Bicarbonate Transport Along the Loop of Henle

I. Microperfusion Studies of Load and Inhibitor Sensitivity

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Abstract

We microperfused the loop of Henle (LOH) to assess its contribution to urine acidification in vivo. Under control conditions ($Na HCO_3^-$ = 13 mM, perfusion rate ~ 17 nl/min^{-1}) net bicarbonate transport ($JHCO_3^-$) was unsaturated, flow- and concentration-dependent, and increased linearly until a bicarbonate load of 1,400 pmol · min^{-1} was reached. Methazolamide ($2 \cdot 10^{-4}$ M) reduced $JHCO_3$ by 70%; the amiloride analogue ethylisopropylamiloride (EIPA) ($2 \cdot 10^{-4}$ M) reduced $JHCO_3$ by 40%; neither methazolamide nor EIPA affected net water flux (Jv). The H^+-ATPase inhibitor bafilomycin A_1 (10^{-5} M) reduced $JHCO_3$ by 20%; the Cl^- channel inhibitor 5-nitro-2'-(3-phenylpropylamino)-benzoate ($2 \cdot 10^{-4}$ M) and the Cl^--base exchange inhibitor diisothiocyanato-2,2'-stilbenedisulfonate ($5 \cdot 10^{-5}$ M), had no effect on fractional bicarbonate reabsorption. Bumetanide (10^{-6} M) stimulated bicarbonate transport (net and fractional $JHCO_3^-$) by 20%, whereas furosemide (10^{-4} M) had no effect on bicarbonate reabsorption; both diuretics reduced Jv.

In summary: (*a*) the LOH contributes significantly to urine acidification. It normally reabsorbs an amount equivalent to 15% of filtered bicarbonate; (*b*) bicarbonate reabsorption is not saturated; (*c*) Na^+-H^+ exchange and an ATP-dependent proton pump are largely responsible for the bulk of LOH bicarbonate transport. (*J. Clin. Invest.* 1991. 88:430–437.) Key words: loop of Henle • bicarbonate transport • amiloride • bafilomycin • diisothiocyanato-2,2'-stilbenedisulfonate

Introduction

Although the proximal tubule is the major site of acid-base transport (1), the distal nephron also makes a significant contribution to net urine acidification. Both free-flow micropuncture and in vivo perfusion studies show that the distal tubule, under appropriate conditions, can reabsorb a significant fraction of filtered bicarbonate (2) and that the collecting duct, either by reabsorbing or secreting bicarbonate (3), plays an important role in determining the final concentration of bicarbonate in the urine.

Two recent in vitro microperfusion studies in the rat demonstrated that the pars recta (S2 and S3) (4) and the thick ascending limb (TAL)[1] (5) reabsorb significant amounts of bicarbonate and secrete ammonium (6). The S2 and S3 as well as the TAL segments are part of the loop of Henle (LOH), a heterogeneous segment between the late proximal and early distal tubule. This part of the nephron includes: a part of the "late" proximal convoluted tubule (pars convoluta; S2); the S3 segment; the thin descending and ascending limbs of the LOH; the TAL; and a small portion of the early distal convoluted tubule (7).

In this study we used in vivo renal microperfusion, which allows a portion of the renal tubule to be studied in the absence of changes in GFR, and under conditions of defined tubular electrolyte loads. This approach permits one to assess the capacity and integrated ion transport response of a defined anatomical, but histologically variable, nephron segment. We microperfused the LOH to determine the acid-base transport characteristics of this composite nephron segment since the LOH is accessible to in vivo microperfusion, but only portions have been perfused in vitro.

Over the last few years a better understanding of the molecular mechanisms of ion transport modes and their distribution along the nephron has also emerged. The luminal processes mediating bicarbonate transport depend on the presence of cytosolic and/or luminal carbonic anhydrase (8) and include a Na^+-H^+ antiporter (9), a H^+-ATPase (proton pump) (10), and a Cl^--base exchanger, the latter operating primarily to reabsorb NaCl in parallel with Na^+-H^+ exchange (11). There is biochemical (12, 13), immunocytochemical (14), and functional evidence (5, 15) that these acidification mechanisms are active along the LOH. In this study we have carried out tubule perfusion experiments to: (*a*) assess the capacity and contribution of the LOH to whole nephron bicarbonate transport; (*b*) define the behavior of bicarbonate transport along this segment during changes in luminal (HCO_3^-) and flow rate; and (*c*) elucidate the cellular transport mechanisms involved in loop bicarbonate reabsorption.

Parts of the results of this paper have appeared in abstract form (1989, *Kidney Int.* 35:452; 1990, *Proc. Physiol. Soc.* 424:15P; and *Proton, Bicarbonate and Chloride Transport in the Kidney,* Satellite Symposium, XI International Congress of Nephrology, 22–24 July 1990, Nara, Japan.

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1. *Abbreviations used in this paper:* ANOVA, analysis of variance; DIDS, diisothiocyanato-2,2'-stilbenedisulfonate; EIPA, ethylisopropylamiloride; $JHCO_3$, net bicarbonate transport rate; Jv, net water flux; NPPB, 5-nitro-2'-(3-phenylpropylamino)-benzoate; LOH, loop of Henle; TAL, thick ascending limb; TF/P, tubule fluid/plasma.

