Factors influencing Renal Ammonium Excretion in Man, particularly in relation to Urinary pH

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Abstract

The excretion of ammonium (NH$_4^+$) by the kidney is regulated by a variety of factors which fall into two main categories; those which affect renal ammoniagenesis and those which affect renal ammonia transport from the tubular cell to the urine. The aim of this work was to investigate, in man, the principal influences regulating these two control mechanisms, along with any consequent alteration in the relationship between NH$_4^+$ excretion rate and urinary pH. A linear relationship of log NH$_4^+$ excretion rate against urine pH, with a slope of approximately -0.3, was found in all studies of normal subjects experiencing spontaneous changes in urine pH, or submitted to minor acute systemic acid-base changes. This relationship was then compared to that found during

- chronic acidosis and chronic alkali loading,
- administration of glutamine substrate,
- administration of a mineralocorticoid and its suppression,
- water diuresis and osmotic diuresis,
- administration of different pharmacological diuretics.

It was discovered that, in all these circumstances, whether the NH$_4^+$ excretion rate increased, decreased or remained the same, the negative correlation with slope -0.3 persisted.

Chronic acidosis increased NH$_4^+$ excretion rate more than could be explained simply by a low urine pH facilitating diffusion trapping, and conversely long term alkali loading reduced NH$_4^+$ excretion rate in relation to urine pH. An adaptive change in the ammonia-generating metabolic pathway may have been induced.

Increasing the availability of glutamine substrate substantially enhanced the rate of NH$_4^+$ excretion in relation to urine pH, indicating that substrate transport into the renal proximal tubular cell may be a factor rate-limiting to ammoniagenesis.

The administration or suppression of a mineralocorticoid, whilst the subject remained normokalaemic, produced no change in the relationship between NH$_4^+$ excretion rate and urinary pH, implying that potassium availability is the more important factor controlling renal NH$_4^+$ excretion.

Both water and osmotic diuresis substantially augmented the rate of NH$_4^+$ excretion, possibly by enhancing the concentration gradient for NH$_4^+$ diffusion into the collecting duct. Osmotic diuresis gave rise to a greater increase in NH$_4^+$ excretion rate than did water diuresis. It is suggested that this was due to the production of a steep concentration gradient for NH$_4^+$ secretion into the proximal tubule.

The administration of both thiazide and loop diuretics reduced the rate of urinary NH$_4^+$ excretion in relation to pH, an effect which may have been caused by the inhibition of potassium-linked mechanisms which can also transport NH$_4^+$. Administration of a potassium-sparing diuretic had no effect on NH$_4^+$ excretion rate.

Overall the constancy of the negative slope in the relationship between NH$_4^+$ excretion rate and urine pH indicates that the mechanism of diffusion trapping is a consistent factor in renal ammonium excretion. Also important in determining the rate of excretion are ammoniagenesis in the proximal tubule and factors influencing ammonia movements in and out of the tubule.
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Chapter 1

Introduction

1.1 Renal regulation of acid-base state

The kidney plays a major role in the regulation of the hydrogen ion (H\(^+\)) concentration, or pH, of body fluids. In a healthy person the pH of extracellular fluid is maintained within a narrow range, with a mean normal value of 7.40 ± 0.02 in arterial plasma and 7.38 ± 0.02 in mixed venous plasma. This range represents a H\(^+\) concentration, or [H\(^+\)], of 36-42 nmol.l\(^-1\) (Du Bose, 1983). The strict control of pH is essential for health because of the marked effects of pH change on enzyme reactions, protein conformation and the function of the central nervous system. The kidney participates in the regulation of pH by regulating the concentration of bicarbonate (HCO\(_3^-\)) in plasma. It does this in two ways:-

1. by regulating the amount of HCO\(_3^-\) reabsorbed from the glomerular filtrate, and

2. by generating HCO\(_3^-\) to replace that lost in buffering strong acids formed in the body.

Both of these functions are accomplished by a single process, the secretion of H\(^+\) derived from the dissociation of carbonic acid by the tubular epithelial cells (Warnock and Rector, 1979). For every H\(^+\) ion secreted, a HCO\(_3^-\) is returned to the systemic circulation. The excess H\(^+\) that is secreted in order to generate new HCO\(_3^-\) cannot be excreted from the body as free H\(^+\) ion, but must combine with urinary buffers. The predominant buffer in tubular fluid is HCO\(_3^-\), but since the reaction between H\(^+\) and HCO\(_3^-\) results in the reabsorption of HCO\(_3^-\) (see section 1.1.1), H\(^+\) cannot be excreted by combining with HCO\(_3^-\). The excess H\(^+\) must therefore combine with nonbicarbonate buffers in the tubular fluid, the most important of which are phosphate and ammonia.
1.1.1 Reclamation of filtered bicarbonate

The kidney must reclam virtually all of the $\text{HCO}_3^-$ filtered through the glomerulus each day (usually > 4300 mmol). This function is achieved by the reaction of $\text{H}^+$ secreted into the tubular fluid with $\text{HCO}_3^-$ to form $\text{H}_2\text{CO}_3$, which in turn dissociates into $\text{CO}_2$ and water. $\text{H}^+$ secreted by the tubular cell is derived from $\text{H}_2\text{CO}_3$ formed from $\text{CO}_2$ and water within the tubular cell, catalysed by the enzyme carbonic anhydrase, and the $\text{HCO}_3^-$ formed by this reaction is released into the plasma. The secretion of each $\text{H}^+$ ion is therefore accompanied by the neutralisation of an $\text{HCO}_3^-$ ion in the glomerular filtrate, which would otherwise be excreted in the urine, and the release of an $\text{HCO}_3^-$ ion into the plasma.

Approximately 90% of the filtered $\text{HCO}_3^-$ is reclaimed (or reabsorbed) in the proximal tubule (Cogan, 1984). Figure 1.1 summarises the profile of bicarbonate reclamation along the rat nephron. In the proximal tubule the tubular fluid is acidified to approximately pH 6.7 (Du Bose et al., 1979) and the $\text{HCO}_3^-$ concentration is reduced to 8 mmol.l$^{-1}$ (Cogan et al., 1979). Both pH and $[\text{HCO}_3^-]$ then rise towards the bend of the loop of Henle to 7.4 and 16–24 mmol.l$^{-1}$ respectively (Du Bose, 1983). Water removal from the descending limb may cause this increase by concentrating $\text{HCO}_3^-$ and consequently alkalinising the tubular fluid (Gottschalk et al., 1960). Along the length of the thick ascending limb of Henle’s loop a significant portion of the $[\text{HCO}_3^-]$ is reabsorbed and fluid delivered to the distal nephron is normally low in $\text{HCO}_3^-$ (5–7 mmol.l$^{-1}$) with a pH of 6.5–6.7 (Buerkert et al., 1983). The remaining bicarbonate is either reabsorbed in the collecting duct or appears in the final urine. Urinary excretion of bicarbonate is typically less than 0.1% of that which is filtered.

The kidney must generate 50–100 mmol of $\text{HCO}_3^-$ per day to replace the $\text{HCO}_3^-$ lost in buffering the strong acids, such as sulphuric acid from sulphur-containing amino acids, produced by metabolism. It achieves this by secreting 50–100 mmol of $\text{H}^+$ in excess of the 4300+ mmol needed to reabsorb filtered $\text{HCO}_3^-$. The excess $\text{H}^+$ is excreted in combination with buffers, as explained in the following two sections (1.1.2 and 1.1.3).

1.1.2 Urinary titratable acidity

Some secreted $\text{H}^+$ reacts with $\text{HPO}_4^{2-}$ in the tubular fluid to form $\text{H}_2\text{PO}_4^-$. This excreted $\text{H}_2\text{PO}_4^-$ represents the major component of the urinary titratable acids (TA); these are defined as those weak acids in urine that can be titrated, to bring the pH of an acidic
Abbreviations:

- [HCO$_3^-$] bicarbonate concentration in mmol.l$^{-1}$
- PCT proximal convoluted tubule
- DTL descending thin limb
- TAL thick ascending limb
- DCT distal convoluted tubule
- CD collecting duct

Figure 1.1: Luminal bicarbonate and pH changes along the nephron.
urine back to the pH of the glomerular filtrate (usually 7.4). The pK for the phosphate buffer system is 6.8, and from the Henderson-Hasselbalch equation

\[ \text{pH} = 6.8 + \log \left( \frac{[\text{HPO}_4^{2-}]}{[\text{H}_2\text{PO}_4^-]} \right) \]

the ratio of HPO_4^{2-} to H_2PO_4^- is 4:1 in the glomerular filtrate (pH 7.4), i.e., 80% of the buffer is in the form that can react with H^+. In the proximal tubule the effectiveness of this buffer system is limited by luminal pH, but it is important in the distal nephron, and in a maximally acidic urine (pH 4.5) the ratio [HPO_4^{2-}]:[H_2PO_4^-] is 1:200. The normal range for phosphate excretion is 15–50 mmol per day (standard UCH figures), indicating that the amount of phosphate normally delivered to the distal nephron allows 12–40 mmol of H^+ to be excreted as H_2PO_4^- per day. TA is often calculated from phosphate excretion and urine pH, but the TA fraction of urine also includes H^+ ion combined with small amounts of urinary buffers such as uric acid (a weak acid with pK 5.75) and creatinine (a weak base with pK 4.97).

### 1.1.3 The role of ammonium

The ammonia buffer system has greater capacity than the phosphate system for excess H^+, and thus has a very important role in renal H^+ excretion. The dissociation reaction for the ammonia buffer system is

\[ \text{NH}_4^+ = \text{NH}_3 + \text{H}^+ \]

This reaction has a pK of 9.1 (Bank and Schwartz, 1960a), which is more alkaline than any body fluid, so virtually all of the total ammonia in the body fluids is present as the ammonium ion (NH_4^+). It may seem, therefore, that the amount of NH_3 available to react with secreted H^+ would be insignificant. However, NH_3 is a highly effective buffer for secreted H^+ because of two important factors:

1. NH_3 is synthesised in the epithelial cells of the proximal tubule and secreted into the tubular fluid. Thus the amount of NH_3 available to function as a buffer is not limited to the tiny amount, approximately 0.02 mmol.l^{-1} (Newsholme and Leech, 1984), entering the tubular fluid by glomerular filtration. Almost all of the NH_3 synthesised in the epithelial cells is derived from glutamine (see section 5.1.1 for a review of this subject). The enzyme glutaminase is abundant in the mitochondria of tubular epithelial cells. The remaining NH_3 synthesised in the proximal cells is derived from other amino acids, in particular alanine and glycine (Pitts, 1971).
2. NH₃ and NH₄⁺ have markedly different solubility characteristics. NH₃ is moderately lipid soluble and can diffuse passively across cellular membranes. In contrast, NH₄⁺ is polar and crosses cell membranes poorly.

Until recently it was accepted that the transfer of ammonia from the proximal tubular cell to the urine was entirely passive (Pitts, 1973). Thus, as NH₃ was synthesised and its concentration in the epithelial cell rose, NH₃ diffused out of the cell. Although NH₃ could diffuse into either the tubular lumen or the peritubular capillaries, diffusion into the lumen (NH₃ 'secretion') was favoured because NH₃ entering the lumen immediately reacted with previously secreted H⁺ to form NH₄⁺. The concentration of NH₃ in the tubular fluid therefore remained low, maintaining an NH₃ concentration gradient between tubular cell and lumen which was favourable for further diffusion of NH₃. The NH₄⁺, because of its non-diffusible nature, remained in the tubular fluid and was excreted. Thus ammonia was said to be excreted by non-ionic diffusion followed by ion trapping, a process commonly referred to as diffusion trapping.

Exciting developments in the last few years have shown that the above concepts are too simple. Ammonia movements in the kidney are not entirely passive and cannot be explained purely by non-ionic diffusion of NH₃. There is strong evidence that ionic movement of NH₄⁺ may occur by mechanisms involving paracellular diffusion or mediated transport; in fact, NH₄⁺ can be transported by several carriers of potassium. The subject is reviewed in detail in section 5.1.2. Ammonia is synthesised in the proximal tubule and enters the lumen by both non-ionic diffusion and by ion exchange. It then enters the loop of Henle and much of it is reabsorbed in the thick ascending limb (TAL) both by non-ionic diffusion and by active transport. Absorption in the TAL results in accumulation of ammonium in the medullary interstitium by countercurrent multiplication, i.e., part of the ammonia reabsorbed in the TAL is secreted into the descending limb to create a recycling pathway for ammonia between the two limbs of Henle's loop. The ammonium in the interstitium also enters the collecting duct, principally by non-ionic diffusion of NH₃, to produce a high concentration of NH₄⁺ in the urine. The countercurrent multiplication of ammonia is depicted in Figure 5.2 on page 95.

The excretion of ammonium is regulated partly through control of ammoniagenesis in the proximal tubule and partly through control of ammonia transport within the kidney. These two factors are discussed in the following section.
1.2 Factors controlling urinary ammonium excretion

There are several factors believed to control NH$_4^+$ excretion in health and disease; these can broadly be divided into those which produce changes in ammoniagenesis and those which influence the transfer of total ammonia from the renal tubular cell to the urine.

1. Influences on ammoniagenesis.

(a) Systemic acid-base state. Urine pH varies with changes in systemic acid-base state, and in turn will regulate urinary ammonia by diffusion trapping (see 2a). However, chronic acid administration raises excretion of NH$_4^+$ more than can be explained simply by increased diffusion trapping; there is also increased activity of the proximal tubular enzymes involved in ammoniagenesis (Pitts, 1973). Conversely, chronic alkali administration appears to reduce renal ammonium excretion (Lemieux et al., 1985) to a greater extent than can be explained by a reduction in diffusion trapping caused by increased urine pH.

(b) Availability of substrate. Urinary NH$_4^+$ is derived principally from plasma glutamine in the proximal cell (see section 5.1.1). The amount of substrate available could directly influence the level of ammoniagenesis (Squires et al., 1976; Welbourne, 1987). A lowered renal plasma flow, and consequent reduction in rate of supply of substrate for ammoniagenesis, may account for a subnormal level of NH$_4^+$ excretion as observed in renal failure (Steinmetz et al., 1965), see also 1d), and in sodium chloride depletion (Clarke et al., 1955).

(c) State of potassium homeostasis. Plasma potassium (K$^+$) appears to be an important regulator of ammoniagenesis. K$^+$ depletion results in an adaptive increase in the renal capacity to produce ammonia (Tannen, 1977), which is virtually identical to the changes found in response to chronic metabolic acidosis (see 1a). K$^+$ deficiency has been shown to lower renal intracellular pH (Adam et al., 1986) which may provide the adaptive stimulus to ammoniagenesis. Whether a low plasma K$^+$ concentration per se stimulates renal ammoniagenesis remains controversial. The influence of a high K$^+$ intake on ammonia metabolism has received less attention than the potassium-depleted state. Nevertheless, a high plasma potassium concentration has been shown to suppress the synthesis of ammonia (Tannen, 1977).
(d) **Nephron mass.** In chronic renal failure there is a reduced ability to synthesise ammonia due to the diminished number of nephrons (Walls et al., 1975). There is also a reduced renal blood flow (RBF), thus the filtered substrate load is lower which may contribute to the subnormal rate of production of NH$_4^+$ (see 1b). In many patients with renal disease, it is difficult to know whether a reduced ammonium excretion is due to a reduced number of nephrons or a reduced RBF.

(e) **Prostaglandin availability.** Inhibition of prostaglandin synthesis has been reported to accentuate the renal ammoniagenic response in the rat to an acute decrease in systemic pH (Jones et al., 1984). *In vitro* studies using isolating perfused kidney, renal cortical tubules and LLC-PK$_1$ cells indicate that PGF$_{2\alpha}$ may be the inhibitory metabolite (Tannen and Goyal, 1986; Sahai et al., 1992). The precise possible mechanism of action of PGF$_{2\alpha}$ and the metabolic steps involved require further investigation.

(f) **Adrenal hormones.** Adrenal insufficiency impairs urinary acid and ammonia excretion and reduces renal ammonia production. Increased renal ammoniagenesis does occur in adrenalectomised animals subjected to chronic metabolic acidosis, although the response is less than in normal animals (Welbourne, 1989). These findings indicate that adrenal hormones play a permissive role in renal ammoniagenesis, but it is not certain whether they are involved in a regulatory fashion in the adaptive response. An excess of mineralocorticoids may cause hypokalaemia and a deficiency is often associated with hyperkalaemia. Thus the effects of mineralocorticoids on ammonium excretion may be mediated by potassium (see 1c).

(g) **Other humoral influences.** *In vitro* studies using isolated dog renal cortical tubules indicate that insulin stimulates renal ammonium production and concurrently reduces glucose production (Chobanian and Hammerman, 1987), and that growth hormone stimulates ammonium production from glutamine (Rogers et al., 1989). *In vitro* studies using isolated tubules from rats have demonstrated that angiotensin II stimulates ammoniagenesis and gluconeogenesis (Guder, 1979). The metabolic sites of action of these hormonal effects have not been identified. Furthermore, the physiological implications of these effects have yet to be defined.
2. Influences on the transfer of total ammonia to the urine.

(a) *pH of urine.* Non-ionic NH$_3$ in the tubular cell diffuses down a concentration gradient into the urine where it combines with H$^+$ to form ionized NH$_4^+$. In this latter form it is "trapped" in the tubular fluid and cannot diffuse out (Pitts, 1973). As NH$_3$ leaves the cell, NH$_4^+$ within the cell dissociates to replace it. The rate of diffusion of NH$_3$ is determined by its concentration gradient, which in turn is controlled by the pH of the tubular cell and the tubular urine. When H$^+$ secretion by the tubular cells reduces urine pH, the urine NH$_3$ concentration is reduced as it is converted to NH$_4^+$. The concentration gradient between cell and urine is then increased and the rate of NH$_3$ diffusion into the urine rises.

(b) *Urine flow at the site of tubular entry of NH$_4^+$.* Reduced NH$_4^+$ concentrations in the tubular lumen, caused by increased rates of tubular flow at various points in the nephron, may lead to greater efflux of NH$_3$ from the tubular cell by non-ionic diffusion (Robinson and Owen, 1965) and consequently increased ammonium excretion in the urine.

(c) *Metabolic inhibitors or stimulants.* In addition to non-ionic diffusion of NH$_3$, transport of NH$_4^+$ as such can occur across the tubular epithelia. This mechanism may involve paracellular diffusion or mediated transport. There are three proposed active transporters of NH$_4^+$ in the kidney:-

i. Both membrane vesicle studies (Kinsella and Aronson, 1981a) and *in vitro* perfusion studies (Nagami et al., 1986) have demonstrated that NH$_4^+$ may be secreted into the lumen on the apical membrane Na$^+$-H$^+$ exchanger of the proximal tubule, most likely by substituting for H$^+$ to produce Na$^+$-NH$_4^+$ exchange.

ii. Active NH$_4^+$ absorption by the thick ascending limb occurs predominantly by substitution of NH$_4^+$ for K$^+$ on the apical membrane Na$^+$-K$^+$-2Cl$^-$ cotransporter (Garvin et al., 1988).

iii. Some evidence for NH$_4^+$ uptake into renal cells by substitution for K$^+$ on the basolateral Na$^+$-K$^+$-ATPase in the thick ascending limb has been provided (Garvin et al., 1985).

Inhibition or stimulation of any of these transporters could reduce or enhance the transfer of NH$_4^+$, with a consequent effect on urinary ammonium excretion.
(d) **Mechanical factors.** There are several diffusion barriers through which ammonia must pass before it enters the final urine. Ammoniagenesis occurs in the proximal tubule, where $\text{NH}_3$ enters the luminal fluid. In the thick ascending limb, $\text{NH}_3$ enters the interstitium by non-ionic diffusion. That which enters in the outer medulla can diffuse into the descending limb and be carried to the inner medulla (by the countercurrent multiplier system). $\text{NH}_3$ in the interstitium also diffuses into the collecting tubule, where it is trapped and excreted as $\text{NH}_4^+$ (Buerkert et al., 1982). The permeability of these various barriers might be influenced by renal disease.

(e) **Potassium availability.** Levels of plasma potassium outside the normal range, associated with a total body deficit or excess of $\text{K}^+$, may regulate ammoniagenesis (see 1c) but may also influence the transfer of $\text{NH}_4^+$ along the nephron. As mentioned in 2c, $\text{NH}_4^+$ may be transported by several carriers of $\text{K}^+$. The concentration of $\text{K}^+$ competing with $\text{NH}_4^+$ for the carrier site may therefore influence the transport of $\text{NH}_4^+$ into the lumen (Du Bose et al., 1990).

The prevailing view is that *diffusion trapping* is the most important factor influencing the transfer of ammonium to the urine whilst *systemic acid-base state* is the most important factor influencing ammoniagenesis. This classical explanation of ammonium excretion occurring via non-ionic diffusion of $\text{NH}_3$ and subsequent trapping of $\text{NH}_4^+$ is presented in current physiological texts (Ganong, 1991; Guyton, 1991; Lamb et al., 1991). These and other texts all explain physiological changes in ammonium excretion as being *entirely* due to (i) acid-base changes influencing ammoniagenesis, and (ii) changes in diffusion trapping secondary to urine pH. This view of ammonium excretion can be criticised since the evidence for *diffusion trapping* is based on relatively few experiments. Major points of criticism can be summarised as follows:-

1. That the experimental protocols employed to produce the acid-base disturbance used to influence urine pH may also have influenced ammoniagenesis.

2. That quantitative changes of urinary ammonium excretion with respect to changes in urine pH do not fit the ideal model of diffusion trapping. Work on various mammalian species, including man, has related urinary ammonium to urine pH by its excretion *rate*; if diffusion trapping is the prevailing mechanism one might expect that urinary *concentration* of ammonium would be a variable closely related to urine pH,
and as such would provide an alternative model. In man, there is a doubling of \( \text{NH}_4^+ \) excretion rate per unit fall in pH. The theoretical basis of non-ionic diffusion of weak acids and bases has been thoroughly examined (Milne et al., 1958) and would predict a tenfold increase in urinary \( \text{NH}_4^+ \) concentration per unit fall in pH.

### 1.3 Aims of this investigation

Ammonium excretion was re-examined in man to assess the roles of: (i) ion trapping, (ii) systemic acid-base state, (iii) other factors previously mentioned, e.g., rate of flow along tubular lumen, (iv) ammoniagenesis, and the possible interaction of these factors.

If the passive process of diffusion trapping is the primary mechanism determining ammonium excretion it should influence urinary ammonium content to a greater extent than other processes. Few attempts to validate this proposal have been performed except during brief manipulations of systemic acid-base state. The theory of diffusion trapping predicts a linear relationship between the logarithm of the concentration of ammonium and urine pH (Milne et al., 1958). However, a linear relationship has been described between the logarithm of ammonium excretion rate and pH (see chapter 3).

The analysis of \( \text{NH}_4^+ \) diffusion trapping involves frequent timed urine samples and deliberate manipulations of urine pH, and is therefore very suitable for human experimentation. The following studies were carried out to investigate the importance of various factors believed to regulate \( \text{NH}_4^+ \) excretion. In the protocols listed below, urine pH was varied over the whole physiological range in order to examine if the relationship still held and could be interpreted as evidence of the primary role of the diffusion trapping mechanism in ammonium excretion.

The factors investigated were as follows.

1. **The systemic acid-base state.** Acute acidosis may increase \( \text{NH}_4^+ \) excretion by stimulating ammoniagenesis as well as by increasing diffusion trapping. Conversely, acute alkali loading may suppress ammoniagenesis. The effects of sodium sulphate and acetazolamide, both being agents producing opposing changes in urine pH and systemic pH, were compared to those when both urine and systemic pH were changed in the same direction.

2. **The chronic acid-base state.** Studies involving both chronic acidosis and chronic alkali loading were carried out to evaluate the possibility that the chronic acid-base
state may directly influence ammoniagenesis in the proximal tubular cell, independently of any action in influencing diffusion trapping through an effect on urine pH.

3. The availability of glutamine substrate. Oral administration of glutamine, the main precursor of urinary ammonium, was used to increase plasma glutamine content in order to assess whether its availability was rate-limiting to renal ammonium excretion.

4. Metabolic states which may influence ammoniagenesis. The effects of mineralocorticoid supplementation and deficiency, using 9α-fludrocortisone and spironolactone respectively, were studied to investigate whether these metabolic alterations can influence urinary ammonium excretion.

5. The rate of fluid flow at various sites in the nephron. Studies were performed involving both water and osmotic diuresis to evaluate any influence on ammonium secretion. Water diuresis can influence luminal flow only beyond the proximal tubule whereas osmotic diuresis increases flow throughout the nephron.

6. Metabolic inhibition by pharmacological diuretics. Four commonly prescribed diuretics, known to inhibit renal ion transport mechanisms, were studied. The compounds employed were the benzothiadiazide bendrofluazide, which acts mainly in the distal convoluted tubule, the loop diuretics bumetanide and frusemide, which act in the loop of Henle, and the potassium-sparing diuretic amiloride, which acts in the late distal tubule.

The precise criteria for each study are discussed in the relevant sections of chapters 2 and 4. The effects of renal disease and of ageing on ammonium excretion were also investigated. However, these are discussed in appendices rather than in the main text, because much of the information used was not collected by myself.
Chapter 2

Procedures and Methods

2.1 Standard procedure

Studies were performed using volunteers of both sexes, with an age range of 22–61 years. Permission to perform the studies was obtained from the joint University College Hospital/Medical School Ethical Committee, and the fully informed consent of all the subjects was given.

Control data were obtained from volunteer laboratory personnel performing their usual daily routine and consuming food and fluids ad lib.. None of the subjects smoked at any time and caffeine-containing drinks and alcohol were avoided during the short-term studies. Spontaneously voided urine samples were collected over periods of time ranging from 7–183 minutes, depending on urine flow, the flow rate being corrected by a creatinine measurement (see section 2.2.3). No catheters were used. Urine likely to be more alkaline than pH 7.00 was collected under mineral oil. The Henderson-Hasselbalch equation for the bicarbonate buffer system in the body can be simplified to \( \text{pH} = 6.1 + \log\left(\frac{[\text{HCO}_3^-]}{[\text{CO}_2]}\right) \).

