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Role of Luminal Buffers in Renal Tubular Acidification

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Summary. The acidification kinetics of artificial solutions containing buffers of different permeancy were studied in rat proximal tubules by means of stationary microperfusion techniques. Luminal pH changes were measured by antimony microelectrodes and used to calculate net rates of acidification and the approach to steady-state pH levels. For most buffer species, tracer efflux out of the lumen was compared with changes in buffer concentration as derived from calculations based on the Henderson Hasselbalch equation. Steady-state luminal pH was similar for most buffer systems studied. However, secretory hydrogen ion fluxes into the lumen were significantly higher for permeant than for less permeant buffers. The most likely explanation is that permeant buffers behave as "open" systems maintaining constant low diffusible acid levels in the lumen, whereas impermeant buffers behave as "closed" systems in which non-ionized acid levels are maintained at higher levels. A behavior consistent with this thesis was directly demonstrated for glycodiazine and, to a lesser degree, for DMO. In contrast, phosphate and creatinine behave like buffers in a "closed" system. Characteristics of proximal tubular acidification, of buffer reabsorption, and the effect thereupon of carbonic anhydrase inhibitors are satisfactorily explained by an essential role of (1) hydrogen ion secretion, (2) pK differences, and (3) different permeance of the non-ionized buffer species. However, specific transport mechanisms may, in addition, also contribute to differences in transepithelial buffer movement.

Key words. Acidification, renal tubule, hydrogen ion secretion, buffer permeability, anion transport, titratable acid

Previous studies in which luminal pH changes of bicarbonate and phosphate buffered solutions were measured as a function of time in stopped-flow microperfusions have shown that both buffer species are significantly and rapidly acidified. It was uniformly observed that rates of tubular acidification increased with the buffer load. This functional behavior could be due to a pH-sensitive luminal H-ion secretory mechanism (Malnic & de Mello Aires, 1971; Giebisch, Malnic, de Mello & de Mello Aires, 1977; Cassola, Giebisch & Malnic, 1977) such that a high buffer load, by maintaining an alkaline luminal pH, stimulates H ion secretion.

In the course of such studies it was also observed that the net flux of H^+ into luminal phosphate corresponded to about half of that of the reabsorption rate of an equal tubular load of bicarbonate and that both acidification rates were significantly inhibited by acetazolamide (Malnic & de Mello Aires, 1971; Malnic, de Mello Aires, de Mello & Giebisch, 1972; Giebisch et al., 1977). These differences in the acidification kinetics of the two buffer species could be due either to the presence of an additional transport mechanism for ionic bicarbonate, or both processes could be mediated by hydrogen ion secretion, different rates of acidification resulting from specific properties of the two buffer systems. Thus, the bicarbonate system is titrated at constant *acid* concentration (CO_2 and H_2CO_3) whereas phosphate is titrated at nearly constant *total buffer* concentration in the lumen. This difference is due to different permeability properties of the tubular epithelium toward the acid components of the two buffer systems: CO_2 and H_2CO_3 are non-ionic molecules which readily traverse the proximal tubular wall, while acid phosphate is an anion which is reabsorbed at much slower rates (Malnic & de Mello Aires, 1971; Dennis, Woodhall & Robinson, 1976; Cassola & Malnic, 1977; Lang, Greger, Marchand & Knox, 1977; Warnock & Burg, 1977). Thus,

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the permeability properties of the luminal buffer could exert an important effect upon the kinetics of tubular acidification.

In the present study we have compared several buffers with respect to both their ability to sustain tubular acidification and their molecular reabsorption rates. The results presented are consistent with the thesis that net acidification rates are strongly dependent both on the permeability of the tubular wall to the nondissociated buffer species and their pK values.

Materials and Methods

Male Wistar rats weighing from 200 to 350 g were anesthetized with Pentobarbital (50 mg.Kg^{-1} BW). They received a standard rat-pellet diet and were allowed free access to food and water until the time of the experiment. They were prepared for micropuncture as previously described (Malnic, de Mello Aires & Giebisch, 1972). As a maintenance infusion during the experiment, the animals received 0.9% NaCl, to which 3% mannitol had been added, at a rate of 50 µl min^{-1}.

The techniques of luminal stopped-flow microperfusion, of peritubular capillary perfusion, and of the use of antimony microelectrodes to monitor proximal tubular pH changes have been described previously (Malnic & de Mello Aires, 1971; de Mello Aires & Malnic, 1975). Care was taken to use the same buffer solutions for the calibration of antimony electrodes *in vitro* as those used in the perfusion experiments. The calibration solutions were made up by titrating the respective perfusion solutions from the initial pH values (pH = 7.4) to pH 7.0 and pH 6.5 by addition of HCl and standardized by macroglass pH electrodes (Radiometer PHM72/BMS-3 MK2).

Transepithelial potential differences were measured by means of Ringer-agar-filled micropipettes of about 3 µm tip diameter. Potential values were corrected for tip potentials generated by perfusion solutions, and recorded by previously described methods (De Mello, Lopes & Malnic, 1976).

The *capillary* perfusion solutions had the following composition (in mM): NaCl, 105; KCl, 5; $CaCl_2$, 1; $MgSO_4$, 1.2; Na acetate, 5; plus 30 mM of the test buffer species. The following buffers were used. DMO (5,5 dimethyl-2,4 oxazolidinedione) obtained from Sigma Chemical Company (St. Louis), glycodiazine (sodium salt, obtained from Schering Company, Berlin), acetate, creatinine, TRIS (tris-hydroxymethyl-aminomethane), barbital (sodium 5,5-diethylbarbiturate) from E. Merck (Darmstadt), and sodium tetraborate from Eastman-Kodak (Rochester). We have assumed that the relatively small concentration of acetate (5 mM) in all perfusion fluids does not significantly affect the different results obtained with the various test buffers used at much higher concentration (30 mM).

The pK values of some of these buffers and the oil-water partition coefficients of their conjugate buffer acid are given in Table 1[1]. ^{14}C-DMO, ^{14}C-barbital, ^{14}C-Tris, ^{14}C acetate, and ^{14}C creatinine were obtained from New England Nuclear (Boston) and ^{3}H-glycodiazine from Schering (Berlin). To minimize net fluid reabsorption, luminal perfusions were prepared which had 40 meq/liter of NaCl substituted by raffinose. In the experiments with different buffers the assumption has been made that raffinose reabsorption is rate limiting for the process of fluid transport. This seems justified in view of the generally much slower $t1/2$ values of raffinose (~50 sec) compared to most of the other buffers. In a series of experiments with DMO, acetate, and glycodiazine buffers, acetazolamide (Lederle Laboratories, Pearl-River) was added to capillary

[1] The pK for sodium tetraborate/tetraboric acid was obtained from a titration curve of a sodium tetraborate solution with HCl. A pK of 8.9 was obtained at 25 °C and ionic strength of 0.15 and used in this paper.

