An In Vivo Microperfusion Study of Distal Tubule Bicarbonate Reabsorption in Normal and Ammonium Chloride Rats

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Abstract

For many years it has been thought that distal nephron hydrogen ion secretion can be importantly modulated by factors such as sodium delivery, sodium avidity, and potassium stores. Free flow micropuncture studies have also indicated that the rate of bicarbonate delivery may also alter the rate of bicarbonate reabsorption. The present studies were undertaken to examine possible luminal influences on total CO_2 reabsorption in microperfused distal tubules in the rat in vivo. Tubules from normal and acidotic rats were perfused with five solutions in a manner that induced changes in bicarbonate load, sodium and potassium fluxes (J_{Na}, J_K), and luminal sulfate concentration. In each collected perfusate, simultaneous analyses were undertaken to determine water reabsorption, Na, and K concentrations using graphite furnace atomic absorption spectroscopy and total CO_2 by microcalorimetry. Using factorial analysis of covariance to account for confounding effects on total CO_2 flux (J_{tCO_2}) such as water reabsorption, distal tubules of acidotic rats reabsorbed CO_2 in the range of 50–112 pmol $\cdot$ min$^{-1} \cdot$ mm^{-1}. These J_{tCO_2} values were not significantly correlated with HCO_3 load, J_{Na}, or J_K despite changes in the latter from net reabsorption to net secretion. Distal tubules of rats with normal acid-base status had J_{tCO_2} values which were neither significantly different from zero nor correlated with changes in J_K and J_{Na}. Further, doubling the load from 250–500 pmol/min (by doubling the perfusion rate of 25-mM HCO_3 solutions) did not stimulate J_{tCO_2} in these normal animals. Accordingly, these acute in vivo microperfusion studies indicate for the first time that neither load nor potassium or sodium fluxes are important modulators of distal tubule bicarbonate reabsorption.

Introduction

For more than 30 yr, it has been thought that renal H$^+$ secretion may depend on Na and K transport. The cation exchange hypothesis first proposed by Pitts and Alexander (1) and then by Berliner et al. (2) was modified by Schwartz and his coworkers (3) to explain observations on chronic acid-base disturbances. It was suggested that distal nephron hydrogen ion secretion is modulated by luminal factors such as distal

Na delivery, Na avidity, and anion effects. Perhaps the most provocative aspect of this view was the specific denial that blood pH is a regulator of hydrogen ion secretion.

There are, of course, many studies which have attempted to define factors which may regulate distal nephron hydrogen ion secretion and which bear on the proposals noted above. For example, volume expansion has been considered to suppress hydrogen ion secretion while extracellular fluid (ECF)[1] volume contraction enhances it (4, 5). The load of bicarbonate presented to reabsorptive sites was considered to be a "key" factor in determining hydrogen ion secretion and bicarbonate reabsorption in micropuncture studies on rat distal tubules (6). Evidence from direct studies on the papillary collecting duct of the rat also indicates that bicarbonate load importantly determines its reabsorptive rate (7). Finally, potassium handling has been suggested to be another regulator: potassium loading in dogs increases K secretory fluxes and suppresses the rate of distal nephron hydrogen ion secretion (4). However, in rat distal tubule micropuncture studies, no evidence of a reciprocal relationship between distal tubular K and hydrogen ion secretory rates could be obtained (8).

The present studies were initiated in the context of the foregoing reports. Bearing in mind that ECF volume, bicarbonate load, and potassium and sodium fluxes (J_K, J_{Na}) might play a role in hydrogen ion secretion of the distal nephron, we undertook to directly study in the rat the portion of the distal tubule accessible to micropuncture. Our specific objective was to test the hypothesis that distal tubule bicarbonate reabsorption, measured as total CO_2 flux (J_{tCO_2}), is linked either to bicarbonate load or J_K, J_{Na}. By in vivo microperfusion, we changed both the flow rate and composition of the perfusion fluid without alteration in ECF volume. In each collection of the perfusate we measured simultaneously J_{tCO_2}, J_K, and J_{Na}. Our results from normal and NH$_4$Cl acidotic rats indicate that the normal rat distal tubule does not reabsorb CO_2 significantly, and this cannot be enhanced by either doubling the CO_2 load or altering J_{Na} or J_K by perfusate changes. NH$_4$Cl acidotic rats, in contrast, have distal tubules which consistently show positive J_{tCO_2} values, and which cannot be suppressed by either reduction in load or by inducing wide swings in J_K or J_{Na}. Accordingly, distal tubule J_{tCO_2} does not appear to be linked to these luminal factors. Rather, at least for the case of NH$_4$Cl rats, distal tubule J_{tCO_2} may be responsive to the low pH of systemic blood, or to changes in the cytoplasm of renal tubular cells.

1. *Abbreviations used in this paper:* ANOVA, analysis of variance; BP, blood pressure; BW, body weight; ECF, extracellular fluid; FANCOVA, factial analysis of covariance; FE, flame emission; GFR, glomerular filtration rate; GFAAS, graphite furnace atomic absorption spectroscopy; J_K, potassium flux; J_{Na}, sodium flux; J_{tCO_2}, total CO_2 flux; $\dot{V}$, initial and collected tubular fluid flow rate; J_{H_2O}, water flux.

Methods

These studies were performed on male Sprague-Dawley rats weighing ~300 g. The animals were bred and raised in climate-controlled facilities at the University of Ottawa. The rats were allowed free access to water and food (Rat Chow 5012, Ralston Purina of Canada, Ltd., Whitby, Ontario, Canada) until the time of experimentation. The rats were anesthetized with 100 mg/kg Inactin (BYK, Gulden Konstanz, Federal Republic of Germany) and prepared for micropuncture as previously described (9).

Experimental design

Group I experiments. 27 normal and 52 NH_4Cl acidotic rats were studied. Normal animals were infused with 0.9% NaCl at 0.5% BW/h. Rats were made acidotic by a single gavage of 4 meq/100 g BW NH_4Cl 18 h before the micropuncture experiment. These animals were then infused at the rate of 3% BW/h with 0.9% NaCl to which 50 meq/l HCl was added. Animals so treated were usually acceptable for micropuncture and had low but stable plasma bicarbonate concentrations (see Results), blood pressure (BP) > 100 mmHg, and normal whole kidney glomerular filtration rate (GFR).

