# RENAL GLUTAMINE METABOLISM: STUDIES WITH THE PERFUSED KIDNEY AND WITH ISOLATED CORTICAL TUBULES

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HENRI PHILIP LANCTIN

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MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

# RENAL GLUTAMINE METABOLISM: STUDIES WITH THE PERFUSED KIDNEY AND WITH ISOLATED CORTICAL TUBULES

A thesis

by

© Henri Lanctin

Submitted in partial fulfilment of
the requirements for the degree of
Master of Science

Department of Biochemistry
Memorial University of Newfoundland

March, 1983

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# LIST OF ABBREVIATIONS

ADP - adenosine diphosphate

AMP - adenosine monophosphate

3-AP - 3-aminopicolinic acid

ATP - adenosine triphosphate

dpm - disintegrations per minute

EDTA - ethylenediamine tetroaacetic acid

GDH - glutamate dehydrogenase (EC 1.4.1.2)

gdw - gram dry weight

GFR - glomerular filtration rate

gln - glutamine

glu - glutamate

GS - glutamine synthetase (EC 6.3.1.2)

gww - gram wet weight

-kg - ketoglutarate

KGDH - ketoglutarate dehydrogenase

MSO - L-methionine-D.L.-sulfoximine

NADH - nicotinamide adenine dinucleotide, reduced form

$NH_3$ - ammonia

$NH_4Cl$ - ammonium chloride

$P_{CO_2}$ - partial pressure $CO_2$

PDG - phosphate-dependent glutaminase (EC 3.5.1.2)

PEP - phosphoenolpyruvate

PEPCK - phosphoenolpyruvate carboxykinase

PIG - phosphate-independent glutaminase (EC 2.3.2.1)

pK - dissociation constant of an acid

$P_{O_2}$ - partial pressure $O_2$

RCR - respiratory control ratio

## ACKNOWLEDGEMENTS

The research in this thesis was completed in two laboratories. It was initiated in September 1978 to August 1979 at Memorial University of Nfld., during which time I was the recipient of a Medical Research Council of Canada Graduate Fellowship. From June to August 1981 I completed the work at the Department of Clinical Biochemistry Radcliffe Infirmary, Oxford, England.

I am indebted to a number of people who made it possible for me to complete this thesis. My supervisor, Dr. J.T. Brosnan was also available as a source of information and his patience and encouragement stimulated me to finish. Excellent technical assistance was provided by Ms. B. Hall and Ms. A. Colbourne also, along with Drs. D. Parry and K.C. Man made the lab a stimulating environment to work in.

While in Oxford, Drs. B. Ross, M. Lowry and P. Lund were invaluable, not only for their contributions to the study but also for their interest and enthusiasm.

Amino acid analysis was performed by Mr. D. Hall, electron microscopy by Mr. P. Highanc, EM Unit, Health Science Complex, St. John's, and the figures and graphs were drawn by Ms. C. Logan, A-V Department, Camp Hill Hospital, Halifax, N.S.

Assistance in the preparation of this manuscript was provided by Drs. G. Herzberg, P. Hansen, Dept. of Biochemistry, M.U.N., Dr. P. Lund, Dept. of Clinical Biochemistry, Radcliffe Infirmy, Oxford, England and Dr. B. Issekutz, Dept. of Physiology, Dalhousie Univ, Halifax

Finally I would like to thank Pat for being understanding and supportive during the five years it took me to complete this work.

H.P. Lanctin

## ABSTRACT

The isolated perfused kidney system was developed in our lab and functionally defined. With this technique kidneys from rats which had been subjected to an $NH_4CL$ induced metabolic acidosis for 7-days and 24-hour recovery from this condition were perfused with glutamine. Rates of ammmoniagenesis and gluconeogenesis were measured. Ammoniagenesis was back to normal after 24 hours of recovery which is what occurs with *in vivo* $NH_3$ excretion. This implies an intrarenal adaptation in the 24-hour recovered situation. Acidotic rats were subjected to a metabolic alkalosis following a 7-day acidotic challenge. Isolated kidneys perfused 8-hours after this accelerated recovery was initiated maintained acidotic rates of ammoniagenesis. This is contrary to *in vivo* $NH_3$ excretion in rats treated in a similar manner in which a return of $NH_3$ excretion to normal is observed. Thus, an acute, reversible, extrarenal influence is responsible for the supervision of ammoniagenesis under these conditions.

With the isolated cortical tubule preparation, rates of ammoniagenesis from glutamine were found to be stimulated by acute acidosis (pH 7.06) and with L-methionine-s-sulfoximime (M.S.O.) an inhibitor of glutamine synthetase (G.S.). In tubules incubated with glutamate $NH_4CL$ and lactate acute acidosis was found to inhibit $NH_3$ uptake and glutamine synthesis. This reaction to acute acidosis was also demonstrated in cortical tubules from acidotic and 24-hour recovery animals. From these results it is clear that in some way the acute change in $H^+$ concentration effectively decreases the activity of G.S. resulting in an apparent stimulation of ammoniagenesis.

A model is presented which correlates this observation with anatomical heterogenicity and *in vivo* responses to acute acidosis.

# 1. INTRODUCTION

## 1.1 Importance of pH Control

The effectiveness of the body's defences to changes in pH was first observed by Walter, who in 1877 discovered that on administration of very large acid loads to his experimental animals, death always occurred before he could demonstrate blood acidity with litmus paper (1). This observation is one of the most important aspects of homeostasis as properties and net charges of proteins are highly dependent on pH. Optimal activity of enzymes and proper function of the central nervous system must be maintained.

Under normal conditions, despite the continual barrage of acids and bases from the diet and metabolism into extracellular fluid, the $H^+$ concentration of blood of mammals is normally maintained between 4.47 and $3.55 \times 10^{-8}$ meq/l, or more conventionally, pH 7.35 to 7.45. Below pH 7.0 and above 7.7 a person can expect to survive no more than several hours as severe acidosis eventually leads to coma and alkalosis predisposes to tetany or convulsions.

To effect this precise regulation an elegant defense plan has evolved which encompasses action of the body fluids, lungs and kidneys. These function together employing chemical and physiological buffering, along with all levels of metabolic control. Due to the complexity and numerous clinical applications of this control system much research has followed Walter's initial observation. The mechanism of buffering and actions of the body fluids and lungs are well understood and will be dealt with only briefly. What remains to be elucidated is the regulation of renal response, or more precisely, the regulation of renal glutamine metabolism.

## 1.2 Sources of Acid Loads

The ingestion of preformed acids in the diet is a relatively minor source of acid-base disruption as most acids result from metabolic activity (2), that is the formation of lactic ,acetoacetic or $\beta$-hydroxybutyric acids. In normal physiological states these incomplete oxidations may account for 20-40 meq/day of the total acid excretion (2). During exercise or periods of hypoxia, lactic acid production increases and in abnormal conditions such as diabetes mellitus production of acetoacetic and $\beta$-hydroxybutyric acids may reach 500 meq/day (3).

In addition, there are various other sources of acid production, including the oxidation of organic sulphur to sulphuric acid, hydrolysis of organic phosphoesters to phosphoric acid and the metabolism of nucleoproteins to uric acid. Hence, the production of acid in the form of sulfuric and phosphoric acids is increased by the ingestion of a high protein diet or acceleration of protein catabolism. The average North American person excretes 40-80 meq $H^+$ per day (1).

In 1923, the great self-experimenter, J.B.S. Haldane, set out to induce a metabolic acidemia in himself. Following unsuccessful attempts to lower his blood pH by drinking HCl he worked out a number of chemical tricks "to smuggle the HCl into his blood disguised as something else" (4). One method is the administration of large doses of ammonium chloride. The $NH_4Cl$ is taken up by the liver and converted to urea:

$$2\ NH_4Cl + CO_2 \longrightarrow 2H^+ + 2Cl^- + (NH_2)_2CO + 2H_2O$$

The urea is excreted in the urine leaving the acid behind. This protocol is in wide use today as a means of inducing metabolic acidosis in experimental animals.

## 1.3 Defences Against Acid Challenges

The narrow range of pH is controlled by three main lines of defense: the acid-base buffer systems of body fluids, the respiratory system and the kidneys. Buffers, the first line of defense, are capable of partially neutralizing acids and thus preventing large fluctuations in pH. The phosphate buffer system at physiological pH is:

$$Na_2HPO_4 + H^+A^- \longrightarrow NaH_2PO_4 + NaA^-$$

Since the pK of this system is 6.8 it operates near its maximum buffering power. Despite the efficiency of this chemical buffering system its overall importance in the regulation of plasma pH is minimal due to its low concentration in extracellular fluids (2 mM). The concentration of phosphates in intracellular fluid is about 40 mM making them an important component of intracellular buffering. Due to the high concentration of proteins in the plasma and in cells, and their ability to act as buffers, proteins act as another important buffering component. The two most notable are albumin in the plasma and hemoglobin in the erythrocytes.

The most important physiological buffering system is the $CO_2$-bicarbonate system:

$$H_2O + CO_2 \longleftrightarrow H_2CO_3 \longleftrightarrow H^+ + HCO_3^-$$

The pH of body fluids therefore is determined by the Henderson-Hasselbalch equation:

$$pH = 6.1 + \log \frac{(HCO_3^-)}{(H_2CO_3)}$$

The apparently limited function of this system to operate at physiological pH (pK of 6.1) is reconciled when one considers the degree of control to which $CO_2$ and $HCO_3^-$ concentrations are subjected. $CO_2$ is constantly being produced as a product of metabolism and the respiratory system strives to achieve a constant $P_{CO_2}$ of 40 mm Hg by increases or decreases in ventilatory tidal volume. This control mechanism coupled with a relatively high concentration of plasma bicarbonate (26-28 meq/l) makes this buffer an important means of eliminating acid challenges. The carbon dioxide produced by the reaction of acids with bicarbonate is eliminated by a compensatory increase in ventilation. This form of control is not as rapid as direct chemical buffering and may take several minutes to readjust acid-base balance to normal, following a single insult (5). The respiratory system, in this manner, plays a major role in day to day maintenance of acid-base status, exhaling 13,000 meq $H_2CO_3$ as $CO_2$ daily (6).

Since the total buffering capacity of the body fluids is only about 1000 meq (3), or twenty times normal daily acid production, the overall defense system must become more complex and must contain a component which ensures replenishment of the bicarbonate levels. This important function is achieved by renal action.

