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## Research Article

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Inhibition of proximal tubular cytoplasmic and luminal carbonic anhydrase could well explain the diminished bicarbonate reabsorption and a fraction of the diminished sodium reabsorption noted in these studies. The fall in chloride reabsorption, and a portion of the fall in sodium reabsorption, however, may be a direct or indirect consequence of carbonic anhydrase inhibition or of an influence of benzolamide on a transport mechanism not dependent upon carbonic anhydrase.

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# The Influence of the Carbonic Anhydrase Inhibitor, Benzolamide (CL-11,366), on the Reabsorption of Chloride, Sodium, and Bicarbonate in the Proximal Tubule of the Rat

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**A B S T R A C T** Benzolamide (CL-11,366), a potent carbonic anhydrase inhibitor, was used to examine the influence of carbonic anhydrase inhibition on the reabsorption of chloride, sodium, and bicarbonate in the rat proximal convoluted tubule. Administration of 2 mg/kg benzolamide was associated with a decrease in the tubular fluid/plasma (TF/P) chloride ratio from  $1.19 \pm 0.10$  (SEM) to  $1.06 \pm 0.01$ , and an increase in the TF/P bicarbonate ratio from  $0.181 \pm 0.02$  to  $0.584 \pm 0.02$ . This dose of benzolamide significantly reduced proximal fractional reabsorption of chloride by 29.14%, of sodium by 34.3%, and of bicarbonate by 35.64%. These results indicate that benzolamide administration inhibits the reabsorption of all three electrolytes in the proximal convoluted tubule. Although 20 mg/kg benzolamide accentuated the changes in fractional reabsorption, the differences between 2 and 20 mg/kg were not statistically significant.

Inhibition of proximal tubular cytoplasmic and luminal carbonic anhydrase could well explain the diminished bicarbonate reabsorption and a fraction of the diminished sodium reabsorption noted in these studies. The fall in chloride reabsorption, and a portion of the fall in sodium reabsorption, however, may be a direct or indirect consequence of carbonic anhydrase inhibition or of an influence of benzolamide on a transport mechanism not dependent upon carbonic anhydrase.

## INTRODUCTION

Inhibition of the enzyme, carbonic anhydrase, has been demonstrated to influence bicarbonate reabsorption in

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the proximal convoluted tubule of the dog (1), rat (2-4), and rhesus monkey (5). Despite definite evidence of an influence of carbonic anhydrase on proximal tubular bicarbonate reabsorption, there is less agreement as to what role carbonic anhydrase subserves in influencing proximal tubular sodium and chloride reabsorption. The results of the micropuncture studies which have examined the influence of acetazolamide, a potent carbonic anhydrase inhibitor, on proximal sodium reabsorption in the dog are equivocal (6). Studies in the rat have shown acetazolamide administration both to increase (7) and decrease (8) fractional sodium reabsorption in the proximal tubule. Similarly, the fractional reabsorption of chloride within the proximal tubule of the rat has been observed to be reduced (8) and, conversely, enhanced (7) after acetazolamide administration.

The present study was therefore designed to characterize the influence of the administration of a carbonic anhydrase inhibitor, benzolamide (CL-11,366) on the reabsorption of chloride, sodium, and bicarbonate in the proximal tubule of the rat. The results of these studies would indicate that renal carbonic anhydrase inhibition reduces the proximal tubular reabsorption of chloride and sodium, as well as that of bicarbonate.

## METHODS

Female Simonsen rats weighing 155-264 g were used in all studies. Before the micropuncture study the animals were allowed free access to water and normal rat pellet diet. The rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and placed on a heated pad where a tracheostomy was performed and polyethylene catheters were placed into the left femoral artery, left femoral and external jugular veins, and bladder. After transfer to a thermostatically heated micropuncture platform which maintained body temperature at 37°C, the left kidney was exposed through a lateral abdominal incision, placed in a lucite cup where it was bathed with mineral oil at 37°C, and illuminated

with a fiber-optic light source. During the surgical preparation of the animal, 0.5 ml of isotonic saline was infused to replace extracellular fluid (ECF<sup>1</sup>) lost or sequestered during surgery.

All proximal tubular punctures were made at the end of the accessible portion of the proximal tubule (50–65% of proximal tubular length) after localization by the administration of 40–50  $\mu$ l of 5% lissamine green through the external jugular catheter (9). That this was the last proximal convolution on the surface available for puncture was verified by the injection of a small droplet of mineral oil. If this droplet disappeared from the surface and shortly thereafter returned to the surface in another convolution another proximal tubule was located. After the puncture site was located, 6–8 tubular diameters of mineral oil were placed in the tubule to block tubular flow. Collection of tubular fluid was then initiated by gentle aspiration with care taken to permit spontaneous flow into the pipette and maintain the oil column in a constant position. Separate tubules were selected for each tubular fluid sample and each sample was used for a single analysis. In this study, tubular fluid samples obtained from the most distal convolutions of superficial proximal tubules obtained in the first period of individual experiments were contrasted to those obtained in the second period of the same experiment. In most experiments tubular fluid samples for inulin measurement were obtained with samples for either chloride or bicarbonate determination. The tubular samples for inulin and chloride were collected in micropipettes beveled to 6–8  $\mu$  (o.d.) and filled with water-equilibrated, Sudan black stained mineral oil. The tubular samples collected for *in vitro* bicarbonate concentration measurements were collected in pipettes of identical size but were filled with and kept until use in clear, water-equilibrated mineral oil maintained at 37°C. The latter mineral oil was constantly perfused with a gas mixture containing calibrated quantities of CO<sub>2</sub>, O<sub>2</sub>, and N<sub>2</sub>, in the approximate ratios of 5:20:75. When superficial nephron glomerular filtration rates (GFR/nephron) were determined, the tubular fluid samples for inulin determination were collected during accurately timed intervals.

Three groups of rats were studied.

*Control group (group A).* For 1 hr before the collection of tubular fluid samples the rats were infused with a solution comprised of 125 mM NaCl and 25 mM NaHCO<sub>3</sub>/liter, containing inulin methoxy-<sup>3</sup>H (200  $\mu$ Ci/ml) at 20  $\mu$ l/min with a calibrated infusion pump. After this interval, four to six tubular fluid samples were obtained together with two blood samples (250  $\mu$ l) (period I). Frequently bladder urine was collected under water-equilibrated mineral oil in a preweighed vial for subsequent determination of total kidney glomerular filtration rate (GFR). After period I, which usually lasted 45 min, an interval of 30–45 min was allowed to pass, after which another series of proximal tubular, blood, and urine samples were obtained (period II). The same infusion solution and infusion rate were contained throughout the interval between periods and during period II.

*Benzolamide (2 mg/kg) group (group B).* These rats were infused with a solution containing 125 mM NaCl and 25 mM NaHCO<sub>3</sub>/liter and inulin methoxy-<sup>3</sup>H at 20  $\mu$ l/min for 1 hr before period I which was performed as in group

<sup>1</sup> Abbreviations used in this paper: "c," absolute reabsorption of filtrate in nl/min; ECF, extracellular fluid; FR, fractional reabsorption; GFR, glomerular filtration rate; TF/P, tubular fluid/plasma; TFR, tubular flow rate.

A. Benzolamide<sup>2</sup> (CL-11,366) 2 mg/kg, was then given as a prime dose and infused at 2 mg/kg per hr with 300 mM NaHCO<sub>3</sub>/liter and inulin at 20  $\mu$ l/min. This dosage was selected as it has been reported to maximally inhibit renal carbonic anhydrase without affecting red cell carbonic anhydrase (10). The infusion solution was changed to minimize the fall in plasma bicarbonate which occurs after carbonic anhydrase inhibition. After an interval of 30–45 min, another series of tubular fluid, blood, and urine samples was obtained (period II).

