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Distal Tubule Bicarbonate Reabsorption

by

David H. Vandorpe

Ottawa, Ontario, 1990

A thesis
presented to the University of Ottawa
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Physiology

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ABSTRACT

The purpose of this study was to elucidate certain aspects of the control of bicarbonate reabsorption in the surface distal tubule of rats made acidotic by ammonium chloride treatment. During acute metabolic acidosis, the effects of sodium delivery, water reabsorption, inhibition of carbonic anhydrase, and addition of the anion channel blocker SITS were determined. In addition, bicarbonate reabsorption was assessed during recovery from metabolic acidosis.

Studies during the acute phase of acidosis:

Sprague-Dawley rats were made acidotic by ammonium chloride gavage and then prepared for micropuncture. It was hypothesized that distal tubule bicarbonate transport might have components dependent on direct or indirect Na/H exchange, water reabsorption, carbonic anhydrase activity and chloride conductance. To test these hypotheses surface distal tubules were then microperfused in vivo at 8 nl/min with 4 different isoosmotic, bicarbonate containing solutions. These solutions were designed to 1) provide an index of the increased bicarbonate reabsorption evident in this model and assess the effect of the following on bicarbonate reabsorption: 2) sodium replacement with choline 3)sodium substitution augmented by pharmacologic means (amiloride) 4) inhibition of carbonic anhydrase by acetazolamide. At 8 nl/min, collection of fluid from tubules perfused with all 4 solutions revealed significant bicarbonate reabsorption. Net distal tubule bicarbonate reabsorption was significantly increased in acidotic animals. Perfusion of tubules with sodium free solutions did not decrease this brisk reabsorptive flux, nor did perfusion of sodium free solutions which also contained

amiloride. However, perfusion of tubules with the carbonic anhydrase inhibitor DIAMOX significantly decreased bicarbonate reabsorption. This is the first in vivo demonstration of such an effect in the distal tubule of the acidotic rat when bicarbonate load was held constant.

The effect of sodium replacement at high perfusion rate was also studied, but again was not found to significantly alter bicarbonate reabsorption, although more bicarbonate was found to be reabsorbed at 25 nl/min than at 8 nl/min.

Since some reports have suggested that a chloride conductance may play a role in acidification by proton pumps, the effects of perfusion with a chloride channel blocker was studied. Paired collections at 25 nl/min, with and without SITS, revealed a significant inhibitory effect of SITS.

Studies during recovery from metabolic acidosis:

Animals allowed to recover from the acidosis exhibited a rebound metabolic alkalosis. It was hypothesized that distal tubule bicarbonate reabsorption might contribute to this apparent overshoot of blood bicarbonate concentration. Tubules perfused 44 hours post gavage exhibited significant bicarbonate reabsorption at 8, 15, and 25 nl/min, despite the concurrent metabolic alkalosis. These are the first reported in vivo data of distal tubule bicarbonate reabsorption during rebound metabolic alkalosis.

It is concluded that bicarbonate reabsorption in distal tubules of acidotic rats perfused in vivo at 8 nl/min was not affected by reduced sodium delivery, or water movement, but is partly dependent on carbonic anhydrase activity and may also be partly dependent on chloride permeability. In addition, bicarbonate reabsorption continues during the recovery phase, despite the existence of systemic metabolic alkalosis.

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For putting up with many abscences in the evenings and weekends, I thank my wife, Betty, who was my constant support. Finally, I would like to acknowledge my parents, who always encouraged me in my education.

DEDICATION

This thesis is dedicated to Dr. Graham Mainwood, of whom it truly could be said that here was a scholar and gentleman.

LIST OF IMPORTANT ABBREVIATIONS

J, flux of solute across the membrane in units of amount (picomoles) per unit time (minutes) per unit length (millimeters).

tCO_2, total carbon dioxide, composed of dissolved carbon dioxide and bicarbonate ion.

$JtCO_2$, net bicarbonate transport (the contribution of dissolved carbon dioxide is recognized as being small). In the distal tubule of acutely acidotic rats $JtCO_2$ is a positive value, reflecting the predominance of reabsorption of bicarbonate from the lumen to the blood side of the tubule.

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Chapter I

INTRODUCTION

Processing of the normal diet and metabolism generate acid which is a constant challenge to the body's acid-base status (Halperin and Jungas,1983). Preservation of the normal acid-base status of the body entails reclamation of the bicarbonate filtered by the kidney, and net elimination of hydrogen ions as indicated by urinary excretion of titratable acid and ammonium. As much as 90% of the filtered bicarbonate load is reabsorbed by transport in the proximal tubule (Koeppen et al., 1985). The remaining amount of reabsorption occurs in the more distal parts of the nephron, that although small quantitatively, is importantly modulated by changes in acid-base status, and generate the steep pH gradients required for titratable acid excretion. The following study has as its main goal, the elucidation of certain factors that may affect distal tubule bicarbonate reabsorption.

1.1 Net acid excretion in the nephron

Net acid excretion is defined as the rate of excretion of ammonium plus titratable acid minus bicarbonate, which in the steady state equals the rate of endogenous acid production (Relman et al. 1961). The rate of endogenous acid production is normally determined by the protein content of the diet. High protein intake is associated with a

slight systemic acidosis (Kunau and Walker, 1987), whereas the vegetarian diet may cause a small net alkali addition.

With 'normal' protein intake, the kidney must generate net acid. The first task is to reabsorb the filtered bicarbonate, largely taken care of by the proximal tubule. Titratable acid and ammonium are buffers that trap secreted hydrogen ion and represent de novo bicarbonate generation (Halperin and Jungas, 1983).

Control of net acid excretion is largely dependent on ammonium secretion, and to a lesser extent on increased bicarbonate reabsorption. The anion of titratable acid is largely filtered phoshate, which has a relatively fixed concentration during normal parathyroid gland function. Titratable acid is generated in the lumen of the proximal tubule, where hydrogen secretion (from both Na/H exchange and proton pumping) reduces the luminal pH close to the pK of the phosphate buffer system (Koeppen et al. 1985). Ammonia production can increase greatly during metabolic acidosis, and so is of paramount importance in adaptation (Sartorius et al. 1949). The cells of the proximal tubule are the major sites of the production of ammonia, and the enzymes involved in ammoniagenesis here show adaptation during acidosis (Parry and Brosnan, 1978). Ammonium is reabsorbed in the ascending loop of Henle and ammonia concentration gradients are generated in the cortex (Koeppen et al. 1985). These gradients drive ammonia secretion into the collecting duct system where parallel secretion of hydrogen ions trap the ammonia as ammonium and ensure excretion in the urine (Wall and Knepper, 1990).

1.2 Role of the distal tubule in the excretion of net acid

As in the nephron as a whole, the distal tubule must generate net acid by reabsorbing bicarbonate delivered to the early distal tubule and by titrating luminal buffers with the appropriate pK. Wilcox et al. (1984) studied the surface distal tubule of the acidotic rat by free-flow micropuncture technique and found that the superficial distal tubule did not generate titratable acid, but did secrete significant amounts of ammonia during acute metabolic acidosis. The mechanism of ammonia secretion by isolated cortical collecting ducts of rabbits has been described by Knepper et al. (1984). They concluded that total ammonia secretion occured primarily by diffusion of ammonia and was dependent on a luminal acid pH disequilibrium (vide infra).

Presently, controversy exists in the literature as to the contribution of the distal tubule to bicarbonate reabsorption under normal conditions. Lucci et al. (1982) assessed the contribution of the distal tubule under normal conditions using in vivo microperfusion methods and found that net secretion of bicarbonate occurred. These results are in contrast to results of free-flow micropuncture by Capasso et al. (1986) who report net reabsorption of bicarbonate by the distal tubule. Levine (1985) studied bicarbonate reabsorption under control conditions using in vivo microperfusion and found that no significant bicarbonate reabsorption occurred and in further studies showed that high perfusion rates caused bicarbonate secretion (Iacovitti et al. 1986). A subsequent study (Levine et al. 1988) has shown that fasting causes distal tubules to reabsorb bicarbonate, suggesting that subtle hormonal and/or acid-base changes may have important modulatory effects on distal tubule transport, and may explain some of the discrepancy in the literature. This is supported by the findings of Kunau and Walker (1987) that changes in protein intake caused the distal tubule to change from a low

rate to a higher rate of bicarbonate reabsorption. Clearly, these studies indicate the importance of the definition of what constitutes an adequate control state.

The fact that the distal tubule reabsorbs bicarbonate during metabolic acidosis is generally accepted (Lucci et al. 1982; Levine, 1985; Giebisch et al., 1977). However, the finding that the distal tubule is reabsorbing bicarbonate in the normal state makes its role in the adaptation to the acidotic state unclear (Capasso et al., 1986). The present study finds that under normal conditions the distal tubule reabsorbs little bicarbonate but that this increases greatly during metabolic acidosis, in agreement with previous studies by Lucci et al. (1982) and Levine (1985). Under these circumstances, the microperfused distal tubule shows a capability to reabsorb what would be almost 10% of the filtered load of bicarbonate. These findings implicate the distal tubule as an important site of adaptation in bicarbonate transport.

1.3 The model of acidosis

The mechanism of ammonium chloride acidosis was delineated by J.B.S. Haldane (1921) who performed "Experiments on the regulation of the blood's alkalinity". He proposed that ammonium chloride combined with carbon dioxide to produce urea, water and hydrochloric acid. This model of acidosis was furthur studied by Sartorius et al. (1949) who documented the renal regulation of acid-base balance in human studies. These early studies demonstrated the fall in plasma bicarbonate concentration, plasma pH, and urine pH that followed ingestion of ammonium chloride.

The method of induction of the acidosis is of interest. Most animal studies allow NH_4Cl to be ingested along with the drinking water, requiring several days to produce

a significant acidosis (Guern et al. 1982). Levine et al. (1983) studied the time profile of plasma bicarbonate concentration in rats following administration of NH_4Cl by single gavage of a relatively large amount of ammonium chloride. The profile was found to follow a characteristic fall and return to normal that could be modeled using gamma distribution analysis. A very interesting phenomenon occurs during further recovery from metabolic acidosis caused by NH_4Cl. Guern et al. (1982) have documented a rebound metabolic alkalosis that occurs during recovery and explained that this was due to the persistent increased excretion of hydrogen ions as ammonium during the first days of recovery.

NH_4Cl was administered by gavage in the present study which allowed the study of bicarbonate transport in the distal tubule when this particular system was 'under load' and during recovery allowed the study of bicarbonate reabsorption during rebound metabolic alkalosis.

1.4 Microperfusion

Richards and Walker (1937) first used micropuncture to perfuse the lumen of a single tubule of an amphibian kidney, demonstrating a powerful new technique to study ion transport in the kidney. Nearly 50 years ago Ellinger (1940) perfused nephrons of rats treated with NH_4Cl and ascribed a role for the distal nephron in acidification based on his visual observation of pH induced colour changes of a fluorescent dye (Ellinger, 1940).

The manual injection of solutions of these earlier studies was later refined to become the split oil droplet method, where a droplet of aqueous perfusion fluid is injected between two droplets of castor oil. Using this technique, Ullrich and Papavassiliou (1981) made important observations about bicarbonate reabsorption in the papillary collecting duct. The use of continuous microperfusion of nephrons to assess transport function was first performed by Sonnenberg and Deetjen (1964). Lucci et al. (1982) subsequently became the first investigators to assess distal tubule bicarbonate reabsorption using continuous microperfusion in vivo.

Continous microperfusion permits the regulation of both luminal flow and perfusate composition with minimal change in renal blood flow or other indices of renal function. One of the strengths and one of the weaknesses of the study is the fact that tubular function is studied in vivo. In this day of increasing use of the in vitro isolated perfused tubule (Burg, 1988) it should be realized that in vivo methods allow no direct control of the basolateral environment. However, the in vivo study still warrants attention as a 'closer to real life' situation: the intact animal still surrounds the fragment of nephron in question. The present study employed the technique of continuous microperfusion to study bicarbonate reabsorption in the surface distal tubule in vivo in order that various flow rates and different perfusion solutions could be employed.

1.5 The distal tubule

The surface distal tubule is a heterogeneous epithelium, consisting of the distal convoluted tubule, the connecting segment, and the initial collecting duct (Stanton et al. 1981;Kaissling, 1982). The cells of each segment are different in structure and

function. The best characterized cells are those in the initial collecting duct: principal cells and intercalated cells. Principal cells reabsorb sodium and secrete potassium while intercalated cells reabsorb or secrete bicarbonate (see figure 1). Figure 1 shows the model of epithelial transport that is currently accepted for the cortical collecting duct and that probably is the same in the initial collecting duct of the surface distal tubule (Gluck, 1989). The reader is cautioned that the contributions of the distal convoluted cells and connecting segment cells are not included in this model.

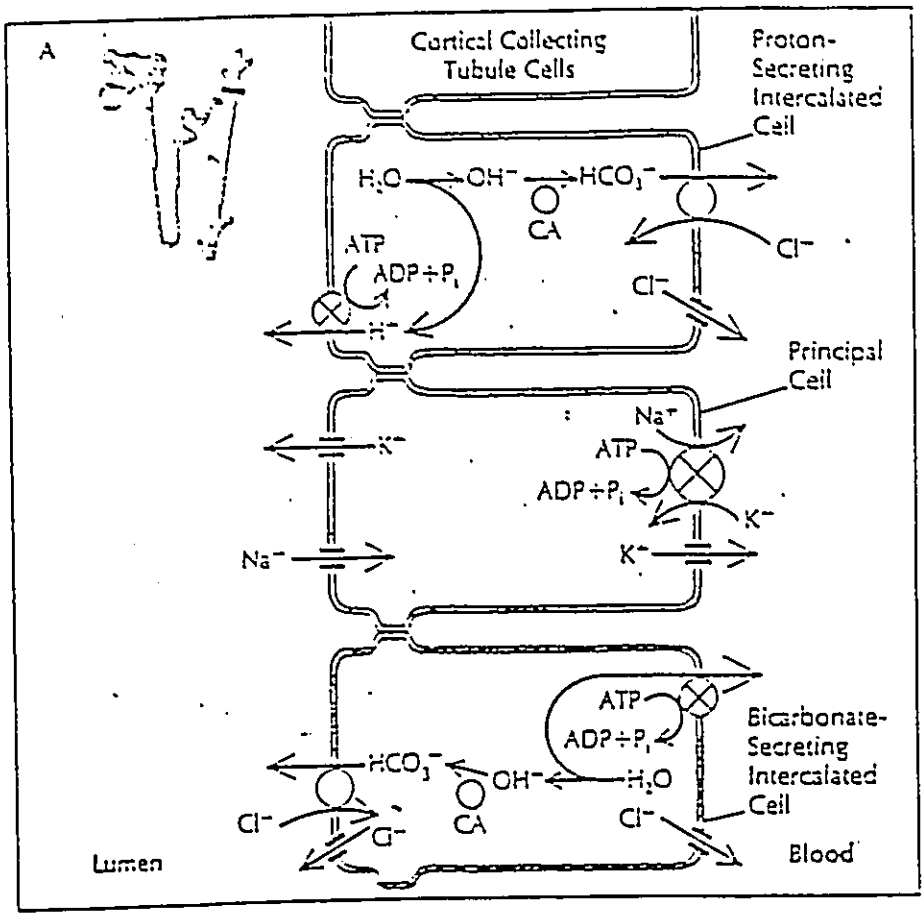
Net transepithelial sodium reabsorption is thought to occur by a pump-leak system that was originally proposed by Koefoed-Johnsen and Ussing (1958) to explain their observations of Na transport by frog skin. Sodium is thought to enter the principal cell by apical channels, down a concentration gradient maintained by the activity of a Na/K ATPase on the basolateral side of the cell. Potassium is thought to be secreted by principal cells, moving out of the cell through apical channels, again down a concentration gradient maintained by the basolateral Na/K ATPase (Gluck, 1989).

Transepithelial bicarbonate reabsorption is thought to be carried out by intercalated cells having a luminal hydrogen ion pump. The energy from ATP is used to pump hydrogen ions out of the cell against an electrochemical gradient (Steinmetz, 1986). (Intracellular carbonic anhydrase is required to form carbonic acid from water and carbon dioxide -see below). Bicarbonate uptake by the blood side is via an anion exchanger - chloride moves into the cell in exchange for bicarbonate. Stetson and Steinmetz (1985) proposed that a variant of the intercalated cell with the opposite polarity was responsible for bicarbonate secretion. Net bicarbonate transport in a kidney segment having both of these cell types is then dependent on the relative numbers and activities of the two intercalated cell types.