## 1.4 Control of $HCO_3^-$ Concentration

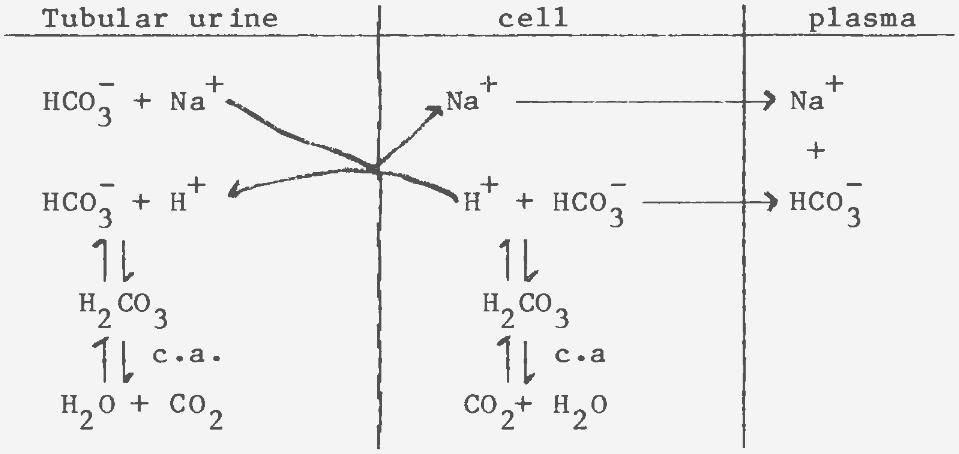
This action of the kidneys is a slower form of control than those previously discussed, requiring several hours to days to readjust the $H^+$ concentration to normal (1). That is, in a severe metabolic acidosis the $HCO_3^-$ concentration may drop drastically to a level which would necessitate respiratory action to restore the $HCO_3^-:CO_2$ ratio to 20:1 and therefore returning the pH to normal (Henderson-Hasselbalch equation). The function of the kidneys in restoring $HCO_3^-$ to its original concentration is achieved in two ways: by reabsorption and net formation of $NaHCO_3$ (Fig. 1). Both actions feature an exchange of $Na^+$ for $H^+$. It is believed that reabsorption is threshold limited with the cellular concentration of $H^+$ ions being the limiting factor (7), although there is evidence for a separate transport mechanism that does not involve $H^+$ excretion (8). In the exchange hypothesis, tubular $NaHCO_3$ is not reabsorbed directly. $Na^+$ is exchanged for $H^+$ across the luminal membrane of the tubular cells with the $H^+$ being buffered by tubular $HCO_3^-$. $CO_2$ diffuses into the cell and by action of carbonic anhydrase, $HCO_3^-$ is reformed within the tubular cell with subsequent diffusion across the antiluminal membrane. The threshold limitation of this reabsorption of $HCO_3^-$ is 25-28 meq/l (6). In metabolic alkalosis this threshold mechanism serves to decrease plasma bicarbonate as sufficient $H^+$ is not readily available for exchange with all the bicarbonate and the excess is excreted resulting in an alkaline urine.

In acidosis the kidney is capable of producing urine with a higher degree of acidity than the blood (pH 4.5-5.0). Concurrent with this excretion of acid is the formation of bicarbonate and reabsorption of $Na^+$ (Fig. 1b). Two notable features of this process, in addition to the elimination of $H^+$, are the prevention of $Na^+$ loss and the net production of $NaHCO_3$ which serves to increase plasma pH. Since the overall process depends on the formation of $H_2CO_3$ inhibition

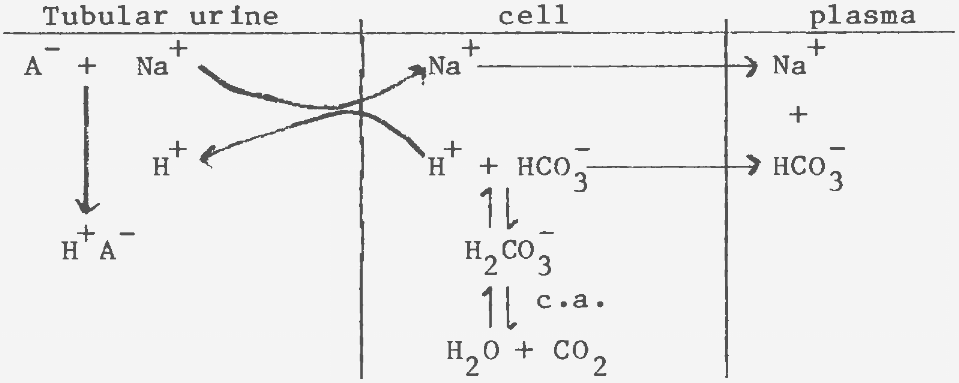
Fig. 1.1 (a) Reabsorption of $HCO_3^-$

(b) Formation of $NaHCO_3$

Figure 1.1(a) Reabsorption of $HCO_3^-$


Tubular urine
cell
plasma
$HCO_3^- + Na^+$
$Na^+$
$Na^+$
+
$HCO_3^- + H^+$
$H^+ + HCO_3^-$
$HCO_3^-$
$H_2CO_3$
c.a.
$H_2O + CO_2$
$H_2CO_3$
c.a
$CO_2 + H_2O$

(b) Formation of $NaHCO_3$


Tubular urine
cell
plasma
$A^- + Na^+$
$Na^+$
$Na^+$
+
$H^+$
$H^+ + HCO_3^-$
$HCO_3^-$
$H^+A^-$
$H_2CO_3^-$
c.a.
$H_2O + CO_2$

of carbonic anhydrase diminishes the secretion of $H^+$ (7).

## 1.5 Acid Excretion

Of the 40-80 meq acid excreted per day in man, 10-30 meq is in the form of titratable acid, that is bound to buffers (6). The salts formed by the buffering of acids with plasma $NaHCO_3$ are transformed into acid salts in the tubules (Fig. 2a):

$$Na_2HPO_4 + H_2CO_3 \longrightarrow NaH_2PO_4 \text{ (excreted)} + NaHCO_3 \text{ (reabsorbed)}$$

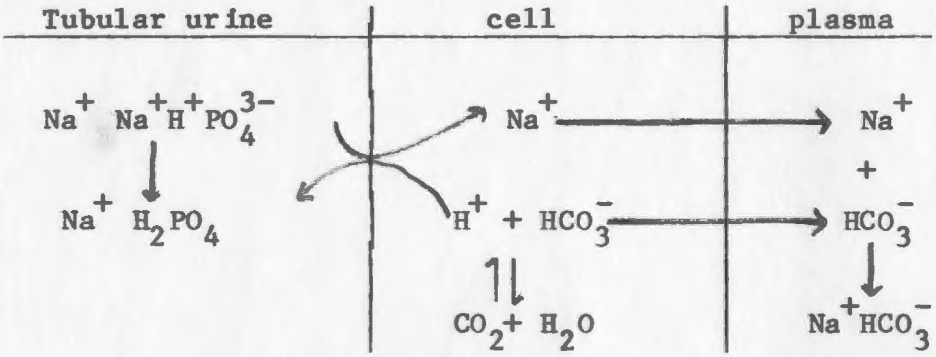
Because the pK of the phosphate buffer system (disodium phosphate to monosodium phosphate) is relatively close to the pH of the urine, $NaH_2PO_4$ can be excreted as free acid. This formation of titratable acid is limited by the concentration of phosphate and the fact that only one of the two sodium ions can be spared from excretion. As the pH of urine does not fall below 4.5 the low ionization constants of some important anions limit their excretion as free acids. Thus chloride and sulfate cannot be excreted as HCl or $H_2SO_4$. The kidney deals with this by forming ammonium salts (Fig. 2b). Although the ability to carry out this exchange of $Na^+$ for $H^+$ exists throughout the entire length of the nephron, the proximal tubular cells are specially adapted for carrying out a high volume exchange against a low pH gradient (with a concomitant reabsorption of bicarbonate) while the distal tubule cells secrete $H^+$ against a large gradient. Acidification of the urine therefore is a process which mainly occurs in the distal tubule and collecting ducts (Fig. 2b).

The relative contribution of the two means of acid excretion can be expressed as a ratio: $NH_3$/titratable acid which in normal man

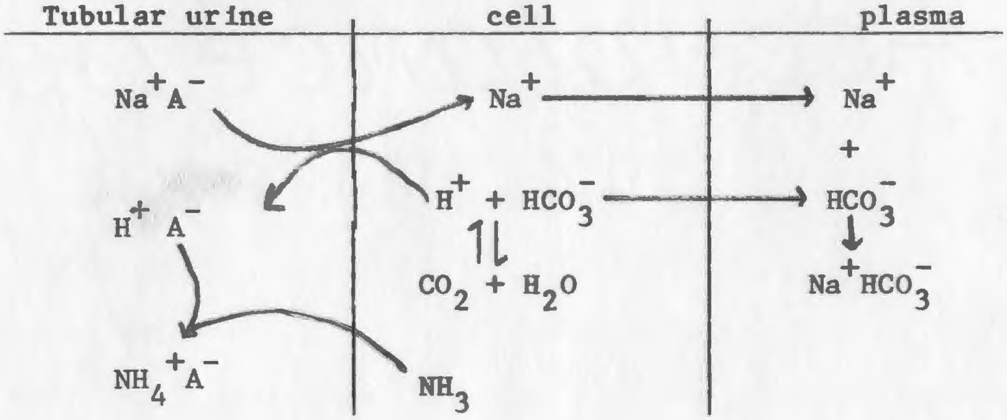
Fig. 1.2. (a) Formation of Titratable acid.

(b) Formation of Ammonium salts.

Figure 1.2(a) Formation of Titratable Acid:


Tubular urine
cell
plasma
$Na^+$ $Na^+H^+PO_4^{3-}$
$Na^+$ $H_2PO_4$
$Na^+$
$H^+ + HCO_3^-$
$CO_2 + H_2O$
$Na^+$
+
$HCO_3^-$
$Na^+HCO_3^-$

(b) Formation of Ammonium Salts


Tubular urine
cell
plasma
$Na^+A^-$
$H^+$ $A^-$
$NH_4^+A^-$
$Na^+$
$H^+ + HCO_3^-$
$CO_2 + H_2O$
$NH_3$
$Na^+$
+
$HCO_3^-$
$Na^+HCO_3^-$

