THE ROLE OF METABOLIC ACIDOSIS IN CHRONIC RENAL INJURY

Thesis submitted for the Degree of Doctor of Medicine at the University of Leicester.

by

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Details of Home Office Licences

All experiments described in this thesis were approved under the Animals (Scientific Procedures) Act 1986 according to the following licences:

Project licence (Licence Holder Professor John Walls) : PPL 40/00529
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Personnel Responsible for Practical Procedures

All animal procedures, including renal surgery, post-mortem in situ perfusion of kidneys, harvesting of organs and collection of aortic blood samples were performed exclusively by the author.

Urine total protein, lysozyme and NAG assays were performed in conjunction with Mr F Baker and Mr J Brown. All other assays, including urine ELISAs for IgG and albumin and the determination of kidney total protein and collagen content, were carried out by the author.

Formalin fixation, sectioning and H & E staining of renal tissue was performed by Mr J Brown. Electron micrographs were prepared in the Department of Histopathology, Leicester Royal Infirmary, and Dr A Howie stained kidney sections from the SA study with antibodies to Tamm Horsfall protein and brush border antigen, and examined the sections on a blinded basis. All other histological procedures, including immunohistochemical staining, processing of renal tissue for identification of anionic binding sites on the glomerular basement membrane and examination of tissue sections to calculate histological injury scores, quantify numbers of infiltrating macrophages and calculate the density of anionic binding sites were performed by the author.
Abbreviations used in the Text

ACEI  Angiotensin Converting Enzyme Inhibitor
ADH  Antidiuretic hormone
AG  Anion Gap
ANOVA  Analysis of Variance
BCKAD  Branched Chain Ketoacid Dehydrogenase
BSA  Bovine Serum Albumin
CCD  Cortical Collecting Duct
CRF  Chronic Renal Failure
CRG  Compensatory Renal Growth
DTPA  Diethylenetriamine Penta-Acetic acid
ELISA  Enzyme-linked Immunosorbent Assay
ET-1  Endothelin 1
FSGHS  Focal Segmental Glomerular Hyalinosis & Sclerosis
GBM  Glomerular Basement Membrane
GCSF-1  Granulocyte Colony Stimulating Factor 1
GDH  Glutamate Dehydrogenase
GFR  Glomerular Filtration Rate
ICAM-1  Intracellular Adhesion Molecule 1
IGF-1  Insulin-Like Growth Factor 1
IgG  Immunoglobulin G
LDL  Low Density Lipoprotein
MCP1  Macrophage-Specific Chemoattractant Protein 1
NAG  N-acetyl-β-glucosaminidase
NAE  Net Acid Excretion
NEM  N-ethyl Maleimide
NHE  Sodium / Hydrogen Antiporter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMCD</td>
<td>Outer Medullary Collecting Duct</td>
</tr>
<tr>
<td>PAH</td>
<td>Para-Amino Hippuric Acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PDG</td>
<td>Phosphate-Dependent Glutaminase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimmine</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>pK</td>
<td>Dissociation constant (pH at which acid 50% dissociated)</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid Hormone</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal Replacement Therapy</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SITS</td>
<td>4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid</td>
</tr>
<tr>
<td>SNGFR</td>
<td>Single Nephron Glomerular Filtration Rate</td>
</tr>
<tr>
<td>TAL</td>
<td>Thick Ascending Limb of Loop of Henle</td>
</tr>
<tr>
<td>THP</td>
<td>Tamm Horsfall Protein</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximum Velocity (of transporter or reaction)</td>
</tr>
</tbody>
</table>
Chapter 1

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Section 1. Acid Disposal by the Normal Kidney

The excretion of hydrogen ions by the mammalian kidney plays a fundamental role in the maintenance of acid-base balance. The process responsible for the majority of new acid generation is the metabolism of dietary constituents by the liver. Since the oxidation of carbohydrates or triglycerides to carbon dioxide and water produces no acid, the vast majority of hydrogen ion presented to the kidney results from the breakdown of dietary proteins. Rare exceptions to this principle include the formation of lactic acid from carbohydrate in hypoxia, or the generation of ketones from fat in diabetic ketoacidosis.

Metabolic acid production

In general terms, hydrogen ions are generated when a substrate is converted into a product with a greater net anionic charge, and consumed when a substrate is converted to a product with a greater net cationic charge. Although the net effect of hepatic protein metabolism is acid generation, the breakdown of amino acids does not invariably generate hydrogen ion, and in some cases involves consumption, rather than generation, of protons. In respect of their effect on acid-base balance, amino acids are therefore divisible into three groups based upon the structure of their side chains (R groups):

a) Neutral amino acids except methionine and cysteine. This group includes amino acids with nonpolar R chains (alanine, valine, leucine, isoleucine, proline, phenylalanine and tryptophan) and those with uncharged polar R chains (glycine, serine, threonine, tyrosine,
asparagine and glutamine). Metabolism of these amino acids neither generates nor consumes protons, and can be summarised as follows:

\[ \text{Amino acid} \rightarrow \text{Glucose (or triglyceride) + Urea} \]

b) Cationic (basic) amino acids (lysine, arginine and some histidine residues) and sulphur containing neutral amino acids (methionine (Met) and cysteine (Cys)). Metabolism of these amino acids generates hydrogen ion as follows:

\[
\begin{align*}
\text{Arginine}^+ & \rightarrow \text{Glucose (or triglyceride) + Urea + H}^+ \\
\text{Lysine}^+ & \rightarrow \text{CO}_2 \text{ (or triglyceride) + Urea + H}^+ \\
\text{Met or Cys} & \rightarrow \text{Glucose (or triglyceride) + Urea + SO}_4^{2-} + 2\text{H}^+
\end{align*}
\]

In the case of arginine and lysine, the anionic counterion present at the pH of food is usually Cl\(^-\), and the acid generated is therefore predominantly hydrochloric acid. As shown above, sulphur-containing amino acids generate sulphuric acid.

c) Anionic (acidic) amino acids (aspartic and glutamic acids). Metabolism of these amino acids consumes hydrogen ions as follows:

\[ \text{Aspartate}^- + \text{H}^+ \rightarrow \text{Glucose (or triglyceride) + Urea + CO}_2 \]

In addition to removal of protons by anionic amino acids, other dietary organic acids (eg acetate, citrate or lactate) also consume hydrogen ions during their conversion to glucose (or triglyceride) and carbon dioxide.
From the above, the approximate net daily production of hydrogen ion by the human liver can be estimated. Consider an individual consuming a western diet of 3300 kcal/day comprising 45% as carbohydrate, 40% as fat and 15% as protein. This diet will contain approximately 1000 mmol of amino acids, which if predominantly of animal origin will contain around 12 mmol cysteine, 24 mmol methionine, 123 mmol of lysine and arginine, 30 mmol of histidine (half of which will be cationic at neutral pH) and 100 mmol of glutamate and aspartate [1]. In addition, dietary organic acids which when metabolised to neutral end products consume protons would total around 60 mmol/day. From these data, a balance sheet can be constructed (Table 1.1).

It is clear from the above discussion that net daily acid generation does not depend directly upon total dietary protein, but rather upon the amino acid composition and dietary organic acid content of a given diet. Since animal diets are rich in sulphur containing amino acids, whereas vegetarian diets have lower levels of these amino acids but greater concentrations of organic acids such as lactate and citrate, it follows that net production of hydrogen ion by the liver is proportional to the meat content of a given diet.

The net effect of nonvolatile acid production by the liver is a reduction in bicarbonate concentration and an increase in pCO$_2$ of hepatic venous blood. Excess carbon dioxide is excreted by the lungs, but the bicarbonate deficit must be repaired by the kidneys, and the mechanisms of this process are discussed below.
Table 1.1  Net acid generation in a typical western diet

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Daily intake</th>
<th>Hydrogen ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>12 mmol</td>
<td>+ 24 mmol</td>
</tr>
<tr>
<td>Methionine</td>
<td>24 mmol</td>
<td>+ 48 mmol</td>
</tr>
<tr>
<td>Lysine</td>
<td>60 mmol</td>
<td>+ 60 mmol</td>
</tr>
<tr>
<td>Arginine</td>
<td>63 mmol</td>
<td>+ 63 mmol</td>
</tr>
<tr>
<td>Histidine</td>
<td>30 mmol</td>
<td>+ 15 mmol</td>
</tr>
<tr>
<td>Glutamate</td>
<td>50 mmol</td>
<td>- 50 mmol</td>
</tr>
<tr>
<td>Aspartate</td>
<td>50 mmol</td>
<td>- 50 mmol</td>
</tr>
<tr>
<td>Organic acids</td>
<td>60 mol</td>
<td>- 60 mmol</td>
</tr>
</tbody>
</table>

**Net acid generation**  
+ 50 mmol
Mechanisms of urine acidification

The role of the kidney in the maintenance of acid base balance is twofold. Firstly, it reclaims bicarbonate ions from the glomerular filtrate, and secondly, it generates new bicarbonate to repair the deficit resulting from the extrarenal metabolic processes described above. Whilst these two processes are distinct, they have two features in common:

i) Both are achieved by acidification of the glomerular filtrate

ii) Failure of either results in metabolic acidosis.

The rationale for point ii) is clear from examination of the equation defining net acid excretion (NAE) by the kidney:

\[
NAE = U_{NH4} + V + U_{TA} V - U_{HCO3} V
\]

where \( U_{NH4} \) is the rate of ammonium excretion

\( U_{TA} \) is the rate of titratable acid excretion (see below)

\( U_{HCO3} \) is the rate of HCO\(_3\)\(^-\) excretion

Any failure to reclaim bicarbonate causes an increase in \( U_{HCO3} \) and a consequent reduction in net acid excretion.

The nephron segments responsible for the majority of urine acidification are the proximal tubule and the collecting duct. Protons secreted into the renal tubule have three potential fates:
1) Conjugation with bicarbonate ions and reabsorption as CO$_2$ and water.
2) Combination with ammonia (NH$_3$) and excretion as ammonium ions.
3) Combination with alkaline phosphate to generate acid phosphate which constitutes the titratable acidity of the urine.

Conjugation of protons with HCO$_3^-$ effects the reabsorption of filtered HCO$_3^-$, whilst combination with alkaline phosphate generates new HCO$_3^-$ to repair bicarbonate deficits. Although H$^+$ ions are excreted with NH$_3$ as ammonium, these protons are generated during the process of ammoniagenesis, and their excretion in urine does not directly accomplish removal of acid from the body (discussed below).

Each of the above processes will now be considered in turn.

1. Bicarbonate reabsorption

Approximately 75% of filtered bicarbonate is reabsorbed in the proximal tubule. Glomerular filtrate has a bicarbonate concentration of 24mEq/l, which falls to 8mEq/l by the end of the proximal tubule, and a pH of 7.25, which falls to 6.7 at the end of the proximal tubule [2]. In theory, bicarbonate reabsorption could occur either directly, as HCO$_3^-$ uptake from the tubular lumen to the tubular cell, or indirectly by secretion of protons to form luminal carbonic acid which is then dehydrated allowing reabsorption of CO$_2$ into tubular cells. Since the dehydration of carbonic acid is a slow reaction, the second scenario would require the presence in the tubular lumen of the enzyme carbonic anhydrase. Establishing which of these mechanisms occurs
in vivo requires the use of the concepts of equilibrium pH (pH\text{eq}), pH in situ (pH\text{is}) and disequilibrium pH (pH\text{de}).

The equilibrium pH is the pH of tubular fluid which would occur assuming complete equilibration of HCO$_3^-$ and CO$_2$ within tubular fluid, and is calculated as follows:

$$\text{pH}_{\text{eq}} = \text{pK} + \log \frac{[\text{HCO}_3^-]}{\alpha \text{pCO}_2}$$

where $\alpha$ is the CO$_2$ solubility coefficient.

The in situ pH is the pH predicted on the basis of tubular [HCO$_3^-$] as follows:

$$\text{pH}_{\text{is}} = \text{pK} + \log \frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]}$$

Since HCO$_3^-$ and H$_2$CO$_3$ instantaneously achieve equilibrium in solution, this equates with the measured pH of a solution.

The disequilibrium pH is defined as:

$$\text{pH}_{\text{is}} - \text{pH}_{\text{eq}}$$

Using these concepts, together with experimental measurements of tubular fluid pH, [HCO$_3^-$] and pCO$_2$, the precise mechanism of bicarbonate reabsorption can be deduced. Theoretically, three possible scenarios could exist in relation to carbonic anhydrase, bicarbonate reabsorption and H$^+$ secretion:
i) No luminal carbonic anhydrase; acidification by H⁺ secretion. In this situation, secreted hydrogen ion would react with luminal HCO₃⁻ to form carbonic acid. This reaction occurs instantly, and in the absence of carbonic anhydrase, the resulting H₂CO₃ would accumulate, such that:

\[
pHi_{is} < pH_{eq} \text{ ('acid disequilibrium pH')} \]

ii) No luminal carbonic anhydrase; acidification by HCO₃⁻ absorption. Removal of HCO₃⁻ would liberate protons in the tubular fluid and reduce luminal [H₂CO₃] below that predicted from the pCO₂, such that:

\[
pHi_{is} > pH_{eq} \text{ ('alkaline disequilibrium pH')} \]

iii) Luminal carbonic anhydrase present. In this situation, pH_{is} would always equal pH_{eq} and the disequilibrium pH would be zero.

In 1965, Rector et al measured tubular [HCO₃⁻] and pH_{is} in superficial proximal tubules of rat kidney, and assuming a tubular pCO₂ equivalent to that of arterial blood, calculated the equilibrium pH using the Henderson-Hasselbalch equation [3]. They found no significant disequilibrium pH under resting conditions, but after addition of carbonic anhydrase inhibitors to tubular fluid, an acid disequilibrium pH of 0.4 - 0.85 was generated. These findings were reproduced by Vieira and Malnic in 1968 [4], and more recently have been confirmed in studies using direct measurements of tubular
fluid pCO₂, rather than an assumption that tubular pCO₂ is equal to that of arterial blood [5]. Two conclusions can be drawn from these experiments:

i) Proximal tubular acidification occurs by H⁺ secretion.

ii) Proximal tubular fluid is normally exposed to carbonic anhydrase.

**Carbonic anhydrase**

From the above discussion, it is clear that rapid dehydration of carbonic acid (CA) is essential to the process of bicarbonate reabsorption. Since spontaneous dehydration of H₂CO₃ occurs only slowly, effective reabsorption of HCO₃⁻ is possible only if this reaction is catalysed by carbonic anhydrase. CA is a metalloprotein enzyme containing one zinc atom per molecule, and existing in four isomeric forms, of which two occur in the kidney. It is inhibited by monovalent anions and sulphonamides, and has one of the highest turnover numbers of any enzyme [6]. The two isoenzymes described in the kidney are renal cytoplasmic CA (type II), which constitutes the majority of renal CA, and membrane bound CA (type IV), which makes up 3-5% of renal cortical CA. The other two isoenzymes, types I and III, predominate in red blood cells and muscle respectively, but play no role in bicarbonate reabsorption in the kidney. Although the majority of bicarbonate reabsorption occurs in the proximal tubule, CA has been identified histologically in most nephron segments.
CA has two functions in the proximal tubule, depending upon its position (Figure 1.1):

i) Luminal CA: allows buffering of secreted protons by \( \text{HCO}_3^- \), thereby preventing the development of an acid disequilibrium pH and the consequent slowing of \( \text{H}^+ \) secretion.

ii) Cytoplasmic CA: generates cytoplasmic \( \text{HCO}_3^- \) allowing basolateral secretion of \( \text{HCO}_3^- \) via the \( \text{Na}/3\text{HCO}_3^- \) cotransporter.

In addition, CA on the basolateral membrane may facilitate removal of \( \text{HCO}_3^- \) by catalysing its conversion to \( \text{H}_2\text{CO}_3 \), thereby preventing a local accumulation of \( \text{HCO}_3^- \) ions which would inhibit activity of the \( \text{Na}/3\text{HCO}_3^- \) cotransporter (discussed below).

Mechanisms of hydrogen ion secretion in the proximal tubule

Having established that \( \text{HCO}_3^- \) reabsorption is achieved by secretion of hydrogen ions, the mechanisms responsible for \( \text{H}^+ \) secretion in the proximal tubule require further consideration. Two functionally significant mechanisms of hydrogen ion secretion have been identified in proximal tubular epithelial cells: \( \text{Na}^+/\text{H}^+ \) exchange and electrogenic \( \text{H}^+ \) secretion.

1. \( \text{Na}^+/\text{H}^+ \) exchange.

Apical \( \text{Na}^+/\text{H}^+ \) exchange is performed by the sodium hydrogen antiporter (NHE), which was first identified in renal cortical vesicles in 1976 [7]. \( \text{Na}^+/\text{H}^+ \) exchange is electoneutral (1:1), and is driven by the \( \text{Na}^+ \) gradient
Figure 1.1 Diagrammatic representation of the functions of carbonic anhydrase in the proximal tubule. Discussion of apical Na/H exchange, basolateral Na/3HCO$_3^-$ cotransport and basolateral Na$^+$/K$^+$ exchange appear later in this chapter.
resulting from basolateral Na$^+$ removal by Na$^+$/K$^+$ ATPase [8]. NHEs occur in most, if not all, mammalian cells and can be divided functionally into two groups:

i) NHEs responsible for maintenance of cell volume and pH: ubiquitous in distribution

ii) NHEs mediating transepithelial transport: confined to secretory/absorptive epithelia

In 1988, using LLC PK$\_1$ proximal tubular cells in vitro, Haggerty et al identified two types of intrarenal NHE which differed in their location and inhibitor sensitivity [9]. An antiporter situated on the apical membrane demonstrated inhibitor sensitivity similar to that previously identified in the proximal tubule NHE, whilst a basolateral NHE shared inhibitor sensitivities with NHE from nonpolar growing cells. Consistent with the functional heterogeneity seen in experimental studies of NHE, four isoforms of the antiporter have now been identified and their cDNAs cloned (Table 1.2; reviewed in [10]). These isoforms differ in their molecular weights, tissue distribution, localisation in polarised cells and phosphorylation sites in their cytoplasmic domains.

Two lines of evidence suggest that the proximal tubule apical Na$^+$/H$^+$ exchanger corresponds to the NHE3 isoform:

i) If NHE isoforms are expressed in Na$^+$/H$^+$ antiporter-deficient fibroblasts, only NHE2 and NHE3 demonstrate amiloride sensitive Na$^+$/H$^+$ activity resembling that seen in the apical membranes of proximal tubular cells [11]
Table 1.2 Expression and localisation of NHE isoforms 1-4. Data from Reference 9.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Predicted mol weight (kDa)</th>
<th>mRNA expression</th>
<th>Chromosome sites</th>
<th>Phosphorylation sites</th>
<th>Immunolocalisation</th>
<th>Response to amiloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE 1</td>
<td>91.5</td>
<td>Ubiquitous</td>
<td>1p</td>
<td>PKC</td>
<td>Basolateral (K)</td>
<td>Sensitive</td>
</tr>
<tr>
<td>NHE 2</td>
<td>79.1</td>
<td>I &gt; S &gt; K &gt; K, B, U, L</td>
<td>2q11.2</td>
<td>PKC, PKA</td>
<td>Apical (I)</td>
<td>Resistant</td>
</tr>
<tr>
<td>NHE 3</td>
<td>93.0</td>
<td>Kc &gt;&gt; I, Km</td>
<td>5p15.3</td>
<td>PKC, PKA</td>
<td>Apical (K)</td>
<td>Resistant</td>
</tr>
<tr>
<td>NHE 4</td>
<td>81.4</td>
<td>S &gt;&gt; I &gt;&gt; U &gt;&gt; B, K, M</td>
<td>2</td>
<td>PKC, PKA</td>
<td>Basolateral (K)</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

PKC: Protein Kinase C, PKA: Protein Kinase A
ii) The distribution of NHE3, which occurs predominantly within renal cortex and has been immunolocalised to the apical membrane of proximal tubular cells (see Table 1.2), is consistent with a role in apical Na+/H+ exchange.

In the rabbit, NHE1 has been immunolocalised to the basolateral surface of proximal tubular cells [12], and NHE1 mRNA is expressed in S1 and S2 segments of juxtamedullary nephrons, the thick ascending limb of Henle's loop, the distal convoluted tubule and the cortical collecting duct. These observations, together with the demonstration that the kinetics of basolateral NHE are identical to those of NHE 1 [10], confirm that basolateral NHE corresponds to the ubiquitous 'housekeeping' NHE1 isoform, and is therefore not directly involved in proton secretion into the tubular lumen.

2. Electrogenic H+ secretion.

Several lines of evidence suggest that apical membranes of proximal tubular cells can secrete H+ using an energy dependent transporter. In 1982, Kinne-Safrin et al identified an ATP-dependent, Na+- independent, oligomycin-resistant electrogenic proton transporter in rat brush border membrane vesicles [12], and in 1988 Brown et al demonstrated positive staining with antibody to a vacuolar H+-ATPase in proximal tubular apical membranes [13]. In addition, a number of functional studies (eg [14]) have demonstrated the ability of tubular cells to defend their intracellular pH against an acid load without the use of apical Na+/H+ exchange, which was inactivated by removal of Na from the bathing medium.
The relative contributions of \( \text{Na}^+ / \text{H}^+ \) exchange and electrogenic \( \text{H}^+ \) secretion to proton secretion in the proximal tubule are difficult to assess experimentally, since removal of extracellular \( \text{Na}^+ \), or inhibition of \( \text{Na}^+ / \text{K}^+ \) ATPase by agents such as ouabain inhibit basolateral \( \text{HCO}_3^- \) efflux as well as apical \( \text{Na}^+ / \text{H}^+ \) exchange. In 1987, Preisig et al found that by using luminal amiloride and \( \text{tert-} \) butyl amiloride, apical \( \text{Na}^+ / \text{H}^+ \) exchange could be almost completely inhibited [15]. Using this system they calculated that apical \( \text{Na}^+ / \text{H}^+ \) exchange accounted for 65% of the apical \( \text{H}^+ \) secretion necessary for \( \text{HCO}_3^- \) reabsorption. The remaining 35% was mediated by a \( \text{Na}^+ \) independent, amiloride insensitive mechanism which was assumed to be the electrogenic ATP-ase.

**Base exit across the basolateral membrane.**

As is clear from examination of Figure 1.1, for every proton secreted into the tubular lumen during the process of urine acidification, a bicarbonate ion is generated within the tubular cell and must subsequently be secreted across the basolateral membrane to maintain constancy of intracellular pH. Studies using both amphibians and mammals have identified two functionally important mechanisms by which this is achieved: electrogenic \( \text{Na}^+ - \text{HCO}_3^- \) cotransport and electroneutral chloride-base exchange.

1. Electrogenic \( \text{Na}^+ - \text{HCO}_3^- \) cotransport

The presence of a high \( \text{HCO}_3^- \) conductance in basolateral membranes of the rat PCT was first demonstrated by Burckhardt et al in 1984 [16]. Rapid reduction of peritubular [\( \text{HCO}_3^- \)] was followed by cell depolarisation which
was reduced by carbonic anhydrase inhibition, and prevented by treatment
with 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS), an
agent known to inhibit the red blood cell Cl⁻/HCO₃⁻ exchanger. An
association between basolateral HCO₃⁻ and Na⁺ transport was first suggested
by studies of the proximal tubule in the salamander [17]. Lowering
peritubular [Na⁺] or [HCO₃⁻] resulted in cell depolarisation, intracellular
acidification and decreased intracellular [Na⁺]. These effects occurred in the
complete absence of chloride, and were blocked by SITS. Similar observations
were subsequently reported in the rabbit proximal tubule [18], suggesting
that the basolateral membrane of the mammalian proximal tubule possesses
an electrogenic Na⁺- HCO₃⁻ cotransport mechanism which is Cl⁻ independent
and transports at least two HCO₃⁻-ions for each Na⁺ ion. The stoichiometry of
the Na⁺- HCO₃⁻ cotransporter was has been evaluated using microelectrodes
in the in vivo perfused rat proximal tubule [14]. The intracellular voltage
response to a given reduction in extracellular [HCO₃⁻] was 2.95 times greater
than the response to a similar reduction in extracellular [Na⁺], consistent with
a stoichiometry of three HCO₃⁻ to one Na⁺.

The above data demonstrate that at least one of the anionic species
transported by the Na⁺- cotransporter must be HCO₃⁻, but do not rule out
transport of an alternative anion in association with HCO₃⁻. Examining this
possibility, Soleimani & Aronson showed in 1989 that sulphite (SO₃²⁻), a
structural analogue of carbonate (CO₃²⁻) can act as a substrate for the
transporter, and in the presence of HCO₃⁻ can stimulate Na⁺ uptake into
basolateral membrane vesicles [19]. Based on these findings, they concluded
that the Na⁺- HCO₃⁻ cotransporter carries Na⁺, CO₃⁻ and HCO₃⁻ in a 1:1:1
ratio on separate sites.
2. Electroneutral chloride-base exchange.

In 1981, Edelman et al identified basolateral Cl⁻/HCO₃⁻ exchangers in the Necturus proximal tubule [20]. Subsequently Alpern & Chambers (1987), using rat proximal tubules perfused without exposure to Cl⁻ on either basolateral or luminal surfaces, demonstrated that the addition of Cl⁻ to peritubular capillaries caused a fall in intracellular pH of 0.14 units which was blocked by SITS [21]. If Na⁺ was also removed from the lumen and peritubular capillaries, the Cl⁻-related fall in pH was reduced to only 0.02 pH units, suggesting that Cl⁻/base exchange was Na⁺-dependent. Since it is known that more than 90% of basolateral membrane H⁺/HCO₃⁻ permeability is Na⁺-dependent [14], and Preisig and Alpern have demonstrated that complete removal of Cl⁻ from luminal and peritubular solutions reduces basolateral H⁺/HCO₃⁻ permeability by 35% [22], it follows that a proportion of basolateral H⁺/HCO₃⁻ movement is coupled to both Na⁺ and Cl⁻. In 1983, Guggino et al demonstrated that lowering extracellular [Na⁺] led to an increase in intracellular [Cl⁻] in Necturus proximal tubular cells. Since basolateral Cl⁻ transport in proximal tubules of this species is known to be electroneutral, it was suggested that Cl⁻/HCO₃⁻ exchange was mediated by a Na(HCO₃)₂⁻/Cl⁻ exchanger. In perfusion studies using rabbit proximal tubules in vitro, Sasaki and Yoshiyama (1988) demonstrated that reducing peritubular [Cl⁻] increased intracellular pH, and that reducing peritubular pH increased intracellular [Cl⁻]: both these effects were abolished by peritubular SITS [23]. Na⁺ removal abolished the effect of bath pH on intracellular [Cl⁻], and Cl⁻ removal slowed the intracellular pH response to lowering extracellular [HCO₃⁻] by 26%. All these results are most consistent with the presence of basolateral Cl⁻/HCO₃⁻ exchange which is predominantly Na⁺ dependent.
Several lines of evidence suggest that of the two mechanisms discussed above, the Na\(^+\)-3HCO\(_3\)- transporter is the predominant system of base exit across the basolateral membrane:

i) The majority of basolateral H\(^+\)/HCO\(_3\)- permeability is Na\(^+\) dependent and Cl\(^-\) independent [14].

ii) Proximal tubular HCO\(_3\)- absorption is almost entirely inhibited by removal of peritubular and luminal Na\(^+\), but is unaffected by removal of Cl\(^-\) [24].

iii) Tubular cell depolarisation inhibits transepithelial HCO\(_3\)- absorption [25], consistent with a predominantly electrogenic mechanism of HCO\(_3\)- efflux from the basolateral membrane.
2. Ammoniagenesis and ammonium excretion.

Ammonium synthesis

Ammonium is produced in the kidney by deamination of glutamine to \( \alpha \) ketoglutarate via glutamic acid. In 1984, Good & Burg, using a microassay for ammonia production, showed that all nephron segments can produce ammonia, but the majority of production occurs in the proximal tubule [26]. Studies measuring tissue levels of the two enzymes responsible for the conversion of glutamine to \( \alpha \) keto glutarate (phosphate-dependent glutaminase (PDG) and glutamate dehydrogenase (GDH)) have identified the proximal tubule as the nephron segment with the highest concentration of both. Consistent with this, it has been demonstrated that the majority of ammonia appearing in the urine is present in tubular fluid by the end of the proximal tubule [27].

The biochemistry of ammoniagenesis in the renal tubule is summarised in Figure 1.2. The important feature of \( \alpha \) ketoglutarate in respect of bicarbonate regeneration is its possession of two carboxyl residues. Destruction of these residues during the subsequent conversion of \( \alpha \) ketoglutarate to glucose or \( \text{CO}_2 \) consumes two protons, allowing their conjugate \( \text{HCO}_3^- \) ions to be released and secreted across the basolateral tubular membrane (Figure 1.3). This regeneration of \( \text{HCO}_3^- \) occurs irrespective of the fate of the ammonium ions produced, and urinary ammonium excretion does not therefore per se eliminate net acid from the body. As ammonium ions are generated stoichiometrically with every carboxyl group destroyed, however, urinary ammonium can be used as an index of proton removal occurring within the kidney, justifying its inclusion in the equation defining total urinary net acid
Figure 1.2 Biochemical pathway of ammoniagenesis in the proximal tubule.