*Benzolamide (20 mg/kg) group (group C).* This group was studied in a manner identical to group B except that the dose of benzolamide was increased to 20 mg/kg.

In all studies, the plasma total CO<sub>2</sub> content was measured with a Natelson microgasometer (Scientific Industries, Inc., Mineola, N. Y.). Arterial blood pH was measured at 37°C with an IL pH meter (Instrumentation Laboratory, Inc., Lexington, Mass.). Plasma and urine sodium concentrations were determined with an IL flame photometer (Instrumentation Laboratory, Inc.) utilizing lithium as an internal standard; plasma and urine chloride were determined with a Cotlove chloridometer (Laboratory Glass and Instruments Corp., New York).

The quantity of tubular fluid collected for inulin determination was measured in a calibrated quartz capillary of constant internal diameter. After this measurement, the entire contents of the quartz capillary was transferred to a scintillation vial containing 1 ml of distilled water to which was added 10 ml of scintillator. Each liter of the latter contained: Spectrafluor PPO (2,5-diphenyloxazole) (Amer sham/Searle, Des Plaines, Ill.) 22 ml, Dimethyl POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)]) 150 mg, and toluene and Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.) in a ratio of 3.5:1. 5  $\mu$ l of plasma were pipetted into a similar vial containing 1 ml distilled water to which the scintillator was added. The urine samples collected in preweighed vials were weighed after which a microsample was handled using the above procedure for tubular fluid samples. All vials containing inulin methoxy-<sup>3</sup>H were counted in a Beckman Liquid Scintillator (Beckman Instruments, Inc., Schiller Park, Ill.). Quenching was monitored by external standardization. All samples were counted to at least 5000 counts; the tubular fluid samples contained at least twice the background count.

Tubular fluid chloride concentrations were determined using a modification of the method described by Ramsay, Brown, and Croghan (12). The tubular fluid samples collected for pH determination and subsequent bicarbonate calculation were analyzed at 37–38°C in an *in vitro* system. The system was contained on a small glass chamber heated by a remote circulating water pump and, with the external circuitry used, is illustrated schematically in Fig. 1. The mineral oil used in this system was mixed with distilled deionized water and then filtered through Whatman (No. 4) filter paper. The oil was maintained in equilibration with water before use. Under mineral oil in one separate compartment (A) were placed standard phosphate buffers (pH 7.384, 6.98, 6.84, 6.663–6.667) or, in a few studies, only buffers of pH 7.384 and 6.84. The second compartment (B) was perfused constantly with mineral oil warmed and equilibrated at 37–38°C with the previously described gas mixture. In compartment B, under mineral oil, were placed the tubular fluid samples together with isotonic solutions con-

<sup>2</sup> 2-benzenesulfonamide-1,3,4-thiadiazole-5-sulfonamide, supplied through the courtesy of Dr. Thomas Maren.

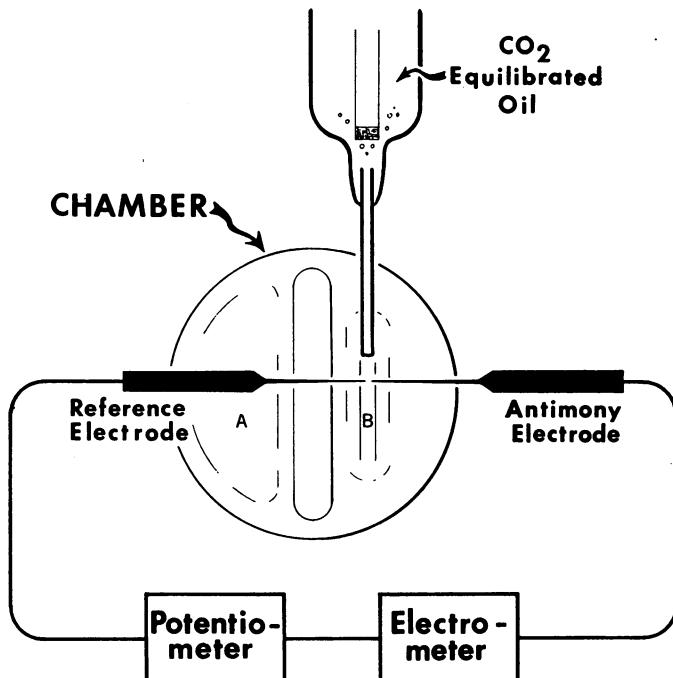


FIGURE 1 The glass chamber used to hold the microsamples for in vitro micro pH determination and the external circuitry of the system are illustrated schematically. The chamber was maintained at 37-38°C by a remote water circulating pump. The standard phosphate buffers were placed under mineral oil in compartment A. The tubular fluid samples and isotonic solutions of known bicarbonate concentration were placed under mineral oil in compartment B. Compartment B was continuously perfused with mineral oil, heated to 37-38°C, and equilibrated with CO<sub>2</sub> of known tension. Micromanipulators permitted movement of the antimony and reference electrodes from one compartment to another.

taining known bicarbonate concentrations. With the use of an antimony micro pH electrode and a reference electrode a standard curve (mv/pH U) for each antimony electrode was constructed utilizing the phosphate buffers as standards. If the electrode system proved to be stable and linear, the antimony and reference electrodes were moved to compartment B of the chamber and the pH of the standard bicarbonate solutions and tubular fluid samples was determined. After these measurements the phosphate standards were again studied. It was always possible to repeat the individual measurements in duplicate and usually all standards and tubular fluid samples were studied three or more times.

The antimony micro pH electrode was constructed similar to the one described by Lacaz Vieira and Malnic (3). Potash soda lead glass tubing (Corning No. 0010, Corning Glass Works, Corning, N. Y.) which has a coefficient of linear expansion more suitable for use with antimony was found to be preferable to Pyrex (Corning Glass Works, Corning, N. Y.) capillary tubing for construction of the electrode. The electrode was drop pulled over a small flame reducing the tip size to approximately 10-15  $\mu$ , a size found to be small enough to use in the in vitro system described. The antimony electrode was connected to the high

impedance input (approximately  $10^{14}$  ohms) of a General Radio model 1230 A electrometer (General Radio Company, West Concord, Mass.). The electromotive force (mv) measured when the reference and antimony electrode were inserted into the microsamples on the glass chamber was nulled by a bucking potentiometer and therefore could be measured and recorded with greater precision than by direct reading of the electrometer. The bucking potentiometer could be read to 0.5 mv, and the electrometer could be nulled to this degree of accuracy. The stability of the antimony electrodes varied; some were used for only one experiment, others were stable for as long as 1 wk. Only those analytical results were accepted which were obtained by electrodes which proved to be stable during at least duplicate determinations of the pH of the standard buffers and of the tubular fluid samples.

The reference electrode in some studies was of the Ling-Gerard type constructed with Pyrex (Corning Glass Works, Corning, N. Y.) capillary tubing and having a tip size of approximately 0.5-1.  $\mu$  (o.d.). It was filled with 2.5 M KCl and 0.5 M KNO<sub>3</sub> and maintained in contact to a Ag-AgCl electrode via a 2.5 M KCl and 0.5 M KNO<sub>3</sub> solution in a teflon pipette holder. In most studies the reference electrode had a tip size of approximately 6  $\mu$  and was filled with 2.5

$0.3 \text{ M}$  KCl and  $0.5 \text{ M}$   $\text{KNO}_3$  in 2% agar. These latter electrodes had resistances of approximately  $5 \times 10^6$  ohms. The tip potentials of representative samples of these latter reference electrodes were measured by determining the difference in potential observed in a solution of saturated KCl from that observed in the phosphate and bicarbonate standard solutions,  $0.3 \text{ M}$  NaCl, and  $0.3 \text{ M}$   $\text{NaHCO}_3$ . The largest tip potential present was 2.1 mv, and in most cases was negligible. The tip potentials were similar in the phosphate and bicarbonate standard solutions. The external circuitry of this system (Fig. 1) had an average impedance of 30–80 megohms.