In this first group of experiments, superficial distal tubules of both normal and acidotic rats were perfused with one of three solutions at either 10 or 20 nl/min, as described below. Each collected sample was analyzed for total CO_2, Na, K, and inulin concentrations. After microdissection for perfused distal tubule length, J_{tCO_2}, J_K, J_{Na}, and water fluxes (J_{H_2O}) were determined. The data obtained were treated as 12 individual groups in a $2 \times 2 \times 3$ matrix. This represented normal and acidotic rats, high and low perfusion rates, and three different perfusion solutions. Each solution contained 25 mM $NaHCO_3$, 0.12% lissamine green, and 25 μCi/ml of inulin-methoxy 3H (New England Nuclear, Boston, MA). The different solutions in addition contained the following (in mM): solution 1: 95 Na, 65 Cl; solution 2: 95 Na, 65 Cl, 15 K, 7.5 SO_4; and solution 3: 33 Na, 15 K, 7.5 SO_4, 101.5 urea.

Solution 1 allowed K secretion to continue with normal reabsorption of Na. Solution 2 was designed to provide a continuing Na reabsorptive flux while superimposing three factors which might, a priori, be construed to enhance H^+ secretion. The addition of K_2SO_4 would be expected to retard K secretion (10) and increase the transepithelial voltage (11), thereby enhancing H^+ secretion, particularly if there was an element of distal $K^+ - H^+$ competition as already suggested by Pichette et al. (4). The presence of the nonreabsorbable anion $SO_4^=$ might also be expected to facilitate H^+ secretion (3). Solution 3 was chosen to reduce Na reabsorption in the presence of K_2SO_4. Urea was added to provide comparable osmolality with solutions 1 and 2. The perfused rates used and the 25 mM HCO_3 content of each solution ensured sufficient sample for the three separate analyses required (total CO_2, Na, and K, inulin), while permitting a favorable hydrogen ion secretory gradient as HCO_3^- delivery increased from 250–500 pmol/min.

Group II experiments. A second set of distal tubule perfusion studies was carried out to confirm conclusions derived from the first set of experiments described above. NH_4Cl acidotic rats, after gavage, as described for group I animals, were subjected to protocols intended to widen the range of K fluxes as well as to control for the presence of sulfate in solution 2 (above). One group of acidotic rats ($n = 7$) was infused systemically with a solution containing 75 mM KCl, 75 mM NaCl, and 15 mM HCl at 3% of the BW/h. Distal tubules of these animals were perfused at the rate of 15 nl/min using solution 1 as described above. A second group of acidotic rats was infused with 0.9% NaCl plus 50 mM HCl (as for group I experiments described above) but a different microperfusion solution was employed. This microperfusion solution (solution 4) was identical to solution 2 except chloride was substituted for sulfate. The perfusion rate was 15 nl/min.

Microperfusion methods. To improve visualization within the neph-

ron, perfusion pipettes were coated with a platinum resin (Matthey Bright platinum, Johnson, Matthey and Co. Ltd., Cresswell Staffordshire, England), baked in a muffle furnace at 350°C for 1–2 h, and then ground to 7–8 μm outer diameter.

The perfusion pipette was attached to a servo-regulated microperfusion pump (Hampel type III, Wolfgang Hampel, Frankfurt, Federal Republic of Germany). The microperfusion pump chamber was fitted with a Kulite 1S pressure transducer (Durham Instruments, Pickering, Ontario) which permitted continuous measurement of intratubular pressure during perfusion. The pressure device used in these studies was originally described by Wunderlich and Schnermann (12), and subsequently modified for use during microperfusion by Lohfert et al. (13). The pressure device was calibrated in vitro using a Gauer manometer before and after intratubular pressure measurements. Tip resistance was 0.21±0.03 mmHg/nl per min determined in surface fluid ($n = 34$). All measurements were made using a multi-channel Linseis flat-bed recorder which permitted continuous display of perfusion rate and intratubular pressure recordings.

In hundreds of perfusions, we have found this technique of continuous intratubular pressure recordings very useful. We have been able to determine whether intratubular pressure departs from normal both during and between sampling. Although incomplete collections could be obtained despite evidence of stable intratubular pressure values, we have only rarely been able to document adequate collections (i.e., within 10–15% of nominal perfusion rate) in the presence of wide variations in intratubular pressure. For this reason, samples associated with erratic or very abnormal pressure readings were discarded. Collections that departed from nominal perfusion rates by more than ~15% were discarded. The microperfusion pump was calibrated in vitro after each pipette mounting by collection of a timed aliquot of perfusate pumped directly into a scintillation vial over the range of 0–50 nl/min. Such calibrations closely approximated perfusion rates determined in vivo. For a setting of 10 nl/min, in vitro calibration in 50 experiments yielded a value of 9.3±0.3 nl/min compared to calculated in vivo perfusion rates of 9.9±0.2 nl/min in 74 samples. For 20 nl/min, corresponding values were 19.9±0.4 nl/min in 50 experiments vs. in vivo values of 20.4±0.3 nl/min in 88 samples. Finally, for settings of 15 nl/min used in 15 group II experiments noted above, the in vitro values were 15.1±0.3 nl/min vs. in vivo values of 14.0±0.3 nl/min in 26 samples.

The technique of in vivo microperfusion of distal tubules was similar to that already described by Good and Wright (10). Briefly, using randomly punctured proximal tubules, boluses of lissamine green dye were injected in order to locate surface distal tubules with two or more loops suitable for puncture. Upon identification of such a nephron, the microperfusion pipette was placed in the first distal segment and perfusion was allowed to proceed at a low rate. Then, using a larger bore, castor oil-filled pipette, the late proximal segment of the same nephron was punctured, and over a period of several minutes the loop was filled with castor oil. After microperfusion proceeded for several minutes at the designated rate of 10, 15, or 20 nl/min, the second segment of the distal tubule was entered with a 6–7 μm Sudan stained mineral oil pipette. The mineral oil was equilibrated with Hepes buffer (as was the oil in the microperfusion pipette) so as to attempt to preserve an ambient pCO_2 of ~65 mmHg (14, 15). Thereafter, sampling took place by careful insertion of an oil block into the distal segment and constant monitoring of intratubular pressure.