Glutamine → Glutamic acid → α-ketoglutarate

PDG: phosphate-dependent glutaminase (glutaminase 1)
GDH: glutamate dehydrogenase
Figure 1.3 Schematic representation of ammonium synthesis in the proximal tubule

\[
\begin{align*}
\text{Glutamine} & \quad \text{Glutamic acid} \\
\text{NH}_4^+ & \quad \text{H}^+ & \text{NH}_4^+ \quad \text{NH}_3 \\
\text{Na}^+ & \quad \text{Na}^+ & \quad \alpha\text{-ketoglutarate} & \quad \text{Glucose} \text{ or CO}_2 \\
\text{2CO}_2 + \text{2H}_2\text{O} & \quad \text{2HCO}_3^- & \quad 2\text{H}^+ \\
\end{align*}
\]
excretion (NAE). From an acid base balance standpoint it is nevertheless important that ammonium is excreted in urine, since the alternative route of removal is via the bloodstream to the liver, where its conversion to urea liberates protons as follows:

\[ 2\text{NH}_4^+ + \text{CO}_2 \rightarrow \text{Urea} + 2\text{H}^+ \]

A second important role of urinary ammonium excretion is in the conservation of \( \text{Na}^+ \) and other cations, for which it is exchanged across the luminal membrane (discussed below). This function is of particular importance in the ketoacidosis of fasting.

**Ammonium transport**

Based on early experimental data demonstrating that \([\text{NH}_3]\) was similar in the tubular lumen, proximal tubular cells and the interstitium [28], it was believed for many years that all ammonia secretion into the proximal tubule resulted from nonionic diffusion of free \( \text{NH}_3 \). Increases in total ammonia concentration within the tubule were thought to result entirely from 'diffusion trapping' of \( \text{NH}_4^+ \) due to luminal acidification. More recent data (discussed below) have confirmed the importance of nonionic diffusion of \( \text{NH}_3 \), but have also demonstrated transport of ammonium on the apical \( \text{Na}^+/\text{H}^+ \) antiporter. In considering these data, it is important to note that studies of ammonia transport are complicated by a number of methodological problems. Firstly, the inability to measure \( \text{NH}_3 \) and \( \text{NH}_4^+ \) independently, such that simultaneous measurements or estimates of pH must be performed; secondly the difficulty of distinguishing coupled \( \text{NH}_3/\text{H}^+ \) transport from
NH$_4^+$ transport; and thirdly the lack of a method for measuring intracellular ammonium concentration.

Evidence from a number of experimental studies has confirmed that nonionic diffusion of NH$_3$ is an important mechanism of ammonia transport in the proximal tubule. Studies using rabbit proximal tubules have demonstrated NH$_3$ permeabilities which are high and comparable to those in other membranes. Similarly high permeabilities to NH$_3$ have been demonstrated in the proximal tubule of the rat, where it has also been shown that basolateral membrane NH$_3$ permeabilities are similar to those of the apical membrane ($\approx 7 \times 10^{-2}$ cm$^2$/sec) [29]. In 1987, Garvin et al used proximal straight tubules from the rabbit to measure ammonia fluxes resulting from NH$_3$ or NH$_4^+$ gradients in the absence of a gradient for the other species. They calculated that NH$_3$ permeability was 330 times greater than NH$_4^+$ permeability, and was independent of temperature between 22 and 37$^\circ$C [30], suggesting that passive diffusion of NH$_3$ is a significant method of transmembrane ammonia transport. In the same year, Simon and Hamm demonstrated that ammonia entry into the rat proximal tubule increased in inverse proportion to the pH of tubular fluid [31]. This finding is also consistent with nonionic diffusion, since falling luminal pH reduces the effective [NH$_3$] by promoting conversion to NH$_4^+$, thereby increasing the concentration gradient for NH$_3$.

In addition to the well recognised movement of ammonia by nonionic diffusion, a number of studies have suggested that ammonium can move into the tubular lumen on the apical Na$^+$/H$^+$ antiporter. In 1981, Kinsella and Aronson first demonstrated transmembrane exchange of NH$_4^+$ for Na$^+$ using rabbit brush border membrane vesicles in which the pH changes resulting from NH$_4^+$ movement had been minimised [32]. The physiological relevance
of this mechanism was investigated in perfused mouse proximal tubules by Nagami et al in 1986 [33]. They demonstrated that the majority of ammonia synthesised in proximal tubular cells was secreted into the tubular lumen. This preferential luminal secretion of ammonia could be accounted for by one or more of the following:

i) Lower ammonia permeability of the basolateral membrane

ii) Movement of ammonia down a concentration gradient generated by an acidic luminal compartment ('diffusion trapping')

iii) Specific apical transport of ammonia

Since it is known that the apical and basolateral tubular membranes have similar ammonia permeability (discussed above), the first of these possibilities can be discounted. In 1986, Nagami et al investigated the contribution of diffusion trapping to preferential luminal ammonia secretion by perfusing tubules with low Na+/0.1mM amiloride solutions to block luminal acidification. Apical ammonia secretion was completely abolished by this manoeuvre, and was only partially restored by reducing luminal pH to 6.2. These findings demonstrated a greater role for the Na+/H+ antiporter in ammonia transport than would be predicted from its effects on diffusion trapping, and were therefore consistent with specific transport of NH$_4^+$ by the apical Na$^+/H^+$ exchanger. In 1990, in the course of their measurements of apical and basolateral ammonia permeability, Preisig & Alpern also found evidence of Na$^+$/NH$_4^+$ exchange [29] Addition of NH$_4^+$ to luminal fluid caused alkalinisation of tubular cells in proportion to the luminal [NH$_4^+$], and this was inhibited by 0.1 mM amiloride.

The relative importance of these two transport mechanisms in proximal tubular ammonium secretion has not been established. As both are high
capacity systems, it is likely that the factor limiting luminal ammonia concentration is the efflux of ammonia as luminal concentrations rise. It is probable that NH₃ efflux is transcellular, whilst efflux of NH₄⁺ occurs via a paracellular pathway [34].

Micropuncture studies have demonstrated that the concentration of ammonia in the distal convoluted tubule is very low. Analyses of luminal ammonia concentrations in other nephron segments have shown that after synthesis in the proximal tubule, ammonia leaves the luminal fluid in the thick ascending limb of the loop of Henle and diffuses through the interstitium to the collecting duct, where it is trapped and excreted [35]. Two features of the loop of Henle encourage passive movement of ammonia from the loop into the interstitium. Firstly, the alkaline pH resulting from reabsorption of water without bicarbonate in the descending limb of the loop of Henle, which encourages NH₃ movement from the tip of the loop into the interstitium by nonionic diffusion; and secondly the positive voltage in the thick ascending limb (generated by reabsorption of more Cl⁻ than Na⁺ in this segment) which promotes passive NH₄⁺ movement into the interstitium down a voltage gradient. In addition, several investigators have described movement of NH₄⁺ across the frusemide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter in the thick ascending limb (TAL). In 1988, Garvin et al evaluated the contribution of active and passive movement of ammonia out of the loop of Henle in a series of experiments in the rabbit TAL. Passive movement of NH₄⁺ was prevented by abolition of the transmembrane voltage, and movement on the Na⁺-K⁺-2Cl⁻ cotransporter was inhibited by frusemide [34]. They concluded that 35% of absorption was passive down a voltage gradient, 35-40% was mediated by the Na⁺-K⁺-2Cl⁻ cotransporter, and the remaining 25-30% occurred across another apical transporter, probably a K⁺ channel.
Movement of ammonia out of the interstitium and into the collecting duct occurs mainly by nonionic diffusion driven by luminal acidification. The predominance of this mechanism has been demonstrated by measurement of concentration gradients for NH$_3$ and NH$_4^+$ across the cortical collecting duct of the rabbit [36], and between the tip of the loop of Henle, the vasa recta and the collecting duct in the rat [37].

In summary, ammonia is synthesised during the conversion of glutamine to α-ketoglutarate in the proximal tubular cell. It is secreted into the tubular lumen on the Na$^+$/H$^+$ antiporter and by nonionic diffusion, and is subsequently reabsorbed from the loop of Henle by a combination of nonionic diffusion, passive NH$_4^+$ reabsorption and active movement across the Na$^+$-K$^+$-2Cl$^-$ cotransporter and probably a K$^+$ transporter. From the interstitium, it re-enters the collecting duct by nonionic diffusion where it is trapped in the lumen as NH$_4^+$ and excreted in urine.

3. Excretion of titratable acid

The titratable acidity of urine is measured by determining the quantity of alkali which must be added to restore urinary pH to 7.4, i.e. the pH of the glomerular filtrate. The predominant constituent of titratable acid in urine is H$_2$PO$_4$- (dibasic phosphate or 'acid phosphate'). The second ionic form of PO$_4^{3-}$ occurring in biological fluids is HPO$_4^{2-}$ (monobasic phosphate or 'alkaline phosphate') and the pK of the H$_2$PO$_4^-$ / HPO$_4^{2-}$ buffer system is 6.8. In plasma (pH 7.4), the ratio of alkaline to acid phosphate (derived from the Henderson Hasselbalch equation) is therefore 4:1, whereas at pH 6.8, slightly higher than that in the early distal tubule, this ratio is 1:1. Acid phosphate is formed in the tubular lumen by the reaction of alkaline phosphate with a
proton. This releases a bicarbonate ion in the tubular cell which when secreted across the basolateral membrane contributes to repair of the total body base deficit. Since the distal nephron is the segment responsible for lowering filtrate pH below 6.8, it is also the main site of acid phosphate formation.

Although all urinary $\text{H}_2\text{PO}_4^-$ contributes to net urine acid excretion, Halperin & Jungas (1983) estimated that only half of the titratable acid appearing in urine eliminates protons generated by hepatic amino acid metabolism [1]. On the basis of $pK$ values of the phosphate groups which occur in a mixed diet, and estimates of the intracellular pH of foodstuffs in this diet, they calculated that 20mmol of $\text{H}_2\text{PO}_4^-$ and 19 mmol of $\text{HPO}_4^-$ are consumed per day. Since the $\text{PO}_4^{3-}$ consumed in the form of $\text{HPO}_4^-$ is unable to accept protons, it cannot act as a vehicle for acid excretion in the kidney, and cannot therefore contribute to the net elimination of hydrogen ions produced by the liver.

As the formation of acid phosphate depends upon luminal acidification in the distal nephron, this will now be considered in more detail. The distal convoluted tubule contributes little to distal luminal acidification, which mainly takes place in the collecting duct and the connecting tubule, and is performed by type A intercalated cells. As in the discussion of proximal tubular acidification, the mode of net $\text{H}^+$ secretion into the lumen ($\text{H}^+$ secretion or $\text{HCO}_3^-$ absorption) and the mechanisms of apical and basolateral acid/base transport will be considered in turn.

Micropuncture studies have demonstrated an acid disequilibrium pH in the collecting duct of both the rat and rabbit [38; 39]. In both species, this occurred spontaneously (without acetazolamide treatment) and was abolished by infusion of CA. This demonstrates that luminal acidification in
this segment is achieved by secretion of protons, and that carbonic anhydrase is absent from the lumen of the collecting duct.

Mechanisms of hydrogen ion secretion

In 1982, Koeppen and Helman were the first of several investigators to demonstrate electrogenic Na⁺-independent H⁺ secretion into the perfused cortical collecting duct (CCD). They measured a fall in luminal pH along the CCD which exceeded that predicted from passive movement of H⁺ down the lumen-negative voltage gradient generated by Na⁺ absorption [40]. Perfusion with Na⁺-free solutions, or addition of ouabain to the peritubular fluid in the presence of Na⁺ reversed the voltage gradient to lumen-positive, but did not prevent luminal acidification. The addition of ouabain did, however, cause the luminal pH to increase. Addition of ouabain in the absence of Na⁺ affected neither the lumen-positive voltage nor the luminal pH. Since inhibition of Na⁺ transport reduced H⁺ secretion only via a secondary effect upon luminal voltage, these findings are consistent with luminal acidification by an electrogenic, Na⁺-independent proton pump. To look directly for evidence of Na⁺- H⁺ exchange in cells of the CCD, Chaillet et al (1985) monitored rates of pH recovery after cell acidification [41]. Addition of Na⁺ to the luminal fluid did not influence rates of alkalisation, but addition to the peritubular fluid accelerated pH recovery. It can be concluded that cells of the CCD possess a basolateral, but not an apical Na⁺ / H⁺ exchanger. In contrast, Wang et al (1993) demonstrated that amiloride inhibits luminal acidification in the distal convoluted tubule, suggesting that in this segment, bicarbonate reabsorption is mediated by an apical Na⁺/H⁺ exchanger [42].
Two classes of H+ transporting ATPases have been identified in mammals. The first class is exemplified by the H+ K+ ATPases of the gastric and colonic mucosae, which are inhibited by the agent Sch 28080. The second class, the vacuolar H+ ATPases, were first identified in lysosomes and are inhibited by the alkylating agent N-ethyl maleimide (NEM) and by bafilomycin A₁. In 1990, Hays & Alpern [43] measured the pH recovery of outer medullary collecting duct (OMCD) cells following an acid load in the absence of Na⁺. Recovery was inhibited by luminal, but not peritubular NEM, but Sch 28080 and K⁺ removal had no effect on either cell surface, consistent with H⁺ transport by a vacuolar ATPase. In addition to this evidence of vacuolar ATPase activity, a number of studies have demonstrated H⁺ K⁺ ATPase activity the collecting duct. This activity is inhibited by Sch 28080 and omeprazole, but not by ouabain, and appears to be significant only in K⁺ deficient states. [44] The findings of Hays & Alpern (discussed above), who found no effect of Sch 28080 on H⁺ secretion in the OMCD of K⁺ replete animals [43] are also consistent with a role for apical H⁺ K⁺ exchange in states of K⁺ -deficiency, but not under control conditions. It can therefore be concluded that apical membrane H⁺ secretion in the collecting duct is mediated by a vacuolar ATPase, and in conditions of K⁺ deficiency, a H⁺ K⁺ ATPase.

Base exit across the basolateral membrane

Data from a number of studies have demonstrated that HCO₃⁻ crosses the basolateral membrane by Cl⁻/HCO₃⁻ exchange [44]. The mechanism of this exchange was investigated in the OMCD of the rabbit by Hays & Alpern [43]. They found that intracellular pH was inversely proportional to bath basolateral [Cl⁻], even if voltage effects were abolished by valinomycin (a K⁺
ionophore) and K+. Cl⁻/HCO₃⁻ exchange continued in the complete absence of Na⁺, but removal of Cl⁻ from apical and basolateral solutions reduced basolateral HCO₃⁻ permeability by 90%. These data demonstrate that the great majority of basolateral base secretion is mediated by a voltage and Na⁺ independent Cl⁻/HCO₃⁻ exchanger. Because the Cl⁻ permeability of the apical membrane is very low, whilst the basolateral membrane is highly Cl⁻ conductive [45], Cl⁻ exchanged for HCO₃⁻ is believed to leave the intercalated cell via the basolateral membrane, before entering the tubular lumen across the intercellular tight junction in response to the lumen positive potential.

Erythrocyte cell membranes contain a Cl⁻/HCO₃⁻ exchanger, the 'band 3 Cl⁻/HCO₃⁻ exchanger'. Antibodies raised against this exchanger also stain the intercalated cell basolateral membrane in the human kidney [46]. Analysis of cDNA libraries in rat and mouse has located only a single gene for both renal and red blood mRNAs, and in both species, the mRNA for the two proteins was almost identical [47]. It can therefore be concluded that the Cl⁻/HCO₃⁻ exchanger responsible for basolateral base transport in the renal collecting duct is essentially the same as that previously described in the membrane of the red blood cell.

Under normal circumstances, the mechanisms of acid disposal described above are sufficient to repair the bicarbonate deficit resulting from metabolism of dietary constituents, and plasma pH and bicarbonate concentration are thereby maintained within a narrow physiological range. Disturbance of the balance between acid generation and ingestion and its disposal, or an increase in bicarbonate loss either from the gastrointestinal tract or kidneys result in metabolic acidosis (MA), which is considered in detail in the following section.
Section 2. Metabolic Acidosis

Definition and aetiology

Metabolic acidosis is defined by the combination of a high plasma [H+] (> 44nEq/l; equivalent to pH < 7.36) and a low plasma [HCO₃⁻] (< 20mmol/l). In general terms, three fundamental mechanisms, occurring alone or combination, are responsible for the development of metabolic acidosis.

i) Net gain of acid, secondary to increased endogenous acid production (eg lactic acidosis) or exogenous acid administration (eg intoxication with ethylene glycol).

ii) Net loss of alkali, either from the gastrointestinal tract (eg in protracted and severe diarrhoea) or from the kidneys (proximal renal tubular acidosis).

iii) Inadequate renal acid disposal, with consequent incomplete correction of metabolic bicarbonate deficit (eg chronic renal insufficiency).

Calculation of the plasma anion gap (AG) is fundamental to the identification of the underlying cause of metabolic acidosis in a particular case. AG has been defined in two ways, which differ in their inclusion or exclusion of [K+] from the equation. Authors including [K+] (eg [48]) define AG as:

\[ AG = ([Na] + [K]) - ([Cl] + [HCO₃⁻]) \]

with a normal range of 12 - 20 mEq/l
Other authors have argued that serum $[K^+]$ is low and relatively constant [49], and should therefore be excluded from the equation, resulting in the following definition which appears in most North American texts:

$$AG = [Na] - ([Cl] + [HCO_3^-])$$

with a normal range of 8 - 16 mEq/l.

As serum is electroneutral (the sum of $[\text{anions}]$ or $[\text{cations}]$ is approximately 152 mEq/l), the term anion gap is a misnomer, as it incorrectly implies that there is a difference between plasma $[\text{total anions}]$ and $[\text{total cations}]$. The AG is in fact determined by the concentrations of unmeasured anions ($UA$) and unmeasured cations ($UC$) as follows:

$\text{total serum cations} = \text{total serum anions}$

$$\therefore [Na] + [UC] = ([Cl] + [HCO_3^-]) + [UA]$$

$$\therefore [Na] - ([Cl] + [HCO_3^-]) = [UA] - [UC]$$

and since $[Na] - ([Cl] + [HCO_3^-]) = \text{the anion gap}$:

$$[UA] - [UC] = AG$$

The unmeasured anions and cations concerned, and their normal plasma concentrations are detailed in Table 1.3.

Calculation of the anion gap allows metabolic acidoses to be divided into two groups: normal anion gap (hyperchloraemic) acidoses, and high anion gap (normochloraemic) acidoses. In normal anion gap MA, increased $[Cl^-]$
Table 1.3  Unmeasured anions and unmeasured cations and their normal concentrations. Data from Reference 49a.

<table>
<thead>
<tr>
<th>Unmeasured cation (mEq/l)</th>
<th>Unmeasured anion (mEq/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>Protein</td>
</tr>
<tr>
<td>4.5</td>
<td>15.0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>PO₄²⁻</td>
</tr>
<tr>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>SO₄²⁻</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Organic acids</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>Total</strong></td>
</tr>
<tr>
<td>11.0</td>
<td>23.0</td>
</tr>
</tbody>
</table>

Anion gap = 23.0 - 11.0 = 12.0 mEq/l
compensates for the reduced \[\text{HCO}_3^-\], such that no overall change in [measured anions] occurs. This compensation results from one or more of the mechanisms listed in Table 1.4, and causes of a normal anion gap acidosis corresponding to these mechanisms are shown in Table 1.5

Halperin and coworkers (1988) have emphasised the importance of urinary ammonium excretion in establishing the cause of a normal anion gap metabolic acidosis [50]. Normal individuals excrete 30 - 40 mmols NH$_4^+$ /day, but in chronic MA, this can rise to 200 mmol/day. In Groups 1 and 3 in Table 1.4, urinary NH$_4^+$ excretion is increased to compensate for acidosis of extrarenal origin. In group 2, however, where the kidney is the source of the acidosis, urine NH$_4^+$ excretion is reduced. Since clinical laboratories do not routinely measure urine ammonium, its rate of excretion must be established indirectly, and this has traditionally been by measurement of urine pH. The use of urine pH as an index of ammonium excretion is based upon two assumptions:

i) Medullary \([\text{NH}_3]\) is constant

ii) \(\text{NH}_3\) is freely available in tubular fluid.

Under these circumstances, a rise in free \([\text{H}^+]\) will cause a predictable rise in urine \([\text{NH}_4^+]\) according to the equation:

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

Whilst \([\text{NH}_3]\) is approximately constant in acute MA, in chronic MA the production of ammonia is upregulated (discussed later), such that the above equation is displaced to the right and free \([\text{H}^+]\) falls. Under these circumstances, urine pH is no longer a useful index of ammonium excretion.
Table 1.5 Causes of a normal anion gap metabolic acidosis

1. a) Diarrhoea, external pancreatic or small bowel draininge.
   b) Ureterointestinal anastamoses (urine Cl- exchanged for HCO\textsubscript{3} in the intestinal segment).
   c) Administration of cholestyramine (given as the chloride salt, which exchanges for HCO\textsubscript{3} in the intestinal lumen) Significant only in patients with underlying renal impairment.
   d) Proximal (HCO\textsubscript{3} - wasting; type II) renal tubular acidosis, including administration of carbonic anhydrase inhibitors.
   e) Early uraemic acidosis.

2. Distal (types I & IV) renal tubular acidosis.

3. Administration of HCl, NH\textsubscript{4}Cl, arginine hydrochloride or lysine hydrochloride

4. Administration of sulphuric acid, phosphoric acid, or sulphur-containing amino acid whose anion rapidly undergoes renal excretion.

5. Rapid intravenous administration of saline.
Table 1.4  Mechanisms of compensation for low $[\text{HCO}_3^-]$ by increased $[\text{Cl}^-]$ in hyperchloraemic acidosis.

1. Gastrointestinal or renal $\text{HCO}_3^-$ loss with subsequent $\text{Cl}^-$ retention.
2. Defective acid excretion by the kidney. The conjugate base of the metabolically produced acid is excreted as the sodium salt, and $\text{NaCl}$ is retained secondarily.
3. Addition of chloride-containing acid to body fluids.
4. Addition of other acid to body fluids, with consequent reduction of $\text{HCO}_3^-$ and renal excretion of the accompanying anion which is replaced by $\text{Cl}^-$. 
5. Dilution of plasma $\text{HCO}_3^-$ by infused $\text{Cl}^-$ containing fluids.
Richardson and Halperin (1988) have confirmed this theoretical argument by demonstrating that urine pH can be 6 when ammonium excretion is low (in acute MA) or high (in chronic MA) [51].

An alternative index of \( \text{NH}_4^+ \) excretion, which is accurate both in acute and chronic MA, is the urine net charge, or anion gap. As in plasma, the sum of anions and cations in urine is equal. Therefore:

\[
\begin{align*}
\text{Na}^+ + \text{K}^+ + 2\text{Ca}^{2+} + 2\text{Mg}^{2+} + \text{NH}_4^+ \\
= \\
\text{Cl}^- + \text{H}_2\text{PO}_4^{2-} + 2\text{HPO}_4^{2-} + \text{SO}_4^{2-} + \text{organic anions}
\end{align*}
\]

The excretion of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) is small, and excretion of phosphate, sulphate and organic ions is approximately constant. Since the difference in excretion of these anions and cations is approximately 80 mEq anions/day [50], the equation can be simplified to:

\[
\text{Na}^+ + \text{K}^+ + \text{NH}_4^+ = \text{Cl}^- + 80
\]

The urine net charge, defined as \( ([\text{Na}^+] + [\text{K}^+] - [\text{Cl}^-]) \) therefore represents the concentration of the one important unmeasured urine cation, \( \text{NH}_4^+ \). In urine with a net negative charge \( ([\text{Cl}^-] > [\text{Na}^+] + [\text{K}^+]) \), excretion of \( \text{NH}_4^+ \) exceeds 80 mmol/day, consistent with the causes of hyperchloraemic acidosis in groups 1, 3 and 4 above. In urine with a net positive charge \( ([\text{Na}^+] + [\text{K}^+]) > [\text{Cl}^-] \), \( \text{NH}_4^+ \) excretion is less than 80 mmol/day, suggesting defective ammonium excretion as found in distal renal tubular acidosis.
When an acid load whose attendant non-Cl" anion is not rapidly excreted by the kidneys is added to the body fluids, a normochloraemic, high anion gap MA results:

\[ \text{H}^+ - \text{anion}^{-} [a] + \text{NaHCO}_3 \rightarrow \text{Na}^+ - \text{anion}^{-} + \text{H}_2\text{O} + \text{CO}_2 \]

This acidosis will persist as long as the anion [a] remains in the blood, which may occur in three situations:

i) The anion is not filtered at the glomerulus (eg uraemic anions in chronic renal failure).

ii) The anion is filtered at the glomerulus but almost completely reabsorbed (eg lactate).

iii) The anion cannot be utilised because of deranged metabolic pathways (eg ketoacids in diabetic ketoacidosis).

The common causes of high anion gap acidosis are demonstrated in Table 1.6.

Although calculation of the anion gap is the standard method of categorising the causes of MA, Halperin et al (1992) proposed an alternative pathophysiological classification [52], which categorises MA according to whether or not acids are overproduced (Tables 1.7 & 1.8). Broadly speaking, overproduction of acid causes a high anion gap acidosis, whilst acidosis with normal acid production causes a normal anion gap acidosis. Exceptions to this rule, in which the classifications do not overlap, are as follows:
Table 1.6  Common causes of metabolic acidosis with a high anion gap (normochloraemic) acidosis. Classification based on Reference 51.

1. Lactic acidosis:

   a) Type A (due to poor tissue perfusion)
      i) Cardiogenic, septic or haemorrhagic shock
      ii) Acute hypoxaemia
      iii) Carbon monoxide poisoning
   b) Type B
      i) Feature of various common disorders: mechanisms variable. Examples include liver disease, leukaemia, infection, diabetes mellitus especially treated with biguanides.
      ii) Ingestion of drugs / toxins, eg biguanides, ethanol, salicylates, isoniazid.
      iii) Hereditary forms, eg glucose 6 phosphate deficiency

2. Ketoacidosis

   a) Diabetic
   b) Alcoholic
   c) Starvation
   d) Inborn errors of metabolism (eg Maple Syrup Urine disease)

3. Intoxication

   a) Salicylates
   b) Methanol
   c) Ethylene glycol
   d) Paraldehyde

4. Advanced uraemia
Table 1.7  Pathophysiological classification of metabolic acidosis 1: overproduction of acids

a) Accumulation of anions in plasma (→ increased anion gap)

- Lactic acidosis types A and B
- Ketoacidosis
- Poisoning (e.g., methanol, ethylene glycol)
- Miscellaneous (e.g., hyperosmolar hyperglycaemia)

b) Excretion of anions in urine (no increase in anion gap)

- Secretion of anions, e.g., hippuric acid in toluene abuse
- Failure to reabsorb anions, e.g., ketoacidosis + lactic acidosis or ketoacidosis + ingestion of aspirin
Table 1.8  Pathophysiological classification of metabolic acidosis 2: no overproduction of acids

1. Loss of NaHCO₃

   a) GI losses (urinary NH₄⁺ excretion ≥ 200 mmol/day):
      • diarrhoea
      • vomiting with achlorhydria

   b) Renal losses (urinary NH₄⁺ excretion ~ 40 mmol/day)
      • proximal renal tubular acidosis
      • Acetazolamide

2. Reduced NH₄⁺ excretion

   a) Low [NH₃] in medullary interstitium (urine pH low)
      • low NH₄⁺ production
         - chronic renal failure
         - hyperkalaemia
         - low glutamine availability
      • low NH₄⁺ reabsorption in TAL
         - hyperkalaemia
      • defect in countercurrent system
         - infection
         - infiltration
         - interstitial nephritis
         - congenital lesions eg medullary cystic disease
         - postobstruction

   b) Low net secretion of H⁺ into lumen of collecting duct (urine pH high)
      • pump defect
         - distal renal tubular acidosis
      • failure to stimulate pump
         - aldosterone deficiency
         - ↓ lumen -ve transepithelial PD
      • backleak of H⁺
         - amphotericin B
1. Uraemic acidosis (AG increased; acid production normal)
2. Ketoacidosis with inhibition of tubular ketoacid reabsorption by agents such as lactate or salicylate (AG normal; acid overproduced)
3. Toluene poisoning: toluene oxidised to hippuric acid which is secreted into tubules (AG normal; acid overproduced)

In summary, MA results from net gain of acid, net loss of bicarbonate or inadequate renal H⁺ disposal, and can be classified according to urine biochemistry into high or normal anion gap acidosis, or pathophysiologically based upon whether or not it results from increased acid production. Chronic renal failure (CRF) is probably the commonest cause of stable metabolic acidosis seen in clinical practice. Depending upon the severity of renal impairment, it can be of normal or high anion gap type, but is not associated with an increase in acid production. The pathophysiology of this form of MA will now be considered in more detail.

