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FACTORS AFFECTING PROXIMAL TUBULAR ACIDIFICATION OF NON-BICARBONATE BUFFER IN THE RAT

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SUMMARY

1. The effect of peritubular P_{CO_2} and pH changes within the physiological range on proximal tubular acidification of non-bicarbonate (phosphate) buffer was evaluated with and without carbonic anhydrase inhibition by stopped-flow microperfusion and Sb micro-electrode techniques.

2. Luminal steady-state pH was reduced from 6·69 to 6·37 and H ion fluxes (J_{H^+}) increased from 0·63 to 1·57 nmol cm^{-2} s^{-1} by increasing capillary CO_2 from 0 to 9·6 % at pH 7·2.

3. After acetazolamide a marked, although attenuated, effect of CO_2 on acidification was still observed; J_{H^+} increased from 0·088 nmol cm^{-2} s^{-1} at 0 % CO_2 to 0·78 at 9·6 % CO_2. Most of this effect can be explained by titration of luminal buffer by CO_2, uncatalysed CO_2 hydration and H_2CO_3 recirculation.

4. An increase in capillary CO_2 reduced acidification half-times ($t/2$), which, according to an analogue circuit model, may be due to increased H ion access to the pump.

5. Peritubular pH changes at 0 % CO_2 also modified tubular acidification, increasing J_{H^+} from 0·73 nmol cm^{-2} s^{-1} at pH 7·6 to 0·99 at pH 7·0. After acetazolamide, J_{H^+} still increased from 0·11 nmol cm^{-2} s^{-1} at pH 7·6 to 0·57 at pH 7·0.

6. In conclusion, both peritubular CO_2 changes at constant pH and pH changes at 0 % CO_2 were effective to modify J_{H^+}, in the presence and absence of carbonic anhydrase activity. In the studied range, capillary CO_2 induced larger changes in J_{H^+} than pH. The data show substrate (H ion) is a limiting factor for tubular H ion secretion.

INTRODUCTION

It is recognized that capillary pH and P_{CO_2} are important factors for the modulation of renal tubular acidification. However, the individual role of these factors has not been clearly identified. Almost three decades ago, Pitts (1952/53), presented evidence indicating that changing P_{CO_2} at constant blood pH caused larger modifications of bicarbonate reabsorption than changing pH at constant P_{CO_2}. However, in his experiments plasma bicarbonate levels varied widely and this alone could affect bicarbonate reabsorption, since bicarbonate reabsorption is markedly dependent on its filtered load (Malnic & Mello-Aires, 1971; Alpern, Cogan & Rector, 1982).

More recently, using microperfusion and micro-electrode methods, a significant relationship has been demonstrated between capillary P_{CO_2} at constant pH and tubular acidification; a more marked effect was observed when pH was varied at constant P_{CO_2}, albeit over a very wide range of capillary pH (5·6–8·4) (Mello Aires & Malnic, 1975). An important role of bath P_{CO_2} in bicarbonate reabsorption was demonstrated by Jacobson (1981*b*) in isolated perfused rabbit proximal tubules, at constant bath bicarbonate levels, a condition where pH modification acted in the same direction as P_{CO_2}. On the other hand, in turtle bladder studied '*in vitro*', serosal P_{CO_2} has been shown to have a more important effect than pH on mucosal acidification (Schwartz & Steinmetz, 1971).

The present work studies the influences of variations of capillary P_{CO_2}, and the effect of pH in a narrower range than used before (Mello Aires & Malnic, 1975) on proximal tubular acidification of non-bicarbonate (phosphate) buffer. It also analyses the effect of these factors after carbonic anhydrase inhibition, when the role of CO_2 in tubular acidification probably depends largely on uncatalysed H ion generation, which is a function of cellular CO_2 hydration.

Our data confirm that both changes in capillary P_{CO_2} at constant pH and changes in pH at constant P_{CO_2} exert an important effect in tubular acidification. After carbonic anhydrase inhibition H ion secretion is still stimulated by increased capillary CO_2 and also by an increase in H ion concentration.

An analogue model is presented for the interpretation of the process of tubular H ion secretion, which allows for an understanding of the origin of changes in acidification half-time as well as of the nature of modification in acidification rates under the described experimental conditions.

METHODS

Male Wistar rats weighing 250–300 g were anaesthetized with pentobarbitone (40 mg kg^{-1}) intraperitoneally. They had free access to water and food up to the time of the experiment. The jugular vein was cannulated with a PE 90 polyethylene tubing, and tracheostomy was performed. The animals received an infusion of 0·154 M-NaCl or, when acetazolamide was given, 0·154 M-$NaHCO_3$ (to both of which 3% mannitol was added), at a rate of 0·08 ml min^{-1}. The left kidney was exposed through a flank incision. Micropuncture techniques followed standard methods (Mello Aires & Malnic, 1975). Briefly, peritubular capillaries were perfused with pipettes having tip diameters of 10 μm made of thin walled, 1·5–2 mm outer diameter Kimax glass tubing. Proximal convoluted tubules within the perfused area were impaled by a double-barrelled micropipette, one barrel containing Sudan Black coloured castor oil, and the other the perfusion solution. An oil column was split by a fluid column extending over several loops in the proximal lumen, and impaled by an Sb micro-electrode several loops down-stream. The voltage between the Sb micro-electrode and a reference $Cu/CuSO_4$ electrode placed in contact with the kidney surface through an isotonic NaCl/agar bridge was shown to be proportional to the pH of the luminal fluid (Giebisch, Malnic, de Mello & Mello Aires, 1977). The preparation and calibration of Sb micro-electrodes have been described in detail elsewhere (Vieira & Malnic, 1968; Malnic & Vieira, 1972). The voltage difference between Sb and reference electrode was measured by a Keithley model 615 electrometer and recorded on a Beckman RP dynograph. Simultaneously, voltages were stored in a Hewlett–Packard computer by using an appropriate data acquisition system (mod. 21MX), and processed subsequently. Table 1 shows the composition of the solutions used in this work.