After collections were complete, perfused tubules were filled with Microfil (Canton Biomedical, Boulder, CO). The kidney was removed from the animal, stored at 4°C in saline overnight, and digested in NaOH. After digestion, the microfil cast was photographed, teased free, and total length from perfusion to collection site measured with a calibrated eyepiece micrometer.

Analysis of microperfusion samples

Volumes of collected samples were determined in constant bore quartz tubing. After total volume was determined, the sample was divided

into three aliquots for analyses of [³H]inulin activity, total CO_2, and Na and K concentrations. The perfusate was similarly treated.

Total CO_2 in perfusate and samples was determined by microcalorimetry (14) using oil equilibrated with a Hepes buffer to attain a pCO_2 of 65 mmHg (15). For each analysis, duplicate standards of 15, 20, 25, 30, and 35 mM $NaHCO_3$ were run. The perfusion solution containing 25 mM total CO_2 was determined on the same sample tray as the collected samples which were in the range of ∼20–30 mM. The precision of this method is ±5%.

Graphite furnace atomic absorption spectroscopy (GFAAS) analysis of Na and K in micropuncture samples. Concentrations of sodium and potassium in perfusates and tubular fluid samples were determined by GFAAS using the IL 951/655 (Instrumentation Laboratories, Lexington, MA). Our technique was a modification of that described by Good and Wright (10) and Kuntziger et al. (16). In brief, 0.10–0.20-nl aliquots of standards, perfusate, and micropuncture samples were deposited onto a removable microboat. The microboat is a portable pyrolytically coated graphite platform which can be removed and reinserted into a companion graphite cuvette in the furnace atomizer. The sample aliquots were dried at 75°C, pyrolyzed at 750 and 850°C, and atomized at 2,000°C in the furnace atomizer (IL 655). Sodium and potassium were measured simultaneously in the double-beam mode. Potassium was measured at 766.5 nm and sodium at 589.6 nm. Atomization signals for both Na and K were displayed simultaneously on a video tube and the signals of interest recorded on an Axiom EX-850 video printer. Repeat determinations were within 3% coefficient of variation, often <1% for both Na and K.

Standards were prepared as aqueous solutions containing both sodium and potassium chloride in a concentration range appropriate for the sample analyzed. Atomization signals, however, were sensitive to matrix composition, as evident from the major perturbations in atomization signal with alteration of the chemical form of the analyte. It was therefore necessary to evaluate the extent to which standard curves using sodium and potassium chloride would accurately estimate the total Na and K in complex matrices.

Complete anion substitutions of chloride with acetate, nitrate, fluoride, citrate, hydroxide, sulfate, phosphate, carbonate, and bicarbonate were carried out for Na at 90 meq/liter and K at 15 meq/liter. Complete substitution of chloride by these anions resulted in characteristic alterations in residence time, appearance time, peak shape, and notably peak height for the sodium atomization signals. Sodium peak heights were consistently higher with all chloride substitutions, ranging from 18% enhancement for carbonate to 88% enhancement for both sulfate and phosphate. Peak area, however, was not sensitive to anion substitutions. Phosphate had no effect on peak area sodium signal in agreement with Good and Wright (10). Sulfate showed 7% suppression, and bicarbonate had variable effects (see below). Potassium signals were not as sensitive to changes in chemical form of the analyte for both peak height and peak area as sodium. Peak area absorbance for K for all substitutions was identical to KCl except for carbonate (+4%). Peak height absorbance was also insensitive to anion substitutions with the exception of enhancement of sulfate (+17%) and phosphate (+12%). Peak area absorbance was therefore used to generate calibration curves for both Na and K in all analyses.

Validation of the accuracy of Na and K determination was undertaken for every set of samples analyzed. Perfusates were analyzed daily by flame emission (FE) and compared with GFAAS analysis. Data for Na and K were only acceptable if the relative error (defined as the mean error expressed as a percentage of the accepted value [FE]) was within 3%. Sodium for solution 1 ($n = 28$) was 95.2±0.77 meq/liter by FE compared with 94.6±0.74 meq/liter by GFAAS. Solution 2 ($n = 18$) gave 96.1±0.30 meq/liter by FE for Na and 14.7±0.05 meq/liter for K by FE compared with Na of 95±0.53 with 15.3±0.10 meq/liter for K. Solution 3 ($n = 16$) had sodium concentration of 33.0±0.25 meq/liter and 14.8±0.06 meq/liter for K determined by FE vs. 33.3±0.7 meq/liter for Na and 14.8±0.08 meq/liter for K by GFAAS. Solution 4 in nine experiments gave 93.4±0.4 meq/

liter Na and 14.7±0.05 meq/liter K by FE vs. GFAAS values for Na and K of 95.0±0.53 meq/liter and 14.7±0.3 meq/liter, respectively.

Extensive studies with simulated samples revealed an effect of bicarbonate on enhancing the sodium signal which was constant over a wide range of sodium concentration (60–150 meq/liter) and varied as a function of the bicarbonate concentration. Insofar as the perfused bicarbonate concentrations and collected concentrations were always within a narrow range, and since the perfusion solution was always analyzed along with unknown samples, the presence of such bicarbonate interference was always detectable by measurement of the perfusion standard. Such interference was corrected by changing the microboat or, on occasion, machine parameters. In short, in our hands, bicarbonate enhancement of the Na signal was very infrequent, always detectable, and remedied successfully. The precise explanation for such enhancement or its correction continues to elude us.

To preclude other possible interferants that may be endogenous to tubular fluid or collected perfusate, recovery studies were carried out on representative samples. Table I shows the results for recovery of added Na and K to five micropuncture samples. Recoveries for sodium varied from 96.6 to 107.7% while potassium recoveries varied from 96.9 to 101.8%.

Calculations and statistical methods

The collected tubular fluid flow rate ($\dot{V}$) was determined by measuring total volume and time for collection. The initial perfusion rate ($\dot{V}$) was calculated as the product of $\dot{V}$ and the quotient of the [³H]inulin concentration in the sample and the perfusate. Absolute fluxes were calculated as the difference between load (i.e., $\dot{V}$ times initial concentration of tCO_2, Na, or K) and collected amounts (i.e., $\dot{V}$ times final concentration). Flux values, J, are expressed as $pmol \cdot min^{-1} \cdot mm^{-1}$.