**Uraemic acidosis**

Metabolic acidosis develops when the glomerular filtration rate (GFR) falls below approximately 30 mls/minute. In moderate renal impairment (GFR 20-30 mls/min) the acidosis is of normal anion gap, hyperchloremic type, but it progresses to a high anion gap acidosis when GFR falls below 15-20 mls/min [53]. Since metabolic acid is not overproduced in chronic renal failure [54] it follows that uraemic acidosis must result from impairment of renal acid excretion.
1. Normal anion gap uraemic acidosis

In 1979, Widmer et al retrospectively studied 41 patients with chronic renal failure and serum creatinine in the range 175 to 1270 µmol/l [54]. They demonstrated an inverse correlation between serum total CO₂ and serum creatinine, and a direct correlation between [unmeasured anions] and serum creatinine. These findings confirm that serum [HCO₃⁻] falls, and [unmeasured anions] rises, as renal failure progresses. In addition, they showed that in moderate renal impairment (defined as serum creatinine 175 - 350 µmol/l) serum [Cl⁻] was significantly higher than in non-uraemic controls (106 ± 1.0 vs 102 ± 0.3 mEq/l, p < 0.01), whilst [unmeasured anions] was similar to controls (13 ± 0.9 vs 12 ± 0.3 mEq/l, NS). In severe renal impairment (serum creatinine > 350 µmol/l), both serum [Cl⁻] and [unmeasured anions] were higher than controls (107 ± 1.0 vs 102 ± 0.3 mEq/l and 16 ± 0.4 vs 12 ± 0.3 mEq/l respectively; p < 0.01 vs controls for both parameters). These data demonstrate that a significant reduction in serum bicarbonate occurs in moderate renal impairment, but is entirely offset electrically by a rise in serum [Cl⁻], such that [unmeasured anions] remains unchanged. In severe renal impairment, the hyperchloraemia persists, but is accompanied by a rise in unmeasured anions and a consequent high anion gap acidosis.

Three explanations were offered for the development of hyperchloraemia in moderate CRF:

1. Hyperparathyroidism. Micropuncture data from the dog have shown that parathyroid hormone (PTH) inhibits bicarbonate reabsorption in the proximal tubule [55]. Although hyperparathyroidism is an almost invariable feature of chronic renal impairment, Widmer et al considered this mechanism insufficient to cause MA in CRF.
Consistent with this conclusion is more recent evidence demonstrating that in contrast to acute rises in PTH, chronic hyperparathyroidism stimulates renal acidification and causes metabolic alkalosis [56].

2. Hyporeninaemic hypoaldosteronism. This syndrome can accompany moderate renal insufficiency, particularly in older patients. It would normally, however, be accompanied by hyperkalaemia, whereas in Widner’s patients with moderate renal insufficiency, mean serum [K+] was only 4.9. In addition, it is unlikely that this relatively uncommon disorder would affect the majority of a group of unselected patients with chronic renal failure.

3. A primary enhancement of tubular chloride reabsorption with consequent reduction in bicarbonate reabsorption. It was speculated that such an increase in chloride reabsorption might result from increased luminal concentrations of anions (eg phosphate and sulphate) whose tubular reabsorption was reduced because of decreased nephron mass. This speculative mechanism has not been confirmed experimentally.

2. High anion gap uraemic acidosis

As discussed above, net acid excretion is defined as:

\[ NAE = U_{\text{NH}_4^+}V + U_{TA}V - U_{\text{HCO}_3^-}V \]

The MA of severe CRF results from impaired net acid excretion [57]. Urine acidification, ie the distal H⁺ secretory capacity, remains intact, such that
urine pH is usually less than 5.4, and the capacity of the distal nephron to respond to increased buffer delivery is maintained. This contrasts with distal renal tubular acidosis, in which distal H⁺ secretory capacity and urine acidification are impaired.

The effect of CRF on the excretion of ammonium, titratable acid and bicarbonate will now be considered in turn.

a) Ammonium excretion.

Inadequate NH₄⁺ production and excretion are the main causes of reduced net acid excretion in CRF. Since the proximal tubule can increase ammonium production four or fivefold in MA in the human, overall renal ammonia excretion is reduced only when the GFR falls below 20% - 25% of normal [57]. In 1983, Buerkert et al measured urinary ammonium excretion in the 5/6ths renal ablation (remnant) model in the rat, and demonstrated systemic acidosis (pH = 7.3) despite the excretion of an acid urine [58]. The measured fall in renal ammonium excretion in this study was sufficient to account for the entire reduction in net acid excretion (Table 1.9).

In 1980, Tizianello et al suggested that most of the renal ammonia generated in chronic renal impairment results from catabolism of substrates other than glutamine [59]. In a study of patients with chronic renal failure, they demonstrated that total renal ammonia production was reduced by 50%, whereas glutamine uptake was reduced by 90% compared with controls. It was proposed that most of the ammonia generated in chronic renal failure results from protein breakdown within the kidney.
Table 1.9  Ammonium and net acid excretion in the remnant kidney. Data from Ref 58.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Remnant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFR (mls/min)</strong></td>
<td>1.37 ± 0.11</td>
<td>0.52 ± 0.06</td>
</tr>
<tr>
<td><strong>Blood pH</strong></td>
<td>7.37 ± 0.01</td>
<td>7.31 ± 0.02</td>
</tr>
<tr>
<td><strong>Urine pH</strong></td>
<td>5.51 ± 0.21</td>
<td>5.51 ± 0.06</td>
</tr>
<tr>
<td><strong>Ammonium excretion (nEq/min)</strong></td>
<td>0.87 ± 0.08</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td><strong>Net acid excretion (nEq/min)</strong></td>
<td>1.22 ± 0.11</td>
<td>0.80 ± 0.08</td>
</tr>
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</table>
A further mechanism by which ammonia production may be reduced in CRF is via hyperkalaemia, which frequently accompanies CRF and is most pronounced in patients with hyporeninaemic hypoaldosteronism (type IV renal tubular acidosis) [60]. Hyperkalaemia suppresses mitochondrial ammoniagenesis [61], and the potential importance of this mechanism in controlling ammonia production is demonstrated by the clinical observation that administration of cation exchange resins to patients with type IV renal tubular acidosis improves MA and hyperkalaemia despite reducing aldosterone levels [62].

In addition to changes in ammonia production, intrarenal handling of ammonia is also altered in CRF. In 1987, Buerkert et al demonstrated a threefold increase in end proximal tubular ammonium delivery in remnant kidneys compared with controls (Table 1.10). In both control and remnant kidneys, end distal was less than end proximal ammonium delivery, due to ammonium absorption in the loop of Henle. End distal ammonium, however, was markedly greater in remnant animals than controls. Since whole kidney ammonium excretion was greater in the control animals (Table 1.9), it can be concluded that ammonium is reabsorbed, not secreted, from the collecting duct in uraemic acidosis. It is of note that in addition to increased ammonium delivery, bicarbonate delivery out of the proximal and distal tubules was also increased in the remnant kidneys (Table 1.9: discussed further below). It follows that the collecting duct is presented with a relatively alkaline fluid in CRF, and since ammonium trapping in the collecting duct depends upon an acid tubular pH, a further cause for decreased ammonia excretion in CRF may be impaired ammonium trapping resulting from increased collecting duct \( [\text{HCO}_3^-] \).
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Remnant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>End proximal delivery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium (pmol/min)</td>
<td>18 ± 2</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>HCO$_3^-$ (pmol/min)</td>
<td>54 ± 7</td>
<td>134 ± 20</td>
</tr>
<tr>
<td><strong>End distal delivery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium (pmol/min)</td>
<td>11 ± 2</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>HCO$_3^-$ (pmol/min)</td>
<td>9 ± 7</td>
<td>59 ± 20</td>
</tr>
</tbody>
</table>
b) Excretion of titratable acid.

Phosphate delivery to the distal nephron is well maintained in CRF because of two mechanisms. Firstly, a reduction in fractional phosphate reabsorption resulting from hyperparathyroidism; and secondly a rise in serum phosphate which offsets the effect of a falling GFR on the total filtered phosphate load. Since distal H⁺ secretory mechanisms respond normally to increased buffer delivery in CRF (see above), and delivery of phosphate to the distal tubule is also preserved, titratable acid formation is not significantly reduced by chronic renal injury [60].

c) Bicarbonate reabsorption.

In the remnant kidney, HCO₃⁻ delivery to the end of the proximal tubule and accessible distal tubule is increased (Table 1.10), though the mechanism of this increase is controversial. In 1959, Schwartz et al demonstrated significant urinary HCO₃⁻ excretion in 5 out of 7 patients with chronic renal impairment, and suggested that impaired renal HCO₃⁻ absorption is common in CRF, even in patients without overt bicarbonaturia, after normalisation of plasma [HCO₃⁻] [63]. In contrast, several studies have demonstrated increased absolute proximal HCO₃⁻ reabsorption in experimental CRF, though fractional HCO₃⁻ reabsorption was still slightly reduced. This reduction in fractional HCO₃⁻ reabsorption may result from several pathophysiological features of CRF [60]:

i) Secondary hyperparathyroidism
ii) Hyperfiltration in residual nephrons
iii) Osmotic diuresis
Although the precise mechanisms remain to be established, it can be concluded that HCO$_3^-$ handling is abnormal in CRF, and that this is likely to contribute to the development of uraemic acidosis.

**Nephron adaptation to metabolic acidosis**

The nephron responds to MA by upregulating the enzymes and transport systems responsible for acid disposal. The most important mechanisms to be upregulated in this way are:

i) Ammonium production and excretion  
ii) Proximal tubular apical Na$^+$/H$^+$ exchange  
iii) H$^+$ excretion in the distal nephron

The mechanisms by which MA upregulates each of these processes will now be considered.

i) Ammonium production and excretion

The three most important regulatory steps in ammonia production in the rat are the conversion of cytoplasmic glutamine to mitochondrial glutamate (comprising the transport of glutamine from cytoplasm to mitochondria, followed by the deamination of glutamine by phosphate dependent glutaminase); the conversion of mitochondrial glutamate to $\alpha$ keto glutarate; and the conversion of oxaloacetate to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK) [64].
Several studies have demonstrated upregulation of glutamine transport into mitochondria in chronic MA, though the precise mechanism has not been established [65]. Rector et al showed in 1954 that both PDG and GDH are upregulated in NH$_4$Cl acidosis [66]. More recent data have confirmed this observation, and demonstrated that the increased enzyme activity occurs only in the early proximal convoluted tubule. PEPCK activity and mRNA levels are increased in acidotic LLC-PK$_1$ proximal tubular cells, consistent with the observation made in 1966 that renal gluconeogenesis is upregulated in chronic MA [67].

In 1994, Schoolwerth et al described changes in the abundance and distribution of mRNA for PDG, GDH and PEPCK in kidneys of rats made acidotic by NH$_4$Cl gavage [68]. PDG, GDH and PEPCK mRNA levels began to rise 4 hours after induction of acidosis, peaked at 10 hours and returned to baseline by 30 hours. In situ hybridisation studies of PEPCK mRNA before NH$_4$Cl supplementation showed that it was confined to the medullary rays, consistent with the known localisation of the enzyme in the pars recta of the proximal tubule. After induction of acidosis, PEPCK mRNA was demonstrated throughout the superficial cortex. Similar, though less dramatic increases in area of distribution were seen for PDG and PEPCK mRNA after NH$_4$Cl supplementation. These changes in mRNA were followed by increased immunohistochemical staining for the respective enzymes, which in contrast to mRNA staining persisted for several days. This immunohistochemical evidence of increased activity and wider distribution of ammoniagenesis in MA is consistent with the findings of Good and DuBose (1987), who measured ammonium secretion in the proximal convoluted tubule (PCT) of the rat using free flow micropuncture techniques [69]. They demonstrated that in normal rats, ammonia underwent net secretion in the early PCT, but net absorption in the late PCT. In contrast, rats
made chronically acidotic by dietary NH₄Cl showed increased ammonium secretion throughout the PCT, such that net secretion occurred in both early and late segments of the tubule. It was concluded that since the rate of production of ammonium is a major determinant of its rate of secretion, ammoniagenesis is increased throughout the proximal tubule in chronic MA, and this increase is more prominent in the early part of the tubule. Similar alterations in the distribution of ammoniagenesis in uraemic acidosis have also been described, and are discussed above (see 'uraemic acidosis').

The factor responsible for upregulation of ammoniagenesis in chronic MA has not been established. In rats with chronic respiratory acidosis, Carter et al (1959) found a transient early rise in urinary ammonium excretion after enrichment of inspired air with CO₂, but this returned to control levels after approximately two days despite persistent acidosis with systemic pH as low as 7.15 [70]. In the same animals, renal glutaminase activity showed no increase over controls. Both these observations argue against a role for systemic pH in the control of ammoniagenesis. Because renal cortical intracellular pH falls in MA but is unchanged in respiratory acidosis, it has been suggested that intracellular, rather than systemic, pH may be responsible for alterations in rates of ammoniagenesis in chronic MA [64].

ii) Proximal tubular apical Na⁺/H⁺ exchange

Proximal tubular fluid from animals with MA has a lower [HCO₃⁻], and therefore a smaller amount of bicarbonate available for absorption, than that from normal animals. A simple comparison of rates of bicarbonate reabsorption, and by implication apical Na⁺/H⁺ exchange, in acidotic animals and controls will not therefore accurately reflect the HCO₃⁻
reabsorptive capacity of proximal tubular cells in the two groups. To overcome this problem, Kunau et al (1985) performed in vivo microperfusion studies of acidotic and control rats in which proximal tubular pH and \([\text{HCO}_3^-]\) were normalised to 7.24 and 22 mM respectively [71]. Using this system, they demonstrated that acidotic rats (blood pH 7.25 ± 0.02) absorbed bicarbonate at a rate 2.5 times greater than controls (blood pH 7.43 ± 0.01). Although the mechanism of increased bicarbonate reabsorption in the acidotic group was not directly examined, the authors concluded that it was likely to result from increased apical \(\text{Na}^+/\text{H}^+\) exchange.

The response of the \(\text{Na}^+/\text{H}^+\) antiporter to MA has been examined more directly in vitro using brush border membrane vesicles. Aronson et al demonstrated that reducing intravesicular pH stimulated \(\text{Na}^+/\text{H}^+\) exchange, and that this stimulation was greater than would be expected from a simple increase in intravesicular \(\text{H}^+\) availability [72]. It is therefore probable that increased cell \([\text{H}^+]\) stimulates \(\text{Na}^+/\text{H}^+\) exchange by two mechanisms: firstly because of increased driving force for \(\text{Na}^+/\text{H}^+\) exchange resulting from greater availability of intracellular \(\text{H}^+\), and secondly via an allosteric effect of intracellular \(\text{H}^+\) on the \(\text{Na}^+/\text{H}^+\) antiporter, which accounts for the upward curve of the graph of intracellular \([\text{H}^+]\) vs \(\text{Na}^+\) influx reported in Aronson’s study.

As discussed earlier, tubular reabsorption of \(\text{HCO}_3^-\) requires secretion of apical \(\text{H}^+\) into the tubular lumen and basolateral \(\text{HCO}_3^-\) into the peritubular capillary. It follows that to maintain constant intracellular pH during upregulation of apical \(\text{Na}^+/\text{H}^+\) exchange, basolateral secretion of base must also be increased. In 1987, Akiba et al measured rates of apical \(\text{Na}^+/\text{H}^+\) exchange and basolateral \(2\text{Na}/3\text{HCO}_3^-\) cotransport in cortical membrane vesicles isolated from acidotic and control rabbits [73]. They demonstrated
that the maximum transport velocity ($V_{\text{max}}$) of both transport systems was inversely proportional to the plasma [HCO$_3^-$], and that the $V_{\text{max}}$ of the Na$^+$/H$^+$ antiporter was therefore proportional to that of the 2Na/3HCO$_3^-$ cotransporter ($r = 0.648$, $p < 0.001$). Two possible mechanisms were suggested for this parallel adaptation of apical acid secretion and basolateral base exit. First, that cell pH falls during MA, stimulating the apical Na$^+$/H$^+$ antiporter (as suggested above by Aronson et al) and secondarily upregulating basolateral 2Na/3HCO$_3^-$ cotransport; and second, that increased 2Na/3HCO$_3^-$ exchange is the primary event in MA, and is followed by intracellular acidosis and a subsequent compensatory increase in $V_{\text{max}}$ of the Na$^+$/H$^+$ antiporter. It was concluded that whichever of these mechanisms proved to be correct, the co-ordinated regulation of the two transport systems would defend systemic pH in acidosis by enhancing proximal proton secretion and bicarbonate reabsorption despite continued intracellular acidosis. It noteworthy that the alterations in $V_{\text{max}}$ of the membrane transporters were observed in vitro after removal of membranes from the acidotic animals, demonstrating a 'memory effect' which persisted after removal of the initial pH stimulus.

The mechanism of upregulation of Na$^+$/H$^+$ exchange and 2Na/3HCO$_3^-$ cotransport in MA was further investigated in rabbit cortical vesicles by Soleimani et al in 1994 [74]. They demonstrated that MA - induced upregulation of Na$^+$/H$^+$ and 2Na/3HCO$_3^-$ transport in brush border vesicles was partially prevented by staurosporine, an inhibitor of protein kinase C, suggesting that phosphorylation of inactive membrane proteins might account at least in part for upregulation of membrane exchangers in MA. As a second mechanism of increased $V_{\text{max}}$ of Na$^+$/H$^+$ exchangers in acidosis, they proposed that acidosis may stimulate recruitment of endosomal vesicles containing Na$^+$/H$^+$ exchangers to the luminal membrane. In support of this argument, they cited work by Schwartz et al (1985), who demonstrated
incorporation of acid-extruding endosomes into the cell membrane of isolated perfused renal tubules from rats with CO₂-induced acidosis [75].

Recent evidence suggests that synthesis of new Na⁺/H⁺ antiporters contributes to the increased \( V_{\text{max}} \) of NHE3 during MA. In 1995, Amemiya et al demonstrated an increase in both Na⁺/H⁺ exchanger activity and NHE3 mRNA abundance in opossum kidney (OK) cells incubated in acid media [76]. Analysis of the time course of this response showed that increased Na⁺/H⁺ activity preceded the increase in mRNA abundance. This observation, together with the demonstration that inhibitors of protein synthesis blocked the effects of acid only partially, led the authors to conclude that there are at least two mechanisms of antiporter activation in the proximal tubule: one related to increased mRNA and a second related to posttranslational modification.

In summary, evidence from a number of studies has demonstrated that MA upregulates Na⁺/H⁺ exchange in the apical membranes of proximal tubular cells. This upregulation is associated with a proportional rise in basolateral 2Na/3HCO₃ cotransport, and is mediated by both increased synthesis and posttranslational modification of NHE3.

iii) H⁺ secretion in the distal nephron

A number of studies analysing the effects of systemic pH on distal acidification have used the urine minus blood pCO₂ (U-B pCO₂) as a surrogate for H⁺ secretion into the distal nephron. Provided the tubular [HCO₃⁻] is high, the vast majority of H⁺ secreted into the distal nephron will be buffered by HCO₃⁻ to form carbonic acid, and after dehydration, CO₂. U -
B pCO₂ provides an index of distal acidification because countercurrent trapping prevents the diffusion of this CO₂ from the renal medulla to the blood, and its concentration is therefore the same in the final urine as in the medullary collecting duct. Because the technique requires a high urine pH, it cannot be used to measure distal acidification in metabolic acidoses induced by NH₄Cl or acids, since these are accompanied by an acid urine. To overcome this problem, and allow the effect of alterations in systemic pH on distal H⁺ secretion to be analysed, Gougoux et al (1980) induced proximal renal tubular acidosis in dogs by infusing lysine, thereby obtaining an alkaline urine in the presence of acidaemia [77]. Using this technique, they demonstrated a direct correlation between U - B pCO₂ factored for urine [HCO₃⁻], and blood [H⁺] (r = 0.86; p < 0.01), and concluded that alterations in systemic acid-base status influence distal nephron H⁺ secretion. A further study by the same group explored the mechanism by which systemic acidaemia promotes distal acidification [78]. Using the same model of proximal renal tubular acidosis, the component of distal acidification resulting from lumen negativity (due to reabsorption of Na⁺ without an accompanying anion) was abolished by treatment with amiloride. Under these conditions, the direct correlation between U - B pCO₂ factored for urine [HCO₃⁻], and blood [H⁺] persisted. It was argued that in the absence of a transtubular potential difference, increased H⁺ secretion could result from either decreased luminal or increased intracellular [H⁺]. Since the acidaemic animals had slightly lower urine pH than controls in this study, it was concluded that acidaemia increased distal H⁺ secretion by reducing intracellular pH. This conclusion is consistent with data from a study by Cohen and Steinmetz (1980), in which the turtle urinary bladder was used as a model of the distal nephron [79]. Luminal H⁺ secretion was shown to be a saturable function of intracellular [H⁺], which in turn was inversely proportional to serosal [HCO₃⁻]. Since alteration of serosal pCO₂ and [HCO₃⁻]
did not influence the relationship between intracellular [H+] and H+ secretion, it was concluded that cell pH was the prime determinant of luminal acidification.

The obvious mechanism by which acute alterations in intracellular [H+] might influence proton secretion is via an effect on the driving force across the H+ pump. In addition, a number of studies have demonstrated chronic morphological adaptations in medullary and cortical intercalated cells in response to altered acid/base status. In 1991, Bastani et al examined the levels and distribution of H+ ATPase and its mRNA in kidneys from rats given acid or alkali loads [80]. Whilst finding no change in total levels of H+ ATPase or its mRNA in acidosis, they demonstrated dramatic alterations in intracellular distribution of the enzyme. In the medullary collecting tubules, there was almost complete loss of vesicular cytoplasmic H+ ATPase staining, whilst staining along the apical membrane dramatically increased (an appearance described as "rim staining"). After two weeks of acidosis, 89% of intercalated cells in the inner stripe of the outer medulla showed rim staining, compared with approximately 12% of controls. In the cortical collecting duct, a similar apical shift of staining for H+ ATPase was observed. It was concluded that the intercalated cell responds to changes in acid base status by cycling a constant total pool of H+ ATPase between cytoplasmic vesicles and the apical membrane.

Levine et al assessed the functional importance of H+ ATPase in MA by measuring the effects of the H+ ATPase inhibitors bafilomycin and Sch - 28080 on HCO₃⁻ reabsorption in microperfused distal tubules of acidotic rats [81]. The inhibitors had no effect on distal HCO₃⁻ reabsorption in rats which had been severely acidotic (blood pH 7.16 ± 0.04) for 18 hours, but did reduce HCO₃⁻ reabsorption in rats which had been mildly acidotic (blood pH 7.26 ±
0.02) for three days. Several explanations were offered for the apparent disparity between these findings and those of Bastani et al.

i) Severe acidosis confers resistance to the effects of H\textsuperscript{+} ATPase inhibitors.

ii) pH sensitive intracellular H\textsuperscript{+} ATPase modulators modify the kinetics of H\textsuperscript{+} ATPase without affecting its overall quantity or distribution.

iii) Changes in H\textsuperscript{+} ATPase structure occur with time, causing alterations in responsiveness to inhibitors.

iv) Severely acidotic rats reabsorb HCO\textsubscript{3}\textsuperscript{-} by a mechanism independent of H\textsuperscript{+} ATPase, possibly by HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} exchange.

Whatever the precise explanation for these findings, they raise the possibility that mechanisms other than apical H\textsuperscript{+} ATPase contribute to distal acidification in severe MA.

In summary, peritubular acidosis reduces intracellular pH and stimulates collecting duct acidification. This acidification is mediated by increased driving forces across the apical H\textsuperscript{+} ATPase, migration of H\textsuperscript{+} ATPase from cytoplasmic vesicles to the apical membrane, chronic adaptations of intercalated cells, and possibly recruitment of one or more alternative mechanisms of H\textsuperscript{+} secretion.
Section 3: Effects of Metabolic Acidosis on the Kidney

In addition to the functional adaptations by which renal acid disposal is increased in MA, studies in the 1960s demonstrated that rats made chronically acidotic by dietary supplementation with NH₄Cl develop proteinuria and renal hypertrophy. The findings of these studies will now be reviewed.

Von Hoesslin proposed in 1909 that the protein content of human urine was inversely proportional to its pH [82]. Gardner, in 1961, designed a study which aimed to formally test this hypothesis by asking two questions. First, do changes in the rate of urinary protein excretion accompany variations in systemic and urinary [H⁺]; and second, if such changes do occur, are they the result of changes in the filtration and/or reabsorption of protein within the kidney? [83]. Systemic and urinary pH of Wistar rats was altered by supplementation of drinking water with 1% ammonium chloride (NH₄Cl), 5% sodium bicarbonate (NaHCO₃), or 0.04% acetazolamide. Measurement of urinary protein excretion confirmed that it was inversely proportional to urine pH across the range 5.0 to 9.0. The origin of urinary protein in the acidotic animals was investigated by intravenously injecting Evans blue dye (T-1824) prior to NH₄Cl supplementation. This dye binds to serum proteins, allowing any protein filtered at the glomerulus to be detected histologically as protein absorption droplets during reabsorption by proximal tubular cells. In acidotic rats, a pronounced increase was reported in cell droplet formation, suggesting either increased protein filtration at the glomerulus, or decreased ability of the proximal tubular cell to clear reabsorbed protein. Since administration of T-1824 24 hours after the establishment of acidosis was followed by rapid clearance of undyed protein droplets from proximal
tubular cells with replacement by dyed protein, it was concluded that the ability of the proximal tubule to reabsorb protein was not reduced, and that proteinuria in these animals must therefore result from increased filtration at the glomerulus. As further evidence of the glomerular origin of urinary proteins in this study, animals pretreated with the tubular toxin uranyl acetate still showed a reduction in proteinuria when given NaHCO₃, consistent with an effect of pH on protein filtration at the glomerulus rather than on protein reabsorption in the proximal tubule. Rats made acidotic by acetazolamide showed no increase in proteinuria, but did demonstrate increased numbers of protein droplets, suggesting a smaller increase in glomerular protein filtration accompanied by an increase in tubular reabsorption sufficient to prevent overt proteinuria.

In 1965, Lotspeich demonstrated that chronic acidosis causes renal growth [84]. After replacing the drinking water of Holtzman rats by 0.28 M NH₄Cl for 7 days, kidney wet weight, dry weight and total nitrogen content all increased significantly. In a separate experiment, similar increases in kidney weight and nitrogen content were seen in the remaining kidney of rats uninephrectomised one week earlier. In rats subjected to both nephrectomy and NH₄Cl supplementation, the effect of the two stimuli on renal growth was additive. To correct for any effect of ammonium ion on growth, a group of non-nephrectomised rats were given a solution of ammonium citrate (which does not induce acidosis since metabolism of the citrate anion generates HCO₃⁻) calculated to provide the same nitrogen load as 0.28 M NH₄Cl. This group showed no increase in wet or dry kidney weight after one week of supplementation, suggesting that ammonium itself does not stimulate renal growth. To define the nature of growth in acidosis and unilateral nephrectomy, kidney digests were assayed for RNA and DNA. Both acidosis and nephrectomy increased kidney DNA and RNA, and it was
concluded that both hypertrophy (increased cell size) and hyperplasia (increased cell number) contributed to increased renal mass in these models. As an index of ammoniagenesis, glutaminase activity was measured in kidney homogenates from acidotic and nephrectomised rats. Increased activity was found in acidotic animals and in nephrectomised animals supplemented with NH$_4$Cl, but not in the nephrectomised non-supplemented group. In addition, a group of rats pretreated with NH$_4$Cl to upregulate ammoniagenesis and subsequently nephrectomised showed a further increase in urinary ammonium after surgery without any increment in glutaminase activity. This suggested that renal growth in acidosis can increase ammoniagenesis by a mechanism independent of glutamine oxidation. In summary, Lotspeich demonstrated that acidosis induces true renal growth involving synthesis of new DNA, RNA and protein, and that ammoniagenesis is upregulated both by increased glutaminase activity and by an unexplained mechanism related to increased renal mass.

**Proteinuria and chronic renal injury**

The identification of increased quantities of urinary protein is a fundamental diagnostic technique in clinical nephrology, and may indicate glomerular or tubulointerstitial disease, either alone or in combination [85]. From first principles, four mechanisms may account for increased urinary protein excretion:

i) Disruption of the glomerular capillary wall barrier, allowing filtration of high molecular weight proteins (eg immunoglobulin G) which subsequently appear in the urine ("glomerular proteinuria").
ii) Tubule cell dysfunction, with consequent reduction in tubular protein reabsorption and appearance of predominantly low molecular weight proteins (eg lysozyme) in the urine ("tubular proteinuria").

iii) Disruption of tubular cell membranes with consequent release of normal cell proteins (eg N-acetyl glucosaminidase) into the tubular lumen and urine.

iv) An increase in the serum concentration of normal or abnormal proteins (eg IgG light chains in multiple myeloma) which are filtered at the glomerulus, overwhelm the reabsorptive capacity of the proximal tubule and subsequently appear in the urine ("overload proteinuria").