Investigations using electron microscopy have found that structural adaptation occurs in intercalated cells in response to acute metabolic acidosis and to acute metabolic alkalosis (Dorup, 1985). The surface density of the luminal membrane in intercalated cells was significantly higher in cells of acid treated rats. This phenomenon is supportive of Madsen and Tisher's hypothesis that intracellular vesicles containing hydrogen ion pumps are inserted into the luminal membrane when hydrogen ion secretion is stimulated (Madsen and Tisher, 1985). Stetson and Steinmetz have hypothesized that there are two variants of the intercalated cells: an alpha type (A) modified for hydrogen ion secretion and a beta type (B) specialized for bicarbonate secretion (Stetson and Steinmetz, 1985). Currently, controversy exists with regard to the existence of distinct types or if one type is able to be changed depending on the cell environment. Recent studies suggest that cell remodeling of bicarbonate secreting cells occurs in response to acid exposure: apical chloride/bicarbonate exchangers may be removed or inactivated (Satlin and Schwartz, 1989).

In vivo studies of bicarbonate transport can only discern net bicarbonate movements. This acknowledges that no specific information on unidirectional flux is made available. i.e. the net reabsorption in these studies could be due to a combination of reduced activity of bicarbonate secreting cells or increased activity of bicarbonate reabsorbing cells. Largely on the basis of histologic studies (Dorup, 1985) it is thought that bicarbonate reabsorption under the circumstances of the present study is primarily due to the activity of the A-type cells.

Figure 1: Model of epithelial transport in the collecting duct. (from Gluck (1989)).


A
Cortical Collecting Tubule Cells
Proton-Secreting Intercalated Cell
H_2O
OH^-
HCO_3^-
CA
Cl^-
ATP
$ADP + P_i$
H^+
Cl^-
Principal Cell
Na^+
ATP
$ADP + P_i$
K^+
K^+
K^+
Na^+
ATP
$ADP + P_i$
Bicarbonate-Secreting Intercalated Cell
HCO_3^-
OH^-
H_2O
Cl^-
CA
Cl^-
Cl^-
Lumen
Blood

In vivo studies of the surface distal nephron must contend with the fact that the segment perfused encompasses all the cell types mentioned. Although the preponderance of evidence has implicated the intercalated cell as having the major role in bicarbonate reabsorption, the possible role of other cell types cannot be yet discounted.

1.6 The regulation of bicarbonate transport

Intracellular carbonic anhydrase catalyzes the formation of hydrogen ion and bicarbonate ion from carbon dioxide and water (Maren, 1974). Distal tubule bicarbonate reabsorption is thought to occur by hydrogen ion secretion into the lumen, with concomitant movement of bicarbonate across the basolateral membrane into the blood. This conclusion is largely based on the results of disequilibrium pH studies, which found that in situ pH measurements were more acidic than equilibrium pH measurements (Rector et al. 1965). An acid disequilibrium pH indicates a steady state accumulation of carbonic acid relative to the equilibrium concentration, due to the slow rate of the uncatalyzed dehydration reaction producing carbon dioxide and water. These earlier studies have been more extensively studied by Dubose (1983) who found that a spontaneous disequilibrium was not present in the distal tubule during control conditions, but was demonstrated during combined respiratory acidosis-metabolic alkalosis (conditions designed to maximally increase bicarbonate reabsorption).

The movement of a proton could easily be affected by simultaneous movements of other charged ions. Possible interactions are direct (exchange mechanisms) or indirect (magnitude of lumen potential is altered). Potassium secretion, sodium reabsorption, and chloride movements have all been proposed to have effects on distal hydrogen ion secretion (Levine and Jacobson, 1986).

Potassium excretion has been shown to influence distal hydrogen ion excretion in the dog (Pichette et al., 1982) and evidence has been recently provided for the existence of a hydrogen ion/ potassium ion ATPase in the collecting duct of the rat (Wingo, 1989). However, this latter observation seems only to occur in K depleted animals. In view of the large volume of literature proclaiming the hydrogen ion pump as an electrogenic process, the following treatise largely ignores the existence of K/H cotransport.

Pitts and Alexander (1945) explained distal tubule bicarbonate reabsorption as a mechanism dependent on Na/H exchange and many subsequent whole animal studies have supported this association (Schwartz and Cohen, 1978). Subsequent in vitro studies of the distal nephron have changed the interpretation of these studies. This topic is dealt with in detail below.

Chloride has been also implicated in animal clearance studies as having an influence on urinary excretion of acid (Schwartz and Cohen, 1978). In those studies it was thought that chloride played a passive role by accompanying sodium reabsorbed in the distal tubule and so affecting the transepithelial potential. In vitro studies have now shown that chloride may play a much more active role in the process of net bicarbonate transport (Schuster and Stokes, 1987).

In the proximal tubule, the influence of water transport on bicarbonate transport is well established (Alpern, 1984). Bicarbonate permeability in the distal tubule is thought to be much lower than in the proximal tubule, suggesting that the passive movement of bicarbonate probably makes a much smaller contribution to net bicarbonate transport here. However, the possibility exists that significant volume flow caused by water reabsorption in parallel with bicarbonate transport might interact (vide infra).

Recently, the concept of the intercalated cell as a target for extracellular signals has emerged (Morel and Doucet, 1986). Aldosterone has been implicated as having an influence on net acid excretion (Dubrovsky et al. 1981) and receptors have been demonstrated using autoradiography in rat DCT (Farman and Bonvalet, 1983). Peptide hormones have now been identified as also playing a role: antidiuretic hormone has been shown to increase and glucagon to inhibit distal tubule bicarbonate transport (Bichara et al. 1987; Paillard and Bichara, 1989). In additon, innervation has recently been shown to play a role in bicarbonate transport here, suggesting the presence of receptors for neurotransmitters (Wang and Chan, 1989). Hormonal changes may well be necessary for the adaptation to metabolic acidosis and might also explain events during recovery. However, hormonal profiles were not followed in the present study.

The present study proposed to alter bicarbonate transport in the surface distal nephron by altering sodium delivery, and by administration of the drugs amiloride, SITS, and acetazolamide. These potential influences on bicarbonate reabsorption are discussed furthur below.

1.6.1 Influence of sodium transport

Much of the literature about the function of the distal tubule draws upon studies of the turtle bladder, thought to be a model of a tight epithelium, analagous to that of the cortical collecting duct. Steinmetz points out that these epithelia have a common embryologic origin, a possible reason that they seem to have analogous functions. This will explain the constant references to these different models in the following discussion (Steinmetz, 1986).

The state of distal sodium delivery and reabsorption by the distal nephron are regarded to have major impact on net acidification (Batlle and Kurtzman, 1985). One influential hypothesis even states that Na/H exchange is a major determinant of the steady state body fluid pH (Schwartz and Cohen, 1978). However, studies of CCT in vitro have emphasized the importance of acute changes of peritubular pH in controlling tubular transport (Breyer et al. 1986). The present discussion will focus on acute studies of distal epithelia and their analogues.

Turtle bladder studies by Steinmetz have shown that hydrogen ion secretion and sodium absorption exhibit indirect electrical interaction, since both the hydrogen ion pump and the sodium pump are electrogenic transport systems (Steinmetz, 1986). This same model of electrogenic proton pumping that can be potenitally modified by the inhibition of sodium transport has been shown to work in the rabbit CCT by Koeppen and Helman (1982), and so should presumably work in the late distal tubule of the rat nephron. Stoner, Burg and Orloff (1974) have measured distal tubule transepithelial potentials (lumen negative) and found the magnitude to be related to sodium transport.

Recent electrophysiological studies of the rabbit outer medullary collecting duct have demonstrated differences in the electrical characteristics and the response to pharmacologic agents in intercalated and principal cells (Koeppen, 1987). Koeppen found that principal cells had a greater basolateral membrane voltage than intercalated cells, and that amiloride increased the fractional resistance of the apical membrane to current flow. Intercalated cells were characterized by sensitivity to SITS and acetazolamide. As the same cell types are present in the late distal tubule, the same mechanism of inhibition of bicarbonate transport by amiloride may occur here.

In contrast to the bulk of studies that conclude that sodium reabsorption does affect distal bicarbonate handling are a few notable exceptions. McKinney and Burg

(1978) attempted to manipulate the sodium delivery to isolated cortical collecting tubules that were reabsorbing bicarbonate. They found an (unexpected?) increase in bicarbonate reabsorption when choline was substituted for sodium. In an in vivo study, Levine (1985) found no effect of using either 95 or 35 mM Na in the perfusate of microperfused distal tubules of acidotic rats. In conclusion, it appears that the majority of studies suggest that sodium transport should affect distal bicarbonate transport. One of the aims of the present study was to determine the effect of zero perfusate sodium concentration on distal tubule bicarbonate reabsorption in vivo.

1.6.2 Amiloride and acidification

Amiloride has been reported to inhibit bicarbonate reabsorption in both in vivo and in vitro studies. Turtle bladder studies show that the application of amiloride inhibits sodium transport and thereby indirectly inhibits hydrogen ion secretion (Steinmetz, 1986). McKinney and Burg (1978) found that the same result occurred when the drug was included in the perfusate of isolated perfused cortical collecting tubules from acid treated rabbits. Kunau and Walker (1987) have recently reported that amiloride inhibited in vivo bicarbonate reabsorption in surface distal tubules of rats fed a high protein diet.

The effect of amiloride on bicarbonate reabsorption is potentially by 2 different mechanisms: inhibition of the sodium hydrogen exchanger or by indirectly affecting hydrogen ion secretion by blocking sodium channels and thereby reducing the favourable transmembrane potential (Frelin et al., 1987). Na/H exchange is known to take place in the proximal tubule and the thick ascending limb of Henle (Burg and Green, 1977; Good, 1985). Kinsella and Aronson (1981) found that amiloride was a reversible, competitive inhibitor for the sodium binding site of the rabbit renal microvillus

membrane Na/H exchanger. Amiloride concentration in the millimolar range is required for this action (Kinsella and Aronson, 1981). In the cortical collecting duct Na transport can be blocked while bicarbonate transport can continue, strongly suggesting that direct Na/H exchange does not occur here (Laski and Kurtzman, 1983). However, recent studies of the Amphiuma diluting segment (thought to be analagous to the early distal tubule of the mammalian nephron) have demonstrated Na/H exchange (Stanton, 1988). Thus, there is a case for the action of amiloride to be via inhibition of direct Na/H exchange in part of the surface distal tubule of rats in micropuncture studies.

The distal mechanism for bicarbonate reabsorption has been characterized as including an electrogenic hydrogen ion ATPase pump (vide supra). The electrogenic nature of this pump is presumably responsible for its sensitivity to amiloride. Amiloride inhibits sodium entry at the apical membrane and secondarily inhibits the sodium transport that generates the lumen negative potential (Sariban-Sohraby and Benos, 1986) and electrogenic proton pumping (Steinmetz, 1986). The dose of amiloride needed to do this is much lower than that required to eliminate Na/H exchange. (A patch clamp study has shown that the probability that sodium channels in the apical membrane of the cortical collecting duct are open decreases when amiloride is present in micromolar quantities, and that the actual unit conductance of each channel is not reduced (Palmer and Frindt, 1986)).

The present study used amiloride in conjunction with low sodium perfusate in order to furthur define the relationship between bicarbonate transport in the distal tubule and sodium transport.

1.6.3 The role of carbonic anhydrase

Acetazolamide has long been known to decrease bicarbonate reabsorption in the kidney (Pitts and Alexander, 1945). This has been attributed to its role as an inhibitor of the enzyme carbonic anhydrase. The classic view presented by Pitts and Alexander was that carbonic anhydrase catalyzed the formation of carbonic acid in the cells of the distal tubule involved in the acidification of urine. Lonnerholm and Ridderstrale (1980) studied the intracellular distribution of carbonic anhydrase in the rat kidney and found that in the late distal tubule there were intercalated cells that contained abundant cytoplasmic enzyme. Many free-flow micropuncture studies have assessed the effect of systemic infusion of acetazolamide on bicarbonate reabsorption (Malnic et al., 1972). These studies show that bicarbonate reabsorption is reduced under these conditions, but the interpretation of these studies is complicated by the large increases in bicarbonate load that occur during these maneuvers. McKinney and Burg (1978) showed that acetazolamide completely abolished bicarbonate reabsorption in the isolated perfused cortical collecting duct.

It is of considerable interest that recent studies have implicated other ways that acetazolamide might inhibit bicarbonate reabsorption. Dixon et al. (1988) have shown that the inhibition of transport by acetazolamide is associated with a decrease in apical membrane surface area, resulting from transient alterations in the rates of endocytosis and exocytosis. This is thought to be significant because Gluck and coworkers (1982) have suggested that one means of regulating the rate of proton secretion is by altering the number of proton pumps present in the apical membrane.

The present study attempted to determine the effect of acetazolamide inhibition on bicarbonate transport in the distal tubule in vivo when bicarbonate load and flow were controlled.

1.6.4 Possible contribution of water movements

Water reabsorption in the kidney is the passive consequence of active solute transport and may be either transcellular or transjunctional (Berry, 1983). Water transport in parallel with solute reabsorption allows the possibility of solute-solvent interaction: solvent drag or an unstirred layer effect have been proposed to be important determinants of epithelial transport.

Bicarbonate transport and its relation to water transport has been extensively studied in the proximal tubule (Cogan and Alpern, 1984). Studies by Jacobson et al. (1982) and Corman and Stefano (1983) have argued against the existence of solvent drag as a significant mediator of bicarbonate reabsorption here. The existence of an unstirred layer effect on the apical side of the proximal tubule has been discounted by geometric considerations (Berry, 1983), but such a layer may exist on the peritubular side (ibid). Alpern (1984) found that there was a relationship between volume flow and bicarbonate reabsorption in the proximal tubule. These results were explained by the existence of a diffusion barrier on the peritubular side of the cell that caused local bicarbonate concentration to increase and secondarily inhibited proton secretion. Volume reabsorption reduces this gradient by convection, and so enhances proton secretion (Alpern, 1984).

Water reabsorption has also been associated with bicarbonate reabsorption in free flow micropuncture studies (Capasso et al., 1986). The few estimates of bicarbonate permeability in the distal tubule seem to point out that the contribution of passive transport of bicarbonate is likely to be very small. However, Dubose and Lucci have been able to estimate that sizeable bicarbonate movements down concentration gradients may occur under certain circumstances (Dubose and Lucci, 1983). An earlier microperfusion study has shown that water movement was correlated with bicarbonate

reabsorption (Levine, 1985). In order to eliminate the effects of water reabsorption on distal tubule bicarbonate reabsorption, the present study employed isoosmotic solutions in the perfusates.

1.6.5 Role of chloride

Proton secretion is thought to be an electrogenic process (Steinmetz, 1986), the secretion of an anion into the lumen might influence hydrogen ion secretion by shunting a positive potential at the apical face of an intercalated cell. Chloride ions might be able to do this in the distal tubule, so a number of studies have employed chloride substitution or anion channel blockers to assess effects on acidification (Stone et al. 1983). Ullrich and Papavassiliou (1981) used SITS, a blocker of anion transport in epithelia, when perfusing papillary collecting ducts of rats in vivo. A significant reduction in bicarbonate reabsorption was noted. Stone et al. (1983) have proposed a model of bicarbonate reabsorption that included a role for cellular chloride transport in parallel with the hydrogen ion pump. Koeppen (1987) did not find a significant chloride conductance in isolated perfused collecting ducts from rabbits. However, Light et al. (1987) have found that the apical surface of cultured intercalated cells had a chloride channel that was not very active, allowing the speculation that such a conductance might be missed by macroelectrode methods, but could be demonstrated using patch clamp techniques. Hilden et al. (1988) have studied the hydrogen pump in vitro and have demonstrated the existence of parallel chloride conductance to the pump in vesicles. Whether or not this choride conductance is lost when the pump is inserted into the luminal membrane is an important observation that has not yet been determined.

1.6.6 SITS

SITS (4-acetamido-4-isothiocyanostilbene-2,2-disulfonate) is a stilbene disulfonate derivative that is known to block Cl/HCO_3 exchange by the band 3 protein of the red blood cell membrane (Jennings, 1984). The same protein has been located by antigenic means on the basolateral membrane of A-type intercalated cells (Drenckhahn et al. 1985) and basolateral application of SITS has been shown to inhibit acidification (Stone et al. 1983). In contrast, in vivo luminal application of 5×10^{-4} M SITS has been found to be not effective in blocking apical anion exchange in the cortical collecting tubule (Schuster and Stokes, 1987). This is surprising, because a recent study of cultured B-type intercalated cells has shown that bicarbonate secretion was inhibited by apical application of DIDS, a closely related stilbene derivative (Van Adelsburg et al. 1989). The effect of luminal SITS on apical anion exchange in animals adapted to metabolic acidosis should be minimal as Satlin and Schwartz (1989) have shown that apical anion exchangers are removed from intercalated cells some hours after exposure to the systemic acidosis.

SITS has been shown to have effects other than inhibition of anion exchange, including blockade of chloride conductances in various tissues. Miller and White (1980) showed that the voltage gated chloride conductance from Torpedo electroplax was SITS sensitive. Nelson et al. (1984) showed in a patch-clamp study that chloride channels from an epithelial cell line could be blocked by 1 mM SITS. If proton secretion in the apical membrane of intercalated cells is accompanied by chloride movement through a channel, as originally proposed by Stone et al. (1983) (and supported by studies such as that of Hilden et al. (1988)), than luminal application of SITS may have an inhibitory effect on bicarbonate reabsorption in the distal tubule. The present study employed collections with and without SITS in the same tubule in order to assess the role of chloride conductance in bicarbonate reabsorption in the distal tubule.

