J. Membrane Biol. 118, 121–130 (1990)

The Journal of
Membrane Biology

pH-Stat Experiments in Proximal Renal Tubules

G. Malnic, A.G. Lopes, A.C. Cassola, A.L. Berardi, M. Mello Aires, and G. Giebisch†
Departments of Physiology, Institute of Biomedical Science, University of Sao Paulo, Sao Paulo, Brazil, and †Yale University School of Medicine, New Haven, Connecticut 06510

Summary. The pH-stat technique has been used to measure H^+ fluxes in gastric mucosa and urinary bladder "in vitro" while keeping mucosal pH constant. We now report application of this method in renal tubules. We perfused proximal tubules with double-barreled micropipettes, blocked luminal fluid columns with oil and used a double-barreled Sb/reference microelectrode to measure pH, and Sb or 1 N HCl-filled microelectrodes to inject OH^- or H^+ ions into the tubule lumen. By varying current injection, pH was kept constant at adjustable levels by an electronic clamping circuit. We could thus obtain ratios of current (nA) to pH change (apparent H^+-ion conductance). These ratios were reduced after luminal 10^{-4} M acetazolamide, during injection of OH^-, but they increased during injection of H^+. The point-like injection source causes pH to fall off with distance from the injecting electrode tip even in oil-blocked segments. Therefore, a method analogous to cable analysis was used to obtain H^+ fluxes per cm^2 epithelium. The relation between J_H^+ and pH gradient showed saturation kinetics of H fluxes, both during OH^- and H^+ injection. This kinetic behavior is compatible with inhibition of J_H by luminal H^+. It is also compatible with dependence on Na^+ and H^+ gradients of a saturable Na/H exchanger. H^+-ion back-flux into the tubule lumen also showed saturation kinetics. This suggests that H^+ flow is mediated by a membrane component, most likely the Na^+-H^+ exchanger.

Key Words pH stat · H^+ fluxes · H^+ microelectrodes · H^+ secretion kinetics

Introduction

The pH-stat technique has been widely applied to "in vitro" studies of acidifying epithelia such as gastric mucosa [19] and turtle [23] as well as toad bladder [7]. The method allows us to study H-ion secretion at constant luminal (or mucosal) pH, that is, under conditions of constant transepithelial H-ion gradients and fluxes. In the turtle bladder, the pH-stat method defined the slope of H-ion secretory rate and mucosal pH, a relationship described by Steinmetz et al. as "active H-ion conductance" [23]. This conductance depends on a number of factors such as carbonic anhydrase activity, the acid-base status, the CO_2 level and the availability of metabolic substrates [23]. On the other hand, the maximal pH gradient of this epithelium — the protonmotive force — is remarkably constant.

Acidification in the renal tubule has mostly been studied in conditions where luminal pH varies with time or distance. For instance, in free-flow micropuncture or in continuous microperfusion experiments, pH falls along the segment under study [15], whereas in stopped-flow microperfusions luminal pH changes with time [5, 8, 13].

These problems can be circumvented by the pH-stat method, and the present study evaluates the relation between H^+ secretion and luminal pH in the proximal tubule under steady-state conditions. We present evidence that H^+ secretion depends on luminal pH and that both secretory and reabsorptive H^+ fluxes show saturation kinetics.

Materials and Methods

Male Wistar rats weighing 250–300 g were anesthetized with pentobarbital and prepared for "in vivo" micropuncture as described previously [13]. Animals had access to a standard rat pellet diet and to water until the time of the experiment. The kidney was prepared by a lumbar approach, and standard micropuncture techniques were used [5, 10, 13].

Figure 1 provides a schematic description of the method as applied to proximal tubule segments. A double-barreled theta glass micropipette was used to perfuse the lumen with two solutions in *A* and *B* (R&D Optical Systems, Spencerville, MD), one solution being colored by addition of 0.05% Lissamine Green. Another micropipette blocked the tubule by injecting it with colored castor oil. A double-barreled microelectrode impaled the tubule at a distance of one or more loops downstream in order to measure pH. One barrel of this microelectrode assembly (diameter 3–5 μm) was an antimony electrode, the other a glass reference micropipette filled with 1 M NaCl/Agar at the time of the experiment. Luminal pH was set at the desired levels by injecting either OH ions via a single Sb microelectrode (making the electrode negative with respect to ground), or H^+ ions via a 1 N HCl-filled

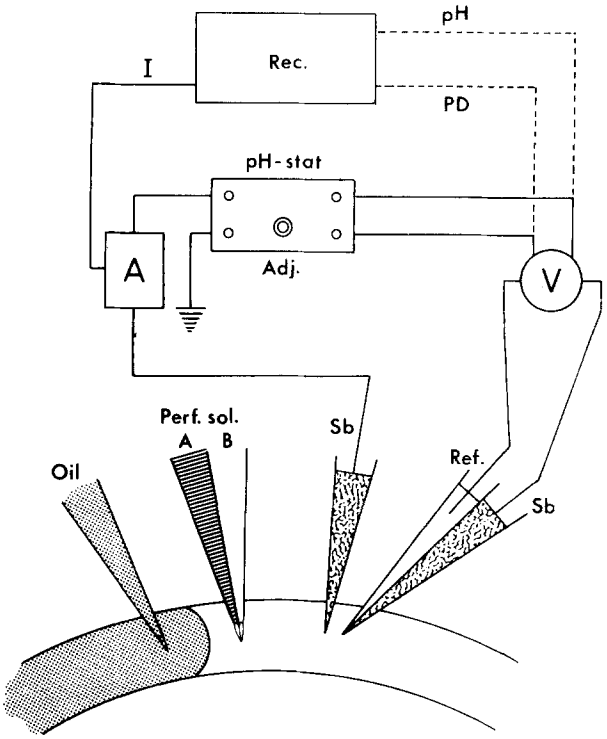

Fig. 1. Schematic representation of the pH-stat method. Shown are: an oil-filled micropipette for tubule blocking; perfusion solutions contained in double-barreled micropipette; *Sb*, antimony microelectrode to inject OH ions; double-barreled microelectrode for pH measurement (Sb/reference); *V*, differential amplifier; *A*, current meter; *Rec*, recorder; *pH-stat*, automatic feed-back system for pH-stat adjustment

conventional microelectrode (not shown in Fig. 1). The HCl-filled electrode was made of filament-containing glass capillaries, and the electrode was rendered positive with respect to ground.

