Axial Heterogeneity of Intracellular pH in Rat Proximal Convoluted Tubule

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Abstract

In the proximal convoluted tubule (PT), the HCO_3^- reabsorptive rate is higher in early (EPS) compared with late proximal segments (LPS). To examine the mechanism of this HCO_3^- reabsorption profile, intracellular pH (pH_i) was measured along the superficial PT of the rat under free-flow and stationary microperfusion using the pH-sensitive fluorescence of 4-methylumbelliferone (4MU). With 4MU superfusion, pH_i was found to decline along the PT. Observation with 365-nm excitation revealed that EPS were brightly fluorescent and always emerged away from their star vessel. Midproximal segments were darker and closer to the star vessel which was surrounded by the darkest LPS. Decreasing luminal HCO_3^- from 15 to 0 mM lowered pH_i in both EPS and LPS, but pH_i remained more alkaline in EPS with both perfusates. Thus the axial decline in pH_i along the PT is due to both luminal factors and intrinsic differences in luminal H^+ extrusion in PT cells.

Introduction

Maintenance of acid-base homeostasis depends on the ability of the proximal convoluted tubule to reabsorb most of filtered HCO_3^- by a process that involves H^+ secretion into the tubular lumen. Several studies have shown that HCO_3^- reabsorption does not proceed at the same rate along the proximal convoluted tubule (1-4). In all these reports the magnitude of HCO_3^- reabsorption is greater in early than in late proximal segments. Because HCO_3^- reabsorption increases as a function of luminal HCO_3^- concentration (5), augmented HCO_3^- reabsorption in the early proximal tubule could be explained by the higher luminal HCO_3^- concentration in early segments compared with late proximal segments. Recently, however, studies by Alpern et al. (6, 7) in the Munich-Wistar rat have argued that the axial heterogeneity for HCO_3^- reabsorption in the proximal tubule cannot be accounted for solely on the basis of differences in luminal HCO_3^- concentration. In the early proximal tubule HCO_3^- reabsorption was about twofold greater than that in the late proximal tubule when microperfused with a HCO_3^- concentration equal to that in glomerular ultrafiltrate. The mechanism responsible for this axial heterogeneity in proximal HCO_3^- reabsorption (H^+ secretion) is unknown.

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The purpose of the present study was to develop a technique for measuring intracellular pH (pH_i) in the superficial proximal tubule, and to examine the importance of pH_i in explaining HCO_3^- reabsorption in this nephron segment. To accomplish this, cells of the proximal tubular epithelium were loaded with the pH-sensitive fluoroprobe 4-methylumbelliferone (4MU), and observed by epifluorescence microscopy. The results demonstrate that under free-flow conditions there is a declining profile of pH_i along the entire superficial proximal tubule. To investigate the etiology of this pH_i heterogeneity, early and late proximal segments were microperfused in situ. At equal HCO_3^- concentrations, late segments were again acidic relative to proximal segments, supporting the results of fluorescence under free-flow conditions. In both early and late proximal segments, pH_i fell when HCO_3^- in the perfusate was reduced. These results demonstrate that the declining pH_i profile along the proximal tubule reflects both luminal factors and intrinsic differences in the mechanisms of pH_i regulation in early compared with late tubular cells.

Methods

Sprague-Dawley rats maintained on normal rat chow and weighing 220-280 g were anesthetized by an i.p. injection of sodium nembutal (50 mg/kg). Rats were placed on a feedback-regulated heating table. A tracheostomy was performed and catheters were inserted in the external jugular vein for the administration of 0.85% NaCl solution at a rate of 40 $\mu\text{l}/\text{min}$, and in the femoral artery for blood pressure monitoring and arterial blood gas measurement. The left kidney was exposed for micropuncture as previously described (8) and catheters inserted in both ureters for urine collection.

Nephron segment identification

Random proximal segments were selected by transit time measurement after i.v. injection of FD&C green dye. Proximal sites belonging to the same nephron were identified by intratubular injection of isotonic saline stained with FD&C green dye into random proximal convolutions with a 6-7- μm outer tip diameter pipette. Early proximal sites were selected by retrograde injection while mid- and late proximal sites were identified by noting the sequence of appearance of dye in downstream convolutions. Localization was confirmed by i.v. bolus injection of dye. Early distal convolutions were selected as those convolutions in which dye first appeared after its transit through the loop of Henle, whereas late distal convolutions were chosen as those in which the dye appeared last before reaching the ureter.

Cell loading

Delivery of fluoroprobes (4-methylumbelliferone acetate [4MUA], 5-6 carboxyfluorescein diacetate [CFA_2]) (Molecular Probes, Inc., Junction City, OR) to the tubular epithelium was accomplished by renal superfusion or intratubular administration. These acetylated derivatives of 4MU and 5-6 carboxyfluorescein (CF) have higher lipid solubility and exhibit min-

1. Abbreviations used in this paper: CF, 5-6 carboxyfluorescein; CFA_2 , 5-6 carboxyfluorescein diacetate; 4MU, 4-methylumbelliferone; 4MUA, 4-methylumbelliferone acetate; pH_i , intracellular pH.

imal (4MUA) or negligible (CFA₂) fluorescence compared with the fluorescent compounds.