1.6.7 Time course of recovery of bicarbonate transport adaptation

No data are available regarding surface distal tubule bicarbonate transport during recovery from metabolic acidosis. However, a recent study of the inner medullary collecting duct by Bengele et al. (1987) has noted that net acid excretion rates remained as high as control excretion rates during recovery, despite a systemic alkalosis. That study concluded that under these conditions systemic pH and bicarbonate concentration do not regulate bicarbonate transport.

Laski and Jackley (1989) have documented that the isolated perfused rat cortical collecting tubule can adapt to increase bicarbonate reabsorption in response to acidification of the basolateral environment in a very short time. Corresponding information on the 'off' phase during recovery is currently lacking. Xie et al. (1989) have recently presented some preliminary data on distal tubule bicarbonate transport during recovery from metabolic alkalosis. This type of study demonstrates the need for information during the transient stages of various acid-base disorders, information that may be quite distinct from that which could be obtained from study of the steady state. The present study set out to determine if distal tubule bicarbonate reabsorption continues during the recovery phase when systemic pH and bicarbonate are elevated (Parry and Brosnan, 1978).

Previous studies of acidification in ammonium chloride acidotic rats have demonstrated the presence of net bicarbonate reabsorption in the in vivo microperfused distal tubule (Lucci et al, 1982; Levine, 1985). With the foregoing in mind, the present study attempted to characterize aspects of the persistent bicarbonate reabsorption that occurs in this model.

1.7 Hypotheses

The specific hypotheses of the study include:

1. Tubular bicarbonate reabsorption is limited by peritubular bicarbonate diffusion in the absence of convective removal by water flow from the tubular lumen.

This hypothesis will be tested by limiting water reabsorption by the use of isoosmotic perfusates.

2. A component of bicarbonate reabsorption is due to direct or indirect Na/H exchange.

This hypothesis will be tested in two different ways: by using a zero sodium perfusate and by using amiloride.

3. Bicarbonate reabsorption in the distal tubule is dependent on the activity of the enzyme carbonic anhydrase.

This hypothesis will be tested by perfusing the tubule with acetazolamide and simulataneous systemic infusion of acetazolamide.

4. A component of distal tubule bicarbonate reabsorption is dependent on chloride conductance.

This hypothesis will be tested by perfusing tubules with and without SITS

5. Bicarbonate reabsorption will continue to occur in the surface distal tubule of rats recovering from ammonium chloride induced metabolic acidosis, despite an elevated systemic bicarbonate concentration.

This hypothesis will be tested by measuring distal tubule $JtCO_2$ 44 hours post gavage.

Chapter II

METHODS

2.1 Animal preparation

Male Sprague-Dawley rats weighing between 250-300 g were obtained from the National Research Council and housed in a climate controlled facility at the University of Ottawa. Rats ate Purina 5012 chow (in groups 1 - 4) and drank tap water ad libitum. Rats studied during recovery from metabolic acidois and in the control state ate Prolab RMH 4020 chow and drank tap water. (Protein content, fat content, and fiber content are essentially the same in both diets. Control rats eating both diets had the same blood electrolyte pattern and acutely acidotic rats on both diets had the same urine electrolyte pattern). The rats were made acidotic by gavage with 40 mmol/kg NH_4Cl approximately 16 hours before the experiment. Rats studied during recovery from acidosis also had access to chow and water.

2.1.1 Urine collection

Urine was collected by housing the animals in individual metabolic cages equipped with a funnel for the direction of urine into collection jars. Contamination by feces was minimized by the use of a wire grid in the funnel and contamination by food was also minimized by use of ground chow that the rat could only reach by climbing through a short tunnel. Contamination by drinking fluid was minimized by use of a small drip tray under the drinking tube. (For a detailed explanation see Dubrovsky et al., 1981).

2.1.2 Surgical preparation

Rats were anesthetized with 100 mg/kg Inactin, the sodium salt of ethyl-(1-methyl-propyl)-malonyl-thio urea (BYK, Gulden Konstanz West Germany), and prepared for micropuncture. The body temperature of the rat was also kept at approximately 38°C by means of a servo-regulated heated animal table and a rectal thermistor probe. A tracheostomy was performed and catheters were placed in the external jugular vein for infusion and in the carotid artery for blood pressure measurement. 3 catheters were placed in the jugular vein: one provided constant saline infusion by a Sage 352 syringe pump, one provided Somnotol (sodium pentobarbital) for the maintenance of anesthesia and euthanasia at the end of the experiment, and one allowed boluses of 10 % lissamine green dye to be injected systemically for the assessment of transit time in the kidney. Blood pressure was recorded from a Statham pressure transducer that was connected to a Litton Medical products recorder. A dorsal approach was used for a laparotomy that was made by making a subcostal incision about 4 cm long on the left flank of the rat's abdomen. The kidney was then freed of perirenal fat and placed in a kidney cup that was attached to the surgical board. The ureter was then catheterized and mineral oil allowed to drip on the kidney surface (Andreucci, 1978). Preparations were discarded if the blood pressure was less than 100 mm Hg.

Transit time was determined by injecting a small bolus of lissamine green dye in the jugular vein and noting the times of appearance of the dye in the proximal star and first distal tubules (Andreucci, 1978). Rat surgical preparations were discarded if the proximal tubule star formation (last appearance of dye in the superficial proximal

tubules, which converge in groups in the form of a star before descending below the renal surface to form the pars recta) was more than 12 seconds or the appearance of distal tubules was more than 45 seconds after the first appearance of dye in the kidney.

2.2 Infusion solutions

Normal animals were given a bolus of 0.5 % body weight of donor plasma and then infused with 0.9% NaCl at a rate of 1% body weight per hour.

Acidotic animals were infused with 0.9% NaCl at the rate of 3% body weight per hour. The acidosis was maintained by adding 50 mEq/L HCl to the infusates of all groups except Group 4.

Group 4 animals received a bolus of acetazolamide (20 mg/kg, in 0.5 mL of 0.9% saline and a sustaining infusion of 20 mg/kg per hour of acetazolamide (Diamox, sodium salt of acetazolamide, from Lederle Products Department, Cyanimid of Canada Ltd., Montreal)). Group 4 did not receive the additional HCl in the infusate because it was found that the combination of carbonic anhydrase inhibition and acid infusion resulted in precipitous declines in arterial pH (unpublished observations). One study that combined acetazolamide and metabolic acidosis was that of Giebisch et al. (1977). However, they reported micropuncture results from that group of rats who had systemic pH < 7.0, which was a protocol that we were anxious not to repeat. Also, the acetazolamide infusion resulted in similar plasma bicarbonate concentrations to those of the other 3 groups during the experiment. (Some readers may be aware that bicarbonate is infused during diamox administration in normal rats, as in Richardson and Kunau (1982). However, the renal response to acidosis is attenuated during metabolic acidosis. For a detailed review, see Maren (1974).).

Rats perfused with NEM or SITS also were infused with 3% BW/hr saline and HCl as in Group 1.

Rats studied during the recovery phase of acidosis were infused with 0.9% saline at the rate of 1% BW/hr.

2.3 Blood sampling

Arterial blood from the carotid artery was sampled at the end of the neck surgery and at the end of the experiment by clamping the carotid catheter and detaching the end of the catheter attached to the blood pressure recording apparatus. The catheter was kept filled with heparinized saline.

2.3.1 Plasma tCO_2 (total carbon dioxide) determination

500 microliters of blood was spun down by centrifuge for the determination of plasma total carbon dioxide concentration. This was measured by a Corning 965 carbon dioxide analyzer (Corning Medical and Scientific, Medfield MA). 50 microliters of plasma was pipetted into a reaction chamber where lactic acid released the plasma carbon dioxide. The amount of this carbon dioxide was detected by change in thermal conductivity in the detection chamber. A two point calibration of the machine was achieved by use of zero and a 15 mM sodium bicarbonate standard. Before each plasma tCO_2 determination the standard was checked. The reproducibility of the method is such that 10 replicate samples will equal $\pm$ 1 mmol/l. The relative accuracy has been assessed by comparison with the Van Slyke method, with no significant bias (965

Instruction Manual, 1979). Remaining plasma was stored at 4 degrees C for the measurement of sodium and potassium by flame photometry (IL 443, Instrumentation Laboratory, Lexington, MA.) and the measurement of chloride concentration by electrotitration (CMT10, Radiometer, Copenhagen). One capillary tube was spun by centrifuge for determination of hematocrit and blood protein concentration by refractometry. Two additional tubes of arterial blood were taken for duplicate determination of pH (PHM72, Radiometer, Copenhagen).

2.3.2 Determination of arterial pH

Two heparinized Clinitubes (Radiometer) were filled with carotid arterial blood for duplicate determination of arterial pH. Arterial pH was determined by aspirating these 85-100 microliter samples into a thermostatted capillary glass electrode, that was then immersed in a KCl salt bridge for assessment of potential difference with a Calomel electrode. Buffers of pH 6.841 and 7.383 were used to calibrate the electrode before use. The method can be reproduced with a standard deviation of about .003 pH units (Siggaard-Andersen, 1963).

2.3.3 Measurement of Na and K in plasma samples

Na and K in plasma samples were measured by flame photometry (IL443, Instrumentation Laboratory, Lexington, Maine). Standards and samples were diluted with a 15 mEq/l lithium solution. Plasma samples were determined in duplicate after first doing a 2 point calibration with a blank and a 140/5 mEq/l (Na/K) standard. Urine samples were determined in duplicate after 2X dilution and standardization with a 100 mEq/l (Na and K) standard.

2.3.4 Measurement of chloride in plasma samples

Chloride concentration in plasma samples was measured by electrotitration (CMT10, Radiometer, Copenhagen). A single point calibration was done with a 100 mEq/l NaCl solution. Duplicate 10 microliter samples were pipetted into the titration chamber.

2.3.5 Operational definition of metabolic acidosis

When studying acute metabolic acidosis, rat preparations were discarded if the arterial total carbon dioxide concentration was less than 10 or more than 20 mEq/l. These preparations occured with low frequency (about 5%), but such variability in the face of a constant acid load provoked some consideration. It is known that overnight fasting can cause up to a 5 mM decrease in tCO_2 (Levine et al. 1988) and most of the rats with tCO_2 less than 10 mEq/l appeared to have empty stomachs. High plasma tCO_2 concentrations might be due to acclerated time course of recovery or poor gavage.

2.4 Preparation and filling of the perfusion pipette

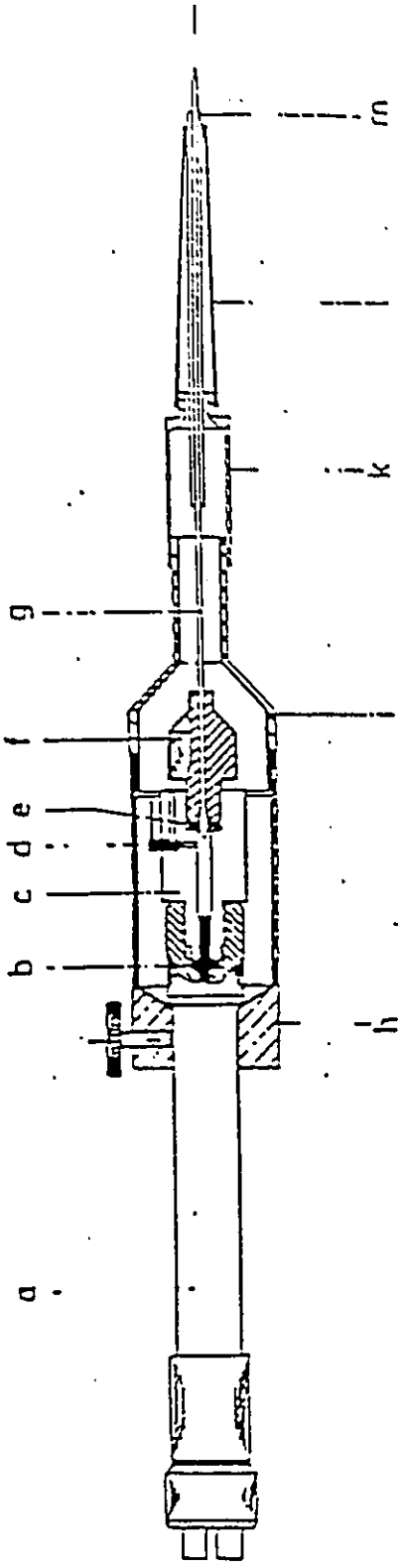
Perfusion pipettes were double pulled on a horizontal micropipette puller (Klaus Effenberger, model KE-HP) to generate a tip shape that minimizes flow resistance. The tip of these pipettes was then immersed in a platinum resin (Blythe Matthey, Brampton, Ontario) and the pipettes were baked in an oven at 360 degrees Centigrade for one hour. These pipettes were then ground on a motor driven grinder (Klaus Effenberger, model GRDR-1) to a tip outer diameter (OD) of 5-7 micrometers. Per-

fusion pipettes were then backfilled with HEPES buffered mineral oil and then mounted on a pipette holder, connected by Tygon tubing to a 60 mL syringe. Gassed perfusate (9% CO_2, 91% O_2) was filtered (0.45 micrometer Millipore) and a small bubble was injected into a plastic Petri dish under HEPES buffered oil. The tip of the perfusion pipette was immersed into the perfusion bubble and several hundred nanoliters of perfusion fluid was sucked into the pipette by pulling back on the syringe. The pipette was then partially backfilled with silicone oil and a sheath of larger diameter glass was slipped over the back of the pipette and pushed forward to protect the tip.

2.5 Mounting the Hampel nanoliter drive pump

See figure 2 for a diagram of the components of the nanoliter drive pump. The back of the perfusion pipette was put through a knurled screw and then through an O-ring. The pipette was then placed in the Lucite holder filled with silicone oil and the knurled screw was tightened. The cock was then tightened to seal the system, taking care to ensure that no air bubbles were enclosed. A heat shield was then placed over the pipette to prevent temperature induced fluctuations in tip resistance and hence flow rate. The mounted pipette was then placed in the Nanoliter drive pump on a Leitz micromanipulator and the tip was immersed in a Petri dish under distilled water. The pump was then turned on at high flow to warm up prior to calibration and use.

Figure 2: Hampel nanoliter drive pump. a, pump. b and e, O rings. c, pump chamber. d, cock. f, knurled screw. g, pipette. m, protective cover for pippette tip. l, plastic cover. h, i, k, l, thermo-protection.

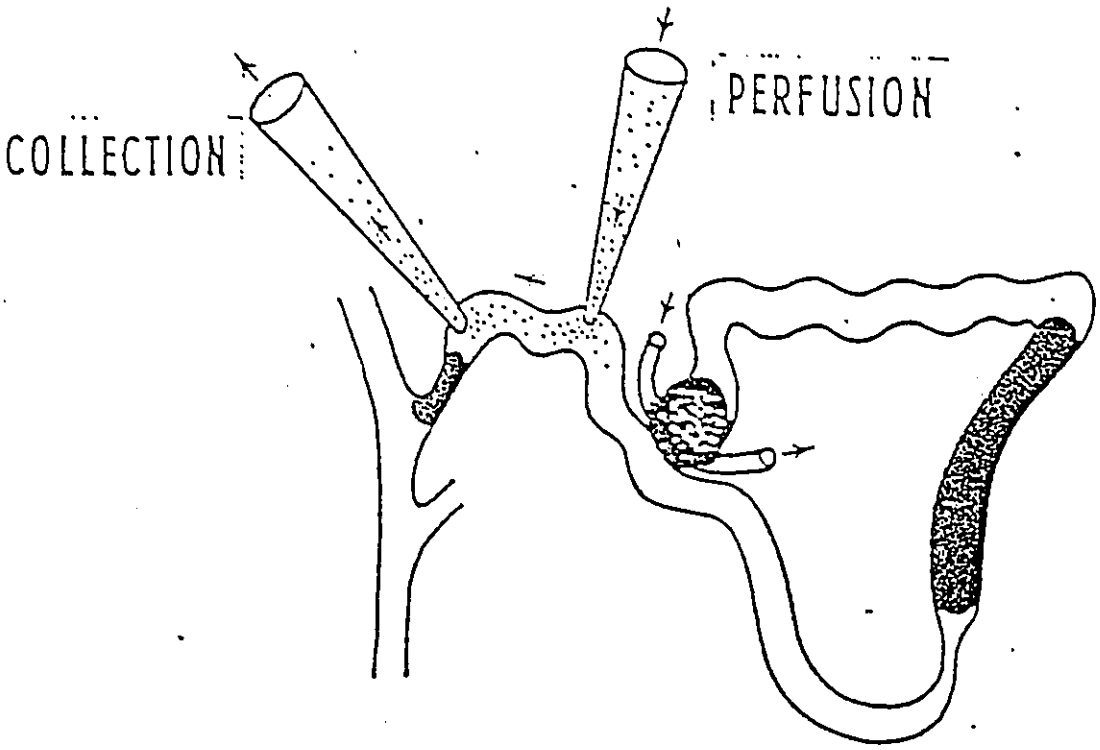

m
k
g
f
e
d
c
b
h
a

2.6 Micropuncture

Nephrons with double surface loops in the distal tubule were located by random puncture of proximal tubules and injection of a 1% Lissamine green dye solution from a micropipette that had a tip approximately 3 micrometers OD. The first convolution of the distal tubule was then punctured with the perfusion pipette connected to the Hampel nanoliter drive pump. The proximal tubule was then filled with castor oil from a pipette with a tip diameter of 9-10 micrometers OD to block the flow of endogenous filtrate. Distal tubule fluid was then collected from the second loop with sampling pipettes having tip diameters of 5-7 micrometers OD that were backfilled with HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) equilibrated mineral oil, stained by Sudan black. All collections were timed. Spontaneous collections were made, as discussed by Schnermann et al. (1969).