The electronic device for pH clamping consisted of a current generator made of a matched pair of voltage followers built into small high-impedance probes to which the electrodes were connected. Luminal pH was changed to preset levels by current modulated by voltage steps proportional to (i) the pH measured in the tubular lumen, and (ii) an offset voltage set to achieve different pH values in the lumen. Current injected via the antimony or the HCl electrode was measured by a Keithley Mod. 602 electrometer, isolated from ground by a Mod. 399 isolating amplifier. Current, pH and transepithelial voltage (recorded from the reference barrel) were recorded on a Beckman Mod. R511A Dynograph. The measured voltages were digitized and pH calculated by a Hewlett-Packard Mod. 9603A computing system.

The transport number of OH^- and H^+ of the current-delivering microelectrodes was determined routinely "in vitro" using a Beckman Mod. 9873B integrator coupler to obtain the total charge passed through the electrode. For the Sb microelectrode (OH^- injection): $t = 0.92 \pm 0.017$ ($n = 5$), and for the HCl electrode: (H^+ injection) $t = 1.09 \pm 0.040$ ($n = 9$), where t = the ratio of OH^- or H^+ delivered/total current passed through the electrode. Standardization of the OH^+ or H^+-injecting electrodes was carried out using 5-nl samples of K^+ acid phtalate or CO_2 equilibrated bicarbonate solutions.

Because OH^- and H^+ ions are injected into the tubule lumen from a point source, the pH varied markedly with distance from the point of impalement. As shown below, the space constant for pH (obtained from pH measurements at increasing distance from the site of insertion of the current electrode) is in the range of 15 to 20 μm, and it is not feasible to work on isolated fluid columns short enough to keep luminal pH constant. To correct for pH decay along the tubule, we used an application of cable analysis where voltage is analogous to pH and current to H ion flow. The relations used are the following [11]:

Table 1. Velocity of pH front in model system with 5 mM phosphate Ringer's solution at different pH

pH	μm/sec	n
4.8	24.8 ± 1.23	(9)
5.7	23.0 ± 0.53	(4)
6.5	26.8 ± 0.99	(6)
7.5	30.0 ± 2.11	(6)
8.3	29.2 ± 1.64	(8)

$$R_m = (\pi^2 \cdot d^3 \cdot R_0^2)/R_i \quad (1)$$

$$R_m = (2 R_i \cdot \lambda^2)/r \quad (2)$$

where R_m is the specific epithelial resistance to H^+, R_o the effective resistance (i.e., pH/I, where I is the H^+ or OH^- current injected into the lumen); R_i, the resistivity of luminal fluid to H^+; λ, the length constant; r, the tubular radius, and d, the tubule diameter. For the calculation of R_o, pH is expressed in volts (61.5 mV per pH unit) and I in amperes.

Two approaches can be used to evaluate the value of R_i. First, the value of R_i can be calculated from the equivalent H ion conductance at the pH of luminal fluid [16]. By this method, values of the order of $2.5 \times 10^7\ \Omega \cdot$ cm are obtained. Using this value of $2.5 \times 10^7\ \Omega \cdot$ cm for H^+ resistivity of luminal fluid in Eqs. (1) and (2), two markedly differing R_m values are obtained. However, by equating Eqs. (1) and (2), an experimental value of R_i of $1 \times 10^4\ \Omega \cdot$ cm can be *calculated*. A likely explanation of the much lower value of R_i obtained in this way is that H^+ current is not only carried by H^+ ions in free solution, but mostly by buffer molecules, for instance, $H_2PO_4^-$ [14]. Second, R_i can also be *calculated* from the equivalent conductance of a solution containing 5 mM phosphate. Using this method, a value of R_i or $4 \times 10^3\ \Omega \cdot$ cm is obtained (the equivalent conductance of phosphate, $\lambda = 50\ \Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{Eq}^{-1}$) [16]. At a phosphate concentration of 5×10^{-3} M, the conductivity of the solution, K is $(\lambda \cdot C)/1000 = (50 \times 5 \times 10^{-3})/1000 = 250 \times 10^{-6}$ mho/cm. Thus, $R_i = 1/K = 4 \times 10^3\ \Omega \cdot$ cm).

We tested the assumption that R_i remains constant during the variations of luminal pH that were induced in the perfusion experiments. We used an experimental model system in which the speed of the movement of a pH wave was used to evaluate the apparent H^+ ion conductivity at different pH of buffer solutions. It consisted of a 2-mm o.d. glass capillary into which an antimony microelectrode was cemented at one end for injection of current (OH^-) and into which another electrode was introduced at the opposite end by means of a micromanipulator. The capillary was filled through a side tube with 5 mM phosphate Ringer's solution, adjusted to different pH levels, and similar to that used for tubule perfusion. This buffer solution contained 1.5% agar to avoid convective fluid movement. Constant current (0.5 μA) was injected, and after a given interval of time, an alkaline pH wave reached the measuring pH electrode. The rate of change of pH

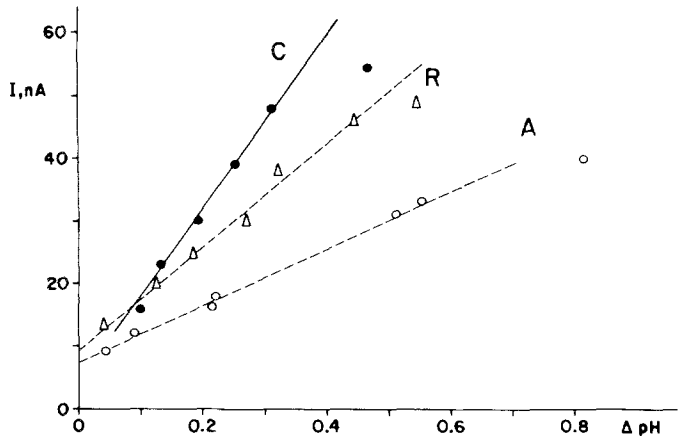

Fig. 2. Relationship between injected OH ion current (I) and effected pH change in control (C), acetazolamide perfused (A) and control after acetazolamide (recovery, R) tubules

was recorded via a Keithley Mod. 616 electrometer by means of a dV/dt coupler of a Beckman Dynograph. Table 1 shows the results obtained at different pH of the buffer solutions. Although there is a tendency for the velocity of the pH front to increase with increasing pH, the observed differences are not significant. Accordingly, no variations of R_i occur with the solutions used in our experiments.