Table I illustrates the results of a typical experiment. The slope of this antimony electrode of approximately 48 mv/pH U is typical. Listed are the actual measurements of the standard phosphate buffers, bicarbonate standards, and of several tubular fluid samples determined in this experiment. The determinations in each column were done in sequence. Included in this table are the measured and calculated pH of the bicarbonate standard solutions used in this experiment.

The quinhydrone micro pH electrode has been a standard method by which the *in vitro* pH of bicarbonate containing micro samples has been measured and from which the bicarbonate concentration could subsequently be calculated. As this electrode is commonly constructed, the wire containing the quinhydrone is inserted near the tip of a standard micropuncture micropipette. The micropipette is filled with mineral oil previously equilibrated with  $\text{CO}_2$  of known tension. A bicarbonate-containing fluid is then aspirated into the pipette and is assumed to come into equilibration with the  $\text{CO}_2$  in the mineral oil. Rector, Carter, and Seldin have demonstrated that the size of the bicarbonate sample is critical (preferably less than  $0.01 \mu\text{l}$ ) if complete equilibration of the sample and the  $\text{CO}_2$  in the oil is to occur (2). Furthermore, as pointed out previously, a loss of  $\text{CO}_2$  from the mineral oil in the micropipette would greatly influence the measurement of high tubular fluid bicarbonate concentrations, but have less influence when the tubular fluid bicarbonate concentration is low (9). For example, if the true bicarbonate concentration were 3 mEq/liter, and if the  $\text{CO}_2$  tension were reduced to one-half its expected

value, the calculated bicarbonate concentration would only rise to 6 mEq/liter. In contrast, if the true bicarbonate concentration were 20 mEq/liter, a comparable loss of  $\text{CO}_2$  from the micropipette would result in a rise in the calculated bicarbonate concentration from 20 to 40 mEq/liter. As separate quinhydrone electrodes are used for individual samples, it is difficult to monitor the  $\text{CO}_2$  tension of the mineral oil in the individual samples. In the present study, the pH of known bicarbonate concentrations was measured together (in compartment B) with the tubular fluid samples. This provides a check both on the adequacy of equilibration of the micro samples with  $\text{CO}_2$ , and of the  $\text{CO}_2$  tension of the mineral oil. If the measured pH of the bicarbonate standard is in agreement with the pH calculated from the  $\text{CO}_2$  tension of the mineral oil and the known bicarbonate concentration of the standard, it can be reasonably assumed that equilibration of the sample has occurred and that the  $\text{CO}_2$  tension of the mineral oil is appropriate.

The Henderson-Hasselbalch equation was used to calculate the expected pH of the bicarbonate standards as well as the bicarbonate concentrations of the tubular fluid samples ( $\text{pK} = 6.1$ , solubility coefficient [aqueous solution] = 0.0322) (11).

Plasma chloride and bicarbonate concentrations were corrected for Donnan factor,  $1.02\text{-Cl}^-$ ,  $1.01\text{-HCO}_3^-$  (12), and plasma water. The latter was determined by refractometry and ranged from 0.93 to 0.95.

**Calculations.** (a)  $\text{GFR}/\text{nephron} (\text{nl}/\text{min}) = \text{tubular flow rate (TFR)} (\text{nl}/\text{min}) \times \text{tubular fluid to plasma (TF/P)} \text{ inulin ratio}$ . (b) Absolute reabsorption of filtrate ("c") ( $\text{nl}/\text{min}$ ) =  $\text{GFR}/\text{nephron} - \text{TFR}$ . (c) Fractional reabsorption (FR) : Sodium =  $1 - (\text{P}/\text{TF inulin}) \times 100$ . Chloride and bicarbonate =  $1 - (\text{P}/\text{TF inulin} \times \text{TF}/\text{P}_{\text{CO}_2} \text{ or } \text{HCO}_3^-) \times 100$ . Total kidney GFR was calculated by the usual means.

The values in Table II and in the figures are the mean  $\pm 1 \text{ SEM}$ . Student's *t* test was used in the statistical analysis of the results.

## RESULTS

The results of these studies are summarized in tabular form in Table II. For statistical purposes in calculating

TABLE I  
Typical Results of pH Measurements with Antimony Micro pH Electrode

	pH	Millivolts			Mean	pH	
		Measured	Calculated				
Phosphate standards of known pH	7.384	199.0	198.0	197.5	198.5	198.3	
	6.98	213.5	215.0	213.5	214.5	214.1	
	6.84	224.5	224.5	224.0	225.0	224.5	
	6.63	234.0	232.5	234.0	235.5	234.0	
Bicarbonate standards	200.5	201.5	201.5	202.0	201.4	7.31	7.335
	210.5	211.5	210.5	211.5	211.0	7.11	7.085
	230.0	229.5	230.5	230.5	230.1	6.72	6.778
Six tubular fluid samples	243.5	242.5	242.5	244.0	243.1	6.46	
	245.0	245.0	244.5	245.0	244.9	6.41	
	239.5	239.0	239.5	239.5	239.4	6.52	
	237.5	237.0	237.5	237.5	237.4	6.57	
	213.0	214.0	213.5	214.0	213.6	7.06	
	216.0	216.5	216.5	216.5	216.4	7.00	

TABLE II  
Summary of Data under Control

	Filtrate			Sodium		Bicarbonate		
	TF/P inulin	GFR/ nephron	"c"*	F. R.	% $\Delta$	Plasma	TF	TF/P
<b>Group A, Controls</b>								
Period I	2.47	29.97	17.63	57.6		26.00	5.04	0.192
	$\pm 0.08$	$\pm 1.20$	$\pm 0.64$	$\pm 2.0$		$\pm 0.49$	$\pm 0.44$	$\pm 0.02$
Period II	2.11	31.57	16.14	51.0		24.96	6.41	0.261
	$\pm 0.07$	$\pm 1.20$	$\pm 0.65$	$\pm 2.5$	-11.46	$\pm 0.49$	$\pm 0.10$	$\pm 0.03$
n = 17	n = 15	n = 15						n = 7
	$P > 0.2$			$P < 0.05$				
<b>Group B, 2 mg/kg benzolamide</b>								
Period I	2.48	32.41	18.88	58.6		25.49	4.63	0.181
	$\pm 0.08$	$\pm 1.18$	$\pm 0.76$	$\pm 2.0$		$\pm 0.78$	$\pm 0.40$	$\pm 0.02$
Period II	1.64	30.15	11.12	38.5		22.25	12.90	0.584
	$\pm 0.05$	$\pm 1.33$	$\pm 0.96$	$\pm 2.4$	-34.30	$\pm 0.82$	$\pm 0.64$	$\pm 0.02$
n = 12	n = 11	n = 11						n = 5
	$P > 0.2$			$P < 0.001$				
<b>Group C, 20 mg/kg benzolamide</b>								
Period I	2.58	33.13	20.06	60.7		24.22	4.95	0.202
	$\pm 0.07$	$\pm 1.22$	$\pm 0.80$	$\pm 1.6$		$\pm 0.66$	$\pm 0.52$	$\pm 0.02$
Period II	1.58	26.35	9.38	36.0		21.55	17.02	0.789
	$\pm 0.05$	$\pm 0.69$	$\pm 0.71$	$\pm 3.0$	-40.7	$\pm 0.53$	$\pm 1.05$	$\pm 0.05$
n = 10	n = 10	n = 10						n = 6
	$P < 0.001$			$P < 0.001$				

Values are expressed as the mean  $\pm 1$  SEM.