Paired collections were made in the SITS group by perfusing the same nephron with 2 different perfusates. Two Hampel pumps were used, and the first distal loop was punctured in sequence. Paired perfusions were carried out at high flow (25 nl/ min) to minimize artefacts created by puncturing the same tubule twice. The perfusion with SITS was always done last because of the possibility of lingering effects during the 'no SITS' perfusion.

Figure 3: Schematic diagram of distal tubule microperfusion. The proximal tubule is blocked with castor oil. A perfusion pipette is inserted in the first loop of a double looped distal tubule. Collection is made from the second loop. A distal oil block ensures a quantitative collection.


COLLECTION
PERFUSION

2.7 Perfusate compositions

2.7.1 Gassing and use of HEPES buffered mineral oil

Dubose et al., (1978) measured the distal tubule pCO_2 and found that it was approximately 60-67 mm Hg. Subsequent studies of tubular bicarbonate transport employing artificial solutions have attempted to perfuse with solutions having a similar pCO_2 and have attempted to maintain this pCO_2 in collected samples of tubular fluid (Cogan et al., 1979). This is important as the dissolved CO_2 must have a significant concentration that could generate significant artifactual errors in bicarbonate transport if allowed to dissipate (theoretically, dissolved carbon dioxide could make up 1.8 mmol/l i.e. 60 mm Hg X .0301). Accordingly, all solutions in this study were gassed for 15 minutes with gas from a tank containing 9% carbon dioxide and 91% oxygen that was first passed through a water chamber.

In order to maintain the pCO_2 of collected samples, tubular fluid was collected in pipettes containing HEPES buffered oil. HEPES buffered oil was mineral oil that was equilibrated with a solution of 48 mM $NaHCO_3$ and 100 mM HEPES, pH 7.6 (Cogan et al., 1979).

2.7.2 Perfusates used in normal rats

Distal tubules from normal rats were perfused with a solution which contained in mM : Na 60, Cl 50, HCO_3 12, K 3, H_2PO_4 1, HPO_4 1, and urea 173.

2.7.3 Perfusates used in rats with acute acidoisis

Group 1 perfusate contained 60 mM Na, which was substituted with choline in group 2. In Group 3, 3 X 10^{-4} M amiloride hydrochloride (Sigma Chemical Company, St.Louis) was added to the choline containing perfusate. Group 4 perfusate contained 10^{-3} M acetazolamide. All perfusates contained in mM: HCO_3, 25, Cl 35, H_2PO_4 1, HPO_4 1, K 3, and urea 173. In addition, all perfusates were gassed with 9% CO_2, 91% oxygen, and contained .05% FD&C dye. Distal tubules in groups 1 to 4 were studied at 8 nL/min., while tubules in groups 1 and 2 were also perfused at 24 nL/min.

In another group tubules were perfused in paired fashion with and without

5 X 10^{-4} M SITS.

Tubules were also treated with NEM in the following concentrations: 1, 5, and 10 mM.

2.7.4 Perfusates used in rats with rebound metabolic alkalosis

Rats studied with rebound metabolic alkalosis were studied with the following perfusate: Na 60, Cl 35, HCO_3 25, H_2PO_4 1, HPO_4 1, K 3, and urea 173.

2.8 Sample handling

Samples were injected into a quartz capillary tube for volume determination and then divided into aliquots. ^{3}H-inulin concentration was determined by liquid scintilla-

tion, carbon dioxide content by microcalorimetry, and sodium concentration by atomic absorption spectroscopy.

95% of the animal preparations and micropuncture was carried out by the author of this thesis, but initially all of the analyses were carried out by Ms. Lori Nash. In particular, Ms. Nash is responsible for all of the atomic absorption spectroscopy analyses. Approximately 50% of the total carbon dioxide analyses can be attributed to the author, who only gradually acquired the analytical skills while initially concentrating on obtaining the often elusive successful collections of distal tubular fluid. I am also indebted to Michelle Iacovitti who was responsible for teaching me the surgical and micropuncture techniques and also conducted some of the analyses.

2.9 Volume determination

The measurement of the volume of the collected tubular fluid was made by transferring the collecting micropipette to a constant bore capillary tube. Volume was determined by noting the linear displacement by the sample in the HEPES equilibrated mineral oil, as measured by a calibrated eyepiece. A Leitz stereo-microscope was used with 16X oculars and 4X objective. The graticule on the eye-piece was labeled 0 to 100 and at that magnification 40 spaces of the graticule equaled 1 mm of displacement in the focal plane. Pyrex tubing with an outer diameter of .60 mm and inner diameter of .20 mm was used, yielding a slope of .908 nl/unit. Glass was obtained from Drummond Scientific Company, Broomall, Penn. Care was taken to ensure that no air contacted the sample.

2.10 Constriction pipettes

For the measurement of volume, total CO_2, and sodium, aliquots of tubular fluid were transferred by using constriction pipettes. The technique of making constriction pipettes is described in detail by Andreucci (1978). Briefly, capillary glass was hand-pulled over an open flame, and a hook was fashioned. This was then transferred to a vertical microforge, where a constriction of the desired dimensions was formed with the aid of a calibrated eye- piece in a Leitz stereomicroscope. Typical volumes of constriction pipettes were 10-20 nl for tCO_2 determination, 5-10 nl for volume determination, and < 1 nl for sodium concentration determination.

2.11 Liquid scintillation counting

Liquid scintillation counting was done by a Beckman LS 3801, a benchtop, microprocessor-controlled spectrometer for radionuclide measuring. Calibration was done routinely to assure that the window setting used really covered the energy spectrum for the isotope. The system used a self-calibration feature, based on using an unquenched calibration standard of tritium provided by Beckman. Distentegrations per minute were recorded and used in the calculation of experimental results to avoid errors caused by the assumption that counting efficiency did not vary. Approximately 5-10 nL of the collected sample was pipetted into a glass vial containing 3.5 mL of distilled water. 11 mL of an aqueous cocktail were then added to the vial, which was then agitated until the mixture gelled (Formula A963, New England Nuclear, Boston Massachusetts). Samples were counted for 15 minutes.

2.12 Tritiated inulin

Inulin used in this study was tritiated methoxy inulin, obtained from New England Nuclear Research Products via Dupont Canada Inc. Approximately 2 ml of nanopure water was added to 5 mCi of inulin and this solution was exhaustively dialyzed for approximately 48 hours at 4 degrees centigrade against nanopure water (18 megohm-cm). Bath changes were made at 2 hour intervals during the first day (3-4 changes) and then at longer intervals during the second day (2 changes at approximately 4 hour intervals). 50 microliter aliquots were pipetted into glass scintillation vials that were then put in a dessicator, subjected to vacuum, and put in a freezer for 24 hours. These scintillation vials were then removed from the freezer, capped and stored at 4 degrees Centigrade until the experiment. When approximately 1.5 ml of perfusate was added to the contents of these vials the final inulin concentration resulted in counts of approximately 150 disintegrations/minute per nanoliter.

If a 6.66 nanoliter constriction pipette was used for tritiated inulin measurement, then approximately 1000 dpm would be recorded for the 6.66 nanoliters of perfusate counted by the liquid scintillation detector. Counting efficiency was approximately 35%, so counts per minute would be approximately 350 for this sample. With a counting time of 15 minutes, the total number of counts in this example would be more than sufficient to ensure that the actual DPM results obtained in 95 out of 100 times were within 5% of the mean. For every experiment, replicate counts of perfusate inulin were determined with a coefficient of variation < 2 %.

2.13 Measurement of total carbon dioxide in nanoliter samples

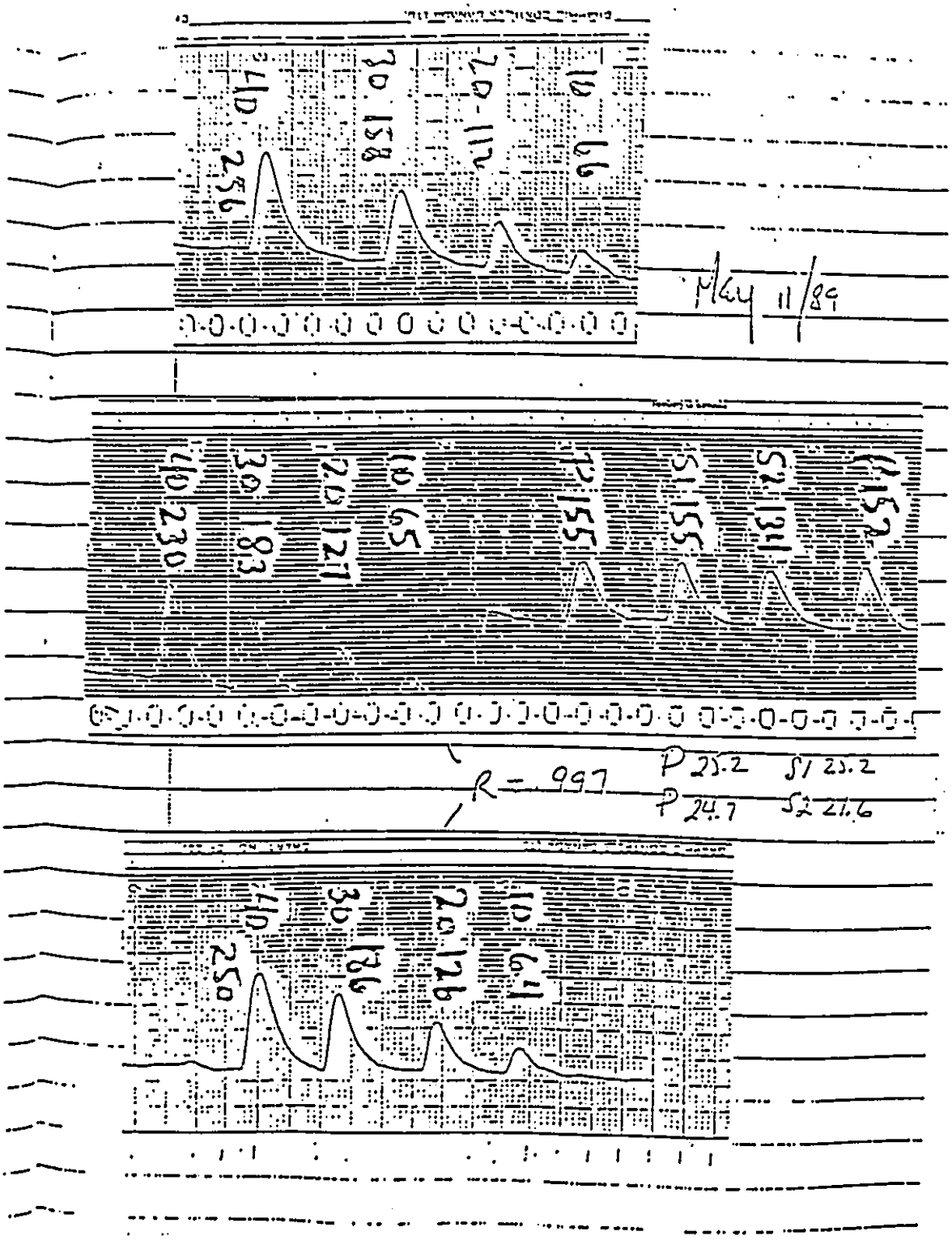
Total CO_2 was measured by microcalorimetry, using a picapnotherm. The method, invented by Vurek et al. (1975), is based on the principal that carbon dioxide gas will react with lithium, causing the liberation of heat. This heat change can be used to cause a change in electrical resistance of a thermistor, allowing a change in current flow. The resulting signal is then integrated. The special adaptations of the method, necessitated by the use of small volumes, include the use of a mercury bulb to allow pipetting into a small sulphuric acid chamber and the use of a microscope to allow accurate pipetting. The sample is dispensed onto the surface of the sulphuric acid and the evolved gas is carried to the thermistor chamber by a freon gas flow.

For each total CO_2 analysis, standards of 10, 20, 30, and 40 mM $NaHCO_3$, and the perfusate were run.

The average regression line for the standard curves was $0.99 \pm .001$ and the coefficient of variation for replicate determination of the perfusate was $2.8 \pm .3$ %. This corresponds to an average standard deviation of 0.7 mM when a 25 mM standard is replicated, which corresponds well with other reports of picapnotherm precision of measurement in the literature (e.g., Breyer et al. (1986) report a standard deviation of 0.8 mM.).

Picapnotherm methodology undergoes continuous scrutiny in our lab and minor modifications of assay have been suggested. The illustration of the standard curve points out the recommended protocol: replicate determinations of standards in water equilibrated mineral oil, followed by the sample run (with perfusate determinations) in HEPES buffered oil, and repeat determinations of standards in water equilibrated mineral oil.

Figure 4: A typical tCO_2 determination using the picapnotherm


40 256
30 158
20 112
10 66
May 11/89
40 230
30 183
20 127
10 65
72 155
51 155
52 131
11 52
R = .997
P 25.2 S1 25.2
P 24.7 S2 27.6
40 250
30 136
20 126
10 61

2.14 Special concerns of picapnotherm methodology

In the course of gaining experience handling the picapnotherm, the operator becomes aware of some methodologic concerns that are rarely mentioned tin the methods sections of papers. The mercury ball hanging over the pool of sulfuric acid suggests that the operator is indeed, on the brink of disaster, but the following section will deal with more subtle details that are no less important in attaining valid results.

2.14.1 Sensitivity of the crystal

A correlation has been noted between the size of the lithium crystal used and the response to carbon dioxide. Less obvious characteristics of the crystal that are reputed to be important are: shape and degree of water saturation. Some labs even go to the extreme effort of carving their own lithium crystals to attain the desired size and shape (Schuster, 1985). Hydration is hopefully standardized by storing the crystals over drierite. It is interesting that length of crystal use is variously reported as 'weeks', 'days', to '30 pipettings'. Stabilization time of 1 hour may be required if the crystal is changed.

2.14.2 Hydration of the acid

Concerns have been raised about the hydration of the acid used to release the bicarbonate contained in samples pipetted into the chamber. Overhydration results in a staircase phenomenon, whereby the baseline moves up with every pipetting. Our lab policy has been to use fresh acid every day, although the WPI manual suggests that 1/4 chamber capacity be left full of acid to maintain the hydration state of the crystal. The WPI manual suggests that the acid can be changed, (Picapnotherm Model GV-1, Instruction Manual, World Precision Instruments, p.19) but the standard curve should then be checked to ensure that the sensitivity did not change.

2.14.3 Concerning dissolved CO_2

Laboratories measuring CO_2 transport by micropuncture techniques report various degrees of concern about dissolved CO_2. Gassing of perfusates has been done with 9% CO_2, 5% CO_2, or no gassing has been used (Levine, 1985; Capasso et al., 1986). The rationale behind gassing the perfusate is to match the pCO_2 of the renal cortex, determined by Dubose and co-workers to be approximately 60-67 mm Hg (Dubose et al., 1978). In our lab, efforts are made to ensure that the perfusate CO_2 is gassed and kept in an environment of the same pCO_2. Similarly, collected samples are collected under HEPES buffered oil with $NaHCO_3$ that has a pCO_2 of approximately 60 mm Hg. This oil is also used in the volume capillary, where the sample is injected for volume determination and divided into aliquots for the various subsequent analyses.

The sample is placed in a tray flooded with HEPES buffered oil for picapnotherm measurement. A separate tray is used for the 10 mM, 20 mM, 30 mM, and 40 mM

$NaHCO_3$ standards that are dispensed under water equilibrated mineral oil. The rationale is that standards are not to be contaminated with dissolved CO_2, while every effort is made to ensure that samples are placed in an environment that has a similar pCO_2 as the kidney cortex.

2.14.4 Maintenance of adequate carrier gas flow

It is obviously important to ensure that an adequate flow of carrier gas is maintained. The freon gas carries released CO_2 to the thermistor bead, a process that should be complete by the end of the integration time. Two good reasons why one should distrust long low peaks: 1) entire signal not integrated 2) susceptibility to baseline correction errors. For these reasons extra effort is made in our lab to maintain a patent air line from release chamber to thermistor chamber. Periodically, the line is flushed with acetone, removing oil, acid, etc. that might retard flow.