Glomerular proteinuria is a cardinal feature of glomerulonephritis, which even if susceptible to immunosuppressive treatment, frequently results in glomerulosclerosis and chronic renal injury [86]. Tubulointerstitial disease, and consequent tubular proteinuria, is also predictive of chronic renal injury, and a number of studies have demonstrated that tubular atrophy and interstitial disease are better predictors of renal impairment than glomerular abnormalities (reviewed in [87]). In addition to its use as a marker of glomerular and tubulointerstitial disease, it has been suggested that proteinuria may independently cause chronic renal injury. Evidence for this hypothesis comes from the demonstration in a wide range of renal diseases of a close correlation between the magnitude of proteinuria and subsequent rates of renal deterioration. The modification of diet in renal disease (MDRD) study prospectively examined the effect of varying degrees of dietary protein restriction and blood pressure control on the progression of chronic renal disease in 840 patients with GFRs ranging from 13-55 mls/min/1.73m² [88]. Irrespective of the baseline GFR, the rate of deterioration of renal function
correlated with the magnitude of baseline proteinuria, even when controlled for potentially confounding covariates. In addition, protein excretion in patients with ≥ 0.25g proteinuria at baseline fell during the first 4 months of intervention, and the magnitude of this reduction was inversely proportional to the rate of fall of GFR seen from 4 months onwards. More recent intervention studies, in which proteinuria in non-diabetic patients with ≥ 3g protein excretion / 24 hrs was reduced by treatment with angiotensin converting enzyme inhibitors (ACEIs), have confirmed the correlation between baseline proteinuria and subsequent rate of decline of GFR, but more importantly have demonstrated that the percentage reduction in proteinuria following ACEI treatment is inversely proportional to the subsequent rate of decline of GFR [89, 90]. This effect is independent of antihypertensive effects of ACEIs.

A number of explanations for the mechanism by which proteinuria might cause renal damage have been offered, and include potential effects both on the mesangium and the tubulointerstitium [87]. Proteins including low density lipoproteins (LDL) have been identified in the mesangium in proteinuric states. In vitro, LDL stimulates both the proliferation of mesangial cells, and the production by these cells of the chemoattractant macrophage-specific chemoattractant protein 1 (MCP-1), the fibrogenic cytokine platelet derived growth factor (PDGF), and the extracellular matrix protein fibronectin. Since these agents are directly or indirectly fibrogenic, they could potentially cause glomerulosclerosis and chronic renal injury in response to the accumulation of serum proteins in the mesangium. Potentially damaging effects of protein on tubular epithelial cells have been divided into two groups: those mediated by direct tubular toxicity and those causing damage indirectly via an effect on normal tubular cell function. Evidence of direct toxicity comes from the observation that urinary excretion of N-acetyl-β -
glucosaminidase (NAG), an enzyme present in proximal tubular cells, correlates with the degree of proteinuria in children with glomerulonephritis [91], suggesting that proximal tubular injury, and consequent release of intracellular contents including NAG into the tubular lumen, may be caused by filtered proteins. The mechanism of such direct toxicity remains speculative, but it has been proposed that intracellular lysosomal enzymes might be implicated. It is known that small quantities of low molecular weight proteins filtered by the normal glomerulus are reabsorbed by endocytosis in the proximal tubule and subsequently broken down by intralysosomal proteolysis [92]. It has been proposed that the increased protein and lysosomal traffic associated with a large tubular protein load result in leakage of lysosomal proteases into the cell cytoplasm with consequent cell damage, which in turn could incite inflammation and tubulointerstitial scarring [93]. Consistent with this was the demonstration in renal biopsies from rats with adriamycin nephrosis of filtered proteins in proximal tubular cell cytoplasm, accompanied by breaks in the tubular basement membrane and associated leakage of tubular contents into the interstitium [94]. In addition to this general mechanism of direct tubular damage by protein overload, high tubular concentrations of specific endogenous proteins may be cytotoxic. Filtered transferrin-iron complexes may dissociate in the acidic environment of proximal tubular fluid, releasing free iron which damages cell and lysosomal membranes by peroxidation [95].

Three broad mechanisms have been proposed to explain how filtered protein might influence tubular cell metabolism and consequently cause renal injury: stimulation of cytokine and matrix protein production, increased ammoniagenesis and tubular hypoxia [87]. Like mesangial cells, tubular epithelial cells can synthesise pro-inflammatory cytokines and matrix proteins, including interleukin 6, PDGF, endothelin 1 (ET-1) and granulocyte
colony stimulating factor (G-CSF), and rates of production are higher in tubular cells from diseased kidneys [96]. The experimental models purine aminonucleoside (PAN) nephrosis (in which administration of a glomerular epithelial toxin causes proteinuria) and protein overload nephropathy (in which proteinuria is induced by intraperitoneal injection of large amounts of bovine serum albumin) are both characterised by a dense interstitial inflammatory infiltrate, even though neither PAN nor intraperitoneal albumin are themselves injurious to the tubulointerstitium. The severity of the infiltrate correlates with the degree of proteinuria, and in PAN nephrosis, in which proteinuria peaks at 14 days before resolving, mirrors its time course [97]. Since tubulointerstitial inflammation has been observed in proteinuric states, and tubular cells can synthesise pro-inflammatory cytokines, several investigators have examined cytokine production in tubular cells exposed to protein on their apical surface. In 1995, Zoja et al demonstrated a dose-dependent increase in ET-1 release from the basolateral surface of tubular cells exposed to high protein concentrations [98], and Burton et al (1996) found that human proximal tubular cells release MCP-1, PDGF and fibronectin from their basolateral surface when the apical surface is exposed to serum proteins in concentrations seen in the tubular fluid of nephrotic patients [99]. mRNA for RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted) is also upregulated in porcine tubular cells exposed to albumin and IgG [100], and this response is abrogated by treatment with inactivators of the nuclear transcription factor NF-κB. Since the transcription of MCP-1 and several other cytokines is also NF-κB-dependent, Abbate et al (1999) have proposed a common intracellular mechanism whereby protein overload activates NF-κB, subsequently upregulating the transcription of several cytokines which when released from the basolateral surface of the tubular cell cause interstitial inflammation and fibrosis [101].
A second potential effect of proteinuria on tubular cell metabolism is via upregulation of ammoniagenesis. In 1992, Rustom et al demonstrated that patients with heavy proteinuria had higher rates of proximal tubular peptide catabolism and urinary ammonia excretion than controls with similar renal excretory function but less proteinuria [102]. In a further study in 1998, the same group showed that reducing proteinuria by treatment with the angiotensin converting enzyme inhibitor lisinopril led to a reduction in tubular protein catabolism and ammonium excretion [103]. They proposed that amino acids released by metabolism of filtered proteins act as substrates for ammoniagenesis which is consequently upregulated. Since ammonia activates complement via the alternate pathway ([104]: see below) this may result in tubulointerstitial inflammation and fibrosis.

The final proposed effect of filtered protein on tubular cell function is the development or exacerbation of hypoxia due to the increased work of tubular protein breakdown. In rat models of renal ischaemia, injection of low molecular weight proteins into renal tubules promotes the development of acute tubular necrosis [105]. Since oxygen tension in the kidney is low, any increase in oxygen requirements may cause cell injury, particularly in established renal disease where oxygenation may be further compromised by loss of postglomerular capillaries [106]. In 1997, Combe et al showed that human proximal tubular cells release intercellular adhesion molecule 1 (ICAM-1), a pro-inflammatory cytokine, when cultured under hypoxic conditions, and that this release is blocked by PDTC, an inhibitor of NF-κB activation [107]. In view of the proposed pivotal role of NF-κB in the cytokine response to proteinuria, this observation raises the possibility that hypoxia resulting from protein overload may contribute to tubular cytokine release in proteinuric states.
The above data demonstrate that proteinuria acts as a marker of glomerular and tubulointerstitial disease, an index of the likelihood of progression of chronic renal injury, and a pathogenic factor in the development and progression of tubulointerstitial injury and chronic renal disease. The demonstration that chronic acidosis causes proteinuria therefore raises the possibility that MA may itself cause renal injury and could promote disease progression via effects on the glomerulus, the tubulointerstitium or both.
Renal growth, ammoniagenesis and chronic renal injury

The hypothesis that chronic MA is injurious to the kidney is supported by the demonstration that chronically acidotic rats develop accelerated renal growth (discussed above), a phenomenon which has also been reported in a number of experimental models of chronic renal injury including hypokalaemic nephropathy, diabetic nephropathy and nephropathy induced by anti-oxidant deficiency. Chronic hypokalaemia, which is an occasional cause of chronic renal failure in humans [108], causes proteinuria and renal growth which in the rat is due to hypertrophy and hyperplasia of intercalated and principal cells of the medullary collecting duct and expansion of the interstitium. There is no associated increase in GFR or glomerular size [109]. Streptozotocin-induced diabetes in the rat causes significant increases in dry kidney weight, total protein content, DNA and RNA which begin 36 hours after the development of glycosuria [110]. GFR, glomerular volume and proximal tubular length also increase by 20-40%, but effects of diabetes on the growth of more distal nephron segments have not been investigated. Similar findings have been reported in patients with diabetes mellitus. Glomerular filtration rate exceeds that of age matched controls by 24-40%, and kidney size, determined radiologically or at necropsy, is increased in proportion to the GFR [110]. Correction of hyperglycaemia by insulin is followed by a fall in GFR and subsequent reduction in kidney size in most cases. A third experimental model known to cause accelerated renal growth is nephropathy due to dietary deficiency of antioxidants. In 1990, Nath & Salahudeen induced a pro-oxidant state in normal rats by feeding a diet deficient in selenium, which normally scavenges reactive oxygen species (ROS), and vitamin E, which normally attenuates the oxidant effects of ROS [111]. Blood pressure in the treated animals was greater than controls, and they developed an almost twofold increase in excretion of total protein, IgG, albumin and low
molecular weight proteins. After nine weeks, kidneys had increased wet weight, dry weight, protein and DNA content, and morphometric analysis demonstrated increased volume of the glomeruli and tubular epithelium. No histological abnormalities were seen in glomeruli or tubular epithelia, but focal cellular infiltrates were seen in the interstitium.

Although all these models are characterised by accelerated growth of the whole kidney and subsequent tubulointerstitial damage, it is clear from the above descriptions that the morphological, histological and functional changes are markedly different in each case. Whilst this might suggest different mechanisms of renal injury in each model, Nath et al (1991) proposed a unifying hypothesis based on the observation that the rate of ammoniagenesis per nephron is increased in kidneys from all three models [104]. In hypokalaemic nephropathy it has been demonstrated that NH$_3$ production rate, urinary NH$_3$ excretion and renal vein [NH$_3$] are all increased compared with controls [112]. However, if hypokalaemic rats are given dietary NaHCO$_3$ supplements to reduce rates of ammoniagenesis, renal hypertrophy, proteinuria and histological injury are all reduced [112]. In diabetes mellitus, ammoniagenesis is not directly stimulated by hyperglycaemia, but increases in single nephron glomerular filtration rate (SNGFR) stimulate the production and secretion of ammonium into the proximal tubule. The mechanism of this stimulation is unknown, but it has been suggested that it is mediated by increased flow rate in the tubular lumen, which in the isolated perfused proximal tubule stimulates mitochondrial ammoniagenesis directly [113]. Finally, in nephropathy induced by anti-oxidant deficiency, renal ammonia production is also significantly increased, but dietary sodium bicarbonate supplements influence neither ammoniagenesis nor renal growth [111]. Nath et al have proposed that ammonia induces renal injury by interacting biochemically
with the C3 component of complement [104]. Free ammonia can disrupt a thiolester bond within the α subunit of C3 producing amidated C3, which causes injury by two mechanisms. Firstly, it acts as a convertase for the alternative complement pathway and thereby generates the cytolytic membrane attack complex C5b-9, and secondly, it is a potent stimulus to the generation of ROS by polymorphs and monocytes. In support of a role for ammonia in renal injury, Nath et al (1985) demonstrated that dietary supplementation with NaHCO₃ reduced ammonia production and renal injury in the remnant model of chronic renal failure in the rat [115]. After 1³/₄ nephrectomy, rats were supplemented either with sufficient NaHCO₃ to neutralise their calculated daily nonvolatile acid production (alkalinised group), or with an equimolar quantity of sodium chloride (controls). Plasma bicarbonate concentration was higher (31 ± 0.7 vs 20.4 ± 1.6mEq/l, p < 0.01), and renal vein total ammonia concentration (which correlates with cortical ammonia concentration) was lower (145 ± 20 vs 240 ± 20 μM) in alkalinised animals than controls. After 6 weeks, total urinary protein excretion was 41 ± 13 mg/24hrs in alkalinised animals compared with 74 ± 16 mg/24hrs in controls (p < 0.05), and excretion of low molecular weight proteins was 14 ± 3 mg/24hrs in alkalinised animals and 28 ± 8 mg/24hrs in controls (p < 0.02). GFR, SNGFR, filtration fraction, initial glomerular capillary plasma flow rate and mean transcapillary hydraulic pressure gradient were similar in both groups, but the Transport Maximum for Para-amino Hippurate /Glomerular Filtration Rate (TmPAH/GFR), an index of tubular function, was increased in the alkalinised group. Histological examination demonstrated fewer intratubular casts, less interstitial infiltration and smaller mean tubular diameter in kidney sections from alkalinised animals than in controls. Immunofluorescent studies suggested that these differences in tissue injury might reflect decreased complement activation in the alkalinised group: sections from control rats showed significantly higher levels of peritubular C3
and C5b-9 fluorescence than those from NaHCO₃ supplemented animals. It was proposed that C3 was converted to amidated C3 by the ammonia present in high concentrations in the cortex of control animals, and that this amidated C3, acting as a convertase for the alternate pathway and thereby generating the membrane attack complex C5b-9, induced tubulointerstitial injury manifest as tubular proteinuria, tubular dilatation and interstitial infiltration. The ability of the concentrations of ammonia found in the remnant kidney to trigger alternate pathway activation was confirmed in a further experiment using an in vitro haemolytic assay. Rabbit erythrocytes were incubated with normal human serum containing variable concentrations of ammonia. Exposure of red cells to 0.25mM NH₄Cl increased haemolysis by 15%, but this effect was abolished by incubation with NH₄Cl in the absence of serum. On the basis of these findings, Nath et al proposed that the reduction in renal mass in the remnant kidney causes tubular hyperfunction and increased ammoniagenesis in the residual nephrons. The resulting increased local ammonia concentration then causes alternative complement pathway activation and complement mediated cellular infiltration and tissue injury. In the same paper, this hypothesis was given further support by a comparison of protein excretion in subtotally nephrectomised mice congenitally deficient for the C5 component of complement with C5 sufficient controls. After 30 weeks, 24 hour urinary protein excretion was significantly greater in C5 deficient mice than in controls, (18.18 ± 3.78 vs 6.33 ± 1.13mg/24hrs, p < 0.05) consistent with a pathogenic effect of alternative complement pathway components in this model.

No large-scale clinical studies of the effects of alkalinisation on the progression of renal failure have been published, but in 1998, Rustom et al reported a small study in which 11 patients with a mean GFR of 46 mls/min/1.73m² were given oral sodium bicarbonate for six weeks [116]. At
the end of the study period tubular protein catabolism, urinary NAG and ammonia excretion were all reduced, whilst proteinuria, GFR and renal plasma flow remained unchanged. On the basis of these findings, it was suggested that oral sodium bicarbonate may protect the renal tubule and help delay renal disease progression. As the numbers in the study were small, the protocol was too short to allow 'break point' analysis of any trends in creatinine clearance and there was no untreated control group, it is difficult to draw a clear conclusion about the effects of alkalinisation on the progression of renal disease from these data.

The ability of ammonia to induce growth of renal cells in vitro was demonstrated by Golchini et al in 1989 [117]. By incubating JTC cells, a line derived from the monkey proximal tubule, in media containing varying concentrations of ammonia, they demonstrated a dose-dependent increase in cell protein content across the concentration range 0 - 20 mM NH₄Cl. Since the increased cell protein content was not accompanied by increased [³H]thymidine incorporation, and the percentage of cells in the population that were in the G₀ phase of the cell cycle was reduced by NH₄Cl, it was concluded that ammonia increased cell content by hypertrophy rather than hyperplasia. The increased cell protein was shown to result both from an increase in protein synthesis (assessed by measurement of ³[H]leucine incorporation) and a decrease in protein degradation, and consistent with the latter was the demonstration of reduced activity of the lysosomal proteases cathepsin B & L in cells exposed to high [NH₄Cl]. Since these alterations in protein content, synthesis and degradation were not accompanied by changes in intracellular or extracellular pH, it was concluded that they represented a direct effect of ammonia on protein metabolism independent of cell pH. It has been suggested that increased SNGFR can induce renal hypertrophy by stimulating Na⁺ transport via the Na⁺/H⁺ antiporter [110]. To assess any
contribution of this mechanism to the hypertrophy of JTC cells, activity of the 
Na⁺/H⁺ antiporter was measured after exposure to NH₄Cl. No increase in 
activity was detected, and since co-incubation of ammonium treated cells 
with amiloride failed to prevent the hypertrophic response, it was concluded 
that alterations in Na⁺/H⁺ exchange were not implicated in cell growth in 
this model. The authors agreed with Nath et al (1991) that renal hypertrophy 
in models such as the remnant kidney and streptozotocin-induced diabetes 
are likely to result from an initial increase in SNGFR, resulting in an increase 
in luminal flow rate and consequent upregulation of ammoniagenesis. In a 
subsequent review in 1991 [113], they proposed a hypothesis similar to that of 
Nath et al, in which renal hypertrophy in protein loading, uninephrectomy, 
diabetes mellitus and NH₄Cl loading were attributed directly or indirectly to 
increased rates of ammonium production/nephron (Figure 1.4). In the case of 
metabolic acidosis, it was proposed that hypertrophy resulted from the 
upregulation of ammoniagenesis in proximal tubular cells rather than by a 
direct effect of low pH on protein metabolism.

The hypothesis that metabolic acidosis causes renal growth exclusively via 
upregulation of ammoniagenesis was called into question by the work of 
Bevington et al in 1994 [118]. Using opossum kidney (OK) cells, a tubular cell 
line, they demonstrated that incubation at pH 7.21 for 4 days caused a 
significant increase in protein/DNA ratio over controls (incubated at pH 7.37) 
without significantly changing total DNA per well. In addition, it was shown 
that a similar increase in protein/DNA ratio could be induced by the addition 
of 11mM NaCl to cells cultured at pH 7.2. Since [NH₄⁺] in the medium was 
not significantly affected by lowering its pH, it was suggested that 
hypertrophy might result directly from low pH independently of 
ammoniagenesis. Because proximal tubular cells upregulate NHE activity 
when incubated in acidic media, and the upregulation of NHE in response to
Figure 1.4 Proposed relationship between renal ammoniagenesis and hypertrophy. From Kurtz I, Reference 113.
acidosis is unique to renal cells, it was suggested that the hypertrophy of acidosis might be mediated by increased intracellular [Na⁺]. This theory would provide an alternative explanation for the observation that acidosis causes hypertrophy of proximal tubular cells, but net cellular protein degradation in non-renal cells. In the ammonium based theory of renal hypertrophy, this observation is explained by invoking the unique ability of the proximal tubule to upregulate ammoniagenesis at low pH.
Section 4. Summary and Hypothesis

Summary

The above consideration of the generation and disposal of metabolic acid, the pathogenesis of MA, renal compensation for MA, and the effects of chronic MA on the renal structure and function can be summarised as follows.

1. Hepatic breakdown of the amino acids contained in a typical western diet generates 50 mEq H+/day. The resulting whole body HCO₃⁻ deficit is repaired by renal ammoniagenesis and urinary excretion of acid phosphate. Tubular H⁺ secretion effects HCO₃⁻ reabsorption, conversion of NaHPO₄²⁻ to NaH₂PO₄⁻ and conversion of NH₃ to NH₄⁺.

2. 'Metabolic acidosis' describes the combination of high plasma [H⁺] (pH < 7.36) and low plasma [HCO₃⁻] (< 20mmol/l)). Biochemically, MA is classified as normal anion gap (low plasma [HCO₃⁻], high plasma [Cl⁻]) or high anion gap (low plasma [HCO₃⁻], normal plasma [Cl⁻]).

3. Renal acid disposal is increased in chronic MA. The ammoniagenic enzymes PDG and GDH are upregulated, and proximal tubular apical Na⁺/H⁺ exchange and distal tubular H⁺ secretion are increased.

4. MA complicates CRF when the GFR falls below 30 mls/min. Moderate CRF (GFR 20-30mls/min) causes hyperchloremic acidosis, whilst advanced CRF causes high anion gap acidosis by reducing NH₄⁺.
production and excretion and interfering with tubular \( \text{HCO}_3^- \) handling. Although total \( \text{NH}_4^+ \) production is reduced, rates of ammoniagenesis in residual nephrons and therefore cortical [\( \text{NH}_4^+ \)] increase in CRF.

5. Chronic MA causes proteinuria and accelerated renal growth. Proteinuria is a well-recognised marker of glomerular and tubulointerstitial injury, an adverse prognostic factor in many renal diseases, and may itself contribute to tubulointerstitial injury. Accelerated renal growth precedes renal injury in several models of chronic renal impairment, and increased ammoniagenesis has been implicated in the pathogenesis of both renal growth and tubulointerstitial injury in these models.

**Hypothesis**

Two proven effects of chronic MA demonstrate its capacity to inflict or exacerbate renal injury. Firstly, it causes proteinuria, which is a marker of glomerular and tubulointerstitial injury, a predictor of disease progression in established renal impairment and may itself cause or exacerbate tubulointerstitial disease; and secondly, it causes accelerated renal growth, which precedes the development of chronic renal injury in a number of models of chronic renal failure. Since MA is an invariable complication of moderate and severe CRF, these observations raise the possibility that uraemic acidosis itself contributes to the progression of chronic renal disease, generating a vicious cycle of increasing injury, worsening acidosis and further disease progression. If such a mechanism exists, aggressive and early treatment of uraemic acidosis (at serum creatinine ≤ 200μmol/l) might slow
disease progression and delay the need for Renal Replacement Therapy (RRT).

Clinical relevance of hypothesis

Established chronic renal failure in humans characteristically progresses to end stage even after resolution of the initial disease process [119]. Analyses of groups of patients with chronic renal injury have shown that rates of progression are approximately normally distributed, with mean fall in GFR ranging from 0.2 to 1.2 ml/min/month in different series [120]. Even within groups of patients with a single underlying disease, rates of progression vary widely, and no consistent relationship has been identified between specific diagnoses and rates of decline of GFR. These observations are consistent with the presence of a common mechanism underlying disease progression in most or all types of chronic renal disease [120]. Such a mechanism was proposed by Brenner et al in 1982, who suggested that chronic renal injury is initiated and promoted by maladaptive glomerular haemodynamic changes [121]. They noted that reduced renal mass, the high protein content of typical western diets and diabetes mellitus all cause chronic renal vasodilatation, resulting in glomerular hyperfiltration and impaired glomerular permselectivity. They suggested that the resulting flux of plasma proteins across the glomerular capillary wall caused mesangial proliferation and increased mesangial matrix production, culminating in the development of glomerular sclerosis. The consequent reduction in functioning glomeruli would then cause further compensatory hyperfiltration in remaining glomeruli, setting up a vicious cycle of progressive renal injury.
Patients requiring renal replacement therapy (RRT) have a high mortality. Data from the European Dialysis and Transplant Association (EDTA) registry show that between 1983 and 1992 the 5 year mortality of patients on RRT ranged from 20% (age range 25-34 years at start of treatment) to 90% (age over 75 years at start of treatment) [122]. As a consequence, any treatment which delays the need for renal replacement, in addition to prolonging the time independent of dialysis, would also be expected to improve survival. Although specific immunosuppressive regimes may be beneficial in the treatment of some forms of glomerulonephritis, they appear to have no influence on any chronic renal injury already sustained. To date, only two interventions have been proven to slow the progression of established chronic renal disease in humans. These are tight blood pressure control, and in patients with insulin dependent diabetes mellitus, the maintenance of near normal glycaemia by the use of strict insulin regimes. In the MDRD study, patients given sufficient antihypertensive therapy to lower mean arterial pressure to 92 mmHg ('low MAP group') had a slower rate of fall of GFR from the fourth month onwards than patients with MAP 107 mmHg ('usual MAP group') [88]. This effect was more pronounced in patients with higher baseline proteinuria [123]. In the Diabetes Control and Complications Trial (DCCT) 1441 patients with insulin dependent diabetes were randomised either to conventional therapy (one or two daily injections of insulin with daily self monitoring of urine or blood glucose) or to intensive therapy (three or more daily injections or use of an external insulin pump with dose adjustment 3 or 4 times daily based on glucose monitoring) and followed up for a mean period of 6.5 years [124]. In patients who had been diabetic for 1-15 years, demonstrating mild to moderate nonproliferative retinopathy and with initial albuminuria less than 200mg/24 hrs (secondary prevention cohort: n = 715) intensive therapy reduced the risk of developing microalbuminuria by 43% and that of developing albuminuria by 56%. Since
microalbuminuria predicts and proteinuria indicates established diabetic nephropathy [125], it can be concluded that tight diabetic control slows the progression of nephropathy in insulin dependent patients. Although the use of low protein diets has been shown to slow disease progression in several animal models of chronic renal disease [126], the results of studies in humans have been inconsistent and inconclusive. Initial analysis of the MDRD data found no significant effect of low protein diets (0.6 or 0.3 g/kg/day) on rates of decline of GFR [88], though a post hoc analysis claimed a 29% slower decline of GFR in patients with an initial GFR of less than 25mls/min and a verified reduction in protein intake of 0.2g/kg/day [127]. A recent meta-analysis of five studies of dietary protein restriction (0.4 - 0.6 g/kg/day) showed a significant though modest benefit in both diabetic (relative risk for renal failure or death 0.67) and non-diabetic (relative risk of renal failure or death 0.56) nephropathy [128]. Against this possible benefit must be set the risk of patients commencing RRT programmes with protein calorie malnutrition, which adversely affects survival in dialysis patients [129]. In conclusion, whilst low protein diets may be beneficial in selected well-motivated and well-supervised patients with chronic renal failure, their widespread use as a means of slowing disease progression is not generally recommended. The treatment of hypertension is already accorded a high priority in the management of patients with chronic renal disease. The potential impact of tight diabetic control is obviously restricted to patients with diabetic nephropathy, and the strictness of monitoring and control required to influence progression (blood glucose analysis ≥ four times daily, glycosylated haemoglobin ~ 7% in the DCCT) requires a degree of patient motivation and medical input unlikely to be attainable on a large scale in most clinics. Further impact on the progression of chronic renal disease therefore requires the development of alternative treatment strategies.
Chapter 2

The effects of metabolic acidosis on renal mass, proteinuria and renal histology in the normal rat: The SA Study
Introduction

As discussed in Chapter 1, it has been demonstrated that non-uraemic rats made acidotic by dietary supplementation with ammonium chloride develop renal growth (comprising both hypertrophy and hyperplasia) and proteinuria [83, 84]. The observation that several experimental models of chronic renal disease are also characterised by renal growth and proteinuria raises the possibility that the mechanisms responsible for renal hypertrophy and proteinuria in acidotic, non-uraemic rats also contribute to the progression of established renal disease. A detailed analysis of the effects of metabolic acidosis on renal structure and function in non-uremic animals might therefore help to evaluate its role in the progression of chronic renal disease.

The studies by Gardner (1961) [83] and Lotspeich (1965) [84] of the renal response to acidosis were deficient in three respects:

1. Acidosis was induced by dietary supplementation with NH₄Cl.

   It is well-established that high protein diets cause renal growth [110], and Wilson demonstrated in the 1930s that dietary glycine, glutamic acid and gluten accelerate renal growth in proportion to their nitrogen content [130]. Since dietary NH₄Cl supplements also deliver a nitrogen load, their effects on renal growth cannot confidently be attributed to metabolic acidosis alone.

2. Urinary protein in acidotic rats was not adequately characterised.

   Gardner's conclusion that the proteinuria of acidosis was glomerular in origin was based on a qualitatative assessment of the number of protein
droplets within tubular epithelial cells of acidotic kidneys, and whilst urine electrophoresis was performed, it detected only 'small amounts' of albumin (normally a major contributor to glomerular proteinuria). No specific assays for proteins of tubular origin were performed.

3. Effects of acidosis on renal morphology were not analysed.

Lotspeich made no reference to the histological appearance of kidneys from NH₄Cl - supplemented rats, and Gardner commented only on changes in tubular cell protein droplet formation. Neither author therefore excluded glomerular or tubular injury as the cause of proteinuria in metabolic acidosis. This is an important omission, particularly given Gardner's conclusion that proteinuria in MA is of glomerular origin, and therefore by implication due to glomerular disease.
Aims of the SA study

As discussed above, previous studies have failed adequately to address a number of important issues relating to the effects of MA on renal growth, protein excretion and renal morphology. The experiments described in this chapter examined these issues in more detail, and their specific aims were as follows:

1. To establish whether MA induced without a concomitant nitrogen load causes renal growth and proteinuria in the rat.

2. If MA does cause proteinuria, to establish whether the urinary protein is of glomerular or tubular origin.

3. To examine the effects of MA on glomerular and renal tubular morphology.
Materials and Methods

1. Animal protocols

Two groups of 12 female Wistar rats (Leicester University strain, initial weight approximately 200g), housed in individual cages with constant temperature and humidity, 12 hour light/dark cycle and free access to water, were pair fed a powdered diet (ICN no. 960259; Appendix A), containing 20% casein (w/w), offered as a paste mixed with water (1:1 w/w) and methylcellulose (2g/100g). After a two week induction period, all animals were weighed, and those in the experimental group were made acidotic by reconstituting their feed with 0.8M hydrochloric acid instead of water. This concentration of hydrochloric acid delivered an acid load which in previous studies from this laboratory consistently produced a metabolic acidosis (arterial blood [HCO$_3$] < 19 mmol/l) without rendering the food unpalatable or changing its consistency [131]. The control group continued to take the standard diet without acid supplementation. At baseline, and after 7 and 14 days, 24 hr urine collections were made using metabolic cages. Following the second collection, animals were again weighed, then sacrificed by exsanguination via aortic puncture under anaesthesia (2.7ml/Kg of Hypnorm/Hypnovel/water 1:1:2 v/v given intraperitoneally). Arterial blood was collected in a heparinised, air-free syringe for blood gas determination, and serum was analysed for urea and creatinine. One kidney from each animal was preserved in 10% formol saline for histological examination, and the contralateral kidney was retained for determination of total protein content.
2. Analyses of blood and urine

Arterial blood gases were measured using a Corning 178 Blood Gas Analyser. Serum urea was determined by the urease reaction, and creatinine by the Jaffe reaction, both using a Vitatron 178 SPS system.

