ) and calculated the intrinsic buffering capacity ( $\beta_i$ ),  $\text{NH}_3$  flux across the basolateral membrane ( $J_{\text{NH}_3}$ ), and the permeability of the basolateral membrane to  $\text{NH}_3$  ( $P_{\text{NH}_3}$ ) during the initial alkalinization (addition of  $\text{NH}_4\text{Cl}$  to the bath) and acidification (removal of  $\text{NH}_4\text{Cl}$  from the bath). These calculations were performed as in our previous studies (14, 15) and are described briefly below.

$\text{dpH}_i/\text{dt}$  was measured directly, and  $\beta_i$  (mM  $\text{H}^+/\text{pH}$  U) was calculated from the following equation

$$\beta_i = \Delta[\text{H}^+]_i/\Delta\text{pH}_i \quad (1)$$

In Eq. 1,  $\Delta[\text{H}^+]_i$  is the change in the amount of  $\text{H}^+$  in the cells by virtue of the  $\text{NH}_3$  loading or removal, and  $\Delta\text{pH}_i$  is the change in  $\text{pH}_i$ .

$J_{\text{NH}_3}$  ( $\text{nmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ ) was calculated from the following equation

$$J_{\text{NH}_3} = (\text{dpH}_i/\text{dt} \times \Delta[\text{NH}_3]_i)/(\Delta\text{pH}_i \times S/V) \quad (2)$$

In Eq. 2, the surface area (*S*) and tubular volume (*V*) are calculated from the measured tubule length and diameter and  $\text{dpH}_i/\text{dt}$  and  $\Delta\text{pH}_i$  are as defined above.  $\Delta[\text{NH}_3]_i$ , the change in  $[\text{NH}_3]_i$ , equals the total amount of  $\text{NH}_3$  that has moved across the basolateral membrane. Assuming, as noted above, that intracellular  $\text{NH}_4^+$  is formed from  $\text{NH}_3$  that has entered the cell and is reduced through the movement of  $\text{NH}_3$  from the cell, we can calculate  $\Delta[\text{NH}_3]_i$  from the change in the intracellular  $\text{NH}_3$  and  $\text{NH}_4^+$  concentrations at maximum  $\text{pH}_i$  after  $\text{NH}_4\text{Cl}$  addition and at resting  $\text{pH}_i$  before  $\text{NH}_4\text{Cl}$  removal. In this calculation, the relative intracellular concentrations of  $\text{NH}_3$  and  $\text{NH}_4^+$  are determined from the  $\text{pH}_i$  with the Henderson-Hasselbalch equation on the assumption that the  $\text{pK}'_a$  for the reaction  $\text{NH}_3 + \text{H}^+ \leftrightarrow \text{NH}_4^+$  equals 9.0 at  $37^\circ\text{C}$  (10). Finally,  $P_{\text{NH}_3}$  (cm/s) was calculated from the following relationship

$$P_{\text{NH}_3} = J_{\text{NH}_3}/\Delta[\text{NH}_3]_i \quad (3)$$

Table 2. Resting  $\text{pH}_i$  and response of  $\text{pH}_i$  to  $\text{NH}_4\text{Cl}$  pulse in proximal tubules from chicken loopless reptilian-type nephrons

Status	$\text{pH}_i$
Resting (control tubules)	$7.18 \pm 0.02$ (32)
Resting before $\text{NH}_4\text{Cl}$ addition	$7.20 \pm 0.03$ (32) <sup>a,b,c</sup>
Maximum after $\text{NH}_4\text{Cl}$ addition	$7.67 \pm 0.03$ (32) <sup>a</sup>
Resting before $\text{NH}_4\text{Cl}$ removal	$7.55 \pm 0.02$ (32) <sup>b,d</sup>
Minimum after $\text{NH}_4\text{Cl}$ removal	$6.95 \pm 0.03$ (32) <sup>c,d</sup>

Values are means  $\pm$  SE; nos. in parentheses indicate number of tubules. Differences between intracellular pH ( $\text{pH}_i$ ) values in response to  $\text{NH}_4\text{Cl}$  pulse were analyzed by paired analysis, with each tubule serving as its own control. Values with same letter superscript are significantly different ( $P < 0.05$ ) by paired analysis.

