

Diuretics Stimulate H⁺ Secretion in Turtle Urinary Bladder

PHILIP D. LIEF, BERTRAND F. MUTZ, and NORMAN BANK, *Renal, Electrolyte and Hypertension Division, Department of Medicine, Montefiore Hospital and Medical Center and the Albert Einstein College of Medicine, Bronx, New York 10467*

ABSTRACT The effect of various diuretics on H⁺ secretion was studied in the isolated short-circuited urinary bladder of the turtle. Mucosal (urinary) chlorothiazide stimulated H⁺ secretion promptly, from 1.33 ± 0.24 to 3.03 ± 0.25 $\mu\text{eq/h}$ ($P < 0.001$). The effect was rapidly reversible upon washout of the drug, H⁺ returning to control levels, 1.37 ± 0.26 $\mu\text{eq/h}$ ($P < 0.001$). Similar effects were observed with mucosal hydrochlorothiazide and mucosal ethacrynic acid/cysteine. Stimulation of H⁺ secretion occurred in the presence or the absence of exogenous CO₂, in the presence or absence of mucosal Na⁺ and during inhibition of Na⁺ transport by ouabain. There was no stimulation of H⁺ secretion by uncomplexed ethacrynic acid or by mucosal furosemide. The nondiuretic sulfonamide, sulfasoxazole, and the nonsulfonamide buffer, borate, had no effect on H⁺ secretion. These observations indicate that the stimulatory effect of diuretics on H⁺ secretion is not related to active sodium transport, transepithelial electrical potential, or the buffering capacity of the drugs. Since the transepithelial pH gradient at which active H⁺ secretion was abolished was identical for chlorothiazide-treated tissues (2.68 pH U) as for control tissues (2.65 pH U, NS), the data suggest that the protonmotive force of the H⁺ pump was unaffected by the diuretic. This observation, plus the rapid onset and reversibility of the drugs, is consistent with an effect on the mucosal membrane to increase H⁺ conductance (K). The findings raise the possibility that direct enhancement of renal H⁺ secretion may play a role in the metabolic alkalosis induced by some diuretics.

INTRODUCTION

The administration of certain diuretic drugs to patients may lead to metabolic alkalosis. This electrolyte dis-

turbance is generally attributed to indirect effects of the diuretics such as volume depletion (1), potassium depletion (2), secondary aldosteronism (3), urinary chloride loss (4), "contraction" (5), or an increase in distal tubule delivery of sodium and impermeant anion leading to increased electronegativity (6).

However, a direct effect of these drugs to stimulate renal hydrogen ion secretion has also been suggested by the results of several clearance and balance studies. For example, Beyer (7) demonstrated that chlorothiazide administration results in a fall in urine pH in dogs. The effect was particularly apparent in animals pretreated with NH₄Cl. Gyory and co-workers found that ethacrynic acid lowered urine pH and increased acid excretion in patients with hereditary distal renal tubular acidosis (8), and in normal subjects (9). Recently, Bosch et al. (10) observed that furosemide enhanced hydrogen ion excretion and generated alkalosis in dogs even without volume or electrolyte depletion or an increase in aldosterone secretion. These studies have suggested, but not proven that diuretics may stimulate H⁺ secretion directly.

The purpose of the present experiments was to examine the effects of several widely-used diuretics on H⁺ secretion by the turtle urinary bladder, an organ with a well-characterized H⁺ transport system (11, 12). The results indicate that addition of thiazides and ethacrynic acid (but not furosemide) to the mucosal bathing medium directly stimulates hydrogen ion secretion in this tissue. This occurred in the presence or absence of exogenous CO₂, in the presence or absence of mucosal sodium, and with inhibition of sodium transport by ouabain. pH gradient experiments suggest that the enhanced H⁺ secretion was due to an increase in pump conductance for hydrogen ions rather than an increase in the protonmotive force. Our findings raise the possibility that direct enhancement of renal hydrogen ion secretion may play an important role in diuretic-induced metabolic alkalosis.

Received for publication 12 March 1979 and in revised form 26 December 1979.

METHODS

General. Adult freshwater turtles (*Pseudemys scripta*) were obtained from Lemburger Co. (Div. of Mogul-Ed, Oshkosh, Wis.). On the day of the experiment, the animals were decapitated and the urinary bladders excised. Excised hemibladders were washed three times in CO₂-free turtle Ringer's solution (CO₂-free NaR)¹ (Na⁺, 115.0 mM/liter; K⁺, 3.5 mM/liter; Ca⁺⁺, 0.9 mM/liter; Cl⁻, 119.7 mM/liter; HPO₄⁻, 0.3 mM/liter; dextrose, 2.0 mM/liter; osmolality, 230 molsmol/Kg H₂O) and were mounted between halves of an Ussing-type lucite chamber, providing an exposed membrane area of 7.3 cm². Each side of the mounted bladder was bathed by 15 ml of CO₂-free NaR (pH = 7.4). The mucosal and serosal solutions were bubbled with air passed through a series of three 3 M KOH baths to trap and remove all CO₂, according to the method described by Steinmetz (11). However in some experiments the serosal bath contained 10 mM NaHCO₃ substituted for 10 mM NaCl and was bubbled with 2.4% CO₂ gas in air. In these studies, the Na⁺ and H⁺ concentrations were equal on both sides of the epithelium. Mixing was accomplished by air lifts. Transepithelial potential difference (E) was measured through 3 M KCl agar bridges and calomel half cells, and recorded on a Fluke digital multimeter, model 8000A (John Fluke Mfg. Co., Inc., Mountlake Terrace, Wash.). The bladders were shortcircuited by the method of Ussing and Zerahn (13) using an automatic voltage clamping device; short circuit current was recorded continuously on a servo-graphic recorder (model 2802, Laboratory Data Control, Div., Milton Roy Co., Riviera Beach, Fla.) and is reported in microamperes, the average instantaneous short circuit current during any experimental period. Transepithelial electrical resistance (R) was estimated intermittently as described previously (14). Active sodium transport was calculated as the difference of total short circuit currents and H⁺ secretion (expressed in microamperes).