The loss of CO\(_2\) from an already alkaline urine would produce a falsely high pH reading. Mineral oil delays the loss of CO\(_2\) and thus delays the pH change likely to occur in an alkaline urine (Oster et al., 1975). Immediately after collection the volume of urine and its pH and osmolality were measured (see section 2.2.2) then approximately 10 ml of each sample was frozen in the presence of thymol to prevent bacterial degradation.

In all the studies performed the purpose was to produce as many urine samples with as many different pH values as possible. After resting urine pH and flow rate had been determined for the initial samples, the pH was manipulated in order to produce as wide a range of values as possible. As urine pH is unpredictable, this was not easily achieved. Subjects took oral ammonium chloride (NH\(_4\)Cl) to acidify and oral sodium bicarbonate...
(NaHCO₃) to alkalise the urine. The NH₄Cl was administered orally in units of 1g of powder contained in a gelatin capsule, and NaHCO₃ taken as 1g tablets. The dose taken depended upon how large a stimulus was required, according to the urine pH of the immediately preceding samples, in order to collect samples over a wide range of pH values. The doses of NH₄Cl and NaHCO₃ used were small, typically less than 0.05g.kg⁻¹ body weight (b.w.) being administered each time. This is approximately 50% of the amount of ammonium chloride used as a standard urinary acidification protocol (Wrong and Davies, 1959) in which the plasma total CO₂ fell by 4.1 ± 1.5 mmol.l⁻¹ (mean of 42 paired observations) yet was still usually in the range regarded as normal. The doses of sodium bicarbonate used in the present study were only around 15% of those used previously to examine the effects of prolonged alkali administration in man (van Goidsenhoven et al., 1954); in this previous study plasma total CO₂ rose by an average of 4.6 mmol.l⁻¹ but was still usually in the range regarded as normal. Thus the small amounts of NH₄Cl and NaHCO₃ used in the present studies would produce predictable minor degrees of systemic acid-base change. As the exact degree of alteration produced would not influence the interpretation of results it was not necessary to measure these changes.

Excretory rates are usually expressed as either per 70 kg body weight or per 1.73 m² body surface area. Insofar as almost all the results described in this thesis are comparisons against self, the choice is immaterial; however the former is used throughout since doses of diuretics, steroids etc. are prescribed according to weight, not surface area. The standard calculation of body surface area in Subjects 9 and 10 (the two principal subjects used during these studies), according to a conventional height/weight nomogram (Du Bois and Du Bois, 1916), yielded results expressed per 1.73 m² which are less than 5% different from that expressed per 70 kg. The results from all subjects are presented in this thesis corrected per 70 kg body weight.

2.1.1 Circumstances examined

The normal relationship between urinary ammonium content and pH was established by studies on ten subjects, from whom spontaneously voided urine samples were collected. The whole range of physiological urine pH, approximately 4.5–8.0, was achieved by the use of ammonium chloride and sodium bicarbonate, as detailed above in section 2.1.

The factors listed in section 1.3 were investigated to establish what changes, if any, they produced in the control relationship between urinary ammonium excretion rate and pH.
a. Administration of sodium sulphate. Sodium sulphate was given intravenously as an intense urinary acidifying stimulus. Previous sodium sulphate infusion experiments have shown that the acidifying response to the sulphate load was enhanced by avid sodium retention (Schwartz et al., 1955), yet whether 9α-fludrocortisone, a low sodium diet, or a combination of both were used to stimulate sodium reabsorption, the same level of lowering of urine pH was achieved. On the day before the present infusion 1 mg of a potent mineralocorticoid, 9α-fludrocortisone, was taken orally every 8 hours to stimulate renal tubular sodium reabsorption. Immediately before the infusion 4 g of sodium bicarbonate was consumed orally to prevent ‘dilutional’ acidosis (Asano et al., 1966; Garella et al., 1975), then 2500 ml of 0.1 M sodium sulphate was infused intravenously at a rate of 500 ml every 30–40 minutes, and urine collections made over the following 5–6 hours. Blood was taken, for analysis of creatinine and electrolytes, 90 minutes before the end of the infusion. Infusion of sodium sulphate caused vomiting on two occasions in Subject 10, and because of this on both occasions the study had to be curtailed early.

b. Administration of acetazolamide. Acetazolamide was administered orally, in doses of between 50 mg and 250 mg, to produce highly alkaline urine by increasing urinary bicarbonate excretion. The urine produced following this stimulus was collected under mineral oil.

c. Long term acidosis. Chronic acidosis was induced by the administration of oral ammonium chloride (8 g.70 kg−1.day−1 or 2.2 mmol.kg−1.day−1). This was taken as 1 g gelatin capsules for a period of 7 days, during which time 24-hour urine collections were made. On the eighth day, after several highly acid urine samples had been obtained, venous blood was taken for analysis of plasma bicarbonate and potassium content. Following this, 20 g of oral sodium bicarbonate was administered. Subsequent urine samples obtained were collected under mineral oil. Subject 9 required further small amounts of sodium bicarbonate to render the urine alkaline. Separate urine collections were continued over the following 48 hours, and blood samples were taken 6 hours and 24 hours after bicarbonate administration, to assess how long systemic acidosis was maintained.
d. **Long term alkali loading.** Oral sodium bicarbonate was administered for 21 days as 1 g tablets (11 g.70 kg⁻¹.day⁻¹ or 2.0 mmol.kg⁻¹). 24-hour urine collections were made throughout this period of alkali loading. At the end of this time venous blood was taken for analysis of bicarbonate and potassium, and separate urine collections were continued over the following 52 hours. Urinary pH manipulations were made with up to 3 g of oral ammonium chloride or sodium bicarbonate.

e. **Administration of glutamine.** Glutamine was administered orally both as a single dose and as repeated doses.

   (i) **Single dose administration.** Three control urine samples were collected and blood taken for plasma amino acid analysis (Cynober et al., 1985). A glutamine load of 38 g.70 kg⁻¹ b.w. or 3.5 mmol.kg⁻¹ was taken in 500 ml water over a 15–20 minute period, then urine samples were collected for up to seven hours and further blood taken for amino acid analysis. Subject 10 also performed this study during chronic acidosis, induced as in (c) above.

   (ii) **Repeated dose administration.** The initial glutamine load was taken as above, then further loads of 9.5 g.70 kg⁻¹ b.w. were taken in 100 ml water every 30 minutes for the following six hours. Urine pH was manipulated by up to 0.05 g.kg⁻¹ b.w. of oral ammonium chloride or sodium bicarbonate as samples were collected, and further blood was taken for amino acid analysis.

f. **Administration of a mineralocorticoid.** Oral doses of 0.5 mg of the mineralocorticoid 9α-fludrocortisone were taken every 8 hours for 48 hours. (This amount is five times the maximal clinical dose recommended for adrenocortical insufficiency.) Spontaneous urine samples were collected for the following 36 hours, the pH being manipulated with oral ammonium chloride or sodium bicarbonate. Blood was taken 24 hours after urine collections began.

g. **Administration of a mineralocorticoid antagonist.** The mineralocorticoid antagonist spironolactone was taken orally as 100 mg every 8 hours. After 48 hours, urine collections were begun and these continued for the following 120 hours of spironolactone administration, urine pH being manipulated with oral ammonium chloride or sodium bicarbonate. Blood was taken during the final 24 hours for analysis of plasma bicarbonate and potassium.
h. Water diuresis. For the purpose of these studies, water diuresis was arbitrarily defined as a urine flow of more than 5 ml.70 kg\(^{-1}\).min\(^{-1}\) with a solute excretion rate of less than 1425 \(\mu\)Osm.70 kg\(^{-1}\).min\(^{-1}\). An average value for resting urine flow rate in the two experimental subjects was 0.5 ml.70 kg\(^{-1}\).min\(^{-1}\); a flow rate of ten times this figure was chosen to represent a diuresis. Normal plasma osmolality is approximately 285 mOsm.kg\(^{-1}\), so the figure chosen to represent the upper limit of solute excretion for a water diuresis was 1425 \(\mu\)Osm.70 kg\(^{-1}\).min\(^{-1}\) (corresponding to 0.5 ml.min\(^{-1}\), with an osmolality of 285 mOsm.kg\(^{-1}\)). A water load equivalent to 20 ml.kg\(^{-1}\) b.w. was drunk to achieve a maximal water diuresis, producing flow rates of up to 20 ml.min\(^{-1}\). Water diuresis was sustained with subsequent water intake equivalent to the urine volume passed. The study was continued until at least ten urine samples had been collected.

i. Osmotic diuresis. Osmotic diuresis was, in these studies, arbitrarily defined as above for flow rate, but with a solute excretion rate of more than 2100 \(\mu\)Osm.70 kg\(^{-1}\).min\(^{-1}\). An average resting value for solute excretion rate in the two experimental subjects was 425 \(\mu\)Osm.70 kg\(^{-1}\).min\(^{-1}\) (corresponding to a flow rate of 0.5 ml.min\(^{-1}\) with an osmolality of 850 mOsm.kg\(^{-1}\)). A solute excretion rate of five times this amount was chosen to represent an osmotic diuresis. A solute output of up to 8000 \(\mu\)Osm.70 kg\(^{-1}\).min\(^{-1}\) was achieved by the intravenous infusion of 2500 ml of 10% mannitol over six hours. Spontaneous urine samples were collected, and the urine pH altered by the oral administration of either ammonium chloride or sodium bicarbonate. Blood was taken, for analysis of plasma creatinine and electrolytes, one hour before the end of the infusion.

j. Administration of a pharmacological diuretic. Four commonly prescribed diuretics were examined; one benzothiadiazide (bendrofluazide), two loop diuretics (frusemide and bumetanide) and one potassium-sparing diuretic (amiloride). The doses of diuretics used are given in the relevant parts of section 4.6. Urine pH was manipulated with both ammonium chloride and sodium bicarbonate. Plasma samples were taken for the analysis of creatinine and electrolytes.

k. Renal disease. See Appendix A for details.

l. Ageing. See Appendix B for details.
2.2 Sample analysis

2.2.1 Analysis of blood

Blood samples of 10–20 ml were centrifuged at 3000 r.p.m. (2500×g) for 15 minutes to separate the plasma, which was then analysed for electrolytes and metabolites by Simultaneous Multichannel Analysis with Computer (SMAC). The most important measurements were:

- Creatinine — for the calculation of creatinine clearance (Chasson et al., 1961).
- Bicarbonate — as a measure of metabolic acidosis (Skeggs and Hochstrasser, 1964).
- Potassium — to ascertain if the subject was either hypo- or hyperkalaemic (Rao et al., 1973).

Other electrolyte levels were measured and any anomalies noted. For example, hypocalcaemia was observed to occur during the sodium sulphate infusions; this phenomenon has been noted in sulphate infusion studies by other workers (Cole, 1989). In the glutamine studies, plasma glutamine and other amino acids were measured by column chromatography (Spackman et al., 1958; Cynober et al., 1985).

2.2.2 Analysis of urine

All measurements were done in duplicate; the chemical methods used throughout were standard.

- pH — measured by glass electrode with a digital pH meter at room temperature, calibrated with buffer solutions of known pH.
- Osmolality — measured by freezing point depression with an Advanced digimatic osmometer. Accuracy was checked with calibration standards.
- Ammonium — measured by a microdiffusion method (Conway, 1962).
- Creatinine — measured by the Jaffé reaction (Bonsnes and Taussky, 1945). Absorbance readings were made at 520 nm on a u.v. visible recording spectrophotometer, calibrated with a standard curve.
- Titratable Acid — measured by titration of urine against 0.05 M sodium hydroxide solution to pH 7.4 (Albright and Reifenstein, 1948).
2.2.3 Measurement of renal clearances

- The measurement of Glomerular Filtration Rate (GFR) was by means of $^{51}$Cr EDTA clearance. Clearance was calculated from the fall in plasma concentration of $^{51}$Cr EDTA after a single intravenous injection (Chantler et al., 1969; Russell et al., 1985). Endogenous creatinine clearance is also used as a measure of GFR, as it is simpler to measure than EDTA. Creatinine is continually liberated into the extracellular fluid from muscle cells. It is freely filtered and not reabsorbed, but there is some active secretion into the tubules so that slightly more is present in the urine than is accounted for by filtration alone. However, the standard chemical analysis slightly over-estimates plasma creatinine so that when the ratio of clearance is calculated the two opposing effects tend to cancel out and the value for creatinine clearance is usually extremely close to that for inulin clearance. Despite its limitations, creatinine clearance has been shown to give highly reproducible results, and to provide a good estimate of GFR. When required $^{51}$Cr-EDTA will give a more accurate determination of GFR (Ham and Piepsz, 1992).

- Effective Renal Plasma Flow (ERPF) was measured using $^{125}$I-Hippuran which was injected intravenously. Clearance was calculated from the fall in plasma concentration (Constable et al., 1979; Tauxe et al., 1982), blood samples having been taken at regular intervals. $^{125}$I-Hippuran does not enter blood cells and is rapidly taken up from the plasma by renal tubular cells and secreted into the tubule, so that little or none remains in the plasma entering into the renal vein. The amount of Hippuran entering the tubule and the urine (depending on the level of Hippuran in the plasma, as the tubular maximum can be exceeded) is denoted by RPF$\times$P. As the excretion rate is UV and all of the Hippuran which enters the tubule is excreted, $RPF = U \times (V/P) = CHippuran$. A typical renal plasma flow for a 70 kg man would be approximately 625 ml.min$^{-1}$. Renal blood flow is given by $RPF/(1 - \text{haematocrit})$ where the haematocrit is the fraction of blood volume occupied by red blood cells. For a typical haematocrit of 0.45 and RPF of 625 ml.min$^{-1}$ renal blood flow would be around 1000 ml.min$^{-1}$.

Resting GFR (from $^{51}$Cr EDTA clearance) and ERPF (from $^{125}$I-Hippuran clearance) measurements for the two principal subjects studied in this thesis, Subjects 9 and 10, are given in Table 2.1. These values are well within the normal range. Creatinine clearances
Table 2.1: GFR and ERPF for Subjects 9 and 10

<table>
<thead>
<tr>
<th></th>
<th>Subject 9</th>
<th>Subject 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR</td>
<td>103</td>
<td>140</td>
</tr>
<tr>
<td>ml.min⁻¹</td>
<td>84</td>
<td>143</td>
</tr>
<tr>
<td>ml.min⁻¹.70kg⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERPF</td>
<td>445</td>
<td>647</td>
</tr>
<tr>
<td>ml.min⁻¹</td>
<td>361</td>
<td>662</td>
</tr>
<tr>
<td>ml.min⁻¹.70kg⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

were used as a guide to GFR in the remaining control subjects, and were all found to be in the normal range. Creatinine clearances were also regularly measured in Subjects 9 and 10. Clearance was calculated from the mean urinary creatinine excretion (measured in mg.min⁻¹) divided by the plasma creatinine (measured in mg.l⁻¹). The resulting figure was then multiplied by 1000 to give a clearance value in ml.min⁻¹.

A urinary creatinine correction factor was used in all studies to compensate for the effect of variations in bladder emptying, particularly important for samples collected over short time periods. The daily urinary excretion of creatinine is relatively constant and uninfluenced by diet, urine flow or exercise (Doolan et al., 1962). A daily mean value for urinary creatinine excretion (measured in mg.min⁻¹) was calculated, and urinary ammonium content and osmolality readings adjusted according to this factor, i.e., multiplied by the mean creatinine value and divided by the sample creatinine value. As an example, the formula

\[
\frac{\text{sample urine flow (ml.min}^{-1}) \times \text{mean daily creatinine excretion (mg.min}^{-1})}{\text{sample creatinine excretion (mg.min}^{-1})}
\]

gives a corrected value in ml.min⁻¹ for the sample urine flow rate.
Chapter 3

Statistical Methods and Establishment of Control Data

The purpose of this chapter is to describe the statistical methods which are used in the remainder of the thesis to analyse the experimental data. The principal tool used is the well known method of linear regression. The discussion below is based on a standard medical statistics text (Armitage and Berry, 1987), and all the arguments used can be found there. In this chapter, page numbers refer to this reference text. Further details can be found in a specialist text (Draper and Smith, 1981).

The simplest and best known measure of goodness of fit to a straight line is the correlation coefficient ($r$). Most computer packages, however, quote the quantity denoted by $R^2$, which is in fact the square of the correlation coefficient. By taking the square root, $r$ can be recovered from $R^2$ to within an ambiguity about its sign. In this thesis $R^2$ is used throughout, and is quoted as a percentage of unity. This choice is necessary because, in later sections, regression on several independent variables is needed; in these cases $R^2$ still has an interpretation whereas the correlation coefficient itself does not, since no meaning can be attached to the sign (page 303). The consistent use of $R^2$ enables comparisons to be made between regressions on different variables, though this comparison is only relative and cannot be associated with a probability that one model is better than another (pages 152 and 303).

### 3.1 Urinary ammonium related to pH

Although it is generally accepted amongst renal physiologists that the relationship between urinary pH and ammonium excretion can be well expressed using the logarithm of the excretion rate, it is worthwhile validating this approach as it is used throughout the
analysis of data discussed in this thesis.

3.1.1 Ammonium concentration

Urine pH was studied over the whole physiological range, as observed both with spontaneous changes and by deliberate short term alkalinisation and acidification of the urine. Eight normal volunteers provided between them a total of 100 control, or 'normal', data, i.e., spontaneously voided urine samples covering a wide range of pH. The raw data that was obtained expresses the urinary ammonium concentration, or $U[\text{NH}_4^+]$, in mmol.l$^{-1}$. The simplest relationship to explore is the correlation of these values against pH. This was done for each of the eight subjects individually, and also for their pooled data, the value of $R^2$ being computed in each case. The results are given in the second line of Table 3.1. It can be seen that the correlation to a straight line is generally poor; this fact is further illustrated in Figure 3.1. No general trend can be observed except that the lower the pH the higher the ammonium concentration. As pH is a logarithmic function it also seemed worth plotting ammonium concentration logarithmically (see third line in Table 3.1). Conversely, ammonium concentration was plotted against urinary hydrogen ion concentration (first line in the table). Neither of these variations offered a clear improvement.

<table>
<thead>
<tr>
<th>U[\text{NH}_4^+] v U[H^+]</th>
<th>1/9 33.0 43.3 26.5 20.9 34.4 17.6 37.3 11.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>U[\text{NH}_4^+] v UpH</td>
<td>18.5 38.0 41.9 9.6 18.5 40.7 14.2 47.0 16.1</td>
</tr>
<tr>
<td>log U[\text{NH}_4^+] v UpH</td>
<td>25.0 45.7 43.5 5.4 50.7 60.7 17.8 49.7 24.3</td>
</tr>
<tr>
<td>U NH$_4^+$ rate v UpH</td>
<td>60.0 54.2 66.7 35.4 62.2 92.1 80.3 70.1 53.9</td>
</tr>
<tr>
<td>log U NH$_4^+$ rate v UpH</td>
<td>64.1 55.7 74.3 34.2 72.3 89.1 92.4 80.1 65.1</td>
</tr>
</tbody>
</table>

3.1.2 Ammonium excretion rate

In a plot of the rate of ammonium excretion ($\mu$mol.min$^{-1}$) against pH (Figure 3.2) a more marked trend could be observed. In all individual cases (see fourth line of Table 3.1) much higher correlations were found than when $\text{NH}_4^+$ concentration was used. Nevertheless, in certain cases, such as Subject 7, where points were spread across the whole physiological pH
Subjects 1-8: Ammonium concentration

Figure 3.1: Urinary ammonium concentration vs. pH (Subjects 1-8)
- Subjects 1-8: Ammonium excretion rate (linear scale)

Figure 3.2: Urinary ammonium excretion rate vs. pH (Subjects 1-8)
range, it was noticed that the variance of the data (i.e., the vertical spread of points with similar pH values) decreased with increasing pH. Use of the logarithm of the ammonium excretion rate offered the possibility of a more uniform variance as well as a more linear plot.

When the logarithm of the rate of ammonium excretion was plotted against the pH the $R^2$ value improved yet further (see fifth line of Table 3.1). It can be seen that for all but two subjects the $R^2$ value in this line was the highest, and for the other two subjects it came a close second. The $R^2$ value for the whole group was also increased; this pooled data is plotted in Figure 3.3. The data for Subject 7, from whom the data was most evenly spread across the pH range, is plotted in Figure 3.4. From this figure it can be seen that plotting ammonium concentration against pH produced a fairly random distribution, and that the plots of ammonium excretion rate are much more closely related to urine pH, the logarithmic one being the better. Figures 3.5 and 3.6 show the ammonium excretion rates for Subjects 9 and 10 plotted against urine pH both linearly and logarithmically. For these subjects also the logarithm of the rate offers a better fit to a straight line; the values of $R^2$ improved after the logarithmic transformation from 66.2% to 75.0% in Subject 9 and from 71.1% to 75.5% in Subject 10.

Nevertheless, owing to the influence of R.F. Pitts, the relationship between urinary ammonium excretion rate and pH is still sometimes described arithmetically. Dr. Pitts' original illustration showing an arithmetic relationship between these two variables in normal and chronically acidotic dogs (Pitts, 1948) is reproduced even now in texts of renal physiology. In both groups, when urine was acutely alkalinated by an intravenous infusion of sodium bicarbonate, ammonium excretion decreased; urinary ammonium excretion was shown to fall in a linear fashion as urine pH increased. I have converted this illustration into numerical data. The comparison of the linear rate versus pH has an $R^2$ value of 88.9%, and that of the logarithmic rate an $R^2$ value of 85.9%. These values are both large and by no means suggest that the linear form is in general preferable to the logarithmic (the figures are similar to those achieved by Subject 6 in Table 3.1). Moreover Pitts' pH values never drop below 5.3, and the low spread of pH values reduces the contrast between the two forms. What is more interesting is that the data given by Pitts for excretion under chronic acidosis, when expressed logarithmically, lie on a straight line parallel to the normal data line (see below in section 3.3). This fact is completely hidden in Pitts' illustration of the arithmetic rate versus pH.
Figure 3.3: Urinary ammonium logarithmic excretion rate vs. pH (Subjects 1-8)
Figure 3.4: Ammonium concentration, linear and logarithmic excretion rates (Subject 7)

- Subject 7: Ammonium concentration
- Subject 7: Ammonium excretion rate (linear scale)
- Subject 7: Ammonium excretion rate (log scale)
Figure 3.3: Linear and logarithmic ammonium excretion rates vs. pH (Subject 9)

- Subject 9: Ammonium excretion rate (linear scale)
- Subject 9: Ammonium excretion rate (log scale)
Figure 3.6: Linear and logarithmic ammonium excretion rates vs. pH (Subject 10)
From this discussion it appears to be the logarithmic rate of ammonium excretion, rather than the urinary concentration of ammonium or linear rate of excretion, which is the variable most closely related to urine pH. For the sake of completeness, it was checked whether the use of a non-linear function (such as a quadratic or cubic function of the form $y = a + bx + cx^2 + dx^3$) of the logarithm of the excretion rate might better fit the data. Standard methods of multiple regression (see section 10.3 of the reference text) allow the significance of the contribution of these extra terms to be tested. These tests were carried out on the data from Subjects 1 to 8, and also on that from two further Subjects, 9 and 10, from whom more data were available (39 and 80 points respectively), but the extra terms proved to be entirely insignificant, and the linear model therefore appears to be adequate.

It thus appears to be very reasonable to model the logarithm of urinary ammonium excretion rate as a linear function of pH. Therefore, for statistical purposes, this model is used from now on, i.e., a linear relationship between the two quantities is assumed.

3.2 Establishment of a set of control data

Spontaneous minute to minute changes in urine pH are not thought to be caused by systemic acid-base changes (Bank and Schwartz, 1960b; Tannen, 1980). In order to provide a wide range of urine pH for the control, or ‘normal’, data used in these studies, ammonium chloride ($\text{NH}_4\text{Cl}$) and sodium bicarbonate ($\text{NaHCO}_3$) were taken (see section 2.1). These agents produce an acute systemic pH change and correspondingly alter urine pH. These acute acid-base changes lasted for less than 8 hours, which is regarded as too short a time to cause adaptations in renal tubular ammoniagenesis (Pitts, 1973). It has, however, been suggested that such short term acid-base changes may influence cellular ammoniagenesis (Tannen and Sastrasinh, 1984). The $\text{NH}_4\text{Cl}$ and $\text{NaHCO}_3$ data were therefore statistically compared to the data collected without any stimuli being used (see below in section 3.3). The relationship between ammonium excretion rate and urine pH was found to be the same whether or not $\text{NH}_4\text{Cl}$ or $\text{NaHCO}_3$ were used. This is interpreted as an indication that these acute systemic acid-base changes had not produced significant alterations to renal ammoniagenesis. The data set used as a control for the comparison of experimental data in this thesis therefore consists of the $\text{NH}_4\text{Cl}$ and $\text{NaHCO}_3$ data combined with that obtained without using any stimuli.

Further studies were carried out in which either sodium sulphate or acetazolamide were used to produce particularly low or high urine pHs respectively without inducing corre-
sponding systemic acid-base changes (see sections 2.1.1a and 2.1.1b for details). When these data were compared to those collected without using any stimuli, a small but arguably significant difference was detected between the two sets of data. (This difference is discussed later in section 6.1.1. Statistical differences were also observed if the sodium sulphate and acetazolamide data were separately compared with the unstimulated data, although the interpretation is more difficult due to the very different pH ranges covered by these sets.) The data obtained from Subject 9 with sodium sulphate or acetazolamide was plotted in Figure 3.7 after having been combined with the data obtained without stimulation; the figure also shows the NH₄Cl and NaHCO₃ data after combination with the unstimulated data. Hence the unstimulated data is that which appears in both plots. Figure 3.8 gives the corresponding plots for Subject 10.