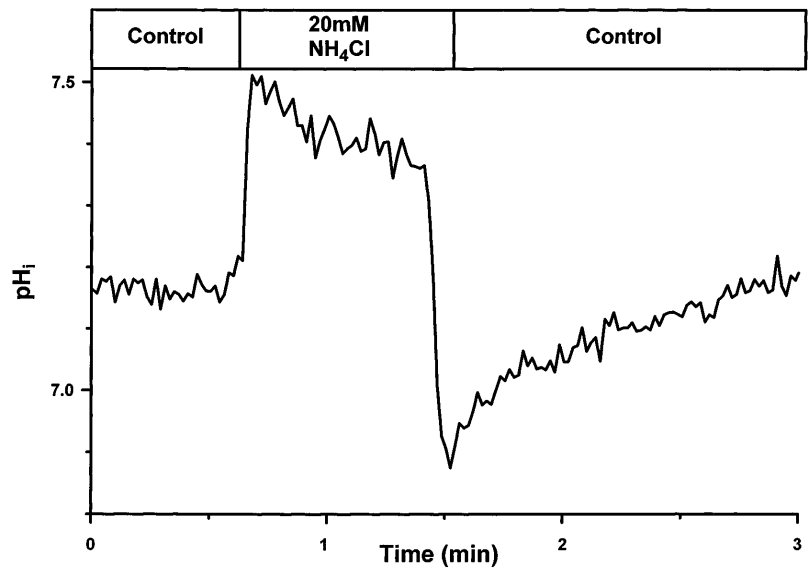


Fig. 1. Intracellular pH ( $pH_i$ ) in single proximal tubule from chicken superficial loopless reptilian-type nephron during various modifications in bathing medium. Boxes above tracing indicate when medium is control Ringer (Control) or Ringer with addition of  $NH_4Cl$ .

in which the terms have been defined above. The measurements of  $dpH_i/dt$  and the results of the above calculations of  $\beta_i$ ,  $J_{NH_3}$ , and  $P_{NH_3}$  are shown in Table 3. The values for the chicken loopless reptilian-type nephrons are very similar for both the alkalization and acidification phases. For comparison, the values obtained on proximal tubules from chicken short-looped transitional nephrons and snake and rabbit nephrons in our previous studies (14, 15) are also shown in Table 3 (see DISCUSSION).

*Effects of  $Na^+$ ,  $Cl^-$ , or  $Na^+$  and  $Cl^-$  removal on rate of recovery from acid  $pH_i$  to control resting  $pH_i$ .* We examined rate of recovery of  $pH_i$  ( $dpH_i/dt$ ) from the acid value after removal of  $NH_4Cl$  to the control resting value in these renal proximal tubules from chicken loopless reptilian-type nephrons. The average control value for this recovery rate is given in Table 4, in which it is compared with the values for proximal tubules from chicken short-looped transitional nephrons and snake and rabbit nephrons obtained in our previous studies (14, 15) (see DISCUSSION). The control recovery is also shown for individual tubules in Figs. 1–4.

In those tubules with completely collapsed lumina, recovery should take place through ion fluxes across the basolateral membrane. A number of basolateral  $Na^+$ -coupled acid or base transporters have been identified in various segments of amphibian or mammalian proximal renal tubules (11). These include 1)  $Na^+/H^+$  exchange that can be inhibited by amiloride or amiloride derivatives (4, 11), 2)  $Na^+$ -coupled  $Cl^-/HCO_3^-$  exchange moving  $HCO_3^-$  into the cells (3, 11, 12) that can be inhibited by DIDS and other disulfonic stilbene compounds (11), and 3) electrogenic  $Na^+-HCO_3^-CO_3^{2-}$  cotransport moving  $HCO_3^-$  out of the cells (1, 5, 11, 18, 28) that can also be inhibited by DIDS (5, 11). Basolateral  $Na^+$ -independent  $Cl^-/HCO_3^-$  exchange moving  $HCO_3^-$  out of the cells that can be inhibited by DIDS has also been described (2, 3, 23). The  $Na^+/H^+$  exchanger and probably one or more of these other basolateral mechanisms appear to function to regulate  $pH_i$  in proximal tubules from chicken short-looped transitional nephrons (14). However, because nothing was known about the regulation of  $pH_i$  in proximal tubules

Table 3.  $dpH_i/dt$ ,  $\beta_i$ ,  $J_{NH_3}$ , and  $P_{NH_3}$

Tubule	$dpH_i/dt$ , pH U/s $\times 10^{-2}$	$\beta_i$ , mM H <sup>+</sup> /pH U	$J_{NH_3}$ , nmol $\cdot$ cm <sup>-2</sup> $\cdot$ s <sup>-1</sup>	$P_{NH_3}$ , cm/s $\times 10^{-3}$
<i>During change of <math>pH_i</math> from control resting to maximum value after <math>NH_4Cl</math> addition</i>				
Chicken loopless proximal tubules	$13.6 \pm 1.19$ (32) <sup>a</sup>	$28.11 \pm 3.95$ (32)	$1.85 \pm 0.16$ (32) <sup>b,c,d</sup>	$3.77 \pm 0.33$ (32) <sup>e,f,g</sup>
Chicken short-looped proximal tubules	$14.6 \pm 0.73$ (59)	$20.36 \pm 1.61$ (48)	$2.43 \pm 0.20$ (59) <sup>b</sup>	$4.95 \pm 0.41$ (59) <sup>c</sup>
Snake distal-proximal tubules	$8.3 \pm 0.50$ (25) <sup>a</sup>	$22.03 \pm 4.83$ (27)	$1.27 \pm 0.16$ (25) <sup>c</sup>	$6.43 \pm 0.82$ (25) <sup>f</sup>
Rabbit proximal S2 tubules	$11.5 \pm 1.80$ (6)	$51.06 \pm 13.25$ (6)	$4.04 \pm 0.78$ (6) <sup>d</sup>	$8.83 \pm 1.70$ (6) <sup>g</sup>
<i>During change of <math>pH_i</math> from resting before <math>NH_4Cl</math> removal to minimum value after <math>NH_4Cl</math> removal</i>				
Chicken loopless proximal tubules	$13.1 \pm 0.90$ (32) <sup>h,i</sup>	$26.99 \pm 2.88$ (32) <sup>j,k</sup>	$1.88 \pm 0.15$ (32) <sup>l,m</sup>	$3.83 \pm 0.32$ (32) <sup>n</sup>
Chicken short-looped proximal tubules	$11.4 \pm 0.69$ (38)	$25.37 \pm 2.08$ (48)	$3.52 \pm 0.37$ (38) <sup>l</sup>	$7.19 \pm 0.76$ (38) <sup>n</sup>
Snake distal-proximal tubules	$5.5 \pm 0.40$ (25) <sup>h</sup>	$17.72 \pm 2.60$ (27) <sup>j</sup>	$0.80 \pm 0.10$ (25) <sup>m</sup>	$4.07 \pm 0.50$ (25)
Rabbit proximal S2 tubules	$6.8 \pm 1.30$ (6) <sup>i</sup>	$54.09 \pm 11.63$ (6) <sup>k</sup>	$2.77 \pm 0.86$ (6)	$6.05 \pm 1.87$ (6)