pH STAT method. The pH of the aerated bathing solutions was precisely adjusted to 7.400±0.001. Combination pencil electrodes (Markson Science Inc., Bliss & Laughlin Industries, Del Mar, Calif.) monitored the pH in mucosal and serosal

¹Abbreviations used in this paper: CO₂-free NaR, CO₂-free turtle Ringer's solution; CTZ, chlorothiazide; HCTZ, hydrochlorothiazide; RSSC, reverse short circuit current.

reservoirs, placed well above the lucite chamber containing the short-circuited membrane so that the current from the voltage clamp did not affect the pH reading. Continuous readings were taken on an Orion digital pH meter, model 801 (Orion Research, Inc., Cambridge, Mass.). Changes in mucosal pH from 7.400 were sensed by an automatic Orion digital controller, model 872. When the mucosal pH fell below 7.400, due to secretion of H⁺ by the bladder, the servo-controller activated a pump (Sage infusion pump, model 355, Orion Research Inc.), which delivered 0.01 N NaOH into the mucosal bath until the pH returned to 7.400. From the time interval and the volume of NaOH delivered, the H⁺ secretion rate, expressed as microequivalents per hour per 7.3 cm² membrane area was calculated.

Reverse short circuit current (RSSC) method. In some experiments, noted below, active mucosal to serosal Na⁺ transport was abolished by (a) addition of 0.1 mM ouabain to the serosal bath or (b) by substitution of Cs⁺ for Na⁺ in the mucosal bathing solution. The reverse electrical current, RSSC, previously shown to be equivalent to active H⁺ secretion (15), was continuously recorded in these experiments. For clarity of presentation, the RSSC has been expressed as micro equivalents per hour to be comparable to the direct titration data described above.

Miscellaneous methods. Results are expressed as mean ±SEM. Comparisons of means was made by paired *t* test. Chlorothiazide (CTZ), hydrochlorothiazide (HCTZ), and ethacrynic acid were supplied by Merck Sharpe & Dohme, Canada Ltd., Montreal, Quebec, Canada. Furosemide was supplied by Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N. J.

RESULTS

Time-control experiments. In six bladders, bathed on both sides by CO₂ free NaR, H⁺ secretion was measured during 60-min consecutive periods. After the first 60 min, 20 μl of 3 N HCl and 20 μl of 3 N NaOH were added to the mucosal bathing solution by infusion pump. pH of the mucosal solution was unchanged by this maneuver and H⁺ secretion was determined for another 120 min. H⁺ secretion was 1.39±0.31 μeq/h during the first 60 min of observation (Table I). Addi-

TABLE I
Effect of Addition and Removal of CTZ from Mucosal Bathing Medium on H⁺ Secretion

	60-min periods		
	1	2	3
	μeq/h		
Control n = 6*	1.39±0.31‡	1.48±0.21	1.29±0.18
	NS		NS
CTZ-washout n = 8	1.33±0.24	3.03±0.25	1.37±0.26
	P < 0.001		P < 0.001
CTZ-no washout n = 4	1.10±0.11	2.77±0.33	2.27±0.34
	P < 0.001		NS

* Number of bladders.

‡ Data expressed as mean±SEM.

tion of diluent (3 N NaOH + 3 N HCl) in period 2 had no effect on H⁺ secretion, which was 1.48 ± 0.21 $\mu\text{eq/h}$ during the second 60-min period, and 1.29 ± 0.18 $\mu\text{eq/h}$ during the third 60-min period. Active sodium transport declined slowly throughout the course of the experiment, and was not affected by addition of diluent.

Effect of mucosal CTZ on H⁺ secretion rate. In eight bladders (four with CO₂-free serosal baths and four with CO₂ containing serosal baths) CTZ (1,000 $\mu\text{g/ml}$, final concentration 3.38 mM) was added to the mucosal bath after a 60-min control period. CTZ was dissolved in 20 μl 3 N NaOH and this solution was delivered with an equivalent amount of 3 N HCl to prevent any change in mucosal pH. Results are depicted in Fig. 1. Control H⁺ secretion rates were markedly increased by mucosal CTZ. This increase occurred in tissues bathed by CO₂-free NaR (closed circles) or by CO₂-containing NaR (open circles). In every instance, washout of the diuretic restored H⁺ secretion to control levels in the third hour. The mean values for each period are presented in Table I. Initial H⁺ secretion rate was 1.33 ± 0.24 $\mu\text{eq/h}$, a value not significantly different from that in the time-control bladders. Mean H⁺ secretion rate increased significantly to 3.03 ± 0.25 $\mu\text{eq/h}$ after CTZ ($P < 0.001$) and decreased significantly upon washout to 1.37 ± 0.26 $\mu\text{eq/h}$ ($P < 0.001$).