Table 1. pK and partition coefficients of some of the used buffer species

Buffer	pK	Solutions	Distributed form	K	Ref.
Acetic acid	4.75	Chloroform/H_2O	Non-ionic	0.020	1
		Chloroform/H_2O	Non-ionic	0.058	[b]
		Benzene/H_2O	Non-ionic	0.022	[b]
		Heptane/H_2O	Non-ionic	0.00025	1
Barbital	8.10	Heptane/H_2O	Non-ionic	0.002	2
		Heptane/H_2O	Non-ionic	0.002	3
		Chloroform/H_2O	Non-ionic	0.7	3
Boric acid[a] (ortho)	9.13	Ether/H_2O	Undef.	0.0015	4
DMO	6.13	Ether/H_2O	Non-ionic	1.3	5
Glycodiazine	5.70	Toluol/H_2O	pH 7.4	0.22	6
		Isobutanol/H_2O	pH 7.4	1.00	6
		Ethylene Cl/H_2O	pH 7.4	4.88	6
		Chloroform/H_2O	pH 7.4	11.5	6
TRIS[a]	8.23	Chloroform/H_2O	Undef.	0.0001	7
		Benzene/H_2O	Undef.	0.00018	7
		Olivine oil/H_2O	Undef.	0.0007	7

[a] Values calculated as solubility ratios, and therefore only estimates of order of magnitude. Note that only values for ortho-boric, and not tetra-boric acid are available.

[b] K.J. Ullrich, *personal communication.*

References: 1: Jackson, Williamson, Dombrowski & Garner, 1978; 2: Brodie, Kurz & Schanker, 1960; 3: Hogben, Tocco, Brodie & Schanker, 1959; 4: Hodgman, 1961; 5: Butler, 1953; 6: Kramer et al., 1964; 7: Nahas, 1962.

perfusate in concentrations varying from 10^{-6} to 10^{-3} M. In another series of perfusions with glycodiazine solution, 10^{-3} M acetazolamide was added to the luminal perfusate.

The luminal disappearance rates of ^{14}C DMO, ^{14}C barbital, ^{14}C TRIS, ^{14}C acetate, ^{14}C creatinine, and ^{3}H-glycodiazine were measured by stationary microperfusion or by means of continuous microperfusions using a Hampel microperfusion pump (Frankfurt a.M., Germany) with isotope-containing solutions in a separate series of experiments. To achieve steady-state specific activity in the proximal tubule under study, peritubular capillaries were perfused with radioactive, buffer-containing solutions for at least one minute, a period several times longer than the equilibration half-times of these substances across the epithelium. Following such isotope equilibration, fluid samples were collected after perfusing tubular segments of different lengths, or reaspiration from the lumen after variable periods of time, their volume measured by determination of their diameter[2] and their radioactivity measured after transfer into counting vials containing 10 ml of Bray's solution (5 g PPO, 100 g naphthalene, dioxane to 1 liter) in a Beckman LS-100 liquid scintillation spectrometer. In order to maintain counting rates above 3 times background, samples of equal perfusion time were pooled if their volume was less than 1 nl. Samples were counted for time periods yielding an error of at most 3%. Net rates of hydrogen ion (J_H) secretion were calculated according to the equation:

$$J_H = \frac{\ln 2}{t/2} \cdot \frac{r}{2} \cdot (B_o - B_\infty) \quad (1)$$

where $t/2$ are half-times of the approach of luminal buffer base concentration to their steady-state levels, r is the luminal radius, and B_o and B_∞ are initial and steady-state luminal *buffer base concentrations* (Giebisch et al., 1977). The variability of J_H between different rats (SD/mean) was of the order of 12% for glycodiazine, and similar in other series of experiments. Hence the results obtained from different rats in the pH and tracer disappearance studies were pooled.

Buffer reabsorption rates of ^{14}C DMO and ^{3}H glycodiazine were similarly obtained from a plot of the difference of buffer base concentrations at time t and their steady-state level against time. These data, plotted on a semi-log scale, yield straight lines and thus are compatible with the mode in which net acidification rates follow first-order kinetics (Cassola et al., 1977). Buffer reabsorption rates of ^{14}C acetate, ^{14}C creatinine, ^{14}C barbital, and ^{14}C TRIS were measured by continuous microperfusion techniques (Green & Giebisch, 1975). Tubules were perfused at a rate of 15 nl/min and buffer fluxes (J_b, nm/cm^2·sec) calculated according to:

$$J_b/A = \dot{V}(C_i - C_f)$$

where A is the tubular area, $\dot{V}$ the perfusion rate, and C_i and C_f are the initial and final buffer concentration. The tubular area was calculated from measurements of the length of the perfused tubular segment and diameter by means of an ocular micrometer. The data, including pH values, are presented as means ± SEM. The significance of differences was evaluated by Student's t-test.

Results

Figure 1 shows a representative example of the approach of buffer base concentration of DMO and glycodiazine to their steady-state level with time. Data points for these two buffers species were calculated by the Henderson-Hasselbalch equation assuming luminal acid concentrations equal to those found in perfused capillaries. The fact that a straight line could be fitted to the points supports application of first-order kinetics to the acidification kinetics of these buffers.

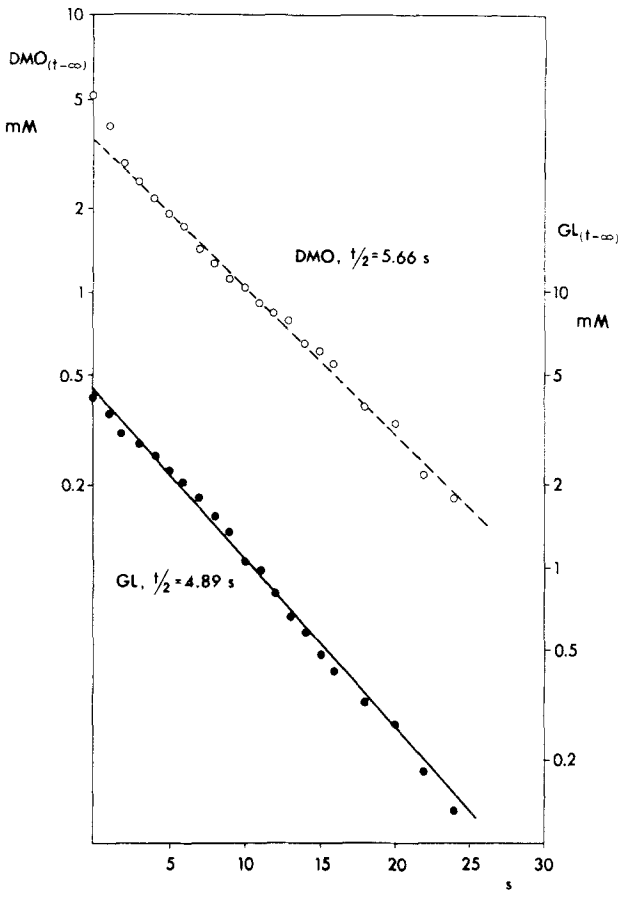

Fig. 1. Acidification of DMO and glycodiazine in proximal tubule of the rat. *Ordinates:* differences between concentrations of anionic form of buffers at time t and at steady state. *Abscissa:* time in seconds

Figure 2 gives examples of similar acidification data of creatinine and borate. We assumed relatively small reabsorption rates for these molecules and hence calculated luminal buffer base concentrations based on a constant luminal total buffer content. As can be seen in these experiments as well, luminal buffer concentrations approach their steady-state value in an exponential manner.