Had the sodium sulphate or acetazolamide produced no significant change in the relationship between ammonium excretion rate and urine pH, those data could have been included in the control set to provide a wider range of urine pH. In practice, the effect of the inclusion of these data is very small, and would make no material difference to the statistical interpretation of the experimental data.

3.3 Statistical analysis of data

It was demonstrated in section 3.1 that it is reasonable to view the relationship between the logarithm of ammonium excretion rate and urine pH as a linear one. The problem here is to decide whether two sets of data, obtained under different circumstances, lie on the same or different straight lines. This problem is fully discussed in sections 9.4 and 9.5 of the reference text (Armitage and Berry, 1987), and again in section 10.2. Recall that page numbers in parentheses refer to this text.

3.3.1 Linear regression

A linear relationship is expressed by the equation \( y = a + bx \) where, in the present case, \( y \) represents the logarithm of ammonium excretion rate, \( x \) represents urine pH, \( a \) is the intercept of the line on the \( y \) axis and \( b \) is the slope of the line. Both ammonium excretion rate and urine pH vary randomly. Linear regression of \( y \) on \( x \) provides the values of \( a \) and \( b \) which best predict the value of ammonium excretion rate corresponding to a given urinary pH. Regression of \( x \) on \( y \) provides the best values of \( a \) and \( b \) for the converse prediction. In this thesis no such predictions will be made; rather, the functional relationship between
Figure 3.7: Different urinary acidifying and alkalising stimuli (Subject 9)
- Subject 10: Control, Sodium Sulphate, Acetazolamide
- Subject 10: Control, Ammonium Chloride, Sodium Bicarbonate

Figure 3.8: Different urinary acidifying and alkalinizing stimuli (Subject 10)
the two variables is of interest. Both regression lines should then be considered (page 271). In the present case it is probably more natural to think of ammonium excretion rate as a function of pH, and thus only the regression equations of y on z have actually been quoted, although those for x on y have also been calculated. In fact, the ratio of the slopes of the two lines is precisely $R^2$, so for data sets with high linear correlation the two lines will be close together, and the conclusions drawn from the different lines are likely to agree. For data sets with a low value of $R^2$ it is helpful to bear both lines in mind. In general, the conclusions pointed to by the regression analyses for y on x and those for x on y are the same if the value of $R^2$ for the experimental data is high. The values for the data sets used in this thesis are given in Table 3.2. It can be seen that the values are in general high, though the values for water diuresis are, for both subjects, no more than moderate, and for the glutamine data very low; these data are discussed later.

The comparison of regression lines for different data sets is discussed from two points of view in the reference text, although the results are exactly the same (page 312), and the following description is that based on the use of dummy variables (page 314). As an example, the comparison of the NH$_4$Cl and NaHCO$_3$ data for Subject 9 with the data obtained without stimuli is now considered. There are 39 pairs $(x, y)$ obtained without stimuli and 35 pairs with NH$_4$Cl and NaHCO$_3$. These data are first combined into a collection of 74 triples $(z, y, i)$, where i is a dummy variable indicating whether the triple came from the unstimulated data or from the NH$_4$Cl and NaHCO$_3$ data ($i = 0$ for the first 39 triples and $i = 1$ for the remainder; the letter i is intended to stand for intercept difference). If the data are now fitted, by means of multiple regression analysis, to the formula $y = a + bx + ci$, the values of $a$, $b$ and $c$ are found which fit best. This yields the two parallel lines which best fit the data sets, namely $y = a + bx$ for the unstimulated data and $y = (a + c) + bx$ for the NH$_4$Cl and NaHCO$_3$ data. If it is desired to find the two best lines without the constraint that they be parallel, a further variable $s = i \times x$ (for slope difference) can be added, and the formula $y = a + bx + ci + ds$ fitted by regression analysis. The two lines will then be $y = a + bx$ and $y = (a + c) + (b + d)x$. These two lines will be the same as the two lines found by doing simple regression analysis on the data sets separately. The rationale for doing the analysis in the way described is that standard methods, which are supplied by computer packages such as Minitab, enable the coefficients $d$ and $c$ to be tested for significant difference from zero. In this way the data sets can be tested for differences.
Table 3.2: Correlations of regression lines for data sets

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Data set description</th>
<th>Data points</th>
<th>Value of $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>unstimulated</td>
<td>39</td>
<td>75.70</td>
</tr>
<tr>
<td>9</td>
<td>chloride/bicarbonate</td>
<td>35</td>
<td>86.51</td>
</tr>
<tr>
<td>9</td>
<td>control</td>
<td>74</td>
<td>82.81</td>
</tr>
<tr>
<td>10</td>
<td>unstimulated</td>
<td>80</td>
<td>75.77</td>
</tr>
<tr>
<td>10</td>
<td>chloride/bicarbonate</td>
<td>30</td>
<td>78.96</td>
</tr>
<tr>
<td>10</td>
<td>control</td>
<td>110</td>
<td>76.81</td>
</tr>
<tr>
<td>9</td>
<td>sulphate/acetazolamide</td>
<td>26</td>
<td>92.36</td>
</tr>
<tr>
<td>10</td>
<td>sulphate/acetazolamide</td>
<td>33</td>
<td>74.01</td>
</tr>
<tr>
<td>9</td>
<td>long term acidosis</td>
<td>19</td>
<td>84.64</td>
</tr>
<tr>
<td>10</td>
<td>long term acidosis</td>
<td>21</td>
<td>84.50</td>
</tr>
<tr>
<td>9</td>
<td>long term alkali loading</td>
<td>33</td>
<td>92.98</td>
</tr>
<tr>
<td>10</td>
<td>long term alkali loading</td>
<td>22</td>
<td>77.42</td>
</tr>
<tr>
<td>9</td>
<td>glutamine</td>
<td>31</td>
<td>08.78</td>
</tr>
<tr>
<td>10</td>
<td>glutamine</td>
<td>38</td>
<td>49.51</td>
</tr>
<tr>
<td>9</td>
<td>9α-fludrocortisone</td>
<td>16</td>
<td>81.40</td>
</tr>
<tr>
<td>10</td>
<td>9α-fludrocortisone</td>
<td>17</td>
<td>75.76</td>
</tr>
<tr>
<td>9</td>
<td>spironolactone</td>
<td>36</td>
<td>89.13</td>
</tr>
<tr>
<td>10</td>
<td>spironolactone</td>
<td>64</td>
<td>79.12</td>
</tr>
<tr>
<td>9</td>
<td>water diuresis</td>
<td>58</td>
<td>61.67</td>
</tr>
<tr>
<td>10</td>
<td>water diuresis</td>
<td>98</td>
<td>62.43</td>
</tr>
<tr>
<td>9</td>
<td>osmotic diuresis</td>
<td>20</td>
<td>51.60</td>
</tr>
<tr>
<td>10</td>
<td>osmotic diuresis</td>
<td>17</td>
<td>80.16</td>
</tr>
<tr>
<td>10</td>
<td>bendrofluazide</td>
<td>20</td>
<td>77.55</td>
</tr>
<tr>
<td>9</td>
<td>frusemide</td>
<td>14</td>
<td>55.24</td>
</tr>
<tr>
<td>10</td>
<td>frusemide</td>
<td>21</td>
<td>86.27</td>
</tr>
<tr>
<td>10</td>
<td>bumetanide</td>
<td>19</td>
<td>80.63</td>
</tr>
<tr>
<td>9</td>
<td>amiloride</td>
<td>12</td>
<td>65.16</td>
</tr>
</tbody>
</table>
### Table 3.3: Comparison of control with acute systemic acid-base change (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>83.8</td>
<td>83.1</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>83.1</td>
<td>82.6</td>
</tr>
<tr>
<td>Single line</td>
<td>82.8</td>
<td>82.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of $s$</th>
<th>$i$</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>90.79%</td>
<td>71.66%</td>
<td>[-3% to 17%]</td>
</tr>
<tr>
<td>for X on Y</td>
<td>42.09%</td>
<td>50.95%</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.4: Comparison of control with acute systemic acid-base change (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>76.8</td>
<td>76.2</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>76.8</td>
<td>76.4</td>
</tr>
<tr>
<td>Single line</td>
<td>76.8</td>
<td>76.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of $s$</th>
<th>$i$</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>21.55%</td>
<td>16.03%</td>
<td>[-9% to 12%]</td>
</tr>
<tr>
<td>for X on Y</td>
<td>7.13%</td>
<td>26.77%</td>
<td></td>
</tr>
</tbody>
</table>

### 3.3.2 Summary table

Each time two sets of data are compared, the results are summarised in a table. The results for the comparison just described appear in Table 3.3. Table 3.4 gives the corresponding summary for Subject 10. The tables give the two best lines, the two best parallel lines, and the best single line for the data as given by regression of $y$ on $x$. For each equation a value of $R^2$ is supplied. This value will inevitably be larger the more variables are used, so it is customary to supply also an “adjusted” value of $R^2$ (page 303) which takes this into account. For example, the adjusted value of $R^2$ for the double lines for Subject 10 is not much larger than that for the parallel lines, and in the case of Subject 9 it is actually smaller, indicating that the double lines do not fit significantly better than parallel lines. However, this is no more than an indication, as mentioned at the start of this chapter, and the precise tests of significance for the slope difference (in the case of double lines) and for
intercept difference (in the case of parallel lines) are given in the table. As stated above, the calculations have been done for both the regression of \( y \) on \( x \) and for the regression of \( x \) on \( y \), even though the equations for \( x \) on \( y \) are not quoted.

A significance level below 95% is regarded throughout as insignificant. Thus Table 3.3 should be interpreted as follows. The test is made first for a significant difference in slopes of the two \( y \) on \( x \) lines, the null hypothesis being that the lines are parallel. Since the coefficient of \( s \), the slope difference, is insignificant (90.79%) two parallel lines are fitted. The difference in intercepts is then tested for significance, the null hypothesis being that the lines coincide. The coefficient of \( i \), the intercept difference, is again insignificant (71.66%) and the conclusion is that the data sets may be regarded as lying on the same straight line. As usual, the same conclusion is reached by examining the regressions of \( x \) on \( y \) (slope difference insignificant at 42.09%, intercept difference insignificant at 50.95%). The two corresponding data sets for Subject 10 may also be regarded as agreeing (Table 3.4).

The remaining part of the tables gives a 90% confidence interval for the percentage increase in ammonium excretion rate in the second data set. This increase is of interest only if the data sets are shown to lie on parallel but distinct lines; it is derived from the confidence interval for \( c \), the intercept difference, since an additive increase in the logarithm of excretion rates translates into a percentage increase in the rates themselves. Finally, the excretion rate corresponding to pH 6.0 in the second of the two parallel lines is given in order to illustrate the difference in actual excretion rates, the value 6.0 being used because it is in the middle of the physiological range of urine pH.

Tables for the comparison of the unstimulated data with that obtained with sodium sulphate or acetazolamide are given in Table 3.5 for Subject 9 and Table 3.6 for Subject 10. It can be observed that for Subject 9 the slope difference for the \( y \) on \( x \) lines is insignificant (at 73.48%) but the intercept difference is significant (99.38%). Similar remarks apply to Subject 10, for whom the slope difference for the \( x \) on \( y \) lines is also just significant.

As a final illustration the data given by Pitts (see section 3.1), under both normal and chronically acidotic conditions, is compared. The results of the computation are shown in Table 3.7. It can be seen that, in the regressions for both \( y \) on \( x \) and \( x \) on \( y \), the slope difference is entirely insignificant, whereas the intercept difference is strongly significant. This parallelism agrees with the results of similar data provided by Subjects 9 and 10, as described later in this thesis.
Table 3.5: Comparison of control, sodium sulphate and acetazolamide (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>91.0</td>
<td>90.6</td>
</tr>
<tr>
<td>$y = 2.983 - 0.312x + 0.173i - 0.043s$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parallel lines</td>
<td>90.8</td>
<td>90.5</td>
</tr>
<tr>
<td>$y = 3.182 - 0.346x - 0.083i$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single line</td>
<td>89.6</td>
<td>89.5</td>
</tr>
<tr>
<td>$y = 3.238 - 0.361x$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significance of $s$ for Y on X: 73.48%
Significance of $i$ for Y on X: 99.38%
Significance of $s$ for X on Y: 44.57%
Significance of $i$ for X on Y: 91.07%

Increase in rate: [-8% to -26%]
Rate at pH 6.0: 10.5 $\mu$mol.min$^{-1}$

Table 3.6: Comparison of control, sodium sulphate and acetazolamide (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>81.8</td>
<td>81.3</td>
</tr>
<tr>
<td>$y = 3.000 - 0.269x + 0.215i - 0.061s$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parallel lines</td>
<td>81.2</td>
<td>80.9</td>
</tr>
<tr>
<td>$y = 3.162 - 0.299x - 0.155i$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single line</td>
<td>78.3</td>
<td>78.1</td>
</tr>
<tr>
<td>$y = 3.288 - 0.328x$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significance of $s$ for Y on X: 93.00%
Significance of $i$ for Y on X: 99.99%
Significance of $s$ for X on Y: 95.72%
Significance of $i$ for X on Y: 77.14%

Increase in rate: [-19% to -39%]
Rate at pH 6.0: 16.4 $\mu$mol.min$^{-1}$

Table 3.7: Comparison of normal and chronic acidosis data, after R.F. Pitts

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>87.6</td>
<td>86.6</td>
</tr>
<tr>
<td>$y = 4.513 - 0.532x + 0.307i + 0.034s$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parallel lines</td>
<td>87.5</td>
<td>86.9</td>
</tr>
<tr>
<td>$y = 4.390 - 0.514x + 0.539i$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single line</td>
<td>65.8</td>
<td>65.0</td>
</tr>
<tr>
<td>$y = 4.875 - 0.545x$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significance of $s$ for Y on X: 35.39%
Significance of $i$ for Y on X: 100.00%
Significance of $s$ for X on Y: 9.71%
Significance of $i$ for X on Y: 100.00%

Increase in rate: [171% to 342%]
Rate at pH 6.0: 69.8 $\mu$mol.min$^{-1}$
Chapter 4
Experimental Results

In this chapter the results of the studies described in Chapter 2 are presented. For each subject a plot of the experimental data compared to control is shown, and the statistical analysis is summarised in a table as described in section 3.3.

4.1 Long term acidosis

Subjects 9 and 10 participated in this study. 24-hour urine collections were made for a period of seven days; during this time a fixed daily amount of ammonium chloride was taken in divided doses as 1 g gelatin capsules. Subject 9 (85 kg) took 10 g per day whilst Subject 10 (61 kg) took 7 g per day, so that the dose was approximately $8 g \times \frac{70}{b.w.} kg$ for both subjects. 24-hourly measurements of urinary ammonium were made and the results are given in Table 4.1 (in which Day 0 denotes a mean resting value of two control days).

Subject 9 continued to increase urinary ammonium output daily whereas the output from Subject 10 levelled off after Day 4. Note also that pH reached a minimum on the third day for both subjects. At the start of the eighth day a plasma sample was taken for bicarbonate and potassium analyses. The results were $\text{HCO}_3^-$ 18.0 mmol.l$^{-1}$, $\text{K}^+$ 4.1 mmol.l$^{-1}$ for Subject 9, and $\text{HCO}_3^-$ 19.6 mmol.l$^{-1}$, $\text{K}^+$ 4.5 mmol.l$^{-1}$ for Subject 10. The potassium values are within the normal range and the low bicarbonate values show that both subjects were acidotic. Administration of ammonium chloride was then stopped, and oral sodium bicarbonate was taken, in divided doses, over the following 24 hours to produce alkaline urines; Subject 9 required 45 g in total, whilst Subject 10 needed 20 g. Consecutive urine samples were collected under mineral oil over the 36 hour period following the cessation of the ammonium chloride and pH, ammonium and creatinine content measured. Acidosis started to diminish as soon as the ammonium chloride intake
Table 4.1: Ammonium excretion rates during acidosis (Subjects 9 and 10)

<table>
<thead>
<tr>
<th>Day</th>
<th>Subject 9 NH₄⁺ mmol.day⁻¹.70kg⁻¹</th>
<th>pH</th>
<th>Subject 10 NH₄⁺ mmol.day⁻¹.70kg⁻¹</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19.4</td>
<td>5.92</td>
<td>30.1</td>
<td>5.55</td>
</tr>
<tr>
<td>1</td>
<td>41.1</td>
<td>5.11</td>
<td>74.1</td>
<td>5.05</td>
</tr>
<tr>
<td>2</td>
<td>55.3</td>
<td>4.94</td>
<td>91.6</td>
<td>4.56</td>
</tr>
<tr>
<td>3</td>
<td>69.7</td>
<td>4.90</td>
<td>117.4</td>
<td>4.52</td>
</tr>
<tr>
<td>4</td>
<td>94.3</td>
<td>5.00</td>
<td>148.0</td>
<td>4.69</td>
</tr>
<tr>
<td>5</td>
<td>130.5</td>
<td>5.07</td>
<td>129.3</td>
<td>4.81</td>
</tr>
<tr>
<td>6</td>
<td>135.9</td>
<td>5.19</td>
<td>129.2</td>
<td>4.91</td>
</tr>
<tr>
<td>7</td>
<td>147.3</td>
<td>5.22</td>
<td>139.3</td>
<td>4.83</td>
</tr>
</tbody>
</table>

Table 4.2: Comparison of control and long term acidosis data (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>%R²</th>
<th>%R²adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.271 - 0.360x + 0.869i - 0.077s$</td>
<td>84.9</td>
<td>84.4</td>
</tr>
<tr>
<td>$y = 3.370 - 0.377x + 0.399i$</td>
<td>84.4</td>
<td>84.0</td>
</tr>
<tr>
<td>$y = 3.045 - 0.307x$</td>
<td>54.5</td>
<td>54.0</td>
</tr>
</tbody>
</table>

Significance of

- for Y on X: 92.30% 100.00%
- for X on Y: 88.23% 100.00%

Increase in rate [123% to 182%]
Rate at pH 6.0 32.2 μmol.min⁻¹

was stopped; for Subject 9 it gradually became less between 24–48 hours after the cessation of ammonium chloride, whilst for Subject 10 urine pH rose from 4.57 to 7.95 within the three hours subsequent to the last dose of sodium bicarbonate. Further plasma samples were also taken and measured for bicarbonate and potassium. Plasma from Subject 10 taken six hours after the start of sodium bicarbonate had HCO₃⁻ of 28.5 mmol.l⁻¹, showing no apparent acidosis, with a plasma K⁺ content of 3.3 mmol.l⁻¹.

The different responses of the experimental subjects to long term acidosis as noted above are reflected in the statistical analyses. Figure 4.1 plots the data for Subject 9. From Table 4.2 it is seen that the parallel lines model is quite acceptable and that the control and long term acidosis lines differ strongly. The data for Subject 10 is shown in Figure 4.2, from which it can be seen that only two urine samples had pH values below 5.2, both of these corresponding to high ammonium excretion rates. Consequently, when the data are analysed statistically, as in Table 4.3, a significant slope difference appears
Figure 4.1: Control vs. long term acidosis (Subject 9)

- Subject 9: Normal Data
- Subject 9: Long Term Acidosis Data

Urine pH

Urine NH₄⁺, µmol/min
Figure 4.2: Control vs. long term acidosis (Subject 10)
with the regression of $y$ on $x$, although not with $x$ on $y$. This slope difference is due to the large influence exerted by the two points at low pH. In fact removal of either data point causes the differences in the slopes to become insignificant (not shown in a table). More data in the acid range would be required to determine whether the slope change is a real phenomenon, i.e., whether for this subject the increase in ammonium excretion rate during chronic acidosis is always greater for acid urines than for normal to alkaline ones. For the remainder of the thesis it will be assumed there is no such variation with pH. Thus, although there was a substantial increase in the daily rate of urinary ammonium excretion in Subject 10 (an increase of nearly fivefold over four days, as shown in Table 4.1) this increase was rapidly abolished by sodium bicarbonate, and an overall change in excretion rate was not shown once urine pH had been manipulated. The results of these chronic acidosis studies are discussed in section 6.1.2.

### 4.2 Long term alkali loading

Subjects 9 and 10 participated in this study. A fixed amount of sodium bicarbonate was taken daily for 21 days in divided doses as 1 g tablets; 14 g per day for Subject 9 and 10 g per day for Subject 10, equivalent to approximately 11 g.70 kg$^{-1}$ b.w. per day.

24-hour urine collections were made by Subject 10, and these were analysed for NH$_4^+$ and titratable acid (TA). Results are shown in Table 4.4. TA was determined by titration of the urine to pH 7.4 with 0.05 M sodium hydroxide. H$^+$ excretion was then calculated as $(TA - HCO_3^-) + NH_4^+$. Urine more alkaline than pH 6.0 contains appreciable amounts of bicarbonate and calculation of hydrogen ion excretion in this way results in signifi-
Table 4.4: Daily TA and NH$_4^+$ excretion rates (Subject 10)

<table>
<thead>
<tr>
<th>Day</th>
<th>TA − HCO$_3^-$ mmol</th>
<th>NH$_4^+$ mmol</th>
<th>Total H$^+$ mmol</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52.7</td>
<td>59.5</td>
<td>112.2</td>
<td>5.38</td>
</tr>
<tr>
<td>2</td>
<td>9.8</td>
<td>32.2</td>
<td>42.0</td>
<td>6.24</td>
</tr>
<tr>
<td>3</td>
<td>57.1</td>
<td>34.9</td>
<td>92.0</td>
<td>6.26</td>
</tr>
<tr>
<td>4</td>
<td>-42.1</td>
<td>14.3</td>
<td>-27.8</td>
<td>6.94</td>
</tr>
<tr>
<td>5</td>
<td>-41.8</td>
<td>24.1</td>
<td>-17.7</td>
<td>6.99</td>
</tr>
<tr>
<td>6</td>
<td>-32.0</td>
<td>16.5</td>
<td>-15.5</td>
<td>6.75</td>
</tr>
<tr>
<td>7</td>
<td>-50.5</td>
<td>13.3</td>
<td>-37.2</td>
<td>7.08</td>
</tr>
<tr>
<td>8</td>
<td>-112.6</td>
<td>12.8</td>
<td>-99.8</td>
<td>7.26</td>
</tr>
<tr>
<td>9</td>
<td>-24.3</td>
<td>20.4</td>
<td>-3.9</td>
<td>6.87</td>
</tr>
<tr>
<td>10</td>
<td>-51.1</td>
<td>25.4</td>
<td>-25.7</td>
<td>6.98</td>
</tr>
<tr>
<td>11</td>
<td>-127.1</td>
<td>17.4</td>
<td>-109.7</td>
<td>7.48</td>
</tr>
<tr>
<td>12</td>
<td>-4.3</td>
<td>29.7</td>
<td>-25.4</td>
<td>6.66</td>
</tr>
<tr>
<td>13</td>
<td>-124.7</td>
<td>14.1</td>
<td>-110.6</td>
<td>7.28</td>
</tr>
<tr>
<td>14</td>
<td>-81.6</td>
<td>16.2</td>
<td>-65.4</td>
<td>7.00</td>
</tr>
<tr>
<td>15</td>
<td>-76.7</td>
<td>8.1</td>
<td>-68.6</td>
<td>7.13</td>
</tr>
<tr>
<td>16</td>
<td>-61.6</td>
<td>9.4</td>
<td>-52.2</td>
<td>7.15</td>
</tr>
<tr>
<td>17</td>
<td>-33.4</td>
<td>21.2</td>
<td>-12.2</td>
<td>6.84</td>
</tr>
<tr>
<td>18</td>
<td>-97.1</td>
<td>9.2</td>
<td>-87.9</td>
<td>7.52</td>
</tr>
<tr>
<td>19</td>
<td>-10.2</td>
<td>18.4</td>
<td>8.2</td>
<td>6.73</td>
</tr>
<tr>
<td>20</td>
<td>-60.0</td>
<td>18.2</td>
<td>-41.8</td>
<td>7.04</td>
</tr>
<tr>
<td>21</td>
<td>-115.8</td>
<td>8.5</td>
<td>-107.3</td>
<td>7.62</td>
</tr>
<tr>
<td>22</td>
<td>-88.5</td>
<td>17.4</td>
<td>-71.1</td>
<td>7.16</td>
</tr>
</tbody>
</table>

cant error, because the TA depends both on bicarbonate and dissolved CO$_2$ content, and variable amounts of the latter may be lost by volatilisation. As almost all of the urine specimens here were more alkaline than pH 6.0, the following procedure for determination of (TA − HCO$_3^-$) was followed (Albright and Reifenstein, 1948). A standard excess (1 ml) of strong acid was added, and the urine sample evacuated with a vacuum pump to drive off CO$_2$, and back titrated to pH 7.4. As shown in Table 4.4, when (TA − HCO$_3^-$) is determined in this way, specimens more alkaline than pH 6.6 have a negative value because of their high concentration of bicarbonate. The results show that the dose of sodium bicarbonate used in this study, 11 g.70kg$^{-1}$.day$^{-1}$ (equivalent to 130 mmol.70kg$^{-1}$.day$^{-1}$), markedly increased the daily amount of bicarbonate excreted.

The dose of sodium bicarbonate used in this study was approximately one eighth of that used in a previous study of long term alkali loading (van Goidsenhoven et al., 1954) in which plasma HCO$_3^-$ remained in the normal range. Thus it was not deemed necessary
to monitor plasma $\text{HCO}_3^-$ during the three weeks of alkali loading in the present study. At the end of the period of 24-hourly urine collections a plasma sample was taken for bicarbonate and potassium. $\text{HCO}_3^-$ was 26.0 mmol.l$^{-1}$ and $\text{K}^+ 4.7$ mmol.l$^{-1}$ in Subject 9, and 29.9 mmol.l$^{-1}$ and 4.3 mmol.l$^{-1}$ respectively in Subject 10; these values are all within the normal range. For this reason the study is described as long term alkali loading and not long term alkalosis. Continuous urine collections were then made over the following 2 days, and ammonium chloride taken in small doses (less than 3 g) to promote a wide range of urine pH. Further plasma samples were taken at the end of the study for bicarbonate and potassium. Values obtained for both subjects were found to lie in the normal range.