Superfusion studies

The kidney surface with capsule intact was superfused via 0.023-inch polyethylene tubing. An ultrathin layer was formed by perfusing under a submicron thick sheet of copolymer (Dow Chemical Co., Midland, MI) placed over the cortical surface. The composition of the superfusate was (millimolar): NaCl 145, CaCl₂ 2.5, MgSO₄ 1.8, KCl 4.5, and Tris 4.5. 4MUA and CFA₂ were first dissolved in methanol and then added to the superfusate to achieve concentrations of 2×10^{-4} M (1% methanol). A superfusate pH of 6.5 was chosen to minimize the fluorescence signal from the superfusate layer over the renal surface. With this procedure, the contribution of this fluid layer under the copolymer sheet to the overall signal measured over the tubular epithelium was < 10% as assessed by comparing fluorescence before and after flushing the renal surface with superfusate devoid of fluoroprobe. Measurements to be reported here were not corrected for the contribution of the superfusate fluid layer. Superfusates were bubbled with 7% CO₂ to obtain a PCO₂ of 60 mm Hg, similar to that in superficial rat renal cortex (9). Appropriate amounts of NaHCO₃ were added to the perfusate to maintain pH constant at 6.5.

Intravascular administration

Studies with CFA₂ by luminal microperfusion. Early and late proximal convolutions of the same nephron were injected manually with Tris buffer containing 10^{-4} M of the fluoroprobe for periods of 2–3 min and at a rate low enough to avoid dilatation of the lumen.

Studies with 4MU by luminal microperfusion. Early proximal and late proximal convolutions belonging to the same nephron were identified as described above. An 8–10- μ m outside diameter pipette was inserted in the distal end of an early proximal convolution and perfusion carried out at 40 nl/min using a modified Hampel pump. Two perfusates were used: 15 mM HCO₃⁻ (high HCO₃⁻), pH 7.0, and 0 mM HCO₃⁻ (low HCO₃⁻), pH 5.6. Both perfusates were bubbled with 7% CO₂ (60 mm Hg) and contained (millimolar) 4.5 KCl, 2.5 CaCl₂, and 1.8 MgCl₂. Appropriate amounts of NaCl were added to achieve a Na concentration of 145 mM. 4MU at a concentration of 10^{-3} M dissolved in 1% methanol or 1% dimethyl sulfoxide (DMSO) was added to perfusates containing FD&C green dye or nigrosin (1%). The proximal end of the convolution was then entered with a 10–12- μ m outside diameter pipette filled with Sudan Black-stained bone wax and a short wax column introduced using a Wells hydraulic microdrive (Trent Wells, Southgate, CA) to interrupt tubular filtrate flow. After fluorescence measurements were obtained, the perfusion pipette was removed and inserted into the late proximal convolution of the same nephron and perfusion carried out at this site. In some nephrons early and late proximal convolutions were perfused sequentially with the two HCO₃⁻-containing perfusates. In this case early and late proximal convolutions were reentered with a separate pipette containing the second perfusate. In some cases sequential perfusion was performed using a double-barreled pipette filled with the two bicarbonate perfusates. pH and PCO₂ were measured in arterial blood, superfusates, and luminal perfusates using a Corning 150 blood gas analyzer (Corning Medical, Medfield, MA).

Fluorescence measurements and optical system

For fluorescence measurements the rat and heating table were mounted on a modified stage of a Labophot microscope (Nikon Inc., Garden City, NY) fitted with an epifluorescence attachment and a trinocular body tube. A 100-W mercury lamp was used as the light source. The epifluorescence attachment was modified to accommodate appropriate band-pass excitation filters (Ditric Optics, Hudson, MA) and Inconel-coated quartz windows (Corion Corp., Holliston, MA) to decrease the intensity of excitation light when indicated. In the studies with 4MUA, light was first filtered using a UG 11 filter (Ealing Corp., S. Natick, MA) and narrow band excitation was then obtained using 365- and 334-nm filters. For 365-nm excitation a neutral-density filter with 1% transmittance was used. Because the intensity of 334-nm light was ~ 100 -fold less than

that at 365 nm, a neutral density filter was not used for 334-nm excitation. Epillumination was achieved using a dichroic mirror and barrier filter assembly (400- and 410-nm transmittance, respectively) and a $\times 20$ Nikon-Fluor objective. In the studies with CFA₂ excitation was performed with 460- and 490-nm bandpass filters using a Nikon B filter cube cassette and $\times 20$ CF objective.

The optical system for both 4MUA and CFA₂ studies included a $\times 2.5$ relay lens inserted in the phototube of the microscope head. Fluorescence was measured using a Farrand microscope spectrum analyzer (Farrand Optical Co., Valhalla, NY) with target spotting capability mounted on the phototube of the microscope. Emission wavelengths corresponding to maximal fluorescence intensity were 460 and 535 nm for 4MU and CF, respectively. Magnification of the combined optical system including lenses in the Farrand analyzer was $\times 625$. A 0.13-mm pinhole and a 15-nm slit were placed in the apertures of the emission-grating monochromator and photomultiplier tube, respectively. With the $\times 20$ objective, the area from which fluorescence was measured was 4 μ m², allowing fluorescence measurements to be localized just to the tubular epithelium while avoiding the lumen and peritubular capillaries. To further ensure that measurements of epithelial fluorescence were not contaminated by fluorescence from luminal dye, we tested the ability of our system to reject a luminal fluorescence signal when the field of measurement was localized over the epithelium. Random proximal convolutions were perfused with a fluorescent but cell-impermeant compound, fluorescein isothiocyanate-labeled dextran (70,000 mol wt, Sigma Chemical Co., St. Louis, MO). Moving the target spotter from the lumen to the epithelium caused fluorescence to fall abruptly to background level.