Values are means  $\pm$  SE; nos. in parentheses indicate number of tubules. Data on chicken short-looped nephrons are from Ref. 14, and data on snake and rabbit tubules are from Ref. 15.  $dpH_i/dt$ , rate of  $pH_i$  change;  $\beta_i$ , intrinsic buffering capacity;  $J_{NH_3}$ ,  $NH_3$  flux across basolateral membrane;  $P_{NH_3}$ , permeability of basolateral membrane to  $NH_3$ . Values for chicken short-looped, snake, or rabbit tubules that differ significantly ( $P < 0.05$ ) from corresponding values for chicken loopless tubules are shown by matching letter superscripts.

Table 4. Control values for  $dpH_i/dt$  from acid  $pH_i$  to control resting  $pH_i$

Tubule Segment	$dpH_i/dt$ , pH U/s $\times 10^{-3}$
Chicken loopless proximal tubule	4.98 $\pm$ 0.38 (32)
Chicken short-looped proximal tubule	5.67 $\pm$ 0.40 (48)
Snake distal-proximal tubule	2.52 $\pm$ 0.37 (21)
Rabbit proximal S2 tubule	8.77 $\pm$ 1.68 (3)

Values are means  $\pm$  SE; nos. in parentheses indicate number of tubules. Data on chicken short-looped tubules are from Ref. 14. Data on snake and rabbit tubules are from Ref. 15. Value for snake tubules is significantly different ( $P < 0.05$ ) from values for chicken and rabbit tubules.

from these chicken loopless reptilian-type nephrons, we decided to examine the effects of removing  $Na^+$ ,  $Cl^-$ , or both  $Na^+$  and  $Cl^-$  simultaneously from the bathing medium on the rate of recovery from acid  $pH_i$  to control resting  $pH_i$ . The results are summarized in Table 5 and shown for an individual tubule in Fig. 2.

When all the  $Na^+$  alone was removed from the bathing medium (solution 4, Table 1), the rate of recovery was significantly depressed by  $\sim 25\%$  (Table 5, Fig. 2). On the other hand, when all the  $Cl^-$  alone was removed from the bathing medium (solution 5, Table 1), the rate of recovery was not significantly different from the control value (Table 5, Fig. 2). When both  $Na^+$  and  $Cl^-$  were removed from the bathing medium (solution 6, Table 1), the rate of recovery was significantly depressed by almost exactly the same amount as with the removal of  $Na^+$  alone (Table 5, Fig. 2). It appears that the removal of  $Na^+$  only, not  $Cl^-$ , affects the rate of recovery.

**Effects of EIPA and DIDS on rate of recovery from acid  $pH_i$  to control resting  $pH_i$ .** As indicated above, both our previous data on chicken short-looped transitional nephrons (14) and data on renal tubules from other species (4, 11) indicate that  $Na^+/H^+$  exchange across the basolateral membrane could be important in regulating  $pH_i$ . Therefore, in view of the effect of  $Na^+$  removal on the rate of recovery of  $pH_i$ , we examined the

Table 5. Effect of treatments on  $dpH_i/dt$  from acid  $pH_i$  to control resting  $pH_i$  in proximal tubules from chicken loopless reptilian-type nephrons

Treatment	$dpH_i/dt$ , pH U/s $\times 10^{-3}$	
	Before treatment	During treatment
0 $Na^+$	5.26 $\pm$ 0.55 (10) <sup>a</sup>	3.92 $\pm$ 0.63 (10) <sup>a</sup>
0 $Cl^-$	5.26 $\pm$ 0.55 (10)	6.14 $\pm$ 1.00 (10)
0 $Na^+$ , 0 $Cl^-$	5.26 $\pm$ 0.55 (10) <sup>b</sup>	3.83 $\pm$ 0.50 (10) <sup>b</sup>
EIPA (1.0 mM)	4.04 $\pm$ 0.72 (13)	4.71 $\pm$ 0.87 (13)
DIDS (0.25 mM)	4.04 $\pm$ 0.72 (13) <sup>c</sup>	2.86 $\pm$ 0.46 (13) <sup>c</sup>
High $K^+$ (75 mM)	4.86 $\pm$ 0.50 (22) <sup>d</sup>	6.45 $\pm$ 0.79 (22) <sup>d</sup>
$Ba^{2+}$ (5 mM)	6.04 $\pm$ 0.43 (9)	6.02 $\pm$ 0.61 (9)