In a separate group of four bladders, the identical protocol was followed, except that CTZ was left in the mucosal bath and the H⁺ secretion rate was determined for 120 min in the presence of the drug. When CTZ

was not removed, the stimulation of H⁺ secretion persisted for at least 120 min (Table I). In these experiments, initial H⁺ secretion rate was 1.10 ± 0.11 $\mu\text{eq/h}$ and increased significantly to 2.77 ± 0.33 $\mu\text{eq/h}$ ($P < 0.001$) during the first hour after addition of CTZ and remained markedly elevated during the second hour at 2.27 ± 0.34 $\mu\text{eq/h}$, a value significantly higher than that observed at a comparable time period in the control experiments (Table I) ($P < 0.01$) or after washout of CTZ ($P < 0.01$).

Stimulation of H⁺ secretion by CTZ was not accompanied by an effect on active sodium transport. In these experiments, there was a slow decline in active sodium transport, which was similar to the observations in the time-control studies.

Effect of serosal CTZ on H⁺ secretion rate. In four bladders, after 60-min of control observation, CTZ (1,000 $\mu\text{g/ml}$) was added to the serosal bathing solution and H⁺ secretion was measured during the next 120 min. Serosal CTZ markedly inhibited H⁺ secretion by the tissues. Control H⁺ secretion, 1.05 ± 0.14 $\mu\text{eq/h}$ was reduced to 0.44 ± 0.08 $\mu\text{eq/h}$ after exposure to serosal CTZ ($P < 0.01$). In contrast to the results with mucosal CTZ, serosal addition of the diuretic markedly inhibited active sodium transport.

Since mucosal and serosal CTZ had opposite effects on H⁺ secretion, studies were performed adding the diuretic simultaneously to both bathing solutions. In seven bladders after 60 min of control observation CTZ (1,000 $\mu\text{g/ml}$) was added to the mucosal bathing solution at the same time that CTZ (100 $\mu\text{g/ml}$) was added to the serosal bathing solution. Control H⁺ secretion, 0.98 ± 0.28 $\mu\text{eq/h}$, was increased significantly to 1.51 ± 0.30 $\mu\text{eq/h}$ after CTZ ($P < 0.01$) demonstrating that the mucosal effect of CTZ was predominant.

Effect of CTZ on H⁺ secretion rate in the presence of 5% CO₂. In five bladders, with serosal baths bubbled with 5% CO₂ in air, CTZ (1,000 $\mu\text{g/ml}$) was added to the mucosal bath after 60-min of control observation. Control H⁺ secretion rate, 1.81 ± 0.32 $\mu\text{eq/h}$ was higher than in previous controls, probably reflecting stimulation of H⁺ secretion by CO₂. Despite this higher control H⁺ secretion rate, mucosal CTZ produced a further significant increase in H⁺ secretion to 2.86 ± 0.34 $\mu\text{eq/h}$ ($P < 0.01$).

Effect of HCTZ on H⁺ secretion rate. In seven bladders (four with CO₂-free serosal bath and three with CO₂-containing serosal bath), HCTZ (1,000 $\mu\text{g/ml}$) was added to the mucosal bath after a 60-min control period. Mucosal HCTZ increased initial H⁺ secretion from 0.99 ± 0.16 to 1.58 ± 0.17 $\mu\text{eq/h}$ ($P < 0.01$). Washout of HCTZ restored H⁺ secretion to control values, 1.03 ± 0.18 $\mu\text{eq/h}$ ($P < 0.01$). These results are similar to those obtained with CTZ, although of lesser magnitude. Moreover, as with mucosal CTZ, mucosal HCTZ had no significant effect on active sodium trans-

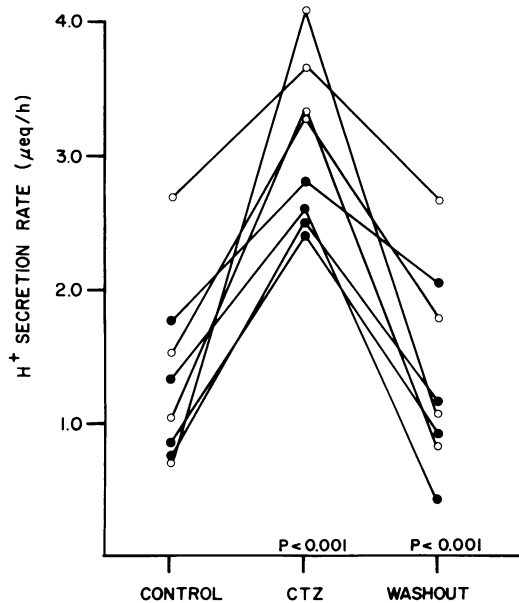


FIGURE 1 Effect of mucosal CTZ on H⁺ secretion by turtle bladder under short-circuit conditions. Closed circles, CO₂-free NaR bathing medium; open circles, HCO₃⁻ + CO₂ NaR bathing medium.