Both subjects showed a significant reduction in ammonium excretion rate with long term alkali loading as compared with their control data (Figures 4.3 and 4.4, with the usual statistical analyses in Tables 4.5 and 4.6). These results are discussed in section 6.1.2.
Figure 4.3: Control vs. long term alkali loading (Subject 9)

- Subject 9: Normal Data
- Subject 9: Long Term Alkali Data

Urine pH

Urinary NH₄⁺, μmol/min
- Subject 10: Normal Data
- Subject 10: Long Term Alkali Data

Figure 4.4: Control vs. long term alkali loading (Subject 10)
4.3 Oral glutamine administration

Urinary ammonium is derived principally from plasma glutamine in the kidney (see section 5.1.1). Administration of glutamine orally in man has been shown to increase ammonium excretion (Welbourne et al., 1972). Oral glutamine was given in these studies as a substrate for renal ammonium production, to investigate whether the amount of substrate available in the proximal tubular cell could directly influence the level of ammoniagenesis (see section 1.2). Both subjects were studied under normal acid-base conditions. Subject 10 was also given glutamine during chronic acidosis to see if the effects of these two stimuli were additive.

4.3.1 Effects of a single glutamine load

In both subjects, a priming load of 500 ml water was drunk at the start of the experiment. The bladder was then emptied and two or three timed urine collections made during the following 1 1/2 - 2 hours. Blood was then collected, using heparin as an anticoagulant, for analyses of basal levels of plasma amino acids. A glutamine load of 38 g.70kg\(^{-1}\) body weight, or approximately 3.5 mmol.kg\(^{-1}\) b.w., was then taken orally in 500 ml of water, over a 20 minute period. Urine samples were collected at intervals of 30 minutes during the following 2 hours, heparinised blood being taken for amino acid analysis after one hour and after two hours. Hourly urine collections were continued for the next 5 hours, producing samples covering a pH range of 5.1 to 7.2.

Subject 10 also underwent a similar treatment during a period of chronic acidosis, achieved by the oral administration of ammonium chloride as described in section 4.1. On the morning of the experiment, as in the study under normal acid-base conditions, two 30-minute urine collections were made and blood taken for amino acid analysis. A glutamine load of 33 g (equivalent to 3.5 mmol.kg\(^{-1}\) body weight) was then taken in 500 ml water during the next 15 minutes. Urine samples were collected at intervals of 30 minutes over the next two hours, heparinised blood samples being taken after one and two hours. Hourly urine specimens were collected for the next five hours. The pH range of the urine produced was 4.35 to 6.15. Urine flow rates and osmolalities were within the normal range (see section 2.1.1) for all the single glutamine load studies, i.e., not high enough to be classed as either water or osmotic diuresis.

In both subjects, under normal acid-base conditions, plasma glutamine rose after one
hour to a value which was approximately three times above the basal level and which
was maintained for two hours. Plasma glutamate reached almost threefold the basal level
within one hour, and declined to little over twice the basal value after two hours. Actual
values are shown in Table 4.7.

In Subject 10, under conditions of chronic acidosis, plasma glutamine content rose
after one hour to more than five times its basal value, and was almost six times basal level
after two hours. Plasma glutamate levels had doubled after one hour and after two hours
were almost 2½ times the basal value (see Table 4.7 for actual values).

### 4.3.2 Effects of repeated glutamine loads

This study was performed on each of Subjects 9 and 10. An initial timed urine sample was
collected over approximately one hour, and then a blood sample was taken for analysis of
basal levels of plasma amino acids. A priming dose of glutamine substrate of 38 g.70 kg⁻¹
body weight, or approximately 3.5 mmol.kg⁻¹ b.w., was taken, as in the experiments
described in section 4.3.1, and a second blood sample taken after approximately one hour.
Timed urine samples were collected, and the pH manipulated by the ingestion of 1–3 g
of ammonium chloride or sodium bicarbonate, to provide as wide a range as possible of
urine pH values. Additional glutamine loads of 9.5 g.70 kg⁻¹ b.w., i.e., one quarter of the
original priming dose, were taken in 100 ml water over the following 5 hours, each load
being taken gradually over consecutive 30 minute periods, so that there was a continuous
ingestion of glutamine. A third blood sample was taken after approximately three hours,
and a fourth taken one hour after the final load of glutamine, i.e., after six hours. Two
Table 4.8: Plasma glutamine and glutamate levels (repeated doses) (Subjects 9 and 10)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time (min)</th>
<th>Plasma Glutamine μmol.l⁻¹</th>
<th>Plasma Glutamate mg.100ml⁻¹</th>
<th>Plasma Glutamine μmol.l⁻¹</th>
<th>Plasma Glutamate mg.100ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject 9</td>
<td>0</td>
<td>1200</td>
<td>17.52</td>
<td>72</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>3348</td>
<td>48.88</td>
<td>162</td>
<td>2.38</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>3897</td>
<td>56.89</td>
<td>106</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>3236</td>
<td>47.25</td>
<td>111</td>
<td>1.63</td>
</tr>
<tr>
<td>Subject 10</td>
<td>0</td>
<td>564</td>
<td>8.23</td>
<td>49</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>3336</td>
<td>48.71</td>
<td>129</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>195</td>
<td>3304</td>
<td>48.24</td>
<td>134</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>3765</td>
<td>54.97</td>
<td>106</td>
<td>1.56</td>
</tr>
</tbody>
</table>

more urine samples were collected after this final blood sample had been taken.

Plasma glutamine content increased to almost three times its basal level after one hour in Subject 9, and over the same period was raised almost six fold in Subject 10. As glutamine continued to be administered, these elevated levels remained fairly constant in both subjects, as can be seen in Table 4.8. Plasma glutamate levels had risen to approximately 2 1/2 times their respective basal values after one hour in both subjects. In Subject 9, glutamate had fallen to less than double the basal value after three hours, and remained at the same level for a further three hours. Plasma glutamate in Subject 10 continued to rise to reach almost three times the basal level after three hours, but had declined to twice the basal value after six hours. The actual values are shown in Table 4.8.

4.3.3 Analysis of glutamine data

For both subjects, there was no significant difference in the urinary ammonium excretion data between the single and the repeated glutamine loading procedures. Neither was a significant difference found in the data obtained from glutamine loading under conditions of chronic acidosis (Subject 10). The experimental data were therefore combined to form one population for each subject. Figures 4.5 and 4.6 show these data plotted against the respective normal data. There is a large dispersion of the data, which is probably due to the variation in the levels of plasma glutamine, which were increased by between three and six times basal levels (see Tables 4.7 and 4.8).

When the data from Subject 9 is subjected to statistical analysis (Table 4.9) it appears not to lie on a line parallel to the control data. This is not surprising in view of the very
Figure 4.5: Control vs. glutamine (Subject 9)

- Subject 9: Normal Data
- Subject 9: Glutamine Data

Urine pH

Urineary NH₄⁺, µmol/min
Figure 4.6: Control vs. glutamine (Subject 10)

- Subject 10: Normal Data
- Subject 10: Glutamine Data

Urine pH

Urinary NH$_4^+$, µmol/min
Table 4.9: Comparison of control and glutamine data (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$%R^2$</th>
<th>$%R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>67.8</td>
<td>66.9</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>61.9</td>
<td>61.2</td>
</tr>
<tr>
<td>Single line</td>
<td>33.9</td>
<td>33.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of $s$ for Y on X</th>
<th>100.00%</th>
<th>100.00%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Significance of $i$ for X on Y</td>
<td>100.00%</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

Increase in rate at pH 6.0: 28.8 $\mu$mol.min$^{-1}$

Table 4.10: Comparison of control and glutamine data (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$%R^2$</th>
<th>$%R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>66.2</td>
<td>65.5</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>65.9</td>
<td>65.4</td>
</tr>
<tr>
<td>Single line</td>
<td>58.0</td>
<td>57.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of $s$ for Y on X</th>
<th>71.09%</th>
<th>100.00%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Significance of $i$ for X on Y</td>
<td>100.00%</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

Increase in rate at pH 6.0: 38.1 $\mu$mol.min$^{-1}$

The data from Subject 10 (Figure 4.6) show a more recognisable trend. Table 4.10 shows that the glutamine and control data lie on parallel but distinct lines. Even for this subject, however, the correlation of the experimental data to a straight line is not good (see Table 3.2). These results, which indicate that administration of glutamine increases ammonium excretion rate at all pH values, are discussed in section 6.1.3.
Table 4.11: Comparison of control and 9α-fludrocortisone data (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>84.2</td>
<td>83.6</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>84.1</td>
<td>83.7</td>
</tr>
<tr>
<td>Single line</td>
<td>84.0</td>
<td>83.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of s, i for Y on X</th>
<th>60.78%</th>
<th>14.95%</th>
<th>Increase in rate</th>
<th>[-11% to 16%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>for X on Y</td>
<td>51.15%</td>
<td>88.98%</td>
<td>Rate at pH 6.0</td>
<td>13.3 μmol.min$^{-1}$</td>
</tr>
</tbody>
</table>

Table 4.12: Comparison of control and 9α-fludrocortisone data (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>76.9</td>
<td>76.3</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>76.7</td>
<td>76.3</td>
</tr>
<tr>
<td>Single line</td>
<td>76.6</td>
<td>76.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of s, i for Y on X</th>
<th>67.88%</th>
<th>44.34%</th>
<th>Increase in rate</th>
<th>[-8% to 20%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>for X on Y</td>
<td>73.05%</td>
<td>83.58%</td>
<td>Rate at pH 6.0</td>
<td>25.2 μmol.min$^{-1}$</td>
</tr>
</tbody>
</table>

4.4 A mineralocorticoid and its antagonist

Subjects 9 and 10 participated in these studies. In the first study, the mineralocorticoid 9α-fludrocortisone was taken orally, at a dose of 0.5 mg at 8-hourly intervals for 4 days. Spontaneous urine samples were collected continuously after the first 48 hours, urine pH being manipulated by the administration of up to 4 g ammonium chloride or sodium bicarbonate. A plasma sample was taken 24 hours after urine collections had begun, for analysis of K$^+$ and HCO$_3^−$. Potassium was 3.8 mmol.l$^{-1}$ in Subject 9 and 4.2 mmol.l$^{-1}$ in Subject 10, both values within the normal range. Bicarbonate values were 32.0 mmol.l$^{-1}$ (slightly above the normal range) and 27.0 mmol.l$^{-1}$ respectively.

The results from each subject have been plotted against their respective normal data and appear to come from the same population (Figures 4.7 and 4.8). In both statistical analyses (Tables 4.11 and 4.12) the differences between the experimental and control lines
Figure 4.7: Control vs. 9α-Fludrocortisone (Subject 9)
Figure 4.8: Control vs. 9α-fludrocortisone (Subject 10)

- Subject 10: Normal Data
- Subject 10: 9α-Fludrocortisone

Urine pH

Urine NH₄⁺, μmol/min

40 45 50 55 60 65 70 75 80
Table 4.13: Comparison of control and spironolactone data (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$% R^2$</th>
<th>$% R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>$y = 3.371 - 0.360x - 0.285i + 0.043s$</td>
<td>85.5</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>$y = 3.161 - 0.340x - 0.031i$</td>
<td>85.2</td>
</tr>
<tr>
<td>Single line</td>
<td>$y = 3.166 - 0.343x$</td>
<td>84.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of</th>
<th>$s$</th>
<th>$i$</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>87.75%</td>
<td>85.36%</td>
<td>[1% to -14%]</td>
<td>12.2 $\mu$mol.min$^{-1}$</td>
</tr>
<tr>
<td>for X on Y</td>
<td>98.57%</td>
<td>59.39%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

are not significant. From these calculations it is concluded that there is no significant change in ammonium excretion rate with 9α-fludrocortisone from control.

In the second study, the mineralocorticoid antagonist spironolactone was taken orally, in a dose of 100 mg, at 8-hourly intervals for 48 hours. Urine collections were then begun. The delay between taking spironolactone and the collection of urine was introduced because it is recognised that 24–48 hours are needed for the drug to exert its full effect (Richards, 1969). Spironolactone administration was continued three times per day for the remainder of the study, during which time consecutive spontaneously voided urine samples were collected. Urine pH was manipulated by doses of up to 4 g of oral ammonium chloride or sodium bicarbonate, and collections were continued for 65 hours (Subject 9) and 72 and 103 hours (Subject 10). Approximately 10 hours before the end of the study, blood was taken for plasma analysis of potassium, bicarbonate and creatinine; $K^+$ was 4.8 mmol.l$^{-1}$ and $HCO_3^-$ 24.0 mmol.l$^{-1}$ in Subject 9 and 4.5 mmol.l$^{-1}$ and 31.0 mmol.l$^{-1}$ respectively in Subject 10, creatinine clearance was 104.2 ml.min$^{-1}$.70 kg$^{-1}$ in Subject 9 and 86.0 ml.min$^{-1}$.70 kg$^{-1}$ in Subject 10, all values within the normal range.

The spironolactone data for both subjects were plotted against their respective normal data (Figures 4.9 and 4.10). These figures suggest that the experimental data differ little from the control data. The statistical analyses of Tables 4.13 and 4.14 confirm this suggestion. For Subject 9 a slope difference in the regression lines for $x$ on $y$ appears, though not for $y$ on $x$. This difference appears to arise from the presence of three points of very alkaline urine pH, for which no there are no corresponding control data. The removal of one of these points removes the slope difference (not shown in a table). It is
Figure 4.9: Control vs. spironolactone (Subject 9)
Figure 4.10: Control vs. spironolactone (Subject 10)

- Subject 10: Normal Data
- Subject 10: Spironolactone Data

Urine pH

Urine NH₄, µmol/min
Table 4.14: Comparison of control and spironolactone data (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$% R^2$</th>
<th>$% R^2_{\text{adj}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.016 - 0.272x + 0.020i - 0.013s$</td>
<td>80.1</td>
<td>79.8</td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.053 - 0.279x - 0.057i$</td>
<td>80.1</td>
<td>79.8</td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.074 - 0.286x$</td>
<td>79.3</td>
<td>79.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of $s$ for Y on X</th>
<th>$s$</th>
<th>$i$</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>43.80%</td>
<td>98.84%</td>
<td>[-5% to -19%]</td>
<td>21.1 μmol.min$^{-1}$</td>
</tr>
<tr>
<td>for X on Y</td>
<td>17.17%</td>
<td>51.07%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

therefore not possible to determine from these data if there is any departure from normal in the alkaline pH range. For Subject 10 it can be seen that the intercept difference for the regression lines for $y$ on $x$ is significant, but that for $x$ on $y$ is not. However the size of the change, even for the $y$ on $x$ line, is modest, circa 10%, and it therefore appears that for both subjects the administration of spironolactone causes no change, or only an extremely small change, in ammonium excretion rate. The results of both of these studies are discussed in section 6.1.4.

4.5 Water and Osmotic Diuresis

Subjects 9 and 10 participated in these studies. The procedure for water diuresis was followed as detailed in section 2.1.1h. All subjects were able to achieve a urine flow of at least 5 ml.70kg$^{-1}$.min$^{-1}$ with solute excretion rate of less than 1425μOsm.70kg$^{-1}$.min$^{-1}$, thus satisfying the criteria adopted for water diuresis. The ranges of solute excretion for Subjects 9 and 10 are shown in Table 4.15.

The data collected for each subject have been plotted against their respective control data in Figures 4.11 and 4.12. From these figures it appears that in both subjects the
Figure 4.1: Control vs. water diuresis (Subject 9)

- Subject 9: Normal Data
- Subject 9: Water Diuresis Data
Figure 4.12: Control vs. water diuresis (Subject 10)

- Subject 10: Normal Data
- Subject 10: Water Diuresis Data

Urine pH

Urine NH₄⁺, umol/min
Table 4.16: Comparison of control and water diuresis data (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>73.0</td>
<td>72.3</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>71.3</td>
<td>70.9</td>
</tr>
<tr>
<td>Single line</td>
<td>63.9</td>
<td>63.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of</th>
<th>$s$</th>
<th>$i$</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>99.40%</td>
<td>100.00%</td>
<td>[29% to 58%]</td>
<td>19.1 $\mu$mol.min$^{-1}$</td>
</tr>
<tr>
<td>for X on Y</td>
<td>6.49%</td>
<td>100.00%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.17: Comparison of control and water diuresis data (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>71.4</td>
<td>71.0</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>71.4</td>
<td>71.1</td>
</tr>
<tr>
<td>Single line</td>
<td>69.2</td>
<td>69.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of</th>
<th>$s$</th>
<th>$i$</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>25.65%</td>
<td>99.99%</td>
<td>[13% to 34%]</td>
<td>29.9 $\mu$mol.min$^{-1}$</td>
</tr>
<tr>
<td>for X on Y</td>
<td>94.33%</td>
<td>100.00%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Water diuresis produced a rate of urinary ammonium excretion greater than or equal to that found under control conditions.

Table 4.16 gives the statistical analysis for Subject 9. A significant difference in slopes is shown for the regression lines for $y$ on $x$, though not for $x$ on $y$. This disparity is a result of the low correlation of the experimental data to a straight line (see Table 3.2), as discussed in section 3.3. All four regression lines (both $y$ on $x$ and $x$ on $y$ for each set of data) are shown in Figure 4.11. For both regressions the difference in intercepts is very significant, and the correlation is better if more than one line is used, so there is a definite difference between the experimental and control data. No explanation is readily apparent for the low linear correlation of the experimental data, and the consequent slope difference in the regressions for $y$ on $x$. In Subject 10 (Figure 4.12) the water diuresis data appear to be similar to the control data. Nevertheless statistical analysis (Table 4.17) reveals a significant increase in the rate of ammonium excretion with water diuresis. These results
are discussed in section 6.2.1.

The procedure followed for the osmotic diuresis study was as detailed in section 2.1.1. Both subjects achieved urine flows of at least 5 ml·70 kg⁻¹·min⁻¹ with solute excretion rates greater than 2100 µOsm·70 kg⁻¹·min⁻¹, thus satisfying the requirements for osmotic diuresis, as shown in Table 4.15. The data are plotted against the corresponding control values in Figures 4.13 and 4.14, and it is clear that osmotic diuresis produced consistently higher rates of ammonium excretion at all pH values. The statistical analyses in Table 4.18 and Table 4.19 confirm this impression. In both subjects there is a clear and highly significant difference between the osmotic diuresis data and the control data. These results are discussed in section 6.2.1.
- Subject 9: Normal Data
- Subject 9: Osmotic Diuresis Data (Mannitol)
Figure 4.14: Control vs. osmotic diuresis (Subject 10)

- Subject 10: Normal Data
- Subject 10: Osmotic Diuresis Data (Mannitol)
Table 4.20: Plasma metabolites and electrolytes with bendrofluazide (Subject 10)

<table>
<thead>
<tr>
<th></th>
<th>Study 1 mmol.l(^{-1})</th>
<th>Study 2 mmol.l(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>4.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>27.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Chloride</td>
<td>100.0</td>
<td>99.0</td>
</tr>
<tr>
<td>Sodium</td>
<td>138.0</td>
<td>138.0</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.2</td>
<td>4.8</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.083</td>
<td>—</td>
</tr>
</tbody>
</table>

4.6 Pharmacological diuretics

The diuretics commonly prescribed fall into three main categories; benzo thiadiazides (‘thiazides’) which act in the distal convoluted tubule, loop diuretics which act principally in the thick ascending limb, and potassium-sparing diuretics which act in the late distal tubule. The aim of this study was to examine the possible inhibitory effects of these on ammonium excretion. One thiazide (bendrofluazide) was investigated, two loop diuretics (frusemide and bumetanide), and one potassium-sparing diuretic (amiloride).

4.6.1 Bendrofluazide

Bendrofluazide is a commonly prescribed benzo thiadiazide diuretic, and its effects were studied on two separate occasions in Subject 10. In both studies 5 mg of bendrofluazide, the standard therapeutic dose, was taken orally following bladder emptying at the start. Consecutive urine samples were collected and pH manipulated by the oral administration of up to 3 g of sodium bicarbonate or ammonium chloride. In the first study, a further 5 mg of bendrofluazide was taken five hours after the first. In the second study the dose was increased, because of no observable diuretic action in the first study, and 5 mg of bendrofluazide was taken at 2-hourly intervals. Both studies continued for ten hours. A blood sample was taken before the final urine sample was collected, and analysed for urea, creatinine and electrolytes. The results of this plasma analysis are shown in Table 4.20. The plasma creatinine value of 83 \(\mu\)mol.l\(^{-1}\) is equivalent to 9.38 mg.l\(^{-1}\). Creatinine clearance was calculated to be 104.5 ml.min\(^{-1}\) in Study 1 and 100.2 ml.min\(^{-1}\) in Study 2, both values within the normal range.

Urinary flow rates and minute excretion of solutes both fell within the normal range, permitting the experimental data to be plotted against the control data for Subject 10.
Table 4.21: Comparison of control and bendrofluazide data (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.016 - 0.272x - 0.176i - 0.009s$</td>
<td>79.7</td>
<td>79.2</td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.026 - 0.274x - 0.225i$</td>
<td>79.7</td>
<td>79.4</td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.044 - 0.283x$</td>
<td>71.5</td>
<td>71.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of</th>
<th>$s$</th>
<th>$i$</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>19.91%</td>
<td>100.00%</td>
<td>[-33% to -47%]</td>
<td>14.4 µmol.min$^{-1}$</td>
</tr>
<tr>
<td>for X on Y</td>
<td>30.07%</td>
<td>100.00%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Figure 4.15). Inspection of this figure suggests that there is a downward shift of the bendrofluazide data from the control values. Statistical analysis (Table 4.21) confirms this. Thus, in these experiments, bendrofluazide significantly reduced ammonium excretion rate in this subject. These results are discussed in section 6.2.2.

4.6.2 Frusemide

Subjects 9 and 10 participated in this study, frusemide being taken by Subject 10 in three separate experiments. In all cases a timed urine collection was taken over a period of about one hour at the beginning of the study and then frusemide was taken by mouth. In the first study Subject 9 took 40 mg and Subject 10 took 30 mg, amounts which are equivalent to approximately 0.5 mg.kg$^{-1}$ or 35 mg.70kg$^{-1}$ of body weight. The standard therapeutic dose is 40 mg per day. In the second and third studies, Subject 10 took an increased dose of 60 mg of frusemide (approximately 1.0 mg.kg$^{-1}$ or 70 mg.70kg$^{-1}$ body weight) as the standard dose did not produce any diuretic response. 3 g of sodium chloride, well within the normal daily intake, was taken by mouth with the frusemide to prevent sodium depletion. Spontaneous urine samples were collected consecutively over the following five hours, manipulation of urinary pH being achieved by the oral administration of up to 6 g sodium bicarbonate. Ammonium chloride was not required, as frusemide stimulates the production of acid urine (Wrong, 1991).

The frusemide data of Subject 9 with urine pH values below 5.8 appear to lie consistently below the control data (Figure 4.16), although the same cannot be said of the four remaining data points. The statistical analysis in Table 4.22 consequently reveals a significant difference in slope between the experimental data and the control, at least.
Figure 4.15: Control vs. bendrofluazide (Subject 10)
Figure 4.16: Control vs. frusemide (Subject 9)
Table 4.22: Comparison of control and frusemide data (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$% R^2$</th>
<th>$% R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.271 - 0.360x - 1.130i + 0.191s$</td>
<td>80.9</td>
<td>80.2</td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.071 - 0.325x - 0.061i$</td>
<td>76.9</td>
<td>76.4</td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.040 - 0.321x$</td>
<td>76.1</td>
<td>75.8</td>
</tr>
</tbody>
</table>

| Significance of $s$ for Y on X | 99.99% | 92.27% | Increase in rate | [-1% to -24%] |
| for X on Y                     | 92.12% | 95.62% | Rate at pH 6.0   | 11.5 $\mu$mol.min$^{-1}$ |

Table 4.23: Comparison of control and frusemide data (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$% R^2$</th>
<th>$% R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.016 - 0.272x - 0.003i - 0.037s$</td>
<td>82.5</td>
<td>82.1</td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.087 - 0.285x - 0.215i$</td>
<td>82.2</td>
<td>81.9</td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.095 - 0.292x$</td>
<td>75.9</td>
<td>75.7</td>
</tr>
</tbody>
</table>

| Significance of $s$ for Y on X | 83.84% | 100.00% | Increase in rate | [-31% to -46%] |
| for X on Y                     | 10.18% | 100.00% | Rate at pH 6.0   | 14.6 $\mu$mol.min$^{-1}$ |

for the regression of $y$ on $x$. If the three most alkaline data points are removed from the analysis, the slope difference disappears and the experimental data in the normal to acidic pH range are shown to lie on a parallel but significantly different line to the control data (not shown in a table). Whether the experimental data in the alkaline range really do lie above the control line cannot be accurately determined from so few points.

The data plotted from Subject 10 (Figure 4.17) suggest a slight downshift of ammonium excretion rate from control across the whole range of urine pH. The statistical analysis from Table 4.23 confirms this. It would thus appear that frusemide causes a significant reduction in ammonium excretion rate from normal.