Values are means  $\pm$  SE; nos. in parentheses indicate number of tubules. Effect of each treatment on  $dpH_i/dt$  was evaluated statistically by paired analysis, with each tubule serving as its own control. EIPA, ethylisopropylamiloride. Values for treatments that differ significantly ( $P < 0.05$ ) by paired analysis from corresponding controls are shown by matching letter superscripts.

effect of 1 mM EIPA, an amiloride analog that is a particularly potent inhibitor of  $Na^+/H^+$  exchange, on the rate of recovery in the presence of  $Na^+$  (standard control solution 3, Table 1) in these tubules. As summarized in Table 5 and shown for an individual tubule in Fig. 3, 1 mM EIPA in the bathing medium had no effect on the rate of recovery.

In studies with rabbit tubules (11) and in our previous work with proximal tubules from chicken short-looped transitional nephrons (14), DIDS had an inhibitory effect on  $pH_i$  recovery even in the nominal absence of  $HCO_3^-$ . Therefore, we examined the effects of 0.25 mM DIDS on the rate of recovery of proximal tubules from these chicken loopless reptilian-type nephrons in the presence of  $Na^+$  and the nominal absence of  $HCO_3^-$  (standard HEPES-buffered control solution 3, Table 1). As summarized for all tubules in Table 5 and shown for an individual tubule in Fig. 3, 0.25 mM DIDS in the bathing medium significantly reduced the rate of recovery by  $\sim 30\%$ .

**Effects of high  $K^+$  concentration or presence of  $Ba^{2+}$  on the rate of recovery from acid  $pH_i$  to control resting  $pH_i$ .** As noted above, at least one basolateral mechanism that might be involved in regulation of  $pH_i$  is the electrogenic  $Na^+-HCO_3^-CO_3^{2-}$  cotransporter that moves  $HCO_3^-$  out of the cells. Its continued function would tend to slow the rate of  $pH_i$  recovery. Because it is electrogenic, its function and thus its ability to delay the rate of recovery would be reduced if the basolateral membrane potential were reduced. To examine this possibility, we undertook maneuvers to reduce the basolateral membrane potential. Although we did not measure membrane potential directly in these studies, we assumed that a high concentration of  $K^+$  in the bathing medium or the addition of  $Ba^{2+}$  to the bathing medium would reduce the membrane potential, as they do in renal tubules from other species (15). Therefore, we examined the effects of increasing the  $K^+$  concentration in the bathing medium to 75 mM (solution 7, Table 1) and of adding 5 mM  $Ba^{2+}$  (solution 8, Table 1) to the bathing medium on the rate of recovery of  $pH_i$ . The results are shown in Table 5 and Fig. 4. As would be expected if the mechanism suggested above were slowing  $pH_i$  recovery, increasing the  $K^+$  concentration to 75 mM produced a significant increase in the rate of recovery (Table 5, Fig. 4). However, in opposition to this expectation, the addition of 5 mM  $Ba^{2+}$  to the bathing medium had no effect on the rate of recovery (Table 5, Fig. 4). Because of the lack of effect of 5 mM  $Ba^{2+}$ , in a few experiments we also examined the effect of the addition of 10 mM  $Ba^{2+}$  to the bathing medium and the effect of the combination of 10 mM  $Ba^{2+}$  and 75 mM  $K^+$  in the bathing medium on the rate of recovery of  $pH_i$ . The addition of 10 mM  $Ba^{2+}$  had no effect, and the combination of 10 mM  $Ba^{2+}$  and 75 mM  $K^+$  produced the same increase in the rate of recovery as 75 mM  $K^+$  alone (data not shown).

**Effects of removal of  $Na^+$ ,  $Cl^-$ , or both  $Na^+$  and  $Cl^-$ , of high  $K^+$  concentration, and of the addition of EIPA, DIDS, and  $Ba^{2+}$  on resting  $pH_i$ .** In view of the effects of some of these treatments on the rate of recovery of  $pH_i$ ,