2.15 Calculation of perfusion rate and $JtCO_2$

The perfused rate ($\dot{V}_o$) was calculated as the product of the collected rate ($\dot{V}_\bullet$) and the the TF/P_{IN} (ratio of inulin in collected fluid and perfusate). The collected rate was determined from the volume of the sample displaced in the capillary tube (see above). The TF/P_{IN} ratio was determined from the liquid scintillation counts of aliquots of the sample and the perfusate, respectively. Collections were discarded if the collected rate was greater or less than 15% of the target perfusion rate, or if the TF/P_{IN} was found to be less than 0.95.

$JtCO_2$, net bicarbonate flux, was calculated as: $(\mathring{V} \times tCO_2\ p) - (\mathring{V} \times tCO_2\ c)$ corrected for length, where tCO_2 was the measured CO_2 concentration in the perfusate and the collected fluid, respectively.

No more than a 2 mM difference between the tCO_2 concentration of the perfusate determined by the Corning analyzer and the picapnotherm was accepted.

2.16 Determination of sodium concentration in nanoliter samples

Sodium concentrations were assessed in some samples by the technique of furnace atomic absorption spectrometry (Instrumentation Laboratories 951 atomic absorption spectrophotometer and model 655 controlled temperature furnace atomizer). The procedure is well described by Nash et al. (1988). Briefly, the sodium atomization signal was integrated for 4 seconds and the peak area absorbance was related to concentration (lamp current was 8 mA, wavelength was 589 nm, slit width was 320 micrometers, bandpass was 1 nm, purge gas was argon at 5 liters per minute.)

A standard curve was generated before each determination of sodium concentration in tubular samples. Standard concentrations of sodium used were 0, 5, 10, 20, and 40 mM. Repeat determinations of standards yielded relative standard deviations of less than 5% (Nash et al., 1988).

The quartz trough used for sample pipetting onto the microboat was meticulously cleaned with Ultrex nitric acid, Ultrex hydrochloric acid, and deionized distilled water, siliconized, and filled with mineral oil. In order to prevent anion interference of sodi-

um concentration determination of sample, the microboat was also pretreated with boric acid. Approximately 5 nL and approximately 50 nL volumes of the standards were transferred to the quartz trough. The quartz trough was viewed through a stereomicroscope and 0.1 nanoliter volumes of standards or samples were pipetted onto a microboat. The microboat was then immediately transferred to the furnace to avoid contamination.

2.17 Tubule dissection

Following perfusion of the distal tubule, the tubule was filled with Microfil (Canton Bio-Medical, Boulder, Colorado). The kidney was removed and stored refrigerated in saline. Prior to dissection the kidney was placed in 25% NaOH for 10 minutes. The tubule was then dissected from the kidney by hand using a stereomicroscope at 50X magnification. Insect mounting needles mounted on macro glass pipettes were used for this task. The dissected tubule was then placed on a plastic coverslip and length was measured using a calibrated eyepiece (Kunau et al. 1983).

2.18 Statistics and curve fitting

More than two groups were compared by analysis of variance with appropriate post-testing (BMDP7d). Unpaired means from 2 different groups were compared by using an unpaired t-test (BMDP3d) and paired comparisons were made by using a paired t-test (different version of BMDP3d). Water reabsorption was regressed on bicarbonate reabsorption using a linear regression procedure (BMDP1r). Statistical

significance was set at $p<.05$. Statistics were performed by BMDP Statistical Software (Los Angeles: University of California Press, 1981).

The curve of blood bicarbonate concentration versus time was constructed using a nonlinear regression program (BMDP 3R). From gross observation of the blood concentration with time it was hypothesized that the system appeared to respond like an underdamped second order system (Glantz, 1979). The solution of a second order system with damping constant less than 1 may be characterized as an exponentially decaying cosine wave, with time course determined by the damping constant and phase lag. Accordingly, the following function was used in the nonlinear BMDP3r program:

$$x(t) = x_{ss} \left[1 - \frac{e^{-t/T}}{(1-L^2)^{1/2}} (\cos wt - O) \right]$$

where x(t) is the blood plasma bicarbonate concentration at any time, x_{SS} is the value of blood plasma bicarbonate concentration at time 0, T is the time constant, w is the angular frequency, O is the phase lag, and L is the damping ratio.

This function was inserted into the nonlinear regression program, initial parameters estimated, and successive iterations performed by the computer resulted in a curve of best fit.

Chapter III

RESULTS

3.1 Blood electrolytes

Table 1 shows the results of the arterial blood sample taken at the end of neck surgery in control rats, acidotic rats in groups 1 to 4, and in rats with rebound metabolic alkalosis (RMA). The group means in table 1 were subjected to a one way analysis of variance, and this resulted in significant F values for arterial pH, arterial HCO_3 concentration and arterial Cl concentration. The results of post-testing (t-test with Bonferroni probability) are discussed below.

3.1.1 Acute metabolic acidosis

Rats in this group were studied approximately 16 hours after gavage with ammonium chloride. Post-testing identified control pH and HCO_3 as being significantly higher, and control Cl as being significantly lower than in the acidotic groups. The NH_4Cl gavage produced a state of metabolic acidosis in all rats studied: arterial blood pH, 7.15 ± .01, HCO_3, 14.8 ± .5 meq/L. Blood electrolyte concentrations are similar to those in a previous study by Levine (1985). Blood pCO_2 and plasma K concentration, which were in the normal range, did not differ significantly among groups 1-4. Plasma bicarbonate concentration was also not different but did fall 2-3 mM in the course of an experiment. However, this decrement was not statistically significant among the

four acidotic groups. Chloride concentrations are also increased over that seen in normal animals (Levine, 1985).

Between the time of gavage and the time of surgical preparation, all acid treated animals in groups 1 to 4 lost approximately 7 gm weight with no differences noted between groups (the target weight of rats used in this study was 300 g, but in practise, the range of weights was between 250 and 320 g). (A subset of animals is shown in table 2 with weight loss of 13 ± 2 g) This is in contrast to the gain in weight that control animals exhibited. Urine pH was 5.43 ± .02 (n=29) for the 16 hour collection, again with no differences between groups.

3.1.2 Rebound metabolic alkalosis

Rats in this group were studied approximately 44 hours post gavage and exhibited a rebound metabolic alkalosis. The same phenomena has been observed in rats by Parry and Brosnan (1978) and by Guern et al. (1982). It is interesting to note that both these studies employed an ammonium chloride drinking protocol to make the animals acidotic. Guern et al. (1982) reported a plasma bicarbonate concentration of 39.0 ± 1.14 on the first day of recovery from a 4 day protocol of NH_4Cl drinking and a remarkable pH of 7.59 ± .01. The present study reports a more modest pH change partly due to higher pCO_2 in the present study. It should be noted that some hypercapnia is to be expected as a respiratory response to metabolic alkalosis. According to Cogan and Rector (1986) the arterial blood pH and bicarbonate concentrations in the alkalotic group would be associated with a pCO_2 of approximately 50 mm Hg, which agrees well with the observed value.

3.2 Balance and urine data

Table 2 shows balance and urine data for 11 normal rats collected overnight (approximately 16 hours) and for 12 rats that were followed for 2 consecutive overnight urine collections following NH_4Cl gavage.

Normal rats gained weight, and ate and drank similar quantities to other normal groups followed (unpublished observations). It is evident that ammonium chloride gavage caused a loss in body weight, decrease in urine pH and bicarbonate excretion and an increase in both sodium and chloride excretion.

RMA rats recover the lost body weight, apparently by drinking more and excreting less sodium in the urine. Urine pH is still dramatically less than in normal rats.

3.3 Micropuncture data in rats with normal acid-base

Surface distal tubules from rats with normal acid-base status were perfused at low and high flow to provide a control for the acidotic groups (see Table 3). At 8 nl/min bicarbonate flux was not different from zero, in agreement with previous studies from this lab (Levine, 1985; Iacovitti et al., 1986). At 25 nl/min, net bicarbonate secretion occured, as originally noted by Iacovitti et al. (1986).

3.4 Micropuncture results at low flow in groups 1 to 4

Results of micropuncture at low flow (8 nL/min) are summarized in table 4. It is evident that bicarbonate reabsorption is significantly different from zero at low flow in groups 1 to 4, in marked contrast to the control group. (65 ± 4 vs -3 ± 4, in acidotic and control rats, $p < .05$, by unpaired t-test).

Group means of the acidotic rats were compared by a one way analysis of variance and showed that there was a significant difference among groups. This was followed by t-testing corrected for multiple comparisons by Bonferroni probabilities. Bicarbonate reabsorption in Group 4 (Diamox) was significantly less than that in Group 1 (High Na) and Group 2 (Low Na) ($p<.05$). The effect of using low sodium concentration in the perfusate and the effect of amiloride in conjunction with low sodium perfusate concentration were not significant. Thus, reducing the concentration of sodium in the perfusate from 60 to 0 mM had a neglible effect on bicarbonate reabsorption during acute metabolic acidosis.

Water movements in groups 1-4 were not significantly different when tested by ANOVA. However, J_V was significantly different from zero in groups 1 and 4. ($p<.05$, by t-test). This point deserves furthur consideration. The average perfusion rate (groups 1-4) was $7.93 \pm .16$ nl/min, the average collected rate was $7.54 \pm .18$ nl/min, and the average collected TF/P_{IN} was $1.06 \pm .02$. As the first number is calculated from the latter two, these results are not too surprising. On first look, one sees a very good calculated perfused rate, that is some 6% higher than the collected rate. One interpretation of the data is that the collections were 'immaculate' and the attempt to stop water movement failed by 6%. It is true that many collections were discarded

if they did not meet the criteria (see Methods), but the average data appear better than they really are. Within this accepted range, collections might be included that are: 1. slightly low due to incomplete collection (i.e. part of the collection leaked out of the tubule at either hole, or may have been lost proximally or distally). 2. slightly high due to contamination with surface fluid. In conclusion, the small water movements reported are negligible in comparison to the nanoliter/min over or undercollection that was tolerated.

Acetazolamide in the perfusate and blood caused a significant inhibition of bicarbonate reabsorption at low flow in distal tubules of rats with acute metabolic acidosis.

3.5 Results of micropuncture at high flow in groups 1 and 2

Results of micropuncture at high flow (25 nl/min) are summarized in table 5. Bicarbonate reabsorption is not significantly different between Group 1 (High Na) and Group 2 (Low Na) (105 $\pm$ 19 vs 91 $\pm$ 20 pmol/min/mm, respectively), as assessed by unpaired t-test.

3.6 Results of Na flux measurements

Results of Na flux measurements are shown in table 6. No significant differences occur among groups (ANOVA). ANOVA indicated that a significant difference existed among group means of the collected concentration of sodium, but individual comparisons were not significant. Evidently, the range of sodium entry was somewhat variable. Net secretion of Na is evident in all groups that were perfused with low sodium

concentration solutions, in marked contrast to the net reabsorption that is normally seen here. It can be seen that amiloride tended to increase the amount of sodium secretion, and that Na concentration seemed to be decreased at high flow (see table 6).

3.7 Effect of SITS

Results of $JtCO_2$ measurements during luminal perfusion with and without SITS in the same tubule (paired samples) are shown in table 7A. There was a significant reduction in bicarbonate reabsorption when SITS was included in the perfusate, as determined by paired t-test ($p < .05$). No time control was included, but the direction of change was opposite to any change that may be due solely to time (Atkins and Burg (1985) have reported that bicarbonate secretion in isolated perfused rat collecting ducts changed to net bicarbonate reabsorption with time). Time of perfusion with either solution ranged from 3-10 min, but time of collections was always 2-3 min in duration.

3.8 Results of NEM treatment

NEM treatment in preliminary experiments are shown in table 7B. This series of experiments cannot yield conclusive results due to the small numbers. The reason that it was not furthur pursued was due to the unphysiological concentrations of NEM needed to inhibit bicarbonate reabsorption. The very unspecific sulfhydryl reaction probably did not have enough time to act during microperfusion experiments. (Collections are made after less than five minutes of perfusion.)

3.9 Micropuncture data in rats with rebound metabolic alkalosis

This study presents the first results of in vivo micropuncture of the distal tubule during rebound metabolic alkalosis. Micropuncture results show that distal nephrons from these animals reabsorb bicarbonate at 8, 15, and 25 nL/min (See table 8). $JtCO_2$ was 18±2, 27±10, and 48±17, pmol/min.mm, respectively. Bicarbonate reabsorption was significantly different from zero and also showed a load dependence, as shown by previous studies (Capasso et al. 1987).

3.10 Modeling of time course of plasma HCO_3

Figure 5 shows the time course of plasma bicarbonate concentration after gavage. The time course may be approximated as a transient response to a second order system responding to a step change in acid-base status (see Glantz, 1979). When the system is underdamped, the solution of the second order equation is a decaying cosine wave. This form of equation was inserted into a nonlinear regression program that performed successive iterations resulting in a curve of best fit. The system is best characterized by a damping constant that was estimated to be approximately 0.8.

Table 1: Blood electrolytes from normal, acidotic, and RMA rats.

These data are from arterial blood sampled from control, acutely acidotic animals (16 hours post gavage) and rats with rebound metabolic alkalosis (44 hours post gavage).

Table 1

Acid-Base and Electrolyte Values

Group	pH	HCO_3	pCO_2	Na	K	Cl	Hct	Pro
		mM	mm Hg	mM	mM	mM	%	g/dL
			Control Group					
control (n=5)	7.41±.01	29.6±.8	48±2	143±1	4.6±.04	102±1	46.8±.4	5.4±.2
			Acute Metabolic Acidosis					
1 Na (n=7)	7.15±02	14.7±.7	43±2	145±1	4.3±.1	118±1	46.6±.5	5.8±.2
2 choline (n=7)	7.14±.02	13.8±.1	42±1	145±1	4.3±.1	120±2	46.6±.5	5.5±.2
3 choline+ amiloride (n=7)	7.13±.02	14.0±.9	43±2	143±1	4.2±.1	119±2	46.1±.5	5.7±.2
4 Diamox (n=6)	7.18±.02	16.7±.8	46±2	145±1	4.1±.3	118±1	47.2±.6	6.3±.1
			Rebound Metabolic Alkalosis					
RMA (n=12)	7.45±.01	35.8±.7	49±1	142±1	4.5±.1	94±1	47.3±.4	5.4±.04

Values are means ± SE, n, number of rats, Hct=hematocrit.
In groups 2,3 choline and choline and amiloride were included in the luminal perfusate, only.
In group 4 diamox was infused systemically and also included in the luminal perfusate. See methods for details.

Table 2: Balance and urine data.

These whole animal data are from rats in control, acute metabolic acidosis, and rebound metabolic alkalosis.

Table 2

Balance and Urine Data

Measurements	Normal rats (n=11)	Acidotic rats (n=12)	RMA rats (n=12)
change in body weight(g)	+10±1	-13±2*	+4±1**
amount drank (ml)	34±3	29±4	49±4**
amount eaten (g)	26±1	16±1*	24±1**
urine			
volume (ml/16 h)	8±1	26±2*	13±2**
pH	6.60±.04	5.46±.02*	5.88±.01**
HCO_3 (uEq/16 h)	80±18	3±1*	75±36
Na (uEq/16 h)	1629±168	2384±150*	519±181**
K (uEq/16 h)	3044±181	3452±231	2488±472
Cl (uEq/16 h)	2256±205	9244±586*	3956±402**

*, significantly different from normal by unpaired t-test.
**, significantly different from acidotic by paired t-test.

Table 3: $JtCO_2$ in normal rats

These data are from tubules perfused at 8 and 25 nl/min. in control rats.

Table 3

Microperfusion Data in Rats with Normal Acid-Base

Group	Length	$\dot{V}_o$	J_v	$[tCO_2]_p$	$[tCO_2]_c$	$JtCO_2$
	mm	nL/min	nL/min	mM	mM	$pmol.min^{-1}.mm^{-1}$
Control (6/5)*	1.4±.1	7.9±.3	.75±.32	11.9±.7	13.8±.8	-3±4
Control (6/5)	1.4±.1	23.2±.5	.55±.33	11.9±.7	13.7±.2	-35±9

Values are means ± SE; $\dot{V}_o$, calculated perfused rate. J_v, water reabsorption. tCO_2, total carbon dioxide concentration measured in perfusate and collected samples, respectively.
$JtCO_2$, bicarbonate secretion.
* numbers in parentheses denote number of tubules/number of rats.

Table 4: $JtCO_2$:acute metabolic acidosis

Data from groups 1 - 4, from tubules perfused at 8 nl/min. Group 4 $JtCO_2$ was significantly less than groups 1 and 2 ($p<.05$).