Since alkaline phosphate buffer was injected into the tubular lumen, the observed pH changes represent the time course of alterations of the Na_2HPO_4/NaH_2PO_4 ratio, that is, alkaline phosphate titration by proton secretion. The rate constant (k) of the approach of NaH_2PO_4 to its steady-state

value was calculated by using a computer program which fits the log of $NaH_2PO_{4_\infty} - NaH_2PO_{4_t}$ against time. Acidification half-times are given by $t/2 = \ln 2/k$. Rates of H ion transport were calculated assuming constant luminal phosphate content, since it has been shown that loss of both acid and alkaline phosphate from the tubular lumen is about five to eight times slower than the rate of pH changes (Cassola & Malnic, 1977). Proton net fluxes were calculated as $J_{H^+} = (NaH_2PO_{4_{t=\infty}} - NaH_2PO_{4_{t=0}}) . k . r/2$, where r is the tubular radius in centimetres. $NaH_2PO_{4_{t=\infty}}$ is the stationary acid phosphate concentration, and $NaH_2PO_{4_{t=0}}$ that at time zero, corresponding to capillary pH in CO_2 experiments, and to 4 mM in the variable pH groups. A mean tubular radius of 15 μm was used (Mello Aires & Malnic, 1975).

TABLE 1. Composition of luminal and peritubular (capillary) perfusion solutions

		Peritubular			
Group	Luminal	0%	2%	5%	10%
NaCl (mM)	75	105	100	95	80
$NaHCO_3$ (mM)	—	—	5	13	26
KCl (mM)	5	5	5	5	5
$CaCl_2$ (mM)	1	1	1	1	1
$MgSO_4$ (mM)	1·2	1·2	1·2	1·2	1·2
Na acetate (mM)	5	5	5	5	5
$NaHPO_4^-$ (mM)	20	20	20	20	20
Raffinose (mM)	90	—	—	—	—
CO_2 (%)	—	—	1·95	4·9	9·6
pH	7·8	7·0–7·6	7·2	7·2	7·2

CO_2 concentrations are actually measured values (Haldane apparatus). In the text and tables, 2, 5 and 10% are used for simplicity.

Experimental groups. In the first group of rats peritubular pH was maintained constant while CO_2 was varied (0, 2, 5 and 10% in air). In order to maintain pH constant, bicarbonate concentrations were progressively increased. The pH was kept at 7·2 in order to be able to work at relatively low bicarbonate concentration (see Table 1). On the other hand, the luminal buffer load was kept constant at 20 mM-phosphate, pH 7·8, in all experimental conditions.

In a second group acetazolamide (20 mg kg^{-1}) was given as a priming dose, and 20 mg kg^{-1} h^{-1} were added to the infusion. 10^{-4} M-acetazolamide was added to capillary perfusion fluid; otherwise, the procedures of the first group were applied.

In a third group of rats transepithelial H ion gradients were directly determined by measuring luminal steady-state pH and the pH in the adjacent perfused capillary. These experiments were done at the same capillary pH of the previous group (7·2) at 0 and 10% of CO_2. Acetazolamide (10^{-4} M) was added to both peritubular and luminal perfusion fluid.

In a fourth group, peritubular perfusion solutions were equilibrated with air (0% CO_2), and pH was adjusted to 7·0, 7·2, 7·4 and 7·6 with 0·1 M-HCl or NaOH. These experiments were performed with and without acetazolamide, giving 20 mg kg^{-1} body weight as priming dose and adding 20 mg kg^{-1} h^{-1} to the infusion. 10^{-4} M-acetazolamide was added to the peritubular perfusion solution.

The stability of the P_{CO_2} of the solutions leaving the capillary pipette was checked in the following way. Small fluid droplets were formed under oil at the tip of a perfusion pipette filled with a test solution which had been equilibrated within a syringe either at 2% or at 10% CO_2 having a final pH of 7·2. Sb and 3 M-KCl electrodes were placed near the oil/fluid interface of the droplet while pH was being recorded. Since HCO_3^- is known and constant, the pH measured in the droplet reflects its P_{CO_2} values. During fluid injection the pH of the droplet did not change. The same constancy is also found when pH is measured in a shallower stream of fluid flowing up the pipette tip into a larger drop of fluid kept at the oil/air interface. Such an experiment is shown in Fig. 1. The pH of the fluid emerging from the pipette was $7·18 \pm 0·01$ ($n = 13$) when the solution (see Table 1, peritubular solution) was equilibrated at 2% CO_2 and $7·22 \pm 0·02$ ($n = 11$) when 10% CO_2 was used;

both values were not significantly different from the pH of the fluid equilibrated within the syringe (7·2). It is concluded therefore that the fluid injected into the capillaries from the micropipette had the expected pH and P_{CO_2}.

On the other hand, it is possible that CO_2 might be modified when perfusing capillaries at P_{CO_2} remote from normal cortical P_{CO_2}, which is of the order of 50–60 mmHg (DuBose, Pucacco, Lucci & Carter, 1978; Gennari, Caflish, Johns, Maddox & Cohen, 1982).

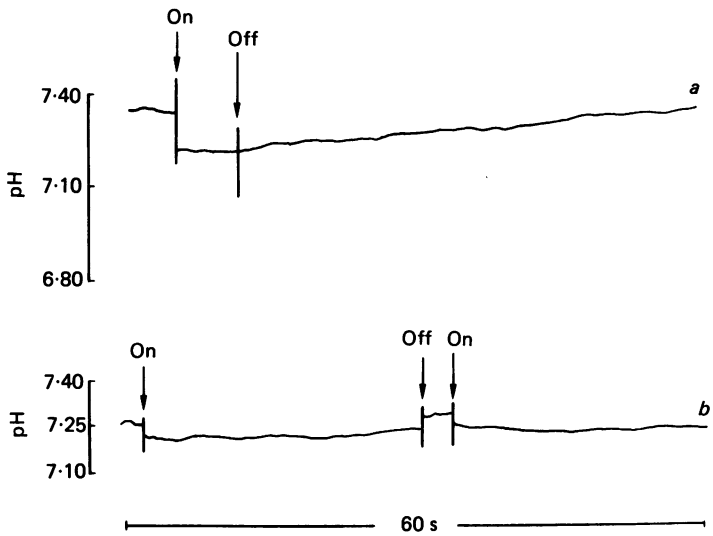

Fig. 1. *In vitro* measurements of pH in a droplet (*a*) or in a stream of fluid (*b*), emerging from a pipette filled with peritubular perfusion solution pre-equilibrated with CO_2. On: perfusion started; off: perfusion stopped. The nominal pH value of solutions was 7·2.