Since the R_i value so calculated is of the order of that obtained from the present experimental data, H ion conductance in solution appears to depend largely on carrier molecules, and the use of R_i obtained from our experimental data is justified.

H ion fluxes induced by lumen pH changes were calculated by

$$J_H = V/(R_m \cdot F) \quad (3)$$

where $V = 61.5 \times 10^{-3} \times \Delta pH$, R_m is obtained from Eqs. (1) and (2), and F is Faraday's constant which converts current (amperes/sec) into ionic flow.

To improve the precision of measurements, we used the same tubule to compare control and experimental conditions, keeping the position of the current injecting and pH measuring microelectrodes unchanged. This can be done by changes in luminal perfusion solution.

Luminal perfusion solutions contained 110 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4 mM KCl and 5 mM phosphate buffer, bringing the osmolality to 300 mOsm with raffinose. Standard solutions for the calibration of antimony microelectrodes had the same composition except for raffinose, pH being adjusted to different levels with NaOH or HCl 0.1 N. Blood pH and pCO_2 were measured in arterial blood samples by means of a Radiometer Mod. PHM 72/MK2/BMS3 pH meter and blood gas system.

Statistical comparisons were made by paired t test or by analysis of variance and contrast determinations by the Scheffe test, as appropriate [22].

Results

OH^+ and H^+ Injection into Single Tubules

Figure 2 summarizes data for a representative perfusion experiment in which luminal pH was changed at different pH values above control pH. During injection of OH^- into the tubular lumen, there is a linear change of luminal pH over a pH range of 0.5 to 1 pH unit above the control level. When larger currents (>40–50 nA) were used, the slope decreased. Table 2 lists the mean ratios of the injected current per change of one pH unit (nA/pH) for the control situation in the linear range. The pH-stat current, necessary to maintain luminal pH at 7.4, and the initial control pH is also given. The relationship I/Δ pH is an "apparent" H ion conductance and includes H^+ ion movement by passive diffusive and carrier-mediated mechanisms [19, 23].

Table 2. H ion fluxes and apparent conductances measured by the pH-stat technique in renal proximal tubules

	Inj. OH^-	Inj. H^+
S, nA/ΔpH	51.1 ± 5.14 (40)	>6.3: 48.0 ± 8.13 (10) <6.3: 21.3 ± 1.88 (16)
J_H (pH = 7.4) (nA)	36.5 ± 2.91 (33)	
pH_o	6.67 ± 0.038 (32)	6.88 ± 0.035 (13)

S, apparent H^+-ion conductance; J_H, pH-stat current for pH 7.4; pH_o, stationary pH before ion injection.

Table 2 and Fig. 3 show apparent H ion conductances during H^+ ion injection. Above a luminal pH of 6.3, the slope of I/Δ pH is similar to that found during OH^- injection, but at lower pH, it is markedly decreased. This observation suggests that, as in the case of OH^- injection, the H^+ ion conductance of the epithelium is dependent on luminal pH and decreases at lower luminal pH.

The Effects of Acetazolamide

We studied the effect of acetazolamide on H^+ ion conductance by perfusing the lumen successively with control solutions and with solutions containing

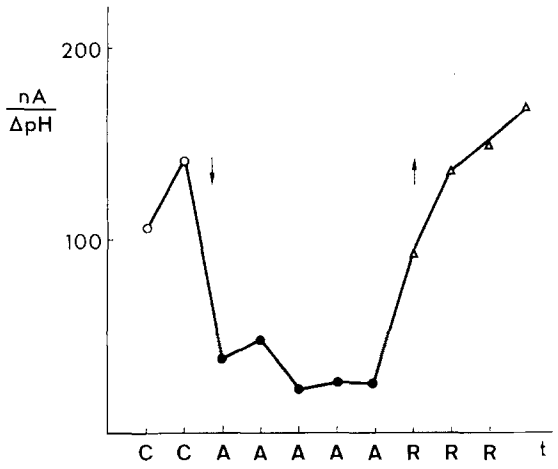

Fig. 3. Changes in apparent H ion conductance (H^+ ion secretion measured during OH^+ injection) during perfusions in different experimental situations, *C*, Control; *A*, acetazolamide; *R*, recovery. Every point is obtained from a line as given in Fig. 2

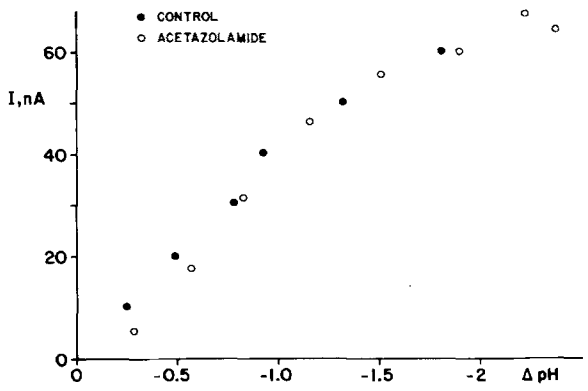

Fig. 4. Relationship between injected H^+ current and effected pH^+ change in proximal tubule

10^{-4} M acetazolamide. Figure 2 and Table 3 show a highly significant reduction in this conductance. This difference is significant both in the pooled data groups and when control and acetazolamide perfusions were performed in a paired way in the same tubule (*see* last line of Table 3). The reduction in H ion conductance by acetazolamide is compatible with the decreased availability of H ions in the tubule cell or with a direct inhibitory effect on the Na/H exchanger.