In our experiments, all data are calculated from luminal pH values, which are the only experimentally measured variable for the data given in Tables 2 to 4. In order to obtain buffer concentrations from this variable by means of the Henderson-Hasselbalch equation, one of the following assumptions has to be made:

[2] Volumes were corrected for small deviations from the assumed spherical droplet shape by measuring diameter and radioactivity of droplets, made up of a ^{3}H-inulin-containing solution of known activity, of similar size as those collected. This correction corresponded to multiplying the calculated volume by 0.89.

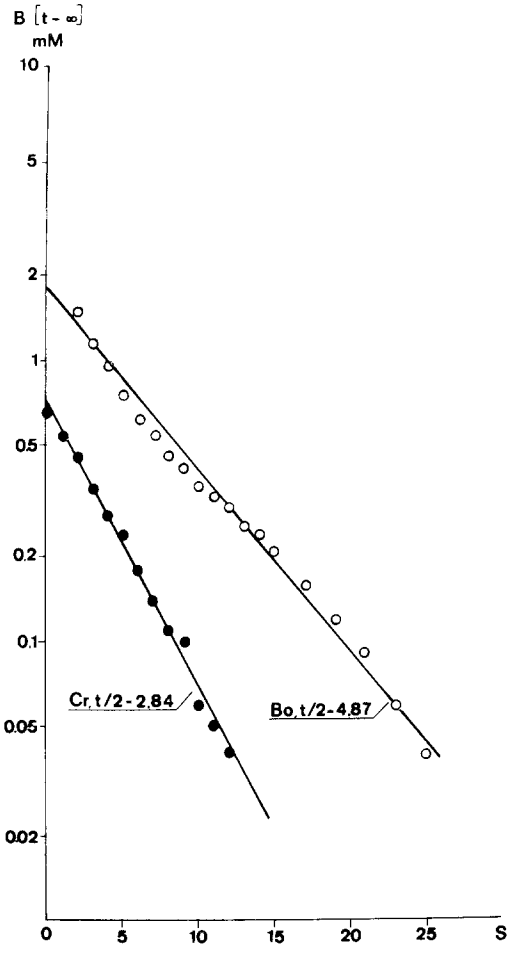

Fig. 2. Acidification of Borate (*Bo*) and Creatinine (*Cr*) in proximal tubule. *B*, buffer base (anionic form)

(a) The buffer injected into the tubular lumen is not lost to a significant extent during the period of observation. This assumption has been shown to hold for phosphate in previous experiments (Cassola & Malnic, 1977). It is the "constant buffer" or "closed system" assumption, and buffer concentrations are calculated according to:

$$\mathrm{pH} = \mathrm{pK} + \log \frac{T-A}{A} \qquad (2)$$

where T = total buffer concentration and A = concentration of the acid form of the buffer. There is only one unknown, A, and the buffer base $= B = T - A$.

(b) The acid form of the buffer is always in equilibrium across the tubular epithelium; that is, buffer acid permeability is so high that a negligible concentration gradient drives the necessary acid flow across the epithelium. This is what is generally assumed when bicarbonate/CO_2 levels are studied using pH microelectrodes.

Using exogenous buffer systems, it is necessary to perfuse the peritubular capillaries with a solution containing known concentrations of buffer at a known pH in order to have a known peritubular buffer acid concentration, assumed to be in equilibrium with the acid concentration, in the tubular lumen. This is the "constant acid" assumption (or "open system"), and buffer base concentrations are calculated by:

$$\mathrm{pH} = \mathrm{pK} + \log \frac{B}{A} \qquad (3)$$

where A is the known peritubular acid concentration and the only unknown is B.

Both these assumptions can be applied operationally to the pH values obtained with any buffer system, but none probably will be entirely valid for any single system. However, it is reasonable, on the basis of experimental data concerning the permeability of the tubular epithelium to buffer components like CO_2 and phosphate, that certain buffer systems behave predominantly like a constant buffer (closed) or constant acid (open) system. Such knowledge about the behavior of buffer systems can be obtained only by measurement of the outflow of the system from the tubular lumen using a specific method, e.g., the determination of radioactive tracers, to measure such fluxes. The data presented in Tables 2, 3 and 4 were obtained by using one or both of the described assumptions. Such data are then compared to the rates of reabsorption of various buffers in Tables 5, 6 and 7. Obviously, the constant acid assumption can only be used during capillary perfusion with a solution containing known concentrations of the involved exogenous buffer.

An example will show that use of these two assumptions will lead to very different results. For glycodiazine, mean steady-state buffer concentrations are 26.3 mM in proximal tubular fluid when the constant buffer assumption is used ($T = 30$ mM), and 4.55 mM when the constant acid assumption is used ($A = 0.59$ mM at a total buffer concentration of 30 mM and a pH of 7.4 in peritubular capillaries). Since the calculated H ion secretory fluxes depend on the difference $B_o - B_\infty$ according to Eq. (1) (Methods, p. 15), net H ion fluxes will be considerably larger using the constant acid assumption (3.03 nmol/cm^2·sec) than using the constant buffer assumption (0.2 mmol/cm^2·sec) (*see* Table 3).

A summary of acidification parameters for luminal perfusions with different buffer systems in the presence of capillary perfusions is given in Table 2. These data correspond to the situation where luminal buffer is titrated in an open (CA) system, with transepithelial equilibration of the diffusible form of the buffer. Data calculated for acidification in a closed (CB) system, i.e., without significant buffer loss from

Table 2. Proximal tubular acidification during luminal and capillary perfusion with different buffer systems

Buffer	Acetate	Glycodiazine	DMO	HCO_3^-/CO_2[a]	Phosphate[b]	TRIS
pK	4.75	5.70	6.13	6.10	6.80	8.23
pH_∞	6.46±0.049(32)	6.59±0.039(31)	6.47±0.04(36)	6.52±0.07(29)	6.31±0.043(24)	6.66±0.080(14)
B_o, mM	29.93	29.41	28.47	28.96	23.98	3.87
B_∞, mM – CA	4.06±0.39(32)	4.55±0.27(31)	3.52±0.21(36)	3.79±0.56(39)	–	–
– CB	29.33±0.10(27)	26.3 ±0.32(31)	20.6 ±0.57(36)	–	7.33±0.33(24)	0.95±0.17(14)
$t/2$, sec – CA	3.99±0.22(32)	4.27±0.20(28)	5.48±0.38(36)	3.96±0.24(29)	–	–
– CB	5.29±0.32(27)	5.65±0.31(27)	8.89±0.72(36)	–	6.19±0.43(21)	4.12±0.39(14)
J_H – CA	3.37±0.28(32)	3.03±0.20(28)	2.37±0.23(36)	3.30±0.30(29)	–	–
– CB	0.059±0.015(27)	0.29±0.048(27)	0.66±0.09(36)	–	1.40±0.14(21)	0.37±0.058(14)

Mean±SE (n). pH_∞: steady-state pH. B_∞: steady-state buffer base conc. $t/2$: acidification half-time. B_o: initial buffer base conc., at pH=7.4. CA: data calculated assuming constant acid (luminal=peritubular) concentration. CB: constant total luminal buffer content. J_H: H ion secretory rate at initial buffer concentration (30 mM) nmol/cm^2·sec.