The data from both subjects were compared also to their respective osmotic diuresis data (Figures 4.18 and 4.19), because the ranges of urinary flow and solute excretion rate overlapped very substantially the corresponding ranges observed during osmotic diuresis (see section 4.5). The flow rates and solute excretion rates are given in Table 4.24. It is
Figure 4.17: Control vs. frusemide (Subject 10)

- Subject 10: Normal Data
- Subject 10: Frusemide Data

Urine pH

Urinary NH₄⁺, μmol/min
Figure 4.18: Osmotic diuresis vs. frusemide (Subject 9)

- Subject 9: Osmotic Diuresis (Mannitol)
- Subject 9: Frusemide Data
Figure 4.19: Osmotic diuresis vs. frusemide (Subject 10)

- Subject 10: Osmotic Diuresis (Mannitol)
- Subject 10: Frusemide Data
Table 4.24: Flow and solute excretion rates with frusemide (Subjects 9 and 10)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Flow (ml/min(^{-1}).70kg(^{-1}))</th>
<th>Solute excretion (mOsm.kg(^{-1}))</th>
<th>Solute excretion (μmol.min(^{-1}).70kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1.8–14.8</td>
<td>324–522</td>
<td>736–5007</td>
</tr>
<tr>
<td>10</td>
<td>1.3–18.0</td>
<td>288–468</td>
<td>585–5455</td>
</tr>
</tbody>
</table>

Table 4.25: Comparison of osmotic diuresis and frusemide data (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>(%R^2)</th>
<th>(%R^2) adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>67.7</td>
<td>64.5</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>66.0</td>
<td>63.8</td>
</tr>
<tr>
<td>Single line</td>
<td>2.2</td>
<td>-0.9</td>
</tr>
</tbody>
</table>

Significance of

<table>
<thead>
<tr>
<th>for Y on X</th>
<th>s</th>
<th>i</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>78.31%</td>
<td>100.00%</td>
</tr>
<tr>
<td>for X on Y</td>
<td>84.80%</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

Increase in rate [-55% to -71%]
Rate at pH 6.0 12.9 μmol.min\(^{-1}\)

Table 4.26: Comparison of osmotic diuresis and frusemide data (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>(%R^2)</th>
<th>(%R^2) adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>87.3</td>
<td>86.2</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>87.2</td>
<td>86.5</td>
</tr>
<tr>
<td>Single line</td>
<td>57.6</td>
<td>56.4</td>
</tr>
</tbody>
</table>

Significance of

<table>
<thead>
<tr>
<th>for Y on X</th>
<th>s</th>
<th>i</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40.00%</td>
<td>100.00%</td>
</tr>
<tr>
<td>for X on Y</td>
<td>5.26%</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

Increase in rate [-61% to -74%]
Rate at pH 6.0 14.5 μmol.min\(^{-1}\)
Table 4.27: Plasma metabolites and electrolytes with bumetanide (Subject 10)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>mmol.l^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>5.3</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>31.0</td>
</tr>
<tr>
<td>Chloride</td>
<td>99.0</td>
</tr>
<tr>
<td>Sodium</td>
<td>140.0</td>
</tr>
<tr>
<td>Potassium</td>
<td>3.8</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.078</td>
</tr>
</tbody>
</table>

Table 4.28: Comparison of control and bumetanide data (Subject 10)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>%R²</th>
<th>%R²adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td>78.8</td>
<td>78.3</td>
</tr>
<tr>
<td>Parallel lines</td>
<td>78.4</td>
<td>78.1</td>
</tr>
<tr>
<td>Single line</td>
<td>72.2</td>
<td>71.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of s, i</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>[-29% to -45%]</td>
<td>15.1 μmol.min^{-1}</td>
</tr>
<tr>
<td>for X on Y</td>
<td>100.00%</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

4.6.3 Bumetanide

Bumetanide is another loop diuretic, and would be expected to act similarly to frusemide. Its effect was studied on two separate occasions in Subject 10. 1 mg of bumetanide, the standard therapeutic dose, was taken by mouth every 3 hours and consecutive spontaneous urine samples collected. Urine pH was manipulated by oral administration of up to 3 g of sodium bicarbonate. In the second study, a blood sample was taken after the third dose of bumetanide to test for plasma urea and electrolytes (results are shown in Table 4.27). From the creatinine figure of 78 μmol.l^{-1} (8.82 mg.l^{-1}) the creatinine clearance was calculated to be 96 ml.min^{-1}.

The bumetanide data for Subject 10 are plotted against the control data in Figure 4.20. Table 4.28 gives the statistical analysis of the results. A significant difference is shown between the experimental and control data, and a noticeable reduction in ammonium excretion rate with bumetanide may be concluded. As with frusemide, the bumetanide results are compared also to the osmotic diuresis data, because the ranges of urinary flow
Figure 4.20h: Control vs. bumetamide (Subject 10)

- △ - Subject 10: Normal Data
- □ - Subject 10: Bumetamide Data

Urine NH₄⁺, μmol/min vs. Urine pH
and solute excretion rate overlapped very substantially the corresponding ranges observed during osmotic diuresis. The values for flow and solute excretion rate are shown in Table 4.29. The data for ammonium excretion rate and pH are plotted in Figure 4.21. Table 4.30 analyses the osmotic diuresis data versus that obtained with bumetanide. The difference between the lines is even more pronounced than when the comparison is with the control data. These results are discussed in section 6.2.2.

### 4.6.4 Amiloride

The effects of a potassium-sparing diuretic, amiloride, on renal ammonium excretion were studied in Subject 9. The standard therapeutic dose, 5 mg, of amiloride was taken at the start of the study and again after 5 hours. Spontaneous urine collections were made continuously over a 9½ hour period, and the pH was manipulated by two separate doses of 3 g ammonium chloride to produce more acidic samples and later three separate doses of 4 g sodium bicarbonate to alkalise the urine. These more alkaline urine samples were collected under mineral oil. A blood sample was taken before the final urine collection, and analysed for urea, creatinine and electrolytes. The results of these plasma analyses are shown in Table 4.31.

Since the urinary flow and solute excretion rates obtained with amiloride were not in
Figure 4.21: Osmotic diuresis vs. bumetamide (Subject 10)

- ▲ - Subject 10: Osmotic Diuresis Data
- □ - Subject 10: Bumetamide Data

Urine pH

Urinary NH₄⁺, μmol/min
Table 4.31: Plasma metabolites and electrolytes with amiloride (Subject 9)

<table>
<thead>
<tr>
<th></th>
<th>mmol.l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>7.2</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>25.0</td>
</tr>
<tr>
<td>Chloride</td>
<td>107.0</td>
</tr>
<tr>
<td>Sodium</td>
<td>143.0</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.4</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.110</td>
</tr>
</tbody>
</table>

Table 4.32: Comparison of control and amiloride data (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>(%R^2)</th>
<th>(%R^2_{adj})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(y = 3.271 - 0.360x - 0.003i - 0.013s)</td>
<td>81.2</td>
<td>80.5</td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(y = 3.284 - 0.362a - 0.085)</td>
<td>81.2</td>
<td>80.8</td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(y = 3.335 - 0.373x)</td>
<td>80.2</td>
<td>79.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of (s) and (i)</th>
<th>(s)</th>
<th>(i)</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>18.95%</td>
<td>96.59%</td>
<td>[-4% to -29%]</td>
<td>10.7 (\mu)mol.min⁻¹</td>
</tr>
<tr>
<td>for X on Y</td>
<td>93.40%</td>
<td>61.28%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

the range of water or osmotic diuresis, the experimental results were plotted only against the control data for Subject 9 (Figure 4.22). From this plot, no differences between the experimental and the control data were observed. The statistical analysis given in Table 4.32 confirms this impression, apart from there being a slight difference in intercept for the parallel regression lines for \(y\) on \(x\). This difference is due entirely to the point with urine pH 7.0 and extremely low ammonium excretion rate and its removal results in the difference disappearing (not shown in a table). It can be concluded that amiloride administration in the dosage used in this study has little, if any, effect on the rate of ammonium excretion, and this is discussed in section 6.2.2.
Figure 4.22: Control vs. amiloride (Subject 9)

- Subject 9: Normal Data
- Subject 9: Amiloride Data

Urine NH₄⁺, μmol/min vs. Urine pH
Chapter 5

General Discussion

5.1 The importance of ammonium

Ammonium is the largest component of net renal H+ excretion and the one most subject to variations with the metabolic needs of the body. A healthy adult on an average mixed diet excretes 50–80 mmol H+ per day, equivalent to the same amount of regenerated HCO₃⁻ returned to the body, of which more than half is NH₄⁺. The ammonia buffer system has greater capacity for H+ excretion than the phosphate buffer system (see section 1.1.2). When net acid excretion is stimulated by an acid load, renal NH₄⁺ excretion can increase from a mean of approximately 35 mmol per day by up to eightfold to 150–300 mmol per day (Wood, 1955; Elkinton et al., 1960), whereas titratable acid excretion, normally around 20 mmol per day, increases no more than threefold (Haldane et al., 1923; Sartorius et al., 1949). This adaptation of ammonia production, which takes 3 to 5 days to fully develop, may be due to increased glutaminase activity and/or facilitation of glutamine transport into the mitochondria (see Figure 5.1).

5.1.1 Pathways of ammonia production

The conclusion that urinary NH₄⁺ was produced in the kidney, and not just extracted from the blood, came from early observations that the NH₄⁺ concentration was higher in renal venous blood than in arterial blood, and that the NH₄⁺ excretion rate was greater than the rate of delivery of NH₄⁺ in arterial blood to the kidney (Nash and Benedict, 1921). Two decades later it was shown that renal extraction of the amino acid glutamine accounted for the major part of the renal NH₄⁺ rate of production (Van Slyke et al., 1943). A further twenty years on, these early observations were confirmed when in vivo glutamine was demonstrated to be the major amino acid extracted by the kidney of the
acidotic dog (Shalhoub et al., 1963).

The main precursor of NH$_4^+$ is glutamine, which contributes both its amide and amino group to NH$_4^+$ formation (Pitts, 1964). Nearly 90% of the NH$_4^+$ produced in the kidney has this origin (Pitts and Pilkington, 1966). Glutamine is extracted from both the blood and the tubular fluid by tubular cells, principally in the proximal tubule. It is deamidated within the mitochondrion (Curthoys and Weiss, 1974) to glutamate and NH$_4^+$ (Goldstein, 1967) by the enzyme phosphate dependent glutaminase (PDG), as shown in Figure 5.1. Glutamate can then be deaminated to α-ketoglutarate (α-KG) and NH$_4^+$ by glutamate dehydrogenase (GDH) in the presence of nicotinamide adenine dinucleotide (NAD) (Simpson, 1972; Bourke et al., 1971). NH$_4^+$ is also formed from the amino acids glycine and ornithine (not shown in Figure 5.1) which contribute their amino groups to α-KG by transamination to glutamate, which can then be deaminated as above. Formation of NH$_4^+$ by this pathway can account for the remaining 10% of NH$_4^+$ produced by the kidney (Stone and Pitts, 1967). Alpha-ketoglutarate can be metabolised by conversion to phosphoenolpyruvate (PEP) as shown on the right hand side of Figure 5.1, which can then be metabolised to glucose via the gluconeogenic pathway or can re-enter the mitochondria to be oxidised to CO$_2$ and water (Pitts, 1973). Glutamine synthetase (GS) may form glutamine from glutamate and NH$_4^+$ although if this equalled the rate of intramitochondrial glutamine deamidation a futile cycle would result.
Although glutamine is an ionically neutral substance, when it is metabolised to α-KG and two molecules of NH₃, two H⁺ ions are released. In order for renal NH₄⁺ excretion to be equivalent to net urinary H⁺ excretion, these two H⁺ ions must be neutralised (Stoff et al., 1976). This is achieved by either the metabolism of α-ketoglutarate to CO₂ and glucose or by its complete oxidation to CO₂ and water. In each case the metabolism of the anion, α-KG, consumes two H⁺ ions, thereby balancing the two H⁺ ions released by the PDG and GDH reactions (see Figure 5.1). Without this metabolic removal of H⁺ ions, the excretion of NH₄⁺ in the urine would result in no net gain of bicarbonate to the body, and the essential acid-base function of ammonia production would be lost. The metabolism of α-ketoglutarate to PEP is a vital part of the ammoniagenic response to acidosis, and there is also evidence that TCA (tricarboxylic acid) cycle intermediates have an impact on the overall rate of ammonia formation. A constant efflux of substrate from the mitochondrion is needed for the removal of the carbon skeleton of α-KG. The transported acid is believed to be malate, and it has been reported that mitochondrial malate transport is stimulated by acidosis (Kopyt et al., 1974).

Experiments with isolated mitochondria of the rat indicate that a low pH of the medium inhibits both glutamine deamidation by PDG and glutamate deamination by GDH (Kunin and Tannen, 1979). However, studies with tissue slices from both the rat and the dog have shown that a low pH appears to stimulate the decarboxylation of α-ketoglutarate to succinate and also glucose production by the renal cortex (Pilkington and O'Donovan, 1971; Irias and Greenberg, 1972). Recent studies in the rat suggest that in various acid-base states the rate of renal gluconeogenesis is linearly correlated with malate efflux from the mitochondria (Nissim et al., 1991). It should be noted that net synthesis of glucose is not a requirement for ammoniagenesis, but transport of malate, and possibly OAA (Gimpel et al., 1973), across the mitochondrial membrane, is essential for the removal of α-KG (see Figure 5.1).

Another, and probably minor, pathway of NH₄⁺ production involves the transamination of glutamine and α-ketoglutarate to yield ketoglutaramate and glutamate (not shown in Figure 5.1) and the subsequent deamidation of ketoglutaramate to ketoglutarate plus NH₄⁺ and the deamination of glutamate via the GDH reaction (Goldstein, 1967; Richterich and Goldstein, 1958). Another cytoplasmic pathway which releases NH₄⁺ is the purine nucleotide cycle (PNC) which involves the transamination of glutamate to aspartate (Bogusky et al., 1976). High activities of glutamate-aspartate transaminase occur in
every tissue (Lowenstein, 1972) ensuring a rapid conversion of glutamate to aspartate and vice versa (see right hand side of Figure 5.1). The contribution made by the PNC to total renal NH₄⁺ production has yet to be determined.

Several different sites within the tubular cell have been considered for the regulation of ammoniagenesis, i.e., production of NH₄⁺ (Tannen, 1978). The entry of glutamine into the mitochondrion and its deamidation by PDG may be rate-limiting steps for NH₄⁺ production. Renal ammoniagenesis can increase in acidosis with or without an adaptive increase in PDG activity, depending upon the species: in the guinea pig and rat PDG rises (Davies and Yudkin, 1952; Leonard and Orloff, 1955) but there is no observable increase in the activity of this enzyme in the dog (Rector and Orloff, 1959). In dogs given ammonium chloride for seven days a fourfold increase in ammonium excretion was found and it was suggested that sufficient PDG and GDH was already present to produce the NH₄⁺ (Pollak et al., 1965). About 60% of the NH₄⁺ would be derived from glutamine, and the rest from other amino acids through amino acid transferases and glutamate (see Figure 5.1). Very few studies of the induction of the enzymes involved in ammoniagenesis have been done in man. No increase in PDG and GDH was found in renal cortical specimens taken from two subjects on the seventh day of an ammonium chloride load (Pollak and Mattenheimer, 1965). In man the activities of GDH were found to be highest in the proximal tubule (Mattenheimer, 1971).

5.1.2 Ammonium movements in the kidney

The Henderson-Hasselbalch equation as applied to the ammonia buffer system

\[
pH = 9.1 + \log_{10}[\text{NH}_3]/[\text{NH}_4^+] \cdot
\]

indicates that the ratio of the two forms in solution will be 1:1 at pH 9.1, and will decrease tenfold for each unit reduction in pH, such that the ratio [NH₃]:[NH₄⁺] will be 1:100 at a pH of 7.10. The pH of proximal tubular cells is approximately 7.2 (Alpern and Chambers, 1986), which would mean that only a small fraction (around 1%) of the total ammonia here would be in the NH₃ form. As a pH of around 8.0 is the greatest achieved in any body fluids (in pancreatic juice and in urine) NH₄⁺ is the predominant form of total ammonia in all body fluids and cells.

The non-electrolyte NH₃ is a small molecule (molecular weight 17) with a relatively high diffusion coefficient in aqueous solution. It is slightly lipid soluble and is thought to
penetrate cell membranes by diffusion through the nonpolar lipid phase. Measurement of partition co-efficients (solubility ratios) across a two phase system consisting of a non-

polar solvent (such as hexadecane) are often used to estimate how easily solutes can enter 
the lipid phase of membranes. It has been shown that NH$_3$ is less lipophilic than carbon 
dioxide (CO$_2$), another small non-electrolyte; the hexadecane-to-water partition coefficient 
of NH$_3$ is only 0.0022 (Walter and Gutknecht, 1986), showing that NH$_3$ is much more 
soluble in water than in the nonpolar solvent hexadecane. By contrast the hexadecane-
to-water partition coefficient of CO$_2$ is 1.5 (Simon and Gutknecht, 1980). Thus NH$_3$ 
may be predicted to be considerably less permeant across lipid membranes than is CO$_2$. 
Nevertheless, NH$_3$ moves principally by passive diffusion and is much more diffusible than 
NH$_4^+$ across renal tubular cell membranes (Good and Knepper, 1985). The epithelial 
permeability of NH$_3$ has been measured in several segments of the renal tubule, and found 
to decrease from relatively high (approximately 0.06 cm.sec$^{-1}$) in proximal convoluted 
tubules to relatively low (approximately 0.004 cm.sec$^{-1}$) in collecting ducts (Hamm et al., 
1985). These differences in permeability are thought to be principally due to differences 
in the surface areas of the plasma membranes of these renal tubule segments (Star et al., 
1987b). The surface area of the apical membrane of the proximal tubular cell is much 
greater than that of the thick ascending limb or the collecting duct owing to the presence 
of a brush border (Maunsbach et al., 1987).

Until recently it was largely accepted that the mechanism by which total ammonia 
passed from its site of production in the proximal tubular cell to the lumen of the collecting 
duct was by passive non-ionic diffusion of NH$_3$. This theory of ammonium movement was 
proposed by Robert F. Pitts and was based on the fact that NH$_3$ is uncharged and the 
suggestion that it could transverse lipid membranes (Pitts, 1973). The free base, NH$_3$, 
could thus diffuse from the proximal tubular cell in both directions, into the urine and into 
peritubular capillary blood. Any NH$_3$ diffusing out of the tubular cell would immediately 
be replaced by the dissociation of NH$_4^+$ because of the steady state existing between H$^+$ 
and NH$_3$. In acid urine, the base would buffer hydrogen ions to form impermeant NH$_4^+$ 
ions. The mechanism could therefore be described as firstly, one of non-ionic diffusion 
(as it is the uncharged species which diffuses from cell to urine) and secondly, as one of 
ion trapping (as the free base diffuses into acid urine where it is trapped as non-diffusible 
NH$_4^+$ ion). A key feature of this secretory mechanism is that no energy is expended in 
moving NH$_3$ from cell to urine or blood as the NH$_3$ diffuses down a concentration gradient.
The concept of non-ionic diffusion is now known to be too simple to fully explain ammonia movements in the kidney; the pathways involved have been found to be far more complicated than originally thought. In recent years there have been rapid developments in the understanding of transport of total ammonia in the kidney; it has been shown that these movements are not entirely passive. The assumption that \( \text{NH}_4^+ \) cannot, because of its charge, easily penetrate the lipid phase of a membrane appears to be correct (Knepper et al., 1989). There is, however, evidence that \( \text{NH}_4^+ \) formed in the proximal tubular cell may be transported via specialised pathways.

Ammonia movement does not occur simply through the apical membrane of the tubular cell in which ammonium is derived from glutamine. In the rat the main site of ammoniagenesis is the proximal tubular cell (Good and Burg, 1984) whereas the main site of tubular entry of ammonium is the collecting duct (Buerkert et al., 1982). Between the two sites total ammonia diffuses from the proximal tubular cell into the proximal tubular lumen (Buerkert et al., 1983), is largely reabsorbed in the loop of Henle to reach the medullary interstitium (Wilcox et al., 1984), and is then secreted into the collecting duct lumen (Sonnenberg et al., 1981), as shown in Figure 5.2 on page 95.

Two mechanisms of ammonia transport into the lumen of the proximal tubule have been established. Firstly, as explained above, \( \text{NH}_3 \) can diffuse out of the cell across either the apical or the basolateral membrane, but tends to move across the apical membrane because of the lower luminal pH (non-ionic diffusion). Secondly, \( \text{NH}_4^+ \) can be transported across the apical membrane on the Na-H antiporter, most likely as Na-NH\(_4^+\) exchange (Kinsella and Aronson, 1981a). The physiological importance of direct Na-NH\(_4^+\) exchange in the proximal tubule has yet to be determined. At least two more specific transport systems probably move ammonia in the proximal tubule. The hydrated ionic radius of \( \text{NH}_4^+ \) is identical to that of \( \text{K}^+ \) (Good and Knepper, 1985) and there is evidence that \( \text{NH}_4^+ \) can traverse some epithelial \( \text{K}^+ \) transport pathways. \( \text{NH}_4^+ \) movement on the Na\(^+-\)K\(^+-\)ATPase, where \( \text{NH}_4^+ \) can substitute for \( \text{K}^+ \), has been demonstrated in both the proximal tubule and the thick ascending limb (Garvin et al., 1985; Kurtz and Balaban, 1986). The Na\(^+-\)K\(^+-\)ATPase is located in the basolateral membrane and transports \( \text{K}^+ \) into the cell. Therefore the Na\(^+-\)K\(^+-\)ATPase could, in theory, serve as an \( \text{NH}_4^+ \) pump to transport \( \text{NH}_4^+ \) in a secretory direction. However, the quantitative importance of the latter process is controversial. The proximal tubule also possesses paracellular \( \text{K}^+ \) channels which represent another possible route of some \( \text{NH}_4^+ \) transport (Good et al.,
1984), although at present there is little information available regarding the ability of \( \text{NH}_4^+ \) to penetrate \( \text{K}^+ \) channels in renal tubular epithelia.

In the loop of Henle, luminal fluid alkalinisation resulting from water abstraction would cause \( \text{NH}_3 \) to enter the medullary interstitium by non-ionic diffusion. \( \text{NH}_4^+ \) is also transported into the interstitium in the thick ascending limb (TAL) by passive voltage-driven diffusion and by active transport. There is a lumen-positive transepithelial potential difference, caused by active chloride absorption, in the TAL (Burg and Green, 1973), and this positive voltage can drive \( \text{NH}_4^+ \) diffusion out of the lumen (Garvin et al., 1988). Apical membrane transport of \( \text{NH}_4^+ \) can occur also on the \( \text{Na}^+\text{-K}^+\text{-2Cl}^- \) cotransporter (Kinne et al., 1986). Ammonia that enters the interstitium in the outer medulla can diffuse into the descending limb and be carried to the deeper medulla by countercurrent multiplication system, as explained below, or diffuse into the fluid in the collecting duct.

### 5.1.3 Countercurrent multiplication of ammonium

After ammonia is produced and secreted by the proximal tubule it is transported to the renal medulla via the Loop of Henle. Absorption of ammonium from the thick ascending limb is predominantly mediated via \( \text{Na}^+\text{-K}^+\text{-2Cl}^- \) cotransporters in the apical membrane (Kikeri et al., 1989), and this active absorption of \( \text{NH}_4^+ \) is the first step in the countercurrent multiplication of ammonia (Good et al., 1987). A transepithelial concentration difference favouring secretion of ammonia into the medullary collecting duct (MCD) is generated, and up to 80% of the ammonia excreted in the urine is secreted along the MCD and cortical collecting ducts (Graber et al., 1981a; Sajo et al., 1981). Other studies have directly demonstrated ammonia addition to the collecting duct (Graber et al., 1981b; Sonnenberg et al., 1981). This entry may be by non-ionic diffusion, as has been widely believed, or may be by transport of \( \text{NH}_4^+ \) as has been postulated for turtle bladder (Arruda et al., 1984) and for the thick ascending limb (Good et al., 1984); however, direct \( \text{NH}_4^+ \) transfer does not appear to be the mode of ammonia secretion across the collecting duct (Hamm et al., 1985; Star et al., 1987a). Thus, ammonia in the interstitium diffuses into the cortical and medullary collecting tubules by non-ionic diffusion (Flessner et al., 1991).

Figure 5.2 summarises modern theories of ammonium transport pathways; where \( \text{NH}_4^+ \) transport is indicated the \( \text{NH}_4^+ \) could be transferred as \( \text{NH}_4^+ \) directly or by a combination of \( \text{H}^+ \) and \( \text{NH}_3 \) movement. Ammonium reabsorbed from the thick ascending limb of the Loop of Henle (TAL) diffuses into the descending limb (DTL) to create a recycling pathway.
Key:  
1. $\text{NH}_4^+$ produced from glutamine in the proximal tubular cell is secreted into the lumen.
2. $\text{NH}_4^+$ is reabsorbed, principally in the thick ascending limb, and concentrated by the countercurrent multiplier.
3. $\text{NH}_4^+$ is secreted into the lumen of the collecting duct.

Abbreviations:  
PCT proximal convoluted tubule  
PST proximal straight tubule  
DTL descending thin limb  
TAL thick ascending limb  
DCT distal convoluted tubule  
CCD cortical collecting duct  
IMCD inner medullary collecting duct

Figure 5.2: Ammonium transfer in the nephron.
for ammonium between the two limbs of the loop (Packer et al., 1991). An accumulation of NH$_4^+$ in the tissues of the renal medulla would be enhanced by this countercurrent multiplier, with eventual diffusion trapping of ammonium occurring in the lumen of the collecting duct.

5.2 The relationship between urinary ammonium and pH

An inverse relationship between urinary pH and the amount of total ammonia excreted in the urine has been recognised for many years (Pitts, 1948). The more acid the urine, the greater the proportion of total ammonia existing in the ionised form, i.e., the lower the urinary concentration of NH$_3$, and thus the greater the concentration difference promoting the passive diffusion of NH$_3$ from renal tubular cell to lumen. This section discusses how the relationship between urinary ammonium and pH may be expressed and what factors are likely to alter the relationship from that which theory would predict.

5.2.1 Urinary ammonium concentration and excretion rate

The theoretical basis of non-ionic diffusion would predict a tenfold increase in urinary NH$_4^+$ concentration per unit fall in pH (Milne et al., 1958). According to this theory, the concentration ratio of total ammonia across the membrane separating the site of ammonia synthesis in the tubular cell from the tubular lumen will be equal to the ratio of the H$^+$ concentration between these two sites, provided that certain conditions (see below) are fulfilled. When both variables are expressed logarithmically the logarithm of the ratio of total ammonia concentration across the membrane will be negatively correlated with the pH difference between the two sites, with a slope of $-1.0$.