The effect of acetazolamide during luminal acid injection, however, is opposite of that obtained during OH^- injection (*see* Table 3 and Fig. 4). H^+ ion conductance — again measured in the linear range — is increased during perfusion with the drug (− by 54% in paired experiments). These results show that H^+ ion back-flux across the epithelium does not

Table 3. Effect of acetazolamide on apparent H ion conductance of proximal tubule

	Inj. OH^-	Inj. H^+
S_c, nA/ΔpH	67.4 ± 9.81 (17)	21.3 ± 1.88 (16)
S_a, nA/ΔpH	29.8 ± 4.22 (22)	33.0 ± 2.89 (26)
S_r, nA/ΔpH	64.6 ± 19.4 (8)	25.8 ± 2.95 (16)
S_a/S_c	0.45 ± 0.046 (7)[a]	1.54 ± 0.17 (27)[a]

C, control; *a*, acetazolamide; *r*, recovery; *Sa/Sc*, same tubule conductance ratios.
[a] Different room 1 ($P < 0.01$).
S_c: control apparent H^+ ion conductance.
S_a: H^+ ion conductance during perfusion with acetazolamide.
S_r: Recovery after acetazolamide.

Table 4. Effect of capillary perfusion with 5 mM phosphate Ringer's on apparent H ion conductance measured by the pH-stat method (nA/ΔpH)

Experiment	Control[a]	Cap. Perfusion
OH^- Inj.	31.4 ± 4.84 (14)	20.1 ± 4.57 (12)
H^+ Inj., pH > 6.3	43.7 ± 3.90 (13)	33.9 ± 6.23 (13)
H^+ Inj., pH < 6.3	21.4 ± 2.31 (12)	14.3 ± 2.09 (13)[b]

[a] Blood in peritubular capillaries.
[b] $P < 0.05$ (difference with controls.

depend on cellular H^+ ion generation, since these ions are furnished by the injecting electrode. The reason for the observed increase in conductance, however, is not entirely clear. The absence of a secretory H^+ ion flow, opposite of that due to H^+ injection, may be a possible cause. The normally present secretory flux would reduce changes in pH when the rate of H^+ ion injection is altered and lower H^+ ion conductance in control conditions.

Effects of Peritubular Perfusion with Bicarbonate-Free Solutions

When an acid solution is injected into the tubular lumen, alkalinization occurs both by efflux of H^+ ions and influx of bicarbonate ions from blood to lumen [12]. Although the magnitude of epithelial bicarbonate permeability is not large, it contributes to the net fluxes of H^+ and bicarbonate across the proximal tubule epithelium [3, 10]. Accordingly, we tested the influence of peritubular changes of bicarbonate on H^+ fluxes measured by the pH-stat system. Table 4 shows that the apparent tubular H ion conductances during injection of OH^- and H^+ are not significantly different during capillary perfusion with blood and with Ringer's solution, with the ex-

Table 5. Cable analysis of H^+ transport in proximal tubules

	Injection of OH^-	Injection of H^+
λ, μm	15.9 ± 1.28 (13)	17.1 ± 1.10 (4)
R_i, $\Omega \cdot$ cm	1.07×10^4	pH>6.3: 1.06×10^4
		<6.3: 2.37×10^4
R_m, $\Omega \cdot cm^2$	35.9	pH>6.3: 41.2
		<6.3: 92.0

λ, length constant; R_i, resistivity of luminal fluid to H^+; R_m, apparent specific resistance of epithelium to H^+.

ception of a small difference in the pH range below 6.3. This confirms the prediction that passive bicarbonate fluxes play only a minor role in determining luminal steady-state pH levels. The reduction of peritubular bicarbonate concentrations should increase the sodium-coupled bicarbonate efflux from the cell, thereby reducing intracellular bicarbonate and thus pH, factors that stimulate H^+ ion secretion. These events, however, do not necessarily affect H^+ ion conductance, the relation between H^+ ion fluxes and pH.

Cable Analysis of pH-Stat Data

Table 5 summarizes our data derived by cable analysis and permits calculation of H^+ ion flows per unit area. The data show that length constants, when OH^- or H^+ ions are injected, are of very similar magnitudes. Tubular fluid resistivity are those of a phosphate-containing solution of 5 mM, as discussed above. Note that the specific epithelial resistance to H^+ is of similar magnitude for OH^- and H^+ injection above pH 6.3, but higher at lower pH. From these R_m values, it is possible to calculate net H ion flux per cm^2 epithelium for a luminal pH of 7.4 according to the expression (*see* Materials and Methods)

$$J_H = V/(R_m \cdot F) = \frac{(7.4 - 6.67) \times 61.5 \times 10^{-3}}{35.9 \cdot 96{,}500}$$
$$= 13 \text{ (nmol)/cm}^2 \cdot \text{sec.} \quad (4)$$

This value can be compared to the rates of bicarbonate reabsorption in rat proximal tubules, either measured by stationary perfusion (3 nmol/cm^2 · sec at 20 mM initial bicarbonate) or by continuous microperfusion (5 nmol/cm^2 · sec at 25 mM initial bicarbonate [2]. The J_H^+ obtained by the pH-stat method for pH 7.4, however, are higher than these values, indicating that at a constant pH of 7.4 the rate of H^+ ion secretion is considerably higher than when luminal pH falls toward its steady-state level (pH = 6.7).

H^+ Fluxes at Constant Luminal pH

Figure 5 shows the relation between steady-state H fluxes and luminal pH levels. Fluxes are presented as the ratio of the measured flux at a given luminal pH over the maximal flux observed. Data obtained during capillary perfusion with 5 mM phosphate Ringer's are included for comparison. These data are similar to those obtained during experiments with blood-perfused capillaries. The Figure shows that there is a linear relationship between H^+ ion fluxes and luminal pH to approximately 0.8 pH units above and below the stationary pH level. Beyond this point, the curve flattens, indicating that stimulation of H secretion by luminal pH or H ion back-flux of the tubule is limited beyond this pH range.