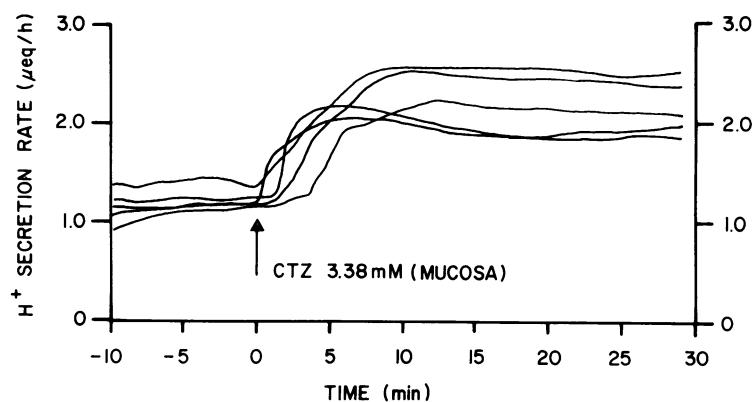


FIGURE 2 Onset of action of mucosal CTZ on H^+ secretion, measured in ouabain-treated bladders by RSCC.

port, which declined slowly throughout, similar to control observations.

Time-course of effect of CTZ on H^+ secretion, measured by RSCC. In five experiments, continuous, instantaneous measurements of H^+ secretion rate was determined in ouabain-treated bladders by recording the RSCC. These results are shown in Fig. 2. After a stable RSCC was established, addition of CTZ (1,000 $\mu\text{g/ml}$) to the mucosal bath at time zero produced a prompt increase in RSCC (H^+ secretion) as early as 15 s, which reached a new plateau in 5–10 min. As noted above (Table I), in other tissues, this response was maintained for at least 120 min, although the H^+ secretion rate tended to decline slowly with time.

Dose-response to CTZ. In five bladders, treated with 0.1 mM serosal ouabain, RSCC was recorded continuously. After a stable control period, increasing concentrations of CTZ (10, 100, 500, 1,000, 2,000, and 3,000 $\mu\text{g/ml}$) were added to the mucosal bath. After each concentration increment, RSCC was recorded for 15–30 min until a stable plateau was reached, before adding the next increment. Results are depicted in Fig. 3. Control H^+ secretion rate in the absence of CTZ in five bladders was $1.36 \pm 0.35 \mu\text{eq/h}$ (not shown). Addition of 10 and 100 $\mu\text{g/ml}$ had no significant effect on H^+ secretion rate. Addition of increasing amounts of CTZ from 500 to 1,000 $\mu\text{g/ml}$ increased H^+ secretion rates from 1.25 ± 0.30 to $1.90 \pm 0.28 \mu\text{eq/h}$ ($P < 0.01$). Further increments of mucosal CTZ up to 3,000 $\mu\text{g/ml}$ had no additional effect on H^+ secretion.

Effect of mucosal pH on H^+ secretion. In six tissues, treated with serosal ouabain, 0.1 mM, RSCC was recorded at various mucosal pH levels. While serosal pH was maintained at 7.40, mucosal pH was lowered in a stepwise fashion by addition of HCl from 7.40 to that value required to reduce RSCC to zero. At each level of mucosal pH, RSCC was recorded until stable, before lowering pH further. After completing this sequence, mucosal pH was raised to 7.40,

CTZ 1,000 $\mu\text{g/ml}$ was added to the mucosal bath, and the stepwise lowering of pH and recording of RSCC was repeated. Results are shown in Fig. 4. In tissues without CTZ, the sequential lowering of mucosal pH produced a linear decrease in H^+ secretion rate (open circles). H^+ secretion was $1.27 \pm 0.12 \mu\text{eq/h}$ at mucosal pH 7.4, and was completely abolished at a mucosal pH of 4.75 ± 0.07 . When these same bladders were then treated with mucosal CTZ (1,000 $\mu\text{g/ml}$), H^+ secretion at mucosal pH 7.40 was increased to $2.23 \pm 0.19 \mu\text{eq/h}$, a value significantly higher than control ($P < 0.001$) (closed circles). The subsequent sequential lowering of mucosal pH produced a linear decrease of H^+ secretion rates in these treated tissues although at each pH level the rates were significantly higher than in the absence of CTZ. H^+ secretion in CTZ-treated tissues was abolished at mucosal pH of 4.72 ± 0.04 ,

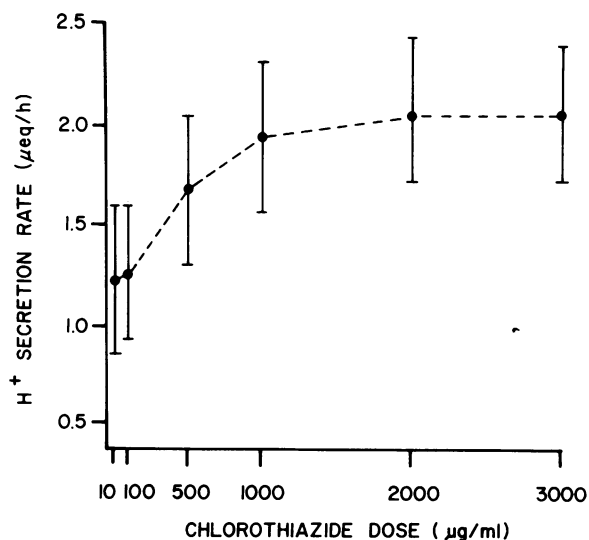


FIGURE 3 Dose-response curve relating mucosal CTZ to H^+ secretion rate. Vertical lines, ± 1 SE.