\* Abbreviations used:

"c," absolute reabsorption of filtrate in nl/min.

% $\Delta$ , per cent change in reabsorption from period I to period II.

V, urine flow in ml/min.

n, number of rats in which these observations were made.

F. R., fractional reabsorption.

the fractional reabsorptions of sodium, bicarbonate, and chloride, the TF/P inulin, TF/P Cl<sup>-</sup>, and TF/P HCO<sub>3</sub><sup>-</sup> ratios of the individual periods of each experiment were averaged and treated as one value. The statistical significance of intragroup comparisons is included in the table.

*Group A.* The changes in the superficial GFR/nephron and proximal TF/P inulin ratio from period I to period II during continued hydropenia (group A) are graphically illustrated in Fig. 2, A and B. The GFR/nephron rose very slightly from  $29.97 \pm 1.20$  (SEM) to  $31.57 \pm 1.20$  nl/min,  $P > 0.2$ , whereas the TF/P inulin ratio fell from  $2.47 \pm 0.08$  to  $2.11 \pm 0.07$ . This slight fall in the TF/P inulin ratio was the consequence of both the rise in GFR/nephron and a small fall in the absolute reabsorption of filtrate ("c") (Table II), although neither change was of itself significant. Correspondingly, fractional sodium reabsorption fell

from 57.6% in period I to 51.0% in period II, ( $P < 0.05$ ). Fig. 3, A and B, demonstrates the changes in the TF/P bicarbonate and chloride ratios observed in group A. The very small fall in the TF/P chloride ratio from  $1.20 \pm 0.01$  to  $1.19 \pm 0.01$  was associated with a slight increase in the TF/P bicarbonate ratio from  $0.192 \pm 0.02$  to  $0.261 \pm 0.03$ . As can be seen from Table II, these ratio changes were the result of slight changes in both the plasma and tubular fluid concentrations of chloride and bicarbonate.

As shown in Fig. 8A, fractional chloride reabsorption fell from 50.5% in period I to 44.9% in period II,  $P > 0.2$ . Likewise, fractional bicarbonate reabsorption, Fig. 9A, fell from 90.8% in period I to 86.3% in period II,  $P > 0.1$ .

*Group B.* As in group A, the data is presented in tabular form in Table II. The effect of the administration of 2 mg/kg benzolamide after period I on the

Conditions and after Benzolamide

Bicarbonate		Chloride				V	Last blood pH	Last blood pCO <sub>2</sub>
F. R.	%Δ	Plasma	TF	TF/P	F. R.	%Δ		
%		mEq/liter		%		ml/min		
90.8		116.8	141.0	1.20	50.5		0.00413	
±2.8		±1.17	±0.8	±0.01	±2.8		±0.0005	
86.3		116.9	139.5	1.19	44.9		0.00503	7.51 33.44
±2.8	-4.96	±1.10	±0.98	±0.01	±3.3	-11.09	±0.0005	±0.02 ±2.01
n = 7				n = 12	n = 12			
P > 0.1					P > 0.2			
90.9		117.2	139.5	1.19	52.5		0.00354	
±1.4		±0.86	±1.27	±0.01	±3.3		±0.0002	
58.6		120.1	126.8	1.06	37.2		0.01887	7.47 30.36
±3.6	-35.64	±0.74	±1.03	±0.01	±2.8	-29.14	±0.0022	±0.01 ±1.32
n = 4				n = 8	n = 7			
P < 0.001					P < 0.005			
92.4		118.8	139.8	1.18	51.6		0.00419	
±1.3		±1.55	±0.55	±0.01	±2.8		±0.0003	
48.7		123.6	123.6	1.01	33.5		0.02978	7.40 34.33
±7.7	-47.29	±0.90	±0.52	±0.01	±5.1	-35.8	±0.00223	±0.01 ±1.42
n = 5				n = 6	n = 5			
P < 0.001					P < 0.025			

superficial GFR/nephron and TF/P inulin ratio is illustrated in Fig. 4, A and B. The GFR/nephron fell slightly from  $32.41 \pm 1.18$  nl/min in period I to  $30.15 \pm 1.33$  nl/min after benzolamide (period II),  $P > 0.2$ . The TF/P inulin ratio fell from  $2.48 \pm 0.08$  to  $1.64 \pm 0.05$ . In contrast to group A, the fall in the mean TF/P inulin ratio in this group was the consequence of a fall in the absolute reabsorption of filtrate ("c"). Fractional sodium reabsorption in group B (Table II) fell from 58.6% in period I to 38.5% in period II,  $P < 0.001$ . The 34.3% (%Δ) fall in the fractional reabsorption of sodium in group B was significantly greater ( $P < 0.001$ ) than the fall of 11.46% noted in group A.

The influence of 2 mg/kg benzolamide on the TF/P bicarbonate and TF/P chloride ratios is demonstrated in Fig. 5, A and B. The increase in the TF/P bicarbonate ratio from  $0.181 \pm 0.02$  in period I to  $0.584$

$\pm 0.02$  in period II is partially the result of a slight fall in the plasma bicarbonate concentration (Table II) but primarily the result of an increase in the tubular fluid concentration of bicarbonate from  $4.63 \pm 0.40$  mEq/liter in period I to  $12.9 \pm 0.64$  mEq/liter in period II. Likewise, the reciprocal fall in the TF/P chloride from  $1.19 \pm 0.01$  to  $1.06 \pm 0.01$  was primarily a result of a decrease in the tubular fluid concentration of chloride from  $139.5 \pm 1.27$  mEq/liter in period I to  $126.8 \pm 1.03$  mEq/liter in period II.

The changes in the proximal tubular fractional reabsorption of chloride and bicarbonate are illustrated in Figs. 8B and 9B, respectively. 2 mg/kg benzolamide reduced the fractional reabsorption of chloride from 52.5 to 37.2%,  $P < 0.005$ . This fall in reabsorption occurred in spite of the marked fall in the tubular fluid chloride concentration, indicating that 2 mg/kg benzolamide induces a relatively greater reduction in the

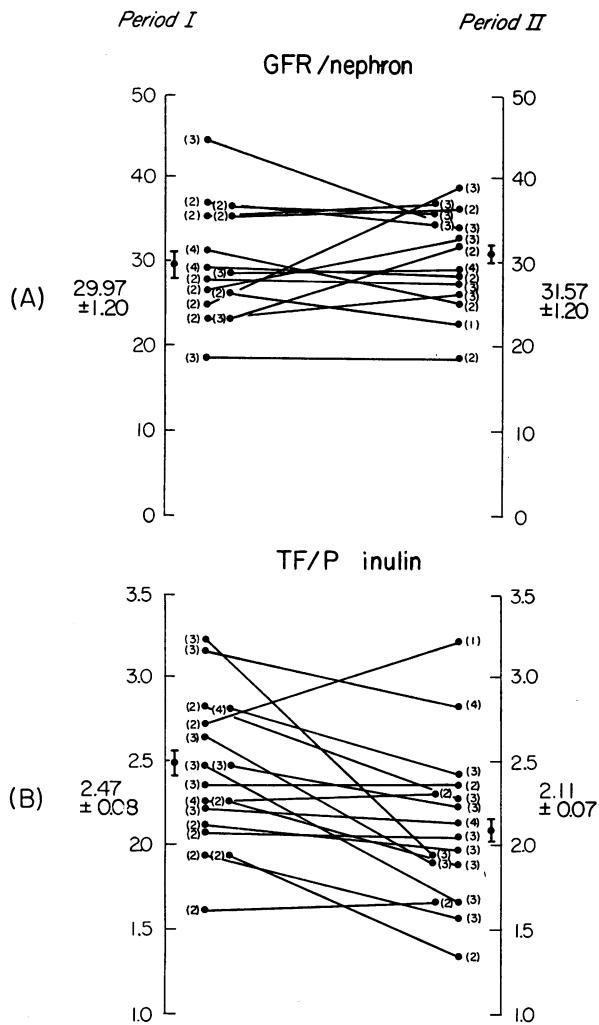


FIGURE 2 Comparison of the superficial GFR/nephron (A) and TF/P inulin ratio (B) measurements in period I and period II of group A (control) studies. Each line connects the mean observation from one experiment. In this and in the following figures the numbers in parentheses indicate the number of individual observations from which the mean values were calculated. The mean value indicates the mean  $\pm 1$  SEM. There was no significant change in the GFR/nephron,  $P > 0.2$ , whereas the fall in the TF/P inulin ratio reflects a significant reduction in fractional sodium reabsorption,  $P < 0.05$  (see text).