[a] Data from Malnic and de Mello Aires, 1971

[b] Data from de Mello Aires and Malnic, 1975.

Table 3. Proximal tubular acidification during luminal perfusion with different buffer systems

Buffer	pK	pH_∞	B_∞ (mM)	B_0 (mM)	$t/2$ (sec)	J_H (nmol/cm^2·sec)
Creatinine	4.97	6.65±0.043(30)	29.25±0.072(30)	29.89	3.66±0.35(30)	0.091±0.019(30)
Phosphate[a]	6.80	6.36±0.045(11)	8.15±0.42(11)	23.98	7.70±1.05(12)	1.069±0.214(11)
Borate	8.90	6.98±0.030(54)	0.40±0.022(54)	0.92	4.65±0.26(54)	0.058±0.0058(54)

Mean±SE (n). pH_∞: steady-state pH; B_∞ steady-state buffer base conc.; $t/2$: acidification half-time; J_H: H ion secretory rate at initial buffer concentration; (30 mM). B_o: initial buffer base conc., at pH=7.4.

[a] Data from de Mello Aires and Malnic, 1975.

the tubular lumen during the period of acidification, are also included. In Table 3, such parameters are given for several impermeant buffer systems in experiments in which capillary perfusion was not performed, i.e., with blood in the capillaries. For calculation of acidification of relatively impermeant buffers, capillary perfusion is not necessary since transepithelial equilibration does not occur.

It should be noted that independent of the nature of the luminal buffer used, steady-state pH levels were quite comparable and fall into the range from 6.4 to 6.7. The slightly higher values observed with borate and barbital (6.85, $n=55$) cannot be explained at present. In Table 2, steady-state buffer base concentrations, $t/2$ values of acidification and net hydrogen ion secretion have been calculated for both "open" (CA) and "closed" (CB) systems. It is clear that, depending on the assumed penetrability of the luminal buffer, dramatically different values were obtained. H ion secretion in an "open" system with fixed acid buffer levels (rapid attainment of transepithelial steady state) results in higher and more efficient acidification at the same initial buffer levels and at the same steady-state pH levels. This is due to lower steady-state buffer-base concentrations as well as faster acidification half-times which result from the more effective translocation of the acid buffer component out of the tubular lumen. Acidification half-times were significantly faster when calculated for an open system than for a closed system in the case of acetate, glycodiazine and DMO ($P<0.01$, *see* Table 2).

It should also be realized that independent of the assumption of an open or closed luminal buffer system, the pK value of the buffers also exerts a powerful effect upon net acidification. This is due to the different segments of the titration curve encompassed by the physiological pH boundaries of the proximal tubular epithelium. Tables 2 and 3 summarize relevant data. Net secretory H-ion fluxes are greatest when the buffer pK is near the physiological pH range. These effects are even more accentuated in the case of these buffers which have pK's in the physiological range and are highly permeant, falling thus into the category of "open" buffer systems.

The effect of different concentrations of the carbonic anhydrase inhibitor acetazolamide upon DMO and glycodiazine acidification kinetics is shown in Table 4. Already at a concentration of 10^{-6} M significant inhibition of H-ion fluxes are found. Larger fractions of these fluxes are inhibited at higher concentra-

Table 4. Effect of acetazolamide on tubular acidification of DMO and glycodiazine

Buffer	Acetazolamide	Δ pH	Δ Buffer base (mM)	$t/2$ (sec)	J_H
DMO	0	$0.93 \pm 0.040(36)$	$24.95 \pm 0.21(36)$	$5.48 \pm 0.38(36)$	$2.37 \pm 0.23(36)$
	10^{-4} M	$0.34 \pm 0.030(53)^b$	$15.46 \pm 0.76(53)^b$	$9.05 \pm 1.27(53)^a$	$0.89 \pm 0.187(53)^b$
Glycodiazine	0	$0.78 \pm 0.039(31)$	$24.85 \pm 0.27(31)$	$4.27 \pm 0.20(28)$	$3.03 \pm 0.201(28)$
	10^{-6} M	$0.75 \pm 0.014(67)$	$24.36 \pm 0.12(67)$	$6.16 \pm 0.22(67)^b$	$2.06 \pm 0.105(67)^b$
	10^{-5} M	$0.43 \pm 0.022(42)^b$	$18.20 \pm 0.36(42)$	$7.79 \pm 0.44(39)^b$	$1.22 \pm 0.101(39)^b$
	10^{-4} M	$0.32 \pm 0.017(30)^b$	$15.60 \pm 0.37(30)^b$	$7.32 \pm 0.46(29)^b$	$1.11 \pm 0.105(29)^b$
	10^{-3} M	$0.25 \pm 0.015(53)^b$	$13.70 \pm 0.42(53)^b$	$8.97 \pm 0.43(51)^b$	$0.79 \pm 0.064(51)^b$
	10^{-3} M, luminal	$0.20 \pm 0.018(50)^b$	$10.63 \pm 0.66(50)^b$	$9.78 \pm 0.94(38)^b$	$0.57 \pm 0.090(38)^b$

Acetazolamide added to capillary perfusion except if otherwise noted. Δ pH, pH difference, capillary perfusate – lumen in steady state. Δ BB, same for buffer base concentration. J_H in nmol/cm^2·sec. Differences with controls (0 acetazolamide): [a] ($0.05 > P > 0.01$); [b] ($P < 0.01$).

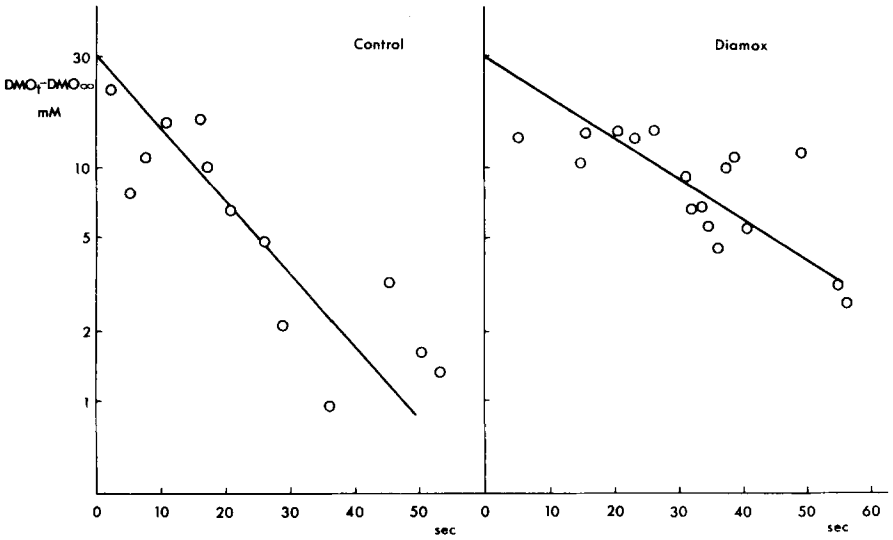

Fig. 3. Reabsorption of DMO in proximal tubule as measured during stopped-flow microperfusion using ^{14}C-DMO, in control and diamox-perfused tubules

tions, and at concentration of 10^{-3} M inhibition reaches reaches only 75% when the drug is added to capillary perfusate, and 81% when added to luminal solution. From inspection of Table 4 it can also be seen that pH and buffer base gradients across the proximal tubular epithelium diminish with increasing inhibitor concentration; at the same time acidification half-times become progressively longer.