In order for a slope of $-1.0$ between urinary pH and urinary ammonium concentration within the tubular lumen to be achieved, certain criteria would have to be met, namely:

- that the concentration of total ammonia inside the tubular cell is constant,
- that the pH inside the tubular cell is constant,
- that diffusion should proceed to equilibrium,
- that diffusion is entirely passive,
- that NH$_4^+$ is non-diffusible,
that the permeability of the cell membrane should remain constant.

If the movement of ammonia to the urine were governed by the above considerations, it would be anticipated that, at the site of its accumulation in the tubular lumen, the concentration of NH$_4^+$ would be a function of pH. To the extent that no further changes in pH, volume and NH$_4^+$ content of the urine occur, the concentration in the final urine would be a function of pH. If the flow rate past the last point of equilibrium were relatively unchanged, the rate of excretion of NH$_4^+$, as well as its concentration, would be a function of pH. However, if changes in volume were to take place after pH and NH$_4^+$ content were established, the relationship between pH and NH$_4^+$ concentration would be distorted (Orloff and Berliner, 1956) but the relationship between pH and rate of NH$_4^+$ excretion maintained. In fact, ammonia behaves similarly to urea, sodium and potassium, the main urinary solutes, in that its minute excretion is moderately constant regardless of flow, whereas the urinary concentrations of these solutes are much less constant (Hai and Thomas, 1969; Atherton et al., 1971). Although an inverse relationship between urinary NH$_4^+$ concentration and pH exists in man, a closer relationship has been observed, in the present studies, between the rate of NH$_4^+$ excretion and pH (see section 3.1), and henceforth this latter relationship will be used. The implication of all these findings is that the amount of solute in the urine is determined proximal to the main site of water removal, i.e., the main volume change in the urine is likely to occur distally to the principal site of addition of ammonia. This point is further discussed in section 6.2.1.

5.2.2 Excretion rate expressed arithmetically and logarithmically

In dog, rat and man, the rate of NH$_4^+$ excretion is approximately doubled for every unit fall in pH. The relationship appears to be logarithmic (Clarke et al., 1955; Orloff and Berliner, 1956) with a slope of log$_{10}$ NH$_4^+$ min$^{-1}$ against pH of approximately $-0.3$. The finding that the slope of the log$_{10}$ NH$_4^+$ min$^{-1}$ against pH was around $-0.3$, rather than $-1.0$ (i.e., ammonium excretion is only doubled instead of increasing tenfold for each tenfold increase of urinary hydrogen ion concentration) contrasts with what would be expected on a theoretical basis for an 'ideal' weak base, completely non-diffusible in ionised form, but diffusing to equilibrium in unionised form. It was, therefore, proposed that ammonia is not an 'ideal' base, nor the tubular epithelium an 'ideal' membrane, in terms of non-ionic diffusion (Milne et al., 1958).

The original evidence for the diffusion trapping model of renal ammonium transport
came from several studies in the rat and the dog, which showed that the rate of ammonium excretion was a negative function of urinary pH (Pitts, 1948; Leonard and Orloff, 1955; Orloff and Berliner, 1956). Studies in man showed a similar close negative relationship between urine pH and the rate of ammonium excretion (Stanbury and Thomson, 1952; Clarke et al., 1955; Wrong and Davies, 1959). Early microcatheterisation studies in rats supported the concept of ammonia trapping; the inner medullary collecting duct (IMCD) was shown to be the site of maximal acidification and maximal secretion of ammonium (Ullrich et al., 1958; Ullrich and Eigler, 1958). Further studies in dogs supported the role of urinary pH as the major factor controlling ammonium excretion (Balagura and Pitts, 1962; Sullivan and McVaugh, 1963). For well over thirty years, ammonia trapping was thought to be the reason behind the close negative relationship between urine pH and the rate of ammonium excretion.

There are several advantages in expressing ammonium excretion rate as a logarithmic function of urine pH:

- pH itself is a logarithmic expression,
- the relationship is approximately linear (see above),
- it is easier to compare experimental conditions with normal,
- a logarithmic relationship is to be expected on physiological grounds.

### 5.2.3 Nature of the logarithmic relationship

The subject of non-ionic diffusion and the excretion of weak acids and bases was thoroughly reviewed over thirty years ago (Milne et al., 1958). The observations of Milne referred to the concentration ratio between tubular cell and urine, rather than the rate of excretion, of these weak acids and bases; however, his conclusions may now be extrapolated to the rate, which is the variable used for the expression of data in this thesis (see section 5.2.1).

There are several theoretical reasons why the observed relationship between logarithmic ammonium excretion rate and urinary pH might differ from the theoretical relationship with its slope of $-1.0$. These reasons are detailed below and their effect on the relationship is illustrated in Figure 5.3 on page 101. The reader may find it helpful to refer to this diagram.
1. Reduced intracellular NH$_3$ concentrations caused by enhanced ammonium excretion into a more acid urine might be expected to reduce ammonium excretion below predicted values and so to alter the slope of the linear relationship between urinary ammonium excretion rate and pH, making it more curvilinear at low pH values (see Figure 5.3). At present little is known about the stability of intracellular NH$_3$ concentration in the various cell structures through which it passes, i.e., proximal tubule, thick ascending limb and collecting duct (see Figure 5.2 on page 95).

2. The concept of diffusion equilibrium of ammonia throughout renal tissues contradicts the fact that a concentration gradient must exist for diffusion to occur. The diffusion trapping model of renal ammonium transport assumes both that tubular epithelia have such high NH$_3$ permeabilities that NH$_3$ concentration gradients do not exist, and also that there is a state of diffusion equilibrium among adjacent parts of the kidney. Diffusion through several membranes and intracellular structures is unlikely to achieve complete equilibrium, and it has been demonstrated that such an equilibrium does not occur (Simon et al., 1985; Good and Du Bose, 1987; Packer et al., 1991). Failure of renal ammonia to reach diffusion equilibrium whilst passing through the multiple diffusion barriers between proximal tubular cell and collecting duct would result in a decreased rate of ammonium excretion, particularly with more acid urines in which ammonium excretion is greater, producing a convex relationship which will approach linearity in the more alkali urine pH range (Milne et al., 1958).

3. Although total ammonia movements in the kidney seem to be predominantly by passive diffusion, evidence has already been presented (see section 5.1.2) that active transport of NH$_4^+$ also takes place, principally via potassium-linked mechanisms. This movement might be limited by the amount of energy (ATP) available or by saturation of the ion transport pathway at high rates of NH$_4^+$ excretion, which would tend to reduce ammonium excretion below that predicted at low urine pH values, producing a curvilinear relationship as shown in Figure 5.3.

4. The NH$_3$:NH$_4^+$ permeability of renal tissues is not fully known, but various figures for related tissues have been calculated. Turtle urinary bladder, which resembles mammalian collecting duct epithelium in its handling of H$^+$, has been shown to have a diffusibility ratio of 50:1 for NH$_3$:NH$_4^+$ (Schwartz and Tripolone, 1983). A ratio of 40:1 has been estimated in the rat proximal convoluted tubule (Simon et al., 1985; Good and Du Bose, 1987; Packer et al., 1991). Failure of renal ammonia to reach diffusion equilibrium whilst passing through the multiple diffusion barriers between proximal tubular cell and collecting duct would result in a decreased rate of ammonium excretion, particularly with more acid urines in which ammonium excretion is greater, producing a convex relationship which will approach linearity in the more alkali urine pH range (Milne et al., 1958).
The mucosa of human large intestine has been calculated to have a ratio of between 5:1 (Castell and Moore, 1971; Bown et al., 1975) and 400:1 (Cohen et al., 1988). (In the latter study lumen electronegativity also appeared to affect flux of total ammonia by retarding NH$_4^+$ diffusion and reducing the NH$_4^+$ permeability coefficient.) Ratios of between 5:1 and 100:1 in renal tissues would permit sufficient diffusion of NH$_4^+$ to lower the rate of ammonium excretion below that predicted at low pH values; by reducing the $y$ (NH$_4^+$) values at low $x$ (pH) values, there would be a curvilinear relationship between the two variables which would approach a linear relationship at higher urinary pH values (Milne et al., 1958).

5. The stability of the tubular cell pH is not yet known. A reduction in proximal tubular pH, as a result of increased H$^+$ accumulation from any cause, would mean that the proportion of intracellular total ammonia available as NH$_3$ would be reduced, tending to lower the concentration gradient of ammonia between the tubular cell and the lumen and consequently reduce the rate of ammonium excretion below that predicted, particularly at low pH values (Denis et al., 1964; Tannen, 1978). This would produce a curvilinear relationship between urinary ammonium excretion rate and pH, as seen in Figure 5.3.

6. A factor influencing ammonia entry in the collecting duct is the existence of an acid disequilibrium pH (Knepper et al., 1985). An 'acid disequilibrium pH' refers to a lower pH of tubular fluid in situ than in fluid removed from the tubule and equilibrated to a similar pCO$_2$. It implies that the dehydration of carbonic acid to CO$_2$ does not reach equilibrium in situ, i.e., there is excess H$_2$CO$_3$ in relation to CO$_2$. (See section 5.2.4 below for a full explanation.) Because a disequilibrium pH depends on the presence of bicarbonate and lack of luminal carbonic anhydrase in the collecting duct, its effect is more marked in alkaline urines containing larger concentrations of bicarbonate. The disequilibrium pH will tend to increase diffusion trapping and a higher level of urinary ammonium excretion than would be predicted from the eventual urine pH will result. This will produce a concave relationship at high $x$ (pH) values by increasing the $y$ (NH$_4^+$) values.
Factors:
1) ammoniagenesis is limited
2) diffusion fails to reach equilibrium
3) transport of $\text{NH}_4^+$ may be limited
4) $\text{NH}_4^+$ has appreciable permeability
5) intracellular pH of tubular cell falls with more acid urines
6) acidic disequilibrium pH

Figure 5.3: Effects of theoretical factors influencing non-ionic diffusion.

5.2.4 Acid disequilibrium pH

Two forms of carbonic anhydrase (CA) exist in the kidney, the first a soluble cytoplasmic enzyme (Wistrand, 1980) and the second a membrane bound form (Wistrand, 1984). The latter, when present in the luminal membrane of the renal tubule, increases the rate of dehydration of carbonic acid in the lumen from the titration of filtered bicarbonate by secreted $H^+$ ions. In the absence of luminal CA, $H^+$ secretion can result in accumulation of carbonic acid.

$$\text{HCO}_3^- + H^+ \rightarrow \text{H}_2\text{CO}_3 \xleftrightarrow{\text{CA}} \text{CO}_2 + \text{H}_2\text{O}$$
Although acidification of tubular fluid begins in the proximal tubule, even during acidosis as much as 10% of filtered bicarbonate may remain to enter the distal tubule (Malnic, 1974). The absence of luminal CA in this tubular segment (Star et al., 1987b) allows carbonic acid formed by the combination of H+ ions with bicarbonate to persist for several seconds, during which time it behaves as a fairly strong acid (pK approximately 3.6) and temporarily makes the pH of the tubular fluid lower, by up to 0.8 units, than it will become after the reaction above has proceeded to equilibrium (Du Bose, 1983). This difference, known as an acid disequilibrium pH, can be measured by comparing pH readings made with a rapidly responding microelectrode in the tubular fluid and with an external pH electrode on a sample of the fluid which has been withdrawn and has had time to come to equilibrium.

Accumulation of carbonic acid lowers both the luminal pH and the luminal ammonia concentration to values less than would occur for the same rate of secretion if carbonic anhydrase were present. The lower luminal ammonia concentration would increase the NH3 gradient across the epithelium and hence enhance ammonia secretion (Good and Knepper, 1985). Infusion of luminal CA eliminates the acid disequilibrium pH and reduces ammonium excretion (Kurtz et al., 1986). In contrast, in tubule segments which possess luminal CA, such as the proximal tubule (Lucci et al., 1983), luminal carbonic acid is rapidly dissipated and ammonium secretion is limited by a relatively high luminal pH and NH3 concentration, i.e., by the lack of an acidic disequilibrium pH. The effect of a disequilibrium pH is more marked in alkaline urines where a significant amount of bicarbonate is present in the collecting duct (Du Bose, 1983). Ammonium transport in this segment occurs by parallel NH3 diffusion and H+ secretion and is enhanced by the disequilibrium pH which results from H+ secretion in the absence of luminal CA (Flessner et al., 1992). Its effect on the relationship between urinary ammonium excretion rate and pH would therefore be to increase ammonium excretion rate and reduce the slope of the line in curvilinear fashion in the alkaline pH range, as shown in Figure 5.3.

Figure 5.3 illustrates the effects of the theoretical factors listed above on the linear relationship between the logarithm of ammonium excretion rate and pH. Of the six factors listed above, five would lower urine NH4+ excretion rate below that predicted in acid urines, and one (acid disequilibrium pH) would increase it above that predicted in alkaline urines. The resulting slope would be less steep than that produced by an ideal model of non-ionic diffusion.
It has been stated that there is no theoretical basis for a straight line relationship except when the slope is $-1.0$ (Orloff and Berliner, 1956). The observations in this thesis that the real relationship approximates to a straight line, although less steep than the predicted $-1.0$, are best explained as the fortuitous result of several factors described above (labelled 1 to 6 in Figure 5.3) influencing non-ionic diffusion. The reasons why the slope is less steep than predicted in theory are more likely to be in the kidney itself than in the ammonia molecule. Studies in dogs using other weak bases with pK values close to that of ammonia, e.g. quinine and some closely related compounds, also showed log $\text{excretion rates}$ which were negatively related to urine pH with slopes of approximately $-0.3$ (Orloff and Berliner, 1956). These other bases were all foreign chemical compounds of considerably larger molecular size than ammonium and not metabolised or synthesised in the body and therefore not likely to be actively transported. The fact that these other weak bases showed this relationship suggests that factor 3 (active transport of $\text{NH}_4^+$) is the least important of the six theoretical factors listed in section 5.2.3, i.e., that passive movements of $\text{NH}_3$ influence the relationship more than active movement of $\text{NH}_4^+$.

5.2.5 Animal models

Throughout this thesis, references are cited to work, both in vivo and in vitro, on animals. Many methods applied to these animals are not possible in man; the invasive procedures required for an analysis of the renal excretion of ammonia precursors, or in vitro studies of kidney slices, kidney homogenates, isolated renal tubules etc., are not feasible. The observations made on animals provide useful information on the possible mode of ammonia production and excretion in man. A degree of caution, however, must be used when extrapolating results based on animal work to man since species differences may occur in the renal handling of ammonia.

Only a few mammalian species have been studied, but it is clear that ammonium excretion shows marked species differences. The most convenient, and therefore the most commonly used, animal models are the dog and the rat, in which metabolism of dietary foodstuffs produces net acid. These species are therefore dependent on ammonium synthesis to maintain acid-base balance. The rabbit, which has very little acid load to excrete, does not have this need. If an acute or chronic acid load is ingested, the rabbit can excrete only small quantities of ammonium, despite producing a urine with a very low pH (Yu et al., 1976). The guinea-pig and rabbit are exceptions amongst mammals so far studied,
as urinary ammonium is not inversely related to urine pH but is greater in both highly acid and highly alkali urines (Richterich van Baerle et al., 1956; Richterich and Goldstein, 1958). This is a difficult relationship to explain and further studies of these species are required. Even in species which excrete large quantities of ammonium, renal pathways exhibit considerable differences, e.g. in the dog the major fate of glutamine is oxidation to carbon dioxide whereas gluconeogenesis is much more important in the rat. The pig and sheep are two other mammals in which the rate of ammonium excretion has been shown to be inversely related to pH (Scott, 1969; Scott, 1971) but insufficient data have been produced to show whether the relationship follows the logarithmic form. The dog and rat are considered to be animal models whose ammonia excretion closely approximates to that of humans.
Chapter 6
Discussion of experimental results

As has already been emphasised, there is evidence showing that urinary ammonia excretion changes in a way which is qualitatively consistent with the trapping model after acute or chronic alteration of urinary pH (Pitts, 1973). There are, therefore, two main ways in which NH\textsubscript{4}\textsuperscript{+} excretion may respond to acid-base changes; firstly by alterations in urine pH which determine NH\textsubscript{4}\textsuperscript{+}-trapping, and secondly by alteration of ammoniagenesis. Another possible way is by changes in tubular flow at sites of NH\textsubscript{4}\textsuperscript{+} secretion affecting the magnitude of the NH\textsubscript{3} gradient required for passive NH\textsubscript{4}\textsuperscript{+} trapping/secretion (see section 6.2.1).

6.1 Influences on ammoniagenesis

There are many factors which can influence renal ammoniagenesis (see section 1.2). The influences of some of these, namely systemic acid-base state, substrate availability and adrenal hormones, were investigated during the present studies. The effects of these parameters on the relationship between urinary ammonium excretion rate and pH are discussed in this section.

6.1.1 Systemic acid-base state

Spontaneous urine pH changes produced in samples collected without any acid-base stimuli being given were used to produce a control relationship between urinary ammonium excretion rate and pH. Minor systemic acid-base changes were produced acutely by administration of ammonium chloride and sodium bicarbonate (see section 2.1). The relationship found between ammonium excretion rate and urinary pH under these conditions was then compared to that produced under conditions where no acid-base stimuli were used (see
section 3.2). No significant difference between the two relationships was found, suggesting that acute acid-base changes had not produced any significant alterations in renal ammoniagenesis. The purpose of these acid-base alterations was to provide a wide range of urine pH for the control data in each of the two experimental subjects (9 and 10). The small doses of ammonium chloride and sodium bicarbonate used were extremely unlikely to produce a change in the systemic acid-base state outside the normal range, but could possibly influence ammoniagenesis. The finding (section 3.2) that the doses used were not sufficient to cause a significant change in ammoniagenesis implied that:

1. these data could be combined with the data obtained without using any acid-base stimuli to provide a set of control data

2. ammonium chloride and sodium bicarbonate could be used in these low doses, during the collection of the experimental data, to provide a wide range of urine pH.

Whilst the results of these studies confirm others that, in man, acute acid-base changes do not influence ammoniagenesis (Schwartz and Cohen, 1978), some observations made during the last ten years on animals, both in vivo and in vitro, have produced contradictory evidence. Studies in vitro in the rat, with both isolated renal tubules (Vinay et al., 1980b) and with the isolated perfused kidney (Terao and Tannen, 1980), have shown that an acute drop in surrounding perfusate pH stimulates renal ammoniagenesis independent of changes in urinary pH and NH₄⁺ excretion. Increased ammoniagenesis in the rat in vivo has been shown to be evident within 15 minutes of an acute change in systemic pH (Narins et al., 1982). The effect of acute acidosis on ammonia production in the dog has not been so intensively studied as in the rat, but there is a suggestion of a stimulant effect in these animals as well (Gougoux et al., 1982). Species differences may account for these discrepancies between animals and man.

Further experiments were carried out in the present study in which the pH of the urine was altered without corresponding systemic acid-base changes being produced, thus excluding the possibility of changes in ammoniagenesis. Intravenous sodium sulphate was used to acidify the urine intensely without correspondingly reducing systemic pH. In previous studies with sodium sulphate infusions (Schwartz et al., 1955), no significant change in either blood pH or serum carbon dioxide was found. The sulphate anion SO₄²⁻ is poorly diffusible and is not actively reabsorbed in the tubule, and as the subjects in the present study were avidly reabsorbing sodium (as they had previously been given
9α-fludrocortisone) the SO$_4^{2-}$ was excreted with H$^+$ producing a highly acid urine (lowest pH value of 4.2). This finding is in agreement with the studies of Schwartz where a minimum pH of 4.3 was achieved. The limited penetrating ability of the SO$_4^{2-}$ anion impedes the called-for retention of the accompanying sodium and thereby promotes the secretion of H$^+$ ions.

When the data from the sodium sulphate infusions were compared with the unstimulated control data, they exhibited a small, but statistically significant, difference in the relationship between ammonium excretion rate and pH (see section 3.2), the excretion rate being generally reduced. Production of urine of very low pH normally results from acute acidaemia. It is therefore possible that the lack of such an ammoniagenic stimulus, as occurs during the sodium sulphate infusion, resulted in a lower rate of ammonium excretion than would be predicted from the final urine pH. Further studies are required to validate this suggestion.

Oral acetazolamide was used to produce extremely alkaline urines (highest pH 7.75) which are difficult to achieve with sodium bicarbonate. Acetazolamide inhibits carbonic anhydrase (Leaf et al., 1954), which results in a lower rate of tubular secretion of H$^+$ ions. The urine contains large amounts of bicarbonate and this loss of HCO$_3^-$ produces systemic acidosis (Maren, 1977). Thus administration of sodium bicarbonate and acetazolamide both lead to production of alkali urines containing high levels of bicarbonate, although they have opposite effects on systemic acid-base state.

The data collected during administration of acetazolamide, when compared with the unstimulated control data, showed a small, yet statistically significant reduction in the rate of ammonium excretion in extremely alkaline urines (see section 3.2). Thus ammonium excretion was not increased by the systemic acidosis produced by acetazolamide, in fact it was reduced below the predicted level, evidence against the possibility of acute acid-base changes influencing renal ammoniagenesis. One possible reason for the suppressive effect of acetazolamide on ammonium excretion is that it may inhibit several components of the TCA cycle (see Figure 5.1) which are important for the ammoniagenic response to acidosis (Gougoux et al., 1987). An alternative explanation is the effect of acetazolamide on intracellular pH ($pH_i$) which has been examined by several groups. When $pH_i$ of rabbit proximal convoluted tubule was measured, it was observed that the $pH_i$ decrease induced by peritubular acidification was inhibited by acetazolamide (Krapf et al., 1987). Acetazolamide was similarly found to alkalinise cells of the rabbit inner medullary collecting
duct (Prigent et al., 1985). A degree of cell alkalinisation greater than would be expected from the corresponding alkalinisation of the urine could, by suppressing ammoniagenesis, explain the slight tailing off of the points observed at the alkali end of the data for Subject 10, where urine samples were collected during the administration of acetazolamide (see Figure 3.8).

6.1.2 Chronic acid-base state

Long term acidosis

Acidosis induced by the ingestion of an acidifying stimulus, usually ammonium chloride (NH₄Cl), has been the major experimental model used for the investigation of ammonia metabolism. It is assumed that similar mechanisms would be applicable in response to normal dietary modifications of acid and alkali intake. There have been many studies, going back over 60 years, on long term acidosis in man (Gamble et al., 1925; Farquharson et al., 1931; Sartorius et al., 1949; Madison and Seldin, 1958). In one study, ammonium chloride was given for 44 days, in doses of 8 g.day⁻¹ (Wood, 1955). Acidosis reached a peak at Day 5 and the phase of chronic acidosis then plateaued from Day 6 to Day 44; acidosis after Day 5 subsided to a lower level and remained there as long as ammonium chloride was given. Investigations in rats (Kopyt et al., 1974) and dogs (Gougoux et al., 1982) have also shown that sustained metabolic acidosis produces large increases in ammonium production. Chronic respiratory acidosis does not seem to provide the same major stimulus to renal ammonium production (Carter et al., 1959; Rodriguez-Nichols et al., 1984).

The two experimental subjects studied in this thesis showed different magnitudes of response to long term acidosis. Referring to Table 4.1 on page 46, it can be seen that the daily ammonium output in both subjects had increased almost fivefold from basal levels by Day 4. However, Subject 9 was still continuing to increase his rate of ammonium output on Day 7, whereas Subject 10 had reached a peak on Day 4 and the response then reached a plateau. It appears, therefore, that ammoniagenesis was continuing to be stimulated in Subject 9 whilst Subject 10 had reached maximum ammoniagenesis. Once ammonium chloride had ceased to be taken and sodium bicarbonate was given to produce more alkaline urines Subject 9 still showed an increased rate of ammonium excretion above control levels whereas this effect did not last in Subject 10 whose excretion rate showed no significant change from control (see section 4.1 on page 45). Creatinine clearance was unchanged from normal values, showing that this result was not a consequence of differences in glomerular
filtration rate. The duration of the effect in Subject 9 was still observable for 24-48 hours after ammonium chloride ceased to be taken. The enhanced ammoniagenesis of Subject 10 was not marked enough to be statistically significant over the whole pH range, perhaps because it disappeared rapidly when sodium bicarbonate was used to alkalinise the urine (see section 4.1). From these results, it is clear that there is an individual response to chronic acidosis and that the increased rate of ammonium excretion cannot be explained simply by non-ionic diffusion and ion trapping. In the present studies both subjects took 8g.70kg⁻¹.b.w. of NH₄Cl daily, but this increased the rate of ammonium excretion of Subject 9 more than Subject 10 whose diet, being principally vegetarian, may have been less acid-producing than that of Subject 9. Previous studies (Elkinton et al., 1960) have also shown a lesser response than normal of ammonium excretion to a daily acid load by a vegetarian (RM McCance). It may be that this subject did not increase his ammonium excretion because his kidneys had sustained very little acid load stimulating ammoniagenesis for many years.

Chronic metabolic acidosis induces an adaptive change in the ammonia-generating metabolic pathway which enhances the renal capacity for ammonia production (Tannen, 1978). Studies in the intact rat (Lowry and Ross, 1980), the isolated perfused kidney (Tannen and Ross, 1979; Terao and Tannen, 1980), and renal tubules *in vitro* (Vinay et al., 1980a) have all shown that decreased intracellular pH stimulates ammoniagenesis from glutamine (Trivedi and Tannen, 1986). An increased rate of ammonia production per nephron in chronic metabolic acidosis may be due, in part, to an increased uptake of glutamine across the basolateral membrane of the cortical cell (Windus et al., 1984). Recent work, using LLC-PK₁ cells, has further suggested that a reduced intracellular pH of proximal tubular epithelium may be the signal for increased NH₃ production (Sahai et al., 1990). Another possibility that has been suggested is that an increased rate of glutamine metabolism is a direct result of urinary acidification. When the urine is acidified during acute acidosis, there is increased trapping of ammonia as NH₄⁺ and a decrease in NH₃ in the renal cortex. As PDG is inhibited by NH₃ (see Figure 5.1), a lowering of tissue NH₃ concentration could increase the rate of glutamine metabolism (Denis et al., 1964; Tannen, 1978). However, this explanation seems unlikely as the increase in the rate of ammonium excretion is greater than would be predicted from the urine pH.