In order to verify CO_2 loss or gain along the perfused capillaries, we performed a series of experiments in which capillary pH was measured at variable distances from the tip of the perfusing pipette. Two different solutions of low buffer capacity (3 mM-phosphate) were used, one equilibrated with air (0 % CO_2) and the other with 15 % CO_2, both containing 10^{-4} M-acetazolamide. If some CO_2 diffusion occurred along the perfused capillaries, changes in the pH of capillary perfusate between pipette tip and periphery of the perfused area would be expected. In these experiments the slope of pH changes *vs.* distance was not significantly different from zero; 15 % CO_2: $r = 0{\cdot}18$, $n = 8$ and 0 % CO_2: $r = 0{\cdot}18$, $n = 13$. This indicates that perfusion velocity is high enough to preclude significant P_{CO_2} changes along the perfused capillaries.

At the end of the experiments samples of blood obtained by puncture of the carotid artery and of urine from the urinary bladder were withdrawn. Blood and urine pH were measured in a BMS 3/PHM72-Mk2 blood pH meter System, Radiometer, Copenhagen.

Results are presented as mean values ±S.E. of mean. Significance between differences of two groups were measured by Student's *t* test. When more than two groups were compared, analysis of variance and the Scheffé test were applied (Snedecor & Cochran, 1971).

RESULTS

Blood pH was $7{\cdot}41 \pm 0{\cdot}02$ ($n = 24$) in control and $7{\cdot}38 \pm 0{\cdot}04$ ($n = 14$) in acetazolamide-treated rats. Urine pH was $6{\cdot}29 \pm 0{\cdot}20$ ($n = 7$) in control and $7{\cdot}97 \pm 0{\cdot}04$ ($n = 18$) in acetazolamide-treated rats.

Titration of luminal buffer by CO_2

In order to evaluate the contribution of CO_2 diffusion into the luminal perfusate to titration of luminal buffer via luminal carbonic acid formation, we performed '*in vitro*' experiments in which air-equilibrated alkaline phosphate buffer with similar pH to the luminal perfusate was equilibrated with an excess of 2, 5 and 10% CO_2 in a chamber through which the gas was bubbled vigorously through a fritted glass disk, which constituted the bottom of the chamber. pH changes were continuously

TABLE 2. '*In vitro*' measurements of acidification half-times and steady-state pH of a solution containing 20 mM-phosphate bubbled with different CO_2 mixtures

	Observed $t/2$ (s)		Observed pH		
CO_2 (%)	Control	Carbonic anhydrase	Control	Carbonic anhydrase	Theoretical pH
1·95	106	42	7·25	7·20	7·23
4·90	53	26	6·97	6·92	6·99
9·60	45	16	6·73	6·84	6·80

Control: solution without carbonic anhydrase. Observed: experimental values. Theoretical: calculated values.

recorded until steady state was reached. The initial and final pH of a solution containing alkaline 20 mM-phosphate and 130 mM-NaCl was measured using a standard glass pH electrode in the presence and absence of carbonic anhydrase (0·1 mg ml^{-1}) (Sigma). This solution was then placed in the equilibration chamber and pH was recorded continuously using a Sb electrode with a 3 M-KCl reference electrode.

Table 2 shows acidification half-times and experimental and theoretical values of steady-state pH obtained in these '*in vitro*' experiments.

A sample calculation of the expected equilibrium is based on the isohydric principle, equating Henderson–Hasselbalch relations for phosphate and bicarbonate buffer:

$$6{\cdot}8+\log\frac{HPO_4^{2-}}{H_2PO_4^-}=6{\cdot}1+\log\frac{HCO_3^-}{2}.$$

The solution is equilibrated with 9·6% CO_2 (2 mM-dissolved CO_2) and total phosphate is 20 mM. Since all $H_2PO_4^-$ is formed by reaction of HPO_4^{2-} with H_2CO_3, we obtain:

$$H_2PO_4^- = HCO_3^- = 10 \text{ mM}$$

and

$$pH = 6{\cdot}1+\log\tfrac{10}{2} = 6{\cdot}8.$$

Both in the presence and absence of carbonic anhydrase, acidification half-times are considerably larger than those found in rat proximal convoluted tubules during capillary perfusion at variable P_{CO_2}. On the other hand, steady-state pH resembles closely the theoretically calculated value, and was different from the steady-state pH measured in rat tubules for each value of peritubular CO_2 (see Table 3).

The applicability of these data to the '*in vivo*' experimental situation depends on complete and rapid equilibration of CO_2 with the titrated fluid in both conditions. The experiment shown in Fig. 2 was performed to evaluate this question. Acidification

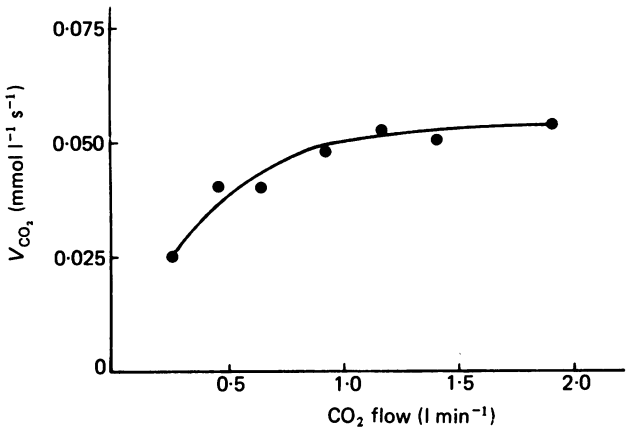

Fig. 2. Changes of $V_{CO_2}\left(NaH_2PO_{4_{t-0}} \cdot \frac{\ln 2}{t/2}\right)$ with increase in CO_2 flux (ml min^{-1}) in an '*in vitro*' system (25 °C, 1 atm). The solution used was 20 mM-phosphate Ringer solution, with an initial pH of 7·9.