Kinetic Analysis of H Fluxes

Since the major portion of proximal tubule acidification depends on the operation of an electroneutral Na^+/H^+ exchanger, the increase in H^+ ion flux with the fall in luminal H ion concentration can be analyzed, assuming inhibition of H fluxes by luminal [H], an approach used by Aronson et al. for brush-border membrane vesicles [6]. For this purpose, we use the Michaelis-Menten relationship for the action of an inhibitor [14]

$$V = \frac{V_{max} \cdot [S]}{[S] + K_m(1 + [I]/K_i)} \quad (5)$$

where V = rate of H^+ or Na^+ flux across the exchanger, S = substrate concentration (Na^+ in the compartment from where the flux originates), K_m = apparent Michaelis constant for substrate S, I = inhibitor concentration, and K_i = Michaelis constant of the inhibitor (for competitive inhibition).

In our experimental conditions, $V = J_{Na}$ into cell = J_H into lumen; $[S]$ = [Na] in lumen; K_m = Michaelis constant for noninhibited Na or H^+ flux; $[I] = [H_L]$; K_i = Michaelis inhibition constant for luminal H^+.

In Fig. 6, this equation is plotted in the linearized form

$$1/V = 1/V_{max}(1 + K_m/[S] + K_m/(V_{max}[S]K_i) \cdot [I]. \quad (6)$$

The experimental data used in Fig. 6 are those in the alkaline range of Fig. 5, V being equivalent to secretory H fluxes in relative units and $[I]$, luminal H ion concentration. The intercept of this line with the x axis is

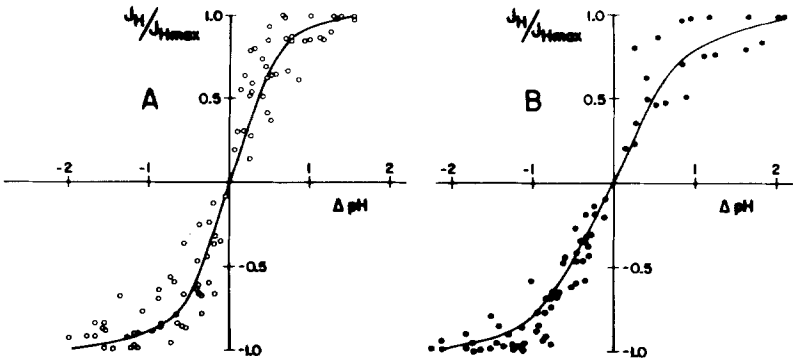

Fig. 5. H^+ secretion ($+J_H$) and back-flux ($-J_H$) relative to the maximal rates (J_{Hmax}) at luminal pH clamped at different levels above and below the stationary level by the pH-stat system. *Left*: experiments with luminal perfusion only; *right*: luminal plus capillary perfusion with 5 mM phosphate Ringer

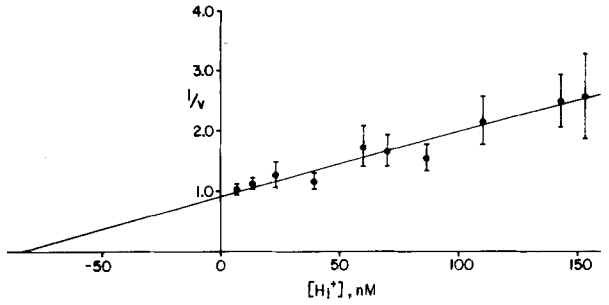

Fig. 6. Inhibition kinetics (Dixon plot) relating $1/J_H$ with luminal H^+ levels in control proximal tubules. The experimental points are means ± SE

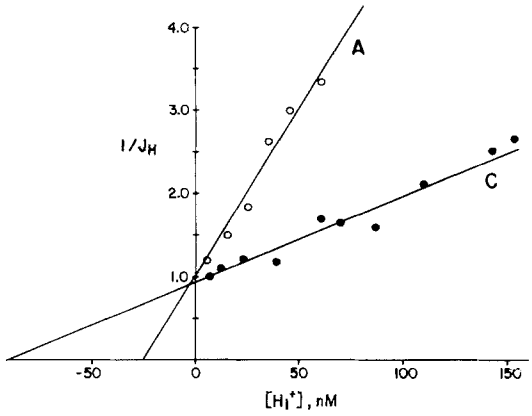

Fig. 7. Comparison of inhibition kinetics in control (*C*) and acetazolamide (*A*) perfused proximal tubules. Points are means of values for a given range of luminal H^+ ion concentration

$$-[I] = K_i\,(1 + [S]/K_m) = K_H \text{ apparent.} \quad (7)$$

In order to obtain K_i, we should perform an experiment at [Na] = 0. Since such a condition is difficult to obtain in vivo, we have used data from experiments on brush-border membrane vesicles in vitro [6]. Using the value of the x axis intercept of Fig. 6 $K_{Happarent} = -I = 86.8$ nM ($x\ 10^{-9}$), [S] = 145 mM for luminal sodium and $K_{m_{Na}} = 50$ mM for a luminal pH of 6.7 (*see* ref. [6]) in equation

$$K_{Happ} = K_{H_{Na=0}}\,(1 + [Na]/K_{Na}) \quad (8)$$

we obtain a value for $K_{H_{Na=0}}$ of 22.3 nM. This corresponds to a pK of 7.65 for the titration site of brush-border H sensitivity. This value compares with a $K_{Happarent} = K_{H_{Na=0}} = 35$ nM in brush-border membrane vesicles, equivalent to an apparent pK = 7.5 [6]. Thus, our in vivo value of 7.65 for the pK of H^+ ion inhibition of the Na-H exchange is very similar to the in vitro data obtained in brush-border membrane vesicles.

Figure 7 depicts the similar relationship in which the values after treatment with acetazolamide are compared to the controls. Here, an intercept of 24.5 nM is found corresponding to a $K_{H(Na=0)}$ of 6.27 nM and pK of 8.2. The higher pK indicates that the site of H^+ ion binding has a greater sensitivity to inhibition by H ions after acetazolamide.

An alternative approach for calculation of the kinetic parameters between luminal pH and H^+ ion secretion involves the use of the chemical driving forces (μ_H) for H^+ movement by a Na^+-H^+ exchanger

$$J_{H^+} = Ls\mu_{H^+} = Ls\,(RT \ln (Na_l/Na_c) + RT \ln (H_c^+/H_l^+)). \quad (9)$$

Here, Ls is a transport coefficient, and C_l and C_c are ion concentrations in lumen and cell, respectively. For the calculation, sodium concentrations are considered to be constant and lumen and cell H levels change. Cell H is calculated based on the experimentally established relationship between lumen and cell pH [2, 25].