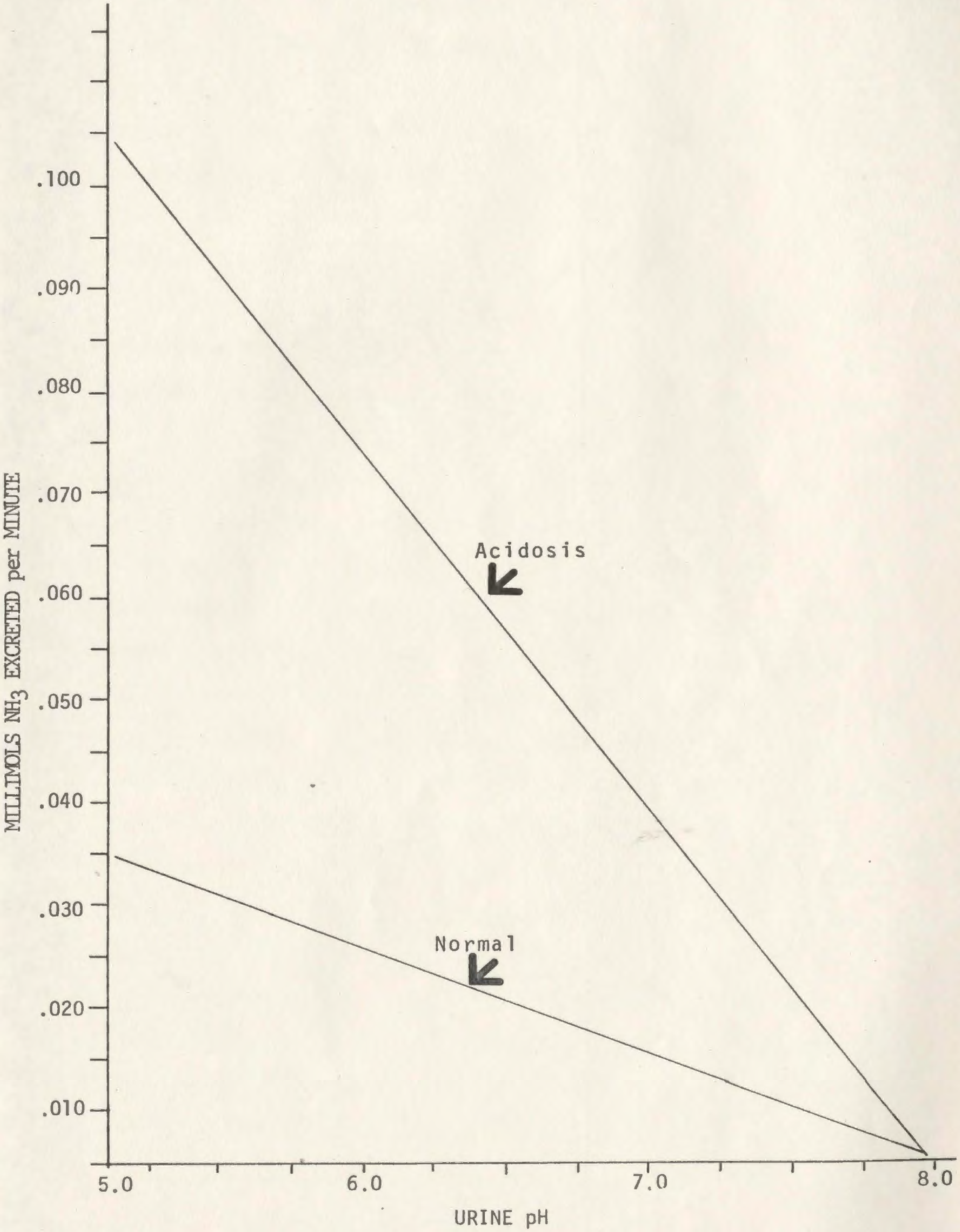
varies from 1.0-2.5. Under normal conditions the daily excretion of ammonium salts is 30-50 meq (6). Although the amount of phosphate buffer is fixed, $NH_3$ supply is adaptable and thus makes it an important component to the overall maintenance of acid-base balance.

## 1.6 Ammonia Production

Ammonia has long been known to accumulate in the urine inversely with pH, and Pitts in 1948 demonstrated this phenomenon by administering sodium bicarbonate to a dog to increase urinary pH during two conditions of acid-base balance: $NH_4Cl$-induced metabolic acidosis and under normal balance (9) (Fig1.3). In both conditions urinary $NH_3$ decreased inversely with pH over the range 5.2-8.0. This anticipated correlation was overshadowed by the approximately 2.5-3.0 fold increase in the urinary ammonia in the acidotic state for each given pH. This was the first indication that the rate of $NH_3$ excretion was under several forms of control, that is, direct pH of the urine and some factor which was dependent on a physiological adaptation mechanism.

That $NH_3$ is not merely extracted from the blood by the kidneys was first demonstrated in 1921 by Nash and Benedict, who showed that more ammonia left the kidney in the renal vein than was found in the renal artery (10). This important discovery established the fact that the kidney itself must be the organ of ammoniagenesis. Following this discovery many precursors were implicated in ammoniagenesis, including urea. This seemed a logical candidate due to the inverse relationship which was observed between urea and $NH_3$ excretion (11). This relationship has since proven not to be causal and is an expression of extra-renal control exerted by the liver (12). The urea normally

Fig. 1.3. Rate of excretion of ammonia by a single dog under two conditions of acid-base balance. In both conditions an infusion of sodium bicarbonate was administered to increase urine pH (1).


.100
.090
.080
.070
.060
.050
.040
.030
.020
.010
MILLIMOLS $NH_3$ EXCRETED per MINUTE
Acidosis
Normal
5.0
6.0
7.0
8.0
URINE pH

produced in the liver for excretion can be decreased allowing nitrogen availability for another synthetic reaction, the formation of glutamine. The major site of this formation in the dog appears to be the liver (13). In man, although this remains poorly understood, the primary site seems to be skeletal muscle (14,15).

Glutamine was first discovered to be the primary source of urinary ammonia in 1943 by VanSlyke et al. (16), who measured the arteriovenous differences across dog kidneys. By measuring all of the amino acids, and ammonia they concluded that the glutamine amide nitrogen could account for two-thirds of the ammonia excreted by acidotic kidneys. Twenty years later, with the advent of column chromatography and amino acid analyzers, Pitts confirmed the work of VanSlyke by measuring arteriovenous differences for twenty-three amino acids (17). In addition it was found that glutamate, the product of glutamine deamidation was not added in significant amounts to venous blood. This suggested the further removal of the amino group of glutamine as a contributory reaction to renal ammoniagenesis. Pitts, with the use of $^{15}N$ infusion studies into renal arteries of acidotic dogs went on to demonstrate that the nitrogens of plasma glutamine contribute 60% to the total ammonia leaving the kidney (18). Small contributions are made by glycine, valine, and proline with the remaining $NH_3$ being extracted from the blood. Similar results have been reported in the rat (19) and in man (20).

## 1.7 Ammonia as a Urinary Buffer

The virtues of ammonia as a buffer predispose it to being a cheap and efficient means of eliminating acid. Ammonia nitrogen is a waste product which would otherwise be excreted as urea. The reciprocity between excretion of urea and ammonia demonstrates that ammonia excretion is primarily an acid-base control mechanism rather than a mere means of nitrogen elimination. Glutamine, the major precursor of ammonia and the amino acid of highest concentration in blood (18,19,20) is synthesized from glutamate in the liver or skeletal muscle in a reaction requiring only one ATP. Deamidation in the kidney requires no energy.

A key factor in ammonia's ability to aid in acid excretion is determined by its diffusion properties. The free base, $NH_3$ readily diffuses throughout the kidney and into the renal tubules, whereas the ionized form, $NH_4^+$, is much less membrane soluble. Thus by "non-ionic diffusion" equilibrium is established between tubular urine $NH_3$ and $NH_3$ in tubular cells (21). The pK of this $NH_3$-$NH_4^+$ system is 9.1, meaning that a proportionally much greater amount of ammonia in the acidic urine will be in the ionized form and thus "trapped" and ultimately excreted.

### 1.8 Lcalization of $NH_3$ Production in the Nephron

Early micro puncture studies suggested that $NH_3$ was added along the entire length of the renal tubule (22). These studies were supported by microanalysis of $NH_3$-producing enzymes in the various segments of the tubule (23,24). Although $NH_3$ production capabilities were demonstrated in the distal straight, proximal convoluted and proximal straight tubules, only in the proximal convoluted did adaptation occur in acidosis (24). Recent studies have indicated that ammonia movement within the nephron is not as simple as had been previously thought. It has been demonstrated by micropuncture techniques that a significant $NH_3$ loss occurs between the proximal convoluted and distal tubules (25). Most of the excreted $NH_3$ is present in the proximal tubule whereas only 20-30% of this excreted amount can be detected in the distal tubule. Microcatheterization of the collecting duct in the same study has demonstrated that this $NH_3$ loss in nephron transit is added to the collecting duct fluid. Thus, it appears that by some mechanism $NH_3$ exits from the nephron, probably in the loop of Henle resulting in an increased medullary concentration which leads to a rapid non-ionic diffusion into the collecting duct as it courses through the papilla.

## 1.9 Pathways of $NH_3$ Metabolism

### 1.9.1 Phosphate independent glutaminase (EC 2.3.2.2, -glutamyl transpeptidase and -glutamyl transferase)

Phosphate independent glutaminase (PIG) catalyzes the deamidation of glutamine with the liberation of ammonia and is activated by maleate (26). This cytosolic enzyme was initially believed to be distinct from -glutamyl transpeptidase, an enzyme found in the brush border membrane but they have since been shown by cellular fractionation to be identical (27). It is non-specific, utilizing either D- or L-glutamine and has a low affinity.

Depending on the conditions, the enzyme can act either as a true glutaminase, converting glutamine to glutamate and ammonia or it can catalyse a transpeptidation reaction (28). Malate stimulates both reactions (28) and a decrease in pH favours the glutaminase reaction (29). Only low activities of PIG have been demonstrated in the kidneys of dog and man (30) and furthermore, its activity does not increase in acidotic conditions (31).

Welbourne has proposed that the cytoplasmic PIG pathway is the predominate pathway in the nonacidotic rat kidney, accounting for 70% of the total ammonia production (29,32,33). He bases his arguments on the fact that when rat kidneys are perfused with higher than physiological concentrations of glutamine (4 mM) a large amount of glutamine nitrogen remains unaccounted for following perfusion. He presumed this nitrogen was in the form of glutamyl peptides which are retained in the kidneys. Ross and Bullock (34) have since shown by measuring metabolites of glutamine recovered in the perfused kidney tissue that 65% of the deficit in nitrogen balance that Welbourne observed can be accounted for by glutamine alone, 19% by 5-oxopyrrolidine-2-carboxylate, 11% as intrarenal $NH_3$ and 5% by aspartate and glutamate. None of the deficit was attributable to glutathione. Thus this pathway is of relatively minor importance in overall ammoniagenesis.

### 1.9.2 Glutaminase II pathway (glutamine ketoacid aminotransferase, E.C. 2.6.1.15, and  -amidase, E.C. 3.5.1.3)

This cytosolic pathway was first localized by Cooper and Meister in 1974 (35). Glutamine ketoacid aminotransferase catalyzes the transamination of an amino group from glutamine to a keto acid with the subsequent formation of the respective amino acid and  -ketoglutaramate. The  -ketoglutaramate is then deaminated by the  -amidase to produce  -keto acid and ammonia. Even though this pathway has been documented in rat (36), dog (37), and human kidneys (38), it appears to contribute little to total ammoniagenesis in the kidney under normal conditions. In chronic acidosis, measured in vitro activity of the pathway in the rat increases (39,40). In the dog it remains controversial with some studies indicating an increase (37), and others suggesting no change (38). The activity of this pathway, however, is not sufficient to account for the increased ammonia production in chronic metabolic acidosis.

### 1.9.3 Phosphate-dependent glutaminase (EC 3.5.1.2)

Phosphate-dependent glutaminase (PDG), believed to be the main $NH_3$ producing enzyme, is stereo-specific with a high affinity for L-glutamine. Its exact localization in the kidney mitochondria remains controversial with some reporting localization to the matrix region (27) and others providing evidence for an inner mitochondrial membrane site (41,42). It is activated by inorganic phosphate and phosphorylated compounds. By gel ultrafiltration and velocity sedimentation the molecular weight of rat kidney PDG was determined in the absence of and presence of inorganic phosphate (43). It was found that dimers form in the activated state, and inhibition, by lowering the phosphate concentration, led to the dissociation of the dimers. Thus from this evidence it is believed that *in vivo* a dynamic equilibrium exists between the monomer and dimer forms and that this balance is regulated by the small molecular activator. The unusual feature of this system is the nonspecificity of the activator.

PDG activity was shown to be inhibited by increased glutamate concentrations by Krebs as early as 1935 (44). Since then this has been demonstrated to be a direct effect on purified PDG (45). This may be a key factor in the regulation of ammoniagenesis and will be discussed below.