The *proximal tubule* is the chief site of renal ammonium production; GDH activities are regulated only in proximal tubular segments in the rat (Wright and Knepper,
1990). GDH is induced in response to chronic metabolic acidosis (Kaiser et al., 1992) and entry of ammonia into the proximal tubular lumen is thereby enhanced (Buerkert et al., 1983). This increased proximal tubular ammoniagenesis and secretion produces an increased ammonium concentration in the luminal fluid in the loop of Henle (Buerkert et al., 1982). The thick ascending limb reabsorbs increased amounts of ammonium (Good and Kurtz, 1989) by the direct transport of NH$_4^+$

This NH$_4^+$ absorption occurs predominantly by active transport mediated by transport of NH$_4^+$ on the K$^+$ site of the Na$^+$-K$^+$-Cl$^-$ cotransporter in the apical membrane (Kinne et al., 1986; Garvin et al., 1988). A minor component of this absorption is also thought to be by diffusion, driven by the lumen-positive transepithelial voltage (Good, 1988). Recent studies provide an additional explanation that there is an adaptive increase in the absorptive capacity of the medullary thick ascending limb (MTAL) for NH$_4^+$ (Good, 1990a) which would lead to increased ammonium secretion into the collecting duct during chronic metabolic acidosis. Active absorption of ammonium by the MTAL serves as a stimulus for countercurrent multiplication of ammonium in the renal medulla (Good and Knepper, 1985). Countercurrent multiplication causes NH$_4^+$ to accumulate in the loop of Henle and interstitial fluid of the renal medulla (see Figure 5.2 on page 95), generating a NH$_3$ concentration gradient favouring secretion of NH$_4^+$ into the collecting duct (Good et al., 1987).

**Long term alkali-loading**

In the experiments with long term loading of sodium bicarbonate, both subjects showed reduced ammonium excretion. Long term alkali-loading was the term used to describe this study, rather than alkalosis, because it is very difficult to maintain an alkalosis by administration of sodium bicarbonate in man. Previous alkali-loading studies used eight times as much sodium bicarbonate per person as in the present study and yet plasma total CO$_2$ was still often in the normal range; the kidney is extremely efficient at excreting bicarbonate (van Goidsenhoven et al., 1954). The dose of sodium bicarbonate used in these experiments, 11g.70kg$^{-1}$, equivalent to 130mmol.70kg$^{-1}$, was not large enough to produce a chemical alkalosis but would significantly reduce the daily need for excretion of H$^+$.

The effect of long term alkali-loading on ammonium excretion was short lived in both subjects. In Subject 9, urine samples were collected for six hours after the cessation of sodium bicarbonate, then 6g of ammonium chloride (NH$_4$Cl) was taken to obtain acid
urines. The rate of ammonium excretion, reduced with long term alkali administration, was observed to rise back to control levels during the following 14 hours. In Subject 10, the effects of chronic alkali-loading on ammonium excretion had ceased to be apparent within 5 hours of the cessation of sodium bicarbonate, without any NH₄Cl being given (see section 4.2).

The reduction seen in ammonium excretion, of between 25% and 40% from control values, was greater than can be explained by a high urine pH alone reducing non-ionic diffusion and subsequent ion trapping. It is suggested that this reduction is mediated by suppression of ammoniagenesis in the proximal tubule, caused by the removal of the H⁺ stimulus present under normal acid-base conditions. These results support those of previous animal studies. It has been shown in the dog and the rat that ingestion of NaHCO₃ appears to decrease renal ammoniagenesis and gluconeogenesis from glutamine (Goodman et al., 1966; Goorno et al., 1967; Kamm and Asher, 1970; Pitts, 1972). Ammonium production is decreased by acute metabolic alkalosis in the rat (Narins et al., 1982) and the dog (Fine et al., 1978). The response to chronic metabolic alkalosis appears to be largely the converse of the response to chronic metabolic acidosis (Lemieux et al., 1985).

### 6.1.3 Availability of substrate for ammoniagenesis

Glutamine is the major substrate for ammonia produced by the kidney of the rat (Lotspeich, 1967), dog (Van Slyke et al., 1943) and man (Owen and Robinson, 1963). Previous studies have shown that giving an experimental subject oral glutamine substrate will markedly increase ammonium excretion (Madison and Seldin, 1958). In healthy subjects, orally administered glutamine raised plasma glutamine concentration markedly for over 2–3 hours (Welbourne et al., 1972), with an accompanying substantial increase in urinary ammonium excretion in relation to urine pH both under normal acid-base conditions and during metabolic acidosis.

In the studies described in sections 4.3.1 and 4.3.2 a two- to three-fold increase in plasma glutamine levels resulted after ingestion of a single dose of glutamine substrate, and with repeated doses the concentration of plasma glutamine was elevated up to six times. There was no significant difference between the ammonium excretion rate against urine pH data for the single and repeated dose studies, so for each of the two experimental subjects these results were combined. The correlation of the data to a linear relationship was poor in both subjects, in particular in Subject 9. However, glutamine administration
appeared to cause an increased rate of urinary ammonium excretion in both subjects. An increase in ammonium excretion rate in both subjects occurred promptly, i.e., it was noticeable in the first urine samples to be collected, 30 minutes after the ingestion of the glutamine. This increase corresponded to the increased plasma level of glutamine during the single dose studies. During the repeated dose studies plasma glutamine was elevated up to six times, but an increase in ammonium excretion rate of this magnitude was not achieved, the increase being a maximum of 90%. This result suggests that when there is an excess of glutamine substrate available, ammonia production may be limited by cellular enzyme activity. Enzymes are rarely saturated with substrate \textit{in vivo} and thus the amount of enzyme is unlikely to be rate-limiting. It is more probable that the accessibility of the enzyme could be limiting, e.g., the uptake of enzyme into the cell, or feedback inhibition of enzyme may limit ammoniagenesis. The results of this study support the suggestion that the availability of substrate may be a rate-limiting factor to renal ammoniagenesis under normal acid-base conditions.

During acidosis the uptake of glutamine by the kidney has been shown to increase in both the dog and man (Pitts et al., 1966; Pitts and Pilkington, 1966; Stone et al., 1967). Studies in rats have shown that renal ammonium production can increase within 30 minutes of acid administration, and that uptake of glutamine into the mitochondria and the generation of glucose can increase within hours, but it is many hours before increased renal ammonia-producing enzymes can be detected (Alleyne, 1970). It appears, therefore, that the actual uptake of glutamine into the cell is the more likely regulator of ammonia production (Pitts, 1973). The fate of glutamate may also be very important for renal control of ammoniagenesis. Several studies have shown that there is an increased glutamate flux through the GDH pathway (see Figure 5.1 on page 89) in the mitochondria of rats with acute metabolic acidosis (Boyd and Goldstein, 1979; Vinay et al., 1980a). The concentration of $\alpha$-ketoglutarate is decreased in acidosis, so it is probable that increased $\alpha$-ketoglutarate metabolism would activate PDG by lowering intramitochondrial glutamate levels; in this way $\text{NH}_4^+$ production would be increased (Goorno et al., 1967). Whatever the extramitochondrial fate of the glutamine carbon skeleton, an increased flux through GDH occurs during chronic metabolic acidosis and more glucose is produced. Cortical slices from animals with chronic metabolic acidosis oxidise more glutamate and generate more ammonia than control slices incubated under the same conditions \textit{in vitro} (Simpson, 1972; Kamm and Strope, 1972).
Studies of individual segments of rat nephron (Curthoys and Lowry, 1973a) demonstrated that during incubation in an acid medium, the glutamine concentration is reduced in all segments of the nephron. This suggests that glutamine metabolism, and therefore ammonia production, is enhanced throughout the nephron during acidosis. However, during chronic acidosis, the activities of PDG (Curthoys and Lowry, 1973b) and of PEPCK (Burch et al., 1978), two enzymes important in the production of ammonia (see Figure 5.1), are increased only in the proximal convoluted tubule. The decrease in tissue glutamine content could be due to an increased rate of glutamine transport across the inner mitochondrial membrane (Adam and Simpson, 1974). An increased rate of transport into the mitochondria would reduce tissue glutamine concentration if a significant proportion of that transported were quickly metabolised (Curthoys and Shapiro, 1978).

6.1.4 Adrenal hormones and potassium homeostasis

It is very difficult to distinguish the effects of mineralocorticoids and potassium (K⁺) on ammonium excretion because an increase of these hormones commonly leads to reduced plasma K⁺, whilst a hormone deficiency is followed by an increase in plasma K⁺. States of mineralocorticoid excess, particularly primary aldosteronism, are associated with hypokalaemia and with metabolic alkalosis (Ledingham, 1987) and a urinary ammonium excretion which is elevated in relation to urine pH (Mills et al., 1961; Wrong, 1964). The administration of mineralocorticoids results in increased net acid excretion (NAE) in normal humans (Sonnenlich et al., 1961; Lemann et al., 1970), including increased ammonium excretion in relation to pH, although there is no evidence that any stimulatory effect of mineralocorticoid hormones on NAE is due to a direct effect on ammoniagenesis (Harrington et al., 1986). Conversely, states of mineralocorticoid deficiency, such as primary adrenal failure or renal tubular insensitivity to the action of mineralocorticoids, commonly lead to hyperkalaemia and metabolic acidosis (Morris and Sebastian, 1983), a state which is recognised as one form of hyperkalaemic renal tubular acidosis, and in which urinary ammonium excretion is markedly reduced in relation to urine pH (Sebastian et al., 1977); it has been suggested that the latter reduction in ammonium excretion is due to suppression of renal ammoniagenesis by hyperkalaemia (Hulter et al., 1977). Studies of adrenalectomised dogs maintained postoperatively with replacement mineralocorticoids showed that when these hormones were stopped hyperkalaemia occurred and NAE decreased (Hulter et al., 1979). The excretion rate of ammonium appears to vary inversely
with the degree of hyperkalaemia. In patients with aldosterone deficiency correction of hyperkalaemia increases ammonium excretion without a reduction in urine pH (Szylman et al., 1976).

In some circumstances administration of exogenous potassium, without a change in mineralocorticoid status, suppresses the production of ammonium by the mammalian kidney (Tannen, 1977). Increases in plasma K+ concentration suppress the synthesis of ammonia (Sastrasinh and Tannen, 1983; Sleeper et al., 1982) and reduce ammonium excretion (Jaeger et al., 1983). In rats, dietary potassium loading can lead to a 40% reduction in ammonium excretion in the urine with no change in ammonium secretion by the proximal convoluted tubule (Du Bose and Good, 1991), the extra ammonium appearing in renal venous blood. It is suggested that this reduction in ammonium excretion is due to an impaired ability to transfer NH$_4^+$ from the proximal tubule to the collecting duct; an increase in the amount of K+ competing with NH$_4^+$ for transport via K+ linked mechanisms could reduce NH$_4^+$ transport and subsequent ammonium excretion. A variety of observations with both clinically and experimentally induced potassium depletion in humans and animals have suggested that potassium depletion may modify renal ammonia metabolism. An increase in urinary ammonium excretion relative to urine pH has been described in situations of potassium depletion in man, including laxative abuse (Schwartz and Reiman, 1953), primary hyperaldosteronism (Leutscher, 1964) and fasting (Sapir et al., 1976), as well as experimentally induced potassium depleted states (Clarke et al., 1955; Huth et al., 1959). In addition to these in vivo observations, several in vitro studies in potassium depleted rats have shown an increase in ammoniagenesis (Pagliara and Goodman, 1970; Adam and Simpson, 1975; Tannen and Kunin, 1976; Tannen and McGill, 1976). Thus changes in plasma potassium (K+) outside the normal range appear to exert an important influence on ammoniagenesis.

The purpose of the present study was to investigate whether mineralocorticoids affected excretion of ammonium independently of plasma potassium levels. In the experiments described in section 4.4 the effects of hypo- or hyperkalaemia were not specifically investigated, because it is extremely difficult to manipulate plasma potassium levels in healthy subjects without weeks of experimental protocol which carry some risk to the subjects involved. Plasma K+ remained in the normal range in both subjects during these studies. Neither administration of the mineralocorticoid 9α-fludrocortisone, nor the aldosterone antagonist spironolactone, produced any significant change in the relationship between
the rate of ammonium excretion and urine pH in either of the experimental subjects. No alteration of the urinary NH$_4^+$ excretion rate to pH relationship by mineralocorticoid hormones alone has been reported, and the results of the experiments in section 4.4 do not support the hypothesis that mineralocorticoids alone produce any changes in ammonium excretion rate. Taken together, these results provide further evidence that potassium availability, rather than mineralocorticoid level, is the more important factor controlling the rate of ammonium excretion.

6.2 Influences on ammonia transfer to urine

The major influence on the transfer of total ammonia to the urine is urine pH. The effect of this variable on urinary ammonium excretion has been examined during all the experiments detailed in this thesis, and is summarised in section 6.3.1. The permeability of the diffusion barriers through which ammonia must pass before entering the urine may be affected by renal disease, which is discussed later (see Appendix A). In this section the effects of urine flow rate and metabolic inhibition on ammonia transfer in the nephron are discussed.

6.2.1 Urine flow rates at different nephron sites

Water diuresis

A higher rate of ammonium excretion in relation to urine pH than control was observed in the water diuresis studies described in section 4.5. This is probably a dilution effect of ammonium passing down a concentration gradient in the collecting duct. The higher the rate of luminal flow the more dilute the tubular fluid will be and therefore the steeper the concentration gradient for diffusion of ammonia from the medulla into the lumen. Experimental work suggests that an increase in tubular fluid flow rate may also enhance ammonium absorption in the thick ascending limb (TAL); studies done in vitro in the rat TAL showed, interestingly, that increasing the luminal flow rate led to a directly proportional increase in the rate of ammonium absorption (Good, 1987). The fall in luminal HCO$_3^-$ concentration which accompanies acidification of the lumen in the rat TAL would be minimised at higher flow (Good et al., 1984) resulting in a higher pH of the lumen. The result of this increase in pH would be an increase in luminal NH$_3$ concentration which would increase net ammonium absorption. Stimulation of ammonium absorption in
the TAL may enhance ammonia excretion by promoting increased medullary ammonium accumulation and consequently increased secretion of ammonium into the collecting duct (see Figure 5.2 on page 95). The data obtained in the present studies support the in vitro observations of Good that, to some extent, urinary ammonium concentration is related to urine pH. However, a much closer relationship is observed in man when the rate of urinary ammonium excretion is related to pH (see section 5.2.1).

Although indirect evidence suggests that ammonium is added to the collecting duct system, direct examination of this issue has been limited. It was first reported, over thirty years ago, that ammonium was added to the medullary collecting duct of the hamster (Ullrich et al., 1958) but no quantitative information was provided. Ten years ago, several studies provided further evidence for net secretion of ammonium into the medullary collecting duct (Graber et al., 1981a; Sajo et al., 1981; Sonnenberg et al., 1981). Water removal occurs throughout the distal convoluted tubule and the collecting duct (Jamison and Kriz, 1982). If the principal site of water removal were distal to the main site of ammonium addition in the collecting duct it would explain the fact that the rate of ammonium excretion is more closely related to urine pH than is urinary ammonium concentration.

Recently, during water diuresis, countercurrent multiplication of ammonium has been shown to persist, creating an interstitium to lumen gradient that could drive NH$_3$ secretion into the medullary collecting duct (Packer et al., 1991). It is not surprising that this should be so, since the rate of active NH$_4^+$ transport in the TAL is not affected by antidiuretic hormone (Good, 1990b). The increase in the rate of ammonium excretion with water diuresis was around 30% above control levels in the present studies. It is suggested that this increase in rate is due to an enhanced rate of diffusion of ammonia, caused by an increased concentration gradient, from the medullary interstitium into the lumen of the collecting duct.

Osmotic diuresis

The results of the present studies on osmotic diuresis (see section 4.5) show a marked increase in the rate of ammonium excretion above control in both subjects. This increase is three to five fold greater than that observed for water diuresis. Osmotic diuresis increases the rate of flow, from the proximal tubule distally, at every point along the nephron. An important way in which luminal flow rate may affect renal tubular transport processes is by affecting the luminal concentration of transported solutes (Wright, 1982). The increased
rate of ammonium excretion above control could therefore be due to a dilution effect on fluid within the tubular lumen, which would lower ammonia concentrations and so increase the gradient between tubular cell and lumen. Osmotic diuresis is likely to have a greater influence on non-ionic diffusion of NH$_3$ than water diuresis as it increases the rate of flow of the luminal fluid in the proximal tubule at critical sites of addition of ammonia, reducing the concentration ratio, and therefore more ammonia will diffuse into the lumen before any equilibrium can be reached.

A direct correlation between luminal flow rate and delivery of ammonium to the late proximal convoluted tubule was first reported in studies of acutely acidotic rats (Simon et al., 1983). It was then demonstrated that increasing the rate of perfusion of the mouse proximal tubule in vitro has a direct effect on ammonium secretion rate (Nagami and Kurokawa, 1985). This increase was seen both with and without glutamine in the perfusate, showing that the stimulus was not solely due to an increase in luminal substrate delivery. Experiments in the proximal convoluted tubule of the rat in vivo showed that an osmotic diuresis which doubled the tubular flow rate increased ammonium excretion by more than 60% (Simon and Hamm, 1987). Thus an increase in luminal flow has been shown to be an effective stimulus for proximal tubular ammonium secretion in vivo as well as in vitro in animal models.

Changes in medullary ammonium concentration have been shown to correlate directly with changes in ammonia excretion during mannitol diuresis (Robinson and Owen, 1965). The medullary countercurrent exchange system generates a transepithelial concentration difference for ammonium that favours medullary NH$_4^+$ accumulation (Atherton et al., 1968; Packer et al., 1991). An increased concentration of ammonium being delivered to the TAL would increase net ammonium absorption, and increase the accumulation of ammonium in the medulla. The result would be increased secretion of ammonium into the collecting duct and consequent increased excretion in the urine (see Figure 5.2).

The effects of osmotic diuresis on the relationship between urinary ammonium excretion rate and pH have not previously been studied in man. The present work supports previous results obtained from animals and provides evidence of a substantial increase in ammonium excretion with an osmotic diuresis. The increase is probably caused by

1. increased secretion of ammonia into the proximal tubular lumen, and
2. increased countercurrent multiplication of ammonium in the TAL.
6.2.2 Metabolic inhibition by pharmacological diuretics

Benzothiadiazide diuretics

Acute administration of the thiazide diuretic bendrofluazide in the present studies resulted in a reduced rate of urinary NH$_4^+$ excretion of approximately 40% at all urine pH values (see section 4.6.1). A possible reason for this reduction is that bendrofluazide inhibits active transport of NH$_4^+$ in the renal tubule.

Thiazide diuretics cause a marked natriuresis by inhibition of Na$^+$ reabsorption in the distal convoluted tubule of the dog (Edwards et al., 1973). An electroneutral NaCl cotransport system located on the apical, or luminal, membrane of the flounder urinary bladder, a model for the distal tubule, can be inhibited rapidly and reversibly by hydrochlorothiazide (Stokes, 1984). Thiazide-sensitive NaCl absorption in the rat has also been demonstrated in the early distal tubule (Costanzo, 1985), the ascending limb of the loop of Henle (Ellison et al., 1987) and the cortical collecting duct (Terada and Knepper, 1990) although its effects are most marked in the distal tubule. Chronic hydrochlorothiazide infusion in rats has been shown to reduce distal Na$^+$–Cl$^-$ transport activity (Morsing et al., 1991) leading to reduced Na$^+$ entry into the tubular cell. Such a reduction of Na$^+$ entry has been shown to reduce the pool of active Na$^+$–K$^+$–ATPase in the medullary thick ascending limb (Grossman and Hebert, 1988).

The concentration of NH$_4^+$ in blood and extracellular fluid is low (0.01-0.02 mmol.l$^{-1}$) compared to that of K$^+$ (3–5 mmol.l$^{-1}$) such that NH$_4^+$ is unlikely to compete with K$^+$ for transport on the Na$^+$–K$^+$–ATPase of most tissues (Newsholme and Leech, 1984). In the kidney, ammoniagenesis results in much higher levels of NH$_4^+$, especially in the medulla, where it accumulates owing to countercurrent multiplication (Good and Knepper, 1985). Total ammonia concentrations in the vasa recta of the inner medulla of rats are in the range of 9–23 mmol.l$^{-1}$ (Stern et al., 1985) and near the bend of the loop of Henle in the inner medulla 11–21 mmol.l$^{-1}$ has been found (Buerkert et al., 1982). A physiologically important interaction between K$^+$ and NH$_4^+$ in renal Na$^+$–K$^+$–ATPase is most likely in both the inner and outer medulla of the kidney where a relatively low ERPF allows accumulation of NH$_4^+$. Thus, in the medulla, direct transport of NH$_4^+$ into the cells by the Na$^+$–K$^+$–ATPase could be important for total ammonia and net acid transport (Garvin et al., 1985). Reduced activity of Na$^+$–K$^+$–ATPase could therefore result in a decrease in NH$_4^+$ uptake into the cell (Kurtz and Balaban, 1986). Thiazides have been
shown to reduce the activity of the basolateral \( \text{Na}^+\text{-K}^+\text{-ATPase} \) in the distal convoluted tubule \textit{in vitro} (Garg and Narang, 1987). Inhibition of this enzyme by bendrofluazide could, therefore, result in less \( \text{NH}_4^+ \) being transported. Such a reduction in ammonium transport may explain the reduced rate of ammonium excretion observed in a subject taking bendrofluazide (see section 4.6.1).

**Loop diuretics**

Frusemide and bumetanide are loop diuretics which are virtually instantaneous in their action and completely reversible in their effects (Friedman, 1988). In the experiments described in sections 4.6.2 and 4.6.3, administration of both of these loop diuretics caused a substantial reduction of approximately 40% less than control values in ammonium excretion rate in relation to urine pH. These diuretics, as their collective name suggests, act principally in the loop of Henle, and specifically on the cortical and medullary segments of the thick ascending limb (TAL) where \( \text{NaCl} \) is reabsorbed (Rocha and Kokko, 1973). \( \text{Na}^+ \) and \( \text{Cl}^- \) transport are coupled at the luminal membrane (Hebert et al., 1981); current evidence suggests an electroneutral \( \text{Na}^+\text{-K}^+\text{-2Cl}^-\text{-cotransporter} \). Loop diuretics act on the luminal side of the thick ascending cells where they inhibit the reabsorption of \( \text{Cl}^- \), \( \text{Na}^+ \) and \( \text{K}^+ \) (Imbs et al., 1987). Frusemide and bumetanide have both been shown to bind to and inhibit the \( \text{Na}^+\text{-K}^+\text{-2Cl}^-\text{-cotransporter} \) in the luminal membrane of the TAL (Oberleithner et al., 1982; Haas and Forbush, 1987; Turner and George, 1988), and thus to inhibit active \( \text{NaCl} \) reabsorption (Greger, 1985; Breyer and Jacobsen, 1990). Frusemide has also been shown to abolish the corticomedullary ammonium gradient in the rat kidney (Packer et al., 1991), thus leading to reduced ammonium excretion.

\( \text{NH}_4^+ \) has been shown to be absorbed by active transport in the TAL as a result of the substitution of \( \text{NH}_4^+ \) for \( \text{K}^+ \) on the \( \text{Na}^+\text{-K}^+\text{-2Cl}^-\text{-cotransporter} \) in the apical membrane (Garvin et al., 1988). As the \( \text{Na}^+\text{-K}^+\text{-2Cl}^-\text{-cotransporter} \) is sensitive to bumetanide and frusemide, \( \text{NH}_4^+ \) absorption could be inhibited by these diuretics. The TAL is also rich in \( \text{Na}^+\text{-K}^+\text{-ATPase} \), but loop diuretics have too slight an inhibitory effect on \( \text{Na}^+\text{-K}^+\text{-ATPase} \) to act via this mechanism (Doucet et al., 1979).

Bumetanide has been shown to have a greater potency than frusemide for the \( \text{Na}^+\text{-K}^+\text{-2Cl}^-\text{-cotransporter} \) (McRoberts et al., 1982; Owen and Prastein, 1985). This is in keeping with the accepted therapeutic dose levels which were followed in the present studies, where, in Subject 10, 1 mg of bumetanide produced a similar inhibitory effect.
Table 6.1: Changes in ammonium excretion rate with loop diuretics (Subject 10)

<table>
<thead>
<tr>
<th></th>
<th>cf. to control</th>
<th>cf. to osmotic diuresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frusemide</td>
<td>-31%—46%</td>
<td>-61%—74%</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>-29%—45%</td>
<td>-59%—74%</td>
</tr>
</tbody>
</table>

on ammonium excretion rate to that produced by 60 mg of frusemide (see Table 6.1). Inhibition of the apical \( \text{Na}^+ - \text{K}^+ - 2\text{Cl}^- \) cotransporter in the TAL, causing reduced active transport of \( \text{NH}_4^+ \), is therefore the probable explanation for the reduction in ammonium excretion rate described in sections 4.6.2 and 4.6.3.