It is reasonable to assume that the physiological buffer, the bicarbonate-CO_2 system, can be treated as an open one in view of the high permeability of the tubular epithelium to CO_2 (Malnic & de Mello Aires, 1971; Warnock & Burg, 1977). Also, from the slow disappearance of phosphate ions, the latter approach a closed system. To extend the knowledge of the penetrability of additional buffer species, tracer disappearance of labeled glycodiazin and DMO were studied. Figure 3 summarizes results from stationary microperfusion experiments using ^{14}C-labeled DMO. Also included are results from experiments in which diamox was added to the capillary perfusate at a concentration of 10^{-4} M. From these graphs, and similar ones obtained for glycodiazine, mean values of steady-state buffer base concentration, half-times of tracer disappearance, and rates of net tracer reabsorption were calculated and are given in Tables 5 and 6. With respect to the rate of tracer disappearance, it is of interest that glycodiazin transport was faster than that of DMO and that both buffer reabsorption rates were significantly inhibited by the administration of diamox. The rate of reabsorption measured for ^{14}C-creatinine was 0.0023 ± 0.0022 ($n=7$) nmol/cm^2·sec, not significantly different from zero. That for acetate was 3.85 ± 0.33 (15), for Tris, 0.33 ± 0.11 (12) and for barbital, 7.93 ± 0.667 (18) nmol/cm^2·sec. In rats receiving acetazolamide (10^{-4} M added to the capillary perfusion) acetate reabsorption was significantly reduced ($0.05 < p < 0.01$) to $2.96 \pm 0.23(17)$ nmol/cm^2·sec.

Transepithelial potential difference measurements during luminal and peritubular buffer perfusions were slightly more negative than in free-flow conditions: PD $= -0.40 \pm 0.14$ ($n=30$) mV for glycodiazine Ringer's and -1.03 ± 0.11 (42) mV for DMO

Table 5. Transport of DMO in proximal tubule of the rat

	DMO_∞ (mM)	b (sec^{-1})	$t/2$ (sec)	J_{DMO} (nmol/cm^2·sec)
Control	8.54±1.06(11)	−0.057±0.0090(13)	12.1±1.92 (13)	0.856±0.210(11)
Diamox 10^{-4} M	16.06±1.89(10)[b]	−0.021±0.0069(17)[a]	33.1±10.85(17)	0.195±0.111(10)[a]

b, slope of DMO concentration decrease with time; $t/2$, half-time of luminal DMO approach to steady-state, J_{DMO}, net flow of DMO from lumen to interstitium at time zero.

[a] $(0.05 > P > 0.01)$.
[b] $(P < 0.01)$.

Table 6. Transport of glycodiazine in proximal tubule of the rat

	GLD_∞ (mM)	b (sec^{-1})	$t/2$ (sec)	J_{GLD} (nmol/cm^2·sec)
Control	1.95±0.15(9)	−0.125±0.020(20)	5.54±0.89(20)	2.58±0.74(9)
Diamox, 10^{-5} M, cap.	2.84±0.26(9)[a]	−0.108±0.015(12)	6.41±0.89(12)	2.15±0.46(9)
D, 10^{-3} M, cap.	5.13±0.89(9)[b]	−0.077±0.013(20)	9.04±1.52(20)	1.40±0.42(9)
D, 10^{-3} M, lum.	9.34±0.78(10)[b]	−0.054±0.018(17)[a]	12.88±4.28(17)	0.81±0.44(10)[a]

See Table 4 for explanations.
Differences with controls: [a] $(0.05 > P > 0.01)$. [b] $(P < 0.01)$.

Ringer's. During luminal perfusion alone these values were: PD = −0.78±0.22(16) for glycodiazine Ringer's, and −1.67±0.19(15) for DMO Ringer's. These small differences are significantly different from zero ($P < 0.01$). However, transepithelial potential differences of this magnitude do not exert important changes in H^+-ion secretion (Malnic & Cassola, 1977).

Discussion

One of the main conclusions of the present studies is the strong dependence of luminal acidification kinetics and of the rate of net acidification of the proximal tubular epithelium upon the permeability properties of luminal buffer species. The net rate of tubular acidification is significantly enhanced with increasing buffer permeability. This is schematically shown in Fig. 4*A* in which titration curves for DMO are plotted assuming high buffer permeability and, as a consequence, rapid transepithelial equilibration of the non-dissociated acid across the tubular epithelium. In such an open system, buffer base concentrations are calculated by the Henderson-Hasselbalch equation, assuming constant luminal acid concentration equal to that of the peritubular capillaries.

Figure 4*B* represents a titration curve based on the assumption that luminal buffer is essentially retained within the tubular lumen. With progressive acidification, buffer base concentration declines concurrent with the equimolecular rise in buffer acid concentration. As a consequence of acidification in such a closed system, the fall in buffer base concentrations

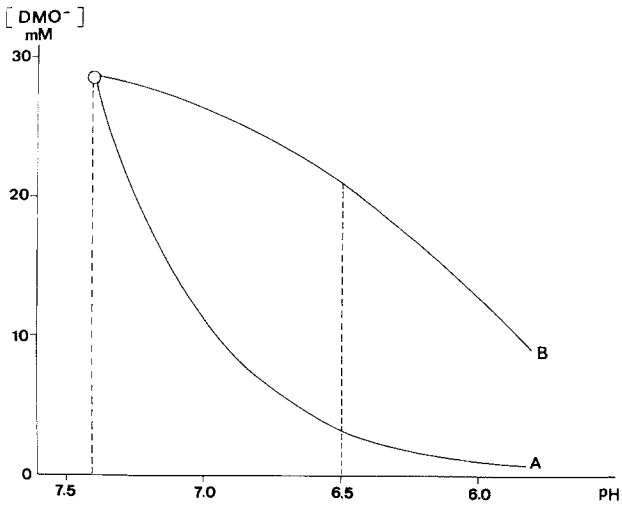

Fig. 4. Titration curves of DMO between pH 7.4 and 6 for open (*A*) and closed (*B*) systems. For the open system, a constant acid concentration of 1.53 mM was assumed, corresponding to the capillary perfusate level at pH 7.4 and total buffer of 30 mM

is not as marked as that expected in the open system. Depending on the permeability of the luminal buffer, dramatically different acidification rates result from identical pH changes. If DMO were to behave as an open system, at a final luminal pH of 6.5, titration would have achieved a buffer base concentration of 3.6 meq/liter. In sharp contrast, in a closed system, the same pH shift would only reduce buffer base concentration to 9.0 meq/liter. Inspection of Eq. (1) shows that the magnitude of net hydrogen ion secretion depends mainly on two factors: (1) the difference between initial and steady-state buffer base level as