Davies and Yudkin in 1952 showed that renal PDG activity in the rat increased in chronic acidosis (46). This increased PDG activity was later shown to be limited to the proximal convoluted tubule (24) and to occur gradually, reaching maximum activity after 3-5 days (9). Increased activity has also been demonstrated in rats fed high protein diets (47), and this adaptation was initially thought to be the rate-limiting factor in renal ammoniagenesis. This apparently logical explanation was shattered when Rector and Orloff found no adaptive increase in PDG activity in the dog kidney (48) and Goldstein demonstrated that administration of actinomycin D, which prevents protein synthesis inhibited PDG induction but did not prevent the increased rate of $NH_3$ excretion in rats made acutely acidotic (49). Clearly, in the normal acid-base situation the rates of flux through PDG are controlled and in acidosis there are metabolic changes which allow these fluxes to increase without increased enzyme synthesis.

### 1.9.4 Glutamate dehydrogenase (EC 1.4.1.2)

Glutamate dehydrogenase (GDH) is a mitochondrial enzyme which deaminates glutamate to $\alpha$-ketoglutarate with the production of $NH_3$. There are studies which show increased activity of this enzyme during acidosis in the rat (37,50). Schoolwerth reported a 50-fold increase in the flux through the enzyme in mitochondria from chronically acidotic rats. The total activity of the enzyme only doubled (50). Glutamate penetrates renal cortical mitochondria poorly (51) and thus hydrolysis of glutamine via PDG principally provides substrate for the GDH reaction. The combined action of PDG and GDH produces two molecules of ammonium. It is thought that GDH operates at near-equilibrium rendering it an unlikely site for initiation of metabolic control. However this near-equilibrium status has been considered an important link in the regulation of glutaminase by events distal to GDH exerting an influence on glutamate concentration. Some recent work has cast doubt upon the belief that GDH is a near-equilibrium reaction (50). In this study isolated mitochondria from rat renal cortex were incubated under normal and acidotic conditions. Changes in the mass-action ratio were not supportive of equilibrium dynamics.

### 1.9.5 Glutamine synthetase (EC 6.3.1.2)

Glutamine synthetase is a cytosolic enzyme found primarily in the proximal straight tubule (5). It irreversibly catalyzes the formation of glutamine from glutamate and $NH_3$ with the expenditure of ATP. The role of this enzyme in the regulation of ammoniagenesis has largely been ignored when compared to the other enzymes of $NH_3$ metabolism. This is due to the conventional belief that GS is not present in the kidney of dog and man (52,53) and that an adaptive increase in ammoniagenesis does occur in these two mammals. On review of the literature, however, measured activities of GS have been detected, although at a much lower activity level than in the rat kidney (Table 1) (30).

In the rat GS activity is greatest in the outer medulla with only 60% of this maximal activity located in the cortex. In acidosis the activity in the medulla falls to the cortical rate (30). Janicki and Goldstein (54) found no effect of metabolic acidosis on GS activity although they used whole kidney preparations and did not make the above distinction. *In vivo* studies by Damian and Pitts (53) demonstrated a decrease in renal glutamine synthesis in chronic metabolic acidosis. Hems reported in 1972 that in the perfused rat kidney glutamine synthesis was inhibited when the perfusate acidity was increased from pH 7.4 to 7.1 (55). It is thus apparent that GS activity is influenced by

TABLE 1.1 Rates of GS Activity in Renal Tissue Homogenate in Rat, Dog and Man.

| | RAT | | DOG | | MAN | |
|---|---|---|---|---|---|---|
| | CORTEX | MEDULLA | CORTEX | MEDULLA | CORTEX | MEDULLA |
| Normal | 230 | 312$^{+}$ | 25 | 45$^{+}$ | 24 | 18 |
| Acidotic | 199 | 182$^{++}$ | 21 | 38 | | |

+ significantly different from cortex

++significantly different from normal acid-base status

units - moles -glutamyl monohydroxymate formed/g tissue per hour

FROM: Guy Lemieux, G. Baverel, P. Vinay and P. Wadoux. GS + GT in the kidney of man, dog and rat, Amer. J. Physiol 231 (4): 1068-1073, 1976.

chronic and acute acid-base alterations and that these changes would be reflected in the measured rates of "ammoniagenesis" by inhibition or stimulation of $NH_3$ utilization. GS can be considered as a component of a single, operationally reversible reaction with PDG, the forward and reverse rates differing with varying microenvironmental changes.

## 1.10 Theories of Control Mechanisms

Study of intermediary metabolism is difficult in the kidney because of problems of cellular and subcellular heterogeneity. In addition the anatomy of the kidney and the interrelations between various parts of the nephron make studies with isolated cells vulnerable to criticism. For these reasons the vast literature which has been accumulated in this field is littered with ambiguities and discrepancies.

### 1.10.1 Tubular transport of glutamine

Glutamine is taken up by the tubular cells via the luminal and antiluminal membranes. In acute acidosis the blood glutamine concentration increases (56) and in chronic acidosis a slight decrease is seen (57). Recently, Alleyne has shown an increased transport of glutamine in brush border vesicles from acidotic rat kidneys (58). However it is unlikely that this is an important factor in the regulation of ammoniagenesis since all of the filtered glutamine is reabsorbed in normal and acidotic rats.

### 1.10.2 Mitochondrial transport of glutamine

PDG, the main ammoniagenic enzyme is a mitochondrial enzyme and therefore much interest has been focused on the control of glutamine entry into the mitochondria through the inner membrane. Adam and

Simpson (59) have shown that although the process is carrier mediated and can be inhibited by mercurials, it is not an active transport mechanism and thus cannot function against a concentration gradient. The carrier was initially thought to be a glutamine-glutamate antiport (60). Brosnan and Hall in 1977 (61) demonstrated, in isolated mitochondria, by measuring the metabolism of [1-$^{14}$C]glutamine in the presence of glutamate, and of [1-$^{14}$C]glutamate in the presence of glutamine that the uptake and metabolism of extramitochondrial glutamate was insufficient to account for the observed rate of glutamine metabolism and uptake. This suggested a uniport transporter which with further studies was found to be specific for L-glutamine (61,62). This site has been implicated and carefully studied as a possible control point in the ammoniagenic response to acid-base changes. The activity of the glutamine transporter has been shown to increase in chronic acidosis (59,63) and this was postulated to be the rate-limiting step in ammonia production. This assumption was supported by the inability to demonstrate a measurable amount of glutamine within the mitochondria. It was felt that the inability to detect intramitochondrial glutamine was due to the high activity of PDG and that the deamination of glutamine may even be associated with the uniport mechanism. This proposed glutamine carrier-glutaminase complex was thought to release glutamate into the matrix and in acidosis the carrier moiety adapts either by an increase in activity or an increase in binding sites with the resultant presentation of more glutamine to PDG. This model would explain the observed increase in ammoniagenesis in isolated mitochondria from acidotic dog kidneys which fail to demonstrate an *in vitro* increased PDG activity. This work has been challenged by

Curthoys *et al*. (52) who repeated these experiments using a more highly purified mitochondrial preparation. Mitochondria isolated by routine differential centrifugation are contaminated with brush border fragments and the resulting activity of phosphate independent glutaminase can cause difficulties in the interpretation of transport studies. Curthoys *et al*. avoided this problem by purifying their mitochondria. When these preparations were incubated with 0.5 mM $[^{14}C]$glutamine significant levels of glutamine which approached the incubation medium concentration in both control and acidosis could be detected within the matrices. These experiments demonstrate that PDG is not linked with the carrier protein and glutamine is not cleaved in the process of transport. They do however support the earlier observation that the rate of transport is increased in acidotic conditions. The regulation of this uptake remains unclear. pH does not appear to be regulatory as the rate of ammonia production from added glutamine was found to be relatively constant between pH 7.0 and 7.7 (41).

### 1.10.3 Metabolic concentration regulation

#### 1.10.3.1 Glutamate

As mentioned above, glutamate is an end-product inhibitor of PDG and it has been postulated that it may play a role in the overall rate of ammonia formation (44,45). It has been reported that renal glutamate concentrations are decreased in acidosis (52,64). Glutamate can be deaminated by the action of GDH or it can undergo transamination with oxaloacetate to form -ketoglutarate and asparatate. The latter reaction which is catalyzed by glutamate-oxaloacetate transaminase (GOT) is not increased in metabolic acidosis (65). Flux through GDH may be controlled by its products -ketoglutarate, NADH and $NH_3$ if the kinetics are near equilibrium (65). Each of the end products could thus be implicated as rate controlling. Schoolwerth _et al_. have demonstrated a stimulated flux through mitochondria from acidotic rats that cannot be attributed to increased activity of the enzyme. By measuring fluxes across PDG, GOT and GDH they have shown that external glutamate is metabolized primarily by transamination in control rat mitochondria and that GDH flux is markedly increased in metabolic acidosis. The mass action ratio changes suggest non-equilibrium regulation and are more consistent with an increased flux which is secondary to the provision of more glutamate from glutamine deamidation. Another problem with the glutamate inhibition theory is that not all studies have shown a decrease in glutamate concentration with acidosis and indeed an increase in glutamate has been observed with a concurrent stimulation of ammoniagenesis by acidosis (66).

It has been suggested that decreased $NH_3$ concentration may be a critical stimulus for increased ammoniagenesis in acute metabolic acidosis (67). In the perfused kidney preparation it was demonstrated that following a control period, the acute lowering of the bicarbonate concentration in the perfusion medium caused an immediate decrease in urine pH. This increase in urine acidity was paralleled by an increase in ammonia production and excretion. From this Tannen and Ross postulated that the lowering of the urine pH caused an immediate trapping of $NH_3$ in the urine and thus decreased $NH_3$ concentration in the tubule mitochondria. This would release end product inhibition on GDH and elicit an increased ammoniagenesis. Although this may prove to be a contributing factor in the _in vivo_ response to acid challenge it certainly is not the sole mechanism involved as evidenced by the response which can be achieved in isolated tubules to acute acidosis.