reabsorption of filtrate than in the TF/P chloride ratio. The fractional reabsorption of bicarbonate was reduced from 90.9 to 58.6% by 2 mg/kg benzolamide,  $P < 0.001$ . The per cent fall ( $\% \Delta$ ) in the fractional reabsorption of chloride, 29.14%, and of bicarbonate, 35.64%, in group B was significantly greater,  $P < 0.05$  and  $P < 0.001$ , respectively, than that observed in group A.

*Group C.* The dosage of benzolamide (2 mg/kg) used in group B has been reported to maximally inhibit

renal carbonic anhydrase activity while not inhibiting red cell carbonic anhydrase (10). Our studies with 2 mg/kg demonstrate a markedly smaller change in the TF/P bicarbonate ratio than noted by others measuring the tubular fluid bicarbonate concentration in vitro with the antimony pH electrode after 15 mg/kg acetazolamide (3). A possible explanation for this difference is that 2 mg/kg benzolamide was not maximally inhibiting renal carbonic anhydrase. Furthermore, if incomplete renal carbonic anhydrase inhibition was present in group B, it is possible that the reciprocal changes in the tubular fluid concentrations of chloride and bicarbonate would be accentuated if more complete inhibition could be achieved. If this situation occurred with minimal or no further change in fractional proximal sodium reabsorption, the relative reabsorption of (sodium) bicarbonate would decrease while that of (sodium) chloride would increase. To examine this possibility, group C studies (20 mg/kg benzolamide) were performed.

Fig. 6A demonstrates that in contrast to groups A and B, 20 mg/kg benzolamide resulted in a significant lowering of the mean superficial GFR/nephron from

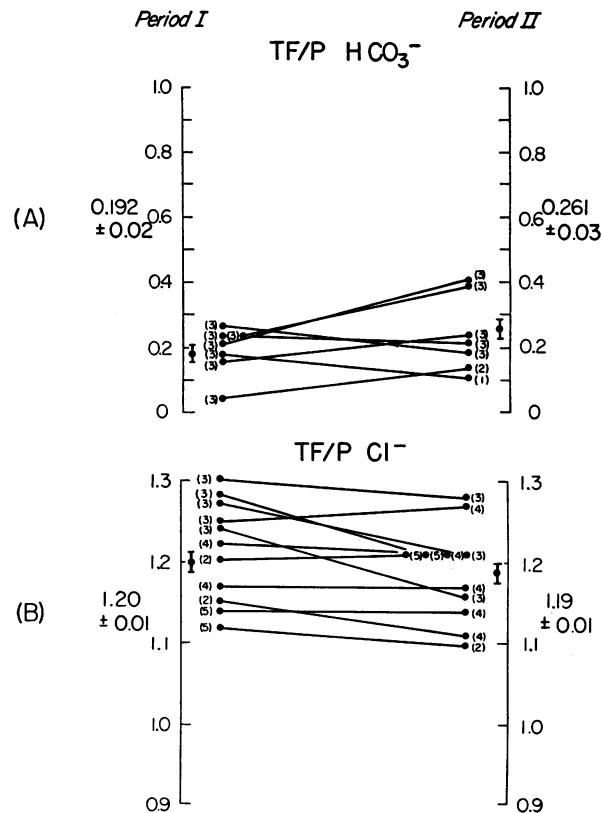


FIGURE 3 Comparison of the TF/P  $\text{HCO}_3^-$  (A) and TF/P  $\text{Cl}^-$  (B) ratios in period I and period II of group A (control) studies.

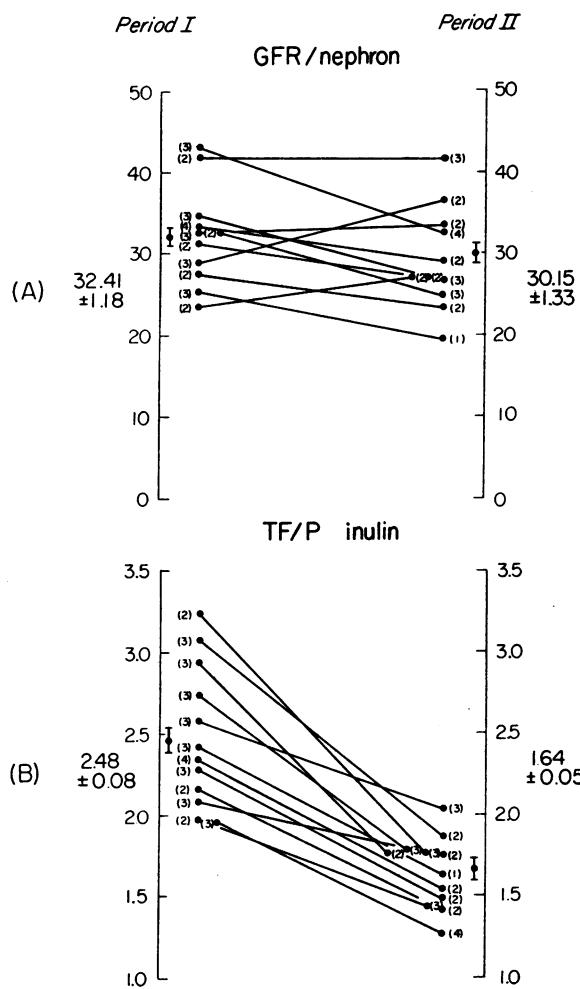


FIGURE 4 Comparison of period I and period II GFR/nephron (A) and TF/P inulin ratio (B) measurements in group B (2 mg/kg benzolamide) studies. The GFR/nephron in period II was not significantly different from the value in period I ( $P > 0.2$ ). The change in the TF/P inulin ratio indicates a significant fall in fractional sodium reabsorption from period I to period II,  $P < 0.001$ . The fall in fractional reabsorption in group B was significantly greater than that observed in group A,  $P < 0.001$ .

$33.13 \pm 1.22$  nl/min to  $26.35 \pm 0.69$  nl/min,  $P < 0.001$ . Associated with the fall in GFR/nephron, the TF/P inulin ratio also fell (Fig. 6B) from  $2.58 \pm 0.07$  in period I to  $1.58 \pm 0.05$  in period II. The fall in TF/P inulin associated with a reduction in GFR/nephron indicates that the relative fall of "c" exceeds the fall in GFR/nephron. This drop in inulin ratio corresponds to a fall in fractional sodium reabsorption from 60.7 to 36%,  $P < 0.001$ . The 40.7% fall (%  $\Delta$ ) in fractional reabsorption observed in group C was significantly greater ( $P < 0.001$ ) than the fall of 11.46% noted in

group A, but was not statistically different from that of group B,  $P > 0.2$ .