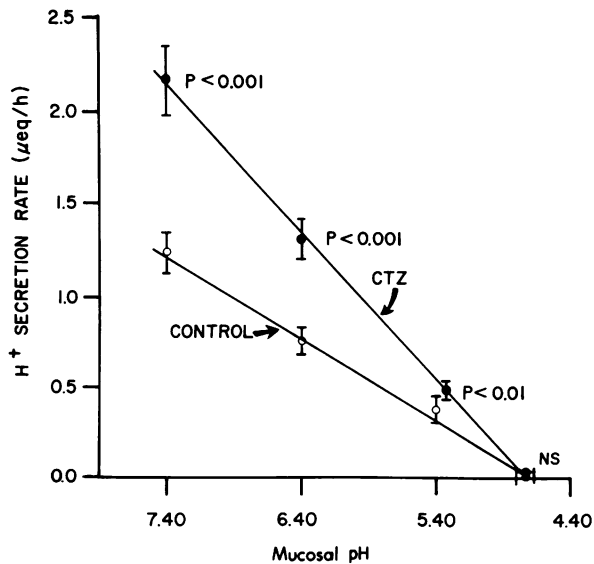


FIGURE 4 Relationship between mucosal pH and H⁺ secretion rate before and after addition of CTZ to mucosal bath.

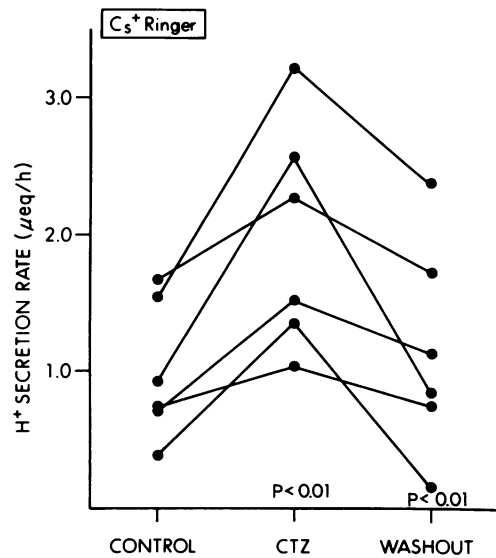


FIGURE 5 Effect of mucosal CTZ on H⁺ secretion in absence of mucosal sodium (Cs⁺ Ringer is solution mucosal bath).

a value not significantly different from that observed without CTZ.

Effect of removal of Na⁺ on CTZ-stimulated H⁺ secretion. H⁺ secretion rate was determined in six bladders bathed by Na⁺-free Cs⁺ Ringer's solution on the mucosal side and Na⁺ Ringer's solution on the serosal side. The serosal bath was aerated with 1.5% CO₂ in air to provide excess exogenous CO₂. Results are presented in Fig. 5. Control rates of H⁺ secretion in the first 60 min averaged 1.00 ± 0.21 μeq/h and were increased significantly by mucosal CTZ, 1,000 μg/ml to 1.91 ± 0.32 μeq/h in the second 60 min (*P* < 0.01), despite the removal of Na⁺ from the mucosal bathing solution. In the third 60 min, washout of the diuretic with fresh Na⁺-free Cs⁺ Ringer's solution returned H⁺ secretion toward control levels (1.08 ± 0.30 μeq/h, *P* < 0.01).

Effect of ethacrynic acid plus cysteine on H⁺ secre-

tion. In eight bladders, after a 60-min control period of H⁺ secretion, ethacrynic acid plus cysteine (at equivalent concentration to CTZ, 1,000 μg/ml, 3.38 mM) was added to the mucosal bathing solution. Results are presented in Table II. Addition of mucosal ethacrynic/cysteine increased H⁺ secretion from 0.79 ± 0.11 to 1.30 ± 0.10 μeq/h (*P* < 0.001). Washout of the diuretic significantly reduced H⁺ secretion to 0.23 ± 0.08 μeq/h (*P* < 0.001). Sodium transport measured simultaneously declined slowly and significantly throughout the experiment, similar to the decline noted in the time-control experiments.

Dose-response to ethacrynic acid plus cysteine. In six bladders, after determining control rates of H⁺ secretion, increasing concentrations of ethacrynic acid plus cysteine (10, 100, 500, 1,000, 2,000, and 3,000 μg/ml) were added to the mucosal bath. After each concentration increment, H⁺ secretion was measured

TABLE II
Effect of Mucosal Ethacrynic Acid Plus Cysteine in CO₂-free NaR on H⁺ Secretion and Na⁺ Transport*

	60-min periods		
	1 Control	2 Ethacrynic/cysteine	3 Washout
H ⁺ secretion, μeq/h	0.79 ± 0.11 † <i>P</i> < 0.001	1.30 ± 0.10 <i>P</i> < 0.001	0.23 ± 0.08 <i>P</i> < 0.001
Na ⁺ transport, μA	326 ± 29 <i>P</i> < 0.001	237 ± 27 <i>P</i> < 0.01	165 ± 23

* Data for eight bladders.

† Data expressed as mean ± SEM.