Table 4

Microperfusion Data (8 nL/min) acute metabolic acidosis

Group	Length mm	$\overset{o}{V}$ nL/min	J_v nL/min	$[tCO_2]_p$ mM	$[tCO_2]_c$ mM	$JtCO_2$ pmol.mm^{-1}.min^{-1}
1 Na=60 (7/7)*	1.3±.1	7.7±.3	.68±.1	26.5±.8	16.6±1.3	65±4
2 choline=60 (7/7)	1.4±.1	8.3±.3	.44±.12	27.3±.8	18.1±.8	59±5
3 choline=60 +amiloride (7/7)	1.3±.1	8.0±.3	.26±.07	26.5±.8	16.9±.8	58±6
4 Na=60 + Diamox (9/6)	1.5±.1	7.8±.4	.74±.21	26.6±1	20.3±.8	40±4**

Values are means ± SE; $\overset{o}{V}$, calculated perfused rate, J_v, water reabsorption, (tCO_2), total carbon dioxide measured in perfusate and collected samples. $JtCO_2$, bicarbonate reabsorption.
* numbers in parentheses denote number of tubules/number of rats.

** Significantly different from Group 1 at $p<.01$ and from group 2 at $p<.05$, by t-test, using the Bonferroni correction for multiple comparisons.

Table 5: $JtCO_2$:acute metabolic acidosis

Data from groups 1 and 2, from tubules perfused at 24 nl/min.

Table 5

Acute Metabolic Acidosis

Micropuncture Data at 24 nl/min

Group	Length mm	$\overset{o}{V}$ nL/min	J_v nL/min	$[tCO_2]_p$ mM	$[tCO_2]_c$ mM	$JtCO_2$ pmol.mm^{-1}.min^{-1}
1 Na=60 (8/6)	1.3±.1	23.3±1.0	.55±.18	28.0±0.8	21.0±1.0	105±19
2 choline=60 (9/6)	1.3±.1	24.1±1.0	.72±.28	29.5±0.4	24.9±0.6	91±20

Values are means ± Standard errors. $\overset{o}{V}$, calculated perfused rate, J_v, water reabsorption, tCO_2, total carbon dioxide in perfusate and collected samples. $JtCO_2$, bicarbonate reabsorption.

* Numbers in parentheses denote number of tubules/number of rats.

Table 6: Na flux measurements.

Tubular fluid Na concentration and Na flux measured during microperfusion at 8 or 24 nl/min using a low Na perfusate in the presence and absence of amiloride.

Table 6

Na Data in microperfusion studies

Group	$[Na]_c$ mM	J_{Na} pmol.min^{-1}.mm^{-1}
2 Low Na (8 nL/min) (9/6)	21±3	-99±16
3 Low Na + amiloride) (8 nL/min) (7/7)	31±3	-199±23
2 Low Na (24 nl/min) (7/7)	15±2	-218±34

Values are means ± SE.
$[Na]_c$, sodium concentration in collected samples, J_{Na}, flux of sodium.
*numbers in parentheses denote number of tubules/number of rats.

Table 7: $JtCO_2$:effect of SITS and NEM.

Data collected from rats with acute metabolic acidosis.

Table 7A

Paired Data from 6 tubules perfused with and without SITS

Group	Length	$\overset{\circ}{V}$	J_v	$[tCO_2]_p$	$[tCO_2]_c$	$JtCO_2$
	mm	nL/min	nL/min	mM	mM	$pmol.min^{-1}.mm^{-1}$
No SITS	1.38±.1	23.5±0.8	.97±.39	25.2±0.8	20.1±0.8	125±17
SITS	1.38±.1	24.8±1.3	.52±.47	25.2±0.8	21.4±0.8	93±16*

* significantly different by paired t-test, $p<.05$.

Table 7B

Preliminary Data of tubules perfused with NEM

Group	Length	$\overset{\circ}{V}$	J_v	$[tCO_2]_p$	$[tCO_2]_c$	$JtCO_2$
	mm	nL/min	nL/min	mM	mM	$pmol.min^{-1}.mm^{-1}$
1 mM NEM (n=3)	1.34±.1	6.8±.2	0.20±.06	28.4±0.9	16.4±3.2	57±13
5 mM NEM (n=2)	1.46	8.1	0.55	26.0	16.7	56
10 mM NEM (n=1)	1.00	8.7	0.0	23.2	19.9	29

n, number of tubules. NEM, N-ethylmaleimide. SITS, 4-acetamido-4-isothiocyanostilbene-2,2-disulfonate.
$\overset{\circ}{V}$, calculated perfused rate. J_v, water reabsorption. tCO_2, total carbon dioxide concentration.
$JtCO_2$, bicarbonate reabsorption.

Table 8: $JtCO_2$ 44 hr post gavage.

All animals were in a state of rebound metabolic alkalosis.

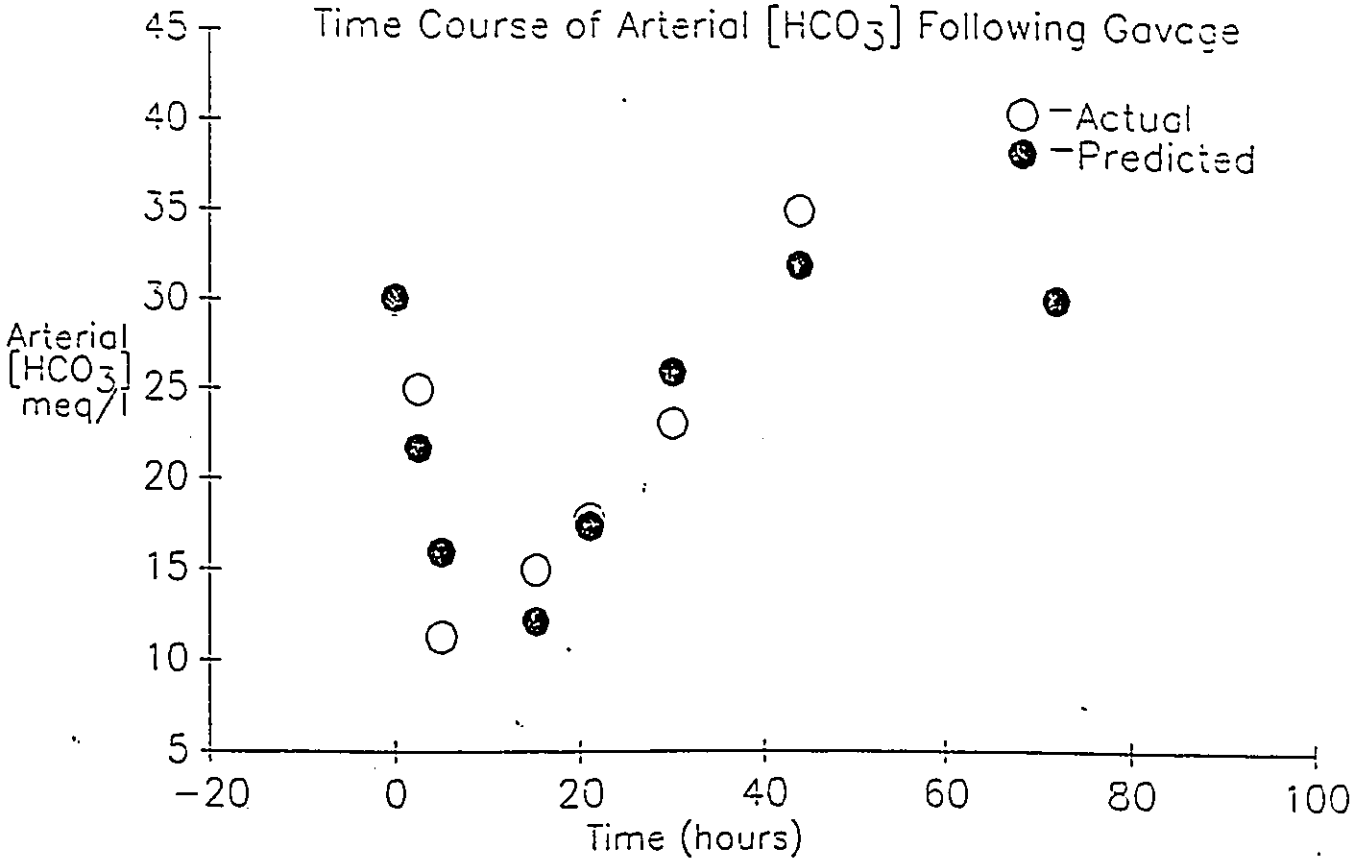
Table 8

Microperfusion data of rats with rebound metabolic alkalosis

Group	Length mm	$\overset{\circ}{V}$ nL/min	J_v nl/min	$[tCO_2]_p$ mM	$[tCO_2]_c$ mM	$JtCO_2$ pmol.min^{-1}.mm^{-1}
1 (4/6)	1.5±.1	8.4±.4	.37±.13	25.8±0.6	23.2±0.6	18±2
2 (7/10)	1.3±.1	14.1±.4	.32±.15	25.9±0.6	23.8±0.8	27±10
3 (4/6)	1.5±.1	26.7±.7	.39±.18*	25.9±0.6	22.3±1.3	47±17

Groups 1 and 3 represent paired data from the same tubule, while group 2 represents a separate group of tubules. * water flux in group 3 was negative in direction.
$\overset{\circ}{V}$, calculated perfused rate. J_v, water reabsorption. tCO_2, total carbon dioxide concentration. $JtCO_2$, bicarbonate reabsorption.

Figure 5: Time course of plasma HCO_3 following gavage. Open circles are from actual data and filled circles represent predicted concentrations from the equation obtained by nonlinear regression. Initial and final actual and predicted values are the same.


Time Course of Arterial [HCO3] Following Gavage
○ –Actual
● –Predicted
Arterial [HCO3] meq/l
45
40
35
30
25
20
15
10
5
–20
0
20
40
60
80
100
Time (hours)

Chapter IV

DISCUSSION

This study has characterized some aspects of distal tubule bicarbonate reabsorption in rats with metabolic acidosis induced by NH_4Cl administration, studied by in vivo microperfusion. Bicarbonate reabsorption was found to persist despite the presence of very low sodium concentrations in the perfusate and reduction of water movement to near zero. The results are consistent with those of a previous study (Levine, 1985) where a different range of sodium concentrations in a hypotonic perfusate were used. Amiloride was demonstrated not to be effective in inhibiting bicarbonate reabsorption when present in low sodium solutions. This is in marked contrast to the results of many studies using amiloride with higher concentrations of sodium in solutions used to perfuse bicarbonate reabsorbing distal epithelia, both in vivo and in vitro. Acetazolamide has been demonstrated to be effective in blocking some of the bicarbonate reabsorption in the distal tubule microperfused in vivo. In paired collections, SITS has been shown to significantly reduce bicarbonate reabsorption when included in the luminal perfusate. During recovery from metabolic acidosis bicarbonate reabsorption was found to persist, despite a transient systemic alkalosis.

Presumably, these results suggest that bicarbonate reabsorption in the alpha-type intercalated cell is stimulated by the metabolic acidosis. This bicarbonate reabsorption does not seem to be dependent on sodium or water transport in the neighbouring principal cells, but does seem to be dependent on intracellular carbonic anhydrase and a postulated chloride conductance. The intercalated cell's stimulated activity seems to

persist despite the appearance of extracellular signals thought to be important inhibitors of bicarbonate reabsorption. These results are discussed in more detail below.

4.1 Anatomic heterogeneity

The heterogeneous nature of the surface distal tubule perfused in the present study has been discussed by Kaissling (1982). The structure of the three segments beyond the macula densa have been described: the distal convoluted tubule (DCT), the connecting tubule (CNT) and the cortical collecting duct (CCD). Kaissling found that in rats, the pattern of segmentation seen is one of gradual transition. The four types of cells recognized in these segments are: the DCT cell, CNT cell, principal cells, and intercalated cells. The latter two cell types are characteristic of the cortical collecting duct. Woodhall and Tisher (1973) have studied rat strain differences and found that the surface distal tubule contained variable amounts of epithelium characteristic of collecting duct: Sprague-Dawley rats had 19%, whereas Munich-Wistar rats had 45% of the epithelia characteristic of this segment. If these data are correct, it is important to consider the possible contribution of the the DCT and CNT cells to bicarbonate transport. The part of the distal tubule that we perfuse consists of all these segments, and, presumably, all these cell types. However, from a variety of histologic and electrophysiology studies it is thought that the intercalated cell is primarily involved in acidification (Steinmetz, 1986).

Dorup (1985) has documented the adaptation of intercalated cells in the rat distal nephron to the acidosis caused by ammonium chloride administration. The surface density of the luminal membrane in intercalated cells was found to be significantly higher in acidotic animals when compared to controls. Stetson and Steinmetz (1985)

have suggested that a carbonic anhydrase rich cell was responsible for H+ secretion and underwent characteristic change in conformation when subjected to acidosis. Stimulation of hydrogen ion secretion by carbon dioxide was found to expand the apical areas of the cell. Schwartz and Al-Awqati (1985) have demonstrated that carbon dioxide administration causes the exocytotic fusion of vesicles with the luminal plasma membrane of collecting ducts. Thus, a variety of histologic evidence implicates the intercalated cell as being involved in bicarbonate reabsorption. We have determined that cells from the distal tubules of rats from our colony contain intercalated cells, as identified by transmission electron microscopy (unpublished observations).

4.2 Influence of water movement

Free flow micropuncture studies show that luminal fluid in the surface distal tubule of normal rats becomes increasingly hypertonic, presumably reflecting the osmotic movement of water in the presence of anti-diuretic hormone (Capasso et al. 1986; Woodhall and Tisher, 1973). At the same time, active sodium reabsorption occurs here (Costanzo and Windhager, 1978). The simultaneous movement of water and solute also creates a situation where water transport could also affect solute transport. Potential mechanisms by which this could occur include solute-solvent coupling or by unstirred layer effects.

In 1957, Andersen and Ussing theorized that water might penetrate toad skin through pores in response to an osmotic difference across the membrane. If this were true, they predicted that solvent drag might occur due to bulk flow of water through the

pores. These authors did find evidence for solvent drag, and concluded that some pores must be common pathways for water and solvents (Andersen and Ussing, 1957).

In the late distal tubule and cortical collecting duct the paracellular pathway is viewed as a very high resistance junction that does not constitute a significant pathway for solute or solvent transport (Hebert and Andreoli, 1985). Although as yet unidentified, transmembrane protein molecules are thought to be inserted into the apical membrane of principal cells in response to the binding of vasopressin to the basolateral membrane (the participation of the intercalated cells is still a major unresolved issue - see Handler (1989)). Handler (1989) has recently reviewed this topic and points out that one of the key pieces of evidence for water channels is the observation that in response to elevation of cell cAMP, particle aggregates appear in coated pits in apical plasma membrane of the collecting duct. These molecules are thought to provide a channel for water reabsorption. If sufficiently large, these channels could provide a potential site for solvent-solute interactions. However, theoretical considerations have constrained these channels to have a diameter of only 2 angstroms, sufficient to allow the passage of the 1.5 angstrom water molecule but too small to allow the passage of larger ions (Hebert and Andreoli, 1985).

Unstirred layers are thin layers of static liquid immediately adjacent to a surface. Within this layer, the concentration of solutes varies with position and does not equal that in the bulk solution (Dainty and House, 1966). These layers have been identified in isolated frog skin preparations (ibid) but are thought not to exist on the luminal side of kidney tubules (Hebert and Andreoli, 1985). However, the existence of boundary gradients on the basolateral side of kidney tubules in vivo cannot be discounted, and has been proposed as one mechanism that may link water and bicarbonate movements in the proximal tubule (see below).

The results of the present study demonstrate that bicarbonate reabsorption can proceed in the absence of water movement in the distal tubule in vivo. This demonstration, and the fact that bicarbonate permeability is thought to be quite low in this segment point out that most of the bicarbonate reabsorption is likely all due to active transport across an intracellular path. Kunau and Walker (1987) have also shown that bicarbonate reabsorption continued when water movement was negligible in an in vivo study.

An earlier microperfusion study using hypoosmotic perfusate has indicated that water movement was significantly linked with $JtCO_2$ (Levine, 1985). Water flux was shown to be a significant covariate of bicarbonate transport in that study. Bicarbonate reabsorption in group 1 of the present study was not different from that measured in ammonium chloride treated rats in Levine's 1985 study (65 ± 4 vs 74 ± 14 pmol/min/mm). The latter group of rats were perfused at 10 nl/min with a hypotonic perfusate. Thus the effect of the reduction of water movement by using an isotonic perfusate seems not to be significant. The present study's findings in group 1 are also very similar to those of a similar group of Lucci et al. (1982). That study also employed microperfusion technique to study distal tubule bicarbonate reabsorption in chronically acidotic rats, at 7 nl/min (65 ± 4 vs 52 ± 13 pmol/min/mm).

Covariance between water and bicarbonate transport was not observed in the in vitro study of McKinney and Burg (1978) who demonstrated that bicarbonate reabsorption could continue in the absence of net water movement. The link with water movement could be a phenomenon that might be explained by bicarbonate reabsorption secondary to water flux. In the proximal tubule Alpern (1984) has demonstrated stimulation of hydrogen ion secretion secondary to increased volume reabsorption and postulated that this was due to removal of a diffusion gradient on the basolateral side

of bicarbonate reabsorbing cells. Thus, theortical arguments seem to be in place for a passive role for water reabsorption in stimulation of bicarbonate reabsorption.