Exposure to excitation light, especially at ultraviolet wavelengths, for periods in excess of 5 min consistently resulted in damage. This was manifested in order of increasing severity by prolonged tubular transit times, decrease in capillary blood flow, and cloudiness of the epithelium. In our studies total exposure times were therefore kept under 1 min and light intensity reduced by use of neutral density filters to levels just high enough to allow measurement in the most sensitive scale of the photometer. As an additional precaution measurements were discarded if tubular transit times taken at the end of the study were prolonged by > 1 s in early proximal and 2 s in late proximal convolutions.

In vitro calibration

Solutions containing 4MU or CF (the nonesterified free acids) were titrated to various pH values and fluorescence emission measured using the same optical system as that for measurement of epithelial fluorescence. To obviate bleaching, fluorescence was measured from the solutions as they were gravity perfused through 55 μ M quartz capillary tubing.

4MU in vivo calibration

In wax-blocked nephrons from a separate group of rats, lumen and capillaries of early and late proximal convolutions were perfused with a solution of the following composition (millimolar): 25 Hepes, 60 K₂HPO₄, and pH adjusted to 6.8, 7.1, or 7.4 (PCO₂ 60 mm Hg, appropriate amount of NaHCO₃ added for each pH value) as reported by Alpern (10). Perfusates contained nigericin (10 μ M) a K⁺/H⁺ antiporter. 4MU (10^{-3} M) was added to the luminal perfusate. K⁺ concentration was chosen to approximate K⁺ activity reported in proximal tubular cells (11). Fluorescence measurements were obtained 5–10 min after initiation of perfusion. Calibration did not differ in early and late proximal convolutions. Furthermore, the calibration curve overlapped with that obtained in vitro.

pH sensitivity of 4MU fluorescence

At excitation wavelengths of 320–340 nm, the fluorescence of 4MU is relatively pH-insensitive whereas a marked increase is observed as a function of pH (6.5–8.0) at wavelengths of 350–380 nm. We chose 334 and 365 nm as pH-insensitive and -sensitive wavelengths, taking advantage of the intense emission of the mercury lamp at these two wavelengths, and the availability of the corresponding bandpass filters. Changes in fluorescence as a function of pH were expressed as the ratio of 365/334 nm to correct for changes in fluoroprobe concentration and light intensity

(Fig. 1 and Graber et al. [12, 13]). Ratio was unaltered by a fivefold increase in light intensity and a threefold increase or decrease in fluorophore concentration. 4MU had no apparent toxic effects on tubular function: Continuous superfusion for up to 4 h, a period far greater than that required to perform the measurements reported here, failed to show changes in tubular morphology, tubular transit times, or arterial blood gases.

pH sensitivity of CF fluorescence

We have previously shown that an increase in pH causes increase in the fluorescence of CF which is more marked at excitation wavelengths near 490 nm than at shorter wavelengths (430–460 nm [13]). We chose 490 and 460 nm as the pH-sensitive and relatively pH-insensitive wavelengths, respectively. A ratio of fluorescence at these two excitation wavelengths is linearly related to pH (13).

Statistical comparisons were made using Student's *t* test for paired or unpaired data, as appropriate.

Results

Qualitative observations

4MUA. Fig. 2 (diagram I) illustrates the fluorescence appearance of a star vessel complex during 4MUA superfusion under 365-nm excitation. Three star vessels are shown surfacing in close proximity to one another surrounded by convolutions belonging to several nephrons. Shown on the right is a limited drawing of an adjacent star vessel complex. Star vessels and peritubular capillaries appear totally dark, reflecting the known absorption by hemoglobin of the excitation wavelengths used (334 and 365 nm) to elicit 4MU fluorescence (14). In small-diameter capillaries, erythrocytes flow in single file with a stop-and-go pattern. Fluorescence of 4MU in plasma is seen between clumps of red cells. Over a given point capillary fluorescence is intermittent related to interruptions in red cell flow. Areas where these capillaries crossed the epithelium were easily identified and avoided. The surface nephron convolutions differ strikingly in their fluorescence intensity, a pattern which is also observed during perfusion of peritubular capillaries with a solution devoid of fluorophore. Some convolutions are bright blue, others appear medium bright whereas still others exhibit dark blue fluorescence. In all convolutions, fluorescence is highly homogeneous and is not affected by light exposure (photobleaching), and tubular lumens are minimally visible, if at all. There is no evidence of increased fluorescence localized to the peritubular space. Convolutions with similar fluorescence intensity group themselves together forming discrete areas. These areas, in turn, adopt a highly organized pattern. Clusters of bright convolutions are far-

thest from the star vessels and form ridges that tend to encircle the latter. In some cases a full crown effect is seen. Darkest convolutions are found in areas near star vessels. Convolutions with moderate brightness are located between the bright and dark convolutions. By the transit time technique bright convolutions are always early proximal segments. Convolutions with intermediate fluorescence correspond to midproximal segments, whereas dark convolutions represent late proximal segments. Distal segments appear dark with early distal segments usually occupying a more central position, near the star vessels, than late distal segments (diagram II). In contrast to these observations at 365 nm, using pH-insensitive excitation at 334 nm, all surface convolutions have approximately the same fluorescence intensity.