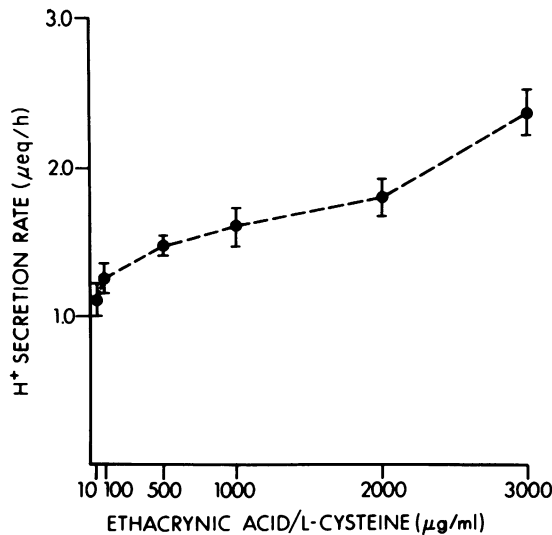


FIGURE 6 Dose-response curve relating mucosal ethacrynic acid/cysteine to H⁺ secretion rate. Vertical lines = 1 SE.

for 30 min before proceeding to the next higher dose of diuretic.

The dose-response curve is presented in Fig. 6. Control H⁺ secretion rate (not shown) was 1.19 ± 0.16 $\mu\text{eq/h}$ and was not significantly affected by 10 or 100 $\mu\text{g/ml}$ of complexed diuretic added to the mucosal bath. At progressively higher concentrations of ethacrynic acid/cysteine, from 500 $\mu\text{g/ml}$ to 3,000 $\mu\text{g/ml}$, there was a progressive increase in H⁺ secretion rate, reaching 2.38 ± 0.15 $\mu\text{eq/h}$ at the highest concentration of the diuretic.

Effect of ethacrynic acid alone on H⁺ secretion. In nine bladders (four with CO₂-free serosal bath and five with the CO₂ and HCO₃ solution used in the CTZ experiments) ethacrynic acid alone (1,000 $\mu\text{g/ml}$, 3.38 mM) was added to the mucosal bath after a 60-min

control period. Results are presented in Table III. In CO₂-free NaR III mucosal ethacrynic acid reduced H⁺ secretion from 1.45 ± 0.21 $\mu\text{eq/h}$ in the first four to 0.30 ± 0.11 $\mu\text{eq/h}$ ($P < 0.05$) in the second hour. Simultaneous Na⁺ transport was immediately and markedly reduced from 401 ± 88 μA in the first hour to 54 ± 13 μA ($P < 0.05$) in the second hour. This rapid and pronounced fall in active sodium transport was very different from the gradual decline noted in the time-control or ethacrynic acid plus cysteine experiments described above. Washout of the drug left the bladders without measurable H⁺ secretion or Na⁺ transport in the third hour.

Because the diminution of Na⁺ transport and the absence of exogenous CO₂ might have indirectly limited H⁺ secretion in these experiments, additional studies with ethacrynic acid were performed with added exogenous CO₂. These results are presented in Table III. Control H⁺ secretion, 1.46 ± 0.19 $\mu\text{eq/h}$ was still inhibited by mucosal uncomplexed ethacrynic acid to 0.41 ± 0.07 $\mu\text{eq/h}$ ($P < 0.001$) despite the provision of exogenous CO₂. Simultaneous Na⁺ transport was reduced from 432 ± 69 to 90 ± 26 μA ($P < 0.01$). With washout, H⁺ secretion remained reduced at 0.44 ± 0.19 $\mu\text{eq/h}$ and Na⁺ transport declined further to 18 ± 10 μA ($P < 0.05$). Exogenous CO₂ failed to prevent the reduction in H⁺ secretion produced by uncomplexed ethacrynic acid. Addition of cysteine alone (not shown) had no effect on H⁺ or Na⁺ transport. Thus, the stimulation of H⁺ secretion by ethacrynic acid required that the drug be present in its complexed form with cysteine.

Effect of furosemide on H⁺ secretion. After a 60-min control period, furosemide (100 $\mu\text{g/ml}$, 0.363 mM in five tissues; 1,000 $\mu\text{g/ml}$, 3.38 mM in four tissues) was added to the mucosal bath. At these concentrations, furosemide had no significant effect of H⁺ secretion (Table IV).

TABLE III
Effect of Mucosal Ethacrynic Acid Alone on H⁺ Secretion and Na⁺ Transport

		60-min periods		
		1 Control	2 Ethacrynic	3 Washout
CO ₂ -free NaR <i>n</i> = 4*	H ⁺ secretion, $\mu\text{eq/h}$	1.45 ± 0.21 ‡	0.30 ± 0.11	0.0
	Na transport, μA	401 ± 88	54 ± 13	-3 ± 2
			NS	$P < 0.05$
Serosal CO ₂ <i>n</i> = 5	H ⁺ secretion, $\mu\text{eq/h}$	1.46 ± 0.19	0.41 ± 0.07	0.44 ± 0.19
	Na transport, μA	432 ± 69	90 ± 26	18 ± 10
			NS	$P < 0.05$

* Number of bladders.

‡ Data expressed as mean \pm SEM.

TABLE IV
Effect of Mucosal Furosemide on H⁺ Secretion

	60-min periods		
	1 Control	2 Furosemide (100 µg/ml)	3 Washout
<i>n</i> = 5*	1.34±0.10‡	1.06±0.18	1.39±0.09
	NS	NS	
	Control	Furosemide (1,000 µg/ml)	Washout
		µeq/h	
<i>n</i> = 5	1.29±0.24	1.37±0.26	1.53±0.21
	NS	NS	

* Number of bladders.

‡ Data expressed as mean±SEM.