Table I. Summary of Baseline Data (Mean±SEM) in Each Group of Rats Studied and Corresponding To Tables IIa, IIb, and IIc, Respectively

Group	n	Weight	Mean BP	Packed cell volume	Blood pH	Blood [HCO_3]
		g	*mmHg*	*%*		*mM*
a	17	216±4	121±2	46±1	7.381±0.007	28.7±0.6
b	16	234±4	115±3	50±1	7.372±0.007	27.2±0.5
c	23	212±4	112±2	47±1	7.377±0.007	28.1±0.7

n = number of rats in each group.

Methods

Preparation of animals. Experiments were done on a total of 56 male Sprague-Dawley rats (180–280 g body wt), grouped in cages at 21°C in controlled daylight, and fed to the time of study. Animals were anesthetized intraperitoneally with Inactin (120 mg · kg^{-1} body wt; Byk Gluden, Konstanz, FRG), tracheostomized, placed in the right lateral position on a thermoregulated table (37°C), and prepared for micropuncture. The right carotid artery was catheterized to record blood pressure and take blood samples for measurements of hematocrit, pH, and total CO_2. The left jugular vein was cannulated with PE-50 tubing and used for infusion, via a syringe pump (Harvard Apparatus Co., Inc., S. Natick, MA), of a Ringer's saline solution (125 mM NaCl + 25 mM $NaHCO_3$) at 4 ml · h^{-1}. The left kidney was exposed through a flank incision, freed of perirenal fat, and immobilized in a lucite chamber with 3% agar in 0.9% saline. The kidney was bathed with prewarmed (37°C) paraffin oil. The left ureter was catheterized with PE-10 tubing for collection of urine.

Microperfusion. Superficial loops of Henle were microperfused to measure bicarbonate and water transport under conditions of controlled luminal flow rate and bicarbonate delivery. A perfusion pipette was inserted in the last surface loop of a proximal tubule and a castor oil block was placed upstream of the perfusion pipette. Microperfusion was started at ~20 nl · min^{-1} with a thermally shielded microperfusion pump (Hampel, Frankfurt, FGR). The control perfusion solution contained the following (in mM): NaCl, 128; $NaHCO_3^-$, 13; KCL, 3.6; $MgCl_2$, 1; NaH_2PO_4, 0.38; Na_2HPO_4, 1.62. FD&C blue dye (0.07%) and 12.5 μCi · ml^{-1} ^{14}C-inulin were added to the perfusion solution. Net water flux (Jv) and net bicarbonate transport rate ($JHCO_3$) were measured under the following conditions: (*a*) during intraluminal perfusion with methazolamide ($2 \cdot 10^{-4}$ M), a lipid soluble carbonic anhydrase inhibitor; (*b*) in the presence of ethylisopropylamiloride (EIPA; $2 \cdot 10^{-4}$ M), a specific blocker of Na^+-H^+ exchange; (*c*) during addition to the perfusate of the loop diuretics bumetanide (10^{-6} M) or furosemide (10^{-4} M).

Load- and flow-dependence of bicarbonate transport were assessed: by raising the bicarbonate concentration in the perfusate at constant flow rate from 13.0 to 75 mM; $NaHCO_3^-$ in the perfusion fluid was increased by replacing NaCl with $NaHCO_3^-$ without altering total osmolality. At the higher bicarbonate concentrations this substitution resulted in a modest decrease of Cl^-. It is unlikely that the fall in Cl^- changed either $JHCO_3$ or Jv since the addition of chloride channel inhibitors 5-nitro-2′-(3-phenylpropylamino)-benzoate (NPPB) did not affect either transport operation. Load was also augmented by increasing, at constant [HCO_3^-] of 13 mM, the perfusion rate from 20 to 40 nl · min^{-1}. In a separate set of experiments, we tested the effects of: the proton pump inhibitor bafilomycin A_1 (10^{-5} M); the Cl^- channel blocker NPPB ($2 \cdot 10^{-4}$ M); and the anion exchange inhibitor, diisothiocyanato-2,2′-stilbenedisulfonate (DIDS; $5 \cdot 10^{-5}$ M). The same rat was used to carry out control and experimental perfusion studies. Transport data are expressed per individual loop since it had been shown that the LOH is a nephron segment of essentially constant length of 6–7 mm (16).

Analytical methods. Tubule fluid total CO_2 concentration was measured by microcalorimetry (Picapnotherm; World Precision Instruments, Inc., New Haven, CT). To avoid loss of CO_2, all mineral oil used (to bathe the kidney surface, to collect tubule fluid samples, and to cover samples for measurement) was equilibrated to cortical carbon dioxide tension (P_{CO_2}) values with a solution containing 100 mM Hepes buffer, 48 mM $NaHCO_3^-$ gassed with 6.7% CO_2 (17). Each analysis was bracketed by analysis of standards of known $NaHCO_3^-$ concentration. The blood acid-base status of each animal was measured with a blood-gas analyzer (model 170; Corning Medical, Medfield, MA). ^{14}C-inulin radioactivity was measured by a liquid scintillation counter (model P2; G. D. Searle & Co., Chicago, IL). Plasma [HCO_3^-] was measured with a carbon dioxide analyzer (model 965; Corning). Plasma [K^+] was measured by flame photometry (model 480; Corning).