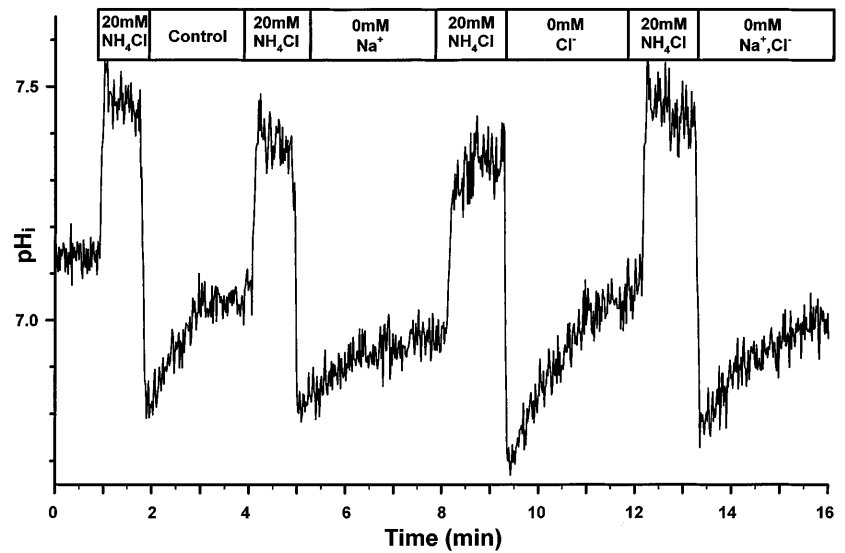


Fig. 2.  $pH_i$  in single proximal tubule from chicken superficial loopless reptilian-type nephron during various modifications in bathing medium. Designations in boxes above tracing indicate same changes as in Fig. 1 with the addition of  $Na^+$ -free Ringer (0 mM  $Na^+$ ),  $Cl^-$ -free Ringer (0 mM  $Cl^-$ ), and  $Na^+$ - and  $Cl^-$ -free Ringer (0 mM  $Na^+$ ,  $Cl^-$ ).

we also examined the effects of all of them on the resting  $pH_i$ . The results are summarized in Table 6. Removal of  $Na^+$  or of both  $Na^+$  and  $Cl^-$  produced an equal decrease in resting  $pH_i$ , whereas removal of  $Cl^-$  alone had no effect. Increasing the  $K^+$  concentration to 75 mM, which should have markedly reduced the basolateral membrane potential, produced a significant increase in resting  $pH_i$ . However, the addition of 5 mM  $Ba^{2+}$  to the bathing medium, which should also have reduced the basolateral membrane potential, had no effect on resting  $pH_i$ . DIDS had no effect on resting  $pH_i$ , but the addition of EIPA produced a significant increase in  $pH_i$ .

#### DISCUSSION

$pH_i$  and its regulation in proximal tubules from chicken superficial loopless reptilian-type nephrons differ distinctly from those in proximal tubules from deeper short-looped transitional nephrons. In the present study, the resting  $pH_i$  ( $\sim 7.20$  or less) in proximal tubules from loopless nephrons in HEPES-buffered

medium was significantly lower than the resting  $pH_i$  ( $\sim 7.30$ – $7.40$ ) in proximal tubules from short-looped transitional nephrons in HEPES-buffered medium measured in our previous study (14). However, the resting  $pH_i$  in proximal tubules from these loopless reptilian-type nephrons was essentially the same as the resting  $pH_i$  in snake and rabbit proximal tubules in HEPES-buffered media, measured by others and by us with the same pH-sensitive fluorescent dye (BCECF) technique (11, 15, 16).

The general pattern of the response of  $pH_i$  to an  $NH_4Cl$  pulse in proximal tubules from these chicken loopless reptilian-type nephrons was similar to the pattern in proximal tubules from chicken short-looped transitional nephrons (14), snake nephrons, and rabbit nephrons (15). However, there were some quantitative differences in the information derived from these responses between the tubules in the present study and the chicken, snake, and rabbit tubules in the other studies (Table 3). In the case of proximal tubules from the two types of chicken nephrons,  $J_{NH_3}$  and  $P_{NH_3}$  were

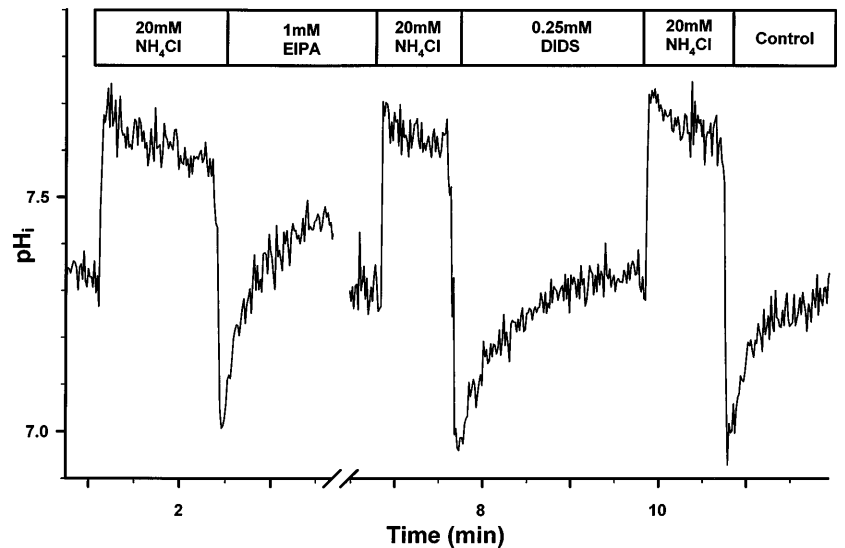


Fig. 3.  $pH_i$  in single proximal tubule from chicken superficial loopless reptilian-type nephron during various modifications in bathing medium. Designations in boxes above tracing indicate same changes as in Fig. 1 with the addition of 1 mM ethylisopropylamiloride (EIPA) and 0.25 mM DIDS.