Effect of sulfasoxazole and borate on H⁺ secretion. After a 60-min control period of H⁺ secretion, sulfasoxazole (1,000 µg/ml, 3.38 mM) in five tissues or Na borate (3.38 mM) in eight tissues was added to the mucosal bath. H⁺ secretion was determined for two 60-min periods after addition of these compounds. Results are shown in Table V. Neither the addition of sulfasoxazole, a nondiuretic sulfonamide, nor borate, a nonsulfonamide buffer with pK similar to CTZ produced any significant effect on H⁺ secretion. Thus, at similar concentrations, neither structural similarity nor similar buffering capacity conferred H⁺ stimulatory effect in these experiments.

DISCUSSION

The results of the present study demonstrate that CTZ, a commonly used sulfonamide diuretic, is capable of direct stimulation of H⁺ secretion in the turtle urinary

TABLE V
Effect of Sulfasoxazole and Borate on H⁺ Secretion

	60-min periods		
	1 Control	2 Sulfasoxazole	3 Sulfasoxazole
<i>n</i> = 5*	1.34±0.29‡	1.60±0.24	1.71±0.16
	NS	NS	
	Control	Borate	Borate
		µeq/h	
<i>n</i> = 8	1.23±0.18	1.35±0.15	1.24±0.16
	NS	NS	

* Number of bladders.

‡ Data expressed as mean±SEM.

bladder. H⁺ secretion in this tissue was also increased by HCTZ and ethacrynic acid/cysteine, although no stimulation was noted with furosemide or ethacrynic acid alone. Stimulation of H⁺ secretion occurred only when the diuretics were applied to the mucosal (urinary) surface, where a rapid onset of action was observed (15 s–3 min), and a maximal effect achieved in about 10 min. Increased H⁺ secretion could be maintained for up to 120–150 min of exposure and was rapidly reversed upon removal of the agents from the mucosal bathing solution. Since the stimulation of H⁺ secretion was observed in short-circuited tissues, bathed by solutions of identical pH and chemical composition, where transepithelial electrical potential, H⁺ and other chemical gradients were abolished, the data suggest that these diuretics increase active H⁺ secretion.

Dose-response data suggest that significant increases in H⁺ secretion occurred at drug concentrations, which are probably achieved in vivo. For example, Costanzo and Weiner (16) observed an effect of CTZ on calcium and sodium excretion in intact dogs with urinary concentrations of 500–1,000 µg/ml. Beyer (7) estimated that, in dogs, 50% of an oral dose and 95% of an intravenous dose of CTZ was recovered in the urine within 6 h of administration. If the drug is handled similarly by humans, then one-half of a 1,000 mg oral dose of CTZ would be excreted in a 6-h urine volume (~50–1,000 ml). This urinary concentration of 500–1,000 µg/ml might be achieved in the distal nephron since the drug is actively secreted into the proximal nephron by the organic acid transport system (17), and water reabsorption by distal nephron sites is reduced under diuretic conditions. A similar estimate suggests that ethacrynic acid, which is also secreted into the urine (18), may achieve urinary concentrations of 500 µg/ml or more. At these concentrations in the present study, ethacrynic acid/cysteine was observed to increase H⁺ secretion significantly (Fig. 6).

While it appears likely that in vivo urinary diuretic concentrations (1,000 µg/ml) are similar to the mucosal concentrations which stimulate H⁺ secretion, it is also very unlikely that in vivo blood concentration ever approaches the serosal concentrations which we observed to inhibit H⁺ secretion in the turtle bladder. For example, a 1,000 mg oral dose of CTZ in a 70 kg human would result in a concentration of only 23 µg/ml (assuming distribution in total body water), a value which would be further reduced by rapid urinary excretion of the drug. Protein binding in the blood would further decrease the free (effective) concentration. Finally, to the extent that the diuretics are extracted by the proximal tubule, distal tubular blood concentration would be reduced even further. Thus, the predominant effect in vivo might be expected to reflect the urinary (mucosal) rather than the blood

(serosal) action of the diuretic. To test this directly, we examined the effect of simultaneous placement of the diuretic in both the mucosal and serosal bathing solutions. The combination of CTZ (100 $\mu\text{g/ml}$ serosal and 1,000 $\mu\text{g/ml}$ mucosal) which more closely approximated *in vivo* conditions, resulted in stimulation of H^+ secretion (i.e., predominance of the urinary effect).

Several possible explanations of the mechanism for H^+ stimulation can be considered. First, the effect on H^+ secretion might have been mediated by changes in Na^+ transport. For example, increased Na^+ transport might have increased metabolic CO_2 production, making more endogenous CO_2 available to the H^+ pump (19). Or, changes in Na^+ transport might have produced changes in transmembrane electrical potential, thus enhancing H^+ secretion (11). In the present experiments, however, Na^+ transport was unaffected by CTZ or HCTZ and was actually reduced by ethacrynic acid/cysteine at a time when H^+ secretion was markedly increased. The reduction in short circuit current produced by ethacrynic acid alone was profound (Table III), as compared with the smaller reduction produced by ethacrynic acid-cysteine, and we assume that this represents a toxic effect on the cells. Burg (20) has demonstrated that in isolated perfused loops of Henle, fluid transport is inhibited by ethacrynic acid/cysteine but not by ethacrynic acid alone. Thus, the pharmacologically significant action of this drug seems to occur only when the lumen is exposed to the complex ethacrynic acid/cysteine. Our observations with regard to H^+ secretion by the turtle bladder are in accord with this view.