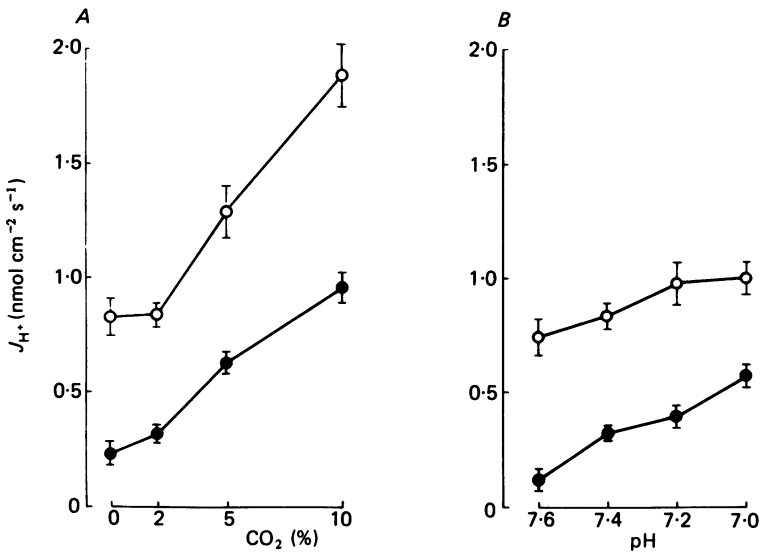

Fig. 3. J_{H^+} plotted against percentage of capillary CO_2 (*A*) or peritubular pH (*B*), in control (○) and in animals receiving acetazolamide (●). Experiments at constant load of luminal buffer.

half-times were measured in a closed chamber containing 20 mM-HPO_4^{2-} Ringer solution bubbled with 5 % CO_2 in air, at variable flow. The experimental approach was similar to that described above, except that CO_2 flux was varied from 260 to 1900 ml min^{-1}.

At low flow rates of CO_2, diffusion appears to be the limiting step since a clear dependence was found between flow and acidification half-times. However, as CO_2 flow was increased, $t/2$ reached a minimum value and no further decrease was found

TABLE 3. Tubular fluid acidification in proximal convoluted tubules during peritubular perfusion with fluids containing variable CO_2 at pH 7·2 and 20 mM-$NaHPO_4^-$. The composition of luminal fluid at $t = 0$ is given in Table 1 (NaH_2PO_4 at $t = 0$ is 5·7 mM)

Capillary CO_2 (%)	0	2	5	10
Control				
pH_∞	6·69 ± 0·029 (69)	6·62 ± 0·028 (69)	6·49*† ± 0·024 (103)	6·37*†‡ ± 0·017 (130)
$NaH_2PO_{4_\infty}$ (mM)	11·14 ± 0·29 (69)	11·89 ± 0·30 (69)	13·24*† ± 0·24 (103)	14·37*†‡ ± 0·16 (130)
$t/2$ (s)	5·37 ± 0·28 (69)	5·58 ± 0·23 (69)	4·66† ± 0·24 (103)	3·71*†‡ ± 0·17 (130)
10^{-4} M-acetazolamide				
pH_∞	7·10§ ± 0·022 (37)	7·02§ ± 0·020 (47)	6·61*†§ ± 0·026 (88)	6·44*†‡§ ± 0·022 (50)
$NaH_2PO_{4_\infty}$ (mM)	6·76§ ± 0·22 (37)	7·61§ ± 0·21 (47)	11·41*†§ ± 0·30 (88)	13·75*†‡§ ± 0·22 (50)
$t/2$ (s)	7·32§ ± 0·59 (37)	7·16§ ± 0·47 (47)	7·03§ ± 0·28 (88)	6·09§ ± 0·30 (50)

pH_∞: luminal steady-state pH. $NaH_2PO_{4_\infty}$: luminal steady-state acid phosphate. $t/2$: acidification half-times.
* ($P < 0·05$) against 0% CO_2.
† Against 2% CO_2.
‡ Against 5% CO_2 and § against control. 0% CO_2 corresponds to use of air equilibrated solution.
Means ± S.E. of mean (n).

TABLE 4. Tubular fluid acidification at variable peritubular pH, using air equilibrated solutions

Capillary $H_2PO_4^-$ (mM)	7·7	5·7	4·0	2·7
Capillary pH	7·0	7·2	7·4	7·6
Control				
pH	6·33 ± 0·032	6·41 ± 0·041	6·64*† ± 0·023	6·66*† ± 0·024
NaH_2PO_4 (mM)	14·74 ± 0·30	13·83 ± 0·39	11·72*† ± 0·24	11·57*† ± 0·27
$t/2$ (s)	6·30 + 0·29	6·19 ± 0·35	5·41 ± 0·19	6·02 ± 0·41
10^{-4} M-acetazolamide				
pH	6·70§ ± 0·022	6·92*§ ± 0·020	6·90*§ ± 0·014	7·24*‡§ ± 0·040
NaH_2PO_4 (mM)	11·14§ ± 0·24	8·60*§ ± 0·17	8·33*§ ± 0·16	5·42*‡§ ± 0·41
$t/2$ (s)	7·15 ± 0·40	7·47 ± 0·49	7·87§ ± 0·34	7·84 ± 0·89

For explanation of symbols see Table 3. (§) $P < 0·05$ against controls.

in this series of experiments, suggesting that at these flows, CO_2 hydration became the limiting step.

In the '*in vivo*' experiments, CO_2 hydration should also be the limiting factor, since geometry (the interface area between capillary perfusate and tubular fluid) is even more favourable for diffusion equilibrium, and since it is known that CO_2 diffusion across tubular epithelium is very fast (Malnic, 1980).

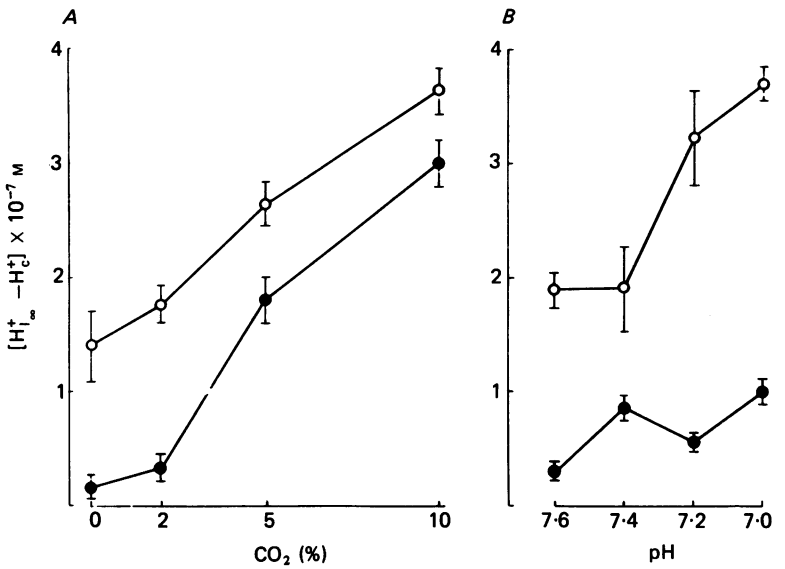

Fig. 4. Changes in transepithelial H ion gradients ($H^+_{l\infty} - H^+_c$) where $H^+_{l\infty}$ is luminal steady-state H ion activity and H^+_c is peritubular H ion activity, during variations of peritubular CO_2 (*A*) or pH (*B*), in control rats (○) and in animals undergoing carbonic anhydrase inhibition (●). For details see text.