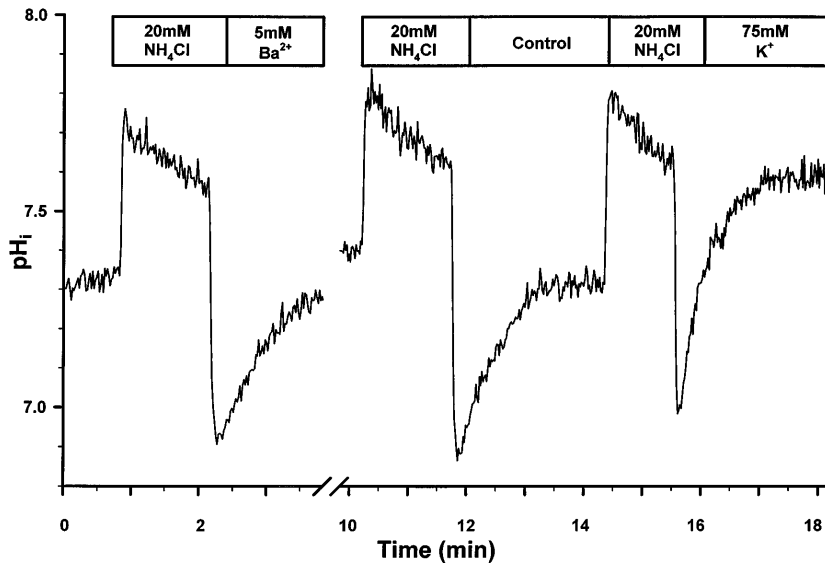


Fig. 4.  $pH_i$  in single proximal tubule from chicken superficial loopless reptilian-type nephron during various modifications in bathing medium. Designations in boxes above tracing indicate same changes as in Fig. 1 with the addition of 5 mM  $Ba^{2+}$  and high  $K^+$  (75 mM).

significantly lower in the loopless reptilian-type nephrons than in the short-looped transitional nephrons during both the initial increase in  $pH_i$  after the addition of  $NH_4Cl$  and during the decrease in  $pH_i$  after the removal of  $NH_4Cl$  (Table 3). Proximal tubules from chicken loopless reptilian-type nephrons, like proximal tubules from chicken short-looped transitional nephrons (14), tended to have a greater  $dpH_i/dt$  than snake proximal tubules and a lower  $\beta_i$  than rabbit proximal tubules (15) (Table 3).

Of particular significance with regard to regulation of  $pH_i$  are the observations on the factors influencing the rate of recovery of  $pH_i$  from an acid value to the control resting, slightly alkaline value. The control rate of recovery is about the same in proximal tubules from these loopless reptilian-type nephrons as in proximal tubules from short-looped transitional nephrons (Table 4) (14). However, there are marked differences between proximal tubules from these two avian nephron populations in terms of factors influencing this rate of recovery. These differences probably relate to differences in mechanisms for acid and/or base fluxes across the

basolateral membrane that may be involved in the recovery process (Fig. 5), although the exact mechanisms involved are not clear for either population of nephrons.

In proximal tubules from the loopless reptilian-type nephrons, the observations that removal of  $Na^+$  from the bath depressed the rate of recovery but that EIPA addition did not affect it indicate that recovery probably does not involve  $Na^+/H^+$  exchange at the basolateral membrane (Fig. 5; *transporter 1*). This is in striking contrast to proximal tubules from short-looped transitional nephrons in which both  $Na^+$  removal and amiloride addition depressed recovery to a similar extent, strongly suggesting that basolateral  $Na^+/H^+$  exchange was an important factor in recovery of  $pH_i$  (14). On the other hand, the pattern observed in these avian reptilian-type nephrons is identical to that observed in true reptilian nephrons from snake kidneys, although the absolute rate of recovery in snake tubules, under these same conditions, is substantially lower than that in avian or mammalian tubules (Table 4) (15). Therefore, an amiloride-inhibitable basolateral  $Na^+/H^+$  exchanger of the type demonstrated in proximal tubules from mammalian, amphibian, and avian short-looped transitional nephrons (4, 11, 14) does not appear to function in proximal tubules from reptilian or reptilian-type avian nephrons (15).

It is, of course, possible that a  $Na^+/H^+$  exchanger completely insensitive to amiloride, such as that described in hippocampal neurons (21), exists in the basolateral membrane of reptilian or reptilian-type avian nephrons. However, although variations in sensitivity of the renal isoforms of  $Na^+/H^+$  exchangers to amiloride and its analogs have been observed (8), no renal isoform completely insensitive to 1 mM EIPA has yet been reported. We have no explanation for the odd increase in resting  $pH_i$  observed with EIPA.