The above anatomical findings indicate that there is a high level of organization of proximal convoluted tubules on the renal surface. Two types of surfacing patterns were identified (Fig. 2, diagrams I–III). In some nephrons, the early proximal tubule reaches the kidney surface and occupies a position farthest from the star vessel (nephrons *A, B, C, F, G* in diagram II and nephrons *H, I, K, L, M, P* in diagram III). These early segments lie in clusters together with early proximal segments from adjacent nephrons. Late proximal convolutions are seen surrounding a star vessel whereas midproximal convolutions occupy an intermediate position between early and late proximal convolutions. In other nephrons (*D* and *E* in diagram II and *J, N, O* in diagram III), the early proximal tubule lies entirely below the renal surface. In these nephrons the first segments to be seen on the renal surface are midproximal, and these lie in areas medial to that occupied by surface early proximal convolutions from other nephrons. This orderly surfacing pattern results in the concentric areas of decreasing fluorescence.

CFA₂. After 3–5 min of superfusion, yellowish-green fluorescence of CF is observed in the epithelium of proximal and distal convolutions. The fluorescence appearance is highly non-homogeneous with striking granularity primarily localized to the basolateral margin. This pattern was more evident in distal convolutions than in proximal segments. Visually, the CF fluorescence is of similar intensity at different proximal tubular segments and the crown effect noted with 4MUA is not observed. Because of the marked granularity of fluorescence and uncertainty as to its meaning, quantitative fluorescence measurements were not obtained with this type of loading. For this purpose we resorted to cell loading by intratubular administration of CFA₂. By this approach CF fluorescence is nearly homogeneous (see below).

Fluorescence intensity measurements in 4MU superfusion studies

The 365/334-nm fluorescence ratios along the proximal and distal tubule are shown in Table I. In the proximal convoluted tubule the 365/334-nm ratio decreased significantly from 0.85 (pH_i 7.14) in early proximal convolutions to 0.65 (pH_i 6.96) in midproximal and fell farther to 0.55 (pH_i 6.87) in late proximal convolutions. These differences were due to significant changes in fluorescence at 365 nm, whereas fluorescence at 334 nm was essentially the same at the different nephron sites (data not shown). These results demonstrate an axial decline in pH_i in this nephron segment. The 365/334-nm ratio in early distal and late distal convolutions was not significantly different (0.20 > *P* > 0.10) and is similar to the ratios in late proximal convolutions.

To further validate the findings in random convolutions,

Figure 1. In vitro relationship between 365/334-nm fluorescence intensity ratio and pH.

Figure 2. Diagram of an area of renal surface as seen during superfusion with 4MUA using 365 nm excitation. (I) Identification of sites along proximal and distal convoluted tubules was performed by transit times during i.v. bolus injections of FD&C green dye. Early proximal convolutions have bright blue fluorescence, midproximal show medium bright fluorescence, and late proximal convolutions exhibited dark blue fluorescence. Nephron identification (II and III) was carried out by intratubular injection of FD&C green dye. Letters refer to individual nephrons. Numbers rank surface convolutions from the first to last.

measurements were obtained sequentially in proximal convolutions belonging to the same nephron and grouped based on transit time as early (3 ± 0.6 s), mid (7 ± 1.2 s) and late (11 ± 1.4 s) convolutions (Table I). The results are qualitatively and quantitatively similar to those obtained in random convolutions.

Fluorescence measurements during luminal perfusion

Background epithelial autofluorescence was homogeneous and relatively low at the excitation wavelengths used for the 4MUA and CFA₂ studies and did not significantly differ at various sites along the nephron. 4MU and CF epithelial fluorescence was at least 10-fold greater than autofluorescence. Ratios reported here were corrected for the measured background fluorescence.

4MU. This series of studies was undertaken with two purposes. First, we wished to further validate the results obtained during superfusion by measuring the fluorescence ratio under conditions where fluoroprobe is solely localized to the epithelium of the injected nephron. Second, we were interested in examining the dependency of pH_i on luminal HCO₃⁻ concentration. 4MU was used in place of 4MUA to avoid the possibility of cell acidification consequent to intracellular 4MUA hydrolysis. Within a few seconds after the initiation of luminal perfusion, blue fluorescence was appreciated in the tubular epithelium of the convolution being perfused. Fluorescence was completely homogeneous. The leak rate of 4MU was very fast, with fluorescence returning to near background levels within 5–10 s of discontin-

Table I. Profile of Fluorescence Ratio (365/334) and pH_i along the Nephron under Free-flow Conditions