Further evidence that the stimulation of H^+ secretion by diuretics was not related to Na^+ transport is that stimulation was noted in tissues in which Na^+ transport was abolished by ouabain (Figs. 2 and 4). Studies were also carried out in short-circuited tissues, which eliminated transepithelial electrical gradients. Nonetheless it was still conceivable that Na^+ entry into the cells across the apical cell membrane might have been altered by the diuretics and that this entry provided a favorable electrical gradient for passive counter movement of H^+ from cell to lumen. To explore this possibility, additional experiments were performed in Cs^+ Ringer's solution where Na^+ was removed from the mucosal bathing solution. Under these conditions, Na^+ movement across the apical membrane was probably abolished, yet the diuretics were still able to stimulate H^+ secretion promptly. Thus the results do not indicate any link between Na^+ transport and the increased H^+ secretion produced by these agents. Steinmetz et al. (21) demonstrated that H^+ secretion by the turtle bladder is independent of transport of other electrolytes, including sodium. Our observations are thus in accord with their findings.

A second possibility is that the effect of the diuretics

might have been related to their buffering properties. The presence of a buffer in an unstirred layer adjacent to the mucosal membrane might increase H^+ secretion by minimizing local pH gradients, or by interacting with the tissue at H^+ exit sites in the apical membrane. While theoretically possible, this alternative does not appear likely. The nonsulfonamide buffer borate, and the nondiuretic sulfonamide, sulfasoxazole, share similar pK_a and solubility characteristics with CTZ and HCTZ, yet had no effect on H^+ secretion. The data thus suggest that the effect of the diuretics was not related to these chemical properties.

Since indirect effects of the diuretics to increase H^+ secretion seemed unlikely, it appeared that the drugs were influencing H^+ secretion directly. By employing the method of Al-Awqati et al. (22), we attempted to evaluate the proton motive force and hydrogen ion conductance (K) of this transport system. The results (Fig. 4) indicate that H^+ secretion is increased by CTZ when the pump is transporting H^+ against little or no pH gradient, but the diuretic has no effect on H^+ secretion when the transmembrane pH gradient is maximal. The data therefore suggest that CTZ increased the conductance of the pump (K) but not its proton motive force. Such an effect might occur either by intracellular mechanisms or by physiologic adjustments in the apical cell membrane. One such intracellular mechanism might have been a diuretic-mediated increase in CO_2 production by the tissue. It has been shown (at least in nondiuretic treated tissues) that H^+ secretion by the turtle bladder is limited by the availability of CO_2 (12). While possible, this mechanism appears unlikely. First, stimulation of H^+ secretion occurred in tissues in which the major source of metabolic CO_2 (Na^+ transport) was inhibited by ouabain (Fig. 2) or by substitution by Cs^+ (Fig. 5). Second, marked variation in available CO_2 (from zero to 2.4%) had no effect on the response of the tissues to the diuretics (Fig. 1 and Results). Finally, significant stimulation of H^+ secretion was noted even under conditions (5% CO_2) where CO_2 was not limiting (Results). Thus it appears that the effect of the diuretic is exerted at some other step in the acidification process, and is not related to an increase in tissue CO_2 production.

Several reasons also make it unlikely that other intracellular effects mediated the increase in H^+ secretion after exposure of the tissues to diuretics. First, most studies indicate that diuretics inhibit rather than stimulate intracellular metabolic processes (23–26). Second, the rapid onset of action when the drug was applied to the mucosal surface (Fig. 2) and the rapid reversibility upon removal (Fig. 1) suggest a mechanism of action unrelated to alterations in intracellular metabolism. Finally, when CTZ was introduced into the serosal bath (where it presumably gained easier access to the cell interior) the drug markedly inhibited active H^+ secretion, an action presumably mediated by its known

effect to inhibit carbonic anhydrase (17). Thus the results of the present experiments support the idea that diuretics increase H^+ secretion by producing a rapidly reversible physiologic alteration in the luminal membrane. Possibilities include increased numbers of pump sites or a specific increase in apical membrane H^+ conductance, but the available data do not permit a choice between these or additional mechanisms.

Whatever the precise mechanism, our observations in the turtle bladder could have important clinical implications. It has long been appreciated that treatment of patients with certain diuretics can lead to increased excretion of acid and the generation and maintenance of metabolic alkalosis (1). Traditionally, this disorder has been attributed to indirect effects of the drugs on H^+ secretion. Electrolyte depletion (potassium or chloride), alterations in sodium handling (increased distal delivery, secondary aldosteronism), changes in plasma volume (volume depletion or contraction) or electrochemical changes at the level of the distal nephron (increased luminal electronegativity or increased delivery of nonreabsorbable anions) have been invoked to explain the systemic alkalosis. While the present experiments do not have any bearing on these indirect mechanisms, they do clearly demonstrate that in the turtle bladder, a tissue analogous to the mammalian distal nephron, CTZ, HCTZ, and ethacrynic acid/cysteine are capable of directly stimulating active H^+ secretion. Furosemide, on the other hand, failed to stimulate H^+ secretion. The data therefore suggest that with some diuretics, alkalosis may be induced in part by direct stimulation of renal H^+ secretion. It is clear that this effect can be dissociated from the usual inhibitory action of the diuretics on $NaCl$ transport, and therefore might occur in segments of the nephron separate from those responsible for the diuresis.

ACKNOWLEDGMENTS

This research was supported by U. S. Public Health grant HL 14720 and a grant from the New York Heart Association.

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