The changes in the TF/P bicarbonate and chloride ratios resulting from 20 mg/kg benzolamide are illustrated in Fig. 7, A and B. The TF/P bicarbonate ratio rose from  $0.202 \pm 0.02$  in period I to  $0.789 \pm 0.05$  in period II. This increase in the bicarbonate TF/P ratio was associated with both a small fall in the plasma bicarbonate concentration and a marked rise in the tubular fluid bicarbonate concentration (Table II). The TF/P chloride ratio, illustrated in Fig. 7B fell from  $1.18 \pm 0.01$  in period II to  $1.01 \pm 0.01$  in period II. This change in chloride ratio was also the consequence of a small increase in the plasma chloride concentration and a large fall in the tubular fluid chloride concentration.

Compared to groups A and B, 20 mg/kg benzolamide (group C) accentuated the reciprocal changes in the TF/P bicarbonate and chloride ratios. However, only the change from period I to period II, in the TF/P

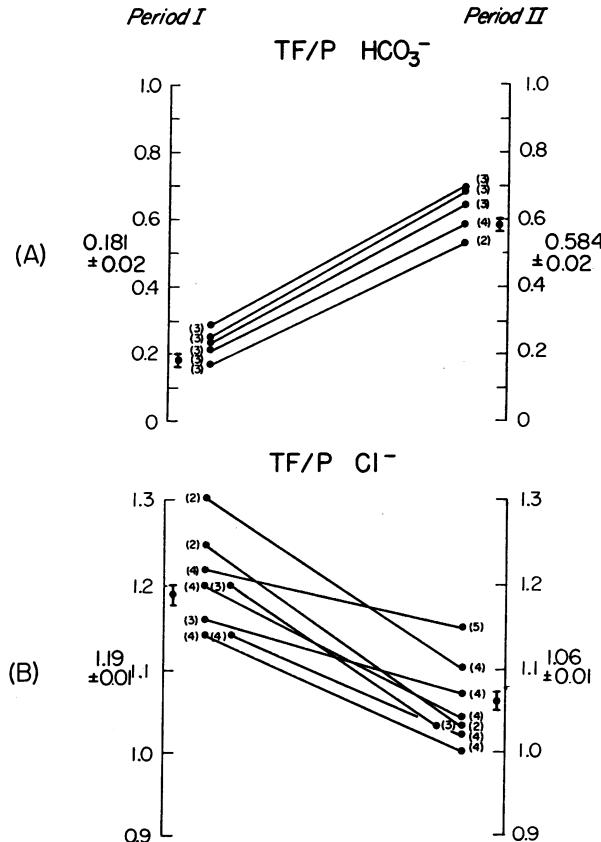


FIGURE 5 Comparison of the TF/P  $\text{HCO}_3^-$  (A) and TF/P  $\text{Cl}^-$  (B) ratios observed in period I and period II of group B studies. 2 mg/kg benzolamide resulted in a marked rise in the TF/P  $\text{HCO}_3^-$  ratio and a reciprocal fall in the TF/P  $\text{Cl}^-$  ratio.

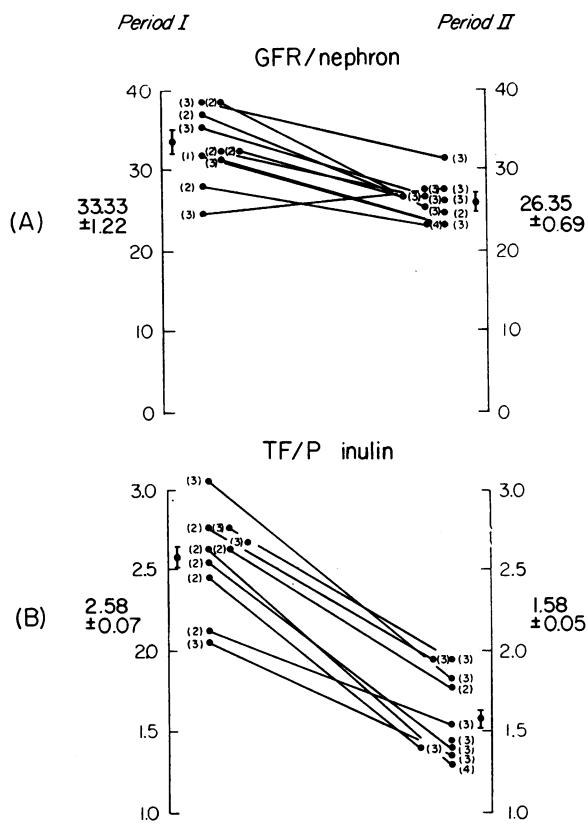


FIGURE 6 Comparison of period I and II GFR/nephron (A) and TF/P inulin ratio (B) measurements in group C (20 mg/kg benzolamide) studies. The GFR/nephron fell significantly from period I to period II,  $P < 0.001$ . The change in TF/P inulin ratio indicates a significant fall in fractional sodium reabsorption,  $P < 0.001$ , which was significantly greater than that observed in group A,  $P < 0.001$ , but not greater than that observed in group B,  $P > 0.2$ .

bicarbonate ratio noted in group C was statistically different from the change observed in group B,  $P < 0.05$ . As illustrated in Figs. 8C and 9C the fractional reabsorption of both chloride and bicarbonate fell significantly after 20 mg/kg benzolamide. The decrease ( $\% \Delta$ ) in proximal tubular fractional chloride reabsorption of 35.8% within group C was greater than the 29.14% observed in group B, although the difference was not statistically significant,  $P > 0.4$ . This does indicate, however, that the fall in TF/P chloride ratio in group C was associated with a greater fractional fall in filtrate reabsorption. Therefore, while the rate of chloride reabsorption relative to that of bicarbonate increased (TF/P bicarbonate increased, TF/P chloride decreased), overall chloride reabsorption fell even further. The per cent decrease ( $\% \Delta$ ) in the fractional reabsorption of bicarbonate in group C of 47.29% was

greater than the 35.64% observed in group B although again the difference was not statistically significant,  $P > 0.2$ .

**Miscellaneous.** Total kidney GFR was measured in most rats in all groups. In group A, it rose from  $1.97 \pm 0.22$  ml/min in period I to  $2.08 \pm 0.16$  ml/min in period II. In both group B and group C, a fall in GFR was noted after benzolamide (period II), from  $2.30 \pm 0.17$  ml/min to  $1.80 \pm 0.16$  ml/min and from  $2.14 \pm 0.30$  ml/min to  $1.70 \pm 0.13$  ml/min in groups B and C, respectively.

Fractional sodium excretion measured in four studies of group C rose from 0.17% in period I to 3.82% in period II. Fractional chloride excretion, likewise measured in four studies of group C, increased slightly from 0.32% in period I to 0.56% in period II.