	Proximal tubule			Distal tubule	
	Early	Mid	Late	Early	Late
Random convolutions	(18)	(16)	(10)	(12)	(5)
365/334	0.85±0.03	0.65±0.03*	0.55±0.02‡	0.59±0.03	0.49±0.05
pH_i	7.14±0.02	6.96±0.02	6.87±0.01	6.91±0.02	6.82±0.04
Same nephron	(5)	(5)	(5)		
365/334	0.83±0.03	0.69±0.03§	0.57±0.02		
pH_i	7.12±0.02	6.99±0.02	6.89±0.01		

Values are means±SEM. Numbers in parentheses denote number of observations. Values are not significantly different from those at preceding site except where noted. * $P < 0.01$. ‡ $P < 0.02$. § $P < 0.05$. || $P < 0.002$. pH_i values were calculated from in vivo calibration in a separate group of rats.

uing perfusion. In measuring epithelial fluorescence care was taken to avoid those areas where capillaries crossed the epithelium. Uptake of 4MU in convolutions adjacent to the one being perfused was barely appreciable, if at all. Significant fluorescence could be seen only if a leak at the puncture site was present as evidenced by appearance of FD&C green dye outside of the punctured convolution. Fluorescence from convolutions below the surface could not be detected. The quantitative results using luminal perfusion of 4MU are shown in Table II. To determine the magnitude of the change in pH_i after changes in luminal HCO_3^- concentration studies were repeated in a separate group of rats in which in vivo calibration analysis was also obtained. Results were very similar to those calculated for the 365/334-nm ratios reported here (see legend to Table II). In random early and late proximal convolutions, decreasing HCO_3^- concentration from 15 to 0 mM caused the 365/334-nm ratio to decrease significantly at both sites. pH_i decreased from 7.23 to 7.13 in early convolutions and from 7.15 to 7.07 in late proximal convolutions. The decrease in 365/334-nm ratio in early proximals ($\Delta = 0.11 \pm 0.04$ ratio units) was similar to that in late proximal

convolutions ($\Delta = 0.08 \pm 0.03$). A second finding is that the 365/334-nm ratio and pH_i are significantly higher in early proximal than in late proximal convolutions belonging to the same nephron with both 15 and 0 mM HCO_3^- perfusates. These results indicate that regardless of the luminal HCO_3^- concentration pH_i is more alkaline in early than in late proximal convolutions, and that at both sites cell pH is altered by variations in luminal HCO_3^- concentration or pH.

CFA₂. To further validate the results obtained with 4MU in free-flowing nephrons, the pH sensitive 490/460-nm fluorescence ratio was measured under free-flow conditions in early and late proximal convolutions loaded with CFA₂. 1–2 min after initiation of luminal perfusion, bright yellow-green epithelial fluorescence was noted in the convolution being injected. Fluorescence was only faintly granular. The leak rate of CF was relatively rapid with a $t_{1/2}$ of 2–3 min in early proximal and 5–7 min in late proximal convolutions. The 490/460-nm fluorescence ratio was measured within 3 min after cessation of luminal perfusion in early and late proximal convolutions (six nephrons). Early proximal convolutions were significantly more alkaline than late

Table II. Effect of Luminal HCO_3^- on Epithelial Fluorescence Ratio (365/334) and pH_i in Random Early and Late Proximal Convolutions, Paired by Perfusate, and in Convolutions Belonging to Same Nephron, Paired by Site

HCO_3^-	Early proximal		Late proximal	
	15 mM	0 mM	15 mM	0 mM
Random convolutions				
365/334	0.95±0.05	0.84±0.05*	0.86±0.06	0.78±0.06*
pH_i	7.23±0.04 (n = 7)	7.13±0.04 (n = 7)	7.15±0.05 (n = 5)	7.07±0.05 (n = 5)
Same nephron				
365/334	0.94±0.05		0.86±0.06‡	
pH_i	7.22±0.04 (n = 10)		7.15±0.05 (n = 10)	
365/334		0.86±0.06		0.74±0.06§
pH_i		7.15±0.05 (n = 7)		7.04±0.05 (n = 7)

Values are means±SEM. n = number of observations. * $P < 0.05$ compared with 15 mM in same convolution. pH_i values were calculated from in vivo calibration in a separate group of rats. In this latter group pH_i values as a function of luminal HCO_3^- were for early proximal cells, 7.26 (15 mM), 7.15 (0 mM); and for late proximal cells, 7.15 (15 mM) and 7.06 (0 mM). ‡ $P < 0.02$ and § $P < 0.05$ compared with early proximal in the same nephron.

proximal convolutions as evidenced by a higher 490/460-nm fluorescence ratio in the former (0.500 ± 0.016 vs. 0.440 ± 0.018 , $P < 0.02$). To ensure that these measurements were not influenced by the falling intracellular concentration of fluoroprobe, in a separate series of studies 490/460-nm ratios were measured immediately and again 3–4 min after epithelial loading. In paired comparisons, sequential ratios were not significantly different (data not shown).

Discussion

Two major findings of these studies are (a) the demonstration of a high level of organization in the proximal convolutions on the cortical surface, and (b) the presence of an axial pH_i gradient along this segment. These two observations account for the striking concentric differences in pH_i expressed on the kidney surface.