However, if J_H^+ is to be limited by the number of Na/H exchangers, a different relationship, based

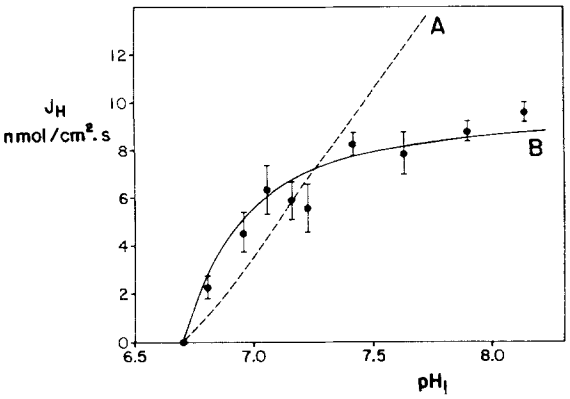

Fig. 8. Dependence of secretory H^+ ion flow (J_H) on luminal pH (pH_l). Line *A* calculated by Eq. (9), line *B* by Eq. (10). The points (means ± SE) are those given in Fig. 5

on saturation kinetics, including μ_H, the driving force for H in this system, must be used

$$J_H = (J_{max}\,\mu_H)/\mu_H + K_m). \qquad (10)$$

In Fig. 8, the theoretical relationships described above (Eqs. (9) and (10)) are compared with our experimental data. The derived line (*A*) predicted by Eq. (9), does not fit the experimental data, whereas a relationship based on Eq. (10) (*B*) fits the data well. This behavior is compatible with ion-gradient driven H^+ flow through the Na/H exchanger together with a limited number of exchanger sites in the membrane.

Kinetics of H^+ Ion Back-Flux

The data shown in Fig. 5 demonstrate that H^+ ion back-flux across the proximal tubule is not a linear function of luminal pH. Therefore, we have also applied Michaelis-Menten kinetics to analyze this transport mechanism. Plotting our data in the form of $[H_l^+]/_V$ as a function of luminal H^+ as shown in Fig. 9 yields a straight line with $K_{Happarent}$ of 194 mM (pK = 6.71). We conclude that H^+ ion back-flux occurs by interaction with a saturable membrane component.

Maximal Transport Rates

Table 6 summarizes mean maximal H^+ ion fluxes obtained from individual pH-stat experiments. V_{max} for H^+ secretion may also be obtained from Figs. 6, 7, and 9. Using the *y* intercept of Fig. 6 and K_{Na} from Aronson et al. [6], a value of 11.04 nmol/cm² · sec is obtained for control rats, similar to the mean experimentally observed value of 9.68 ± 0.16 [19] nmol/cm² · sec (Table 6). For H^+ ion back-flux (Fig. 9), a V_{max} of 10.0 nmol/cm² · sec is obtained, similar to the mean value of the maximal H ion back-flux data observed experimentally, 10.7 ± 0.11 nmol/cm² · sec.

Table 6. Maximal H ion fluxes observed in proximal tubules by the pH-stat method (nmol/cm² · sec)

	H secretion	H back-flux
Control	9.68 ± 0.86 (28)	10.7 ± 0.11 (23)
Acetazolamide	6.53 ± 1.22[a] (13)	12.6 ± 0.60[a] (10)

[a] ($P < 0.01$) with controls.

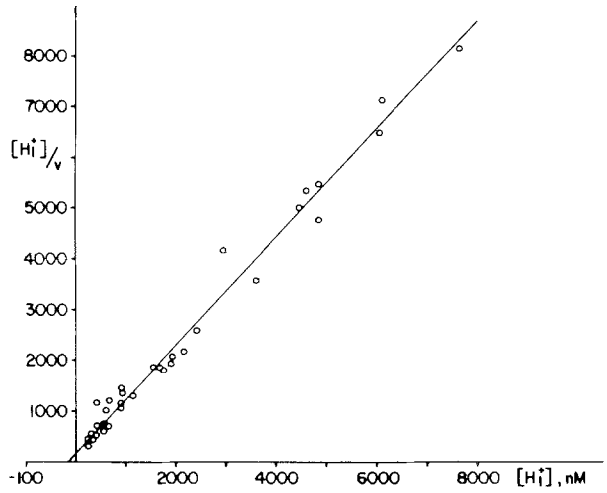

Fig. 9. Michaelis-Menten kinetics of H^+ ion back-flux from tubule lumen. $[H_l^+]$, luminal H^+ ion concentration; *V*, J_H

Discussion

The pH-stat method has been applied to H secreting membranes like gastric mucosa and turtle bladder, because it permits the direct determination of the amount of H secreted per unit membrane area and time during the imposition of known pH gradients. Our current study is the first application to use this method to measure the H^+ ion secretion in renal tubules.

The relationship between transepithelial H^+ fluxes and pH changes in the bathing solutions has been carefully studied in the turtle bladder under a wide variety of experimental conditions. An "active H ion conductance" was defined by Steinmetz [23] and Al Awqati [1] as the slope of J_{H^+}/pH. In terms

of an electrical model elaborated by these authors for the turtle bladder, this conductance corresponds to the series resistance of the electrogenic ATP-driven H^+ ion pump [23]. According to this analysis, the series resistance depends either on the number of active H ion transport sites acting in the membrane in parallel, the turnover rate of the pumps or the availability of H^+ ions to the pump. The nature of this conductance can also be defined by an analog model of H^+ ion secretion in which the process of H ion secretion is represented by an electrical analog circuit consisting of an EMF, its series resistance, a capacitor that is charged by the EMF, and a shunt resistance [5].

In this model and in the analysis of the data of our study, we have used an electrical approach for the interpretation of an essentially electrically neutral process of H^+ ion secretion via the Na^+/H^+ exchanger. In our analysis, voltage is analogous to the transepithelial pH gradient, and current to hydrogen ion fluxes. This allows the use of several standard electrophysiological approaches to the analysis of ion transport, independent of its mechanisms.