### 1.10.3.2 Gluconeogenic theory

Phosphoenolpyruvate carboxykinase (PEPCK) (EC 4.1.1.32) activity was shown to increase in chronic acidosis by Alleyne and Scullard in 1969 (68). This enzyme catalyzes the conversion of oxalacetate to phosphoenol pyruvate (PEP), a rate controlling reaction in the gluconeogenic pathway. Thus -KG produced by the deamination of glutamate is converted to maleate which leaves the mitochondria and enters the gluconeogenic pathway. This increased conversion of -KG to glucose was suggested to be responsible for the stimulation of ammonia production. The importance of this relationship between ammoniagenesis and gluconeogenesis was clarified by Stoff et al. (69). When glutamine, a neutral substance, forms -KG and $NH_3$ there is a release of two protons:

$$\text{Glutamine} \ \underline{\text{PDG}} \ \text{Glutamate}^- + NH_3 + H^+$$

$$\text{Glutamate}^- \ \underline{\text{GDH}} \ \text{-KG}^{2-} + NH_3 + H^+$$

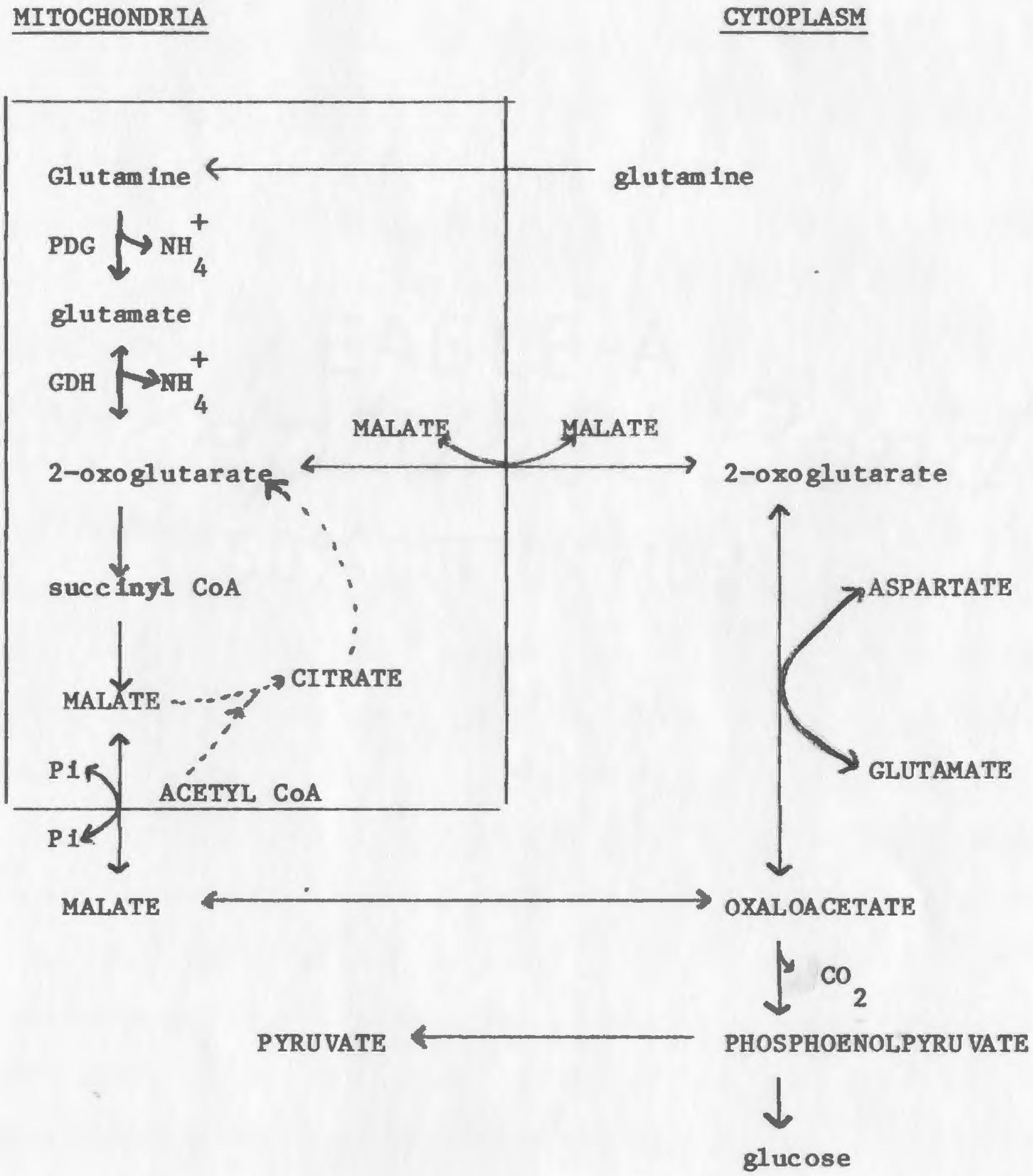
To be an efficient adaptive mechanism for excretion of acid these must be neutralized. This is accomplished by the subsequent conversion of -KG to glucose or to $CO_2$ and $H_2O$:

$$KG^{(2-)} + 2H^+ \quad 5CO_2 + 5H_2O$$

$$2\ KG^{(2-)} + 4H^+ \quad 4CO_2 + \text{glucose}$$

Therefore without this metabolic linking of two pathways there would be no net gain of bicarbonate to the body. As shown on Fig. 4, PEPCK is required for either total oxidation or the conversion to glucose of -KG's carbon skeleton. As PEPCK is a cytosolic enzyme, -KG must be transported out of the mitochondria and this is accomplished in the form of malate.

Fig. 1.4. A diagram of glutamine metabolism in the rat kidney cortex with pathway of glucose formation. The key ammoniagenic enzymes PDG and GDH are included and Krebs cycle reactions are indicated with broken arrows.


MITOCHONDRIA
CYTOPLASM
Glutamine
glutamine
PDG
$NH_4^+$
glutamate
GDH
$NH_4^+$
MALATE
MALATE
2-oxoglutarate
2-oxoglutarate
succinyl CoA
ASPARTATE
CITRATE
MALATE
GLUTAMATE
Pi
ACETYL CoA
Pi
MALATE
OXALOACETATE
$CO_2$
PYRUVATE
PHOSPHOENOLPYRUVATE
glucose

PEPCK is an important enzyme in gluconeogenesis and is probably rate controlling. Increased PEPCK activity is accompanied by increased rates of gluconeogenesis from all substrates that enter the pathway prior to this enzyme. Tissue slices from kidneys of acidotic rats have an increased gluconeogenic capacity from oxaloacetate but not from glycerol or fructose (70). Burch _et_ _al_. have shown that PEPCK activity is highest in the proximal convoluted tubule and the activity in this region increases 4-fold in acidosis (71). The role of PEPCK regulation of ammoniagenesis however is debatable. Originally it was believed that the enhanced activity of PEPCK is responsible for increased ammoniagenesis through the stimulation of glutamine metabolism by the lowering of the gluconeogenic precursors. One difficulty is in the early stages of acidosis during which time ammoniagenesis is stimulated but increased PEPCK activities do not occur until 6-12 hours after induction of acid challenge (72). Tissue -KG and malate levels however decrease after two hours. Possibly in the early stages enzyme synthesis is not necessary for an increased rate of gluconeogenesis. Chemical inhibitors have been employed in studying the role of PEPCK. Kidneys perfused with 3-mercaptopicolinate, a specific inhibitor of PEPCK, demonstrated an inhibition of the adaptive increase in ammonia formation in acidotic kidneys (73). This suggests that the gluconeogenic pathway may be more than merely a salvage reaction for the -KG produced by combined PDG+GDH action and may play a part in the regulation of ammoniagenesis. Major difficulties do exist with the theory that PEPCK activity can regulate ammoniagenesis. It is very unlikely that all reactions between PEPCK and glutamine are poised close to equilibrium so that they may be accelerated by a pull. Tissue concentrations of malate and aspartate are both elevated by the PEPCK inhibitor, probably due to inhibition of oxaloacetate conversion to phosphoenolpyruvate. Despite this, tissue -KG levels fall in response to increased acidity to the same level as in the absence of the inhibitor (73). This indicates a more proximal site of control.

### 1.10.3.3 -ketoglutarate dehydrogenase

The -ketoglutarate dehydrogenase complex (KGDH) catalyzes the oxidation of -ketoglutarate to succinyl-CoA. This reaction has a large decrease in standard free energy and is irreversible. Because of its low *in vitro* activity this site has been implicated as being a regulatory enzyme. It is now well accepted that a low pH decreases renal -ketoglutarate concentrations. Lowered -ketoglutarate concentrations have been demonstrated under acidotic conditions in the intact animal (65,74), the perfused rat kidney (73), and in isolated tubules (75). This alteration is seen in both the chronic model (65) and in acute acidosis (73,74). Boyd and Goldstein (74) in their study of renal metabolite concentrations in acute acidosis found that administration of 5.0 mmol/kg $NH_4Cl$ by stomach tube produced a 44% decrease in mean kidney -ketoglutarate level after one hour and 20.0 mmole/kg $NH_4Cl$ administration produced a 69% fall. Malate concentration was also decreased. Tissue ammonia content increased and no significant change in glutamate concentration was detected. This fall in -KG has been postulated to be a regulatory factor in ammoniagenesis and physiological concentrations inhibit transport of glutamine into renal mitochondria (76). Rat kidneys perfused in the presence of mercaptopicolinate, an inhibitor of PEPCK, demonstrate a lower -ketoglutarate concentration in response to acid challenge (73).

This work clearly indicates that the increased $H^+$ effect is proximal to PEPCK and KGDH has been implicated. In 1979, McCormack and Denton (76) demonstrated that heart muscle KGDH had an increased affinity for $\alpha$-ketoglutarate when the pH was lowered. Lowry and Ross (75) observed the same in rat kidney KGDH and found that in isolated tubules presented with an acid challenge the decrease in $\alpha$-ketoglutarate concentration was coincident with a 64% increase in gluconeogenesis and a 33% stimulation of ammoniagenesis from 1.0 mM glutamine. The acid-base effects on KGDH kinetics appear to be clear, however the implications of the acid stimulation of the enzyme remains uncertain with respect to the control of gluconeogenesis and ammoniagenesis. An apparent discrepancy occurs in the kidney perfused with mercaptopicolinate in which $NH_3$ production is not enhanced despite the decrease in tissue $\alpha$-ketoglutarate. This suggests that additional biochemical stimuli are required for acceleration of ammoniagenesis.

### 1.10.4 Unknown regulatory factors

When urine is allowed to drain freely back into the perfusion medium in the perfused rat kidney preparation the ammoniagenic stimulation in response to acute acidosis is abolished (77). No evidence has been found for the description of this factor. Recently Alleyne *et al*. (78) have shown that a non-protein, dialysable factor is found in plasma of acutely acidotic rats which he claims induces a conformational change on PDG and subsequently activates it. In slices of normal kidneys, preincubated with plasma obtained from acutely acidotic rats he has demonstrated a stimulation of $NH_3$ production. The nature of this factor remains obscure.

## 1.11 Summary of Metabolic Changes in Chronic Metabolic Acidosis

In response to a chronic acid load the rat excretes maximal rates of ammonia in the urine by three days (57). This is paralleled by increased renal arteriovenous differences for glutamine which are maximal after 2 days of acid challenge. In the first few days there is an appreciable acidification of the blood but as the renal compensation occurs the acid-base status improves. PDG activity peaks by day 3 at a level of 3-4 times control activity. The time sequence of PDG activation when compared with $NH_3$ excretion demonstrates some dissociation in acidosis. The peak in ammonia excretion occurs some 24 hours before the peak in glutaminase activity. PEPCK activity elevates in acidosis to about 3 times control levels, peaking in about 2 days. This increase in activity corresponds well with the excretion of $NH_3$. Thus, in chronic acidosis there is good correlation between measured enzyme activities and the observed compensatory renal response to acidosis-$NH_3$ excretion.