## DISCUSSION

Cytoplasmic carbonic anhydrase in the proximal tubule of the mammalian kidney is assumed to aid bicarbonate reabsorption by facilitating a supply of hydrogen ions

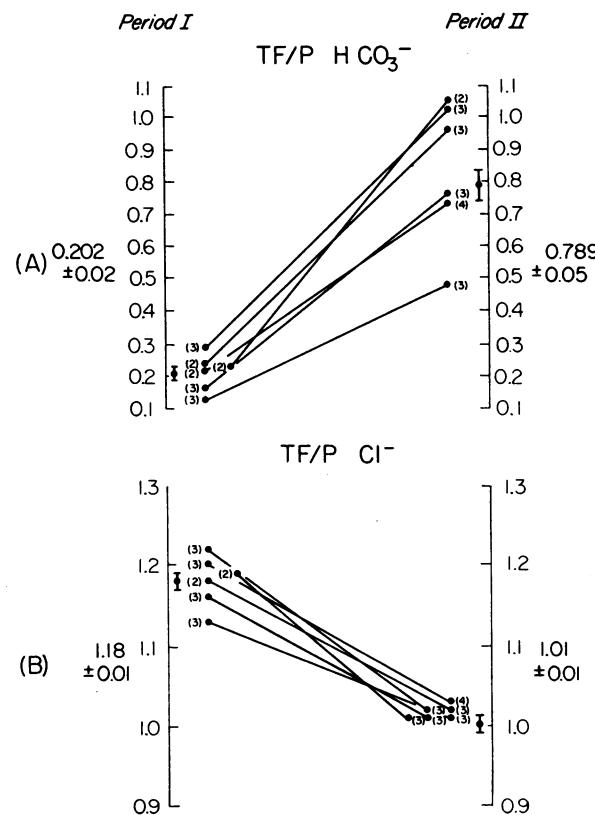
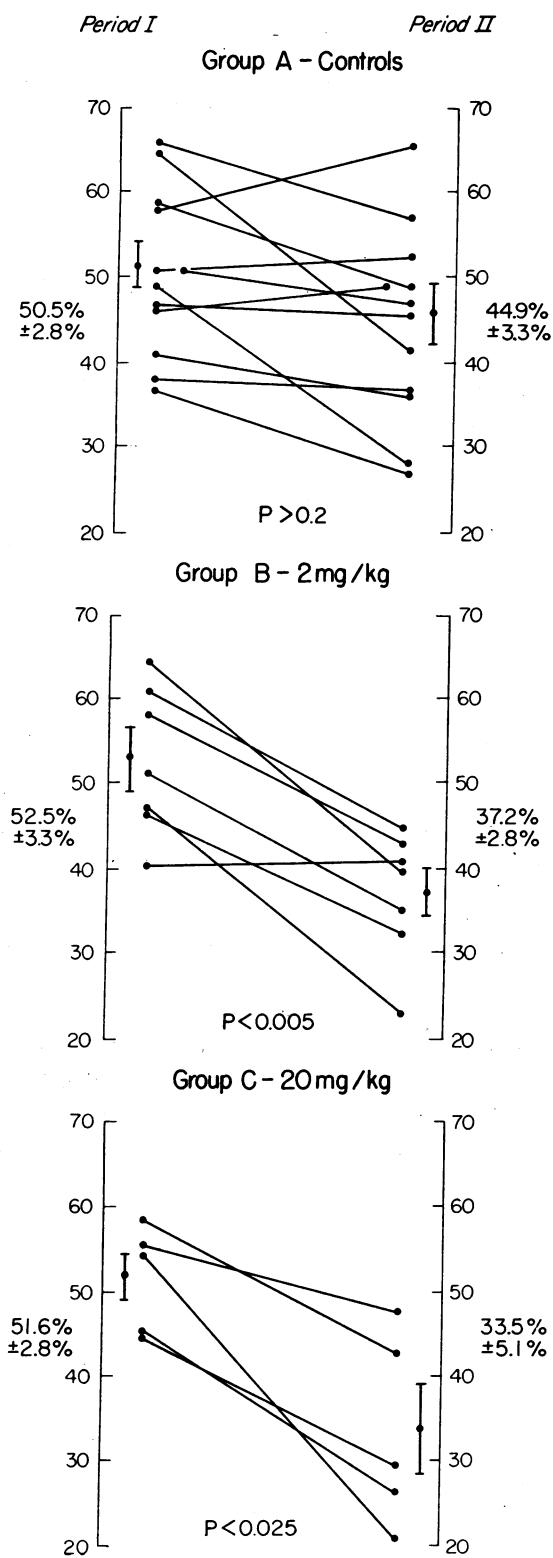


FIGURE 7 Comparison of the TF/P  $\text{HCO}_3^-$  (A) and TF/P  $\text{Cl}^-$  (B) ratios in period I and period II of group C (20 mg/kg benzolamide) studies.

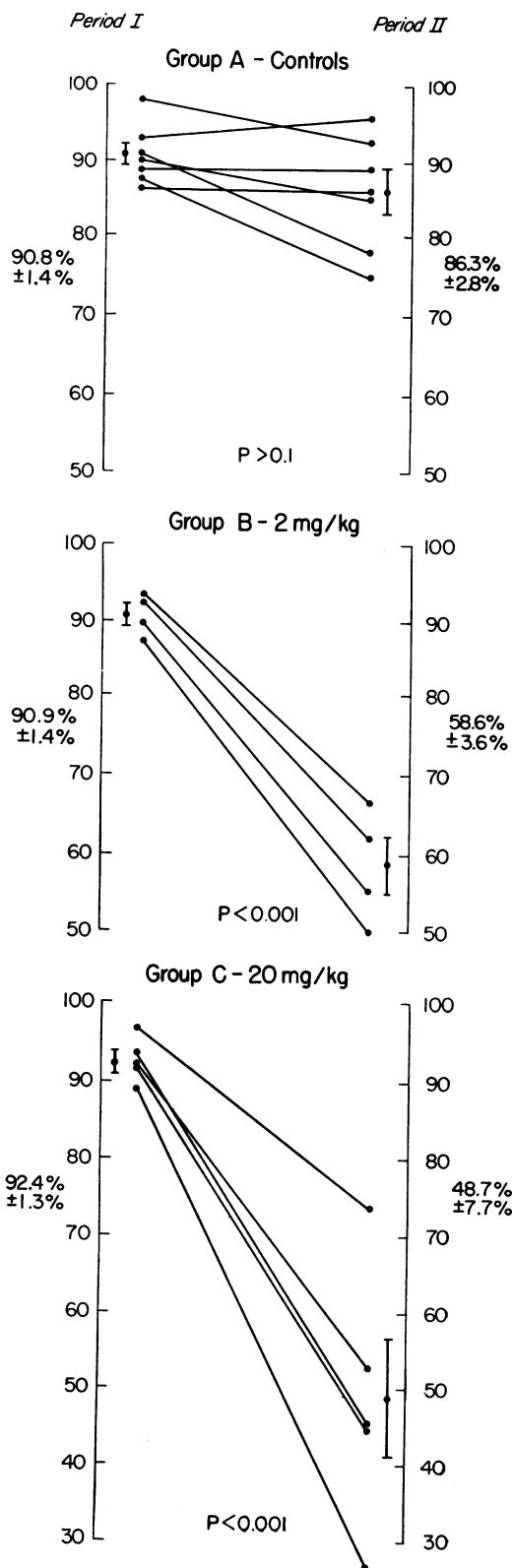


for secretion into the tubular lumen. Luminal carbonic anhydrase, at least in the proximal tubule of the rat, is assumed to aid bicarbonate reabsorption by catalyzing the dehydration of intraluminal carbonic acid formed from a reaction between intraluminal bicarbonate and secreted hydrogen ion (2). As hydrogen ion secretion may be related to the pH gradient between the lumen and tubular cell, this catalytic dehydration would retard the formation of a limiting gradient. Inhibition of carbonic anhydrase at both sites decreases bicarbonate reabsorption by diminishing the supply of hydrogen ion available for secretion and by increasing the pH gradient against which hydrogen ions must be secreted. As sodium reabsorption is felt to be operationally linked to hydrogen ion secretion and/or bicarbonate reabsorption, carbonic anhydrase inhibition would be expected to diminish proximal sodium reabsorption.