A two-level approach was used for drawing statistical inferences from our data. The first approach used the traditional methods of t testing and simple analysis of variance. The second approach involved more sophisticated statistical techniques to more rigorously assess the many factors in our study which simultaneously could influence the rate of bicarbonate reabsorption.

In group I experiments, the effects of J_K, J_{Na}, and J_{H_2O} were considered to be the principal potential modulators of J_{tCO_2}. Systematic differences in these parameters were assessed with respect to three factors: normal acid-base status vs. systemic metabolic acidosis, perfusion rate of 10 nl/min vs. 20 nl/min, solutions 1, 2, and 3. To assess these effects, initially unpaired t testing was used to compare normal vs.

*Table I. Recovery of Sodium and Potassium from Tubular Fluid Samples**

Sample Na	Na recovery after standard addition	Sample K	K recovery after standard addition
meq/l	%	meq/l	%
129.8	104.7±1.6 ($n = 10$)	4.2	99.4±1.10 ($n = 10$)
50.4	96.58±3.2 ($n = 4$)	8.6	100.7±2.2 ($n = 4$)
88.4	104.2±1.2 ($n = 5$)	4.7	101.8±1.06 ($n = 5$)
57.8	99.8±4.2 ($n = 5$)	1.6	99.8±4.2 ($n = 5$)
85.1	107.7±2.5 ($n = 5$)	4.5	96.9±2.7 ($n = 5$)

* See Methods for details.

acidotic rats as well as flow rate. One-way analysis of variance, followed by the Student-Newman Keul's procedure was used to judge the effect of the three solutions.

Since an important goal of this study was to assess the influence of J_{Na}, J_K and J_{tCO_2} upon each other, in each of the 12 treatment cells noted above (two conditions of acid-base status times two flow rates times three solutions) partial correlation coefficients between pairs of these measures were generated, each of which were controlled for the other response measures and the effect of water movements. In this fashion, the confounding influences of the factors were accounted for.

Although the techniques just described provided useful insight into relationships underlying our data, they nevertheless failed to fully exploit the potential of our experimental design. As a further step, nine samples were randomly selected from each of the twelve experimental cells, thereby yielding a sample size of 108 observations from the original pool of 163. The nine observations in each cell generally came from different animals, but occasionally from different tubules within the same animals. Factorial analysis of covariance (FANCOVA) was then used to simultaneously assess the influence of the three experimental factors (acid-base status, flow rate, and solutions) as well as the confounding influence of water movements, sodium, and potassium movements on total CO_2 reabsorptive rates (17). As shown in the Results and in the Appendix, the formal analysis of variance table and the cell means as adjusted for the significant covariance are reported.

To confirm the conclusions from group I experiments, additional experiments were done on two further groups of acidotic rats (see above). As described above, nine samples were allocated to each cell from this two-group portion of the larger study, and a one-way ANCOVA was used to assess the various effects. Adjusted cell means are also presented for this analysis (see Results).

Results

Table II summarizes acid-base and electrolyte values in normal and NH_4Cl acidotic rats with and without systemic KCl infusions. GFR for normal animals was 1.0 ± 0.1 ml/min, for NH_4Cl rats 1.2 ± 0.1 ml/min, and 1.1 ± 0.1 ml/min for NH_4Cl rats infused with KCl. The GFR value for NH_4Cl rats was significantly higher when compared with normal rats ($P < 0.05$).

Group I experiments. In group I experiments, 91 samples (43 tubules) were taken from 30 NH_4Cl acidotic rats and 73 samples (33 tubules) from 21 normal rats. The corresponding values for J_{tCO_2}, J_{Na}, and J_K are presented in Tables III and IV. Table III shows the raw sample data expressed as the sample mean±SEM, whereas Table IV shows the covariance

Table II. Acid-Base and Electrolyte Values for Normal and NH₄Cl Acidotic Rats

	Normal (n = 19)	NH₄Cl acidosis (n = 30)	NH₄Cl acidosis KCl loaded (n = 13)
pH	7.400±0.015	7.125±0.015*	7.039±0.022‡
pCO₂ (mmHg)	45.08±1.42	35.84±0.74*	28.34±1.36‡
HCO₃ (meq/liter)	26.98±0.44	11.80±0.43*	7.51±0.51‡
Na (mEq/liter)	143.8±1.0	141.3±0.50*	140.8±0.88
K (mEq/liter)	4.2±0.10	4.4±0.10	5.77±0.29‡
Cl (mEq/liter)	105.2±0.80	124.9±0.80*	126.0±0.78
Hematocrit (%)	46.1±0.80	48.8±0.60*	50.5±1.09
Protein (g/dl)	5.20±0.05	5.40±0.08	4.70±0.17‡

* $P < 0.05$, vs. normal rats (unpaired t test).

‡ $P < 0.05$, vs. NH₄Cl acidotic rats (unpaired t test).

adjusted mean values which were generated as described in Methods.

In normal rats the mean J_{tCO_2} values at either 10 or 20 nl/min perfusion rates were not significantly different from 0 by t testing ($P > 0.05$). Acidotic rats, however, showed consistently positive J_{tCO_2} values at both perfusion rates for the three solutions used. These values were significantly different from 0 ($P < 0.05$) and are always significantly higher than corresponding values from normal rats ($P < 0.05$) by the unpaired t test. The striking difference between normal and acidotic rats is easily apparent from changes in the total CO_2 concentrations. The tubular fluid (TF)/perfusion fluid (PF) tCO_2 ratio (TF/$_{PF}$) was 1.15 ± 0.11, 1.19 ± 0.05, and 1.04 ± 0.04 for the three solutions in normal rats perfused at 10 nl/min, compared with the corresponding values in acidotic rats of 0.80 ± 0.05, 0.87 ± 0.08, and 0.81 ± 0.04 ($P < 0.01$) in each instance. Similar values were observed during 20-nl/min perfusions.