However, recent in vitro studies have indicated that antidiuretic states may be associated with hormonal changes that may play an active role in the modulation of bicarbonate transport. The recent work of Tomita et al. (1986) has indicated that bicarbonate reabsorption was stimulated in isolated perfused rat cortical collecting duct by the addition of vasopressin to the bath. Bichara et al., (1987) have shown that the surface distal tubule of Wistar rats reabsorbs more bicarbonate when arginine vasopressin was infused systemically. The possibility exists that ADH levels cause an increase in cAMP levels in the intercalated cells which results in increased activity of the luminal H-ATPase. Hays et al.(1986) have shown that cAMP and forskolin increased net bicarbonate reabsorption in isolated perfused rabbit medullary collecting duct. However, Tomita et al. (1986) point out that the effects of vasopressin in CCT could be secondary to changes in sodium reabsorption that result in increased lumen negativity.

In conclusion, the use of isoosmotic perfusates in the present study did not inhibit bicarbonate reabsorption. This suggests that the correlation between water flux and bicarbonate reabsorption seen by Levine (1985) was merely coincidental. (Presumably, the diuretic states of the groups of animals in the present study were similar and so ADH levels were also probably similar to those of Levine (1985)). Furthur studies might employ hypertonic perfusates to cause net water movement into the tubule while bicarbonate reabsorption was stimulated.

4.3 Sodium substitution

We were unable to find a significant effect of reducing sodium concentration in the perfusate on bicarbonate transport in the distal tubule. These results are similar to a previous study where a different range of sodium concentrations were used (95 and 35 mM vs 60 and 0 mM, in Levine (1985) and the present study, respectively. The present study also used isoosmotic perfusate and higher flow rates, efforts designed to furthur reduce sodium concentration in the lumen. Levine (1985) reported a similar range of bicarbonate fluxes at 20 nl/min in acidotic rats perfused with 90 and 35 mM Na perfusates: 119 $\pm$ 22 vs 83 $\pm$ 16 pmol/min/mm in high and low sodium perfusion, respectively. It is interesting to note that both studies showed a small reduction in bicarbonate flux with low sodium perfusion that was not statistically significant. As both studies employed micropuncture results from two unpaired groups of tubules, it is possible that variability among tubules may have obviated the discovery of a significant inhibition. The greater bicarbonate reabsorption seen at high flow is a load dependent effect that has been seen by other investigatorss (Capasso et al, 1987). Ullrich and Papavissiliou (1981) also found no change in HCO_3 reabsorption when they substituted choline for Na in stationary perfused papillary collecting ducts of acidotic rats in vivo.

The lack of an effect of sodium removal is not confined to in vivo studies. McKinney and Burg attempted to manipulate the bicarbonate flux in acid-treated rabbit CCT in vitro by substituting choline for sodium in the perfusate (McKinney and Burg, 1978). Choline perfusion was not found to inhibit bicarbonate reabsorption, in fact in that study it was found to stimulate it.

In vivo experiments have demonstrated the entry of Na into the distal tubule that is perfused in vivo with Na free solutions (Costanzo and Windhager, 1978; Good et al, 1984). The reported concentrations of collected Na in the present study are similar to

those reported by Good and co-workers (1984), who perfused rat distal tubules in vivo. As the K_m of the luminal Na/H exchanger is quite low (Kinsella and Aronson, 1981), it is not possible for us to conclude that Na/H exchange does not occur here. However, the substitution experiments at high flow demonstrate that bicarbonate reabsorption increases when luminal Na concentration decreases, suggesting that these ion movements can be dissociated.

The lower plasma bicarbonate and pH of our animals are important regulating factors of bicarbonate reabsorption. Breyer et al. (1986) have demonstrated that acute (minutes) decreases in bicarbonate concentration and pH in the bath of isolated perfused rabbit cortical collecting duct resulted in increased bicarbonate reabsorption. The hours of systemic acidosis experienced by the distal tubules micropunctured in this study might have caused additional changes contributing to bicarbonate reabsorption. Dorup (1985) documented that the surface density of the luminal membrane of intercalated cells became more dense than control cells only 5 hours following ammonium chloride administration. Principal cell function may also be influenced by metabolic acidosis. Palmer and Frindt (1987) have shown that cytoplasmic pH directly influences sodium channel activity: single channel activity was found to decrease when the cytoplasmic side pH was decreased from 7.4 to 6.4. (It should be kept in mind that in vivo pH changes are probably not as large.) Thus, it is quite possible that prolonged exposure to acidosis might cause adaptation in cells of the surface distal tubule that would make luminal sodium substitution less likely to have an effect on bicarbonate reabsorption.

Recent studies lend more credence to the idea that sodium transport and bicarbonate transport in the distal nephron may proceed independently. Vehaskari et al. (1989) have studied the effect of the mitogen epidermal growth factor on ionic trans-

port in isolated perfused cortical collecting tubule of the rabbit. While peritubular EGF decreased Na transport here, no effect on bicarbonate transport was observed.

In conclusion, the acidotic condition may have caused changes in the distal tubule that made sodium substitiution less likely to inhibit bicarbonate reabsorption. Alternative explanations are that choline has some unexplained stimulatory effect on bicarbonate transport (Arruda et al. 1980) or that enough Na enters down a concentration gradient into the lumen to permit sodium entrance into the cell. Furthur experiments to determine which of these alternatives is correct would involve sodium substitution with a cation other than choline (i.e. tetraethyl- ammonium) and reduction of a gradient for Na entry by simultaneous luminal and peritubular substitution.

4.4 Effect of amiloride and low sodium perfusion

The present study failed to show an effect of amiloride on bicarbonate reabsorption in the surface distal tubule of the acidotic rat when perfused with low sodium concentration. Amiloride has been shown to inhibit the negative transepithelial potential that is normally recorded across the surface distal tubule. This has been interpreted as meaning that the sodium transport that generates the negative transepithelial potential is inhibited by amiloride (Stoner et al. 1974). If bicarbonate transport in the distal tubule proceeds by an electrogenic process (proton ATP ase), then amiloride should theoretically inhibit bicarbonate transport here. This model is supported by studies like that of Laski and Kurtzman (1983) who showed that ouabain reduced both the transmembrane potential and the bicarbonate reabsorptive flux in rabbit CCT perfused in vitro. Kunau and Walker (1987) have indeed shown an inhibition of in vivo bicarbo-

nate reabsorption when amiloride was perfused in distal tubules of rats fed a high protein diet. Possible reasons for disparate results in the present study are the different treatment of the animals and the fact that we only used amiloride in conjuction with low sodium perfusate.

Systemic adminsistration of amiloride has been shown to result in higher urine pH and bicarbonate excretory rates in rats given NH_4Cl. This has been interpreted to mean that amiloride caused a defect in distal acidification (Arruda et al. 1980). In vitro studies have been responsible for much of the mechanistic information on amiloride action in the distal nephron. Amiloride was shown to inhibit bicarbonate reabsorption in one of the first studies of the isolated perfused cortical collecting tubule from acid treated rabbits (McKinney and Burg, 1978). A recent study by Koeppen (1987) has employed intracellular electrical measurements in the medullary collecting duct, allowing the effect of amiloride on different cell types to be discerned. One cell showed a large change in fractional resistance of the membrane, in keeping with the known action of amiloride to block the sodium channel of principal cells (Sariban-Sohraby and Benos, 1986). Another cell, presumably the intercalated cell, was hyperpolarized on the apical membrane by amiloride. Koeppen stated that inhibition of electrogenic transport by one cell type would be expected to alter the membrane voltage in the other cell type owing to circular intraepithelial current flow (an indirect effect). This would be expected to result in decreased bicarbonate reabsorption as the luminal hydrogen ion pump would have to work against a less favourable gradient.

Central to Koeppen's conclusions is the idea that proton pumping is an electrogenic process- that the hydrogen ion is pumped alone and so generates a lumen positive voltage. This idea originated from turtle bladder studies (Steinmetz, 1986), and was supported by studies measuring potential differences in the cortical collecting duct

(Koeppen and Helman, 1982). However, recent preliminary reports have challenged these classical ideas: Okusa et al., (1989) have found that an inhibitor of gastric H-K ATPase, SCH 28080 was effective in reducing potassium flux in surface distal tubules of K deficient rats perfused in vivo. Wingo et al., (1989) have also provided evidence for the existence of a hydrogen ion- potassium ion ATPase in intercalated cells of the rabbit cortical collecting duct. They found that omeprazole (an inhibitor of gastric H-K ATPase) either increased a K secretory flux or decreased a K absorptive flux. In addition, antibodies against gastric H-K ATPase strongly labelled intercalated cells in the cortical collecting duct. All these studies suggest that hydrogen ion secretion may be directly linked to other ion movements.

Data from a recent study by Stanton (1988) suggest that Amphiuma diluting segment (thought to be an analogue of the early distal tubule of the mammalian kidney) transports bicarbonate by Na/H exchange. Since the segment that is perfused in the present study contains a variable portion of the early distal tubule, the possibility that part of the bicarbonate reabsorption is due to Na/H exchange cannot be discounted. Kinsella and Aronson (1981) predicted that amiloride should be more effective in blocking Na/H exchange at low sodium concentration. However, the fact that the dose of amiloride used in the present study is enough to block 85 % of Na/H exchange (Kinsella and Aronson, 1981) suggests that the contribution of the early distal tubule to bicarbonate reabsorption in the present study is minimal. Furthur studies with more specific analogues of amiloride now available would be able to selectively inhibit the Na/H exchanger or the apical Na channel and furthur elucidate the interplay between Na and bicarbonate movements in the distal nephron. Ethylisopropylamiloride could be used to specifically block the Na/H antiporter and subsequent studies could employ dichlorobenzamil to specifically block the epithelial sodium channel (Frelin et al. 1987).

The dose of amiloride used deserves careful consideration. The half maximum effect for amiloride inhibition of the Na/H exchange system was observed to be at 25 micromolar in rabbit cortical brush border membranes (Kinsella and Aronson, 1981). In contrast, the half maximum inhibition of the Na channel by amiloride has been observed to be 70 nanomolar in the rabbit cortical collecting tubule (O'Neil and Boulpaep, 1979). Thus, the dose used in this study was sufficient to inhibit the Na/H antiporter and Na channel activity.

Data from turtle bladder studies have also explored the relationship between amiloride's effect on acidification. Husted and Steinmetz (1979) noticed that amiloride was not effective in reducing turtle bladder acidification if ouabain was administered first. More relevant is the study of Lief and Mutz (1977) who found that amiloride was not effective in blocking hydrogen secretion of turtle bladders that were bathed in Na free media. Frazier (1985) used the toad bladder model to study acidification and found that amiloride inhibited hydrogen ion excretion in the acidotic toad only in the absence of exogenous carbon dioxide. When exogenous carbon dioxide was present there was no inhibition of hydrogen ion excretion. The interesting proposal that Frazier put forward to explain the effectiveness of amiloride to inhibit hydrogen ion excretion in the absence of carbon dioxide is that blockage of the Na transport would significantly reduce carbon dioxide production due to lowering of the metabolic requirements of the bladder (Frazier, 1985).

Reversal of the Na concentration gradient by the use of the low Na perfusate might prevent block of the Na channel by amiloride. Na secretion was observed in these studies, which might indicate movement of Na out of the cell, through the channel (although paracellular movement is another possibility). Induction of reverse flow through active transport pathways in toad urinary bladder treated with ouabain has

been observed by Dawson and Al-Awqati (1978). This might effectively 'knock out' any blocking amiloride molecules. Precedent for this type of block prevention does exist (Hille, 1981). (However, this would only occur if the luminal sodium concentration decreased below intracellular sodium concentration.) All these studies suggest that amiloride is less effective when sodium transport is minimized, and support the present study's findings.

In summary, our results showed that amiloride and low sodium perfusion did not alter bicarbonate reabsorption in distal tubules from acidotic rats. This appears to contradict some of the literature involving amiloride and distal acidification mechanisms. However, the use of amiloride in low sodium perfusate, and the acidotic rat model employed, all appear to reduce the effectiveness of amiloride as previously suggested in the literature (Lief and Mutz, 1977; Frazier, 1985). In addition, very recent studies appear to contradict the classical views of electrogenic acidification and the indirect effect of sodium transport on bicarbonate transport in the distal nephron (Okusa et al., 1989; Vehaskari et al., 1989). These studies bring into question the mechanism of amiloride inhibition of bicarbonate transport in the distal nephron.

4.5 Acetazolamide administration

We found that acetazolamide resulted in a reduction in bicarbonate reabsorption but that there was a remaining bicarbonate reabsorption that could not be inhibited. This is the first study to report this effect when bicarbonate load and flow were held constant. This is surprising if one assumes that bicarbonate reabsorption is via a simi-

lar mechanism in CCT and surface distal tubule. McKinney and Burg (1978) found that acetazolamide administration caused almost total inhibition of acidification in isolated perfused CCT from rabbits. In vivo studies of the distal nephron generally find that inhibition of acidification by acetazolamide is less than complete (Al-Awqati, 1978). Giebisch et al.(1977) showed that there was no difference in the kinetics of phosphate titration in the late distal tubule of acidotic rats and acidotic rats given acetazolamide, but did report longer half times of acidification in the latter group when large concentrations of bicarbonate perfused the late distal tubule. Malnic et al. (1972) found that the relative rate of bicarbonate reabsorption was lower in distal tubules of diamox treated rats, but their results must be viewed with caution as very large increases in bicarbonate load occured during this free-flow study. Richardson and Kunau (1982) have studied the papillary collecting duct by in vivo techniques, and also found that acetazolamide treated animals reabsorbed less bicarbonate, if the increased load was taken into account. Dubose and Lucci (1983) have found that significant reabsorption of bicarbonate remained in acetazolamide treated rats between the distal tubule and the papillary collecting duct. More recently, Laski (1987) has reported the presence of carbonic anhydrase independent acidification in the isolated CCT of fasted rabbits.

Al-Awqati (1978) addressed the issue of carbonic anhydrase independent acidification. He pointed out that the complete inhibition of transport with in vitro addition of the drug raised the possibility that in vivo results could be explained by inadequate drug delivery. If the levels of carbonic anhydrase inhibition are effective, as has been stated to be by others (Maren, 1977), then the question that must be asked is what mediates the remaining bicarbonate reabsorption? Maren (1974) has suggested that an intracellular acidosis allowed bicarbonate reabsorption to proceed without carbonic anhydrase. Dubose and Lucci (1983) have calculated that the uncatalyzed rate of car-

bonic acid production could only account for a few pmol/min of bicarbonate reabsorption. Winaver et al.(1986) have also studied the acetazolamide resistant bicarbonate reabsorption in free flow micropunctured rats and and concurred with Dubose and Lucci that hypercapnia might stimulate reabsorption. They also suggested that H_2CO_3 recycling from lumen to cell might provide a continuous source of hydrogen ions for secretion.

The preceding discussion is based on the classical assumption that acetazolamide works by inhibiting carbonic anhydrase and reducing the supply of protons to be transported. It should be noted that recent work suggests that acetazolamide interferes with the process of exocytosis (thought to be important in luminal insertion of proton pumps) (Dixon et al., 1988). Gluck et al. (1982) have pointed out that acidosis causes increased insertion of proton pumps by exocytosis. Regulation of the rate of this process is now thought to be an important control point in acidfication. In another recent study it has been shown that the action of acetazolamide is independent of change in intracellular pH! (Graber et al., 1989). In view of these recent developements, the persistent acidification that remains after acetazolamide administration might be regarded as that which is independent of insertion of new proton pumps.

4.6 Intervention with SITS

4-acetamido,4-isothiocyanostilbene 2,2-disulfonic acid (SITS) was found to decrease bicarbonate reabsorption in the distal tubule when included in the luminal perfusate. Many studies have found that bicarbonate reabsorption is reduced when SITS is included in the bath, but few studies have assigned a possible role for SITS at the apical membrane. However, Ullrich and Papavassilliou (1981) have found that

SITS reduced papillary collecting duct acidification when included in the perfusate of micropunctured papillary ducts. The magnitude of the inhibition found in the present study was modest in contrast to the 58% decrement noted by Ullrich and Papavasilliou. However, it should be noted that the latter study employed both lumen and capillary perfusion, so some of the inhibitory effect seen in that study could be attributed to the well-known inhibitory effect of SITS on a basolateral anion exchanger.

SITS was originally synthesized as a fluorescent label for the outside of the plasma membrane of cells by Maddy (1964). Maddy designed the requirements of the molecule: it does not pass through the osmotic barrier of the membrane and it reacts with the membrane under physiological conditions of temperature, pH and tonicity without disrupting the cell. It has been recognized that SITS might act as a chloride channel blocker (Nelson et al. 1984). SITS has also been recognized as a blocker of Cl/HCO_3 exhange in the red blood cell membrane and in the basolateral membrane of the intercalated cell (Gluck, 1989).

A role for chloride in acidifying epithelia was suggested by Stone et al. (1983) who found that medullary collecting duct acidification proceeded in parallel with chloride secretion. Stone and Xie (1988) have suggested that chloride may serve as the dominant co-ion that is involved in the counterbalance of electrogenic acidification.