Acidification at variable CO_2

Table 3 shows that most of the measured variables were not significantly different when peritubular perfusion solution was equilibrated with either air or 2% CO_2 at constant tubular buffer load. On increasing peritubular CO_2 to 2%, tubular steady-state pH decreased by only 0·07 pH units, while the acidification rate coefficient and net H ion fluxes were not significantly altered (Fig. 3*A*). At higher values of peritubular CO_2 a clear stimulatory effect on acidification was observed. When 5% CO_2 was used in the capillary perfusate, luminal steady-state pH and acidification half-times fell and steady-state NaH_2PO_4 concentration increased in comparison with the 2% group. In consequence, an increase in net H ion flux was evident. These effects were further enhanced at 10% peritubular CO_2 (see Fig. 3*A*).

The effect of an increase in peritubular CO_2 on transepithelial H ion gradient, obtained from these experiments as the difference between luminal and peritubular steady-state H ion activity, is shown in Fig. 4*A*. An almost linear relationship between peritubular CO_2 and the transepithelial H ion gradient was found.

As in control rats, 5% CO_2 at the basolateral side had a strong stimulatory effect

on H ion flux after acetazolamide administration. A marked fall in steady-state pH was observed. By increasing peritubular CO_2 to 10 % a further decrease in steady-state pH was found. In the experiments conducted during acetazolamide administration acidification half-times were not different.

When a solution containing bicarbonate is injected into the lumen of an acetazolamide-treated animal a luminal acid disequilibrium pH can be formed due to carbonic acid accumulation. Due to the way in which net H ion secretion is calculated (see Methods), this could lead to artifactually increased rates of bicarbonate reabsorption. In the present case, however, where an excess of non-bicarbonate buffer is present in the lumen (20 mM-phosphate), this buffer will be titrated by the H_2CO_3 generated by capillary bicarbonate diffusing into the lumen, and the calculated rate of acidification will correspond to the actual rate of titration of phosphate buffer.

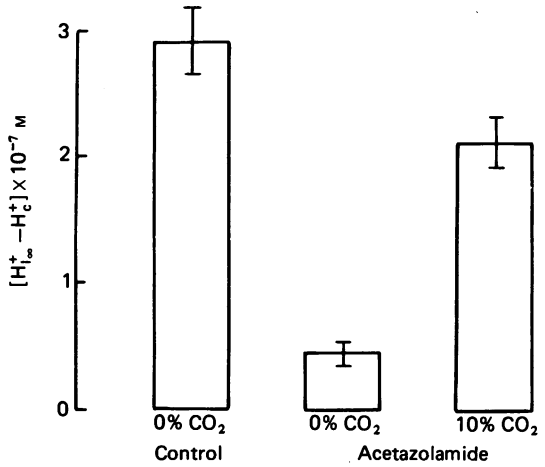

Fig. 5. Transepithelial $[H^+]$ gradients measured between tubular lumen and immediately adjacent capillary vessels, where H^+_c is capillary $[H^+]$ and $H^+_{l\infty}$ is luminal steady-state $[H^+]$, measured at 0 % capillary CO_2 with and without carbonic anhydrase inhibition; and at 10 % capillary CO_2 with carbonic anhydrase inhibition.

The fraction of net H ion fluxes inhibited by acetazolamide decreases as capillary CO_2 is increased (Fig. 3*A*). Proton net fluxes were inhibited by 49 % at 10 % of peritubular CO_2 and by 73 % when solutions were pre-equilibrated with air.

As shown in Fig. 4*A*, after carbonic anhydrase inhibition, the transepithelial proton gradient followed a similar pattern with respect to capillary CO_2 as the untreated group. However, at all CO_2 levels these gradients were lower when compared to controls.

In the data given above, steady-state luminal pH is compared to the pH of the capillary perfusion fluid measured '*in vitro*'. Since these values might be different from those actually present in peritubular capillaries, in a group of perfusions, transepithelial H ion gradients were measured directly by puncturing the lumen and the adjacent capillary in sequence with the same electrode. Results are shown in Fig. 5. At 0 % CO_2, in the presence of carbonic anhydrase activity, a transepithelial proton gradient of 0·73 pH units was found. After acetazolamide administration the gradient was reduced to 0·23. However, when peritubular perfusate was equilibrated with 10 % CO_2, after carbonic anhydrase inhibition, the transepithelial proton gradient

increased to 0·63. In these experiments acetazolamide was added both to the lumen and capillary fluid in a concentration of 10^{-4} M, in order to assure complete inhibition also of brush-border carbonic anhydrase. The ensuing inhibition was of the same order as that found in our other experiments, in which the drug was added only to capillary perfusate; under these conditions the drug has been shown to reach the luminal enzyme by tubular secretion (Cogan, Maddox, Warnock, Lin & Rector, 1979).

Acidification at variable capillary pH

In studies where capillary pH was changed from 7·0 to 7·6 at 0 % CO_2, a progressive increase in luminal steady-state pH was found (Table 4). Stationary pH as well as acid phosphate were significantly increased at capillary pH of 7·4 and 7·6 as compared to 7·2. Acidification half-times did not show a definite trend during these modifications in capillary pH. Net H ion fluxes calculated for constant tubular buffer load increased by 35·6 % when pH was decreased from 7·6 to 7·0 (Fig. 3*B*).

Fig. 4*B* shows the relation between the transepithelial proton gradient and capillary pH; a marked increase of the gradient with the decrease in capillary pH was found.