ANOVA testing (Student-Newman Keul) also showed that in both normal and acidotic rats, J_{tCO_2} values did not differ as a function of solution or flow-rate except for the case of acidotic rats perfused with solution 1 vs. solution 2 at 20 nl/min. J_{tCO_2} was significantly different ($P < 0.05$). This result was reassessed by FANCOVA as described below. Values for water reabsorption, usually in the range of 1–2 nl·min⁻¹·mm⁻¹, are accounted for as well in the FANCOVA (see below). In order to determine whether J_{tCO_2} values are affected by changes in perfusion rate or J_K or J_{Na}, a FANCOVA was undertaken. Confounding variables influencing J_{tCO_2}, particularly water movements, are held constant or accounted for by this technique. The covariance adjusted means (Table IV) show the resulting estimate of J_{tCO_2}, J_K, and J_{Na} values when simultaneous influences of water movement are held constant. This FANCOVA analysis indicated that neither flow rate nor J_K or J_{Na} significantly influence J_{tCO_2} in either normal or acidotic rats.

Generation of partial correlation coefficients between J_{tCO_2}, J_K, and J_{Na} was also undertaken. This evaluation revealed significant linkage only between J_{tCO_2} and J_{Na} in acidotic rats perfused with solution 2 ($r = 0.60$, $P < 0.01$) and in the case of J_{tCO_2} vs. J_K in normal rats perfused with solution 1 ($r = 0.56$, $P < 0.02$). Note that this significant partial correlative effect occurred in only two of the twelve cells tested. It was of further interest to note that whereas FANCOVA treated the twelve subsets as a group, the partial r analysis treated them individually.

Group II experiments. To confirm the conclusions derived from the group I experiments, namely that neither flow rate nor movements of J_K or J_{Na} influence J_{tCO_2}, additional experiments were undertaken in two groups of acidotic rats. Our attention was specifically devoted to broadening the range of J_K and thereby possibly uncovering an effect which might not be detected in the group I experiments. Table V shows that J_K in acidotic rats infused with KCl (solution 1) had higher values for J_K than in the previous twelve subsets studied. The perfusate containing KCl was used to control for effects which may have been engendered by the use of sulfate (solution 2). Thus, with particular reference to different influences on J_K, four separate groups were studied and are presented in Fig. 1, which clearly shows that J_{tCO_2} changes little in the presence of wide swings of J_K.

As presented for group I experiments, Table V shows the mean values for samples (mean±SEM). These data were also

Table III. Distal Tubule tCO_2, K, and Na Fluxes* in NH_4Cl and Normal Rats

| | Normal rats | | | | | |
| | J_{tCO_2} | | J_K | | J_{Na} | |
Perfusion rate (nl/min)	10	20	10	20	10	20
Solution 1						
(mean±SE)	7±12	−4±12	−55±11	−82±10	215±51	146±28
No. rats/no. tubules	7/8	7/10	6/6	7/8	7/7	7/9
Solution 2						
(mean±SE)	4±9	−17±10	−19±13	3±21	53±35	49±35
No. rats/no. tubules	5/6	7/8	5/6	7/8	5/6	7/8
Solution 3						
(mean±SE)	13±11	8±16	35±14	8±15	−116±58	−173±88
No. rats/no. tubules	5/8	6/9	5/7	6/9	5/8	6/10
	NH_4Cl rats					
Perfusion rate (nl/min)	10	20	10	20	10	20
Solution 1						
(mean±SE)	74±14	119±22	−32±4	−44±5	126±50	310±66
No. rats/no. tubules	7/9	7/9	7/9	6/9	7/9	6/8
Solution 2						
(mean±SE)	58±11	43±11	14±14	7±10	103±41	50±34
No. rats/no. tubules	7/8	9/10	7/8	9/10	7/8	9/10
Solution 3						
(mean±SE)	82±8	83±16	0±9	9±21	−28±35	−221±72
No. rats/no. tubules	12/16	12/16	8/11	6/9	8/11	6/9

* $pmol \cdot min^{-1} \cdot mm^{-1}$.

tested by FANCOVA using nine samples from each group. The covariance-adjusted mean values for J_{tCO_2}, J_K, and J_{Na} ($pmol \cdot min^{-1} \cdot mm^{-1}$) for acidotic rats infused with KCl were 53, −118, and 177, respectively. Corresponding values for solution 4 were $J_{tCO_2} = 37$, $J_K = 48$, and $J_{Na} = 119$ $pmol \cdot min^{-1} \cdot mm^{-1}$.

Table IV. FANCOVA-adjusted Means for Distal Reabsorption*

Animal group	Perfusate	Perfusion rate	J_{tCO_2}	J_{Na}	J_K
Normal rats	Solution 1	20	−1	167	−77
	(95 Na, 0 K)	10	11	155	−59
	Solution 2	20	−8	61	−6
	(95 Na, 15 K)	10	19	85	−16
	Solution 3	20	25	−64	17
	(33 Na, 15 K)	10	24	−103	35
Acidotic rats	Solution 1	20	93	321	−59
	(95 Na, 0 K)	10	83	102	−26
	Solution 2	20	56	83	12
	(95 Na, 15 K)	10	52	89	12
	Solution 3	20	112	−270	−5
	(33 Na, 15 K)	10	78	−40	−5

Means for J_{tCO_2}, J_{Na}, and J_K were adjusted for correlation with J_{H_2O}.
* $pmol \cdot min^{-1} \cdot mm^{-1}$.

Analysis of covariance testing in this fashion revealed no influence of J_K or J_{Na} on J_{tCO_2}. Finally, partial correlation analysis was also undertaken as described above, and no linkage between J_{tCO_2} and J_{Na} or J_K could be demonstrated.

Discussion

In these in vivo experiments, we have perfused distal tubules in normal and NH_4Cl acidotic rats in order to determine if bicarbonate reabsorption measured as total CO_2 is linked to intratubular bicarbonate load or fluxes of potassium or sodium. By varying the flow rate and composition of the perfusion solution, we have been able to induce changes in bicarbonate load as well as in J_K and J_{Na}. Simultaneous measurement of these three fluxes in each of 189 samples from five experimental groups enabled us to draw the following conclusions: (a) changing J_K and J_{Na} in distal tubules of normal rats does not stimulate J_{tCO_2}; (b) in NH_4Cl acidotic animals, where significant J_{tCO_2} is consistently demonstrated, reversal of J_K and J_{Na} does not suppress this reabsorptive activity; (c) doubling of the load of bicarbonate perfused does not influence the J_{tCO_2} of normal or NH_4Cl acidotic rats. Accordingly, our in vivo direct measurements show for the first time that neither load nor potassium or sodium fluxes are important modulators of distal tubule bicarbonate reabsorption.