Urine total protein was measured by a dye binding assay using pyrogallol red (Randox Laboratories, UK). The technique depends upon the formation of a blue-coloured product when proteins complex with pyrogallol red in an acid environment containing molybdate ions. Optical density of the product at 600 nm is directly proportional to protein concentration up to 5 g/l. 50µl of well-mixed urine were mixed with 3000µl of reagent (Appendix C) and incubated for 5 minutes at 37°C. Absorbance at 600nm was determined using a Vitatron 178 SPS system. Human albumin was used as standard.

Urinary lysozyme was measured by a turbidimetric assay [132] adapted for automated analysis using a one point standard method on an SPS Vitatron system. Urine was incubated with a suspension of 60mg% Micrococcus lysodeikticus (Sigma, Poole, UK) and the reduction in turbidity resulting from subsequent cell lysis was quantified spectrophotometrically at 540 nm. Lysozyme concentration was then calculated by comparison with a standard curve using crystalline hen egg white lysozyme (Sigma, UK) as standard. Urinary N-acetyl glucosaminidase (NAG) was measured using a colorimetric assay (Boehringer-Mannheim, West Livingston, UK) based on conversion of 3-cresolsulphonphthalein-N-acetyl glucosamine to 3-cresol purple, a coloured product subsequently measured photometrically at 580 nm.

Urinary albumin and immunoglobulin G (IgG) content were determined by a solid phase enzyme linked immunosorbent assay (ELISA) developed in
our laboratory. Disposable polystyrene microtitre plates (Nunc Immunoplate, Denmark) were coated with 100μl of either rabbit anti-rat albumin (10mg/ml, Sigma, UK) or rabbit anti rat IgG (5μg/ml, Cappel, Turnhout, Belgium), dissolved in coating buffer (9:1 mixture of molar sodium bicarbonate and sodium carbonate pH 9.6). After washing, plates were blocked with bovine serum albumin (BSA) as an irrelevant protein, for 1 hour and washed again. The standards (rat albumin and rat IgG, Sigma, UK) and samples were then added in duplicate after appropriate dilution and the plate was incubated overnight at 4°C. After further washing, horseradish peroxidase labelled rabbit anti rat albumin or IgG (1:2000 and 1:3000 respectively) was added and the plate incubated for 1 hour at room temperature. The colour was developed using 50μl of a 1,2-phenylenediamine detection system (0.03 M citric acid, 0.07 M disodium hydrogen orthophosphate at pH 5.0, 2 mg OPD tablet (Dakopatts, Denmark) and 1.25μl of 30% hydrogen peroxide per 3ml of solution). The reaction was stopped after 5 minutes by adding 75μl of 1M sulphuric acid to each well and the plate was then read on a Titretek Multiscan photometer (Labsystems, Finland) at 492 nm.

3. Determination of kidney protein content

Whole kidneys were homogenised in 10% (w/w) perchloric acid, and protein was precipitated by chilling. Samples were centrifuged at 4000 rpm for 10 minutes at 0°C, the supernatant was discarded and the pellet was redisolved in 1ml 0.5M NaOH using a vortex mixer. The solution was then assayed for protein by the Lowry method [133]. To 10μl of the kidney digest was added 40μl NaOH and 10μl deionised water. At time = 0, 600μl of Lowry solution C (Appendix B) was added and the solution was immediately vortexed. After 10 minutes, 60μl Folin-Ciocalteu reagent (BDH) was added.
and the solution again vortexed. After standing at room temperature for a further 30 minutes, the optical density of the solution was determined using a Titretek Multiscan photometer (Labsystems, Finland) at 660 nm. Bovine serum albumin was used as standard.

4. Histological and immunohistochemical analyses

Sections of formalin-fixed tissue (thickness 3µm) from both protocols were stained with Haematoxylin & Eosin (H & E) before examination in a blinded fashion. Using an eye-piece graticule, nuclei contained within an area of 30 small squares (25µm² each) were counted and categorised as "tubular" or "other" (comprising endothelial, interstitial and unidentified types). Nuclear density was then calculated and expressed as number of tubular or "other" nuclei/mm². Reproducibility of densities on duplicate examinations was within 10%.

Immunohistochemical analysis was performed using a technique described by Howie et al [134]. Previous studies using this method have shown a correlation between loss of renal tubular staining for Tamm Horsfall protein and brush border antibody, and renal injury from a variety of underlying causes. Sections were prepared using a two stage immunoperoxidase method. Sheep antiserum to proximal tubule brush border (dilution 1:200) and sheep antiserum to Tamm-Horsfall protein (dilution 1:600, both supplied by The Binding Site Ltd) were applied to formalin-fixed sections, followed by peroxidase-linked donkey anti-sheep IgG (dilution 1:100, Serotec, Oxford, UK). Detection was with diaminobenzene and hydrogen peroxide, and counterstaining was with Mayer's haemalum.
Sections were examined on a blinded basis by Dr A J Howie, Department of
Pathology, University of Birmingham.

5. Statistical analyses

Whole body and kidney weights and serum biochemistries are expressed
as means ± standard error (SEM). Urine biochemical indices at week 2 were
compared with baseline by paired 't' test. Urine albumin and IgG excretion at
baseline, week 1 and week 2 were compared by analysis of variance
(ANOVA). Histological scores are expressed as medians with interquartile
range and were analysed by Wilcoxon rank sum test. P values of less than
0.05 were considered significant.
Results

1. Animal data

At the end of the run-in period (ie at baseline) body weights were similar in both groups (220 ± 5 vs 217 ± 3g, NS), but after two weeks, acidotic animals had lower body weights than controls (Table 2.1). Mean daily food intake during the two week experimental period was 49.2 ± 1.6g in acidotic animals compared with 50.9 ± 0.4g in controls (NS), and acidotic animals consequently received a mean daily dose of 19.7 mmol HCl.

2. Urine data

Acid supplementation was followed by a marked rise in urine output, and there was a small, though non-significant fall in urine pH (Table 2.2). Excretion of total protein, lysozyme, NAG, albumin and less markedly IgG significantly increased by week 2 (Table 2.2; Figure 2.1). The volume, pH and total protein content of urine from control animals remained stable throughout the experiment.

3. Blood and serum data

At sacrifice, arterial blood gases confirmed acidosis in the experimental group (pH 7.24 ± 0.02 vs 7.38 ± 0.01, p < 0.001; base deficit 6.5 ± 1.4 vs base excess 3.4 ± 0.6, p < 0.001). Serum creatinine values were similar in acidotic animals and controls (60 ± 4 and 59 ± 4 μmol/l respectively; NS).
Table 2.1  Kidney and whole body weights of acidotic and control animals at sacrifice.

<table>
<thead>
<tr>
<th></th>
<th>Acidotic</th>
<th>Control</th>
<th>'p' value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>196 ± 3</td>
<td>230 ± 5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Kidney weight (g)</strong></td>
<td>0.92 ± 0.02</td>
<td>0.80 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Kidney/body wt (g%)</strong></td>
<td>0.47 ± 0.01</td>
<td>0.35 ± 0.01</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Table 2.2  Characteristics of urine from acidotic animals at baseline and 2 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 2</th>
<th>'p' value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine volume (mls/24hrs)</td>
<td>11.9 ± 1.18</td>
<td>50.9 ± 5.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urine pH</td>
<td>5.86 ± 0.03</td>
<td>5.75 ± 0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Protein excretion (mg/24hrs)</td>
<td>2.9 ± 0.3</td>
<td>16.4 ± 2.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lysozyme excretion (µg/day)</td>
<td>0.24 ± 0.22</td>
<td>10.1 ± 1.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NAG excretion (U/day)</td>
<td>108 ± 8</td>
<td>218 ± 23</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 2.1 Urinary excretion of albumin and immunoglobulin G in acidotic rats. Values expressed as mean ± SEM. Increases in albumin and IgG were both significant (p < 0.05) by ANOVA.
4. Kidney data

Kidney weights and kidney/body weight ratios were higher in acidotic animals than controls (Table 2.2). Kidney protein content was also greater in the acidotic group (123 ± 3 vs 111 ± 4mg/kidney, p < 0.05) confirming that true renal growth had occurred.

5. Histology and immunohistochemistry.

Sections of H & E stained kidneys from acidotic animals showed no abnormalities of either the glomeruli or tubulointerstitium on light microscopy (Figure 2.2). The density of tubular nuclei, however, was significantly less in acidotic kidneys than in controls, whilst the density of non-tubular nuclei remained unchanged (Figure 2.3; raw data shown in appendix D). Application of antibodies to brush border produced no staining in formalin-fixed kidney sections from acidotic or control groups. On the basis of blinded examination of the sections stained with antibody to THP, however, kidneys were easily classified by Dr A.J. Howie as 'normal' or 'abnormal'. After unblinding, these categories corresponded exactly to control and acidotic groups respectively. In control sections, as previously reported in normal kidneys, the thick ascending limbs of the loop of Henle stained well, and there were no tubular casts (Figure 2.4a, 2.5a). In contrast, the thick ascending limbs of acidotic kidneys stained only lightly, and tubules contained many THP-positive casts (Figure 2.4b, 2.5b).
Figure 2.2

Example of kidney section from acidotic animal stained with H and E (x400)
Figure 2.3 Nuclear density of tubular and nontubular cells in sections from acidotic and control animals. Values expressed as median with interquartile range. * p < 0.01
Figure 2.4
Sections from control (a) and acidotic (b) kidneys stained with antibody to Tamm-Horsfall protein (magnification x4)
Figure 2.5

Figures from control (a) and acidotic (b) kidneys stained with antibody to Tamm-Horsfall protein (Magnification x20). Cells of the thick ascending limb are stained heavily in controls, but in acidotic kidneys staining is less prominent, and numerous THP-positive tubular casts are seen.
Summary of Findings of SA Study

1. Metabolic acidosis, induced in non-uraemic rats without a concomitant nitrogen load, caused an increase in kidney weight, kidney/body weight ratio and kidney total protein content consistent with true renal growth.

2. The density of tubular nuclei / unit kidney area was decreased in acidotic animals, suggesting that hypertrophy, rather than hyperplasia, was the predominant mechanism of growth.

3. Acidosis caused an increase in 24 urinary protein excretion. There was a large increase in excretion of the freely filtered but normally completely reabsorbed protein lysozyme, and significant increases in the excretion of albumin and the lysosomal enzyme NAG. Excretion of immunoglobulin G was only modestly increased. This profile is consistent with a predominantly tubular origin of the urinary protein.

4. The pattern of immunohistochemical staining with antibody to Tamm-Horsfall protein was abnormal in sections from acidotic kidneys. Cells in the thick ascending limb of the loop of Henle stained only lightly, but many THP-positive tubular casts were seen. This pattern has previously been reported in kidney sections from patients with renal impairment.

5. Sections of kidneys from acidotic rats were normal by light microscopy, and serum creatinine was similar in acidotic animals and controls.
Chapter 3

The effects of prolonged metabolic acidosis on renal mass, proteinuria, glomerular filtration rate and renal histology in the normal rat: The LA Study
Introduction

The data reported in Chapter 2 confirm previous reports that metabolic acidosis causes renal growth and proteinuria. They also suggest that the predominant mechanism of growth in acidosis is hypertrophy, and show that the pattern of proteinuria is consistent with a tubular origin. As the protocol lasted for only 14 days, however, it failed to establish whether the renal growth and proteinuria seen after short term acidosis are forerunners of more significant structural and functional abnormalities when acidosis is prolonged. Since patients with chronic renal impairment may be acidotic for months or years, any assessment of the influence of acidosis on progression of their disease must examine its effect over more prolonged periods. In addition, a number of other observations from this study require further investigation. Firstly, since renal excretory function was evaluated only by the measurement of serum creatinine (which rises only when renal function is significantly impaired), it cannot be confidently concluded that acidosis did not influence the glomerular filtration rate. Secondly, the cause of the polyuria observed in acidotic animals, and its potential effect on protein excretion, were not investigated. Thirdly, effects of acidosis on the anionic charge of the glomerular basement membrane (GBM), which influences the filtration of both small and large molecular weight proteins, were not examined. Finally, no assessment was made of the effects of MA on the influx of inflammatory cells, which are central to the development of glomerular and tubulointerstitial injury in many experimental models of renal disease.

The polyuria observed in acidotic rats in Chapter 2 could be due either to polydipsia induced by HCl supplements, or to a defect in urinary concentrating capacity. Defects in urinary concentration may reflect inadequate antidiuretic hormone (ADH) production by the posterior pituitary
gland (neurogenic diabetes insipidus), resistance of the collecting duct to ADH (nephrogenic diabetes insipidus), or an inability to generate a sufficiently hypertonic medullary interstitium [135]. Since the development of nephrogenic diabetes insipidus in acidotic rats would imply a toxic effect of acidosis on the distal tubule (which may be relevant to the development or promotion of chronic renal injury), it is important to evaluate the mechanism of polyuria in this model. In addition, polyuria has been shown to influence rates of excretion of both high and low molecular weight proteins. In 1982, Viberti et al demonstrated in 18 healthy subjects that polyuria induced by both acute and chronic water loading caused a significant (more than twofold), though transient, increase in urinary albumin excretion [136]. Since rates of protein excretion returned to baseline before polyuria resolved, they suggested a 'washout' phenomenon, whereby the onset of diuresis washed out protein from the tubular lumen, transiently increased transglomerular protein traffic or inhibited tubular protein reabsorption. In 1988, Jung et al also demonstrated transient increases in urinary excretion of lysosome (> fivefold) and β2-microglobulin (> 1.5 fold) during diuresis in six healthy men. In contrast, the excretion of ribonuclease was approximately halved. They concluded that the diagnostic sensitivity of low molecular weight protein excretion was reduced in the face of high urine output [137]. In view of these findings, and the demonstration in Chapter 2 that acidotic rats develop polyuria, it is important to establish any effect of polyuria on protein excretion in this model before drawing conclusions about the tubular effects of MA.

Filtration of proteins is restricted by both size and charge barriers within the glomerulus. Fixed anionic charges have been identified both on glomerular epithelial cells (due to sialic acid) and on the laminae interna and externa of the GBM (due to heparan sulphate residues) [138] and these
combine with the physical barrier imposed by the glomerular basement membrane to retain the majority of protein molecules (which are predominantly cationic) within the vascular space. In addition, the density of anionic sites along the glomerular basement membrane (GBM) is reduced in patients with a range of glomerulonephritides, including the congenital nephrotic syndrome [139] and idiopathic membranous glomerulonephritis [140]. Similar observations have been made in experimental animals. Perfusion of rat kidneys with cationic protamine sulphate causes foot process effacement and loss of fixed anionic charges on glomerular epithelial cells: both changes are reversed when anionic heparan sulphate is infused [141]. Intra-aortic infusion of plasma from nephrotic patients into normal rabbits reduces the number of anionic sites on the GBM and increases proteinuria [142], and in streptozotocin-induced diabetes mellitus in the rat, the number of anionic sites progressively decreases from the time of induction of diabetes mellitus, but is normalised by treatment with insulin [143]. These observations demonstrate that components of the plasma circulating through glomerular capillaries can influence epithelial cell morphology and the magnitude of anionic charges on both the epithelial cell and the GBM. Since alterations in pH can induce conformational changes in many proteins, it is conceivable that MA could influence glomerular permselectivity by changing the net anionic charge on epithelial cells and/or the GBM. Foot process effacement mirrors changes in epithelial cell charge [141], and anionic charge on the basement membrane can be evaluated by exposure of the GBM to the highly cationic molecule polyethyleneimmine (PEI) and subsequent examination by electron microscopy [144]. Changes in charge on both epithelial cells and the GBM can therefore be identified by perfusion of kidneys with PEI followed by examination with the electron microscope.
Macrophages accumulate in the glomerulus in most human glomerulonephritides, where they are the predominant infiltrating leukocyte, and secrete a number of products (including reactive oxygen species, Interleukins 1 and 6, Platelet-Derived Growth Factor and Tumour Necrosis Factor) known to mediate glomerular injury [145]. They stimulate mesangial cell proliferation and induce glomerulosclerosis in models of puromycin nephrosis, renal ablation and hyperlipidaemia, and techniques which deplete monocytes in these models also abrogate renal injury. In both immune and non-immune experimental glomerulonephritis, the number of interstitial mononuclear cells (both T cells and monocytes) is proportional to the rate of decline in renal function, and immunosuppressive treatment of rat accelerated anti-GBM disease or puromycin-induced glomerulosclerosis dramatically inhibits interstitial mononuclear cell infiltration and reduces renal functional impairment [145]. Since macrophages play a central role in renal inflammation, measurement of their infiltration into the kidney provides a useful index of both glomerular and tubulointerstitial injury.
Aims of the LA study

The experiments described in this chapter extended the observations made in Chapter 2, and their specific aims were as follows:

1. To establish whether the proteinuria and renal growth seen after 14 days of acidosis progress to more significant structural and functional abnormalities when the period of acid exposure is extended to three months.

2. To evaluate the effect of prolonged MA on the glomerular filtration rate.

3. To establish whether the polyuria seen during HCl-induced MA is the result of acid-induced thirst or a urine concentrating defect.

4. To examine any effect of polyuria on the excretion of high and low molecular weight proteins in the Wistar rat.

5. To establish whether MA influences glomerular epithelial cell morphology or GBM anionic charge density.

6. To evaluate glomerular and tubulointerstitial inflammation in acidotic rats by measuring macrophage infiltration.
Materials and Methods

1. Animal protocols.

53 female Wistar rats (initial weight = 200g), housed in individual cages with constant temperature and humidity, a 12 hr light/dark cycle and free access to water, were fed a powdered diet (ICN no. 960259) containing 20% casein (w/w), offered as a paste mixed with water (1:1 w/w) and methylcellulose (2g/100g). After a two week induction period, 32 animals had the standard paste replaced by feed reconstituted with 0.5 mol/l hydrochloric acid instead of water, whilst 21 pair-fed controls continued on the standard diet without acid supplementation. Since previous studies had shown tolerance to 0.8mol/l HCl only for two weeks, a lower acid concentration was selected for this experiment to ensure tolerance throughout the longer protocol. Food consumption was recorded daily, body weights were measured weekly, and at fortnightly intervals animals were placed in metabolic cages for collection of 24 hour urine samples.

After 8 weeks, 6 acidotic animals and 6 controls were sacrificed by exsanguination under anaesthesia (2.7ml/Kg of Hypnorm/Hypnovel/water 1:1:2 v/v given intraperitoneally) and tissue was prepared for identification of anionic binding sites on the glomerular basement membrane (see below). After 14 weeks, the GFR of the remaining animals was measured using $^{99m}$Technetium diethylenetriamine penta-acetic acid (DTPA) according to the method of Nankivell et al (1992) [146]. A measured, timed dose of approximately 20 MBq of $^{99m}$Technetium DTPA (supplied by Dept of Medical Physics, Leicester Royal Infirmary) was administered by intraperitoneal injection to a depth of no more than 7mm to avoid injection
into bowel or intraperitoneal fat. The residual radioactivity of the syringe and needle was determined. A further dose of 20 MBq \(^{99m}\)Tc DTPA was added to 1000mls water in a volumetric flask for use as a reference standard, and the activity of the syringe residue was recorded. Forty five and 90 minutes after injection, two timed 200\(\mu\)l blood samples were taken from a tail vein under light ether anaesthesia and centrifuged. 50\(\mu\)l aliquots of plasma and a 50\(\mu\)l aliquot of the reference standard were then transferred to 10ml plastic counting tubes, and technetium activity was determined by a sodium iodide crystal well counter (Beckmann), which was programmed to correct activity to a uniform start time. GFR was calculated using the slope-intercept method (Appendix F).

After determination of GFR, the remaining animals were sacrificed by exsanguination under anaesthesia as above. Aortic blood was collected in a heparinised, air-free syringe for blood gas determination, and serum was analysed for urea and creatinine. Hearts and kidneys were removed and weighed, and kidneys were bisected coronally. One half of each kidney was fixed in formol saline, and the other half in Carnoy's solution. Sections of formalin-fixed tissue were stained with haematoxylin and eosin and examined by light microscopy. Kidneys fixed in Carnoy's solution were used to quantify infiltrating macrophages.

As in the previous study, acidotic animals developed polyuria and polydipsia, the cause of which was investigated by a fluid restriction study performed in 6 acidotic animals and 6 controls. During a six hour baseline period, animals were allowed free access to water and their urine output and urinary osmolality were recorded. Drinking water was then removed, though free access to casein paste (which was made up 50% w/w with water) was continued, and urine output and osmolality were measured 6 hourly for 24
hours. Animals were also weighed every 6 hours to ensure that body weight did not fall by more than 5% during fluid restriction.

The effects of polyuria on urinary protein excretion were investigated in 16 control animals made polydipsic by the addition of glucose to their drinking water. Urinary total protein and lysozyme excretion were measured during a baseline 24 hour period, after which drinking water was supplemented with 5% glucose. Urine output and 24 hour total protein and lysozyme excretion were then remeasured for comparison with baseline values.

2. Analyses of blood and urine.

Arterial blood gases, serum creatinine and urine total protein, lysozyme and IgG were determined as described in Chapter 2.

3. Light microscopy

Three μm formalin-fixed tissue sections were stained with Haematoxylin & Eosin (H & E) and examined for glomerular or tubulointerstitial abnormalities by light microscopy. The density of tubular and non-tubular cells was evaluated as described in Chapter 2.
4. Identification of anionic binding sites on the GBM.

Tissue was processed by a method adapted from Schurer et al (1977) [147]: all solutions were used at 4°C. Immediately after death, an incision was made in the inferior vena cava and kidneys were perfused in situ with a solution of 1% polyethyleneimmine (molecular weight 50000-60000) in normal saline. After perfusion, kidneys were removed, weighed and sectioned coronally. A 1mm^3 section of tissue was removed and fixed in 0.5% glutaraldehyde for 1 hour, then washed three times in Sorensen's buffer before immersion in a solution of 2% phosphotungstic acid for 30 minutes. After three further washes, tissue was processed for transmission electron microscopy. Cubes of tissue were fixed for ≥ 4 hours in 0.25% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, adjusted to 300 mOsM with sucrose. They were then washed in buffer and post-fixed in 1% aqueous osmium tetroxide for 1 1/2 hours, dehydrated through six grades of alcohol / water, infiltrated with EMIX resin and polymerised at 60°C for 16 hours. 0.5 μM sections were then cut, examined by electron microscopy and photographed in the Department of Histopathology, Leicester Royal Infirmary. Prints of magnification 6000 were prepared from each section, and the number of anionic binding sites per 10 cm length of lamina rara externa was recorded in each case.

5. Identification of infiltrating macrophages.

Infiltrating macrophages were quantified for use as an index of tubulointerstitial inflammation as follows. Carnoy's-fixed kidneys from acidotic animals and controls and were paraffin wax embedded and cut into 3 μm sections which were then mounted on glass slides, dewaxed in graded
alcohol and xylene and immersed for 10 minutes in a blocking solution of one part 30% peroxidase to nine parts of absolute methanol. Sections were incubated for 30 minutes in a 1:250 dilution of ED1 mouse monoclonal antibodies (Serotec, Oxford, UK), then washed three times with PBS to remove unbound antibody before incubation for 5 minutes in a solution of biotinylated rabbit anti-mouse immunoglobulin G (Zymed, San Francisco). 100 μl of streptavidin peroxidase concentrate were then applied to each slide and incubated for 5 minutes. Slides were washed three times with PBS and incubated for a further 5 minutes with aminoethyl carbazole and hydrogen peroxide. After rinsing with distilled water, slides were counterstained by incubation with haematoxylin solution for 2 minutes.

For each section, the number of ED1-positive nuclei falling within five randomly selected high-power fields was recorded as follows. A high power lens was positioned manually over the centre of each section, and the stage was then moved to the left (even number) or right (odd number) and up (even number) or down (odd number) by an increment corresponding to the third figure of a random number generated by a scientific calculator (Sharp EL 556G). Random number generation and repositioning of the section were continued until 5 high power fields had been obtained from each section.
Statistics

Whole body and kidney weights, serum biochemistries and glomerular filtration rates are expressed as means ± SEM. Serial urine chemistries were analysed by analysis of variance (ANOVA), and compared at each time point with simultaneous control values by unpaired 't' test. Histological scores are expressed as medians with interquartile range and were analysed by Wilcoxon rank sum test. P values of less than 0.05 were considered significant.
Results

Animal protocols.

Somatic growth was significantly slowed by dietary acid (Fig 3.1), and at the end of the study the mean body weight of acid-supplemented animals was 251 ± 4g compared with 313 ± 10g in controls (p < 0.001). Despite a pair-feeding protocol, overall mean paste consumption was slightly higher in the acidotic group than in controls (41.0 ± 0.4g/day vs 38.4 ± 0.5g/day, p < 0.01) since acidotic animals often ate all the food provided during the morning and required supplements to avoid food deprivation at night. Acidotic animals received a mean daily acid load of 10.3 mmol. As in the short term study, urine output was greater in acidotic rats than controls (30 ± 2 vs 19 ± 2 mls/24 hrs at 14 weeks; p < 0.001). Urine pH was at least 0.5 lower in the acidotic group in every urine collection (Figure 3.2), progressively falling from 5.67 ± 0.04 at week 2 to 5.47 ± 0.03 at week 14 (p < 0.001). There was no significant change in the pH of control urine.

In the fluid restriction study, measurements taken before restriction showed a significantly higher urine output (8.1 ± 0.5 vs 5.1 ± 0.7mls/6hrs, p < 0.05) and a lower urinary osmolality (758 ± 19 vs 1109 ± 127 mOsm/Kg, NS) in acidotic animals than controls (Figures 3.3 and 3.4). By 6 hours, urine output in the acidotic group had almost halved, and subsequently fell further such that by 24 hours output was 3.2 ± 0.2mls/hr compared with 2.8 ± 0.2mls/hr in controls (NS). Urine osmolality also responded rapidly to fluid restriction, rising to 1414mOsm/Kg at 6 hrs, and reaching a maximum of 2480 ± 95mOsm/Kg at 24 hours, compared with 2940 ± 130mOsm/Kg in controls.
Figure 3.1 Serial body weights in acidotic and control groups during prolonged acidosis. Values expressed as mean ± SEM.
+ p < 0.05, * p < 0.005
Figure 3.2  Sequential urine pH during prolonged acidosis. Values expressed as means ± SEM. Differences in pH are significant (p < 0.001) from weeks 2 - 4 inclusive.
Figure 3.3 Urine output during fluid restriction in acidotic and control groups. Values expressed as mean ± SEM.  * p < 0.05
Figure 3.4 Urine osmolality during fluid restriction in acidotic and control groups. Values expressed as mean ± SEM.
(NS). These findings demonstrate that acid supplementation did not impair urinary concentrating capacity.

As in the short term study, acidotic animals developed proteinuria. Urine total protein excretion progressively increased from baseline to week 8 before returning to levels no greater than control by week 14 (Figure 3.5). Excretion of lysozyme and albumin followed a similar pattern. Urinary lysozyme increased from undetectable levels at baseline to a peak of $176 \pm 36 \mu g$/day at week 8 ($p < 0.001$) before again becoming undetectable at week 12 (Figure 3.6). Urinary albumin also peaked at week 8, when its excretion was 4.5 times greater than in controls ($p < 0.001$), but by week 12 excretion had returned to baseline. Acidosis caused only a slight increase in the excretion of IgG, such that at 8 weeks excretion in acidotic animals exceeded that in controls by a factor of 1.7 ($p < 0.05$).