Acetazolamide administration led to a clear reduction in luminal steady-state NaH_2PO_4 with respect to control rats, at all peritubular pH values. However, in all cases the tubules still retained the capacity to maintain H ion gradients although these gradients were always lower than in controls. Also here, luminal pH increased significantly when capillary pH was elevated between 7·2 and 7·6. Acidification half-times tended to be higher than in controls, a difference which reached significance only at pH 7·4. Proton fluxes, when compared with those observed in untreated animals, were found to be inhibited by between 37 and 81 % at peritubular pH of 7·0–7·6 respectively (Fig. 3*B*).

In contrast to the results obtained with variable peritubular CO_2 plus acetazolamide, after carbonic anhydrase inhibition transepithelial H ion gradients varied only moderately with capillary pH (Fig. 4*B*).

DISCUSSION

Stimulation of acidification by CO_2

The effect of blood P_{CO_2} on tubular H ion secretion has been described by several investigators. Pitts (1952/1953) suggested a direct effect of CO_2 on bicarbonate reabsorption. Rector, Seldin, Roberts & Smith (1960), were able to show an increase in apparent tubular maximum reabsorption capacity of bicarbonate by enhancing plasma CO_2. Furthermore, results from this and other laboratories (Mello Aires & Malnic, 1975; Jacobson, 1981*b*; Chan, Biagi & Giebisch, 1982; Sasaki, Berry & Rector, 1982) have shown that CO_2 at the basolateral cell side has the ability to stimulate net H ion fluxes and bicarbonate reabsorption. The present data confirm these observations in a physiological range of pH, under conditions where peritubular CO_2 effects were completely dissociated from peritubular pH effects and tubular buffer load was kept constant, as opposed to whole animal studies. Furthermore, these effects are analysed after carbonic anhydrase inhibition.

As is shown in Table 1 (see Methods), peritubular HCO_3^- concentration had to be varied in order to keep pH constant. Since luminal perfusion solution was HCO_3^--free phosphate Ringer solution, a peritubular to luminal HCO_3^- gradient was necessarily built up. The creation of such a gradient could introduce an error in H ion flux calculations, leading to lower J_{H^+} values due to the presence of bicarbonate flux into the lumen (Lang, Quehenberger, Greger, Silbernagl & Stockinger, 1980). However, this effect would decrease the observed action of CO_2 on acidification, and therefore, the stimulation of acidification by CO_2 is, if anything, greater than that observed by us.

The stimulatory role of peritubular CO_2 on net H ion fluxes involved two factors, (1) a decrease in acidification half-times and (2) a fall in luminal pH and increase in luminal steady-state NaH_2PO_4 concentration. Both were markedly altered at peritubular CO_2 concentrations higher than 2%. The effect of increasing CO_2 on acidification half-times has been noted before (Mello Aires & Malnic, 1975) and has been attributed to an increased H ion conductance in the context of a proton pump leak model for proximal convoluted tubules (Cassola, Giebisch & Malnic, 1977) (see below).

Effect of CO_2 after carbonic anhydrase inhibition

One important finding that emerges from our results is the maintenance of a marked stimulatory effect of CO_2 on H ion secretion after carbonic anhydrase inhibition. A preliminary report in which total CO_2 reabsorption was measured by the picapnotherm technique did not detect differences in bicarbonate reabsorption in the presence of acetazolamide at two different levels of bath P_{CO_2}, 37 and 74 mmHg (Jacobson, 1981*a*). The reason for the discrepancy between these results and ours might originate from the different experimental approaches used. During the running perfusion experiments, in which total CO_2 is measured by the picapnotherm technique, tubular fluid is in contact for a much shorter time (2–6 s) than in our stopped-flow conditions (30–60 s); in these latter experiments a lower final pH may be reached. Therefore, this present method may be more sensitive in detecting the capacity of the epithelium to maintain pH gradients.

How could capillary CO_2 affect H ion secretion after carbonic anhydrase inhibition? If intracellular H ion generation is responsible for proton pump activity, as has been suggested (Cohen & Steinmetz, 1980; Sasaki *et al.* 1982) our observations would indicate that exogenous CO_2 must be able to decrease intracellular pH and thus to provide substrate to the pump, in spite of carbonic anhydrase inhibition. The ability of CO_2 to decrease intracellular pH in spite of the presence of acetazolamide has been shown in snail neurones (Thomas, 1976) and in turtle bladder (Schwartz, 1976).

An important consideration is the source of the secreted H ions stimulated by CO_2 after carbonic anhydrase inhibition. Table 5 attempts to account for this secretion considering several possible sources of uncatalysed H ion generation. The first column of the Table (J_HCO_{21}) is taken from '*in vitro*' experiments given in Table 2 and refers to acidification due to luminal phosphate titration by CO_2 diffusing from perfused capillaries. As shown above, this rate depends on CO_2 hydration in the tubular lumen and reaction of H_2CO_3 with luminal Na_2HPO_4. The second column is proton generation by uncatalysed cellular CO_2 hydration calculated from '*in vitro*' kinetic

data (Rubio, de Mello, Mangili & Malnic, 1982). The third column is passive bicarbonate flow from capillary to lumen, which in the steady state could be converted into H_2CO_3 and recycled into the cell, generating H ions without need of carbonic anhydrase catalysis (Rector, 1973). The sum of these values is of the order of the increment ($J\Delta$) in H ion secretion caused by elevated capillary CO_2 levels, indicating that the observed increments could be explained by mechanisms independent of carbonic anhydrase catalysis, but still related to the HCO_3^-/CO_2 buffer system.

TABLE 5. Source of H ions after carbonic anhydrase inhibition at different capillary CO_2

Capillary CO_2 (%)	J_HCO_{2l}	J_HCO_{2c}	$J_{HCO_3^-}$	$J\Sigma$	J_{H+obs}	$J\Delta$
0	—	—	—	—	0·088	0
2	0·017	0·031	0·030	0·078	0·165	0·077
5	0·059	0·107	0·170	0·336	0·480	0·392
10	0·094	0·171	0·370	0·635	0·780	0·692

J_HCO_{2l}: acidification due to titration of luminal buffer by CO_2 during equilibration with capillary CO_2 (data from Table 2; $J_HCO_{2l} = (\ln 2/t/2)\ (\Delta A)$, where ΔA is the difference between initial and final acid phosphate concentration). J_HCO_{2c}: uncatalysed H ion generation. $J_{HCO_3^-}$: bicarbonate leak flow ($J_{HCO_3^-} = P_{HCO_3^-}$). ($\Delta HCO_3^-$), using $P_{HCO_3^-} = 1{\cdot}7 \times 10^{-5}$ cm s^{-1} (Chan, Malnic & Giebisch, 1983). $J\Sigma$: sum of the three sources of H ions or H_2CO_3 independent of carbonic anhydrase catalysis. J_{H+obs}: experimental H ion secretion after carbonic anhydrase inhibition. $J\Delta$: H ion flux due to CO_2 stimulation above base line of J_{H+} (0% CO_2). Fluxes are in nmol cm^{-2} s^{-1}.