Load Dependence of Renal Tubule Acidification

One of the important applications of the pH-stat technique is the analysis of the mechanism of load dependence of proximal renal tubule acidification and bicarbonate reabsorption. Normally, the nephron segment avidly reabsorbs bicarbonate by rapidly reducing its concentration in the lumen. An increased buffer load, induced either by raising GFR or by elevating the filtered buffer (bicarbonate) concentration, leads to increased H secretion and higher bicarbonate reabsorption rates [3, 9, 13]. Such stimulation of acidification is, in part at least, related to increased buffer concentrations along more downstream and hence normally unsaturated tubule segments (axial effect of increased buffer load). Also, an increased flow of fluid along the tubule counteracts limiting bicarbonate gradients that may be present at some site within the epithelium (a radial limitation of bicarbonate reabsorption) [4]. Besides such factors it is, however, well recognized that elevated buffer concentrations, particularly those of bicarbonate, are powerful factors stimulating H ion secretion [3, 9, 13]. We provide evidence that an increased delivery of bicarbonate stimulates H^+ ion secretion by its effect on luminal pH.

Figure 5 demonstrates that as luminal pH is elevated, H^+ ion secretion increased linearly up to approximately 0.8 to 1 pH unit above and below the physiological pH level of 6.7. Using this value, the maximum rates of bicarbonate reabsorption occur at about 1 pH unit above this value and correspond to a luminal bicarbonate concentration of 48 mM at 40 mmHg CO_2 and of 65 mM and 55 mmHg. These values can be compared with the saturation level of bicarbonate transport of 45 mM reported by Alpern et al. [3]. There is a linear relationship between bicarbonate concentration and reabsorption rate [13]. Thus, the present data confirm the linearity of the relation between luminal buffer concentration and H^+ secretory rate over a wide range of physiological concentrations. The data also confirm the existence of transport saturation above this concentration range.

What is the mechanism of the stimulating effect of luminal pH on H^+ ion secretion? In turtle bladder and in renal epithelium acid secretion can be stimulated by CO_2 via exocytosis of apical vesicles containing H ion pumps (H-ATPases) into the apical membrane of H^+-transporting epithelia [21]. It is, however, unlikely that this mechanism mediates the effect of acute luminal pH changes since it is dependent on *intracellular* acidification. In our experiments, an elevation of luminal pH, on the other hand, must be associated with cell alkalinization. Thus, increased H^+ secretion at elevated luminal pH must be due to the more favorable concentration gradient of Na^+/H^+ exchange.

Our analysis of H^+ ion secretion, in which we used either inhibition kinetics [6] or Michaelis-Menten kinetics with substrate concentration replaced by the combined chemical potential gradients of Na^+ and H^+, supports the view that the interaction of the brush-border Na^+/H^+ exchangers with luminal $[H^+]$ determined secretory H^+ ion fluxes across the apical membrane. Note that the kinetic parameters of interaction of luminal H^+ with the Na^+/H^+ exchanger derived from our experiments in intact tubules in vivo are very similar to those obtained in studies on brush-border membrane vesicles.

We further confirm that luminal H ions interact with the Na^+/H^+ carrier at a single site where competition with Na and other cations as well as amiloride takes place [6]. This conclusion is based on the linearity of the relationship 1/(H secretion rate) with luminal H^+ concentration (*see* Figs. 6 and 7).

This behavior is different from the transport properties of the cytoplasmic interface of the Na^+/H^+ carrier, where kinetic studies have indicated the interaction of H ions at two sites, a transport and an activator site [6].

In principle, the interaction of Na^+ and H^+ at

the apical exchanger site could be due to two processes. First, H^+ ion may exert a specific inhibitory action on the external exchange site, an event independent of its cellular concentration. Second, H^+ ion concentration could affect the exchange rate of the carrier by modulating the transmembrane concentration difference of H^+ ions. The fact that our kinetic analysis has established gradient dependence of H^+ secretion and back-flux favors the view that it is the chemical potential difference of H^+ and Na^+ across the apical cell membrane that determines H^+ ion fluxes. An exclusive external inhibitor site where H^+ ions block the carrier is not required.

We have not investigated the role of changes of sodium concentrations in our experiments since we had previously demonstrated [17] in the isolated perfused rat kidney, that solutions containing low Na^+ plus amiloride sharply impair transepithelial H^+ efflux in proximal tubules.

Mechanism of H^+ Ion Back-Flux

The observation that H ion back-flux from the lumen also reaches a saturation level is of interest. Such saturation was also observed in the turtle bladder and explained by a model where the back-flux of H^+ ion flow from serosa to mucosa was postulated to pass through the same pump mechanism responsible for active H ion secretion [24]. Our studies suggest that H flux out of the lumen may likewise flow through a transport path involving specific membrane interactions. Schwartz suggested carrier-mediated H^+ ion back-flux across renal epithelia since he observed a reduction in H^+ flux in isolated perfused rabbit proximal tubules when sodium was removed from the lumen [20]. Rubio et al. found reduced H back-flux in proximal tubules of isolated perfused rat kidney at low Na^+ in the presence of 10^{-3} M amiloride [17]. These authors also observed an effect of temperature on H^+ flux, activation energies of the same order (8–9 kcal/mol) being found for H^+ ion secretion and back-flux [18]. These data suggest that both H ion secretion and back-flux are mediated by the same carrier, the Na^+/H^+ exchanger.[1]

In summary, we have demonstrated that, with special adaptations, it is possible to utilize the pH-stat method for the renal tubule. As in the turtle bladder, an apparent "active conductance" can be measured for H^+ ion secretion by the epithelium; this conductance is markedly reduced by carbonic anhydrase inhibition. In the acid pH range, achieved by H^+ ion injection into the lumen, this conductance is decreased by capillary perfusion with bicarbonate-free solutions, demonstrating back-flux of bicarbonate across renal proximal epithelium. Finally, the analysis of the relationship between luminal pH and H^+ ion secretion by the proximal tubule epithelium shows this relation to be linear over a range of approximately ± 0.8 pH units above and below the stationary pH, reaching a plateau beyond these levels. These and other experimental data support the theory of bi-directional H ion flow across the apical Na^+/H^+ exchanger.