The effect of polyuria on protein excretion was investigated by supplementing the drinking water of control rats with 5% glucose, thereby stimulating fluid intake and increasing urine output. After two weeks of acid feeding at the beginning of the study, excretion of protein and lysozyme in the acidotic group was significantly greater than in controls ($18.8 \pm 2.2$ vs $5.7 \pm 0.7$ mg/24hrs, $p < 0.001$; and $330.8 \pm 113.9$ vs $33.7 \pm 9.4$ $\mu g$/24hrs, $p < 0.02$ respectively). Supplementation of controls’ water with glucose caused an immediate increase in water intake and urine output, and within 5 days there was no significant difference between outputs of the two groups ($33 \pm 3$mls/24hrs in acidotic animals vs $37 \pm 3$mls/24hrs in controls). The induced polyuria did not, however, increase protein excretion and the output of both total protein ($4.8 \pm 0.8$mg/24hrs) and lysozyme ($19.5 \pm 7.0$ $\mu g$/24hrs) showed small reductions compared with the values recorded before glucose
Figure 3.5 Total urinary protein excretion / 24 hrs in acidotic and control animals. Values expressed as mean ± SEM. 
+ p < 0.01, * p < 0.001
Figure 3.6  Total lysozyme excretion in acidotic and control groups during prolonged acidosis. Values expressed as mean ± SEM.
+ p < 0.05, * p < 0.01
Figure 3.7 Urinary protein excretion pre- and post standardisation of urine output $+ p < 0.001$, $^* p < 0.0001$
supplementation (Figure 3.7). Increased protein excretion in the acidotic animals cannot therefore be attributed to acid-induced polyuria.

At sacrifice, mean arterial pH was lower in the acid-supplemented group (7.35 ± 0.02 vs 7.41 ± 0.01; p < 0.05). There was no significant difference in serum creatinine between acidotic and control rats (58 ± 4 vs 60 ± 4μmol/l), and glomerular filtration rate, measured in 10 animals from each group, was 2.2 ± 0.2mls/min in acidotic vs 2.4 ± 0.1mls/min in controls (NS). Kidney weights were very similar in both groups, but kidney/body weight ratios were greater in acidotic animals (Table 3.1). In contrast, heart weights, recorded as an index of growth of organs other than the kidney, were lower in acidotic animals, such that kidney/heart weight ratios were higher in the acidotic group (Table 3.1).

Histological and Immunohistochemical analyses

Histological analysis identified no significant abnormalities in kidneys from acidotic or control animals. Light microscopy of H & E sections was normal, and the density of both tubular and non-tubular cells was the same in acidotic and control groups (Figure 3.8). Electronmicrographs from animals killed at eight weeks showed no evidence of foot process effacement (Figure 3.9). Basement membranes stained well for anionic binding sites (example in Figure 3.10) and the mean density of binding sites was 48.0 (interquartile range 44.0-51.0)/10cms in the experimental group compared with 44.0 (interquartile range 41.8-47.3)/10cms in controls (NS). ED1 staining of Carnoy's fixed sections (example in Figure 3.11) demonstrated no significant difference in macrophage density between acidotic and control groups (median 5 vs 8 positive cells/ 5 high power fields).
Table 3.1 Whole body and organ weights at sacrifice after prolonged acidosis
Values expressed as mean ± SE

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Acidotic</th>
<th>'p' value</th>
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<tbody>
<tr>
<td>Body wt (g)</td>
<td>313 ± 10</td>
<td>251 ± 4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Kidney wt (g)</td>
<td>0.91 ± 0.02</td>
<td>0.94 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Heart wt (g)</td>
<td>0.93 ± 0.03</td>
<td>0.87 ± 0.01</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Kidney/body wt (g%)</td>
<td>0.29 ± 0.01</td>
<td>0.36 ± 0.01</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Kidney/heart wt (g/g)</td>
<td>0.99 ± 0.03</td>
<td>1.08 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Figure 3.8 Nuclear density of tubular non-tubular cells in sections from acidotic and control animals. Values expressed as median with interquartile range.
Figure 3.9

Electron micrograph of basement membrane from kidney of acidotic rat after perfusion with polyethyleneimmine (magnification x40,000).

The epithelial foot processes are normal with no evidence of fusion.
Figure 3.10

Electron micrograph from PEI - perfused kidney showing staining of anionic binding sites along the basement membrane (magnification x40,000)
Figure 3.11

Sections from acidotic (a) and control (b) kidneys stained immunohistochemically for microphages using ED1 antibody
Summary of Findings of LA Study

1. Prolonged acidosis slowed somatic growth, but kidney weights at sacrifice (14 weeks) were similar in acidotic and control animals, such that kidney/body weight ratios were significantly greater in the acidotic group.

2. As in the short term study, acidotic animals developed proteinuria in a pattern consistent with a predominantly tubular origin. The excretion of protein, lysozyme and albumin all increased after acid supplementation, but this increase was not sustained, and after peaking at eight weeks, excretion of all protein components fell to levels no greater than control by week 14. These findings suggest that prolonged acidosis did not cause sustained glomerular or tubulointerstitial injury.

3. Neither serum creatinine nor isotope GFR were significantly influenced by prolonged acidosis.

4. Acidotic animals developed polyuria, but their urine output and osmolality both normalised after 24 hours of fluid restriction. Control animals made polyuric by stimulating their fluid intake showed no significant increase in urinary protein excretion. These observations demonstrate that acidosis does not impair urinary concentrating capacity, and that the proteinuria seen in acid-supplemented animals was not merely a consequence of polyuria.

5. Kidneys from acidotic animals were normal on light microscopy, and electron microscopy showed neither effacement of glomerular epithelial cell foot processes nor a reduction in the density of anionic charges on the
glomerular basement membrane. There was no significant difference in macrophage density between acidotic and control groups. The histological appearances were therefore consistent with the proteinuria data, and demonstrate that prolonged acidosis did not cause sustained glomerular or tubulointerstitial inflammation or injury. Although kidney/body weight ratios were higher in acidotic animals than controls, the density of both tubular and non-tubular cell nuclei was similar in both groups. The normal cell nuclear densities in the acidotic kidneys contrast with lower tubular densities seen after short term acid exposure (Chapter 2) suggesting that renal growth in acidosis may comprise an early hypertrophic phase followed by later hyperplasia.
Chapter 4

The effects of alkalinisation on the progression of chronic renal disease in the remnant kidney model: The RP Study.
Introduction

As discussed in Chapter 1, renal growth and proteinuria are early features of several experimental models of chronic renal disease. Although short periods of metabolic acidosis cause proteinuria and renal growth, data from Chapter 3 demonstrate that when acidosis is prolonged, urinary protein excretion returns to baseline, and neither glomerular nor tubular injury occur. Whilst this demonstrates that isolated acidosis does not cause progressive injury in previously normal kidneys, it does not exclude a role for uraemic acidosis in the progression of established chronic renal disease. Since uraemic animals develop MA spontaneously, the effects of this acidosis on the progression of renal disease can be investigated by comparing the outcome of uraemic animals fed a normal diet with that of a second group whose acidosis is abolished by dietary bicarbonate supplements. Experiments described in this chapter examine the effect of dietary bicarbonate supplements on GFR, blood pressure, histological injury and survival in the remnant model of chronic renal failure in the rat.

Reduction of kidney mass causes increased blood flow, increased intraglomerular pressure and hyperfiltration, proteinuria and interstitial fibrosis in the residual viable renal tissue [148]. The remnant kidney (5/6ths nephrectomy) model is created by uninephrectomy and reduction of the contralateral kidney to 1/3rd of its original size by pole resection, ligation of branches of the renal arteries or ligation of the poles of the kidney. Animals whose renal mass is reduced in this way develop proteinuria, hypertension and a progressive deterioration in renal function culminating in the development of end stage renal failure. In the branch ligation model, in which branches of the renal artery are selectively ligated to infarct approximately 2/3rds of the kidney, hypertension develops in the second post-operative
week and is thought to be central to the subsequent development of progressive renal impairment [149]. Changes in renal function in this model can be divided into three phases: an early period of deterioration due to postoperative acute renal failure, a second period of stable (but impaired) renal function, and a final stage of progressive and eventually terminal renal failure [148]. Plasma urea rises during the first week after 5/6ths nephrectomy, and this increase is usually accompanied by histological evidence of acute renal failure. Subsequently urea and creatinine fall (though do not normalise) due to partial recovery from perioperative acute renal failure and the development of hypertrophy in the remaining viable tissue. In the branch ligation model these processes are complete by the tenth postoperative week and are accompanied by the development of hypertension. The acute phase is followed by a period of relatively stable renal function of variable duration, during which proteinuria increases and blood pressure remains high. Finally, in the third phase, proteinuria and creatinine increase further, culminating in the development of end stage renal failure and death.

Histologically, the remnant kidney is characterised by glomerular hypertrophy, mesangial hypercellularity and matrix expansion, fusion of epithelial foot processes and obliteration of capillary lumens leading to focal and segmental glomerulosclerosis [150]. Tubular dilatation and cast formation (thought to be due to increases in single nephron GFR and proteinuria [148]) are followed by tubular atrophy and interstitial fibrosis.

As previously discussed (see Chapter 1) Nath et al showed in 1985 that the abolition of uraemic acidosis by bicarbonate supplementation reduces urinary total and low molecular weight protein excretion, preserves tubular function and abrogates histological injury in remnant rats [115]. Their paper can,
however, be criticised on a number of grounds. Firstly, since animals were sacrificed after only six weeks, it is questionable whether the findings of this study are relevant to animals or patients with stable chronic renal impairment. As the acute phase of the remnant model is characterised by deterioration and subsequent recovery from perioperative acute renal impairment, Gretz et al (1993) have argued that "During this period of time (ie until the tenth postoperative week) no experiments intended to analyse chronic renal failure should be started" [148]. Secondly, since GFR was the same in alkalinised remnants and controls at the time of sacrifice, it was not established whether the reported differences in proteinuria, tubular function and histological injury would translate into different rates of deterioration of excretory renal function over more prolonged periods. Thirdly, although histological injury including interstitial fibrosis and glomerulosclerosis was assessed by a scoring system, no quantitative comparison of renal fibrosis (ie total renal collagen deposition) was made between the two groups; and finally, no blood pressure measurements were made. Since hypertension is fundamental to the development of renal injury in the remnant kidney, serial blood pressure recordings could provide important information about the relative likelihood of disease progression in the two groups.

To address these concerns, the experiments described in this Chapter examined the effect of prolonged alkalinisation on proteinuria, blood pressure, histological injury, total kidney collagen content, GFR and time to the development of terminal uraemia in the remnant model of chronic renal failure in the rat.
Materials and Methods

1. Surgical procedure

Animals were starved overnight prior to surgery, and anaesthetised using 2.7mls/kg body weight of a solution of midazolam (12.5mg/10mls) and fentanyl (0.8mg/10mls) administered intraperitoneally 30 minutes before the procedure.

After shaving, separate midline abdominal incisions were made in the skin and muscle layers. The small bowel was gently moved to the right side of the abdominal cavity and packed with gauze soaked in sterile saline. A second gauze swab was placed above the left kidney to keep the spleen and omentum out of the operative field. The branches of the left renal artery were identified in the angle between the superior border of the left renal vein and the medial border of the upper pole of the kidney. After freeing from the surrounding fat by blunt dissection, each branch was occluded in turn using blunt forceps. By examining the proportion of the kidney becoming dusky after occlusion of each branch, a combination of branches whose ligation would infarct approximately \( \frac{2}{3} \)rds of the kidney was identified. If this was not possible using branches superior to the renal vein, the angle between the lower border of the renal vein and the medial border of lower pole of the kidney was explored. Branches selected for ligation were then dissected free from the surrounding tissues using blunt forceps, and ligated with 5/0 silk suture. The left and right abdominal packs were removed.

To allow the right nephrectomy, the small bowel was then moved to the left side of the abdominal cavity. A small incision was made in the capsule of
the right kidney and enlarged by blunt dissection to allow delivery of the kidney through the resulting defect. The right renal artery and vein were then clamped with two pairs of Spencer-Wells forceps and ligated with 3/0 silk between the two clamps. The small bowel was returned to the midline and the abdominal muscle layer was sutured with a continuous 3/0 silk stitch. The skin was closed with an interrupted 3/0 silk suture, burying the knots within the wound to prevent damage from biting after recovery. The skin was cleaned with saline swabs, and animals were wrapped in a towel and returned to their cages for recovery.

2. Experimental protocol

24 female Wistar rats (initial weight = 160g) underwent right nephrectomy and ligation of two left renal artery branches as described above. Postoperatively, they had free access to water and were fed 20% casein feed (ICN 960259), offered as a paste mixed with water (1:1 w/w) and methylcellulose (2g/100g). After two weeks, all animals were placed in metabolic cages for collection of 24 hour urine specimens, and blood samples were subsequently withdrawn from a tail vein under light ether anaesthesia. Animals were ranked separately for urea, creatinine and 24 hour urinary protein excretion, and a final overall ranking for severity of renal injury was derived by averaging the ranks for the separate parameters. The rats were then divided into two groups by allocating them alternately to group 1 and group 2 in rank order. Group 1 (alkalinised remnants) were fed a sodium-deficient diet (ICN 960231) offered as a paste mixed with water (1:1 w/w) and methylcellulose (2g/100g) and supplemented with 1.9g NaHCO₃/100g. Group 2 (control remnants) were pair-fed the same diet which to ensure equal sodium intake was supplemented with 1.3g NaCl/100g. Food consumption
was recorded daily, body weights were measured weekly, and at fortnightly intervals animals were placed in metabolic cages to quantify 24 hour urinary protein excretion. After 1, 2 & 3 months, systolic blood pressure was recorded using a Harvard tail cuff sphygmomanometer. Animals were placed in a restraining tube in a darkened room maintained at 30°C, and left for 15 minutes to acclimatise. Three consecutive blood pressure recordings were then made and the mean systolic blood pressure was recorded in each case. At 6 & 12 weeks, glomerular filtration rate (GFR) was measured using the method of Nankivell et al (1992) described in Chapter 3.

Animals were individually examined on a daily basis, and were sacrificed by exsanguination under anaesthesia (2.7ml/Kg of fentanyl/midazolam/water given intraperitoneally) when they developed terminal uraemia, characterised by lethargy, anorexia, and failure to gain weight. Arterial blood was collected in a heparinised, air-free syringe for blood gas determination, and serum was analysed for urea and creatinine. Whole body and kidney weights were recorded, and kidneys were then sectioned coronally. The first half of each kidney was fixed in formol saline for histological examination, and the second half was assayed for collagen as described below.

3. Analyses of blood and urine

Arterial blood gases, urea, creatinine and urine total protein were analysed as previously described.
4. Light microscopy

Histological assessment was carried out on 3μM Kidney sections stained with haematoxylin and eosin. Each was graded 0 - 5 for severity of injury based on a blinded assessment of glomerulosclerosis, tubular dilatation, cast formation and interstitial fibrosis (Appendix G). In addition, cast formation was quantified by recording the number of casts contained within 500 tubules, starting counting in the outer cortex of the midpole and moving in a corticomedullary direction.

5. Calculation of total kidney collagen content

Since hydroxyproline is found exclusively within collagen in all biological systems, and the ratio of hydroxyproline to collagen is virtually constant, assays of hydroxyproline within homogenised and hydrolysed tissue can be used to calculate tissue collagen content and concentration [151]. Total kidney collagen content in animals from this study was determined using a hydroxyproline assay as follows. The portion of each kidney retained for collagen analysis was weighed and cut into small (approximately 1mm³) pieces before being reduced to a pulp in a ground glass organ grinder. The resulting tissue was hydrolysed in 6N HCl at 110° for 16 hours, evaporated to dryness, suspended in deionised water, lyophilised, then resuspended in 2mls of buffer. The hydroxyproline concentration of this solution was determined by the method of Stegemann & Stalder [152] as follows. 1 ml of the sample was mixed with 0.5mls of a 0.05 M solution of Chloramine T, then left at room temperature for 20 minutes to oxidise. After oxidation, 1ml of a 1.2 M solution of Ehrlichs aldehyde reagent (dimethylaminobenzaldehyde) in propan-1-ol (70%) and perchloric acid (30%) was added, and the resulting
solution was incubated at 60° for 15 minutes. After cooling, absorbance of the solution was determined at 550nm using a Cecil CE 2040 spectrophotometer, and hydroxyproline concentration was then calculated by reference to a standard curve. Collagen content of the hydrolysate was estimated by multiplying its hydroxyproline content by 7.42 \[151\], and total kidney collagen was then derived in each case from the tissue collagen concentration and original kidney weight.

Statistics

Values are expressed as means ± SEM. Urinary indices at each time point, data at killing and the numbers of casts per 500 tubules were compared by unpaired t test. Histological scores were analysed by Wilcoxon rank sum test. P values of less than 0.05 were considered significant.
Results

Experimental protocol.

The division of the rats into two comparable groups on the basis of postoperative ranking for serum and urine biochemistry ensured similar initial values of serum creatinine (94.4 ± 6.3 vs 93.7 ± 6.8 µmol/l, NS), urea (21.6 ± 2.1 vs 22.2 ± 2.4 mmol/l, NS) and 24 hour urinary protein excretion (26.5 ± 11.3 vs 31.7 ± 8.3 mg/24hrs, NS) in control and bicarbonate supplemented animals. Two animals died within 2 weeks of surgery and were therefore excluded from the analysis. Food intake was similar in both groups (overall means 33.4 ± 0.7 vs 32.7 ± 0.5 g/day, NS), and mean bicarbonate intake in the alkali-supplemented group was 3.7 mmol/day.

Systolic blood pressure rose to a similar extent in both groups (Figure 4.1) and glomerular filtration rates were 0.77 ± 0.10 vs 0.64 ± 0.10 mls/min at 1 month, and 0.62 ± 0.12 vs 0.66 ± 0.10 at 2 months (both NS). There was no statistically significant improvement in survival in the bicarbonate-treated group, and the difference in average time from surgery to death between the two groups was less than one week (Table 4.1). Because of variable sensitivity to general anaesthesia, which resulted in a range of values for pCO₂ in arterial blood at the time of sacrifice, terminal arterial pH is expressed both as 'raw pH', and as 'corrected pH', adjusted using the Henderson-Hasselbalch equation to represent the pH value at a standard pCO₂ of 5.6. Using both these indices, pH was markedly higher in bicarbonate-supplemented animals than controls (Table 4.1) Alkalinised animals had a negligible base deficit, demonstrating that bicarbonate-supplementation had adequately abolished uraemic acidosis. Serum urea and creatinine were both lower in controls than
Figure 4.1. Systolic blood pressure in alkalinised remnants and controls.

Values expressed as means ± SEM
Table 4.1  Data from bicarbonate-treated and control remnants at time of sacrifice.  
Values represent mean ± SE

<table>
<thead>
<tr>
<th></th>
<th>Bicarbonate-treated remnants</th>
<th>Control remnants</th>
<th>'p' value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Survival (days)</strong></td>
<td>119 ± 17</td>
<td>113 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Arterial blood pH</strong></td>
<td>7.27 ± 0.06</td>
<td>6.95 ± 0.04</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Corrected arterial pH</strong></td>
<td>7.39 ± 0.06</td>
<td>6.87 ± 0.04</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Blood Base excess</strong></td>
<td>-0.7 ± 3.4</td>
<td>-25.4 ± 1.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Plasma urea (mmol/l)</strong></td>
<td>100 ± 11</td>
<td>63 ± 9</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>Plasma Creatinine (μmol/l)</strong></td>
<td>374 ± 23</td>
<td>287 ± 45</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Kidney collagen content (mg/kidney)</strong></td>
<td>67 ± 11</td>
<td>66 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td><strong>No of casts / 500 tubules in each kidney</strong></td>
<td>138 ± 26</td>
<td>142 ± 37</td>
<td>NS</td>
</tr>
</tbody>
</table>
in treated animals, and for urea this difference achieved statistical significance (Table 4.1). Final body weights were the same in both groups (184 ± 9 vs 184 ± 13g, NS), but kidney weights (2.79 ± 0.2 vs 2.21 ± 0.14g, p = 0.053) and kidney/body weight ratios (1.5 ± 0.1 vs 1.2 ± 0.1g%, p < 0.05) were higher in controls than in the bicarbonate supplemented group.

**Blood and urine analyses**

In every 24 hour urine collection from the fourth week onwards, urine pH was significantly higher in the bicarbonate-supplemented group than in controls (Figure 4.2). All animals developed proteinuria, which progressively increased from the time of surgery until a pre-terminal decline approximately 2 weeks before death. Because of this pattern of proteinuria, the peak and overall mean protein excretion was recorded for each animal in addition to calculations of group means from each fortnightly 24 hour urine collection. There was no significant difference in 24 hour protein excretion between the two groups at any time (Figure 4.3), and peak proteinuria (222 ± 23 vs 199 ± 16mg/24 hrs, NS) and overall mean proteinuria (93 ± 8 vs 100 ± 8 mg/24 hours, NS) were also similar.

**Light microscopy**

All kidney sections showed advanced chronic injury, characterised by tubular dilatation, cast deposition, glomerulosclerosis and interstitial fibrosis (Figure 4.4). The severity of injury was the same in both groups, and with the single exception of one control kidney, which scored grade 3, all sections scored grade 5 on the 0 - 5 histological injury scale. There was no statistical
Figure 4.2. Urine pH in alkalinised remnants and controls. Values expressed as means ± SEM.
+ p < 0.005, * p < 0.001
Figure 4.3 Urinary protein excretion (mg/24 hrs) in alkalinised remnants and controls. Values expressed as mean ± SEM.
Figure 4.4

Examples of kidney sections from control (a and b) and alkalinised (c and d: overleaf) remnants stained with H&E (magnification x400). All sections show tubular dilation, thyroidisation, glomerulosclerosis, periglomerular fibrosis and interstitial fibrosis.
difference in the number of casts per 500 tubules in control and bicarbonate-supplemented kidneys (Table 4.1).

Total kidney collagen content

Calibration curves showed good linear correlation between standard hydroxyproline concentrations between 0 and 12.5μg/ml and optical density at 550 nm (example shown in Appendix H). Despite the differences in kidney weights between alkalinised remnants and controls, there was no significant difference in total kidney collagen content between the two groups (Table 4.1).
Summary of Findings of RP Study

1. The quantity of bicarbonate administered to alkalinised remnants was sufficient to ensure abolition of uraemic acidosis at the time of death.

2. All animals became hypertensive, but blood pressure rose to a similar extent in alkalinised and control groups. GFR was similar in both groups at 1 and 2 months. Urea and creatinine were lower in the alkalinised group at sacrifice, and for urea the difference was statistically significant.

3. Urinary protein excretion progressively increased from the time of surgery until approximately two weeks before death. There was, however, no significant difference in proteinuria between alkalinised and control groups.

4. Bicarbonate supplementation did not delay the development of terminal uraemia.

5. Total kidney collagen content and histological injury scores were similar in both groups, but kidney weights and kidney/body weight ratios were statistically lower in alkalinised remnants than controls.

Whilst these findings suggest that the correction of acidosis may reduce renal growth in experimental renal disease, they contrast with the findings of Nath et al (1985) by showing no benefit of alkalinisation on proteinuria, histological injury, collagen deposition or rate of decline of GFR in the remnant model of chronic renal failure.
Chapter 5

The effects of acid supplementation and alkalinisation on the progression of chronic renal disease in the uninephrectomised rat: The NA Study.
Introduction

The experiments reported in Chapters 3 and 4 demonstrate that prolonged metabolic acidosis does not induce renal injury in normal kidneys, and that abolition of uraemic acidosis after 5/6ths nephrectomy does not influence the progression of experimental chronic renal failure. Since the models used represent extremes of the spectrum between normality and severe renal injury, the absence of an effect of pH modification does not exclude a role for acidosis in the progression of renal disease in humans. Deleterious effects of acidosis may be insufficient to cause injury in completely normal kidneys, whilst the severity of injury in the remnant kidney may be too advanced for abolition of acidosis to influence further disease progression. To confidently exclude a contribution of acidosis to the progression of CRF, the effect of alterations in pH in mild and slowly progressive renal disease must also be examined.

Ageing rats develop spontaneous proteinuria due to focal segmental glomerular hyalinosis and sclerosis (FSGHS) which affects both cortical and juxtamedullary glomeruli [153]. A similar lesion appears in the remaining kidney after uninephrectomy (a procedure which also exacerbates FSGHS of ageing [154]) and has been attributed to increased hydrostatic pressure in the afferent arteriole accompanied by lipid deposition in the glomerular mesangium [153]. Since proteinuria and renal impairment progress slowly after uninephrectomy (in contrast to more rapid rates of progression seen in the remnant model) the uninephrectomised rat is an appropriate model to study the effects of prolonged alterations in pH on renal injury. In addition to the slow progression of renal injury, the uninephrectomy model has two further advantages over the remnant kidney in evaluating any influence of pH on progression. Firstly, in contrast to the remnant, in which the severity of
the lesion is inevitably variable, it guarantees a uniform reduction in renal mass in all animals. Secondly, it allows dietary acid supplements to be administered to evaluate any effects of superimposed acidosis on underlying renal injury. In the remnant, such supplementation is not possible since the combination of uraemic and experimental acidosis would be rapidly life-threatening.

The aim of the experiments described in this chapter was to further examine the effects of acidosis on the progression of renal injury by evaluating the effect of dietary acid and bicarbonate supplements on total and low molecular weight proteinuria, renal excretory function and renal morphology in the uninephrectomised rat.
Materials and Methods

1. Surgical procedure

Animals were starved overnight prior to surgery, and anaesthetised using a solution of midazolam and fentanyl administered intraperitoneally 30 minutes before the procedure as detailed in Chapter 4.

A 1.5 cm incision was made in the left flank, approximately 0.75 cms below the costal margin, parallel to the lower ribs and with its most lateral end inferior to the curve of the lower ribs as they rise to meet the vertebrae. The subcutaneous fat beneath the costal margin was then lifted away from the underlying muscle to raise an ellipse of fat parallel to the long axis of the incision. This was resected using scissors to reveal the underlying muscle. The fold of muscle immediately beneath the skin incision was raised with forceps, with the fold of tissue at right angles to the long axis of the incision. A small cut was made in the muscle layer with scissors, and extended to produce a muscle incision immediately beneath, but shorter than, the skin wound.

The left kidney was located by palpation between fingers and thumb of the left hand and gently delivered through the incision. If the left ovary and fallopian tube came through the incision with the kidney, they were moved away from the kidney and covered with a gauze swab before the nephrectomy was performed. The kidney was freed from the renal capsule (and adrenal gland) by breaking the capsule between finger and thumb and squeezing the kidney out through the resulting hole. The renal vessels and ureter were clamped with Spencer-Wells forceps, and tied on the aortic side with 3-0 Mersilk thread. The kidney was removed by running the blade of a
scalpel along the kidney side of the forceps. The forceps were then slowly released, and completely removed only when it was clear that there was no bleeding from the vascular stump. If bleeding was seen, the forceps were reapplied and a second ligature tied around the vascular stump. After haemostasis was confirmed, the suture was cut short.

The vascular stump, fat and if appropriate ovaries were returned to the abdominal cavity by elevating the edges of the muscle layer and allowing them to fall back into the wound. If necessary, this was encouraged by wetting the fat with normal saline. The muscle layer was repaired using a continuous suture, and the skin was closed with two or three interrupted sutures (all 3-0 Mersilk), burying the knots within the wound to prevent damage from biting after recovery. The skin was cleaned with saline swabs, and animals were wrapped in a towel and returned to their cages for recovery.

2. Experimental protocol

40 female Wistar rats (initial weight approximately 185g) housed in individual cages with a 12 hour light/dark cycle, constant temperature and humidity and free access to water, were fed a powdered diet (ICN 960259) containing 20% casein (w/w) offered as a paste made up with water (1:1 w/w) and methylcellulose (2g/100g). After a 2 week induction period all animals underwent left nephrectomy as described above. Postoperatively, they were divided into three groups, which were pair-fed the following diets. Group 1 ('controls') continued to receive the standard diet made up with water; Group 2 ('acidotic') received the same diet made up 0.4M HCl instead of water; and Group 3 ('alkalotic') received a sodium-deficient diet (ICN...
960231) supplemented with 0.5g/100g NaHCO₃, which provided the same sodium load/g as the other two diets. Animals were weighed weekly, and at fortnightly intervals were placed in metabolic cages for the collection of 24 hour urine specimens. After 6 months, a final 24 hour urine collection was made, and creatinine clearance was calculated from this using the final serum creatinine. Glomerular filtration rate (GFR) was measured using the method of Nankivell et al (1992) described in Chapter 3, and all animals were then sacrificed by exsanguination under general anaesthesia. Arterial blood was collected in an air-free syringe for blood gas estimation, and venous blood was sampled for estimation of creatinine and electrolytes. Whole body and kidney weights were recorded, and kidneys were removed, bisected and preserved in 10% formol saline for histological analysis.

3. Analyses of blood and urine

Arterial blood gases, serum urea and creatinine and urine total protein and lysozyme were analysed as previously described.

4. Light microscopy

Histological assessment was carried out on 3μM Kidney sections stained with haematoxylin and eosin. Sections from each kidney were examined individually and morphological abnormalities recorded in each case.
Results

Data during study period

Pair feeding ensured similar food consumption in all 3 groups (gp 1: 35.7 ± 0.6g/day; gp 2: 37.1 ± 0.5g/day; gp 3: 35.9 ± 0.4g/day; NS) but acidotic animals gained significantly less weight than alkalotic animals or controls (Table 5.1). Mean HCl load in gp 2 was 7.4 mmol/day, and mean NaHCO₃ load in gp 3 was 1 mmol/day. From the second postoperative week onwards, urine pH was higher in gp 3, and lower in gp 2 than in gp 1 at every time point except week 10 (Figure 5.1). Acidotic animals developed an early and transient rise in total protein excretion such that proteinuria was significantly greater in Group 2 than in Groups 1 and 3 from weeks 2 - 10 (Figure 5.2). Thereafter, protein excretion in all groups rose in parallel to a final overall mean of 20.3 ± 3.7 mg/24 hours. The excretion of lysozyme also rose after acid supplementation, peaking at week 2, and exceeding excretion in control and alkalotic groups at all time points thereafter (Figure 5.3). It is noteworthy that the excretion of lysozyme in bicarbonate-supplemented animals and controls did not parallel total protein excretion, and remained very low until the end of the experiment (Figure 5.3).