Calculations and statistical analysis. The perfusion rate in vivo was obtained from the rate of fluid collected from the early distal tubule

Table IIa. Effect of Changes in Bicarbonate Load (Perfusate Flow Rate and Bicarbonate Concentration) on Loop of Henle Bicarbonate Reabsorption. Comparison with Control (Set 1) after 1-way ANOVA

Set	n tubules	Perfusate [HCO_3]	Perfusion rate	Collection rate	TF/P inulin	Jv	Bicarbonate load	$JHCO_3$	$FRHCO_3$
		mM	*nl · min^{-1}*	*nl · min^{-1}*		*nl · min^{-1}*	*pmol · min^{-1}*	*pmol · min^{-1}*	*%*
1	30	13.1±0.1	17.1±0.3	9.0±0.4	1.96±0.06	8.1±0.2	222.9±3.9	146.1±3.9	65.9±1.9
2*	18	13.1±0.1	37.4±0.4‡	30.0±0.7‡	1.25±0.02‡	7.4±0.5	486.3±4.7‡	189.4±15.8‡	39.1±3.4‡
3	13	24.2±0.2	19.1±0.4‡	13.4±0.5‡	1.46±0.45‡	5.8±0.5‡	469.9±10.1‡	208.3±14.0‡	44.8±2.9‡
4	9	38.0±0.0	16.9±0.5	10.3±0.3	1.66±0.04‡	6.7±0.4§	643.8±19.9‡	271.7±20.0‡	42.0±2.5‡
5	15	53±0.1	16.9±0.5	10.1±0.4	1.70±0.07‡	6.7±0.4§	892.8±25.6‡	446.3±42.9‡	49.3±4.3‡
6	7	69±0.0	19.1±0.7‡	12.5±0.6‡	1.54±0.02‡	6.6±0.2§	1319.4±48.2‡	524.6±34.5‡	39.6±1.9‡
7	7	75±0.0	20.0±0.3‡	12.6±0.3‡	1.59±0.03‡	7.4±0.3	1498.8±22.4‡	574.4±30.1‡	38.4±2.1‡

* Perfusate flow rate; ‡ $P < 0.01$; § $P < 0.05$.

Table IIb. Effect of Carbonic Anhydrase, Na^+–H^+ Exchange Inhibitors and the Loop Diuretics Furosemide and Bumetanide, on Loop of Henle Bicarbonate Reabsorption. Comparison with Control after 1-way ANOVA

Group	*n* tubules	Perfusate $[HCO_3]$	Perfusion rate	Collection rate	TF/P inulin	Jv	Bicarbonate load	$JHCO_3$*	$FRHCO_3$
		mM	*nl · min⁻¹*	*nl · min⁻¹*		*nl · min⁻¹*	*pmol · min⁻¹*	*pmol · min⁻¹*	%
Control	30	12.5±0.1	20.2±0.3	11.7±0.3	1.73±0.05	8.5±0.3	262.3±3.8	164.7±6.3	62.9±2.3
Methazolamide (2.10^{-4} M)	13	12.5±0.1	19.9±0.3	11.6±0.2	1.72±0.02	8.3±0.2	258.4±3.4	44.5±7.5‡	17.3±3.0‡
EIPA (2.10^{-4} M)	24	12.5±0.1	19.8±0.4	11.8±0.4	1.71±0.05	8.1±0.4	257.7±4.9	100.9±9.8‡	38.6±3.5‡
Furosemide (10^{-4} M)	31	12.5±0.1	19.6±0.2	14.1±0.4‡	1.41±0.03‡	5.5±0.3‡	255.0±3.1	159.5±9.4	62.8±3.7
Bumetanide (10^{-6} M)	14	12.5±0.1	20.4±0.5	13.6±0.5‡	1.52±0.06‡	6.8±0.6‡	264.7±7.0	194.0±10.5§	73.2±3.3§

* $JHCO_3$, covariate, bicarbonate load; ‡ $P < 0.01$; § $P < 0.05$.

multiplied by the tubule fluid/plasma (TF/P) inulin ratio. The perfusion pump was calibrated by timed collections of perfusion fluid delivered directly into counting vials for measurements of known ^{14}C-inulin concentrations. Jv was calculated as the difference between perfusion and collection rates. $JHCO_3^-$ was calculated from the amount of bicarbonate delivered in the perfusion pipette minus the amount collected in the collection pipette. Statistical analysis was by one-way analysis of variance and covariance (ANOVA and ANCOVA; covariate, bicarbonate load) followed by comparison with control (post hoc) by least significant difference. All data are expressed as mean±SEM.

Results

Systemic measurements. Table I summarizes body weight, mean arterial blood pressure, packed cell volume, and systemic acid-base measurements (blood pH and bicarbonate concentration) in the experimental groups corresponding to Tables IIa, IIb, and IIc: groups a, b, and c. No significant changes were observed in the different experimental conditions.

(a) Concentration- and flow-dependence of bicarbonate transport. Table IIa summarizes perfusion rates, collection rates, TF/P inulin ratios, fluid reabsorption (Jv), bicarbonate load, absolute ($JHCO_3^-$) and fractional ($FRHCO_3^-$) bicarbonate reabsorption. Jv was reduced significantly, by ~ 30% (from 8.1±0.2 to 5.8±0.5 nl · min⁻¹; $P < 0.01$), when the luminal bicarbonate concentration in the tubule fluid was raised from 13 to 24 mM. In contrast, Jv was unaffected (8.1±0.2 vs. 7.4±0.5 nl · min⁻¹) by a comparable increase in bicarbonate load (469.9±10.1 {concentration} vs. 486.3±4.7 {flow} pmol · min⁻¹) produced by increasing the perfusion rate from 17.1±0.3 to 37.4±0.4 nl · min⁻¹ at unchanged perfusate bicarbonate concentration (see Fig. 1).