## 1.12 Recovery from Chronic Acidosis

Parry and Brosnan have measured the enzyme activities during induction and recovery from chronic metabolic acidosis (57). When rats are given an acid challenge by drinking 1.5% $NH_4Cl$ for 7 days and the acid challenge then removed, urinary $NH_3$ excretion drops from the high adapted rates to control levels within 24 hours. This is a marked difference from the activity of glutaminase which remains elevated 7 days following withdrawal of challenge. PEPCK activity parallels $NH_3$ excretion and rapidly returns to control values. This important series of observations in recovered animals demonstrates that the *in vivo* regulation of $NH_3$ production is not dependent upon the capacity of the mitochondrial enzymes. Isolated mitochondria have demonstrated elevated rates of ammoniagenesis even after *in vivo* production has normalized. This was thought to be due to extra-mitochondrial control which is supported by data obtained in renal tissue slices. In an attempt to dissociate PEPCK activity from ammonia production Brosnan and Parry (79) accelerated recovery from metabolic acidosis by administrating $NaHCO_3$ by gastric intubation. With this protocol urinary $NH_3$ excretion was back to normal by 8 hours whereas PEPCK activity remained elevated. This study suggests that an alkalosis is an important factor in the normalization of glutamine metabolism following acidosis. A marked alkalosis occurs during the first 24 hours of recovery from acidosis (57). However in 8-hour recovered control animals an alkalosis had yet to be seen, whereas in 8-hour recovered bicarbonate intubated rats a mean blood pH of 7.50 was observed. $NH_3$ excretion remained elevated in the control group at 282 mol $hr^{-1}$ 100 g body $wt^{-1}$, and decreased in the bicarbonate intubated group to 98, with the non-acidotic control excretion rate being 18.7. It is evident from these data that alkalosis may play a role in the decrease in ammoniagenesis seen during recovery. In the same study a decrease in ammoniagenesis from isolated mitochondria was observed after 8 hours of recovery by using physiological concentrations of phosphate. Previous studies which had shown an elevation of glutamine metabolism in mitochondria from recovered rats (47) had used high phosphate concentrations (20 mM). Enzyme flux measurements indicated that the suppression of ammoniagenesis in the mitochondria from bicarbonate intubated rats was almost entirely due to inhibition of GDH.

## 1.13 Acute Acidosis

Despite the fact that enzymatic adaptations do not occur in acute acidosis, a stimulation of $NH_3$ production is obtained in vivo (112) in the perfused kidney (67) and in isolated cortical tubules (75). Several possible mechanisms of regulation have been alluded to in previous sections which may also be important in the acute situation (urinary trapping of $NH_3$, KGDH activation). In the past, most studies have concentrated on the adaptations in chronic acidosis, however currently interest is shifting to the acute situation for insight into the control of ammoniagenesis.

## 1.14 Purpose of this Study

Although much is known about renal response in metabolic acidosis there are many unanswered questions. Some of the more important questions are: What is the rate of glutamine transport into mitochondria and how is it (if it is) regulated? What is the role of GS? What is the mechanism of the acute effects of $H^+$? Does glutamine metabolism in a good *in vitro* system (i.e. kidney perfusion) parallel *in vivo* responses during recovery? What is the role of PEPCK in the regulation of ammoniagenesis?

Obviously these are too varied and involve too many technical approaches to be dealt with in one study. It was decided, however, to concentrate on several of these problems in an attempt to help clarify the understanding of this complex field.

### 1.14.1 Recovery in the perfused kidney

Although it has been demonstrated in tissue slices of rat kidney that glutamine metabolism during recovery does parallel the *in vivo* situation I felt there was a need to repeat this in a better *in vitro* system. Balaban *et al*. (80) have provided evidence for the existence of anoxic zones in slices rendering them of questionable usefulness in the investigation of renal cortical metabolism.

This objective required that the kidney perfusion system, a technically demanding tool, be set up and functionally defined.

### 1.14.2 The role of PEPCK

PEPCK has been studied extensively in an attempt to establish the relationship between gluconeogenesis and ammoniagenesis. Inhibitors of this enzyme have been employed. However because the effect of inhibiting an enzyme in a pathway is so profound, interpretation of the results is difficult. Recently a hyperglycemic agent, aminopicolinic acid has been shown to stimulate PEPCK (81). The effect of this agent on gluconeogenesis and ammoniagenesis in the perfused kidney was felt to be an excellent approach to studying the role of PEPCK in acid-base regulation. Each kidney could serve as its own control by adding the aminopicolinate following a period of perfusion during which base-line metabolic rates could be determined.

### 1.14.3 Study of acute acidosis in isolated renal tubules

This problem was felt to be best approached with the tubule preparation due to the ease of data collection and thus time efficiency. The important question to be addressed here is the role of G.S. in acute acidosis. The specific approach taken was the use of MSO, an inhibitor of G.S. Unpublished data from our laboratory demonstrated significant cycling between glutamine and glutamate and the role of this cycling was investigated.

# 2. MATERIALS AND METHODS

The research in this thesis was completed in two laboratories. The initial work was done in the Department of Biochemistry at Memorial University of Nfld., and included the setting up of the perfusion system, the kidney perfusion study of recovery from acidosis and the study of the effect of aminopicolinic acid on gluconeogenesis. The rest of the laboratory work was completed in the Department of Clinical Biochemistry at the Radcliffe Infirmary, Oxford, England. This included virtually all the work with isolated cortical tubules including rates of ammoniagenesis from glutamine alone and glutamine plus lactate under normal and low pH conditions. The various techniques will be described as performed in Newfoundland with differences, if any, between the same work in the two laboratories noted at the end of each respective section.

## 2.1 Materials

### 2.1.1 Animals

Male albino Sprague-Dawley rats obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, LaPrairie, Quebec were used. They were fed Purina Rat Chow (Ralston Purina of Canada Ltd., Don Mills, Ontario) and weighed 250-500 gms when used.

In the Oxford experiments male albino rats of the Wistar strain were obtained from Charles River Ltd., Margate, Kent. They were maintained on laboratory rat chow (Dixon and Sons Ltd., Ware, Herts).

### 2.1.2 Substrates, enzymes, and cofactors

Obtained from Sigma Chemical Company, St. Louis, Missouri.

### 2.1.3 Radioisotopes

$^{14}C$-inulin was obtained from New England Nuclear, Lachine, Quebec.

### 2.1.4 Other biochemicals

Heparin (1000 units/ml) was obtained from M.T.C. Pharmaceuticals, Hamilton, Ontario. Bovine serum albumin was purchased from Sigma Chemicals Ltd. and Miles Laboratories Ltd., Slough, Bucks.

### 2.1.5 Anesthesia

"Somnotol" (sodium pentobarbital 65 mg/ml) was obtained from M.T.C. Pharmaceuticals, Hamilton and ether from Mallinckrodt Inc., St. Louis, Missouri.

### 2.1.6 Chemicals

Aquasol and Omnifluor were obtained from New England Nuclear, Boston, Massachusetts. All shelf chemicals and buffers were obtained from Fisher Scientific Company, Fair Lawn, N.J. or BDH Chemicals Ltd., Montreal. NCS Tissue Solubilizer was purchased from Amersham Corporation, Illinois.

## 2.2 Methods

### 2.2.1 Treatment of animals

On arrival the animals were housed in standard rat cages, with hardwood chip bedding. Not more than 4 rats were kept in one cage. They were maintained for a minimum of 3 days before use in experiments to allow time to adjust. The animal room was self-contained with a 12 hour diurnal light-dark cycle. The rats had free access to food at all times and the "normal" rats had free access to tap water. In the "acidotic" animals, the tap water was substituted with 1.5% $NH_4Cl$

for a 7-day period. In the "recovered" rats, tap water was reintroduced for 24 or 48 hours following a 7-day course on $NH_4Cl$. The "rapid recovery" group was, following a 7-day induction of acidosis, administered an intragastric bolus of $NaHCO_3$ (1.5 mmoles/100 g body wight). In order to perform this with minimal trauma to the animals, light ether anaesthesia was employed which resulted in immobilization for 1-2 minutes. Control rats were intubated with NaCl (1.5 mmoles/100 g body weight). These rapid recovery animals were sacrificed at 8 hours for subsequent kidney perfusion. During this period they were allowed free access to food and water.

### 2.2.2 Kidney perfusion

#### 2.2.2.1 Operative technique

The surgical technique developed was a modification of the procedures described by Nishiitsutsuji-uwo *et al*. 1967 (83), Ross *et al*., 1973 (84) and Bowman, 1969 (85). Well fed rats were anaesthesized by intraperitoneal injection of sodium pentobarbital, 0.1 ml/100 g rat weight of 65 mg/ml solution. A midline abdominal incision was made along the relatively avascular linea alba from the xiphoid process to a point where the abdominal aorta bifurcates. The incision was retracted laterally, and the intestine placed to the animal's left and covered with saline soaked gauze. The right kidney was used for perfusion because the superior mesenteric artery arises from the aorta at the same level as the right renal artery (Fig. 2.1). Following the localization and clearing of the right ureter from the surrounding adipose tissue it was cannulated with Portex tubing size PE10 (internal diameter 0.28 mm; external diameter 0.61 mm). This was tied in place with surgical thread Surgilene 3.0 (as was used in all ligatures). The superior mesenteric artery, aorta and renal artery were then cleared and loose ligatures placed around them as shown in Fig. 2.1. The right adrenal artery was tied off and cut distally. At this point the kidney was cautiously freed from the surrounding adipose tissue with care so as not to directly traumatize the organ. Heparin, 1000 units, was then injected into a femoral vein. A small Bulldog clamp was then placed on the mesenteric artery between ligature #5 and the aorta. Ligature #3 was then tied and the mesenteric artery was cannulated with a blunted 18 gauge butterfly cannula. This was advanced to the clamp and secured with ligatures #4 and #5. The clamp was then removed. Back flow did not occur through the cannula as the tubing was clamped proximal to the cannula with a hemostat. The cannula was filled to the tip prior to insertion into the mesenteric artery with gassed, warmed (38°C) washout medium (described below) under a gravity pressure head of 76 cm. Once cannula was secured, flow of washout medium was initiated by the removal of the hemostat and within 2 seconds the renal vein was transected near the inferior vena cava. Thus no interuption of renal flow occured and at this point in the procedure (about 10-15 sec) the kidney was perfused simultaneously by perfusion medium and host blood. The kidney blanched from its normal beefy red colour to a yellow-brown within 3 seconds following the initiation of perfusion medium flow. The venous outflow drained freely into the abdominal cavity. The last step involved tightening the ligatures placed about the aorta proximal and distal to the origin of the right renal and superior mesenteric arteries and transecting the aorta so as to free the kidney from the animal. Flow was switched from washout medium to recirculating medium and the kidney transferred to the perfusion cabinet. The entire procedure took from 10-20 minutes from initial incision to installation in the cabinet.

#### 2.2.2.2 Perfusion medium

A 10% solution of bovine serum albumin was made up in standard Krebs-Henseleit physiological saline (86). This was dialyzed for 48 hours at 4°C against a 3.5 times volume of Krebs-Henseleit solution which was gassed with 95% $O_2$ - 5% $CO_2$. The dialysate was changed three times during the 48 hours. The dialyzed product was frozen and stored in 67 ml aliquots. On the day of perfusion an aliquot was thawed and added to 33 ml of Krebs-Henseleit buffer for a final albumin concentration of 6.7%. The solution was gassed and passed through a 0.45 $\mu$m pore size Millipore filter immediately before use. The entire 100 ml was added to the perfusion circuit and recirculated during the surgical operation. This allowed time for temperature equilibrium (to 38°C) and adequate oxygenation (gassed with 95% $O_2$ and 5% $CO_2$).

The initial wash-out perfusion medium was a protein-free solution of Krebs-Henseleit, as employed by Bowman (85).

The pH of the perfusion medium was checked with an Instrumentation Laboratories pH Blood Gas Analyzer, model 213 and adjusted if necessary. $PO_2$ and $PCO_2$ measurements were also done with this instrument.