Table 7. Components of H ion secretion into different luminal buffer species

Buffer	J_T	J_a	J_H	J_{CA}
Acetate	3.85	0.059	3.91	3.37
Creatinine	0.0023	0.091	0.091	–
GLD	2.58	0.29	2.87	3.03
DMO	0.86	0.66	1.52	2.37
Barbital	7.93	0.28	8.21	0.31
TRIS	0.33	0.37	0.37	–

Fluxes in nmol/cm^2·sec. $J_H = J_T + J_a$ (*see* text).
J_{CA}: acidification calculated for open system (Table 2). For barbital, this value was calculated from $pH_\infty = 6.85$ and $t/2$ of 6.0 sec as obtained in experiments in which the tubular lumen was perfused with barbital-buffered solutions.

discussed above, and (2) the $t/2$ of the approach of buffer base concentrations to their steady-state. It is to be noted that the latter, as shown in Table 2, is shorter when buffer is titrated in an open system. Note that in this table the same experimental data, i.e., the same pH curves changing with time, are treated according to the two models, open and closed system. Thus, a given pH curve yields smaller $t/2$ values if treated according to the assumption of titration at constant acid concentration. This is due to the more rapid fall of buffer base concentration in the open system, as shown in Fig. 4. Buffer concentrations fall more rapidly in an open system since, according to Eqs. (2) and (3), the same initial buffer base concentration will fall, in the same period of time, to lower values in an open than in a closed system.

Additional insight into the mechanisms by which a buffer species is acidified can be obtained by comparing the actual rate of acidification as measured by the pH change, with the isotopically measured buffer disappearance rates. Overall H^+ ion secretion rates (J_H) are given by the relationship:

$$J_H = J_T + J_a \tag{4}$$

where J_T is the rate of isotopically measured buffer disappearance due to H^+ ion secretion (formation of a diffusible acid form in the lumen), and J_a is the rate of acidification calculated from luminal pH changes assuming constant buffer, i.e., J_a is a measure of the increase in nondiffusible buffer acid species. Both these rates are calculated for time zero, when maximal luminal buffer concentrations are present. With respect to the measurement of acidification rates, it has been shown that they are independent of the loss of total buffer from the lumen when referred to $t=0$ (Giebisch et al., 1977).

For a "constant acid" system (e.g., bicarbonate), luminal acid concentrations will not increase significantly with time, and $J_a = 0$. Thus,

$$J_H = J_T. \tag{5}$$

For a "constant buffer" system (e.g., creatinine), no buffer will be lost, and $J_T = 0$. Hence,

$$J_H = J_a. \tag{6}$$

If both buffer loss and increase in luminal buffer acid concentration occur, Eq. (4) must be used.

Let us consider the example of the buffers DMO and glycodiazine. As given in Table 7, for DMO $J_T = 0.86$ nmol/cm^2·sec and J_a, calculated for constant buffer, is equal to 0.66 nmol/cm^2·sec. Thus,

$$J_H = 0.86 + 0.66 = 1.52 \text{ nmol/cm}^2\cdot\text{sec}.$$

For glycodiazine, $J_T = 2.58$ and $J_a = 0.29$ nmol/cm^2·sec, and therefore $J_H = 2.87$ nmol/cm^2·sec. The fact that in the case of glycodiazine the acidification rate calculated by Eq. (4) is very similar to that calculated for an open system indicates that the above way of subdividing H ion secretion is a realistic one.

Clearly, assignment of a given buffer species to either open or closed systems necessitates precise knowledge of the permeability of the epithelium to the nondissociated buffer form. With respect to DMO, buffer efflux out of the lumen was found to be slower than acidification. Hence, buffer translocation is not fast enough to yield complete transepithelial equilibration of the nondissociated acid. True acidification rates will be intermediate between the two values listed in Table 2, but the net reabsorptive DMO fluxes obtained in Table 5 are nearer to net H ion fluxes obtained when DMO acidification in a closed system is considered (*see* Table 2). On the other hand, glycodiazine disappearance rates were observed to be similar to those of acidification of solutions containing this buffer. This indicates rapid and virtually complete equilibration of its conjugate acid across the proximal tubular epithelium. It is titrated more nearly according to an open system and, as shown in Tables 3 and 6, its rate of disappearance will approach that of hydrogen ion secretion into this buffer system.[3]

[3] According to the "open system" hypothesis, the permeability of the acid form of the buffer should be high enough to maintain a transepithelial concentration gradient of near zero. This could be verified by the following calculation, which uses our flux data and an assumed buffer acid permeability similar to that of pyridine in isolated perfused cortical collecting tubules (Schafer & Andreoli, 1972). The expected concentration gradient, DA, can be obtained by dividing the glycodiazine flux (*see* Table 6) by the permeability of these tubules to pyridine (of the order of 12.6×10^{-4} cm/sec) and by 40, since the geometrical area of the luminal surface is about 40 times smaller than the effective area of the luminal brush border surface (Al-Awqati, 1978):

The behavior of acetate appears to be similar to that of glycodiazine, with a high rate of buffer reabsorption, also suggesting an open-system acidification. However, it cannot be excluded that a portion of the acetate flow measured by this technique is due to transport of acetate as such or to metabolization of the molecule within the tubular cell. In order to obtain some insight into this possibility, acetate flow was measured also in the presence of 10^{-4} M acetazolamide in the capillary perfusate. Under these conditions, this flow was reduced only to 77% of the control value, while DMO transport was reduced to 23% and glycodiazine to 54% of controls (*see* Tables 5 and 6) in isotope flow experiments, and to 37 to 38% of controls in experiments in which luminal acidification was measured. Since ^{14}C-acetate reabsorption was inhibited much less than reabsorption of other buffers and less than acidification, it is probable that other anion transport processes besides non-ionic diffusion contribute to the apparent acetate flow across proximal tubular epithelium. It is of interest to note that despite the large acetate efflux this system does not appear to behave like a perfect open system. This follows from the observation that the rate of hydrogen ion secretion calculated according to the "constant acid" assumption exceeds quite substantially the acetate efflux that, from its diamox sensitivity, could be related to hydrogen ion secretion and to non-ionic diffusion.

With respect to barbital, reabsorptive fluxes are considerable higher than those of all other buffers tested and exceed acidification calculated for an open system, assuming steady-state pH and $t/2$ values similar to those given in Table 7; this latter calculation gives H ion fluxes of 0.31 nmol/cm^2·sec, considerably smaller than isotope fluxes. It appears that for this species non-ionic diffusion alone, since it is less than the total hydrogen transport rate, cannot account for buffer reabsorption.

Since phosphate (Cassola & Malnic, 1977) and creatinine are but slowly or not at all reabsorbed by the proximal tubular epithelium, their acidification kinetics are expected to follow closely those of a closed system. Accordingly, the rate at which these buffer species sustain acidification will be significantly lower than that of highly permeant luminal buffers. Inspection of Table 7 indicates almost complete impermeability of the tubular wall to creatinine. It should be noted that even though phosphate reabsorption is slow ($t/2$ of luminal phosphate disappearance equal to 30–60 sec compared to a $t/2$ of phosphate acidification of 6–7 sec), a small but significant fraction disappears from the tubular lumen during these experiments (Cassola & Malnic, 1977). As the precise proximal tubular permeability to borate is not known, its acidification rate cannot be precisely assessed. However, its lipid partition coefficient is relatively low (*see* Table 1), and therefore it has been treated as a "closed system" buffer. On the other hand, since its nondissociated conjugate has some lipid solubility they cannot be expected to be acidified in an entirely closed system.