Fig. 2 illustrates the relationship between luminal bicarbonate load and absolute net bicarbonate reabsorption. Bicarbonate reabsorption is unsaturated at physiological bicarbonate loads and increases when either flow rate or luminal bicarbonate concentrations are increased. Loop bicarbonate reabsorption is load-dependent and increases up to a load of ~ 1,400 pmol · min⁻¹, with reabsorption rates reaching a plateau of ~ 550 pmol · min⁻¹.

(b) Effects of transport inhibitors. Table IIb summarizes data on perfusion rate, collection rate, TF/P inulin ratio, Jv, bicarbonate load, total and fractional bicarbonate reabsorption under control conditions, and in the presence of various transport inhibitors, added to the perfusion fluids. The data are graphically represented in Figs. 3–5.

Methazolamide and EIPA. Methazolamide ($2 \cdot 10^{-4}$ M) reduced $JHCO_3^-$ significantly by 70% from 164.7±6.3 to 44.5±7.5 pmol · min⁻¹; $P < 0.01$. Fractional bicarbonate reab-

Table IIc. Effect of H^+-ATPase, Cl^- Channel, and Cl^-–HCO_3^- Exchange Inhibitors on Loop of Henle Bicarbonate Reabsorption. Comparison with Control after 1-way ANOVA

Group	*n* tubules	Perfusate $[HCO_3]$	Perfusion rate	Collection rate	TF/P inulin	Jv	Bicarbonate load	$JHCO_3$*	$FRHCO_3$
		mM	*nl · min⁻¹*	*nl · min⁻¹*		*nl · min⁻¹*	*pmol · min⁻¹*	*pmol · min⁻¹*	%
Control	43	13.1±0.1	17.4±0.2	9.0±0.3	1.98±0.04	8.4±0.2	225.8±3.2	153.5±4.3	68.1±1.8
Bafilomycin (10^{-5} M)	15	13.1±0.1	18.3±0.4	10.1±0.4	1.86±0.06	8.3±0.3	238.3±4.7	121.1±6.5‡	50.7±2.4‡
NBBP (2.10^{-4} M)	8	13.1±0.1	18.1±0.7	9.4±0.3	1.74±0.03	8.8±0.4	235.8±9.2	157.8±8.1	67.4±3.7
DIDS (5.10^{-5} M)	16	13.1±0.1	18.2±0.3	9.2±0.4	2.04±0.09	9.0±0.3	237.1±3.6	175.0±4.6§	74.0±2.2

* $JHCO_3$, covariate, bicarbonate load; ‡ $P < 0.01$; § $P < 0.05$.

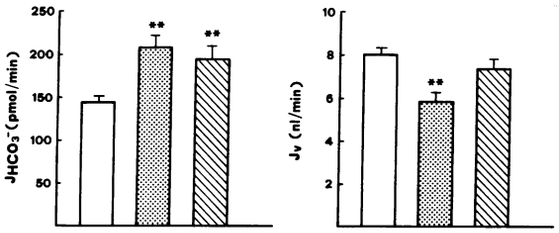

Figure 1. The effect of increasing luminal bicarbonate concentration (load) (from 13.1±0.1 to 24.2±0.2 mM) or flow rate (from 17.1±0.3 to 37.4±0.4 nl · min^{-1}) on bicarbonate and water transport along the loop of Henle. **$P < 0.01$ vs. control. □, Control; □, load; ▩, flow rate.

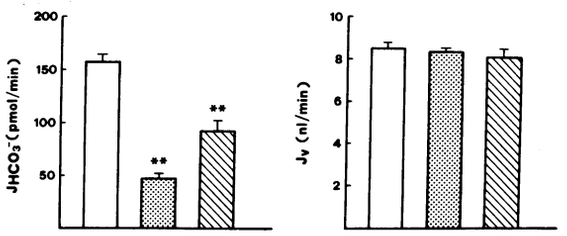

Figure 3. The effect of methazolamide ($2 \cdot 10^{-4}$ M) and EIPA ($2 \cdot 10^{-4}$ M) on bicarbonate and water transport along the loop of Henle. **$P < 0.01$ vs. control. □, control; □, methazolamide (2×10^{-4} M); ▩, EIPA (2×10^{-4} M).

sorption was also reduced 70% (62.9±2.3 vs. 17.3±3.0). When the loops were perfused with solutions containing $2 \cdot 10^{-4}$ M EIPA, bicarbonate reabsorption decreased by 40% (from 164.7±6.3 to 100.9±9.8 pmol · min^{-1}; $P < 0.01$). Neither methazolamide nor EIPA affected Jv (Fig. 3).

Furosemide and bumetanide. The bicarbonate transport rate in the LOH also was measured in the presence of two loop diuretics, furosemide (10^{-4} M) and bumetanide (10^{-6} M). Their effects were evaluated in separate experiments; furosemide did not change $JHCO_3^-$ (164.7±6.3 vs. 159.5±9.4 pmol · min^{-1}), while bumetanide caused a modest but significant increase in absolute (18%; 164.7±6.3 vs. 194±10.5 pmol · min^{-1}; $P < 0.05$) and fractional (16%; 62.9±2.3 vs. 73.2±3.3; $P < 0.05$) bicarbonate transport (Fig. 4). Both diuretics depressed Jv (Fig. 4): furosemide by 35% (8.5±0.3 vs. 5.5±0.3 nl · min^{-1}; $P < 0.91$) and bumetanide by 20% (8.5±0.3 vs. 6.8±0.6 nl · min^{-1}; $P < 0.01$).