Interpretation of data by analogue model

The half-times of acidification show marked and significant modifications in several of the studied experimental situations. They have been interpreted previously as representing a measure of passive H ion conductance within the framework of a constant pump/variable leak model (Cassola *et al.* 1977). More recently, however, evidence has been adduced showing that most of the leak was due to back flow of H ions through the luminal Na/H exchanger; this would not be a passive conductive process (Schwartz, 1981; Rubio *et al.* 1982). Therefore, we will try to interpret variations of $t/2$ in terms of a different model based on a simple analogue electrical circuit (see Appendix and Fig. 6). This circuit is composed of a proton motive force (E), a series resistance (R_H), a capacitance (C) and a shunt resistance (R_s). These components are discussed in more detail in the Appendix. It must be stressed that, although using an electrical circuit, we are not implying an electrogenic H ion pump, but only making use of its kinetic properties. This is what we imply with the concept of an analogue circuit.

In terms of the model (see Appendix, eqn. (3)), acidification half-times could be varied by changes in R_H, R_s or C. Since in our experimental conditions we assume (a) that C remains constant due to the constant luminal buffer load, (b) that R_s is high due to the mostly small gradients of bicarbonate in our experiments and (c) that the conductive H ion component is small (Rubio *et al.* 1982), the major determinant of the half-times would be the internal resistance of the pump, R_H.

In our experimental conditions, R_H might be determined by the availability of H ions for the pump. At high CO_2, when cellular H ion generation is increased by CO_2

hydration, more H ions would be available and so R_H would be less. For turtle bladder, it was suggested that the effect of CO_2 stimulating acidification was mediated by an increase of the number of pump sites, incorporated into the apical cell membrane (Gluck, Cannon & Al-Awqati, 1982). Such an effect would also reduce R_H. On the other hand, R_H would be increased by factors limiting proton generation, like carbonic anhydrase inhibition. The observations in Table 3 show that $t/2$ is reduced as P_{CO_2} increases, whereas $t/2$ is consistently elevated after treament with acetazolamide. In conclusion, a reasonable interpretation of the changes in acidification half-times is that these reflect mostly changes in the internal resistance of the H ion pump, which is dependent on the access of H ions to the transport mechanism. On the other hand, the 'proton-motive force' E will affect the intercept, that is, the transepithelial pH gradient (Appendix, eqn. (2)).

Effect of peritubular pH changes

Transepithelial proton gradients, as shown in Fig. 4, were sensitive to alterations in capillary pH of air-equilibrated phosphate Ringer solutions. In previous work from this laboratory (Mello Aires & Malnic, 1975) changing extracellular pH from pH 5·6 to 8·4 produced a much larger effect on tubular acidification than in the present work. The present experiments were conducted at a much narrower, more physiological range of pH values. After carbonic anhydrase inhibition, however, this sensitivity to capillary pH was markedly reduced, not only in comparison to controls, but also when compared with the experiments in which capillary CO_2 was varied in the presence of acetazolamide. These findings indicate that capillary pH directly affects cell pH. Reducing pH at the peritubular membrane would neutralize base expelled from the cell and activate the bicarbonate/OH^- extrusion system postulated to exist at this membrane; this, in turn, would reduce cell pH. This system has been shown to be sensitive to carbonic anhydrase inhibition (Frömter & Sato, 1976; Garcia & Malnic, 1976), explaining the marked reduction of an effect of peritubular pH changes on the transepithelial proton gradients after carbonic anhydrase inhibition. It has been shown that peritubular buffer capacity acts in this way to alter base transfer, and also that it is dependent on carbonic anhydrase (Amorena & Malnic, 1983).

Comparison of effects of capillary CO_2 and pH

Fig. 3 shows the effect of changing peritubular CO_2 or pH on tubular H ion secretion. During both peritubular CO_2 and pH changes, proton fluxes varied, although the effect observed by modifying pH appeared to be smaller than that caused by changing peritubular CO_2.

It is difficult to directly compare the effect of peritubular pH and P_{CO_2} on tubular H ion secretion, since pH was only changed at 0 % CO_2, a condition where basic rates of H ion secretion are relatively low. Nevertheless, it is clear that both factors are able to modify tubular H ion secretion in a very significant way.

APPENDIX

An analogue model for renal tubular acidification

The analogue model represented in Fig. 6 consists of a pump composed of the electromotive force E and its series resistance R_H, and, in parallel, of a capacitance

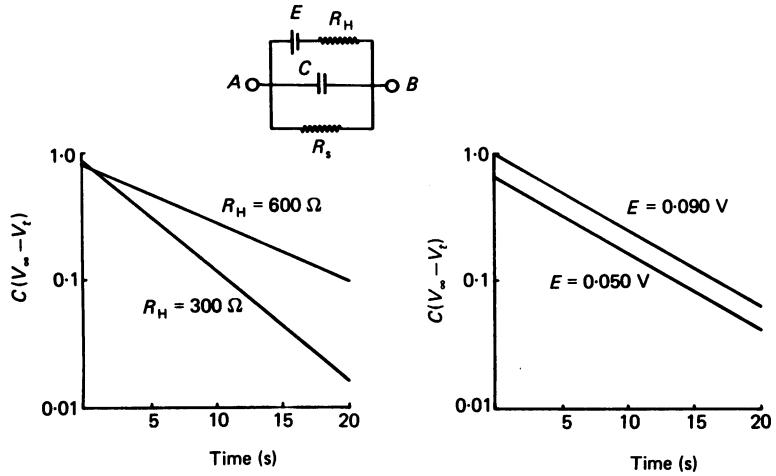

Fig. 6. Simulation of changes in capacitor charge (CV) against time varying only R_H (left) or E (right) for the circuit shown in the insert. For description of circuit components, see text.