Intracellular pH methodology. 4MU has been previously used to measure cell pH in various tissues (15–17) and recently by us in the turtle bladder (12). We used the nonfluorescent derivative of 4MU, 4MUA, which is lipid soluble and readily enters cells where it is converted by cytosolic esterases to the more polar and fluorescent compound 4MU. Similar considerations apply to the use of CFA₂ (18). 4MU fluorescence in the tubular epithelium was completely homogeneous, suggesting that the fluoroprobe is largely localized to the cytosol and does not undergo significant intracellular compartmentalization and/or binding. In mitochondria where pH exceeds 7.3, the pK_a of 4MU (13), accumulation as the dissociated form might be expected but this is apparently precluded by the opposing force of the negative intramitochondrial potential. Failure to observe 4MU accumulation in other organelles may reflect restricted entry or alternatively be due to an inner filter effect created by fluoroprobe in the cytosol. Intracellular binding is also not likely to be a concern with 4MU. This is supported by the finding that in vivo calibration overlaps with that obtained in vitro, an observation that contrasts with the findings reported by Alpern with (2',7')-bis(carboxyethyl) (5,6)-carboxyfluorescein (10). Intracellular binding or high dye concentration may be responsible for the shift to more alkaline values of in vivo calibration compared with in vitro values (13).

Superfusion with 4MUA revealed striking differences in fluorescence intensity in the different nephron segments at 365-nm excitation. That this pattern reflects differences in pH_i rather than concentration differences is supported by three additional findings: (a) Using the pH-insensitive excitation wavelength of 334 nm, fluorescence intensity is comparable along the nephron, indicating roughly equal dye concentrations in the different nephron segments. (b) The gradient of fluorescence ratios along the proximal tubule is also seen during cell loading with luminal 4MU. (c) The same gradient is demonstrable in proximal cells loaded with CF. This fluoroprobe is dissimilar from 4MU, but also exhibits pH-sensitive fluorescence and has been used extensively for this purpose (19, 20). Like 4MU, the 490/460-nm excitation ratio increases with more alkaline pH values. To ensure that differences in the CF fluorescence ratio represent differences in cell pH and not differences in dye concentration, we analyzed only those measurements with comparable fluorescence at the pH-insensitive wavelength of 460 nm.

We did not attempt to quantitate fluorescence ratios when CF was loaded by superfusion because the resultant fluorescence

was not homogeneous and appeared to be confined to the basolateral regions of the tubule. We assume this reflects either a binding phenomenon, or accumulation of CF in alkaline spaces. The conversion of CFA₂ to CF in the basolateral region may represent nonenzymatic hydrolysis in an alkaline environment, or catalyzed hydrolysis by esterases.

Axial profile of pH_i along the superficial proximal tubule.

Intracellular pH in the proximal convoluted tubule of the rat has been measured by Yoshitomi and Fromter (21) with pH-sensitive microelectrodes and by Alpern and Chambers (10, 22) using (2',7')-bis(carboxyethyl) (5,6)-carboxyfluorescein. In these studies the possibility of differences in pH_i along the proximal tubule was not examined as convolutions were selected randomly. Our results demonstrate a declining profile of cell pH from the early to late proximal tubule. This is apparent visually from the pattern of fluorescence seen with pH-sensitive 365-nm excitation, and was confirmed quantitatively using the 365/334-nm fluorescence ratio during superfusion with 4MUA. pH_i in midproximal convolutions, 6.96, is somewhat more acidic than the pH_i of 7.17 reported by Yoshitomi and Fromter (21) at random proximal sites. This discrepancy may reflect differences in segment selection or methodology. One potential drawback of loading by superfusion is that the extremely permeant 4MUA may enter segments below those seen on the cortex surface and thus introduce an artifact in the fluorescence measurements. Because tubular fluorescence with this technique is seen emanating exclusively from superficial tubular segments and fluorescence ratio is essentially identical at different sites in a given convolution, we believe fluorescent contamination from deeper segments is small, presumably due to the inner filter effect of 4MU present in the most superficial segments. To further validate the quantitations obtained during superfusion, we loaded cells instead by luminal perfusion to entirely confine the fluorescence to the single perfused convolution. The axial fall in fluorescence ratio was again seen using either 4MU or CF.

Of further interest is that pH_i in the distal convoluted tubule is similar to that in late proximal epithelium. Because the late distal tubule is an extension of the cortical collecting tubule, the so-called connecting tubule, we specifically looked for presence of intercalated cells. These cells are thought to mediate H^+ secretion in the collecting duct and have been shown to have a more alkaline pH_i than the principal cells that surround them (12, 19, 23). We were unable to detect in the distal tubule areas with increased fluorescence brightness that might represent intercalated cells. This is not very surprising because intercalated cells are few in number, and we sampled only a small number of late distal convoluted tubules.