Substrates were added to the medium prior to filtration. Oleate was prepared by the method of Ross et al. (87). This was done by making a 0.1M solution by placing 1.0 mmole of free fatty acid in a

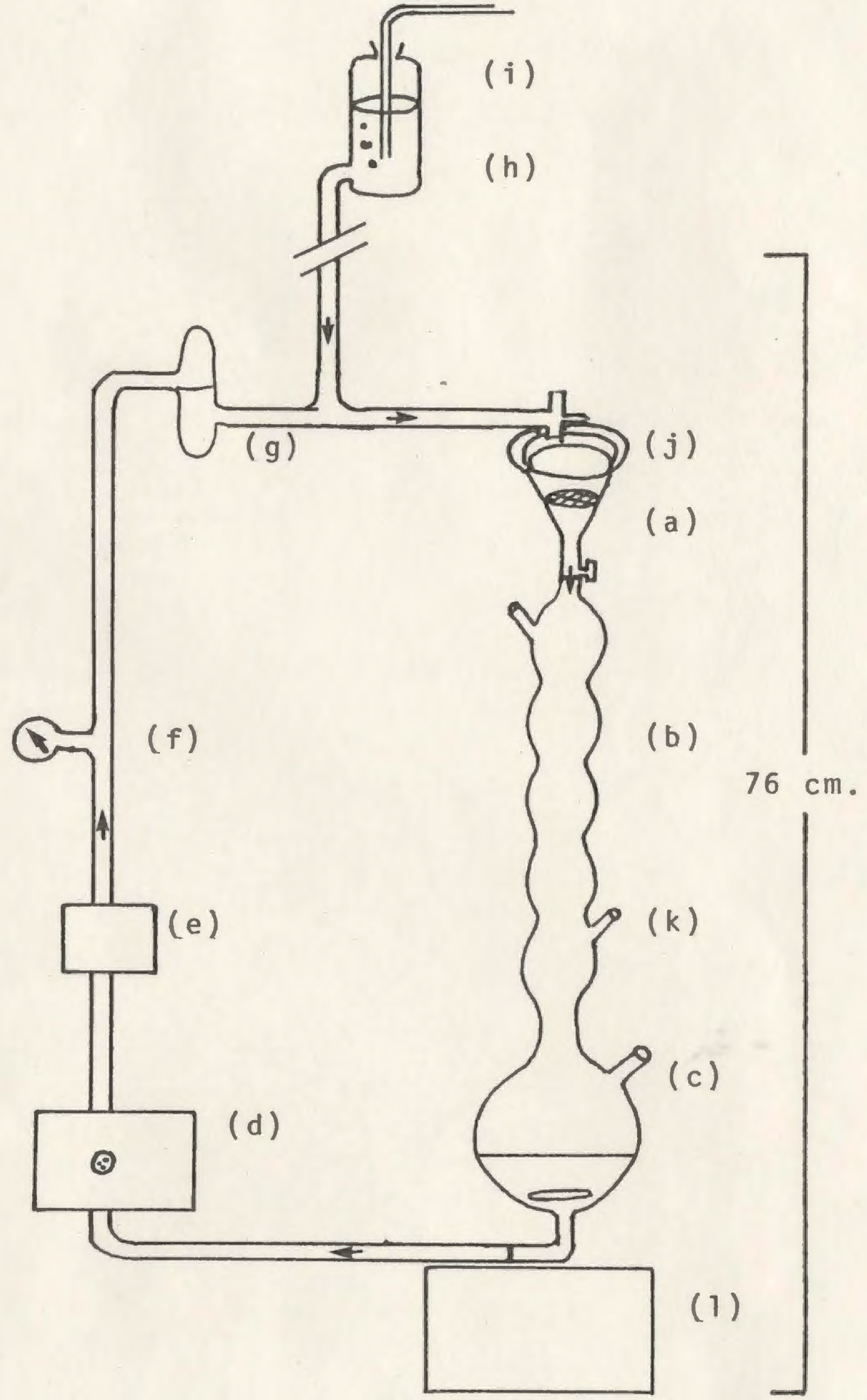
10 ml graduated test tube and adding water to give a total volume of 8.5 ml. The tube was then heated to about 60°C and slightly more than 1 ml of NaOH was added drop-wise while the test tube contents were agitated on a Vortex mixer. The fatty acid dissolved after the addition of a small excess of alkali. The volume was then made up to 10 ml and an appropriate amount was added to the perfusion medium to give a final concentration of 1.2 mM. Perfusion medium concentrations were measured by Dr. Phil Davis on a Packard GLC.

#### 2.2.2.3 Perfusion system

The perfusion system, as diagrammed in Fig. 2.2 was housed in a plexiglass cabinet and maintained at 38°C. The system was composed of a funnel shaped receptacle fitted with a stopcock and a mesh insert on which the kidney rested. The funnel was specially slotted to allow the exit of the PE10 ureter cannula, at a level horizontal to the kidney. The outflow of the funnel was continuous with a glass lung oxygenator, which was constantly gassed with 95% $O_2$ - 5% $CO_2$. A 100 ml reservoir received the oxygenated medium and this pool was stirred magnetically. Samples were drawn from a port in this reservoir. The outflow from this reservoir was drawn through a Millipore 8.0 $\mu$m in-line filters and through an apparatus, especially designed to eliminate air bubbles. A pressure gauge was connected in the system. From the outflow of the bubble trap to the organ tray was a length of tubing sufficient to allow the cannulation in the surgical procedure with subsequent transfer to the organ tray. A T-junction into this length of tubing allowed inflow from the washout reservoir which was located 76 cm above the bench top. With the use of hemostats flow through the appropriate tubing could be controlled.

Fig. 2.2 Isolated rat kidney perfusion system.

a. organ tray; b. oxygenator; c. sample port; d. roller pump; e. in-line filter; f. pressure gauge; h. washout reservoir; i. gas inlet, j. inflow cannula; k. gas inlet; l. magnetic stirrer.


(i)
(h)
(g)
(j)
(a)
(b)
(f)
76 cm.
(e)
(k)
(c)
(d)
(l)

The washout reservoir contained 100 ml of Krebs-Henseleit solution gassed with 95% $O_2$ - 5% $CO_2$. The tubing used in the circuit was of silicone rubber. Pressure was maintained at 100-120 mm Hg by adjusting the perfusion flow rate. Flow rate varied from 25-35 ml/min. The total volume of the circulating medium was about 90 ml.

#### 2.2.2.4 Handling of samples

At timed intervals, samples were removed from the reservoir through the sampling port. Samples for enzymatic analysis of metabolites were deproteinized with 6% $HClO_4$ (.5 ml sample in 1 ml $HClO_4$) and neutralized with 3N $K_3PO_4$. Samples for amino acid analysis were deproteinized with 10% SSA, and adjusted to pH 2.2 with lithium citrate buffer.

Following perfusions the kidney was blotted dry, weighed and placed in a drying oven for a minimum of 48 hours to obtain dry weight.

#### 2.2.2.5 Assessment of renal function

Measurement of GFR was achieved by adding $^{14}C$-inulin to the perfusion medium. Simultaneous perfusate and urine samples were taken and solubilized with NSC Solubilizer (5 parts NCS to 1 part sample). Raioactivity was measured in these samples dissolved in Omnifluor/Toluene with a Beckman Model CS-330 liquid scintillation counter. The GFR was calculated by the following equation:

$$\text{GFR} = \frac{\text{urine inulin (dpm)} \times \text{urine flow rate}}{\text{perfusate inulin (dpm)}}$$

Sodium reabsorption was calculated by measuring sodium in perfusion medium and urine using a Varian Techtron, Model AA-5 Atomic Absorption Spectrophotometer, and applying the following formulae:

$$\text{Na filtered} = \text{plasma Na} \times \text{GFR}$$

$$\text{Na excreted} = \text{urine Na} \times \text{urine flow}$$

$$\text{Na reabsorbed} = \frac{\text{Na filtered} - \text{Na excreted}}{\text{Na filtered}} \times 100$$

### 2.2.3 Preparation of isolated mitochondria

Following perfusion of kidney for 60 minutes, the kidney was freed from the system and the capsule removed. It was quickly cut into quarters and the medulla excised. The cortex was then chopped into small pieces and resuspended in ice-cold homogenization medium consisting of 0.25 M sucrose, 5.0 mM Hepes and 1.0 mM EDTA at pH 7.4. The slices were homogenized by hand and mitochondria isolated by differential centrifugation as previously described (27).

The physiological integrity of the isolated mitochondria was assessed by measuring the respiratory control ratio. This was performed in a Clark-type oxygen electrode at 28°C. The respiratory medium contained 10 mM TRIS-HCl, 120 mM KCl, 25.33 mM $KH_2HPO_4$ 20 mM MOPS and 6.33 mM $MgCl_2$. A substrate ( -ketoglutarate 2.0 mM) was supplied and the rate of $O_2$ uptake was measured during state 3 respiration and during the state 4 (ADP exhausted) respiration that followed. Respiratory control ratio is state 3/state 4.

### 2.2.4 Freeze-clamping procedure and measurement of adenine nucleotides

Following 80 minutes of perfusion, the kidney was immediately clamped (with no prior interruption of perfusate flow) with Wollenberger tongs that had been pre-cooled in liquid nitrogen. The tongs flatten the kidney to wafer thickness and freeze the tissue in as close to an instantaneous fashion as can be achieved. The frozen tissue was then ground to a fine powder in the presence of liquid nitrogen in a mortar and pestle. This powder was then deproteinized with 6% perchloric acid and neutralized with KOH (65). Adenine nucleotides were assayed in the supernatant following the final centrifugation. ATP, ADP and AMP were measured as to the method described by Bergmeyer (88).

### 2.2.5 Electron microscopy

The perfused kidney was examined under the electron microscope for anatomical observations secondary to the perfusion technique. These were compared with unperfused kidneys as controls. The technique followed in the 'unperfused' kidneys involved going through the normal surgical procedure with wash-out period with Krebs-Henseleit buffer. Then instead of switching to the recirculating perfusion medium as is normally done, the kidney was perfused with 100 ml of 1% glutaraldehyde made up in 0.15 M NaCl.

In the 'perfused' kidneys the normal perfusion was performed (60 minutes). A washout with Krebs-Henseleit saline was necessary to remove the albumin and the 1% glutaraldehyde solution (100 ml) was then perfused. The samples were all handled similarly following the fixation with glutaraldehyde. This protocol ensures rapid fixation of the tissue as the fixative is actually perfusing the organ via the normal vasculature. The organ, once fixed with glutaraldehyde was dropped into a beaker of Karnovsky's fixative (a mixture of paraformaldehyde and glutaraldehyde), delivered to the staff of the electron microscopy unit, Health Science Complex, within 30 minutes where the tissue was handled by a rapid processing method (89). Essentially, it was washed in 0.1 M sodium cacodylate, post-fixed in

1% osmium tetroxide, and dehydrated by washing in a series of alcohol solutions. The sample was then embedded in polypropylene capsules for sectioning. Sectioning was done with a Reickart OMU3 ultramicrotome. Sections were examined with a Philips EM300 electron microscope. Photography and developing were also performed in the EM unit.