C and a shunt resistance R_s. This analogue circuit does not imply electrogenic H ion transport, but may be applied also to an electroneutral system like that of proximal tubule, where acidification occurs by Na/H exchange at the brush-border membrane. The equation describing the circuit is the following:

$$\frac{dV}{dt}+\frac{1}{C}\left(\frac{1}{R_s}+\frac{1}{R_H}\right)V=\frac{E}{CR_H}, \tag{1}$$

where V is the voltage across the circuit and represents the pH gradient across the whole epithelium. The equation may be solved for CV, the capacitor charge:

$$\log (CV_\infty - CV_t) = \log \frac{CER_s}{R_s+R_H} - kt, \tag{2}$$

where k is given by:

$$k=\frac{1}{C}\left(\frac{1}{R_s}+\frac{1}{R_H}\right). \tag{3}$$

Charging up a capacitor with electrons transferred by the electron motive force E bears a similarity to titration of luminal buffer by H ions transported by the pump. Both are processes in which a capacitive element is progressively filled with a particle, reaching a steady-state level in an exponential way.

Equation (2) can be used to simulate the variation of CV with time under different conditions. Fig. 6 shows that the slope of these lines, which is equivalent to k, depends on R_H when other components are kept constant; similar graphs are obtained when C or R_s are varied independently. The capacitance C, which is thought to depend on tubular geometry and tubular buffer concentration, can be maintained constant experimentally. Consequently, the slope of the line (k = log 2/(t/2)) will depend on R_s and R_H. Fig. 6 also shows that when E is varied independently, only the intercept of the line is modified.

In an analogue model there is no direct correspondence between the circuit and the biophysical constituents of the transport system. Nevertheless, it is possible to obtain a set of values for these components which may be used for comparisons between the behaviour of the transport system in different experimental conditions. For this purpose, a number of assumptions have to be made. The voltage across the circuit can be obtained from the transepithelial pH gradient; a potential difference factor can be included or omitted corresponding to electrogenic or electroneutral systems, respectively:

$$V = 2{\cdot}303\frac{RT}{F}\Delta\,\mathrm{pH}, \tag{4}$$

where R, T and F have their conventional meaning. C, the capacitance, can be obtained from the ratio between capacitor charge (equivalent to titration of luminal buffer per square centimetre tubular epithelium) and voltage as calculated from eqn. (4):

$$C = \frac{(B_0 - B_s)\,F\,.\,(r/2)}{V}, \tag{5}$$

where B is tubular buffer base at time 0 and at steady state (s), and r the tubular radius.

R_s, the shunt resistance, is thought to depend on leak flow of H ions or HCO_3^- through trans- or paracellular pathways. Especially in a low-bicarbonate medium most passive back-flow of H occurs through the pump, or, as in proximal tubule, through the Na/H exchanger; in other words, it passes through R_H (Schwartz, 1981; Rubio *et al.* 1982). In consequence, R_s is elevated in comparison to R_H. It can be obtained from the relationship between the voltage (pH gradient) driving H flow across the epithelium and the current of H ions (J_H) observed when the Na/H exchanger is blocked by low Na medium and amiloride (Rubio *et al.* 1982):

$$R_s = \frac{2{\cdot}303\,RT/F\,\Delta\,\mathrm{pH}}{J_H\,.\,z\,.\,F}. \tag{6}$$

R_s may be estimated from data obtained by Rubio *et al.* (1982) after blocking the Na/H exchanger by low Na plus 10^{-4} M-amiloride. At a transepithelial pH gradient of 1·2 units, a back-flux of H ions (J_{H+}) of about $0{\cdot}19 \times 10^{-9}$ nmol cm^{-2} s^{-1} was observed. Therefore, according to eqn. (6):

$$R_s = \left(2{\cdot}303 \times \frac{RT}{F} \times 1{\cdot}2\right)/(0{\cdot}19 \times 10^{-9} \times z \times F) \cong 4{\cdot}000\,\Omega.$$

In the presence of bicarbonate, R_s for this ion can be calculated in a similar way. R_H can be estimated from eqn. (3):

$$R_H = \frac{R_s}{kCR_s - 1}, \tag{7}$$

and E by substitution in eqn. (1):

$$E = V\,.\,\frac{R_H + R_s}{R_s}. \tag{8}$$

Table 6 gives a series of such derived circuit values for the experiments in which capillary CO_2 is varied in the presence or absence of acetazolamide. It is seen that C is practically constant, and that R_H falls as CO_2 increases. This fall corresponds to the decreased acidification $t/2$ found under these conditions; it is equivalent to a

faster charging of the capacitor up to a given voltage, corresponding to an increased H flow into the lumen. The Table also shows circuit values for changes in capillary pH, and data for experiments in which acetazolamide was added to the capillary perfusate. During changes in capillary pH, R_H changes much less than during variation of CO_2, the lowest value being found at pH 7·4. The increase of R_H as well as $t/2$ at lower pH is compatible with the more elevated $t/2$ found in metabolic acidosis

TABLE 6. Electrical values of analogue model components calculated from experimental data. Effect of capillary CO_2 at constant pH, capillary pH at $CO_2 = 0$, 10^{-4} M-capillary acetazolamide and luminal buffer concentration

Experiment	C (mF)	R_s (Ω)	R_H (Ω)	E (mV)
Capillary CO_2				
2%	13	2230	770	48
5%	12	1460	615	62
10%	12	1315	450	68
Capillary pH				
7·0	12	4000	814	50
7·2	12	4000	755	58
7·4	12	4000	676	55
7·6	10	4000	776	69
Acetazolamide (10^{-4} M) pH				
7·0	13·5	4000	963	23
7·4	10	4000	1077	39
Luminal buffer				
20 mM*	12	4000	677	54
100 mM*	53	4000	311	50

* Data from Mello Aires & Malnic (1975).

(Giebisch *et al.* 1977). After carbonic anhydrase inhibition, R_H and $t/2$ are also higher than the values found under control conditions; here, the higher R_H may be due to the decreased availability of H ions within the tubular cell. Finally, an increase in luminal buffer concentration from 20 to 100 mM-phosphate leads to a more elevated acidification $t/2$ (10·5 s at 100 mM against 5·41 at 20 mM), reflected in an increased capacitance. At the same time, R_H is reduced at 100 mM-phosphate, possibly due to a greater turnover of the individual pump sites, caused by the more prolonged maintenance of luminal pH at higher levels due to the increased luminal buffer capacity.

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