The absence of a basolateral  $Na^+/H^+$  exchanger extruding  $H^+$  from the cells in reptilian and loopless reptilian-type avian nephrons might explain the lower

Table 6. Effects of treatments on resting  $pH_i$  in proximal tubules from chicken loopless reptilian-type nephrons

Treatment	Resting $pH_i$	
	Control	After change
0 $Na^+$	7.14 ± 0.03 (10) <sup>a</sup>	6.97 ± 0.04 (10) <sup>a</sup>
0 $Cl^-$	7.14 ± 0.03 (10)	7.10 ± 0.04 (10)
0 $Na^+$ , 0 $Cl^-$	7.14 ± 0.03 (10) <sup>b</sup>	6.97 ± 0.05 (10) <sup>b</sup>
EIPA (1 mM)	7.19 ± 0.04 (13) <sup>c</sup>	7.32 ± 0.03 (13) <sup>c</sup>
DIDS (0.25 mM)	7.19 ± 0.04 (13)	7.22 ± 0.03 (13)
High $K^+$ (75 mM)	7.20 ± 0.03 (22) <sup>d</sup>	7.34 ± 0.03 (22) <sup>d</sup>
$Ba^{2+}$ (5 mM)	7.22 ± 0.04 (9)	7.24 ± 0.04 (9)

Values are means ± SE; nos. in parentheses indicate number of tubules. Effect of each treatment on resting  $pH_i$  was evaluated statistically by paired analysis, with each tubule serving as its own control. Values for changes that differ significantly ( $P < 0.05$ ) by paired analysis from corresponding controls are shown by matching letter superscripts.

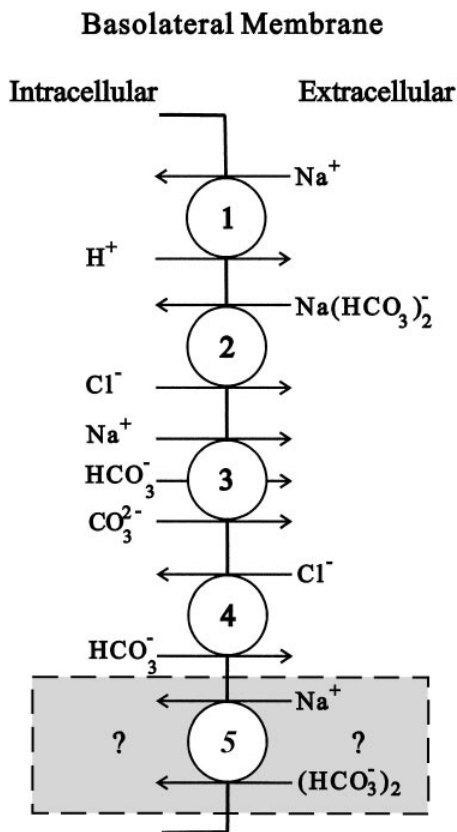


Fig. 5. Diagram of suggested basolateral acid or base transporters (1-5) for regulating  $\text{pH}_i$  in proximal tubules. Shaded transporter in gray indicates suggested new transporter.

resting  $\text{pH}_i$  in these two nephron types compared with that in short-looped transitional avian nephrons (14, 15). However, it does not explain why the resting  $\text{pH}_i$  in loopless reptilian-type avian nephrons and reptilian nephrons is the same as that in mammalian nephrons that do have a basolateral  $\text{Na}^+/\text{H}^+$  exchanger (11, 15).

Another basolateral mechanism for recovery of  $\text{pH}_i$  that might be suggested is a DIDS-inhibitable  $\text{Na}^+$ -coupled  $\text{Cl}^-/\text{HCO}_3^-$  exchanger moving  $\text{HCO}_3^-$  into the cells, as described for mammalian tubules (Fig. 5; transporter 2) (2, 3, 11, 12). Some  $\text{HCO}_3^-$  (largely produced by the tubule cells) must be present even in nominally  $\text{HCO}_3^-$ -free media, such as those used in the present experiments, and  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  transporters can apparently function under these circumstances (11, 16). However, although the inhibition of recovery by  $\text{Na}^+$  removal or DIDS addition supports a mechanism of this type, the lack of effect of  $\text{Cl}^-$  removal does not. Therefore, this does not appear to be a functioning mechanism in chicken loopless reptilian-type nephrons, although it does appear likely in chicken short-looped transitional nephrons (14).

A basolateral DIDS-sensitive, electrogenic  $\text{Na}^+/\text{HCO}_3^-/\text{CO}_3^{2-}$  cotransporter that moves  $\text{HCO}_3^-$  out of the tubule cells, similar to that reported for amphibians and mammals (1, 5, 11, 18, 28) (Fig. 5; transporter 3) could also influence the pattern of  $\text{pH}_i$  recovery. Such a transporter is important in transepithelial  $\text{HCO}_3^-$  reab-

sorption in these other species. However, it is not certain that this is the case in avian proximal tubules (7, 9). Moreover, the present study provides only conflicting evidence for such a transporter in these chicken loopless reptilian-type nephrons. Under control conditions in media nominally free of  $\text{HCO}_3^-$ ,  $\text{HCO}_3^-$  movement out of the cells across the basolateral membrane by this mechanism should be greater than in media containing  $\text{HCO}_3^-$ . Removing  $\text{Na}^+$  from the bathing medium should further enhance this efflux of  $\text{HCO}_3^-$ , thereby reducing resting  $\text{pH}_i$  and the rate of recovery of  $\text{pH}_i$ . That is exactly what happened. Moreover, because this is an electrogenic process, depolarizing the basolateral membrane should increase resting  $\text{pH}_i$  and the rate of recovery of  $\text{pH}_i$ . This also is exactly what happened when the  $\text{K}^+$  concentration in the bathing medium was increased. However, the addition of  $\text{Ba}^{2+}$ , which should have produced a similar effect on membrane potential, had no effect on resting  $\text{pH}_i$  or recovery of  $\text{pH}_i$ . This does not completely rule out such a transporter because there might be no  $\text{Ba}^{2+}$ -sensitive  $\text{K}^+$  channels in the basolateral membrane of these tubule segments and  $\text{Ba}^{2+}$  might have had other effects (see below). However, the effects of DIDS in the present study also do not provide evidence for this transporter. In other species, this transporter is inhibitable by DIDS (5, 11), and such inhibition, by reducing  $\text{HCO}_3^-$  efflux, should increase the rate of recovery of  $\text{pH}_i$  after acidification. Instead, in the current study, DIDS reduced the rate of recovery of  $\text{pH}_i$ . Thus there is no consistent evidence to support function of this transporter in proximal tubules from avian loopless reptilian-type nephrons.