At the outset, it is very important to review some difficult statistical issues which we encountered in analyzing the present

Table V.* Distal Tubule Total tCO_2, K, and Na Fluxes‡ in NH_4Cl Acidotic Rats§

		15 nl/min		
		J_{tCO_2}	J_K	J_{Na}
Solution 1 plus systemic KCl infusion	Mean±SEM	57±8	−112±21	175±32
	No. rats/no. tubules	7/10	7/10	7/10
Solution 4	Mean±SEM	40±8	50±26	127±38
	No. rats/no. tubules	7/9	7/9	7/9

* See also text for details. ‡ $pmol \cdot min^{-1} \cdot mm^{-1}$. § Group 2 experiments.

data. We have already been acutely aware of various statistical problems inherent in the design of micropuncture experiments. In previous publications, we have drawn attention to the mandatory use of appropriate ANOVA techniques as well as the use of partial correlation analysis (18, 19). Thus, in the present study, as in many other micropuncture investigations, the questions asked involve the allocation of an effect of one parameter or perturbation upon another measured parameter. In the present experiments, our specific question can be simply restated as follows: "Are distal tubule total CO_2 fluxes linked with changes in sodium or potassium fluxes?" We have already shown how changes in mean values in different protocols and significance of simple correlation coefficients cannot answer such a question when there are confounding variables such as changes in water reabsorption, perfusion rates, and different experimental protocols (19). As discussed in detail in Methods and Results, an initial factorial analysis of variance matrix was developed using 12 different sets which derived from normal and acidotic rats perfused at two flow rates using three different perfusates. In a separate analysis, to confirm the conclusions of the first set of experiments, the results of a second group of experiments using two further protocols were also analyzed. Factorial analysis of variance indicated that when J_{H_2O}, which strongly influenced movements of J_{tCO_2}, J_{Na}, and J_K, was accounted for, there was no demonstrable effect on J_{tCO_2} induced by either changing the load of perfused bicarbonate or the five different protocols employed. The subsequent evaluation of partial correlation coefficients (partial r) generally confirmed this overall finding. That is, with the exception of a significant partial r of J_{tCO_2} and J_K in normal rats using the high sodium zero K solution, and in acidotic rats of J_{tCO_2} and

J_{Na} using the K sulfate-containing solution, partial r values were not found to be significant in any of the other 12 cells tested. Thus, in view of the very wide swings of potassium and sodium fluxes we intentionally induced, these rigorous tests of statistical significance strongly support the conclusion that J_{tCO_2} is not importantly linked with J_{Na} and J_K or with bicarbonate load.

The most important finding of our studies is the demonstration, in vivo, that rat distal tubule bicarbonate reabsorption is seemingly uninfluenced by the bicarbonate load or by changes of J_{Na} and J_K from net reabsorption to net secretion. It is appropriate to consider this result with regard to the following issues: anatomic heterogeneity of the distal tubule; acidification characteristics of distal nephron segments other than the distal tubule; anion effects; effects of bicarbonate load; and the non-homeostatic hypothesis (3) of renal acidification.

Anatomic heterogeneity of the distal tubule. Of course, it has been long recognized (20) that the distal tubule of the rat is composed of different types of epithelium. The distal tubule on the surface of the kidney that confronts the micropuncturist is known to be composed of epithelium of the distal convoluted tubule, the connecting tubule, and of the initial collecting tubule. As already noted by Lucci et al. (15), this anatomic heterogeneity could contribute to the variability of bicarbonate reabsorptive rates measured in normal rats and rabbits. Since it is possible that the mitochondria-rich intercalated cells found in the initial collecting tubule may secrete protons (20), the length of such epithelium traversed by our perfusions could influence the observed rate of bicarbonate reabsorption. It is important to appreciate that in the present studies no attempt was made to perfuse either early or late surface distal tubule segments. Rather, the length and location of the perfused epithelium was dictated only by the presence of two surface loops (see Methods) and the convenience of placement of perfusion and collection pipettes. There can be no doubt, therefore, that the 189 samples that comprise the present study must have derived from distal tubule segments which were composed of both initial collecting tubule epithelium as well as "true" early distal convoluted tubule epithelium (20). It appears reasonable to conclude that whatever the intimate mechanisms of proton secretion in surface distal tubules, the present studies almost certainly reflect the combined contributions of different epithelial types to acidification in the face of changes in bicarbonate load, J_{Na}, and J_K (see below).

Acidification characteristics of distal nephron segments other than the distal tubule. Recently, several important in

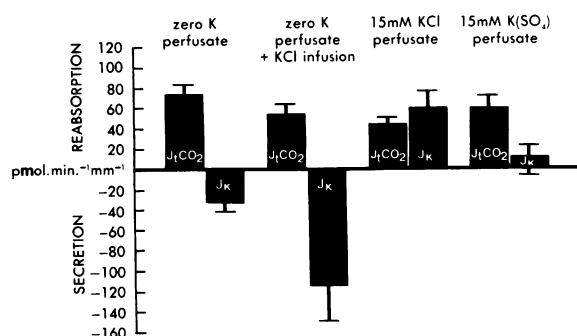Figure 1. J_{tCO_2} and J_K values during distal tubule in vivo perfusion in four experimental settings. Shown are mean values±SEM.