Bafilomycin, NPPB, and DIDS. When 10^{-5} M bafilomycin was added to the luminal perfusion fluid, bicarbonate reabsorption decreased by 20% (153.5±4.3 vs. 121.1±6.5 pmol · min^{-1}; $P < 0.01$). NPPB ($2 \cdot 10^{-4}$ M) in the perfusate had no effect on net bicarbonate transport along the LOH (153.5±4.3 vs. 157.8±8.1 pmol · min^{-1}). DIDS ($5 \cdot 10^{-5}$ M) in the perfusate was associated with a small but significant increase in $JHCO_3^-$ (153.5±4.3 vs. 175.0±4.6 pmol · min^{-1}); the fractional change was not significantly different from control (68.1±1.8 vs. 74.0±2.2%). Since we have demonstrated that $JHCO_3$ is load-dependent, it is reasonable to interpret the modest enhancement of $JHCO_3$ after DIDS to be caused by the small load increment that was observed in this experimental setting. Bafilomycin, NPPB, and DIDS not affect Jv. These results are summarized in Fig. 5.

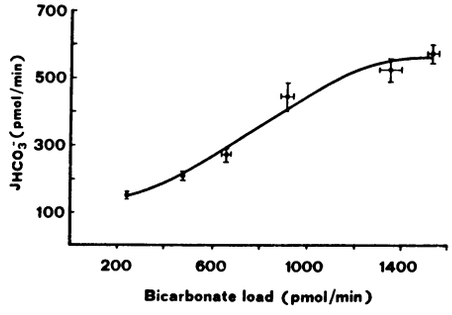

Figure 2. The effect of increasing bicarbonate load from 222.9±22.4 to 1498.8 ± 22.4 pmol · min^{-1} on bicarbonate reabsorption along the loop of Henle.

Discussion

Bicarbonate reabsorption along the loop of henle. Although free-flow micropuncture studies on loops of Henle originating from superficial nephrons have demonstrated significant bicarbonate reabsorption (18–20) the functional behavior of this complex tubule segment in acid-base regulation has not been evaluated extensively. The present microperfusion study of the loop of Henle provides novel information about the properties of the bicarbonate transport system along this heterogeneous nephron segment under conditions in which the tubular perfusion rate and bicarbonate concentration were varied from conditions mimicking physiological loads to significantly elevated flow rates and tubule bicarbonate concentrations. In addition, we attempt to elucidate the cell mechanisms involved in HCO_3^- transport along the loop of Henle.

Our previous free-flow micropuncture studies had shown that the loop of Henle reabsorbs a significant fraction of the filtered bicarbonate (2, 21). Assuming a glomerular load of bicarbonate of 1,000 pmol · min^{-1} (2), our present results indicate that an amount equivalent to ~ 15% (150 pmol · min^{-1}) of filtered bicarbonate are reabsorbed in perfused LOH. This rate of bicarbonate transport is of similar magnitude although somewhat higher than values derived from free-flow micropuncture studies (2, 21).[2] Assuming that 75–80% of filtered bicarbonate is reabsorbed along the proximal convoluted tubule, the further retrieval of some 15% of bicarbonate from the lumen along the loop of Henle would result in the delivery of some 5–10% of filtered bicarbonate to the early distal convoluted tubule, a value quite similar to that observed in free-flow studies (2, 21). From these quantitative considerations we conclude that the loop of Henle plays a significant role in tubule bicarbonate reabsorption.

The loop of Henle is a heterogeneous nephron segment lined with cells of different morphological and functional properties (7). It is made up of the late S_2 (22) and S_3 (4) segment of the proximal tubule, the thin descending and ascending limbs of Henle, the medullary and cortical TAL (5, 15), and the initial portion of the distal convoluted tubule (2). Free-flow micropuncture studies in rat superficial tubules have demonstrated that the bicarbonate concentration in the late proximal

2. The reason for the larger bicarbonate reabsorption along the LOH in this study compared to that in the free-flow micropuncture studies may be that the rats used in the latter studies were significantly smaller compared with those in our study. Tubular length and diameter increase during maturation (16).

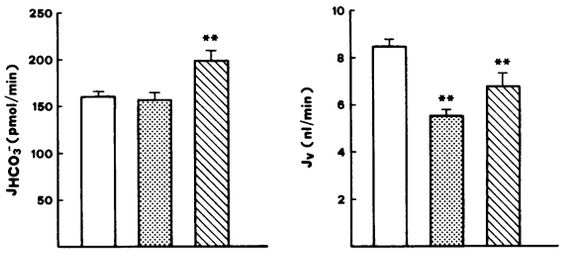

Figure 4. The effect of intraluminal furosemide (10^{-4} M) and bumetanide (10^{-6} M) on bicarbonate and water transport along the loop of Henle. $^{**}P < 0.01$ vs. control. □, control; ▨, furosemide (10^{-4} M); ▩, bumetanide (10^{-6} M).

and early distal tubule are similar (2, 21). It has been assumed, at least in juxtamedullary nephrons, that the bicarbonate concentration rises towards the tip of Henle's loop as a consequence of water removed from the tubule (19); the fall of bicarbonate concentration along the ascending portion of the loop implies the presence of a hydrogen secretory process and of bicarbonate reabsorption in this nephron segment (5).