There is no evidence for a basolateral DIDS-inhibitable,  $\text{Na}^+$ -independent, electroneutral  $\text{Cl}^-/\text{HCO}_3^-$  exchanger like that described in other species (Fig. 5; transporter 4) (2, 3, 22) in proximal tubules from chicken loopless reptilian-type nephrons. If this were functioning,  $\text{Cl}^-$  removal should have decreased basolateral  $\text{HCO}_3^-$  efflux from the cells (or actually produced basolateral  $\text{HCO}_3^-$  uptake by reversing the transporter), thereby leading to an increased resting  $\text{pH}_i$  and an increased rate of recovery of  $\text{pH}_i$ . This did not happen. Again, these data contrast with our previous data on proximal tubules from chicken short-looped transitional nephrons (14).

A possible basolateral transport mechanism compatible with the data on rate of recovery, although not one described in other species, would be an electrogenic, DIDS-inhibitable  $\text{Na}^+$ -coupled  $\text{HCO}_3^-$  cotransporter that moves two or more  $\text{HCO}_3^-$  ions into the cells (Fig. 5; transporter 5). The effects of  $\text{Na}^+$  removal, high  $\text{K}^+$  concentration, and DIDS addition on rate of recovery are all compatible with such a process. However, the lack of effect of DIDS on resting  $\text{pH}_i$  and the lack of effect of  $\text{Ba}^{2+}$  on either rate of recovery of  $\text{pH}_i$  or resting  $\text{pH}_i$  do not appear to support this proposed mechanism. Moreover, clear dependence of regulation of  $\text{pH}_i$  on the presence of  $\text{HCO}_3^-$  in the absence of other buffers has yet to be demonstrated.

The lack of effect of  $Ba^{2+}$ , however, as noted above, could simply indicate that there are no  $Ba^{2+}$ -sensitive  $K^+$  channels in this membrane and that  $Ba^{2+}$  had no effect on membrane potential. This appears most likely in view of the observation that a high  $K^+$  concentration enhanced recovery of  $pH_i$  to the same extent whether  $Ba^{2+}$  was present or not. Because of the very small size and especially the extreme fragility of avian nephrons, we have not attempted to measure directly the membrane potential with microelectrodes as we have in other species (15). It is also possible that  $Ba^{2+}$  had an effect on acid or base flux other than that produced by its effect on membrane potential. For example, during the recovery of  $pH_i$  from acidification, acid might leave the cells as  $NH_4^+$ , probably through  $K^+$  channels. If  $Ba^{2+}$  blocked  $K^+$  channels, it might not only reduce the membrane potential but also reduce or eliminate this  $NH_4^+$  flux and the two effects might cancel each other out. However, as noted above, the lack of effect of  $Ba^{2+}$  on the effect of high  $K^+$  suggests that  $K^+$  channels are not sensitive to  $Ba^{2+}$ , and this alternative explanation involving  $NH_4^+$  flux for some of our observations appears unlikely.

In summary, there appear to be distinct differences between proximal tubules from chicken loopless reptilian-type nephrons and proximal tubules from chicken short-looped transitional nephrons in terms of basolateral mechanisms for the regulation of  $pH_i$  during maintenance in HEPES-buffered media. In proximal tubules from chicken short-looped transitional nephrons, a basolateral  $Na^+/H^+$  exchanger clearly appears to be present and important (14). A  $Na^+$ -dependent  $Cl^-/HCO_3^-$  exchanger, a  $Na^+-HCO_3^-CO_3^{2-}$  cotransporter, and a  $Na^+$ -independent  $Cl^-/HCO_3^-$  exchanger (Fig. 5; *transporters 2-4*) also may play roles in the regulation of  $pH_i$  under these circumstances (14). Among these  $HCO_3^-$  transporters, the  $Na^+$ -dependent  $Cl^-/HCO_3^-$  exchanger appears most likely to be the major one (14). None of these mechanisms is clearly supported by the present data on proximal tubules from the chicken loopless reptilian-type nephrons, and an additional mechanism appears most likely. The reason for the apparent differences in basolateral regulation of  $pH_i$  between proximal tubules of these two nephron types is not apparent, although it may relate to differences in acid secretion and bicarbonate reabsorption as part of overall acid-base regulation. Although the information on proximal tubules from snake kidneys (15) is less complete than that on proximal tubules from chicken kidneys, the reptilian-type avian nephrons (with the exception of the effects of DIDS) appear to resemble true reptilian nephrons more than they do short-looped transitional avian nephrons.

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