Recent studies of H-ATPase activity in membrane vesicles have allocated a more active role for chloride in acidification. Hilden et al. (1988) have found that ATP driven H+ pumping had an absolute requirement for chloride. They concluded that this dependence might derive from a chloride channel whose function was intimately related to hydrogen ion pumping by the ATPase. These investigators also found that chloride channel inhibitors reduced Cl uptake in vesicles containing H+ ATPase.

A recent new thrust of investigation is the assessment of transport processes in cultured epithelial cells. Light and co-workers (1987) have studied intercalated cells in culture and identified a chloride channel in the apical membrane. They used the patch clamp technique to determine the conductive properties of this channel and found that 0.5 mM DIDS (a disulfonic stilbene very similar to SITS) inhibited channel activity. These investigators state that it must be considered that the apical chloride channel regulates electrogenic proton secretion (Light et al. 1990). Very recently, the chloride channel associated with the proton ATPase of clathrin coated vesicles has been solubilized and isolated by Xie and Stone (1988). It is likely that this protein will be found to be the chloride channel that Light et al. patched, and it is tempting to speculate that inhibiting the same apical channel with SITS caused the inhibition of bicarbonate reabsorption seen in the present study by preventing the counterbalance of electrogenic acidification (vide supra).

Furthur study of the anion dependence of proton pumping might include use of more specific blockers of anion channels, such as 5-nitro-2- phenyl-propylamino benzoate, as used by Hilden et al. (1988).

4.7 Intervention with NEM

N-ethylmaleimide (NEM) is a sulfhydryl blocking reagent thought to to block proton transport by luminal hydrogen ion ATPase in the distal nephron (Stone and Xie, 1988). The present study found that NEM only blocked proton transport at very high concentrations. The reasons for this discrepancy are probably related to the length of time that tubules were exposed to this agent. Long exposure times are typical of in vitro studies (as long as one hour in some studies), whereas typical exposure times in the present study were only 2-5 min.

Recently, Garg and Narang (1985) have studied N-ethylmaleimide sensitive ATPase activity in collecting duct segments of the rat nephron. They found that metabolic acidosis significantly stimulated this activity over control conditions. Interestingly, if the 35 pmol ADP/min/mm activity that they found is correct, one can estimate the resultant proton transport that this activity should be associated with. If one assumes that the ATP per hydrogen ion moved stoichiometry is approximately 1 to 2 (Steinmetz, 1986), then the resultant $JtCO_2$ should be about 70. This is very close to the value observed at the higher flow rates in the present study.

Caution is needed in the interpretation of these studies. NEM is such a non-specific inhibitor that Stone and Xie (1988) state that it is not possible to precisely identify proton pump activity- "in any more than a loose quantitative fashion". Despite this, studies continue to use NEM for want of a more specific inhibitor.

Microperfusion with NEM in the present study was a crude attempt to identify one of the reported characteristics of the apical proton pump. Recently, Brown and co-workers have localized proton pump ATPase in the rat kidney by immunocytochemistry. Intercalated cells in the late distal tubule were found to stain heavily for this protein (Brown et al. 1988).

4.8 Metabolic acidosis and recovery

The model of metabolic acidosis used in this study has been well characterized by Sartorius et al., (1949). Ammonium chloride administration via gavage causes a large increase in the delivery of ammonium to the liver via the portal circulation. Ammonium ion is converted to urea, a process that consumes bicarbonate (Oliver and Bourke,

1975). (Hepatic cells avidly take up ammonia from the portal blood, which is then consumed with equimolar amounts of bicarbonate in the synthesis of urea (Haussinger et al., 1984)). This causes a decrease in arterial bicarbonate concentration - a hyperchloremic metabolic acidosis ensues. The particular mode of administration used in this study - gavage of a relatively large load of ammonium chloride - causes a dramatic decrease in blood bicarbonate concentration within hours (Levine et al., 1983).

Restorative forces are soon set into motion to return the sytemic acid-base condition to normal. The role of the kidney is to reclaim all the filtered bicarbonate and to generate new bicarbonate through ammonium excretion (Halperin, 1989). Bicarbonate reabsorption is known to increase in both proximal and distal tubules (Cogan et al., 1979; Levine, 1985). Enzymes involved in the production of ammonium: phosphate-dependent glutaminase and phosphoenolpyruvate carboxykinase activites are known to increase, contributing to an increased rate of glutamine metabolism during metabolic acidosis (Parry and Brosnan, 1978). These restorative forces contribute to a recovery process that returns blood bicarbonate concentrations to normal (Levine et al., 1983).

Parry and Brosnan (1978) commented on the metabolic alkalosis that was seen on recovery from metabolic acidosis due to chronic ammonium chloride ingestion in rats. They felt that this was largely due to continued activity of enzymes involved in the production of ammonia. In an extensive chronic study, Guern et al. (1982) confirmed this idea, and coined the term 'rebound metabolic alkalosis' to describe the transient condition during recovery. A contribution of the present study is that this condition can be seen after a single gavage of acid, instead of the several days of administration in the former studies. Evidently, a more dramatic stimulus administered over a shorter time period must be just as effective in inducing changes in the enzymatic pathways as a moderate stimulus administered over a longer time period.

These studies of the biochemical aspects of recovery from metabolic acidosis did not comment on the time course of blood bicarbonate concentration, except in descriptive terms. It is quite evident that the response of the acid-base system to the acid perturbation resembles the response of a second order system. i.e. the system could be described by the second order differential equation:

$$\ddot{x} + a\dot{x} + bx = f(t)$$

where $\ddot{x}$ is the second derivative of bicarbonate w.r.t. time, $\dot{x}$ is the first derivative of bicarbonate w.r.t. time, x is the blood bicarbonate concentration at any time, a and x are arbitrary constants and f(t) is the function that describes the variation of blood bicarbonate at any time.

Certain transient solutions to the second order equation greatly resemble the blood bicarbonate profile seen following the gavage, including the rebound metabolic alkalois. Underdamped systems exhibit a decaying oscillation following a step change in input (Glantz, 1979). In this scheme, the acid gavage functions like a step input: in a relatively brief period, the acid causes the blood bicarbonate to be reduced to a low level. The 'system' responds by increasing net acid excretion. As we have an underdamped system, the response is fast, but there is oscillation- the blood bicarbonate 'overshoots' the normal arterial concentration. In teleologic terms, the price the organism pays for a fast response to the acid perturbation is a tendency to oscillate.

Levine and co-workers (1983) modelled the response of the blood bicarbonate to the identical perturbation used in the present study. The considerations and the differential equation were identical to those just presented. The crucial difference is that blood bicarbonate concentration was only followed for 26 hours post gavage. The overshoot was not seen, so the data seemed best fitted by a gamma distribution curve.

The reader may well ask what is to be gained by fitting this data into a model that approximates the time course, when verbal discription of the earlier papers seemed sufficient. The beauty of viewing the system in terms of the mathematical model is that it allows certain predictions (and hypotheses). Considerations of clinical acid-base disorders are usually constrained to static situations. The model presented predicts that the output of the system is governed to some extent by the rate of change of bicarbonate and even by its acceleration. Two hypotheses for furthur experiments immediately spring to mind after considering this model: is the degree of overshoot related to the degree of acidosis? and would the adrenalectomized rats in Levine's 1983 study show the overshoot?

4.9 Bicarbonate reabsorption during rebound metabolic alkalosis

The microperfused distal tubule does not reabsorb significant amounts of bicarbonate during normal conditions (Levine, 1985), reabsorbs maximally after some initial delay following acid gavage, and continues to reabsorb bicarbonate during recovery despite the ensuing metabolic alkalosis. Thus, the surface distal tubule bicarbonate reabsorption follows the same time course as the blood bicarbonate concentration, and could be predicted by the second order model presented earlier. Indeed, the action of the distal tubule and/or other segments of the distal nephron may be required for the overshoot of plasma bicarbonate concentration to occur.

Little experimental information is available about tubular function during rebound metabolic alkalosis. Bengele et al. (1987) have studied the function of the inner medullary collecting duct during control, acidotic and during rebound metabolic alkalo-

sis. They found that net acid excretion and bicarbonate reabsorption remained high in this segment during rebound metabolic alkalosis. The present study also found that bicarbonate reabsorption was significant during rebound metabolic alkalosis.

4.10 Implications of studies during rebound metabolic alkalosis

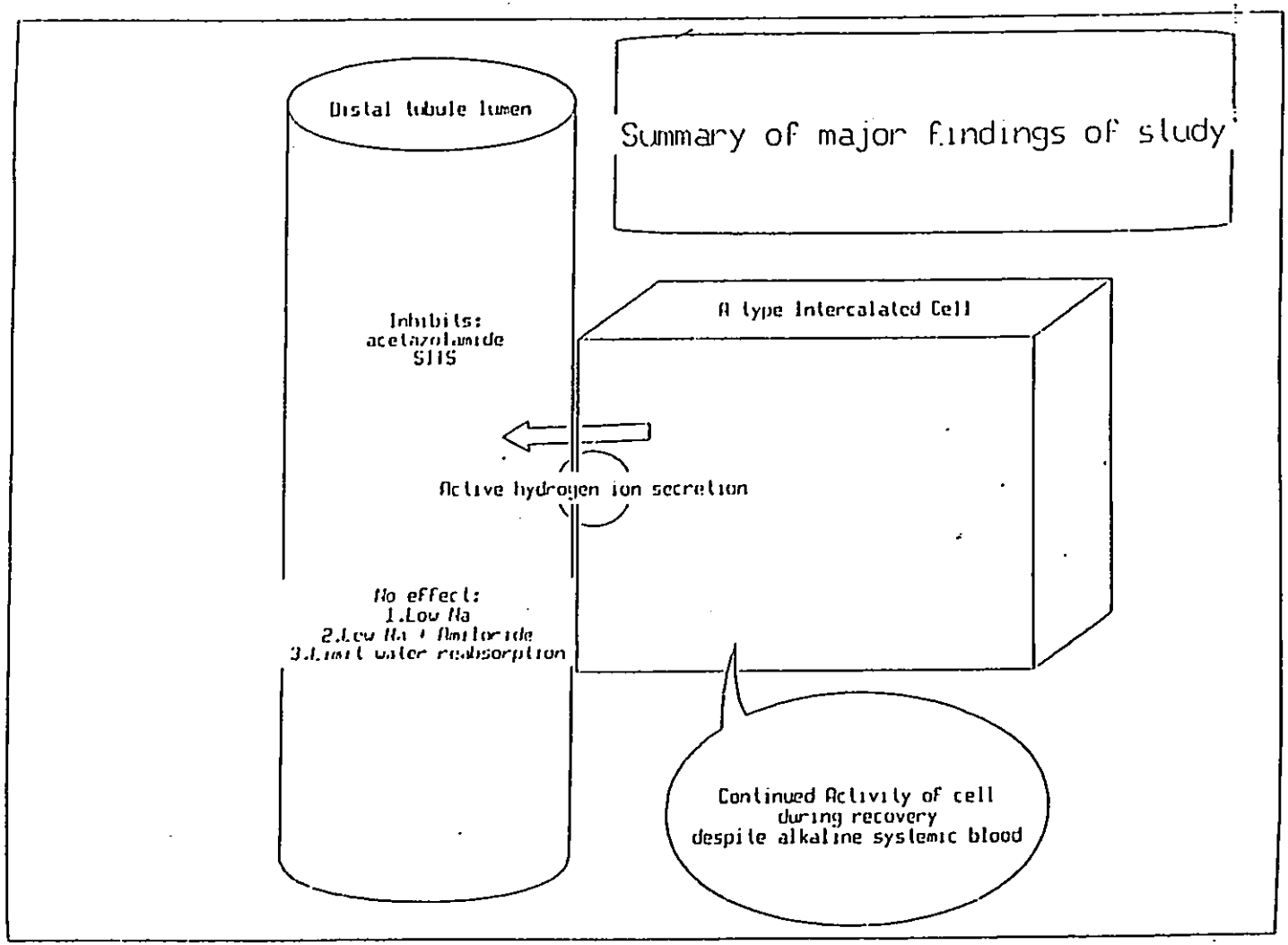
The fact that DT bicarbonate reabsorption continues during RMA suggests that blood bicarbonate and/or blood pH does not regulate intercalated cell function at this time. What then is regulating intercalated cell function ? It is possible that some other extracellular messenger continues to act during rebound metabolic alkalosis. One such candidate would be aldosterone. This hormone has been shown to be elevated in response to NH_4Cl administration (Perez et al. 1977) and is also known to stimulate net acid excretion (Dubrovsky et al. 1981) and increases urine-blood pCO_2 (an index of distal acidification) (Damasco et al. 1989). It is quite possible that the time course of blood aldosterone concentration follows the elevated acid excretion seen in these animals.

The apical membrane of hydrogen secreting intercalated cells has been observed to undergo structural and functional change after acid exposure. Dorup (1985) has documented an increase in surface density of hydrogen ion secreting (type A) intercalated cells after administration of ammonium chloride. Verlander et al. (1987) have observed an increase in apical microprojections and in the surface density of type A intercalated cells following several hours of respiratory acidosis. Vesicles enriched in hydrogen ion pumps fuse to the luminal membrane of cortical collecting ducts exposed to carbon dioxide (Schwartz and Al-Awqati, 1985). Presumably, it will take time for

these structural and functional adaptations to revert to normal, following the removal of the acidotic challenge. Once in place, are these structural adaptations always associated with elevated hydrogen ion secretion?

An alternative explanation is that the important signals for acidification are intracellular, and that these signals remain operant during rebound metabolic alkalosis. Intracellular pH and bicarbonate concentration may still be less than normal in intercalated cells during rebound metabolic alkalosis. Intracellular bicarbonate concentration has recently been found to be an important regulator of bicarbonate transport in isolated rabbit cortical collecting duct (Matsuzaki et al. 1989). Another potential candidate for intracellular regulation of acidification is calcium. Slotki et al. (1989) have found that changes in intracellular calcium are important in the regulation of intracellular pH in cultured cells from the inner medullary collecting duct. Obviously, the study of any of these intracellular messengers during rebound metabolic alkalosis would make an interesting project.

Figure 6: Summary of major findings of study


Distal tubule lumen
Summary of major findings of study
Inhibits:
acetazolamide
SITS
A type intercalated Cell
Active hydrogen ion secretion
No effect:
1.Low Na
2.Low Na + Amiloride
3.Limit water reabsorption
Continued Activity of cell
during recovery
despite alkaline systemic blood

4.11 Conclusion

What is the significance of these findings? The informed reader might well suggest that bicarbonate reabsorption in the present study was higher than normal because of the use of bicarbonate concentrations and perfusion rates that are greater than those normally seen in the distal tubule (i.e. see Capasso et al. (1987) for discussion of the load dependence of distal tubule bicarbonate reabsorption). However, it has been observed in free-flow studies that distal tubules can reabsorb large amounts of bicarbonate when the early distal tubule flow rate was only 10 nl/min and the early distal tubule bicarbonate concentration was approximately 10 mM (Capasso et al. 1986).

Hydrogen ion secretion only continues if sufficient buffer is available to prevent generation of high pH gradients (Steinmetz, 1986). Undoubtedly, in the present study bicarbonate is the major buffer, but in 'real life' ammonia may play a more important role. Wilcox et al. (1984) have found that ammonia secretion increases in the distal tubule of acid-infused rats. The ability of the distal tubule to produce ammonia has been confirmed by Good and Burg (1984). The current view of ammonium excretion is that it occurs by the parallel secretion of protons and ammonia (Wall and Knepper, 1990). Thus, the bicarbonate reabsorption studied in this thesis may be viewed as equivalent to indirectly studying proton secretion, although the protons may leave the late distal tubule in combination with other buffers in the intact animal.

How important quantitatively is the amount of bicarbonate reabsorbed by the distal tubule? If the distal tubule could increase its bicarbonate reabsorption to some 50 pmol/min/mm, then the summed effort of the distal tubules of some 60,000 nephrons in the kidneys of a 300 g Sprague-Dawley rat (Andreucci, 1978) should be able to effect an increase in plasma bicarbonate concentration of 1.2 mM/hour. (Assuming: 30,000 distal tubules/rat kidney, length of distal tubule=1mm, HCO_3 space of the animal =

50%). Even though such a calculation can only be regarded as a rough approximation, the exercise points out that the surface distal tubule can make very significant contributions to the acid-base status of the animal.

In conclusion, we found that acetazolamide reduced $JtCO_2$ in distal tubules perfused in vivo in rats with metabolic acidosis, and that low sodium perfusate and amiloride did not have effects under these conditions. Bicarbonate reabsorption can proceed here in the absence of water movement and is stimulated by high flow. SITS was found to significantly reduce bicarbonate reabsorption. Bicarbonate reabsorption was found to persist during the recovery phase of metabolic acidosis, despite a transient systemic alkalosis. These are all findings consistent with the view that distal tubule bicarbonate reabsorption occurs by the action of hydrogen ion pumps in intercalated cells.

Chapter V

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