Microperfusion studies on isolated segments of the late proximal tubule and the thick ascending limb of Henle's loop confirm that these nephron segments effect bicarbonate retrieval from the tubule lumen. From in vitro perfusion studies of Garvin and Knepper (4) in isolated perfused rat proximal tubules, values of bicarbonate reabsorption of ~ 40 pmol · min^{-1} can be calculated for the total length of the S_3 segments (23). Uncertainties in assigning precise rates of bicarbonate transport to specific tubule segments arise from the fact that the proximal straight tubule of the rat includes not only the S3 but also the terminal portion of the S2 segment. Our perfusion studies in rats indicate that the bicarbonate transport rate along the whole length of the LOH, at similar loads of 250 pmol · min^{-1}, was 150 pmol · min^{-1}. It is virtually certain that in addition to the S_3 (and late S_2) segment bicarbonate transport in the thick ascending limb of Henle also contributes to the overall rate of hydrogen secretion along the LOH. This view is supported by the observations of Good who has reported bicarbonate transport rates of 10 pmol · min^{-1} (20–40 pmol · min · mm^{-1} · total length of tubule) in perfused thick ascending limbs of the rat (5). Since the distal tubule, including its early convoluted portion (Wang, T., G. Giebisch, G. Malnic, and Y. D. Chan, unpublished observations), also reabsorbs bicarbonate, this tubule segment may also make a small contribution to total LOH bicarbonate transport.

Flow- and concentration-dependence of bicarbonate transport. The data presented in Table IIa and illustrated in Fig. 1 show that bicarbonate reabsorption is flow-dependent. Thus,

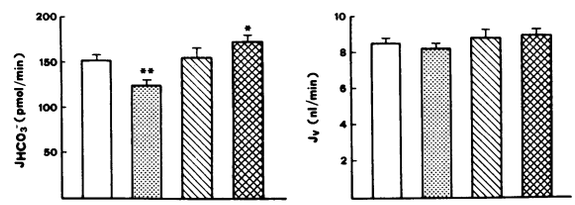

Figure 5. The effect of bafilomycin (10^{-5} M), NPPB ($2 \cdot 10^{-4}$ M), and DIDS ($5 \cdot 10^{-5}$ M) on bicarbonate and water transport along the loop of Henle. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control. □, control; ▨, bafilomycin (10^{-5}); ▩, NPPB (2×10^{-4} M); ■, DIDS (5×10^{-5} M).

an increase in perfusion rate from 17 to 37 nl/min increases HCO_3^- reabsorption from 146 to 189 pmol · min^{-1}. Flow-dependent stimulation of bicarbonate transport has also been shown by Alpern et al. (24) and Liu et al. (22) in studies of proximal tubule and by Chan et al. in rat distal tubule (25). There are two possible explanations for this: (*a*) the increase in tubule flow rate may stimulate an unsaturated bicarbonate transport system by altering the luminal diffusion barrier (24); (*b*) changes in flow rate may attenuate the fall of bicarbonate concentration along the LOH and thus stimulate proton secretion.

Our results further indicate that bicarbonate transport along the loop of Henle is also unsaturated when the luminal bicarbonate concentration is raised. There is an almost linear increase of bicarbonate transport from control loads, achieved at flow rates and bicarbonate concentrations in the physiological range (load 222 pmol · min^{-1}) to loads that exceed control values by a factor of 6 (1,322 pmol · min^{-1}).

The effect of increasing the luminal bicarbonate concentration on net bicarbonate transport is similar to that observed in other tubule segments, such as the proximal and distal tubule, nephron segments in which saturation of net transport of bicarbonate has also been observed in the luminal concentration range of 40–50 mM bicarbonate (1, 26). The functional behavior of the loop of Henle, largely reflecting activities of the S_3 segment of the proximal tubule and the thick ascending limb of Henle's loop, can be explained most reasonably in terms of pump leak systems (see below) with a finite backflux component of bicarbonate ions. The bicarbonate permeability, probably of the order of values measured in proximal and distal tubules (proximal tubule: range $2.6–9.8 \times 10^{-7}$ cm^2 · s^{-1} [1, 24, 26–29]; distal tubule: $2.32 \cdot 10^{-7}$ cm^2 · s^1 [25]) thus would permit bicarbonate to diffuse into the lumen in the low range of luminal bicarbonate concentration (< 24 mM) whereas passive bicarbonate reabsorption would occur by efflux to peritubular blood at luminal bicarbonate concentrations exceeding plasma levels. The presence of a significant bicarbonate permeability is also suggested by our observation that bicarbonate concentration in the collected perfusate was never less than 4 mM. Levine et al. also observed the accumulation of 6 mM bicarbonate in LOH perfused in vivo from the proximal tubule with initially bicarbonate-free solutions (30).

Our data show that increased luminal bicarbonate concentrations tend to decrease Jv (see Table IIa). In view of the very low water permeability of the thick ascending limb of Henle's loop it is reasonable to postulate that the modest but significant reduction occurs predominantly along the S_3 segment of the proximal tubule. Since the reflection coefficient of HCO_3^- in the rat exceeds that of Cl in the proximal tubule (31), a rise in HCO_3^- would be expected to shift the balance of driving force for H_2O in the direction of reduced fluid reabsorption. Alpern et al. also found a decrease of Jv when proximal tubules were perfused with solutions of increasing HCO_3^- concentrations (24). It is noteworthy that increasing the luminal bicarbonate load by doubling flow rate at constant luminal [HCO_3^-] does not affect Jv. Chan et al. also did not observe any effect of increasing the bicarbonate concentration on Jv in cortical distal tubules (25). The different behavior of proximal and distal tubules may be related either to the low P_{H2O} and/or the absence of a solvent drag effect of HCO_3^- in the distal tubule.