Data at sacrifice

Body weights of animals in Group 2 were lower, and kidney weights and kidney/body weight ratios were higher, than those in the other two groups (Table 5.1). Both raw and corrected arterial pH were lower in Group 2 than in the other two groups, though this difference did not achieve statistical
Table 5.1. Data from control, acid- and bicarbonate- supplemented groups at sacrifice
Values expressed as mean ± SEM. Statistical comparison by ANOVA

<table>
<thead>
<tr>
<th></th>
<th>Control (Gp 1)</th>
<th>Acidotic (Gp 2)</th>
<th>Alkalotic (Gp 3)</th>
<th>'p' value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>322 ± 8</td>
<td>265 ± 5</td>
<td>326 ± 8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.39 ± 0.08</td>
<td>1.55 ± 0.04</td>
<td>1.31 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Kidney/body wt (g%)</td>
<td>0.43 ± 0.02</td>
<td>0.59 ± 0.02</td>
<td>0.40 ± 0.01</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>'Raw' pH</td>
<td>7.39 ± 0.02</td>
<td>7.35 ± 0.02</td>
<td>7.36 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Corrected pH</td>
<td>7.40 ± 0.01</td>
<td>7.37 ± 0.01</td>
<td>7.40 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Base excess</td>
<td>4.11 ± 0.41</td>
<td>2.07 ± 0.81</td>
<td>4.23 ± 0.42</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>57 ± 5</td>
<td>56 ± 3</td>
<td>57 ± 1</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 5.1. Sequential urine pH in control, acidotic and alkalotic rats following uninephrectomy. Values represent mean ± SE. + p < 0.05; ‡ p < 0.01; * p < 0.001.
Figure 5.2  24-hr urinary protein excretion in control, acidotic and alkalotic rats following uninephrectomy. Values represent means (error bars omitted for clarity). + p < 0.05; * p < 0.001 by ANOVA
Figure 5.3 24-hr urinary lysozyme excretion in control, acidotic and alkalotic rats following uninephrectomy. Values represent means ± SE $+ p < 0.05$; $* p < 0.001$; $\dagger p < 0.0005$ by ANOVA.
significance. Because of hypercapnia in several animals in Group 3, raw pH was lower in this group than in controls, but after correction for pCO₂, pH was 7.4 as in the controls. The only statistical difference in blood gas indices related to base excess, which was significantly lower in Group 2 than the other two groups. Serum creatinine was similar in all 3 groups (Table 5.1), and whilst creatinine clearance and GFR, both raw and corrected for body weight, were lower in the acidotic group than in alkalinised animals and controls, this difference did not achieve statistical significance (Figures 5.4 and 5.5).

**Light microscopy**

The majority of kidney sections showed no generalised abnormalities, though many contained occasional tubular casts consistent with the degree of proteinuria. Scoring was not possible as these changes were so mild and localised. Sections from 2 control, 3 acidotic and 2 alkali-supplemented kidneys showed significant diffuse abnormalities comprising tubular dilatation, lymphocytic infiltration, cast deposition and diffuse interstitial fibrosis.
Figure 5.4 Creatinine clearance (left panel) and creatinine clearance /100g body weight (right panel) at sacrifice in uninephrectomised rats. Values represent means ± standard error: differences not significant by analysis of variance in both panels.
Figure 5.5  GFR (left panel) and GFR / 100g body weight (right panel) at sacrifice in uninephrectomised rats. Values represent means ± standard error: differences not significant by analysis of variance in both panels.
Summary of Findings of NA Study

1. Animals in all three groups developed progressive proteinuria beginning in the eighth postoperative week. In addition, the acid supplemented group showed an early transient rise in total protein excretion which peaked on week 2 before returning to control/bicarbonate-supplemented levels by week 12. Lysozyme excretion mirrored total protein excretion in the acid-supplemented group, but showed no increase in control or bicarbonate-supplemented animals at any stage.

2. Acid supplementation slowed somatic growth and accelerated renal growth, but somatic and renal growth was similar in bicarbonate-supplemented animals and controls.

3. Creatinine clearances and GFR, both raw and corrected for body weight, were similar in all three groups.

4. Minor histological abnormalities were seen in all kidneys at sacrifice, but their severity was similar in all three groups.

These findings are therefore similar to those seen in normal rats given acid supplements. Although there was an early increase in total and low molecular weight protein excretion in acidotic animals, this increase was neither sustained nor followed by accelerated disease progression when compared with bicarbonate-supplemented animals or controls. Conversely, bicarbonate supplementation did not reduce the late rise in protein excretion, nor influence GFR or histological injury in this model.
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Overview

The studies described in Chapters 2 - 5 provide new information about the effects of changes in systemic pH on renal structure and function in both normal rats and those with varying degrees of experimental renal injury. In the normal Wistar rat, dietary acid supplements caused renal growth and self-limiting tubular proteinuria, but these did not proceed either to tubulointerstitial or glomerular injury, or to impairment of renal excretory function, even when the duration of acidosis exceeded three months. In the remnant model of chronic renal injury, which is characterised by the development of hypertension, progressive renal impairment and uraemic acidosis, correction of acidosis by dietary bicarbonate did not reduce proteinuria, slow the fall in GFR, influence blood pressure, abrogate histological injury, reduce renal collagen deposition or improve survival. In the uninephrectomised rat, a model of slowly progressive renal impairment in which the residual kidney spontaneously develops focal segmental glomerular hyalinosis and sclerosis, the development of renal injury and impaired excretory function were not influenced by dietary acid or bicarbonate supplements, though MA again caused transient tubular proteinuria. These findings demonstrate that although MA may temporarily impair renal tubular function and stimulate renal growth, it neither causes chronic renal injury nor promotes the progression of established renal disease in the rat. It can be concluded that whilst the treatment of uraemic acidosis confers symptomatic relief, improves renal bone disease and mitigates muscle wasting, it is unlikely to influence rates of disease progression in patients with chronic renal failure.
Alteration of systemic pH by dietary manipulation

The dietary acid and bicarbonate supplements used in these studies successfully altered systemic pH and were well tolerated. In the SA study (Chapter 2), animals receiving diet made up with 0.8M HCl received a mean daily acid dose of 19.7mmol, and had significantly lower arterial blood pH and base excess than controls after two weeks (pH 7.24 ± 0.02 vs 7.38 ± 0.01, p < 0.001; base excess -6.5 ± 1.4 vs 3.4 ± 0.6, p < 0.001). Similarly in the LA study (Chapter 3), in which feed reconstituted with a lower concentration of HCl was used to ensure tolerability throughout the prolonged protocol, the mean daily acid dose was 10.3 mmol, and arterial pH at 14 weeks was 7.35 ± 0.02 in the acid-supplemented group vs 7.41 ± 0.01 in controls (p < 0.05). The smaller pH difference between acidic animals and controls in the LA study reflected the lower acid dose used, and the longer period of time available for compensatory upregulation of renal acid disposal. At the end of the RP study (Chapter 4) corrected arterial pH was normal and base deficit was negligible in the bicarbonate-supplemented remnants, in contrast to control remnants which were profoundly acidotic with a corrected pH of 6.87 ± 0.04 and a base deficit of 25.4 ± 1.1. In view of the length of the protocol, regular measurement of arterial blood gases during the study was not possible since it would have required repeated general anaesthetics, but since the quantity of NaHCO₃ administered was sufficient to abolish acidosis at the end of the protocol when animals had advanced uraemia, it can be inferred that acidosis was abolished during the earlier stages when renal failure was less advanced.

Although acidotic animals in the SA, LA and NA studies gained less weight than controls, this was not due to reduced food intake. Acid supplementation did not reduce the palatability of the feed, since acidotic rats ate more than controls when allowed unrestricted access to food. In the SA
and NA studies, there was no significant difference in food consumption between acidotic animals and controls, and in the LA study overall mean food consumption was slightly higher in the acidotic group (see p 94). The effect of reconstitution of feed with HCl on the consistency of the final paste, which was more powdery and therefore more susceptible to 'scatter' than feed made up with tap water, made it important to search bedding for scattered food before weighing the uneaten residue when quantifying daily food consumption. Failure to identify uneaten food would have produced spuriously high estimations of food and acid consumption in the acid supplemented groups.

Acidotic animals in the SA and LA studies consistently developed polydipsia and polyuria. Polyuria is not an expected consequence of MA, since several mechanisms of renal acid disposal which are upregulated in MA reabsorb Na⁺ in exchange for secreted H⁺, and would therefore be expected to be antidiuretic. In the proximal tubule, MA upregulates activity and synthesis of the apical NHE3 (which is responsible for approximately 65% of H⁺ secretion in this nephron segment [15, 73]) and the basolateral 2Na⁺/3HCO₃⁻ antiporter [74, 76] (see pp 44-45), thereby increasing Na⁺ movement from tubular lumen to peritubular capillaries. In the thick ascending limb of Henle's loop, approximately 35% of NH₄⁺ secreted by the proximal tubule is reabsorbed on the K⁺ channel of the Na⁺-K⁺-2Cl⁻ cotransporter ([34]; see p 23). Since MA is accompanied by upregulation of ammoniagenesis with a consequent increase in substrate for the Na⁺-K⁺-2Cl⁻ cotransporter, it would be anticipated that activity of this transporter would also be upregulated with a consequent increase in Na⁺ reabsorption and an antidiuretic effect exactly opposite to that seen after the administration of loop diuretics. A further, though quantitatively less important mechanism of Na⁺/H⁺ exchange has been demonstrated in the apical membrane of the
distal convoluted tubule, and might also result in increased Na⁺ reabsorption during MA [42]. Since MA is not a recognised cause of neurogenic diabetes insipidus [155], the development of polyuria in acid-supplemented rats raised the possibility that acidosis causes a nephrogenic urinary concentrating defect. To investigate this possibility, a fluid restriction test was performed in 6 acidotic animals and 6 controls from the LA study. By 24 hours, there was no significant difference in urine output or urine osmolality between acid-supplemented animals and controls. It can be concluded that polyuria in the acid-supplemented animals was driven by polydipsia resulting from thirst induced by some property of the acid feed, rather than by a previously unrecognised toxic effect of MA on urine concentrating mechanisms in the collecting duct.

Dietary HCl supplementation causes a normal anion gap acidosis (see Chapter 1, Table 1.5) whereas uraemic acidosis is hyperchloremic in mild disease but changes to a high anion gap type as organic anions accumulate (Chapter 1, Table 1.6). It could therefore be argued that the acidosis induced by HCl supplements in the SA and LA studies was not representative of the type of acidosis seen in uraemic animals and patients, and that the only appropriate method of assessing effects of uraemic acidosis is by abolishing it in animals with experimental uraemia (the technique used in the RP study). Such an argument ignores the reason for classifying acidosis as 'normal' or 'high' anion gap in type, which is to help in identifying its underlying cause in a clinical situation (see Chapter 1). Irrespective of the cause of acidosis, its pathophysiological effects result from the capacity of altered [H⁺] to induce conformational changes in proteins and peptides, thereby changing the structure and function of structural proteins, enzymes and receptors [156]. Whilst the inorganic anions accumulating in patients with uraemic acidosis may themselves have deleterious effects, these are independent of and
unrelated to the acidosis, and therefore not relevant to a consideration of the effects of acidosis on the progression of renal disease. It can be concluded that the induction of a normal anion gap acidosis by HCl supplementation was an appropriate method of examining the effects of uraemic acidosis on renal function in normal rats.
Effects of acidosis on somatic growth.

Growth retardation is a well-recognised consequence of both chronic renal failure (CRF) and MA in humans and experimental animals. The adult height of patients who had chronic renal failure in childhood averages three standard deviations below the mean [157], and children with renal tubular acidosis, whose glomerular filtration rate is normal, have low growth rates which can be normalised by correction of acidosis [158]. Because it is a constant feature of moderate CRF and has been shown to slow growth rates in acidotic children with normal renal function, MA is thought to contribute to growth retardation in uraemia. Other contributory factors include renal osteodystrophy, energy, protein and glucose malnutrition and hormonal changes [157].

A number of mechanisms have been identified which contribute to growth retardation in acidosis, including increased protein catabolism [159] and decreased production of growth hormone [160], Insulin-Like Growth Factor 1 (IGF-1), and growth hormone receptor [161]. In 1987, May et al demonstrated that muscle breakdown in acidosis was mediated by an increased quantity and activity of branched chain keto acid dehydrogenase (BCKAD) [162], and in 1991 Williams et al suggested that increased skeletal muscle degradation and decreased somatic growth form part of a co-ordinated multi-organ compensation in MA which provides nitrogen for ammoniagenesis and thereby increases net acid excretion [131]. The same group also showed in 1991 that the correction of uraemic acidosis in patients with chronic renal failure significantly reduced skeletal muscle protein catabolism, and that this abrogated an increase in muscle breakdown caused by dietary protein restriction [162a]. On the basis of these findings, it was calculated that the loss of skeletal muscle protein resulting solely from uraemic acidosis would cause
a fall in muscle mass of 37g/day. In 1993, Reiach et al confirmed that the correction of MA in uraemic patients reduced protein degradation, and showed that this was accompanied by a reduction in amino acid oxidation [163]. Whilst also reducing protein synthesis in these patients, the correction of MA still resulted in a net decrease in negative nitrogen balance. In the same year, Challa et al reported a reduction in serum IGF-1, hepatic IGF-1 mRNA and hepatic growth hormone receptor mRNA in rats made acidotic by dietary ammonium chloride, and concluded that inhibition of IGF-1 and IGF-1 receptor mRNA production was partially dependent on decreased food intake [161].

Leptin is a 167 amino acid protein which is produced predominantly by adipocytes and limits food intake whilst stimulating energy expenditure. It has recently been demonstrated that plasma leptin concentrations are elevated in patients with CRF, suggesting that it may be partially responsible for weight loss in patients with renal impairment [164]. In 1999, Teta et al examined the possibility that uraemic acidosis may be the stimulus for increased leptin production by in vitro and in vivo techniques. Contrary to expectations, they found that leptin production by 3T3 L1 adipocytes in vitro, and plasma leptin concentrations in acidotic rats in vivo, were lower than in control adipocytes and animals respectively [165]. They suggested that acidosis was perceived as a "starvation state", and that reduced leptin production resulted from downregulation of the leptin gene due to reduced glucose transport and flux into the hexosamine biosynthetic pathway.

It is likely that growth retardation in acidotic animals in the SA and LA studies was due in part to upregulation of BCKAD activity and consequent increased muscle catabolism. Using a very similar model of acidosis to that used in the SA and LA studies, Williams et al demonstrated a progressive rise
in urinary creatinine excretion (an index of muscle mass provided renal function is normal) as control animals grew, in contrast to a constant level with time in the acidotic group [131]. In addition, the 3 methylhistidine/creatinine excretion ratio, an index of the fractional catabolic rate of skeletal muscle protein, progressively rose in acidotic animals in contrast to a constant level in controls. Increased muscle catabolism is unlikely, however, to fully account for the difference in body weights between acidotic animals and controls in the SA and LA studies. Although fat mass was not formally evaluated, a marked difference in intra-abdominal fat was evident on inspection of the abdominal contents at sacrifice. Acidotic animals had sparse fat deposits, particularly at the end of the LA study, and contrasted with controls whose intra-abdominal fat was abundant. As a result, considerable blunt dissection was needed before the abdominal aorta could be visualised and cannulated and the kidneys removed from control rats. In contrast, the abdominal aorta and kidneys were easily accessed in acidotic animals simply by displacing the gut from the midline. Since acidotic and control groups were pair-fed, and in the LA study overall mean food consumption was slightly higher in acidotic animals than controls, these differences in adiposity cannot be attributed to reduced food intake in the acidotic groups. From the data outlined above, other possible causes include increased leptin production, or reduced IGF-1 and IGF-1 receptor synthesis. Serum leptin concentrations have not been measured in non-uraemic acidosis, but Teta's data in the remnant [165] suggest that acidosis reduces, rather than increases leptin production. In addition, an increase in leptin production would not explain the findings of the SA and LA studies, in which acidotic animals were not anorexic as would be expected in states of leptin excess, but consistently had to be food-restricted to ensure pair-feeding with controls. As discussed above, the reductions in IGF-1 and IGF-1 receptor reported in acidotic rats by Challa et al (1993) were attributed in part to reductions in
food intake. It is of note that serum IGF-1 concentrations were significantly higher in acidotic animals than in the pair-fed non-acidotic group, and that the reported reduction in IGF-1 referred to the difference between acidotic animals and non pair-fed controls, whose body weights were significantly higher. In addition, no significant difference was found in hepatic IGF-1 mRNA or hepatic growth hormone receptor mRNA between acidotic animals and their non-acidotic pair-fed controls. These data suggest that any reduction in IGF-1 production in acidosis results from reduced food intake rather than from a direct effect of acidosis itself. The demonstration of significantly increased serum IGF-1 in acidotic rats vs pair-fed controls suggests that MA may actually increase rather than reduce IGF-1 production when corrected for food intake. In view of this, differences in weight between acidotic animals and pair-fed controls in the SA and LA studies cannot be attributed to alterations in the production of IGF-1 or its receptor.

Whilst upregulation of BCKAD partly accounts for the reduced weight gain of acidotic animals in the SA and LA studies, further as yet unidentified mechanism(s) seem likely to contribute. In 1995, data from this laboratory demonstrated that increasing calorie intake in acidotic rats abolished the weight difference between acid-supplemented animals and controls, although creatinine excretion/body weight was significantly lower in the calorie-supplemented acidotic group [166]. This suggests that body weight was restored by deposition of fat rather than skeletal muscle. In contrast, body weight was unaffected by increasing protein intake. Fat deposition is independent of BCKAD, but is inhibited by insulin resistance, which DeFronzo and Beckles (1979) have identified by glucose clamping techniques during chronic MA in man [167]. It is possible that the reduction in fat deposition, and a proportion of the reduction in weight gain seen in acidotic rats in the SA and LA studies was partially or entirely due to insulin
resistance. Formal tests of tissue sensitivity to insulin by hyperglycaemic and euglycaemic clamping would be required to confirm or refute this hypothesis in these animals.

Since no animals in the NA study (Chapter 5) were uraemic at sacrifice, the only significant metabolic difference between the three groups was their acid base status. It is therefore reasonable to attribute the significantly lower body weights of the acidotic group in this study to the factors discussed above.

In contrast to the first three studies, body weights in the RP study (Chapter 4) were no lower in the acidotic group (ie non-alkalinised remnants with spontaneous uraemic acidosis) than in the alkalinised group, despite dramatic differences in arterial blood pH which on the basis of the above discussions would be expected to exert a significant effect on weight gain. Since all animals in this study had renal failure, it is likely that the anorexic effect of their uraemia was sufficient to override any additional influences of their acid-base status on weight gain. Consistent with this is the observation that paste intake in the RP study averaged 33.4 ± 0.7g/day and 32.7 ± 0.5g/day in control and alkalinised remnants respectively, compared with averages of 41 ± 0.4g/day and 38 ± 0.5g/day in acidotic and control groups in the LA study.

Confirmation of the growth-limiting effects of MA in rats with normal renal function in the SA and LA studies underlines the importance of comparing kidney size in acidotic rats vs controls by means of kidney/body weight and kidney/heart weight ratios rather than by using absolute organ weights. Kidney/body weight ratios have been shown to rise during starvation [168] and could therefore increase if prolonged acidosis caused late
anorexia. Kidney/heart weight ratios were therefore used in addition to kidney/body weights to ensure that the influence of any late loss of fat or muscle mass on kidney/body weight ratios did not exaggerate the growth-promoting effects of acidosis on the kidney. The importance of this approach was seen in the LA study. Comparison solely of absolute kidney weights in acidic animals and controls (0.94 ± 0.02g vs 0.91 ± 0.02g, NS) would have suggested similar renal growth, whereas the large difference in somatic growth (body weights at sacrifice 251 ± 4g vs 313 ± 10g, p < 0.001) resulted in kidney/body weight and kidney/heart weight ratios which were significantly higher in the acidic group (0.29 ± 0.01 vs 0.36 ± 0.01g%, and 1.08 ± 0.02 vs 0.99 ± 0.03 respectively, both p < 0.001) consistent with a specific effect of acid supplementation on renal growth.
Effects of acidosis and nephrectomy on renal growth

The first aim of the SA study was to confirm that MA, rather than increased dietary nitrogen, was the cause of the renal growth previously reported in rats given ammonium chloride supplements [84]. Acid-supplemented animals in the SA study had higher mean kidney weights, kidney/body weight ratios, kidney/heart weight ratios and total protein content than controls at sacrifice, demonstrating for the first time that MA induced without a concomitant nitrogen load causes true renal growth. This is consistent with Lotspeich's reports that neither ammonium citrate (which has no net effect on acid-base status since the bicarbonate ion consumed by metabolism of each NH$_4^+$ is matched by equal bicarbonate production from the metabolism of the accompanying citrate anion) nor urea influenced renal size when administered to rats in doses isonitrogenous with the ammonium chloride.

Although it was not possible to measure serial kidney sizes in vivo in these studies, final kidney weights in acidic animals were the same in both the SA and LA protocols, suggesting that renal growth was confined to the first two weeks of acidosis. In contrast, the final weights of control kidneys were higher in the LA study than the SA study, consistent with more linear renal growth. Comparison of nuclear densities in the two studies provided further information about the mechanism of renal growth. Kidney total protein content was higher, but tubular nuclear density was lower in acidic animals than controls after two weeks. In contrast, tubular nuclear densities were the same in acidic animals and controls at the end of the LA protocol. The return of nuclear densities to control levels despite unchanged kidney weights implies that initial tubular cell hypertrophy was followed by cell proliferation (hyperplasia) with restoration of a normal nuclear/cytoplasmic
ratio. This is consistent with Lotspeich's conclusion (1965) that both hypertrophy and hyperplasia contribute to renal growth in acidosis [84]. In 1991, Golchini et al proposed that hypertrophy in cultured proximal tubular cells exposed to ammonia results from a pH-independent stimulation of protein synthesis and inhibition of protein degradation [117]. Similar modulation of growth by ammonia has been reported in non-renal cells including rat hepatocytes, rat fibroblasts and mouse macrophages [113]. Since acidosis stimulates ammoniagenesis in proximal tubular cells, the same mechanism could also apply in vivo due to increased tubular ammonia concentrations (discussed in [113], summarised in Figure 1.4). This would hold true whether acidosis was induced directly by dietary HCl (as in these studies) or indirectly due to loss of the HCO₃⁻ consumed by metabolism of supplemental dietary NH₄Cl (as in Lotspeich's study). It therefore provides a plausible explanation for the renal hypertrophy and hyperplasia seen in the SA and LA studies. An alternative explanation for renal growth in acidosis was proposed by Bevington et al (1994), who demonstrated that OK cells incubated in acid media hypertrophied without upregulation of ammoniagenesis. It was suggested that increased intracellular [Na⁺] resulting from acid-induced upregulation of apical NHE might be responsible for cell hypertrophy. It is of note, however, that Golchini et al (1989), whilst confirming that NHE was upregulated in OK cells bathed in 20 mmol NH₄Cl, failed to inhibit hypertrophy of these cells by blocking Na⁺/H⁺ exchange with amiloride. Since renal cells in the SA and LA studies were exposed both to low pH (from systemic acidosis) and to increased local ammonia concentration (from upregulated ammoniagenesis), conclusions cannot be drawn from these studies about the relative contributions of ammoniagenesis or upregulated Na⁺/H⁺ exchange to renal growth in MA. To establish this, further studies would be needed in which renal growth was compared after separate inhibition of Na⁺/H⁺ exchange and ammoniagenesis.
The results of the NA study confirm Lotspeich's finding that renal growth induced by MA is additive to the compensatory growth seen in the remaining kidney after uninephrectomy. At sacrifice, absolute kidney weights and kidney/body weight ratios were significantly higher in rats given acid supplements than in those given normal feed or sodium bicarbonate. Findings in the alkalinised rats, however, challenge previous theories about the mechanism of compensatory renal growth (CRG). It has been shown that reduction of renal mass increases single nephron GFR in the remaining nephrons, and that this is accompanied by upregulation of ammonium production and secretion [58]. In addition, ammonium production by isolated perfused mouse proximal tubular cells increases when tubular flow rates are increased [33]. These observations led Kurtz (1991) to suggest that CRG was mediated by upregulation of ammoniagenesis resulting from increased single nephron GFR [113]. On the basis of this theory it would be anticipated that systemic alkalinisation, which reduces proximal tubular ammonium production, should also reduce rates of CRG. Whilst ammonium production and excretion were not directly measured in the NA study, urine pH in bicarbonate-supplemented animals was consistently higher than in controls. This does not directly prove that ammoniagenesis was reduced [169], but it does demonstrate that renal compensation for alkalosis, of which reduced ammoniagenesis is a fundamental component, was occurring in the alkalinised group. The effectiveness of this compensation was shown by arterial pH values at sacrifice, which were the same in bicarbonate-supplemented animals and controls. Given the similarity of kidney weights in two groups which by implication had markedly different rates of ammoniagenesis, the findings of this study do not support a significant role for ammonia in CRG following uninephrectomy in the rat. This contrasts with Tolins et al's findings in hypokalaemic rats (1987), in which renal growth was significantly inhibited by reducing ammoniagenesis with dietary sodium.
bicarbonate [112]. In this model, however, morphological abnormalities mainly affect the inner medulla, in contrast to CRG which disproportionately affects the proximal tubule [170]. The observation that different morphological patterns of growth are seen in models characterised by increased ammoniagenesis argues of itself against a single ammonium-driven mechanism of growth in all pathophysiological cases of renal hypertrophy. The failure of alkalinisation to suppress renal growth in the NA study is consistent with this, and supports Kurtz's conclusion that "to attribute all causes of in vivo renal hypertrophy to an elevation of tissue ammonia concentration is an oversimplification".

In the RP study kidney weights and kidney/body weight ratios were significantly higher in alkalinised remnants than in those with uncompensated uraemic acidosis. Since the severity of renal injury and total kidney collagen content were the same in both groups (Table 4.1: discussed below) it is likely that differences in kidney weight resulted from different rates of CRG. This suggests that in contrast to CRG after uninephrectomy, renal growth in the remnant depends at least in part on increased ammoniagenesis.

In 1991, Nath et al proposed that renal growth induced by heightened ammoniagenesis contributes to the progression of chronic renal injury [104]. Kidneys from acidotic animals in the NA study showed no evidence of morphological abnormality or reduced GFR after exposure to the hypertrophic influence of acidosis for over 3 months, suggesting that progressive tubulointerstitial damage is not an inevitable consequence of renal growth. In addition, although CRG was greater in control remnants than alkalinised remnants in the RP study, the severity of histological injury was identical in the two groups, demonstrating that manoeuvres which
reduce renal hypertrophy do not necessarily mitigate the associated renal damage. These observations suggest that renal growth and subsequent injury, which co-exist in several experimental models of chronic renal failure, are associated rather than causally related. It follows that manoeuvres which cause renal growth both in experimental animals and humans will not inevitably lead to chronic renal injury. Support for this conclusion comes from studies of patients who have previously undergone nephrectomy, usually for the purpose of kidney donation. Kasiske et al (1995) performed a meta-analysis of 48 cross-sectional studies which examined the long term effects of renal mass reduction on excretory renal function, proteinuria, and blood pressure [171]. Although patients experienced an early decline in renal function after uninephrectomy (mean fall in GFR of 17.1 mls/min) this was followed by an increase in GFR (mean 1.4 ml/min/decade) which was attributed to CRG in the remaining kidney. Proteinuria transiently increased after uninephrectomy before progressively falling with increasing time from surgery, and blood pressure also increased, and in the case of systolic BP continued to increase with time, though not sufficiently to cause hypertension. In the only published longitudinal long-term follow-up study of kidney donors, Saran et al (1997) re-examined 47 donors a decade after an initial (≥ 1 year after surgery) post-operative assessment of GFR, proteinuria and blood pressure [172]. They found no statistical fall in GFR over the ten year period, though albumin excretion rate and blood pressure both rose significantly. Whilst renal biopsy was not possible in any of these studies, and histological abnormalities cannot therefore be ruled out, the long term stability of GFR after uninephrectomy is consistent with the findings of the SA, LA and NA studies in arguing against a role for CRG per se in the development of chronic renal injury.
Effects of acidosis on urinary protein excretion

In the RP study, abolition of uraemic acidosis by dietary bicarbonate supplements did not significantly influence overall mean proteinuria, peak proteinuria or urinary protein excretion at fortnightly timepoints after $5/6$ths nephrectomy (Figure 4.3). This is consistent with the failure of alkalinisation to affect histological injury, collagen deposition or rate of decline of GFR in the same study, and argues against a significant role for MA per se in promoting the progression of established renal injury (discussed further below).