vitro studies have been reported which have specifically addressed the sodium dependency of acidification of distal nephron segments. The study of Laski and Kurtzman (21) has provided evidence that CO_2 reabsorption by the cortical collecting tubule is consistent with a model of electrogenic acidification that is dependent on a luminal potential generated by sodium transport. Some sodium dependence of acidification by the cortical collecting tubule using another technique has also been shown by Koeppen and Helman (22). These suggestions of sodium-dependent cortical collecting tubule acidification are in contrast to the earlier work of McKinney and Burg (23). McKinney and Burg (23) and Lombard et al. (24) have also reported that the cortical collecting tubule appears to reabsorb more bicarbonate during in vitro studies when the antemortem state of the animal was acidosis. Other studies (Laski and Kurtzman [21], Lombard et al. [24], and Stone et al. [25]) seem to agree that direct sodium dependency is not demonstrable in the rather brisk acidification of the medullary collecting tubule. Finally, in vivo reports involving the inner medullary collecting duct did not demonstrate an obvious link between hydrogen ion secretion and sodium or potassium movements (26, 27). How, then, do the present distal tubule studies fit in with these other reports? At the outset, it must be understood that in no sense should our results on distal tubules be interpreted to mean that the CO_2 reabsorptive rates we observed in NH_4Cl rats are sodium-independent in a strict transport sense. Our protocols did not involve lowering the luminal sodium concentration below 33 mM, nor was there any manipulation of the peritubular sodium concentration. Thus, our experiments were not intended to demonstrate a sodium or potassium limitation of net acidification by the distal tubule. Rather, what our studies do show is that over a wide range of sodium and potassium concentrations in the perfusate, and subsequent major alterations of net flux (for J_{Na}, +321 $pmol \cdot min^{-1} \cdot mm^{-1}$ to −270 pmol/min per mm; for J_K, −112 $pmol \cdot min^{-1} \cdot mm^{-1}$ to +50 pmol/min per mm), J_{tCO_2} continued to be brisk and seemingly uninfluenced by such flux changes (Tables IV and V). Accordingly, we conclude, based on the simultaneous measurement of J_{tCO_2}, J_{Na}, and J_K of distal tubule samples in vivo, that there cannot be tightly coupled Na-H or K-H secretion in a net sense. Other in vitro distal tubule perfusions or in vivo simultaneous luminal and peritubular perfusions, perhaps with the use of amiloride and ouabain, could more completely characterize the mechanism of acidification.

Anion effects. In the present studies, in eight experiment subgroups, sulfate was substituted for chloride. Thus, in solution 2 there is partial substitution of sulfate for chloride in the presence of a sodium concentration in the perfusate of 93 mM. In solution 3, sulfate was completely substituted for chloride while perfusate sodium concentration was 33 mM and potassium concentration was such as to prevent K secretion. These substitutions at high and low flow in both normal and acidotic animals did not reveal an obvious effect on J_{tCO_2}. Notwithstanding this result, it is clear that to completely exclude anion effects it would be important not only to study other anions such as gluconate, cyclamate, and tetramethylammonium, but also to conduct substitutions with other permutations of high and low sodium concentrations in the perfusate.

Effects of bicarbonate load. As already noted, doubling of the perfused bicarbonate load from 250 to 500 pmol/min could not elicit an enhancement of bicarbonate reabsorption in normal animals, nor alter the brisk rate of reabsorption in acidotic rats. The perfusions used contained ∼25 mM bicarbonate, which facilitated measurement of tubular fluid total CO_2 concentration and made unlikely a gradient limitation to proton secretion. We recognize that lower rates of bicarbonate perfusion (<250 pmol/min) or lower luminal concentrations of bicarbonate could reveal an effect of perfused load on J_{tCO_2} if the present results in fact represent saturation of the proton secretory rate in acidotic animals. In any case, contrary to previous reports (6), at least in the 12 experimental subsets studied by us, load was not a regulator of J_{tCO_2}.

The non-homeostatic hypothesis (3) of renal acidification. In the Introduction we have drawn attention to the early views of renal acidification and those more recently and elegantly presented by Schwartz and Cohen (3). The non-homeostatic theory of acidification specifically denies that pH is an important determinant of hydrogen ion secretion, but rather sodium delivery and sodium reabsorption by sodium "avid" reabsorbing sites is the principal modulator of acidification. It must be stressed that the experiments which led to the formulation of this theory by Schwartz and his colleagues (3) have been, in our view, impeccably executed and reasonably interpreted. These studies, usually of chronic duration in unanaesthetized dogs and humans, stand in contrast to the present acute perfusion experiments which did not involve changes in sodium avidity, total body potassium stores, extracellular fluid volume, chloride balance, etc. Further, the views of Schwartz and Cohen (3) of necessity were not extended to provide predictions of the acidification characteristics of discrete segments that comprise the distal nephron. Rather, their proposals have drawn attention, in a seemingly satisfactory fashion, to a possible central role for sodium reabsorption in explaining long-term chronic steady state adjustments by the whole kidney. Thus, the present in vivo distal tubule perfusion studies cannot be construed as a test of these views. Rather, the significance of our findings is that for the first time in vivo, a portion of the distal nephron—the distal tubule composed of several types of epithelium (see above)—can be made to drastically alter the magnitude and direction of net sodium and potassium transport without, seemingly, importantly modulating bicarbonate reabsorption. Thus, in acidotic rats where bicarbonate reabsorption is brisk, net sodium secretion did not impair simultaneous bicarbonate reabsorption, whereas in normal animals where bicarbonate reabsorption is difficult to demonstrate, vigorous sodium reabsorption plus partial or complete substitution of sulfate with chloride (thought to favor hydrogen ion secretion by the non-homeostatic theory) was not associated with enhanced bicarbonate reabsorption. A more definitive test of the non-homeostatic hypothesis must involve experiments on tubules from animals subjected to the chronic disturbances studied so successfully by Schwartz and his colleagues (3).

In summary, we have undertaken in vivo distal tubule microperfusion experiments in normal and NH_4Cl acidotic rats. We have been able to show for the first time that the brisk J_{tCO_2} reabsorption by distal tubules of acidotic rats is unresponsive to major changes in J_{Na}, J_K, or bicarbonate load induced by in vivo luminal perfusion.

Appendix
*FANCOVA table for J_{tCO_2} as dependent variable**

Source	Sum of squares	Degrees of freedom	Mean square	F	Tail probability
pH level	114742.11	1	114742.11	49.83	0.000
Solution	15900.47	2	7950.23	3.45	0.036
Flow-rate	71.04	1	71.04	0.03	0.861
pH level × solution	5483.98	2	2741.94	1.19	0.309
pH level × flow-rate	5406.76	1	5406.76	2.35	0.129
Solution × flow-rate	3942.50	2	1971.25	0.86	0.428
pH × solution × flow-rate	160.71	2	80.35	0.03	0.966
Covariate H_2O	61344.80	1	61344.80	26.64	0.000
Error	218770.43	95	2302.85		
Regression coefficient	16.573				

* See Methods and Results for details.