Effect of methazolamide. Carbonic anhydrase is found in all renal tubule cells involved in acid-base transport (8) and can

be inhibited by acetazolamide and its derivatives (32). Segments not containing detectable carbonic anhydrase fail to reabsorb bicarbonate, e.g., the rabbit cortical TAL (33). Immunofluorescence studies show that the LOH in the rat kidney is rich in carbonic anhydrase, apart from the luminal membrane of early distal tubule (14). In our study we used the lipophilic carbonic anhydrase inhibitor methazolamide which inhibits both cytoplasmic (type II) and membrane-bound (type IV) carbonic anhydrase (32). This drug caused a 70% reduction of bicarbonate transport. The incomplete inhibitory effect of methazolamide on bicarbonate transport along the LOH could be due to either incomplete inhibition of carbonic anhydrase and/or uncatalyzed, carbonic anhydrase-independent bicarbonate transport.[3] The concentration of $2 \cdot 10^{-4}$ M methazolamide blocks the bulk of proximal bicarbonate transport but data on the dose/response relationship of this carbonic anhydrase inhibition in the loop of Henle are not available. A loss of methazolamide along the perfused loop segment preceding the thick ascending limb of Henle's loop may also lead to incomplete inhibition of carbonic anhydrase.

Effect of EIPA. After the pioneering work of Pitts (34), Murer et al. (35) provided direct evidence for the presence of a coupled electroneutral Na^+-H^+ exchanger in rat renal brush border membrane vesicles. This countertransporter is an important mechanism of H^+ secretion and HCO_3^- reabsorption in the proximal tubule (9). Amiloride effectively inhibits Na^+-H^+ exchange and has been used as a means of studying the tubule sites, membrane localization, and kinetic properties of this transporter (36). Because amiloride can also affect other ion transport processes, such as the Na^+ channel and Na^+-Ca^{2+} exchanger (37), several amiloride analogues with improved specificity and high selectivity for the exchanger have been synthesized. Ethylisopropylamiloride is such a potent analogue that selectively inhibits Na^+-H^+ exchange (38). EIPA in the loop perfusate inhibited $JHCO_3^-$ by 60%. These results support the presence of Na^+-H^+ exchange along the LOH, and are also consistent with inhibitory actions in isolated nephron segments such as S_2, S_3, and TAL (4, 5, 23). The fact that EIPA led to only partial inhibition of $JHCO_3$ could be explained by: (*a*) incomplete block of Na^+-H^+ exchange, possibly because of loss of EIPA along the perfused segment; or (*b*) the existence of other Na^+ (H^+)-independent bicarbonate transport mechanisms.

Effects of bumetanide and furosemide. A large body of experimental evidence supports the presence of Na^+-$2Cl^-$-K^+ cotransporter along the TAL (39). This cotransport mechanism can be inhibited by loop diuretics such as bumetanide and furosemide. The administration of such loop diuretics that inhibit sodium entry from the luminal side is expected to reduce intracellular sodium concentration, a prediction that has been confirmed by measurements of sodium activity in cells of amphibian dilating segments after furosemide exposure (40). This sequence of events steepens the apical gradient for sodium entry and in turn may increase luminal Na^+-H^+ exchange, luminal acidification (41), and enhance bicarbonate reabsorption. In the presence of bumetanide, we did indeed observe a significant increase in bicarbonate transport, results consistent with coexistence of Na^+-Cl^--K^+ cotransport and Na^+-H^+ exchange along the thick ascending limb. Our observations are also compatible with the in vitro data of Good on isolated rat TAL (5) in which bicarbonate absorption rose in the presence of furosemide.

A likely explanation for the lack of an effect of furosemide on bicarbonate absorption along the LOH in vivo is that furosemide is a less specific transport inhibitor compared with bumetanide. Unlike bumetanide, furosemide inhibits carbonic anhydrase (8, 42). Therefore, any stimulatory effect of furosemide on bicarbonate transport by activation of Na^+-H^+ exchange may have been masked by a reduction of bicarbonate reabsorption through inhibition of carbonic anhydrase.[4]

An interesting action of both loop diuretics on the LOH is their inhibitory effect on water transport. This effect in single tubules cannot be linked directly to inhibition of Na^+-$2Cl^-$-K^+ cotransport along the TAL, since this segment has a low water permeability and normally reabsorbs little fluid. Possible inhibition of carbonic anhydrase by loop diuretics such as furosemide could not be involved because the carbonic anhydrase inhibitor methazolamide had no effect on Jv. Possible explanations for this inhibitory effect of both furosemide and bumetanide are that both diuretics inhibit proximal sodium and water reabsorption, an effect directly demonstrated for furosemide (42), and/or that loop diuretics inhibit water efflux from the thin descending limb. A direct action of loop diuretics upon the metabolism of short loops of descending thin limbs of LOH has been reported by Jung and Endou (43). These authors reported that furosemide and bumetanide sharply reduced the fall of ATP that normally occurs during incubation in the absence of exogenous substrate. It is presently not clear how such an action of loop diuretics is related to the inhibition of Jv that we observed.