**Potassium-sparing diuretics**

Administration of a potassium-sparing diuretic, amiloride, produced no observable difference from control values in the rate of ammonium excretion (see section 4.6.4). Potassium-sparing diuretics inhibit sodium reabsorption in the late distal nephron, which is sensitive to aldosterone and which normally reabsorbs 2–3% of the filtered sodium load. Amiloride, like spironolactone, reduces \( \text{Na}^+ \) absorption and \( \text{H}^+ \) secretion in the distal tubule, but does so by an action independent of aldosterone. Amiloride acts principally in the late distal tubule (Bull and Laragh, 1969) and is not effective in the early distal tubule (Costanzo, 1985; Velazquez and Wright, 1986). The cellular mode of action of amiloride is to block sodium channels at the luminal cell surface, and hence to increase both sodium and water excretion, whilst simultaneously decreasing potassium excretion (Benos, 1982). In addition, amiloride inhibits \( \text{II}^+ \) secretion (Arruda et al., 1980). By the use of renal membrane vesicles, it has been demonstrated that amiloride is a competitive inhibitor at the sodium site of the \( \text{Na}^+ - \text{H}^+ \) exchanger in the proximal apical membrane (Kinsella and Aronson, 1981b) and the renal cortical microvillus membrane (Kinsella and Aronson, 1981a). At high concentrations amiloride can also inhibit \( \text{Na}^+ - \text{K}^+ - \text{ATPase} \) (Zhuang et al., 1984) but there is no evidence that, at clinically achievable concentrations, amiloride inhibits any enzymes involved in the active transport of \( \text{NH}_4^+ \).
6.3 Summary

6.3.1 Renal ammonium transport

The concepts of how ammonium is excreted by the kidney in man have changed drastically in the last few years. The diffusion trapping model of Pitts was for a long time thought to be the principal mechanism by which ammonium was excreted. This model provides the classical explanation for ammonium transport in the kidney. Ammonia is synthesised in the renal tubular cell and transport occurs via diffusion of NH\textsubscript{3} from less to more acidic compartments where it is titrated to NH\textsubscript{4}\textsuperscript{+}. In this ideal model, NH\textsubscript{3} equilibrates instantaneously and NH\textsubscript{4}\textsuperscript{+} is completely impermeable through cell membranes and is therefore trapped in the tubular urine. So, according to the ideal theory, there are no NH\textsubscript{3} gradients across renal epithelia because of its spontaneous conversion to NH\textsubscript{4}\textsuperscript{+}, and NH\textsubscript{4}\textsuperscript{+} is present in the kidney in proportion to the H\textsuperscript{+} concentration.

There has been rapid progress over the last decade in discovering the precise mechanisms of renal ammonium transport. Ammonia produced in the cells of the proximal tubule in the renal cortex is secreted into the urine as a result of specialised transport processes in specialised nephron segments. Two mechanisms of ammonia transport have clearly been established in the proximal tubule: passive NH\textsubscript{3} diffusion and active NH\textsubscript{4}\textsuperscript{+} transport on the Na\textsuperscript{+}-H\textsuperscript{+} exchanger. The relative contribution of these pathways to ammonium transport is still unresolved. Absorption of ammonium by the loop of Henle provides the mechanism by which the ammonia produced and secreted by the proximal tubule is transferred to the collecting duct in the renal medulla. Loop ammonium absorption is largely a result of active absorption of NH\textsubscript{4}\textsuperscript{+} by the thick ascending limb, mediated principally by apical membrane Na\textsuperscript{+}-NH\textsubscript{4}\textsuperscript{+}-2Cl\textsuperscript{-} cotransport. Countercurrent multiplication causes ammonium to accumulate to high concentrations in the medullary interstitial fluid and generates a transepithelial concentration difference for NH\textsubscript{3} that favours the secretion of ammonium into medullary collecting ducts. This secretion occurs by passive diffusion of NH\textsubscript{3} in parallel with active H\textsuperscript{+} secretion. The active H\textsuperscript{+} secretion causes an acid disequilibrium pH in segments which lack carbonic anhydrase and enhances the NH\textsubscript{3} gradient driving NH\textsubscript{3} secretion by reducing the luminal NH\textsubscript{3} concentration.

The excretion of ammonium is regulated partly through the control of ammoniagenesis in the proximal tubule and partly through control of renal ammonium transport. The aim of the present work was to investigate, in man, the main factors which may influence these
two variables and so alter the relationship between ammonium excretion rate and urinary pH. The outcomes of the experiments performed are summarised in Table 6.2. Under the circumstances examined it was found that the negative correlation between urine pH and ammonium excretion was preserved, but that the rate of ammonium excretion was raised or lowered under different experimental conditions. In all the experiments the pH of the urine was varied over as wide a range as possible. The constancy of the negative correlation between ammonium excretion rate and urine pH under the conditions studied is in keeping with the theory of non-ionic diffusion of NH₃ and subsequent diffusion trapping.

*Increased luminal flow rate* produced by water diuresis led to a substantial increase in ammonium excretion rate, probably due to a dilution effect augmenting the concentration gradient for diffusion of ammonium into the lumen of the collecting duct. Osmotic diuresis caused an even greater increase in the rate of ammonium excretion. It is suggested that this was due to production of a steep concentration gradient for the secretion of ammonia from the proximal tubular cell into the lumen, in addition to a more distal action similar
to that of water diuresis. *Inhibition of active NH$_4^+$ transport* in the thick ascending limb and distal tubule was attempted by the use of loop and thiazide diuretics. Coupling of cell uptake of NH$_4^+$ to that of Na$^+$ and Cl$^-$ (by substitution of NH$_4^+$ for K$^+$ on the Na$^+$-K$^+$-2Cl$^-$ cotransporter) results in active absorption of NH$_4^+$ across the apical membrane. The transcellular absorption of NH$_4^+$ is completed by its transfer across the basolateral membrane into the medullary interstitium. NH$_4^+$ substitution for K$^+$ on the basolateral Na$^+$-K$^+$-ATPase could theoretically mean that the Na$^+$-K$^+$-ATPase could serve as an NH$_4^+$ pump to transport NH$_4^+$ in a secretory direction. The inhibition of these mechanisms of active NH$_4^+$ transport could result in reduced countercurrent multiplication and decreased ammonium secretion into the lumen. It is suggested that such inhibition occurred during the administration of loop and thiazide diuretics and consequently ammonium excretion rate fell.

6.3.2 Modulators of renal ammoniagenesis

*Acute acidosis* does not appear, from these studies, to exert a significant stimulatory effect on ammoniagenesis. Its effect in increasing renal ammonium excretion is therefore likely to be due to a reduction in luminal pH, causing increased ion trapping. *Chronic acidosis* increased ammonium excretion rate more than can be explained by a low urine pH alone, increasing non-ionic diffusion and ion trapping. An adaptive change in the ammonia-generating metabolic pathway, which enhances the renal capacity for ammoniagenesis, may have been induced. This adaptation is likely to be a change in the mitochondrial PDG pathway, although more than one rate-limiting step may be altered to account for the overall effect. Increased ammoniagenesis within the renal tubule could lead to an increased rate of renal diffusion of NH$_3$ and more NH$_4^+$ trapping in the tubule. The effects of chronic alkali loading appear to be largely the converse of the response to chronic acidosis.

The principal renal precursor of ammonium is glutamine, and in these studies oral administration of this amino acid was used to increase the availability of substrate. Although raising the level of plasma glutamine produced a substantial enhancement of urinary ammonium excretion rate, the increases were not directly proportional; urinary ammonium excretion rate increased by only 15–20% of the rise in plasma glutamine concentration. When glutamine was continuously given, the level of ammonium excretion rate plateaued, despite the high plasma glutamine concentration. These results suggest that either sub-
strate transport into the mitochondria and/or its conversion to ammonia in the mitochon-
dria can be rate-limiting to ammoniagenesis.

The effects of adrenal hormones and potassium homeostatis on renal ammonium excretion are difficult to distinguish. However, studies involving the administration or suppression of mineralocorticoids, whilst the subject remained normokalaemic, showed no effect on the rate of urinary ammonium excretion, suggesting that availability of potassium is the more important factor controlling renal ammonium excretion.

6.3.3 Countercurrent multiplication and ion trapping

The regulation of ammonium production and excretion is clearly a complex process, and one that is currently undergoing intensive research. The majority of observations on renal ammoniagenesis and transport of ammonium have been made on animals, so the studies described in this thesis provide useful evidence in man towards several regulating factors which may be involved. Most importantly the results confirm a primary role in man for ion trapping of ammonium, controlled by the pH of the urine. The general picture that has emerged to explain concentration of ammonium in the urine is that the overall process involves two independent trapping processes:

1. countercurrent trapping in the renal medulla which raises the ammonium concentra-
tion in the medullary interstitium, and

2. diffusion trapping in the collecting duct which raises the \( \text{NH}_4^+ \) concentration in the collecting ducts to a level greater than in the medullary interstitium.

Active exchange mechanisms also appear to play an important role in the transfer of ammonium through the kidney and its subsequent excretion in the urine. Further studies on more subjects are required to confirm the observations presented in this thesis, and more possible factors need to be examined in man to elucidate the full picture of ammonium excretion.
Appendix A

Effects of Renal Disease

A.1 General renal failure

As renal failure progresses, metabolic acidosis eventually develops and is a universal feature of far advanced kidney disease. It has long been recognised that there is a large reduction in ammonium excretion resulting in excretion of more $H^+$ as titratable acid than as $NH_4^+$ (Henderson and Palmer, 1915; Wrong and Davies, 1959; Simpson, 1971).

In the study of Wrong and Davies, spontaneously voided urine samples were collected, and ammonium chloride given to see if subjects could acidify. I have reexamined the original data from this study: data from nine subjects (Subjects 12–20) was used, all of whom had generalised renal failure of varying degrees. In these subjects, plasma was taken for analysis of bicarbonate, potassium and creatinine. The results are shown in Table A.1. All subjects had plasma $HCO_3^-$ values in the normal range apart from Subject 12, whose values were just below the normal range and so showed slight acidosis. Subject 16 could not acidify normally; his urine pH following administration of ammonium chloride would go no more acid than pH 6.33. He showed evidence of renal tubular lesions, had renal dystrophy, osteomalacia, and hypokalaemia. The potassium levels seen in Subjects 12, 13 and 15 were at the top end of the normal range. Throughout the renal failure group as a whole there were not many alkaline urines and most were in the acid range of pH 4.5 to 5.5, confirming previous studies (Henderson and Palmer, 1915; Van Slyke et al., 1926; Linder, 1927; Salvesen, 1928). The mean reduction in GFR from normal, calculated from the creatinine clearances ($C_{Cr}$), was 75%.
### Table A.1: Urine analysis in renal failure (Subjects 12–20)

<table>
<thead>
<tr>
<th>Subject Number</th>
<th>Age(Sex)</th>
<th>Wt (kg)</th>
<th>Symptoms</th>
<th>HCO₃⁻ (mmol.l⁻¹)</th>
<th>K⁺ (mmol.l⁻¹)</th>
<th>Cₜₚ (ml.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>31(M)</td>
<td>61</td>
<td>Chronic glomerulonephritis</td>
<td>20.7</td>
<td>5.0</td>
<td>11.8</td>
</tr>
<tr>
<td>13</td>
<td>53(M)</td>
<td>69</td>
<td>Malignant hypertension</td>
<td>23.2</td>
<td>5.0</td>
<td>27.5</td>
</tr>
<tr>
<td>14</td>
<td>28(F)</td>
<td>70</td>
<td>Partial recovery from acute glomerulonephritis</td>
<td>26.0</td>
<td>3.6</td>
<td>62.0</td>
</tr>
<tr>
<td>15</td>
<td>41(M)</td>
<td>68</td>
<td>Malignant hypertension</td>
<td>29.1</td>
<td>5.2</td>
<td>18.6</td>
</tr>
<tr>
<td>16</td>
<td>19(M)</td>
<td>31</td>
<td>Chronic pyelonephritis and osteodystrophy</td>
<td>22.5</td>
<td>3.0</td>
<td>7.3</td>
</tr>
<tr>
<td>17</td>
<td>35(M)</td>
<td>71</td>
<td>Malignant hypertension</td>
<td>27.9</td>
<td>3.9</td>
<td>32.9</td>
</tr>
<tr>
<td>18</td>
<td>24(M)</td>
<td>52</td>
<td>Chronic glomerulonephritis</td>
<td>25.0</td>
<td>4.0</td>
<td>10.5</td>
</tr>
<tr>
<td>19</td>
<td>64(F)</td>
<td>72</td>
<td>Gout and chronic pyelonephritis</td>
<td>22.2</td>
<td>4.5</td>
<td>41.0</td>
</tr>
<tr>
<td>20</td>
<td>55(F)</td>
<td>78</td>
<td>Gout and hypertension</td>
<td>29.7</td>
<td>-</td>
<td>61.0</td>
</tr>
</tbody>
</table>

### A.1.1 Analysis of data

These data had not been specifically gathered to examine the relationship between urinary ammonium excretion rate and pH. Consequently the range of urine pH for each subject was relatively narrow, and the correlation of the data to a straight line only moderate (in two cases, where the pH range covered was only 0.3, the value of $R^2$ was less than 10%). Not surprisingly, the combined data (normalised by weight) from the nine subjects has an $R^2$ value of only 7.3%. If the data is factorised instead by GFR, the value of $R^2$ drops to 5.2%. If only the six subjects with the largest pH ranges are considered, these $R^2$ values become 25.4% and 0.1% respectively. Given such poor correlation figures, detailed statistical analysis is futile. However, from the plot of the data from subjects 12–20 against the data from the combined normal population (see Chapter 3), shown in Figure A.1, it can be seen that there is a manifest decrease in ammonium excretion rate from normal among the subjects with renal failure. Table A.2 gives the usual statistical analysis, indicating that an estimate of the average reduction in excretion rate caused by renal failure is between 56% and 63%.
Figure A.1: Normal (Subjects 1-8) vs. renal failure (Subjects 12-20) 

- Subjects 1-8: Normal Data 
- Subjects 12-20 (Renal Failure): Normal Data
Table A.2: Comparison of normal (Subjects 1–8) and renal failure (Subjects 12–20)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$%R^2$</th>
<th>$%R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 2.909 - 0.245x - 1.412i + 0.189s$</td>
<td>73.0</td>
<td>72.5</td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 2.551 - 0.179x - 0.397i$</td>
<td>66.6</td>
<td>66.2</td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 2.217 - 0.152x$</td>
<td>18.4</td>
<td>17.9</td>
</tr>
</tbody>
</table>

A.2 Interstitial nephritis

An additional new subject, Subject 11, was studied specifically in order to gain more information about the relationship between urinary pH and ammonium excretion in renal failure. There were several reasons for doing this:

- the data from Subjects 12–20 had not been gathered specifically to examine this relationship,
- urine specimens from Subject 11 were collected over the whole physiological pH range: in Subjects 12–20 no attempt was made to alkalise the urine pH,
- more precise measurements of GFR and renal blood flow could be made than in Subjects 12–20,
- Subject 11 was known to have dense interstitial nephritis‡, and
- the combined data from Subjects 12–20 showed a poor fit to a straight line, so that statistical analysis was less reliable.

Subject 11 was a twenty-five year old male with nephrotic syndrome (a clinical state in which there is a combination of oedema, proteinuria and hypoalbuminaemia) in addition to dense interstitial nephritis. In the latter, the renal tubules are separated by interstitial infiltrate, e.g., chronic inflammatory cells and fibroblasts. The subject stayed in the

‡Interstitial nephritis was of special interest because of a theory recently put forward, stating that this disease reduces NH$_4^+$ excretion by interfering with NH$_4^+$ transfer from the proximal tubule to the collecting duct (Carlisle et al., 1991).
metabolic ward of University College Hospital throughout the duration of the study. He emptied his bladder at 7am on Day 1, and then 2-hourly urine collections were made over the next sixteen hours, immediately followed by an 8-hour overnight collection. 60 mg of acetazolamide was then administered by mouth, at 7am on Day 2, and three more urine collections made, under mineral oil to prevent any loss of CO$_2$ from what were likely to be urines in the alkali range. On Day 3 he emptied his bladder at 7am and 500 mg of acetazolamide were taken. 2-hourly urine collections were then made, under mineral oil, over the next sixteen hours, and an 8-hour overnight collection made, finishing at 7am on Day 4. Three further 2-hourly urine collections were then made, and the study was complete.

A.2.1 Measurements made

In addition to the usual measurements of urinary pH, osmolality, ammonium and creatinine, GFR was measured with $^{51}$Cr EDTA clearance and ERPF with $^{125}$I-Hippuran (see section 2.2.3), with the results shown in Table A.3. These results gave a filtration fraction of 15.3%. GFR is approximately 25% of normal values; the mean standard value of GFR in a young adult is 125 ml.min$^{-1}$.70kg$^{-1}$.

Daily plasma samples were taken during the study for measurement of potassium and bicarbonate. Plasma K$^+$ was 4.2 to 4.7 mmol.l$^{-1}$ (in the normal range) over the days in question, whilst HCO$_3^-$ was 21 mmol.l$^{-1}$ (the lowest point of the normal range).

Subject 11 also underwent a renal biopsy as part of his clinical management. The biopsy result showed a focal sclerosing glomerulonephritis with massive separation of tubules by interstitial infiltrate.

A.2.2 Analysis of data

In the absence of any control data for Subject 11, the data were plotted against the data from the combined normal population (see Chapter 3) and are shown in Figure A.2. The analysis of Table A.4 shows that Subject 11 shows a huge decrease in ammonium excretion
Figure A.2: Normal (Subjects 1-8) vs. interstitial nephritis (Subject 11)
Table A.4: Comparison of normal (Subjects 1–8) and interstitial nephritis (Subject 11)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$R^2$</th>
<th>$R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 2.909 - 0.245x - 1.194 + 0.070s$</td>
<td>89.1</td>
<td>88.8</td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 2.876 - 0.239x - 0.756i$</td>
<td>89.0</td>
<td>88.8</td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.447 - 0.365x$</td>
<td>52.8</td>
<td>52.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of s</th>
<th>i</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>76.38%</td>
<td>100.00%</td>
<td>-80% to -85%</td>
</tr>
<tr>
<td>for X on Y</td>
<td>7.73%</td>
<td>100.00%</td>
<td></td>
</tr>
</tbody>
</table>

rate, of between 80% and 85%, when compared with the average of the normal subjects. From the results in section A.2.1 it is clear that the reduction in ammonium excretion rate is even greater than the reduction in GFR.

A.3 Discussion of ammonium excretion in renal disease

It has been known for a very long time that urine acidification is preserved in far advanced renal failure. These patients tend to produce more acid urines with less ammonium than those of normal subjects (Henderson and Palmer, 1915; Van Slyke et al., 1926; Linder, 1927; Salvesen, 1928). The current analyses of ten patients confirm these early studies. Nine subjects had generalised renal failure, and showed a combined reduction in ammonium excretion rate of between 56% and 63% when compared with a normal population (see section A.1.1). These patients still showed pH-dependency of ammonium excretion. The decreased ammonium excretion in renal failure is thought to occur as a result of the decrease in nephron mass; reduction in tissue mass results in smaller numbers of tubular cells which can produce ammonia from blood glutamine. At the same time, renal blood flow is greatly reduced without any alteration in blood ammonium levels, reducing the delivery of ammonia precursor to the nephron. The ammonium pool in the kidney shrinks and total ammonia excretion into the urine falls. The decrease in ammonium excretion is far greater than the change in titratable acid, which depends only on a change in phosphate excretion.

Studies of unilateral renal disease in both the dog (Morrin et al., 1962) and man (Steinmetz et al., 1965) showed that ammonium excretion in disease was much less than normal,
but that ammonium excretion per ml of GFR was not reduced. In fact, further results found that ammonium excretion per ml of GFR was almost four times greater in diseased than in control kidneys; an increased rate of $\text{NH}_4^+$ excretion per surviving nephron has been shown in the rat (Schoolwerth et al., 1975). In the combined results of Subjects 12–20, the reduction in GFR from normal was 75%, yet the reduction in ammonium excretion rate was only 56–63%. It therefore appears that the diseased kidney, instead of having a reduced rate of ammoniagenesis from glutamine, chronically produces more ammonia per unit of GFR than the normal kidney during normal acid-base conditions (Dorhout-Mees et al., 1966). One explanation for this finding is that the persistent metabolic acidosis of chronic renal failure stimulates utilisation of glutamine and ammonia production in the nephron in the same way as in a normal subject with ammonium chloride acidosis. It has been shown in the remnant kidney of the rat (MacLean and Hayslett, 1980) and the dog (Fine, 1991) that total renal ammoniagenesis per nephron increased to values found in chronic metabolic acidosis, although plasma potassium and bicarbonate were no different to normal. Further possible reasons for the increased ammonium excretion per unit of GFR seen in renal failure are an increased activation of both $\text{Na}^+\text{--K}^+\text{--ATPase}$ (Schon et al., 1974) and the $\text{Na}^+\text{--H}^+$ transporter (Harris et al., 1984). These have both been described following a reduction in renal mass, but the mechanisms involved are not known, and further study is required.

Subject 11 (see section A.2) had nephrotic syndrome and dense interstitial nephritis. The rate of ammonium excretion produced by this subject was between 80% and 85% less than normal subjects. His GFR was approximately 75% less than normal values, and his ability to excrete ammonium was reduced when factorised, i.e., he showed a greater reduction in ammonium excretion than in GFR. As there is a constant acidotic stimulus towards production of ammonium in renal failure, a higher rate of excretion per unit of GFR would have been expected if ammonium excreting pathways had not been involved in the disease. It has been recently claimed (Carlisle et al., 1991) that transport of $\text{NH}_4^+$ from the loop of Henle to the collecting duct will, when interfered with by interstitial disease, reduce $\text{NH}_4^+$ excretion. Subject 11 showed such a reduction. Possibly interstitial nephritis interferes with ammonium excretion because of the increased distance which $\text{NH}_4^+$ must permeate from the thick ascending limb to the medullary collecting duct (see Figure 5.2 on page 95). It has been suggested that concentrating ability and ammonium excretion rate correlate to tubulo-interstitial change (Schainuck et al., 1970).
Appendix B

Effects of Ageing

B.1 Renal changes with age

At the time the studies discussed in this thesis were undertaken, Subject 9 was aged 60. Data on his urinary pH and ammonium excretion existed dating back 30 years, so it was decided to compare this original data with that collected recently, to see if there was any noticeable change in his rate of urinary ammonium excretion with age.

Renal function, measured with inulin clearances, is known to be reduced markedly with advancing age (Davies and Shock, 1950). Further studies by Shock (Adler et al., 1968) of acute acid loading in young (17–35 years) middle aged (49–67) and old (72–93) men demonstrated a highly significant reduction in GFR and ammonium excretion with age, although ammonium excretion was not markedly reduced per unit of GFR, i.e., a parallel reduction in GFR and ammonium excretion rate was found.

B.1.1 Analysis of data

The current normal data from Subject 9 was plotted against the "young" data from when he was aged 32 (Figure B.1). Both sets of data provided a good fit to a straight line, and from the figure there appears to be a slight upshift of the young compared to the normal line. From Table B.1 it can be seen that the data lie on parallel but distinct lines, the fall in excretion rate with age being between 2% and 19% (with 90% confidence). The endogenous creatinine clearance for Subject 9 as a young man was 117 ml.min\(^{-1}\).70kg\(^{-1}\). His present creatinine clearance is 101 ml.min\(^{-1}\).70kg\(^{-1}\). This represents a 14% reduction in GFR with age. The reduction in ammonium excretion rate is thus very similar to the reduction in GFR.
Subject 9: Normal Data (Mean Age 60 Years)
Subject 9: Normal Data (Mean Age 32 Years)
Table B.1: Comparison of normal data, ages 32 and 60 (Subject 9)

<table>
<thead>
<tr>
<th>Regression Equations (Y on X)</th>
<th>$%R^2$</th>
<th>$%R^2_{adj}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.271 - 0.360x - 0.136i + 0.030s$</td>
<td>85.5</td>
<td>85.2</td>
</tr>
<tr>
<td>Parallel lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.143 - 0.337x + 0.042i$</td>
<td>85.4</td>
<td>85.2</td>
</tr>
<tr>
<td>Single line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 3.128 - 0.331x$</td>
<td>85.0</td>
<td>84.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Significance of</th>
<th>$s$</th>
<th>$i$</th>
<th>Increase in rate</th>
<th>Rate at pH 6.0</th>
<th>Rate at pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>for Y on X</td>
<td>76.16%</td>
<td>96.31%</td>
<td>[2% to 19%]</td>
<td>14.5 µmol.min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>for X on Y</td>
<td>90.87%</td>
<td>99.87%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.2 Discussion of ammonium excretion in the elderly

The reduced rate of ammonium excretion seen in Subject 9 in later years compared with his own data of thirty years previously (see section B.1.1) corresponds with results from previous studies (Adler et al., 1968) in which male volunteers were given an acute acid load consisting of $0.1 \text{g.kg}^{-1} \text{NH}_4\text{Cl}$, and their urinary acid excretion followed over an eight hour period. Further studies were carried out in which subjects were given NH$_4$Cl plus glutamine; all subjects increased their acid excretion, but the old subjects (mean age 83 years) excreted a much smaller percentage of the ingested acid than the young ones (mean age 23 years). The decrease was paralleled by an almost equal reduction in GFR so that acid excretion per unit of GFR was almost identical in young and old subjects. Urinary ammonium accounted for less of the total acid excretion in the old subjects than the young.

In another study of ageing on ammonium excretion old subjects, following an acid load, were unable to increase their excretion of ammonium (Schnück and Nádvorníková, 1987). It may be that in the elderly with reduced renal function, as in chronic renal failure, the nephron in the resting state is secreting more ammonium than normal, and has a blunted response to an acid load. With age there is glomerular hyalinisation with thickening of the arterioles and drop-out of the nephrons. These changes rarely lead to chronic renal failure but are probably responsible for a decrease in renal blood flow (Ladevoged and Pedersen, 1969). It has been well established that in those who are clinically normal, but elderly, RPF, GFR, creatinine clearance and other tubular functions decline linearly with age (Epstein and Hollenburg, 1976). The present observations support previous studies showing that ammonium excretion shows a similar decline.
Bibliography