With regard to the finding of a declining pH_i profile along the proximal convoluted tubule under free flow conditions, this may simply reflect the parallel fall in luminal pH and HCO_3^- concentration present in the tubular lumen. In the early proximal tubule the more alkaline tubular fluid would favor H^+ secretion and alkalization of the cell. In contrast, in late proximal cells the more acidic luminal pH would impose a relatively adverse gradient for H^+ secretion and thus lead to relatively more acidic values of pH_i . The luminal perfusion studies clearly implicate a role of luminal HCO_3^- concentration on pH_i in both early and late proximal segments and in this context demonstrate that the axial decline in pH_i seen along the proximal tubule during free-flow conditions is in part due to the decreasing profile of HCO_3^-/pH in this nephron segment. Decrease in pH_i in early proximal and late proximal convolutions (0.10 and 0.08 pH

units, respectively) is in close agreement with the decrease in pH_i (0.08 units) observed by Alpern and Chambers (22) in random proximal convolutions after lowering luminal HCO_3^- concentration from 25 to 5 mM. The above findings also establish that the fall in pH_i along the proximal tubule is not due solely to a decrease in luminal HCO_3^- concentration in this nephron segment; pH_i in early proximal convolutions remains relatively alkaline compared with pH_i in late convolutions when perfused with solutions having identical HCO_3^- concentration. This latter finding must represent either an effect of the peritubular environment or an intrinsic difference in the mechanisms of H^+ secretion and pH_i regulation in early compared with late proximal cells. Concerning the role of the peritubular milieu, a higher cell pH in early proximal convolutions might be the result of diminished HCO_3^- exit (10) if HCO_3^- concentration in early proximal capillaries were higher than in capillaries surrounding late proximal segments. Several arguments argue against this possibility playing a major role. First, HCO_3^- concentration in early proximal capillaries may actually be lower than in late proximal capillaries because of delivery via the countercurrent system of blood with relatively low HCO_3^- concentration (see below). This is further emphasized by the perfusion studies using 0 mM HCO_3^- perfusate. In this setting during early proximal perfusion addition of reabsorbate with very low HCO_3^- concentration to mid- and late proximal capillaries should have decreased, via the countercurrent arrangement of tubular and capillary flows, early proximal capillary HCO_3^- concentration compared with the much more limited decrease in late proximal capillary HCO_3^- concentration during perfusion of late proximal convolutions. Lastly, it would be difficult to explain how a higher cell pH could account for the observations by Alpern et al. (6) that early proximal segments reabsorb substantially more HCO_3^- than latter segments at equal delivered loads. We therefore conclude that the more alkaline cell pH in early proximal cells reflects an intrinsic difference concerning the cell itself. In this regard, a higher rate of H^+ secretion by the early proximal cells is the most likely explanation, and would account for both the higher acidification rates and more alkaline cell pH . The majority of proximal acidification is thought to represent the activity of an Na^+/H^+ exchanger (24, 25). Therefore, it is conceivable that the higher pH_i in early proximal cells reflects the presence of more numerous exchangers in these cells compared with late proximal cells. Our results argue against this possibility: when the tubule lumen was perfused with acidic 0 mM HCO_3^- , this would be expected to impose a lumen-to-cell H^+ gradient that should acidify the cell. This cellular acidification was seen at both sites, but the early cells remained more alkaline than late proximal cells. If the early cells had a greater number of Na^+/H^+ antiporters, one would predict these cells to become more acidic than late cells, all other factors being equal.

We must therefore propose that the early proximal cells have higher rates of H^+ secretion for reasons other than more Na^+/H^+ exchanger units. One possibility to be considered is that of a higher activity of the Na^+/H^+ exchanger in early proximal convolutions. This could be accomplished by lower Na_i or lesser degree of inhibition of the exchanger by cytosolic OH^- (26). It should be noted that other possibilities could also account for our findings: (a) a greater contribution from a luminal H^+ -ATPase pump (27) (existence of such pump in the proximal tubule is, however, a highly disputed matter), (b) decreased luminal cell entry of H^+ or OH^- exit via the operation of a HCl symporter or Cl^-/OH^- antiporter (28, 29), or (c) a higher met-

abolic production of H^+ . Additional studies will be required to test these various alternatives.

Functional significance of proximal tubular organization. Our finding that late proximal convolutions are located near star vessels confirms our previous observations and those made by others (30–33). The present studies also illustrate a surfacing pattern of proximal convolutions not previously appreciated. Mid and early convolutions do not emerge randomly around late proximal convolutions. Nephrons with early proximal convolutions on the surface appear at points further away from their star vessel. Midproximal convolutions from nephrons with or without early convolution on the surface emerge medial to early proximal convolutions. Another significant organizational feature is that early convolutions from separate nephrons either belonging to the same or an adjacent star vessel complex join together forming an outer crown which exhibits bright blue fluorescence by 4MU centered around the star vessel complex. Likewise, segregation of mid- and late proximal convolutions into discrete areas results in concentric areas of decreasing fluorescence brightness with 4MU. The surfacing pattern of early proximal convolutions suggested to us that in superficial nephrons the initial portion of the proximal tubule must move away from its own glomerulus to later emerge in the outer region of the star vessel complex. This unfolding pattern of early proximal tubule has been shown in deeper cortical nephrons by Weinstein and Szyjewicz (33). The folding back of the proximal tubule toward its star vessel creates a countercurrent pattern of tubular fluid and capillary flows on the cortical surface. Our findings clearly establish that this arrangement does not only involve late convolutions as suggested by other investigators (31, 33) but also applies to early and midproximal convolutions. It should be noted that although this pattern strictly applies only to the tops of superficial convolutions, it is also applicable to those portions that lie below the surface in so far as the predominant direction of flow is still oriented toward the star vessel. We also propose that in addition to this surface countercurrent system there is a vertical countercurrent pattern that involves the initial portion of the proximal tubule (Fig. 3). The vascular portion of this system is composed of peritubular capillaries derived from penetrating branches of surface capillaries and draining into the interlobular vein located below the point where the proximal tubule folds back toward its own star vessel (31, 33).