With respect to buffers that constitute a weak base/strong acid system, e.g. Tris/HCl, the secretion of H^+ does not produce a more permeant moiety. The transepithelial flow of Tris molecules will reduce total luminal buffer content, but will not correspond to H^+ ion secretion. This process does not affect the evaluation of J_a at time zero and does not contribute to J_H. Therefore, at $t=0$ the acidification of such buffers can best be described as a closed system; this is why in Table 2 the "constant acid" calculation was not applied to Tris buffer.

Another factor determining the rate of tubular acidification is the pK value of the luminal buffer. Figure 5 depicts titration curves of some of the buffers studied, calculated for behavior in a closed system. As expected, buffers with pK values in the range between the pH of blood and tubular fluid (7.4 to 6.4) are most efficiently titrated. Figure 6 underscores the quantitative importance of buffer pK for the rate of proximal tubular hydrogen ion secretion. Two points should be noted. First, it can be seen that the highest rates of hydrogen ion secretion occur with luminal buffers having a pK between that of glycodiazine (5.7) and phosphate (6.8). Significantly lower acidification rates are observed with buffer species having pK's further removed from the physiological range of proximal tubular pH. Secondly, at each pK open systems sustain higher acidification rates than closed ones, the two highest values obtaining for bicarbonate and glycodiazine.

Carbonic anhydrase inhibitors have been shown in the present study to inhibit markedly acidification rates in the buffer systems studied. However, the extent of such inhibition varies with the different buffers. As seen from inspection of Table 8, a similar increase in luminal pH results in a considerably higher buffer base concentration in a closed system. Hence,

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$$DA = \frac{2.58 \times 10^{-9} \text{ mol/cm}^2 \cdot \text{sec}}{12.6 \times 10^{-4} \text{ cm/sec} \times 40} = 5.12 \times 10^{-8} \text{ mol/cm}^3$$
$$= 5.12 \times 10^{-2} \text{ mM}.$$

In the presence of acetazolamide – if the permeability were not affected – the calculated gradient necessary to drive the flux of 0.81×10^{-9} mole/cm^2·sec would be 1.6×10^{-2} mM. These values are a small fraction of the total buffer concentration of 30 mM, and even of the peritubular acid concentration, of the order of 0.6 mM. This suggests that transepithelial acid concentration gradients are sufficiently low to support the "open system" hypothesis for this class of buffers.

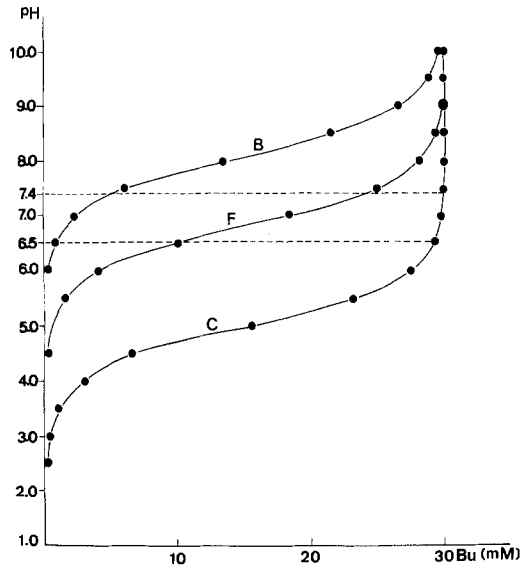

Fig. 5. Titration curves calculated for barbital (*B*), phosphate (*F*) and creatinine (*C*), assuming constant total buffer levels (closed system). The dotted lines correspond to the limits of physiological pH between blood and proximal tubule. *Bu*, buffer base concentration

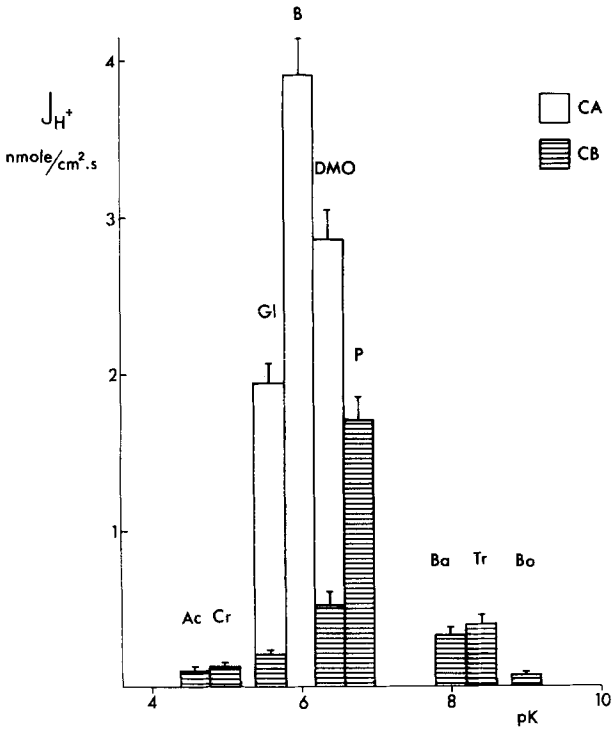

Fig. 6. Net H ion fluxes into buffer solutions in proximal tubule according to their pK. *CA*, constant acid (open system); *CB*, constant buffer (closed system). *Ac*, acetate; *Cr*, creatinine; *Gl*, glycodiazine; *B*, bicarbonate; *P*, phosphate; *Ba*, barbital; *Tr*, Tris; *Bo*, borate

the residual acidification rate, after acetazolamide, is considerably larger in an open system compared with the situation of a closed system in which buffer base concentrations fall much more slowly from their

Table 8. Effect of acetazolamide on renal tubular acidification: role of buffer species

Buffer	Steady-state buffer base (mM)			
	Open system		Closed system	
	C	*D*	*C*	*D*
HCO_3^-/CO_2 [a]	3.79	7.10	–	–
Phosphate [a]	–	–	7.33	19.5
DMO [b]	3.52	13.0	20.6	26.8
Glycodiazine [b]	4.55	13.8	26.3	28.7
Buffer	Net H ion fluxes (nmol/cm²·sec)			
	Open system		Closed system	
	C	*D*	*C*	*D*
HCO_3^-/CO_2 [a]	3.30	1.40 (42.4%)	–	–
Phosphate [a]	–	–	1.40	0.18 (12.9%)
DMO [b]	2.37	0.89 (37.6%)	0.66	0.096 (14.5%)
Glycodiazine [b]	3.03	1.11 (36.6%)	0.29	0.050 (17.2%)

[a] From Giebisch et al., 1977; Malnic & de Mello Aires, 1971; Mello Aires & Malnic, 1975; systemic administration of acetazolamide.

[b] Capillary perfusion with 10^{-4} M acetazolamide. *C*, control; *D*, acetazolamide.