Although micropuncture studies in the dog (1), rat (2-4), and rhesus monkey (5) have clearly demonstrated that carbonic anhydrase inhibition results in an increase in proximal bicarbonate concentrations, the micropuncture studies which have attempted to evaluate the effect of such inhibition on proximal sodium reabsorption have been either equivocal or conflicting. Dirks, Cirksena, and Berliner, utilizing the recollection micropuncture technique, observed that fractional proximal sodium reabsorption in the dog fell slightly after acetazolamide administration (6). However, the observed fall was not greater than that observed in control recollection studies in hydropenic dogs. Weinstein has reported the proximal TF/P inulin ratio to be higher in acetazolamide-treated rats than in the control group (7). These latter studies are in marked contrast to those of Malnic, Mello Aires, and Lacaz Vieira who noted the proximal TF/P inulin ratio to be significantly lower in acetazolamide-treated than in control rats (8).

If the increase in proximal tubular (sodium) bicarbonate concentration noted after carbonic anhydrase inhibition is not associated with a net change in overall proximal tubular sodium reabsorption, sodium chloride reabsorption must be enhanced. The present studies indicate that this is not the case, but that the proximal tubular reabsorption of chloride and sodium, as well as of bicarbonate, is clearly diminished after renal carbonic anhydrase inhibition induced by benzolamide administration. These studies are then in agreement with those

FIGURE 8 The changes which occurred in fractional chloride reabsorption in all three groups. In groups B and C significant reductions in proximal fractional chloride reabsorption occurred. The per cent fall in reabsorption ( $\% \Delta$ ) from period I to period II of 29.14%, group B, and 35.8%, group C, was significantly greater than in group A,  $P < 0.05$  and  $P < 0.025$ , respectively. Groups B and C were not significantly different from each other,  $P > 0.4$ .



of Malnic et al. who observed proximal chloride and sodium reabsorption to be less in acetazolamide-treated rats than in a control group of rats (8).

As indicated in period I by all groups, in the anti-diuretic rat, approximately 50% of the filtered chloride, 90% of the filtered bicarbonate, and 60% of the filtered sodium is reabsorbed by the end of the accessible portion of the proximal convoluted tubule. After 2 mg/kg benzolamide, chloride reabsorption is reduced to 37.2% of the filtered load, sodium to 38.5%, and bicarbonate to 58.5%. 2 mg/kg benzolamide appears to completely inhibit proximal carbonic anhydrase as 20 mg/kg did not result in significantly greater reductions in fractional reabsorption than 2 mg/kg.

A portion of the fall in proximal sodium reabsorption and the fall in bicarbonate reabsorption observed in the present studies likely can be explained within the context of the hypothesis of proximal carbonic anhydrase function as described above. This hypothesis, however, would not appear to readily explain the fall in proximal chloride reabsorption after benzolamide. The administration of carbonic anhydrase inhibitors has been shown to inhibit chloride transport in a number of different tissues. For example, chloride transport has been inhibited in the frog gastric mucosa (14) and turtle bladder (15), tissues which contain carbonic anhydrase (14, 16) and the bullfrog cornea which is thought to be devoid of carbonic anhydrase (17). In addition, Turnberg, Bieberdorf, Morawski, and Fordtran recently have observed acetazolamide administration to inhibit sodium and chloride reabsorption in the human ileum (18), a tissue which possibly contains carbonic anhydrase (19, 20).

Presently there is insufficient information available from the present or previous studies to indicate the mechanism responsible for the decreased proximal tubular sodium chloride reabsorption noted after benzolamide. Although it is attractive to relate the fall in proximal sodium chloride reabsorption to inhibition of carbonic anhydrase, it is possible that benzolamide diminished proximal sodium chloride reabsorption because of an effect not dependent upon carbonic anhydrase inhibition or because of an indirect effect of such inhibition. For example, the elevated tubular fluid bicarbonate concentrations observed after benzolamide

FIGURE 9 The changes which occurred in fractional bicarbonate reabsorption in all three groups. In groups B and C significant reductions in proximal fractional bicarbonate reabsorption occurred. The per cent fall in reabsorption ( $\% \Delta$ ) from period I to period II of 35.64%, group B, and 47.29%, group C, was significantly greater than in group A,  $P < 0.001$ . Groups B and C were not significantly different from each other,  $P > 0.2$ .

may act as a poorly reabsorbable solute and secondarily inhibit chloride and sodium reabsorption.

The maximum increase in the TF/P bicarbonate ratio observed after carbonic anhydrase inhibition in these studies is considerably less than the ratios previously observed in the rat when proximal intraluminal bicarbonate concentrations were measured with the quinhydrone micro pH-electrode (2, 4). The mean TF/P bicarbonate ratio of  $0.789 \pm 0.05$  noted in group C, 20 mg/kg benzolamide (Fig. 7A), is comparable to the ratio of 0.92 calculated from the studies of Lacaz Vieira and Malnic (3). In this latter study the tubular fluid bicarbonate concentrations were determined in a manner comparable to the one used in the present study. The difference between these and earlier studies in which higher TF/P bicarbonate ratios were observed is likely the consequence of the more rigorous control of the  $\text{CO}_2$  tension of the mineral oil used to perfuse these samples and of the equilibrium of these tubular fluid samples with the  $\text{CO}_2$ . In the present study, the use of bicarbonate standards, the pH of which were determined *in vitro* together with the tubular fluid samples, would seem to provide a better index of these parameters than was previously used.

The proximal TF/P chloride ratio in the present study rarely fell below one despite high doses of benzolamide (group C, Fig. 7B). These results are in contrast to the observations of Weinstein who observed a mean TF/P chloride ratio of 0.92 at the end of the proximal convolution after acetazolamide administration in the rat (7). Part of the difference in these results can be attributed to different factors used to correct the plasma chloride concentrations for the Gibbs-Donnan effect. In the present study, a factor of 1.02 was used whereas Weinstein used 1.05.<sup>3</sup> Plasma water corrections were essentially the same in both studies. Had we used a Gibbs-Donnan factor of 1.05 to correct the mean TF/P chloride ratio in group C, it would have fallen from 1.01 to 0.98. However, our results are in close agreement to the TF/P chloride ratio of 1.09, plasma values not corrected, observed by Malnic et al. in acetazolamide-treated rats (8). The mean TF/P chloride ratio, plasma chloride uncorrected, in group C of the present study would be 1.096. Furthermore, the reciprocal changes in the tubular fluid concentrations of bicarbonate and chloride are close to that expected from indirect calculation, assuming the plasma concentrations of chloride and bicarbonate are equal to their tubular fluid concentrations (Table II) (9). Finally, we feel it is unlikely that these differences in the maximal TF/P bicarbonate and chloride ratios noted after

<sup>3</sup>In this regard, 1.02 would appear to be a more appropriate correction factor than 1.05 (13, 21).

carbonic anhydrase inhibition are the result of submaximal doses of benzolamide. According to the studies of Travis, Wiley, Nechay, and Maren, 2 mg/kg benzolamide should result in a maximal inhibitory effect on renal carbonic anhydrase without influencing red cell carbonic anhydrase (10). In the present studies (group C), a dosage 10 times that presumed to be maximally inhibitory to renal carbonic anhydrase was used.

The marked fall in proximal chloride reabsorption after benzolamide administration was associated with a minimal increase in fractional chloride excretion in the four studies in which it was measured. Clearly, the difference reflects reabsorption of chloride in distal parts of the nephron. The micropuncture study of Malnic et al. would indicate that the loop of Henle is the site where the major portion of chloride delivered from the proximal tubule is reabsorbed (8). A similar conclusion is suggested by the recent clearance studies of Rosin, Katz, Rector, and Seldin in the dog (22).

In summary, the results of the present study indicate that benzolamide administration clearly inhibits proximal chloride, sodium, and bicarbonate reabsorption, possibly all through carbonic anhydrase inhibition. Proximal carbonic anhydrase may therefore be fundamentally linked to sodium chloride reabsorption as well as to sodium bicarbonate reabsorption.

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