We further propose that this arrangement of countercurrent flow systems may be important in regulating solute reabsorption in the proximal convoluted tubule in superficial nephrons. In the very early proximal tubule preferential HCO_3^- reabsorption over Cl^- decreases luminal HCO_3^- concentration and increases that of Cl^- . In peritubular capillaries this process will be expected to increase HCO_3^- concentration and decrease Cl^- concentration. The vertical countercurrent system may be important in facilitating HCO_3^- reabsorption by delivering blood with a relatively low HCO_3^- concentration which will reduce peritubular HCO_3^- concentration. In this fashion a relatively high Cl^- concentration gradient (lumen to blood) will be generated, an effect which is a critical driving force for Cl^- reabsorption in mid- and late proximal segments. In these segments the superficial countercurrent system delivers star vessel blood which has a lower Cl^- concentration than systemic blood to peritubular capillaries and serves to maximize the magnitude of the transepithelial Cl^- concentration gradient (lumen to blood) in late proximal segments. If tubular and capillary flows were cocurrent, reabsorbate with a Cl^- concentration higher than in efferent blood will be added

Figure 3. Diagram of outer cortex depicting superficial and deep countercurrent systems. In the superficial system Cl^- reabsorption in mid- and late proximal segments is facilitated by the favorable Cl^- gradient across the tubular wall. Preferential reabsorption of Cl^- over HCO_3^- in mid- and late proximal convolutions (2) will be expected to increase Cl^- concentration and decrease that of HCO_3^- in peritubular capillaries.² The lowering of HCO_3^- in capillary blood should minimize the impact of addition of HCO_3^- -rich reabsorbate in the early proximal region. Delivery of surface blood relatively rich in Cl^- and poor in HCO_3^- to the deep countercurrent system should facilitate HCO_3^- reabsorption in the very early proximal tubule. L, liter.

to peritubular capillaries progressively reducing the transepithelial Cl^- concentration gradient, thus retarding Cl^- reabsorption in downstream segments. The superficial countercurrent flow system may also influence HCO_3^- transport in the superficial proximal tubule. In this case, however, the system may be poised to promote HCO_3^- reabsorption in early convolutions. Because reabsorbate from mid- and late proximal convolutions has a relatively low HCO_3^- concentration its addition to peritubular capillaries should reduce HCO_3^- concentration in capillary blood. Delivery via the countercurrent system of blood with a relatively low HCO_3^- concentration to the early proximal tubule should ameliorate the rise in capillary blood HCO_3^- concentration consequent to the higher rate of HCO_3^- reabsorption in this segment. In this fashion HCO_3^- concentration in early proximal capillaries could be kept at a level lower than in late proximal capillaries. Keeping of HCO_3^- concentration in early proximal capillaries at reduced levels may be necessary to maintain the higher H^+ secretory rate they are capable of as suggested by our pH_i studies.

The nephron organizational pattern found on the renal surface may be also of relevance concerning the declining axial PCO_2 gradient described by Maddox et al. (34) in the lumen and peritubular capillaries along the proximal tubule. Clustering of proximal convolutions into discrete areas will tend to build up local PCO_2 concentration differences. In addition, the countercurrent pattern of tubular fluid and capillary blood flows would also help to maintain luminal PCO_2 differences between early and late proximal convolutions by allowing for a more efficient loss of luminal CO_2 into peritubular capillaries as tubular fluid flows along the proximal tubule. These considerations make it likely that the concentric pattern of pH_i differences on the renal

surface may be mirrored by a similar pattern of luminal and capillary PCO_2 differences which may involve the intracellular compartment as well.

In summary, these results demonstrate that pH_i declines axially along the superficial proximal convoluted tubule. Because surface convolutions emerge in a highly organized fashion, the axial pH_i gradient is expressed as nearly concentric areas with the clustering next to the star vessel having the most acidic values of cell pH . This axial heterogeneity is due in part to the declining profile of luminal pH or HCO_3^- concentration and in part to differences in the mechanisms of pH_i regulation in the two segments. We propose that this difference represents an enhanced rate of active H^+ extrusion across the luminal membrane.

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2. HCO_3^- and Cl^- concentrations in star vessel plasma have been reported (7) to be 28 and 102 meq/liter, respectively (compared with systemic values $\Delta\text{HCO}_3^- = +6$ meq/liter and $\Delta\text{Cl}^- = -8$ meq/liter). Using the values of Liu and Cogan et al. (2) for segmental HCO_3^- , Cl^- and fluid reabsorption in the last 3 mm of the proximal tubule (length of accessible proximal tubule in Sprague-Dawley studies in our laboratory), reabsorbate HCO_3^- and Cl^- concentration in the 1st mm of the superficial proximal tubule can be calculated to be 28 and 165 meq/liter, respectively, and those in mid and late segments (last 2 mm) 22 and 152 meq/liter.

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