References

1. Pitts, R. F., and R. S. Alexander. 1945. The nature of the renal tubular mechanism for acidifying the urine. *Am. J. Physiol.* 144:239–254.

2. Berliner, R. W., T. J. Kennedy, Jr., and J. G. Hilton. 1950. Renal mechanisms for excretion of potassium. *Am. J. Physiol.* 162:348–367.

3. Schwartz, W. B., and J. J. Cohen. 1978. The nature of the renal response to chronic disorders of acid-base equilibrium. *Am. J. Med.* 64:417–428.

4. Pichette, C., Siu-Cheung Tam, Ching-Bun Chen, M. Goldstein, B. Stinebaugh, and M. Halperin. 1982. Effect of potassium on distal nephron hydrogen ion secretion in the dog. *J. Lab. Clin. Med.* 100:374–384.

5. Tam, S. C., M. B. Goldstein, R. M. A. Richardson, W. L. M. Robson, B. J. Stinebaugh, and M. L. Halperin. 1980. Effect of extracellular fluid volume contraction on distal-nephron hydrogen ion secretion in vivo. *J. Lab. Clin. Med.* 96:442–459.

6. Malnic, G., M. de Mello Aires, and G. Giebisch. 1972. Micropuncture study of renal tubular hydrogen ion transport in the rat. *Am. J. Physiol.* 222:147–158.

7. Richardson, R. M. A., and R. T. Kunau, Jr. 1982. Bicarbonate reabsorption in the papillary collecting duct: effect of acetazolamide. *Am. J. Physiol.* 243:F74–F80.

8. Malnic, G., M. de Mello Aires, and G. Giebisch. 1971. Potassium transport across renal distal tubules during acid-base disturbances. *Am. J. Physiol.* 221(4):1192–1208.

9. Levine, D. Z., M. K. Byers, R. A. McLeod, and J. A. Luisello. 1979. Loop of Henle bicarbonate accumulation in vivo in the rat. *J. Clin. Invest.* 63:59–66.

10. Good, D. W., and F. S. Wright. 1979. Luminal influences on potassium secretion: sodium concentration and fluid flow rate. *Am. J. Physiol.* 236(2):F192–F205.

11. Hayslett, J. P., E. L. Boulpaep, and G. H. Giebisch. 1978. Factors influencing transepithelial potential difference in mammalian distal tubule. *Am. J. Physiol.* 234(3):F182–F191.

12. Wunderlich, P., and J. Schnermann. 1969. Continuous recording of hydrostatic pressure in renal tubules and blood capillaries by use of a new pressure transducer. *Pfluegers Arch. Eur. J. Physiol.* 313:89–94.

13. Lohfert, H., I. Lichtenstein, M. Butz, and K. Hierholzer. 1971. Continuous measurement of renal intratubular pressures with a combined pressure transducer microperfusion system. *Pfluegers Arch. Eur. J. Physiol.* 327:191–202.

14. Vurek, G. G., D. G. Warnock, and R. Corsey. 1975. Measurement of picomole amounts of carbon dioxide by calorimetry. *Anal. Chem.* 47:765–767.

15. Lucci, M. S., L. R. Pucacco, N. W. Carter, and T. D. DuBose, Jr. 1982. Evaluation of bicarbonate transport in rat distal tubule: effects of acid-base status. *Am. J. Physiol.* 243:F335–F341.

16. Kuntziger, H., A. Antonetti, S. Couette, C. Coureau, and C. Amiel. 1974. Ultramicro (nanoliter range) determination of calcium concentration ($10^{-3}M$) by atomic absorption. *Anal. Biochem.* 60:449–454.

17. Neter, John, and William Wasserman, editors. 1974. Analysis of covariance for completely randomized designs. *In* Applied Linear Statistical Models. Richard D. Irwin, Inc., Homewood, IL. 685–718.

18. Levine, D. Z., L. A. Nash, T. Chan, and A. H. E. Dubrovskis. 1976. Proximal bicarbonate reabsorption during Ringer and albumin infusions in the rat. *J. Clin. Invest.* 57:1490–1497.

19. Raman, S., G. Mousseau, and D. Z. Levine. 1977. Statistical models for renal micropuncture studies. *Am. J. Physiol.* 233(4):F349–F357.

20. Tisher, C. C. 1981. Anatomy of the kidney. *In* The Kidney. B. M. Brenner and F. C. Rector, Jr., editors. W. B. Saunders Co., Philadelphia. 45–55.

21. Laski, Melvin E., and Neil A. Kurtzman. 1983. Characterization of acidification in the cortical and medullary collecting tubule of the rabbit. *J. Clin. Invest.* 72:2050–2059.

22. Koeppen, Bruce M., and Sandy I. Helman. 1982. Acidification of luminal fluid by the rabbit cortical collecting tubule perfused in vitro. *Am. J. Physiol.* 11:F521–F531.

23. McKinney, T. D., and M. B. Burg. 1978. Bicarbonate absorption by rabbit cortical collecting tubules in vitro. *Am. J. Physiol.* 234(2):F141–F145.

24. Lombard, W. E., J. P. Kokko, and H. R. Jacobson. 1983. Bicarbonate transport in cortical and outer medullary collecting tubules. *Am. J. Physiol.* 244:F289–F296.

25. Stone, D. K., D. W. Seldin, J. P. Kokko, and H. R. Jacobson. 1983. Mineralocorticoid modulation of rabbit medullary collecting duct acidification. *J. Clin. Invest.* 72:77–83.

26. Graber, M. L., H. H. Bengele, E. Mroz, C. Lechene, and E. A. Alexander. 1981. Acute metabolic acidosis augments collecting duct acidification rate in the rat. *Am. J. Physiol.* 241:F669–F676.

27. Ullrich, K. J., and F. Papavassiliou. 1981. Bicarbonate reabsorption in the papillary collecting duct of rats. *Pfluegers Arch. Eur. J. Physiol.* 389:271–275.