Effect of bafilomycin. Recently it has become apparent that, in addition to Na^+-H^+ exchange, proton secretion along the proximal tubule can also occur by a sodium-independent mechanism, the electrogenic proton pump (10, 44). Immunohistochemical (45) and biochemical studies (12, 13) have shown that this transport system is distributed in varying amounts along the entire nephron including key tubule segments lining the loop of Henle. The properties, kinetics, and functional significance of H^+-ATPase have been explored by use of H^+-ATPase inhibitors: Bank et al. (44) reported that proximal electrogenic proton transport was inhibited by dicyclohexyl-carbodimide, while Garg et al. (12) and Ait-Mohamed et al. (13) used *N*-ethylmaleimide to characterize the renal membrane proton ATPase. Recently, Bowman et al. (46) explored the sensitivity of

3. The role of uncatalyzed CO_2^- hydration and H^+ generation along the loop of Henle can be approximated by: $J_H + = K_1 \cdot (CO_2) \cdot$ vol where $K_1 = 0.15\ s^{-1}$, $CO_2 = 1$ mM ($1.2 \cdot 10^{-6}$ mol $\cdot$ cm^{-3}) and the tubule volume (vol) = $5.65 \cdot 10^{-16}$ cm^3 (length: 6 mm, outer diameter 20 μm, inner diameter 10 μm). Thus, $J_H+ = 0.15 \times 1.2 \cdot 10^{-6} \times 5.65 \times 10^{-6} = 1{,}018 \cdot 10^{-12}$ mol/s, or 61 pmol $\cdot$ min^{-1}.

Despite uncertainties such as tubule inhomogeneity and the back reaction of carbonic anhydrase dehydration, the calculated value, probably an overestimate, is in reasonable agreement with the measured rate of bicarbonate reabsorption of 45 pmol $\cdot$ min^{-1} that was observed during perfusion with the carbonic anhydrase inhibitor.

4. In a free-flow micropuncture study in our laboratory the systemic, intravenous administration of furosemide produced a significant decrease in early distal tubule pH (15). This contrasts with the present observations in which furosemide, added to the perfusion fluid, did not affect bicarbonate transport (see Table IIb). One possible explanation is that the concentration of the diuretic in the free-flow studies may not have reached the levels necessary to inhibit carbonic anhydrase.

various membrane ATPases to bafilomycin A_1, a macrolide antibiotic. Their results strongly suggest that bafilomycin, which is highly lipophilic, is a potent and specific inhibitor of vacuolar H^+-ATPases, the enzyme that can generate a proton gradient between cellular compartments. Since incorporation of subsurface vesicular material occurs in the proximal tubule it is likely that this type of H^+-ATPase may also be located in the apical cell membrane. When we perfused the LOH with bafilomycin, it produced a 20% fall in $JHCO_3^-$. This result is consistent, on the one hand, with the view that a modest but significant fraction of LOH bicarbonate transport is not mediated by Na^+-H^+ exchange and, on the other hand, with the immunohistochemical demonstrations of proton pump activity along the LOH (45). To our knowledge, no inhibitory effect of bafilomycin on Na-H exchange has been described. The reduction of $JHCO_3^-$ by bafilomycin can also account for the difference between the inhibitory effects of methazolamide and EIPA: a difference of $\sim$ 50 pmol · min^{-1} compared to a reduction by 35 pmol · min^{-1} of bafilomycin. Despite uncertainties inherent in the use of any transport inhibitors our data are consistent with the presence of a component of hydrogen ion secretion that is mediated by an H^+-ATPase-mediated transport mechanism.

Effect of NPPB and DIDS. Besides Na^+-H^+ exchange and the proton pump, other mechanisms of cell bicarbonate transport should be considered. One of these is the Cl^--HCO_3^- exchanger, linked to the band 3 protein originally described in red cells. This transporter is electroneutral and its direction of transport depends on the relative Cl^- and HCO_3^- concentration gradients. It has been suggested that it may act in parallel with the Na^+-H^+ antiporters to effect net NaCl transport (11). Cl^--base exchange can be inhibited in the kidney by disulphonic stilbene derivatives, which can also block Cl^- channels. Hence we have also used the more specific Cl^- channel blocker NPPB. Our luminal perfusions with DIDS and NPPB showed no reduction of HCO_3^- transport. Thus, unlike the data of Friedman et al. (11) obtained in isolated cortical mouse TAL, we could not find evidence that an apical Cl^--base exchanger or other Cl^--dependent mechanism contributes to LOH bicarbonate transport in the rat.

Conclusion. Our studies demonstrate that under conditions of physiological loads, an amount equivalent to $\sim$ 15% of filtered bicarbonate is reabsorbed along the perfused LOH; this transport is unsaturated and both flow- and concentration-dependent. At normal flow rates, a maximal transport rate of $\sim$ 500 pmol/min^{-1} is reached at HCO_3^- concentrations of $\sim$ 55 mM. Bicarbonate transport is highly sensitive to inhibition by carbonic anhydrase, but a carbonic anhydrase-insensitive transport component remains intact after administration of carbonic anhydrase inhibitors at high concentrations. Anion exchange or chloride channel blockers had no significant effects on LOH bicarbonate transport. We obtained in vivo evidence that Na^+-H^+ exchange and proton pump activity are responsible for the bulk of bicarbonate reabsorption along the LOH.

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