Buffer base concentrations were obtained from the same mean steady-state luminal pH values for open and closed system calculations.

initial levels. This behavior explains the observation that the residual rate of bicarbonate reabsorption exceeds that observed when phosphate buffers are used (Giebisch et al., 1977): net acidification rates after carbonic anhydrase inhibition for bicarbonate and phosphate hold a similar proportion to controls as calculations for open and closed systems in the case of DMO and glycodiazine.

In the present experiments, tubular acidification was studied during symmetrical perfusion of both tubular lumen and peritubular capillaries with nominally bicarbonate and CO_2-free solutions. Figure 7 provides schematic representations of acidification mechanisms in a closed and open buffer system. It is generally accepted that CO_2 plays a key role in the generation of H^+ ions or OH^- buffering in tubule cells (Rector, 1973). In bicarbonate and CO_2-free systems, especially after carbonic anhydrase inhibition, rates of cellular metabolic CO_2 generation are too small to sustain tubular acidification at the rates actually observed (Malnic & Giebisch, 1979).[4] Two possible

[4] The problem whether uncatalyzed H^+-ion generation could account for the acidification of, e.g., glycodiazin after administration of acetazolamide can be assessed in the following manner. A maximal vlaue of acidification can be calculated, multiplying the rate coefficient of CO_2 hydration (Maren, 1978) by a presump-

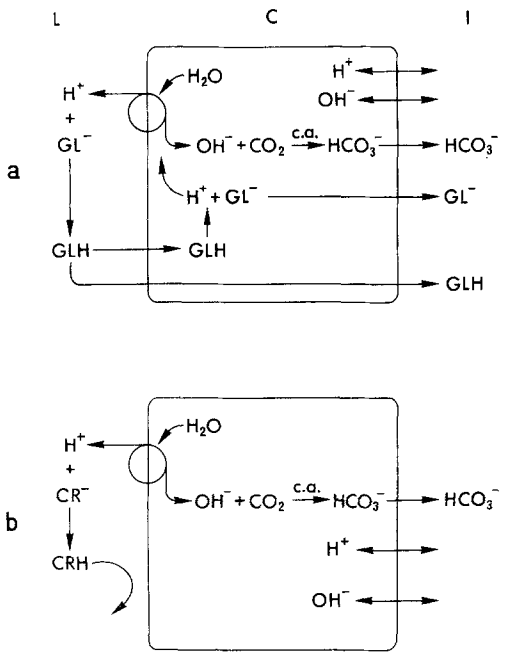

Fig. 7. Schematic representation of possible mechanisms of buffer acidification. (*a*): Acidification and reabsorption of glycodiazine, a buffer with permeant acid; (*b*): Acidification of creatinine, an impermeant buffer. *L*, lumen; *C*, cell; *I*, interstitium

mechanisms deserve comment and could explain the remaining acidification rates. First, it is possible that CO_2 diffuses from nonperfused tubules into the cells of the experimental tubule. An alternative possibility is that in the presence of permeant buffer species continued acidification after carbonic anhydrase inhibition is due to buffer recirculation. As shown in the top panel of Fig. 7, the nondissociated acid could diffuse from the lumen into the tubule cell where it could either generate hydrogen ions by dissociation, or neutralize hydroxyl ions. Given an adequately high permeability of the luminal membrane to the undissociated buffer component, such a process could sustain tubular hydrogen ion secretion. Such a mechanism has been assumed to occur in the case of glycodiazine; Ullrich, Rumrich and Baumann (1975) have actually

tive cellular CO_2 concentration during CO_2-free luminal and peritubular perfusion (0.2 mM), by a geometric factor (tubular radius/2) and by 2.0, the volume ratio of cell to lumen (Malnic & Giebisch, 1979)

$$J_{CO_2} = 0.08\ \text{sec}^{-1} \times 0.2\ \text{mM} \times \frac{15 \times 10^{-4}\ \text{cm}}{2} \times 2.0$$
$$\approx 0.24\ \text{nmol/cm}^2 \cdot \text{sec}.$$

This rate, which is an overestimate since it disregards the back reaction, is considerably smaller than that of 1.40 and 0.81 nmol/cm^2·sec, of glycodiazine reabsorption after acetazolamide (*see* Table 6); when the back-reaction is considered, according to Cogan et al. (1979), a rate of only 0.038 nmol/cm^2·sec is obtained. This indicates that H ion generation via uncatalysed CO_2 hydration is not sufficient to account for the acidification and reabsorption of this buffer species.

Table 9. Role of acid recirculation in proximal tubular acidification

	Control		Acetazolamide		
	J_H	J_B	M	J_H	J_B
GLD (CA)	3.03	2.58	10^{-3}, *c*	1.11	1.40
			10^{-3}, *l*	0.57	0.81
DMO (CA)	2.37	0.86	10^{-4}, *c*	0.89	0.195
(CB)	0.66	0.86	10^{-4}, *c*	0.096	0.195

J_H, H ion secretion; J_B, buffer reabsorption (=acid recirculation) CA, constant acid; CB, constant buffer; M, molar concentration of acetazolamide in capillary (*c*) or lumen (*l*). *J* in nmol/cm^2·sec.

evaluated H ion secretion in renal tubules using the rate of luminal disappearance of this buffer.

Clearly, such a mechanism could not be operative in the presence of impermeant luminal buffer components. This situation is depicted in the lower panel of Fig. 7. Finally, whether diffusional exchange of hydrogen or hydroxyl ions across the peritubular cell membrane can occur at a rate adequate to sustain acidification under these conditions is presently unknown.

At this point, the following question arises: do the present data indicate that acid recirculation could account fot the observed rate of acidification especially after carbonic anhydrase inhibition? In Table 9, some of the data obtained in this work are listed for comparative purposes. It is seen that, for the case of glycodiazine, flow of buffer out of the tubule is similar to the rate of H ion secretion both with and without carbonic anhydrase activity, indicating that for this buffer acid recirculation could account for the observed rate of H ion secretion. For DMO, on the other hand, buffer reabsorption is smaller than H secretion when the buffer is treated as an open system, but larger than H secretion calculated for a closed system. This shows that probably this system operates in an intermediate manner, behaving more like a closed system. In this case, the observed rate of acidification after acetazolamide can still be mediated by acid recirculation.

In conclusion, the present experiments indicate that irrespective of the nature of the luminal buffer, the proximal tubular epithelium establishes similar limiting pH gradients. Luminal buffers are titrated to this limiting hydrogen ion concentration in accordance with their specific titration curves. These depend critically not only upon pK values but also upon the permeability of the luminal membranes to the undissociated buffer components. Both parameters determine the net rate of tubular hydrogen ion secretion as well as the extent of inhibition of acidification by carbonic anhydrase inhibitors. The physiological role of buffers is critically dependent on these proper-

ties. Thus, a permeant buffer will be largely reabsorbed by diffusion of the luminal acid formed by H ion secretion; this is the case for the bicarbonate/CO_2 system. Of the buffers tested in this study specific transport mechanisms need not be invoked for buffers like bicarbonate, glycodiazine, DMO and TRIS. On the other hand, impermeant buffers will be reabsorbed to a much smaller extent. They will sustain lower rates of H ion secretion; however, due to the sustained luminal accumulation of their impermeant acid form, they will contribute importantly to the formation of urinary titratable acid.

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