In contrast to findings in the remnant kidney, results from the SA, LA and NA studies demonstrated a significant effect of MA on protein excretion both in normal kidneys and in the remaining kidney following uninephrectomy. Urinalysis in the SA study confirmed previous reports of proteinuria in short term acidosis [83] and was consistent with a predominantly tubular origin for the urinary protein. N-acetyl-β-D-glucosaminidase (NAG) is a lysosomal glycohydrolase which is not normally filtered at the glomerulus, is present in high concentration in tubular cells, is stable in urine for several months at $-20^\circ$C and is released into tubular fluid (and therefore appears in urine) following tubular injury [173]. Lysozyme is a plasma-derived low molecular weight protein which is freely filtered at the glomerulus, normally completely reabsorbed by endocytosis by proximal tubular cells but which appears in the urine if reabsorptive mechanism in the proximal tubule are impaired by tubular injury [173]. The significant increase in NAG excretion, and 40-fold rise in lysozyme excretion in acidotic animals in the SA study (Table 2.2, Figure 2.1) are therefore both consistent with tubular injury. Whilst there was a small increase in urinary excretion of albumin (Figure 2.1), a higher molecular weight protein usually associated with glomerular injury, such
increases have previously been reported in mild renal tubular injury and attributed to impaired albumin reabsorption. In 1991, Houser & Milner identified albuminuria in rats after administration of the tubular toxins mercuric chloride and maleic acid, and concluded that albuminuria was a more sensitive indicator of subtle renal tubular injury than lysozymuria [174]. The slight increase in IgG excretion, which whilst statistically significant was relatively much smaller than the increases in both albumin and lysozyme excretion (Figure 2.1, Table 2.2) is also consistent with a predominantly tubular origin for proteinuria in MA. Further evidence of tubular abnormalities was obtained from immunohistochemical staining with antibodies against Tamm-Horsfall protein, using a technique developed by Howie et al (1991) [134]. Using biopsy and necropsy material from patients with renal impairment of various causes and severity, they reported a significant correlation between loss of staining for brush border antigen and Tamm-Horsfall protein and the reciprocal plasma creatinine concentration, and between the number of THP positive cortical tubular casts and the reciprocal plasma creatinine. As these findings were independent of the underlying cause of renal impairment, it was suggested that tubular damage was a feature common to all processes causing renal failure. The antibody to brush border, whose loss from tubules correlated most reliably with renal impairment in the study by Howie et al, failed to stain rat sections in the SA study. This was probably due to interspecies variation between human ileal brush border, against which the antibody was raised, and rat kidney brush border. Sections stained well with antibody to Tamm-Horsfall protein, however, and on the basis of differences in their staining pattern, acidotic kidneys could be distinguished from controls with 100% accuracy on blinded examination. These findings, combined with the demonstration that proteinuria was predominantly of tubular origin, suggest that the adverse effects of MA in the normal kidney result from effects on renal tubules rather
than on the glomerulus. This conclusion is at variance with the findings of Gardner & coworkers (1961), who proposed a glomerular origin for the proteinuria of acidosis [83]. Their conclusions were based on the demonstration of increased numbers of protein droplets within tubular epithelial cells of acidotic kidneys. Since a qualitative histological assessment of tubular protein degradation showed no reduction during acidosis, it was concluded that this increase in protein droplets reflected increased protein filtration at the glomerulus. Analyses of urinary proteins were unhelpful in determining the source of proteinuria, since electrophoresis detected only 'small amounts' of albumin and a 7 fold rise in \( \alpha \) globulins, whilst proteins of tubular origin were not analysed. An alternative interpretation of Gardner's findings is that acidosis inhibited proteolysis in tubular cells sufficiently to cause intracellular protein accumulation and tubular proteinuria, but not enough to allow detection by the crude index of proteolysis used. This would be consistent with the failure to identify significant quantities of albumin, which is normally a major component of proteinuria of glomerular origin, in samples from the acidotic animals.

The pattern of protein excretion in the early part of the LA study mirrored that seen in the SA study, with significant increases in urinary total protein, lysozyme, albumin and to a lesser extent IgG (Figures 3.5 & 3.6). A novel finding was that total protein and lysozyme excretion progressively increased during the first 8 weeks of acidosis but subsequently declined, returning to control levels by week 8 (Figure 3.5). As in the SA study, acidic animals developed polyuria, and a possible explanation for proteinuria in this group would be that it resulted from increased urine output. In 1982, Viberti et al reported that 18 subjects given both acute (1 litre over 10 minutes) and chronic (250mls water every 20 minutes for 4 hours) water loads developed increased urinary albumin excretion, though there was no change in urinary
β2-microglobulin (a low molecular weight protein which is freely filtered but normally completely reabsorbed) [136]. In 1988, Jung et al reported an increase in urinary excretion of lysozyme and to a lesser extent β2-microglobulin in six healthy men made polyuric by oral fluid loading [137]. It was suggested by Viberti et al that water loading might cause albuminuria by expanding the extracellular fluid volume and thereby increasing GFR and glomerular albumin filtration, or alternatively by washing out proteins from the tubular lumen. Low molecular weight proteins (eg β2-microglobulin) could then be preferentially reabsorbed 'downstream', resulting in increased quantities of albumin in the final urine. In both studies, proteinuria returned to baseline within four hours, and in Viberti's study this was in spite of a continuing high urine output. It therefore seems unlikely that such a mechanism could be implicated in proteinuria which persisted over 8 weeks in the LA study, but for confirmation of this a water loading study was performed in which 16 control rats were encouraged to increase their fluid intake by supplementing their drinking water with 5% glucose. This technique increased water intake and urine output to levels similar to those in acidotic animals, but did not influence urinary excretion of either lysozyme or albumin (Figure 3.7), confirming that polyuria was not the cause of increased proteinuria in the acidotic group.

Further evidence in favour of a tubular origin for the proteinuria of acidosis came from electron microscopic examination of kidneys perfused with polyethyleneimmine immediately post mortem. As discussed in the introduction to Chapter 3, changes in basement membrane charge, which could conceivably result from conformational changes in BM proteins due to altered pH of the filtrate, could reduce the functional integrity of the glomerular basement membrane in acidosis and cause glomerular proteinuria. Since neither effacement of podocyte foot processes, a
morphological abnormality characteristic of significant proteinuria, nor changes in the density of anionic binding sites along the basement membrane were seen in kidneys from acidotic animals, the morphological appearances agree with analyses of urine proteins in identifying the tubule, rather than the glomerulus as the source of proteinuria in the acidotic group.

Acidotic animals in the NA study showed an early increase in proteinuria similar to that seen in the LA study. Urinary total protein excretion then fell, such that by the twelfth week of the protocol it was similar in acidotic, control and alkalinised groups (Figure 5.2). Animals in all groups then developed a progressive increase in total protein excretion which continued until the end of the protocol (Figure 5.2). In the control and bicarbonate-supplemented groups there was no associated increase in lysozyme excretion (Figure 5.3), demonstrating that the proteinuria was of glomerular origin and signalled the development of early FSGHS. In acid-supplemented animals, the early peak in urinary lysozyme was followed by a small excess of lysozyme excretion for the remainder of the protocol (Figure 5.3), consistent with a continuing, though small, effect of acidosis on the proximal tubule. Although acidosis caused early tubular proteinuria, it did not exacerbate the glomerular proteinuria which developed later in the protocol. Conversely, bicarbonate supplementation did not slow the late rise in protein excretion. These observations demonstrate a lack of correlation between rates of ammoniagenesis and urinary protein, which argues against a role for ammoniagenesis in the development of chronic and progressive proteinuria in this model. This conclusion mirrors comments about the relationship between ammoniagenesis and renal growth discussed above.

As discussed in Chapter 1 (p 51), proteinuria may be caused by one or more of four mechanisms: disruption of the glomerular capillary barrier,
tubule cell dysfunction, disruption of tubular cell membranes, and increased glomerular filtration of proteins whose plasma concentrations are pathologically high. Since the pattern of proteinuria in the LA study was consistent with a tubular origin, and the ultrastructural appearance and anionic charge density of the glomerular basement membranes was the same in acidotic animals and controls, proteinuria in the acidotic groups cannot be attributed to disruption of the glomerular capillary barrier or increased filtration at the glomerulus. Tubular cells were histologically normal at the time of sacrifice, and the development of tubular proteinuria could therefore be explained either by transient tubular cell membrane damage which resolved before sacrifice, or by a short-lived reduction in endocytosis or intralysosomal degradation of tubular proteins. Studies examining the effect of pH on endocytosis by proximal tubular cells have yielded conflicting results. Using a microperfusion technique in rat proximal tubules, Christensen & Bjerke (1986) demonstrated that reducing the pH of perfusion fluid from 7.4 to 6.0 or 4.5 resulted in a 15% increase in albumin reabsorption [175]. Five years later, Schwegler & coworkers (1991) reported that cultured opossum kidney (OK) cells responded to increased or decreased pH by reducing absorption of fluorescein-labelled albumin [176]. Although their specific findings disagreed, these studies established the principle that protein reabsorption by tubular cells can be influenced by alterations in pH. With respect to intralysosomal protein degradation, Golchini and coworkers (1989) reported that NH₄Cl applied to cultured proximal tubular cells caused swelling of lysosomes and a reduction in activity of lysosomal proteases [117]. In 1994, Huang et al demonstrated a similar phenomenon in vivo: proteinase activity in renal tubules from acidotic rats was significantly less than in controls after 1, 3, 6 and 8 days of NH₄Cl supplementation [177]. A suggested mechanism for this effect is that ammonia, acting as a weak base, raises intralysosomal pH and prevents targeting of mannose-6-phosphate coupled
enzymes to lysosomes, thereby reducing intralysosomal protein degradation [178]. Since acidosis is accompanied by large increases in cortical ammonium production, the resulting high local concentration of ammonium ions might influence degradation of tubular proteins in a similar manner. Although mechanisms such as these might increase protein excretion in acidosis, they do not account for the spontaneous resolution of proteinuria which occurred despite continued acid loading in the LA and NA studies. The difference in arterial blood pH between acid-supplemented animals and controls in the SA, LA and NA studies was inversely proportional to the duration of the protocols. After two weeks (in the SA study) arterial pH was 7.24 ± 0.02 in acid-supplemented animals vs 7.38 ± 0.01, in controls (p < 0.001). In the LA study (after 14 weeks of acid supplements) pH was 7.35 ± 0.02 in the acid-supplemented group vs 7.41 ± 0.01 in controls (p < 0.05) and in the NA study (after 24 weeks) there was no statistical difference in pH between acid-supplemented animals and controls (7.37 ± 0.01 vs 7.40 ± 0.01, NS). Since protein and acid intake remained constant throughout all three protocols, this demonstrates that the efficiency of urinary acid disposal increased with increasing duration of acid-loading, and the progressive fall in urine pH as the LA study progressed (Figure 3.2) is consistent with this. Although urinary ammonia was not measured directly, it is well-established that upregulation of ammoniagenesis is the predominant mechanism of renal compensation for MA (discussed in Chapter 1), and it follows that rates of ammoniagenesis must have remained consistently high throughout the LA and NA protocols. Since proteinuria resolved despite continuing high rates of ammoniagenesis in the LA and NA studies, intrarenal ammonia cannot be responsible for the proteinuria of acidosis. An association between protein excretion and systemic acidosis would be more consistent with the data, and the findings of the LA and NA studies could be explained by proposing that proteinuria occurs below a threshold arterial pH, and therefore resolves with the increase
in pH which follows the upregulation of mechanisms compensating for MA. Proximal tubular bicarbonate reabsorption is regulated by systemic pH [179], and protein reabsorption may be regulated in a similar manner, possibly via an effect on endocytosis which as discussed above is a pH-sensitive process in proximal tubular cells.

The mechanisms proposed to explain how proteinuria incites tubulointerstitial inflammation and injury depend upon sustained exposure of mesangial and tubular cells to quantities of filtered proteins sufficient to stimulate release of pro-inflammatory cytokines (discussed in Chapter 1, p 54). Low molecular weight proteins such as lysozyme are filtered by the normal glomerulus, and appear in the urine only when their proximal tubular reabsorption is impaired. Since the glomerular filtration barrier was structurally normal with a normal density of anionic binding sites in acidotic animals in the LA study, it is reasonable to conclude that mesangial cells were exposed to similar quantities of filtered low molecular weights protein in acidotic animals and controls. In addition, only those tubular cells distal to the normal site of reabsorption of these proteins (the proximal tubule) were exposed to abnormally high quantities of protein in the acidotic groups, and the protein was of a different character (low molecular weight vs high molecular weight) and present in much smaller quantities than those reported in experimental models of glomerular disease such as PAN nephrosis [97], or in clinical studies such as the MDRD study [88]. The character of the protein is relevant, since some workers have suggested that specific endogenous proteins (eg transferrin when accompanied by iron in transferrin-iron complexes) may have direct cytotoxic effects on tubular cells [95]. Given all these considerations, it is not surprising that the proteinuria seen in acidotic animals in both LA and NA studies caused neither histological injury nor impaired excretory function.
Effects of altered systemic pH on renal injury

Following the demonstration in the SA study that short periods of acid supplementation cause renal growth and proteinuria (which in other models of experimental renal disease predict the development of chronic renal disease) the LA study tested the hypothesis that prolonged MA causes renal injury in previously normal kidneys. The RP study evaluated the contribution of uraemic acidosis to disease progression after 5/6ths nephrectomy, but because it could be argued that the severity of injury in the remnant kidney is too great for acidosis to influence subsequent rates of progression, the NA study tested the effect of altered systemic pH in a model of more slowly progressive renal injury, the uninephrectomised rat.

Data from all three studies clearly demonstrated that chronic MA neither causes nor exacerbates chronic renal disease. In the LA study, renal histology and GFR remained normal despite 14 weeks of acid supplementation, and as discussed above proteinuria resolved spontaneously by the end of the experiment. It has previously been demonstrated that rates of ammoniagenesis are increased at least threefold in chronically acidotic rats [180]. If, as proposed by Nath et al (1985, 1991), ammonia causes renal injury by activating the alternate complement pathway [115, 104], it would be expected that progressive tubulointerstitial disease would develop in kidneys from the acid-supplemented group. Since histological sections from acidic kidneys showed neither macrophage infiltration (a consistent feature of glomerular and tubulointerstitial inflammation in several models of experimental renal disease [145]) nor structural abnormalities, it can be concluded that high cortical ammonia concentrations do not directly cause injury in normal renal tissue.
In the RP study, abolition of uraemic acidosis by oral bicarbonate produced no improvement in GFR, serum creatinine, proteinuria, blood pressure, histological injury or fibrosis, and the time from surgery to development of terminal uraemia was the same in bicarbonate-supplemented animals as in non-alkalinised controls. If ammonia contributed significantly to the pathogenesis of tubulointerstitial injury in the remnant, it would be predicted that alkalinisation, which reduces ammoniagenesis, would slow disease progression in this model. Since structural and functional parameters were the same in alkalinised and non-alkalinised remnants, it follows that neither acidosis nor ammoniagenesis promote the progression of established renal disease following 5/6ths nephrectomy. This conclusion apparently conflicts with the findings of Nath et al (1985), who reported that postoperative alkalinisation significantly reduced renal injury in the remnant model [115]. They administered a similar dose of bicarbonate to that used in the RP study (2.5meq/100g feed vs 2.3meq/100g in the RP study) and found that protein excretion at 4-6 weeks was almost halved by alkalinisation, and that tubular casts, interstitial infiltration and tubular dilatation were less pronounced in the bicarbonate-supplemented group. Although histological data relating to this period are not available from the LA study, histological appearances at the time of death were similar in both groups, and protein excretion at both 4 and 6 weeks was identical in bicarbonate-supplemented animals and controls (Figure 4.3). It is noteworthy that in Nath's study, GFR at 4-6 weeks was lower (overall mean 0.16 mls/min/100g body wt) than in the RP study (overall mean 0.35mls/min/100g body wt at 6 weeks) consistent with the greater reduction in renal mass (7/8ths nephrectomy in Nath's study vs 5/6ths nephrectomy in the RP study). In the remnant rat, uraemic acidosis is most profound during the first postoperative month, but then improves until a second decline in the preterminal period [181]. The 4-6 week GFR reported by Nath, which is approximately 16% of normal, would be sufficient
to induce uraemic acidosis, and although arterial pH and base excess were not measured in their study, plasma bicarbonate was in the lower range (20meq/l) in the non-alkalinised group, suggesting acidosis. Since it is known from the LA study that acidosis induces transient tubular proteinuria, it is possible that uraemic acidosis contributed to the early proteinuria in Nath's non-alkalinised remnants by a similar mechanism. This would be consistent with the significant proportion (~40%) of total urinary protein which was of low molecular weight, and with the impairment of tubular transport function reported in this group. In contrast, the 6 week GFR in the RP study (35% of normal) would be insufficient to induce significant acidosis, thus explaining the similarity in protein excretion between the bicarbonate supplemented remnants and controls. The subsequent decline in renal function occurred at varying rates in different animals (reflected in the range of survival times), and any proteinuria caused by preterminal uraemic acidosis would consequently have been insufficient to significantly influence the mean protein excretion at any single time point.

The use in the RP study of an end point based partly on a subjective diagnosis of terminal uraemia is open to criticism. The alternative approach, however, of continuing the experiments until uraemic death, would not have allowed the collection of blood samples or the fixation of renal tissue for histological examination. Although objective measurements including weight loss and decreased food intake were taken into account, the lower serum urea and creatinine in non-alkalinised remnants at sacrifice suggest that these animals were culled slightly earlier in the course of their chronic renal disease than the bicarbonate-supplemented group, presumably because their clinical appearance was made worse by acidosis. Despite this effect, however, the survival of alkalinised animals did still not significantly exceed that of
controls, consistent with the conclusion that bicarbonate-supplementation does not influence disease progression in the remnant rat.

As already discussed, dietary acid and bicarbonate did not influence the 'glomerular phase' of proteinuria in the NA study. Consistent with this was the observation that serum creatinine, creatinine clearance/body weight and GFR/body weight were similar in all three groups (Figures 5.4 and 5.5). The majority of kidneys were essentially normal by light microscopy at the time of sacrifice, reflecting the slow development of injury following uninephrectomy, and the seven kidneys showing minor abnormalities were equally distributed throughout the three groups. Although the dose of sodium bicarbonate given to Group 3 was restricted to 1 mmol/day to avoid an excessive sodium load, it was sufficient to raise urine pH above that of controls throughout the study (Figure 5.1). As would be expected, control rats had normal blood pH at sacrifice, consistent with their normal excretory renal function (Table 5.1). The effect of bicarbonate supplementation in Group 3 was not therefore to abolish uraemic acidosis (as was the case in alkalinised animals in the RP study) but to present an additional bicarbonate load to the kidney, to which the normal physiological response is a downregulation of ammoniagenesis [67]. In contrast, the additional acid load presented to kidneys in Group 2 would lead to increased rates of ammoniagenesis. The effectiveness of these compensatory mechanisms in the RP study is confirmed by the normal blood pH at sacrifice in Groups 2 and 3 (Table 5.1). Despite different rates of ammoniagenesis, glomerular proteinuria, GFR and histological appearances were the same in all three groups. These findings are consistent with those of the LA and RP studies, and again suggest that ammonia per se does not cause renal injury.
Clinical implications of experimental findings

The primary aim of the studies reported in Chapters 2 - 5 was to establish whether early and aggressive treatment of uraemic acidosis could slow the progression of chronic renal injury, delay the onset of end stage renal failure and thereby improve the quality of life and longevity of patients with chronic renal impairment. The SA, LA, NA and RP studies clearly demonstrated that MA neither causes chronic renal injury nor influences the progression of established renal disease in the rat. The routine and early administration of sodium bicarbonate to patients with chronic renal failure would not therefore be expected to preserve renal function and cannot be recommended for this indication. Because of the wide variation in rates of progression between patients with CRF (discussed in Chapter 1, p 67) a clinical study to confirm this conclusion would require a very large number of patients to provide adequate statistical power. It would also need to control for protein intake and blood pressure, and require regular assessments of acid-base status to ensure a consistent difference between bicarbonate-treated subjects and controls. Such a study would be similar in scale and methodology to the MDRD study, but would also require the arterial pH of the intervention group to be consistently higher than that of controls. The cost and logistical difficulties involved in organising such a study would be prohibitive, and it is very unlikely ever to be performed.

Although uraemic acidosis does not influence the progression of renal disease, it exerts a number of deleterious effects on extrarenal tissues, most importantly bone and muscle. As discussed in Chapter 1 (p 34), patients with chronic renal disease cannot excrete all the hydrogen ions generated by metabolism of dietary constituents. A proportion of the resulting bicarbonate deficit is corrected by hydrogen ion buffering, predominantly by bone. Two
mechanisms of bone buffering have been identified in chronic acidosis: direct physicochemical exchange of $H^+$ for $Na^+$ and $K^+$ at the bone surface, and increased resorption of bone mineral by osteoclasts (reviewed in [182]). $H^+$ is produced from $H_2CO_3$ within the osteoclast in a reaction catalysed by carbonic anhydrase. Bone is resorbed when osteoclasts secrete $H^+$ onto the bone surface via an electrogenic $H^+$ ATPase. $HCO_3^-$ produced intracellularly by the same reaction is removed from the opposite side of the cell via a $HCO_3^-/Cl^-$ antiporter, reminiscent of the ion movements in the α intercalated cell of the collecting duct (see Chapter 1, Section 1). The dissolution of bone releases $HCO_3^-$ from the hydration shell of hydroxyapatite [182], and also mobilises calcium and phosphate [183]. The effect of acidosis on osteoclast activity in uraemia is additive to the stimulation of osteoclasts caused by hyperparathyroidism [182]. In addition, acidosis increases plasma concentrations of parathyroid hormone [184], and reduces circulating levels of vitamin D3 by inhibiting 1α hydroxylation in proximal tubular epithelial cells [185]. In contrast to its effect on osteoclasts, MA exerts an inhibitory effect on osteoblasts, and the consequent reduction in osteoid deposition blocks the release of hydrogen ions which normally accompanies bone formation [186]. Given these effects of MA on bone cells and on the metabolism of parathyroid hormone and vitamin D, it is not surprising that chronic acidosis contributes to uraemic osteodystrophy. In 1983, Mora-Palma et al reported the findings of over 200 bone biopsies from patients with advanced chronic renal failure [186a]. They showed that bones from patients with more profound acidosis (serum $[HCO_3^-] < 16 \text{mEq/l}$) had more severe histological abnormalities than those from less acidotic patients (serum $[HCO_3^-] >20 \text{mEq/l}$). Several studies have shown that uraemia is accompanied by a reduction in bone carbonate (which has been attributed either to dissolution of bone carbonate stores or replacement of bone $CO_3^{2-}$ by $HPO_4^{2-}$ [182]) and by negative calcium balance [183]. In addition, the
correction of acidosis has been shown to increase bone mineralisation and reduce urinary calcium losses in predialysis patients [187], and improve bone histology in both adynamic and high turnover bone disease in patients on haemodialysis [188].

The effects of acidosis on skeletal muscle have already been discussed (p 128). MA causes negative nitrogen balance, muscle proteolysis, and increased activity and production of BCKAD [131, 162]. In 1989, Jenkins et al showed that the correction of acidosis reduced plasma urea, protein degradation and net negative nitrogen balance in uraemic patients [189]. Reaich et al (1993) confirmed these findings, and found that uraemic patients given oral sodium bicarbonate had lower rates of leucine oxidation than non-alkalinised controls [163]. Whilst patients in Jenkins' study showed no increase in blood pressure, plasma [Na+] or clinical signs of fluid overload, one patient in Reaich's series developed resistant hypertension, and there was an overall increase in mean systolic and diastolic blood pressure.

Uraemic acidosis has a number of other clinical effects, though some are only significant during the severe acidosis which accompanies advanced CRF. Compensatory hyperventilation resulting from the direct stimulatory effect of acidosis on the medullary respiratory centre causes dyspnoea at rest [190], and reduced cardiac contractility and decreased compliance of pulmonary vessels combine to increase the risk of pulmonary oedema in patients with uraemic acidosis [191, 192]. Other metabolic consequences of chronic MA include insulin resistance [167], hyperkalaemia [193] and increased levels of cholesterol and triglycerides [194].

The mainstay of the treatment of uraemic acidosis is the administration of oral sodium bicarbonate. Whilst this is generally well -tolerated, it has the
disadvantage of delivering a sodium load to patients who are already at risk of salt-water overload and hypertension. Calcium carbonate, lactate and acetate are alternative alkalinising agents which have the dual advantages of avoiding a sodium load whilst also acting as phosphate binders. In addition to treatment with drugs, restriction of foods whose metabolism produces large amounts of $H^+$ can improve acid-base status in renal failure. Since the majority of $H^+$ is derived from metabolism of dietary protein, low protein diets reduce $H^+$ generation and improve acidosis [195]. Acid load can be further reduced by increasing the proportion of dietary protein derived from plant sources, which have a lower content of cationic and sulphur-containing amino acids, and also contain significant quantities of 'potential bicarbonate' in the form of organic acids. Despite the theoretical benefits of dietary manipulation in mitigating uraemic acidosis, its role in the clinic is limited for three reasons. Firstly, it requires further alterations in the eating habits of patients who may already be subject to fluid, phosphate and potassium restriction, and compliance is therefore likely to be poor. Secondly, the requirements of a low acid-generating diet may conflict with other dietary restrictions, particularly in relation to potassium, and finally, strict reductions in dietary protein predispose to protein-calorie malnutrition, which may nullify the benefits of improved acid-base status on muscle and bone, and has been shown to reduce survival once patients commence Renal Replacement Therapy [129].

In conclusion, the studies discussed demonstrate that treatment of uraemic acidosis does not influence the progression of CRF. The correction of acidosis nevertheless remains an important priority in the treatment of patients with progressive renal disease. It has beneficial effects on bone and muscle, mitigates some metabolic consequences of CRF and provides symptomatic relief in advanced chronic renal failure. The most effective
alkalinising agents are oral sodium bicarbonate and calcium salts (carbonate, lactate and acetate), and although dietary manipulation can theoretically improve acid-base status in CRF, its practical contribution to the treatment of uraemic acidosis is small.
### Appendix A: Constituents of casein feed ICN 960259

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<th>Ingredient</th>
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<tr>
<td>Casein purified high nitrogen</td>
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<tr>
<td>Corn starch</td>
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<td>Sucrose</td>
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<td>Corn oil</td>
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<tr>
<td>Alphacel, non-nutritive bulk</td>
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<tr>
<td>AIN-76 mineral mix</td>
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(below in g/Kg diet)

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<tr>
<td>Sucrose, finely powdered</td>
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Appendix B: Composition of solutions for Lowry protein assay

Lowry solution A:

20.0g anhydrous Na$_2$CO$_3$
4.0g NaOH pellets
0.2g Potassium sodium tartrate. 4H$_2$O

made up to 1 litre with deionised water and stored at 4°c

Lowry solution B:

5g/l CuSO$_4$. 5H$_2$O

made up to 1 litre with deionised water and stored at 4°c

Lowry solution C:

A freshly mixed solution of 50 parts Lowry solution A with 1 part of Lowry solution B
## Appendix C: Composition of pyrogallol red reagent

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<td>Disodium molybdate</td>
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<td>Succinic acid</td>
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### Appendix D. Chapter 2: raw nuclear counts and densities

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### Appendix E. Chapter 3: raw nuclear counts and densities

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Glomerular filtration rates were calculated using the following equation:

\[
\text{GFR (ml/min)} = \frac{50 \, \text{k}}{1000 \, C_0}
\]

where: 
- \(I\) = injected activity (see below) 
- \(k\) = exponential slope of plasma clearance (min\(^{-1}\)) 
- \(C_0\) = zero time intercept of plasma clearance curve (cpm/50\(\mu\)l)

Injected activity \(I = \frac{\text{Net standard count rate} \times \text{Net animal dose} \times 1000}{\text{Net standard dose}}\)

The exponential slope (k) and zero time intercept (C\(\_0\)) of the plasma clearance curve were calculated from the count rates at times 45 and 90 minutes using a Microsoft Excel spreadsheet.
Appendix G: Histological scoring system used for remnant kidney sections

Sections were scored on scale of 0-5, incorporating tubular dilatation, glomerulosclerosis and interstitial fibrosis as follows:

**Tubular dilatation**
- without intratubular casts: 1
- with intratubular casts: 2

**Glomerulosclerosis**
- 'segmental' - defined as less than half the glomerulus sclerosed: 1
- 'global' - defined as more than half the glomerulus sclerosed: 2

**Interstitial fibrosis** (excludes fibrosis or infiltration within, or arising from, the fibrous cap seen at the edge of some sections and representing the tissue infarcted at the time of arteriolar ligation).
- absent: 0
- present: 1

The score corresponding to the worst injury seen and which involved at least 50% of the glomeruli, tubules or interstitial area was used in each case.
Appendix H. Example of standard curve using [hydroxyproline] 0 - 12.5 µg/l


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Publications and Presentations

The following publications and presentations have been produced from the studies described in this thesis.

Publications


Presentations