This work was supported by Fundaçao de Amparo à Pesquisa do Estado de Sao Paulo, Financiadora de Estudos e Projetos, Conselho Nacional de Pesquisas, and NIH DK 17433-16. Part of this material has been presented in abstract form in VIII Proceedings International Biophysics Congress, Bristol, England, 1984, p. 295A, and Proceedings, Satellite Symposium of International Congress of Nephrology, Fürigen, Switzerland, 1987, p. 57A.

References

1. Al-Awqati, Q. 1978. H transport in urinary epithelia. *Am. J. Physiol.* **235:**F66–F77
2. Alpern, R.J., Chambers, M. 1986. Cell pH in the rat proximal convoluted tubule. *J. Clin Invest.* **78:**502–510
3. Alpern, R.J., Cogan, M.G., Rector, F.C. 1982. Effect of luminal bicarbonate concentration on proximal acidification in the rat. *Am. J. Physiol.* **243:**F53–F59
4. Alpern, R.J., Cogan, M.G., Rector, F.C. 1983. Flow dependence of proximal tubular bicarbonate reabsorption. *Am. J. Physiol.* **245:**F478–F484
5. Amorena, C., Fernandes, D.T., Malnic, G. 1984. Factors affecting proximal tubular acidification of non-bicarbonate buffer in the rat. *J. Physiol. (London)* **352:**31–48
6. Aronson, P.S., Suhm, M.A., Nee, J. 1983. Interaction of external H^+ with the $Na^+ - H^+$ exchanger in renal microvillus membrane vesicles. *J. Biol. Chem.* **258:**6767–6771
7. Arruda, J.A.L., Sabatini, S., Westenfelder, C. 1982. Serosal Na/Ca exchanger and H^+ and Na^+ transport by the turtle and toad bladders. *J. Membrane Biol.* **70:**135–146
8. Cassola, A.C., Giebisch, G., Malnic, G. 1977. Mechanism and components of renal tubular acidification. *J. Physiol. (London)* **267:**601–604
9. Chan, Y.L., Biagi, B., Giebisch, G. 1982. Control mechanisms of bicarbonate transport across the rat proximal convoluted tubule. *Am. J. Physiol.* **242:**F532–F543

[1] Attention has been drawn to the fact that nonlinear behavior of ion fluxes is not unique to carrier-mediated transport processes [10*a*]. For instance, H ion back-flux may obey the following equation describing passive movement:

$$-J_H = L_S [RT \ln^* H_l/H_c + ZFE)] \quad (11)$$

where L_S is a permeability coefficient, H_l and H_c are lumen and cell H ion activities, and E is the PD across the apical cell membrane. The relationship depicted in Eq. (11), similar to Eq. (9), is nonlinear because it contains a logarithmic term. Our data describing passive H ion flux fit Eq. (11). Yet carrier-mediated participation in H ion back-flux is supported by Na dependence and the inhibitory action of amiloride [17].

10. Chan, Y.L., Malnic, G., Giebisch, G. 1983. Passive driving forces of proximal tubular fluid and bicarbonate transport: Gradient dependence of H^+ secretion. *Am. J. Physiol.* **245:**F622–F633
10a. Danisi, G., Lacaz Viera, F. 1974. Non-equilibrium thermodynamic analysis of the coupling between active sodium transport and oxygen consumption. *J. Gen. Physiol.* **64:**372–391
11. Hegel, U., Froemter, E., Wick, T. 1967. Der elektrische Wandwiderstand des proximalen Konvolutes der Rattenniere. *Pfluegers Arch.***294:**274–290
12. Lang, F., Quehenberger, P., Greger, R., Silbernagl, S., Stockinger, P. 1980. Evidence for a bicarbonate leak in the proximal tubule of the rat kidney. *Pfluegers Arch.* **386:**239–244
13. Malnic, G., Mello Aires, M. 1971. Kinetic study of bicarbonate reabsorption in proximal tubule of the rat. *Am. J. Physiol.* **220:**1759–1767
14. Neame, K.D., Richards, T.G. 1972. Elementary kinetics of membrane carrier transport. pp. 56–79. Wiley, New York
15. Rector, F.C. 1983. Sodium, bicarbonate, and chloride absorption by the proximal tubule. *Am. J. Physiol.* **244:**F461–F471
16. Robinson, R.A., Stokes, R.H. 1959. Electrolyte Solutions. pp. 463–467. Butterworths, London
17. Rubio, C.R., DeMello, G.B., Mangili, O.C., Malnic, G. 1982. H^+ ion secretion in proximal tubule of low-Co_2/HCo_3 perfused isolated rat kidney. *Pfluegers Arch.* **393:**63–70
18. Rubio, C.R., DeMello, G.B., Mangili, O.C., Malnic, G. 1982. Effect of temperature on proximal tubular acidification. *Pfluegers Arch.* **393:**71–76
19. Sanders, S.S., Hayne, V.B., Rehm, W.S. 1973. Normal H^+ rates in frog stomach in absence of exogenous CO_2 and a note on the pH-stat method. *Am. J. Physiol.* **225:**1311–1321
20. Schwartz, G.J. 1981. Na^+ dependent H^+ efflux from proximal tubule: Evidence for reversible Na^+/H^+ exchange. *Am. J. Physiol.* **241:**F380–F385
21. Schwartz, G.J., Al-Awqati, Q. 1985. Carbon dioxide causes exocytosis of vesicles containing H^+ pumps in isolated perfused proximal and collecting tubules. *J. Clin. Invest.* **75:**1638–1644
22. Snedecor, G.W., Cochran, W.G. 1971. Statistical Methods. pp. 271. Iowa State University Press, Ames
23. Steinmetz, P.R. 1974. Cellular mechanisms of urinary acidification. *Physiol. Rev.* **54:**890–956
24. Steinmetz, P.R., Andersen, O.S. 1982. Electrogenic proton transport in epithelial membranes. *J. Membrane Biol.* **65:**155–174
25. Yoshitomi, K., Froemter, E. 1984. Cell pH of rat renal proximal tubule in vivo and the conductive nature of peritubular HCO_3-(OH^-) exit. *Pfluegers Arch.* **402:**300–305

Received 5 January 1990; revised 23 April 1990