### 2.2.6 Renal cortical tubules

#### 2.2.6.1 Isolation of tubule cells

The preparation of isolated cortical tubules required that two rats be anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg body wt). The four kidneys were removed and placed in ice-cold Krebs-Henseleit saline. They were decapsulated, sliced longitudinally and demedullated. The procedure is a modification of Guder *et al*. (90). Each half-kidney cortex was sliced by hand with a razor blade and the resulting slices were placed in 10 ml Krebs-Henseleit saline containing 10 mg collagenase (BCL, Lewis, Sussex) in a 250 ml Erlenmeyer flask, gassed continously with 95% $O_2$: 5% $CO_2$ and incubated for 45 minutes in a 37°C water bath with vigorous shaking (100 cycles/min). The incubation was stopped by addition of 40 ml ice-cold Krebs-Henseleit saline and the suspension filtered (1 mm pore) to remove undigested tissue. The tubules were washed in ice cold Krebs- Henseleit, centrifuged for 15 sec at 140 g and resuspended three times. The final volume of the suspension was made to six to seven times the volume of the packed cells to yield a tubule concentration of about 10-15 mg dry weight/ml.

#### 2.2.6.2 Incubation of cortical tubules

Tubular incubations were performed in stoppered 25 ml siliconized Erlenmeyer flasks in a 37°C shaking water bath at 50 cycles/minute.

The flasks were prepared while tissue digestion incubation was in progress (as seen in fig. 2.3 for sample experiment). When the flasks were prepared and the tubules ready, 0.5 ml of the tubule suspension was added to each flask, and the flask was gently gassed with 95% $O_2$ - 5% $CO_2$ for 45 sec. The flask was then stoppered and the 30 minute incubation begun. The total incubation volume was 2.0 ml. Agitation of the shaking bath insured sufficient oxygenation of the tubules. The incubations were terminated by the addition of 0.2 ml of 30% (v/v) perchloric acid. Initial metabolite concentrations were obtained by the addition of tubules to two flasks containing perchloric acid (i.e. time = 0 minutes). The deproteinized samples were centrifuged and the supernatant neutralized with $K_3PO_4$. Since each cortical tubule preparation varied in the concentration of tubules the procedure was standardized by the determination of the dry weight of each suspension by drying an aliquot for 24 hours. This weight was corrected for solute weight (from Krebs-Henseleit saline) by a factor of 10.1 mg/ml.

### 2.2.7 Measurement of metabolites

All glucose, $NH_3$ and glutamate assays were measured by the enzymatic methods as described by Bergmeyer (88) on a Gilford Model 240 spectrophotometer in Newfoundland. In Oxford, a Zeiss spectrophotometer was used. Glutamine and some of the glutamate measurements were performed on a Beckman Model 121 Automatic Amino Acid Analyzer.

### 2.2.8 Statistical analysis

Results of experiments are presented as mean plus or minus one standard deviation. Statistical analysis was performed with

Fig. 2.3. Typical cortical tubule experiment.

| H38<br>Flask # | .2M lactate | .1M glutamine | .2M glutamate,Na | .05M $NH_4Cl$ | 1.0N HCl | MSO | K-HS | tubule suspension | time | total vol. | $HClO_4$ | $K_3PO_4$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1,2 | .05 ml | .02 ml | | | | | 1.430 | 0.5 | 30 min | 2.0 | 0.17 ml | 0.20 ml |
| 3,4 | ↓ | ↓ | | | .034 | | 1.396 | ↓ | ↓ | ↓ | ↓ | (after 30 min) |
| 5,6 | ↓ | ↓ | | | | .02 | 1.410 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 7,8 | ↓ | ↓ | | | .034 | ↓ | 1.376 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 9,10 | ↓ | | .02 | | | | 1.430 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 11,12 | ↓ | | ↓ | | .034 | | 1.396 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 13,14 | ↓ | | ↓ | | | .02 | 1.410 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 15,16 | ↓ | | ↓ | | .034 | ↓ | 1.376 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 17,18 | ↓ | | ↓ | .015 | | | 1.415 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 19,20 | ↓ | | ↓ | ↓ | .034 | | 1.381 | ↓ | ↓ | ↓ | ↓ | ↓ |
| 21,22 | ↓ | | ↓ | | | | 1.410 | ↓ | 0 | ↓ | ↓ | (at 0 min) |
| 23,24 | ↓ | | ↓ | .015 | | | 1.395 | ↓ | ↓ | ↓ | ↓ | |

appropriate use of paired or unpaired t-test with $p = .05$ being taken as the upper limit for statistical significance.

Rates of glutamine uptake, $NH_3$ and glucose production in the perfused kidney were calculated as linear regression analysis from 5 to 60 minutes in all experiments.

# 3. RESULTS

## 3.1 Assessment of perfused kidney system

Using $^{14}C$ inulin the urine/perfusate ratios in our perfusions with 1 mM glutamine was 9.0 with a resulting GFR of 0.31 ml/min. In perfusions with 4 mM glucose as well as 1 mM glutamine the urine/perfusate ratio increased to 20.0 and the GFR to 0.70 ml/min. $Na^+$ reabsorption in the perfusions with glucose was found to be 94%. The most variable parameter with respect to renal function proved to be the rate of urine formation. An important factor in this large variation was found to be the albumin. Miles Laboratories produced an albumin which was better suited for maintaining physiological function in the perfused kidney, that is, more consistent urine flow rates (with perfusion flow rate and pressure being constant). Albumin obtained from Sigma caused the urine production to decrease to very low values. This may be a reflection of impurities in the prepared protein. For this reason we routinely used the Pentex brand albumin from Miles. Consistent rates of gluconeogenesis were obtained which were linear over the perfusion period (Fig. 3.1). Rates of glucose production from 1 mM glutamine in the well fed, normal rat averaged 3.65 moles $gww^{-1}$ $hr^{-1}$ (Fig. 3.2).

Ross et al. (84) report a glucose production under the same conditions which is comparable, 4.40 moles $gww^{-1}hr^{-1}$. Thus our perfusions were similar to those of other workers.

The perfusion system was assessed with respect to oxygen, pH and $CO_2$ status during the perfusion period. By cannulating the renal vein in several perfusions it was found that the renal $PO_2$ did not fall below 160 mm Hg and that the $PCO_2$ rose slightly in the venous flow but did not exceed 36 mm Hg. The pH was maintained close to 7.4. Thus $O_2$ delivery to the perfused kidney was adequate. To test this presumption at the biochemical level adenine nucleotides were measured in a freeze-clamped kidney following 30 minutes of perfusion. The total nucleotide concentration was found to be 1.678 moles/gww (ATP = .960; ADP = .550; AMP = .163). This is a somewhat lower value (by 27%) than concentrations measured in our laboratory in *in vivo* freeze clamped kidneys (ATP = 1.520; ADP = .670; AMP = .120; total 2.310). However, when one considers the higher wet/dry ratio in the perfused kidney compared to the *in vivo* situation and corrects for this, the values for total adenine nucleotides are nearly identical (perfused = 8.76 mole/gdw; *in vivo* = 9.10 mole/gdw).

Cortical mitochondria were isolated following 30 minutes of perfusion to test their biochemical integrity. This was thought to be a crucial parameter as the mitochondria play a central role in renal glutamine metabolism. A respiratory control ratio was determined using -ketoglutarate as a substrate. Ratios obtained were high (RCR = 9.2 ± 1.05; n = 3) indicating good metabolic function.

The perfused kidney has been shown to retain fluid, resulting in elevated wet/dry weight ratios. Nishiitusutsujiuwo *et al*. (83) observed that the ratio increases from 3.94 in the unperfused kidney to 5.63 in the perfused. This change, also obtained in this study (5.22) is probably due to increased interstitial fluid volume because of the greater flow rate.

In addition to these physiological and biochemical parameters we examined the perfused kidney under the electron microscope to detect

Fig. 3.1. Perfusion with 10 mM pyruvate

1. pyruvate ●
2. glucose ▲
3. lactate x

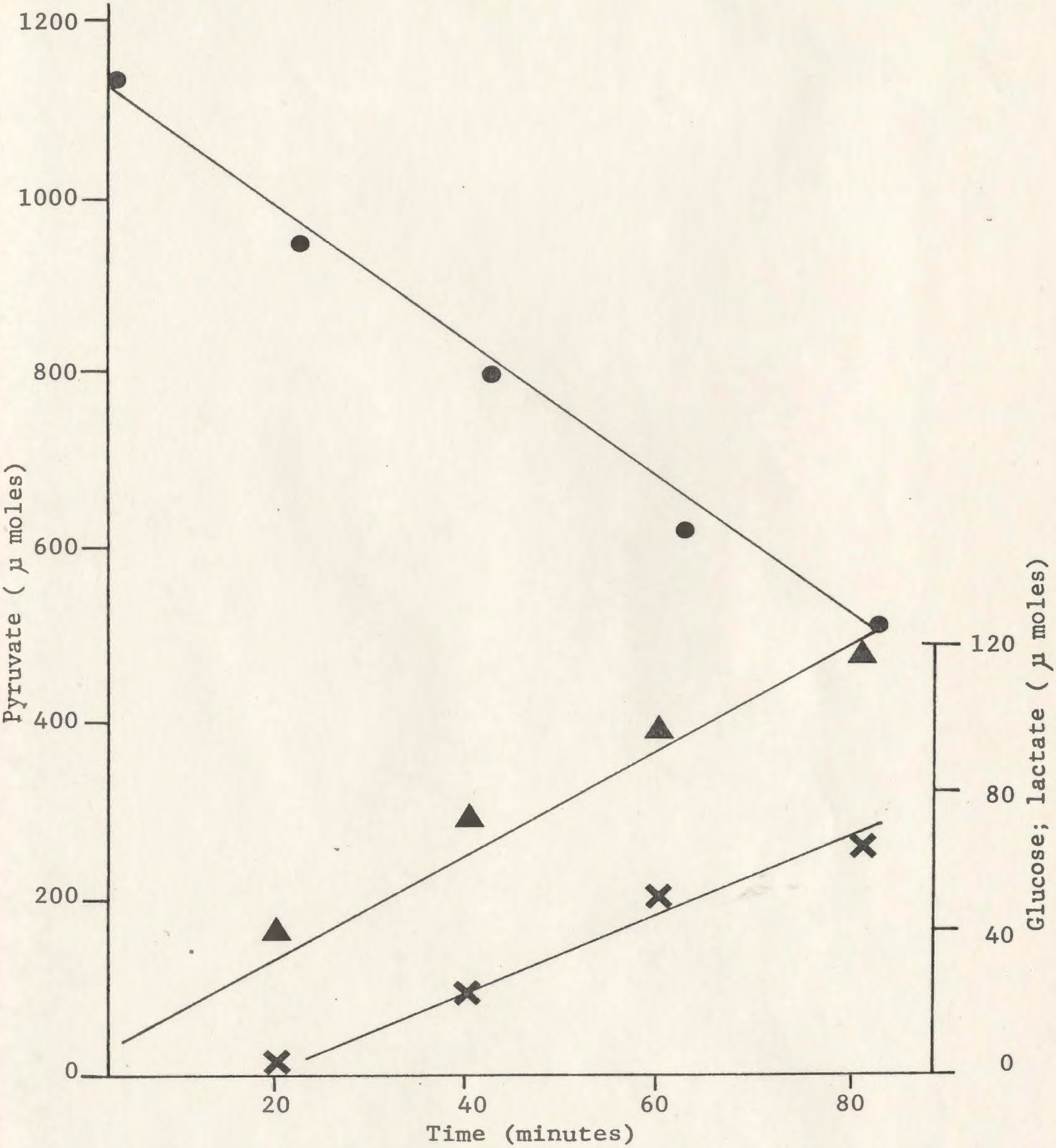
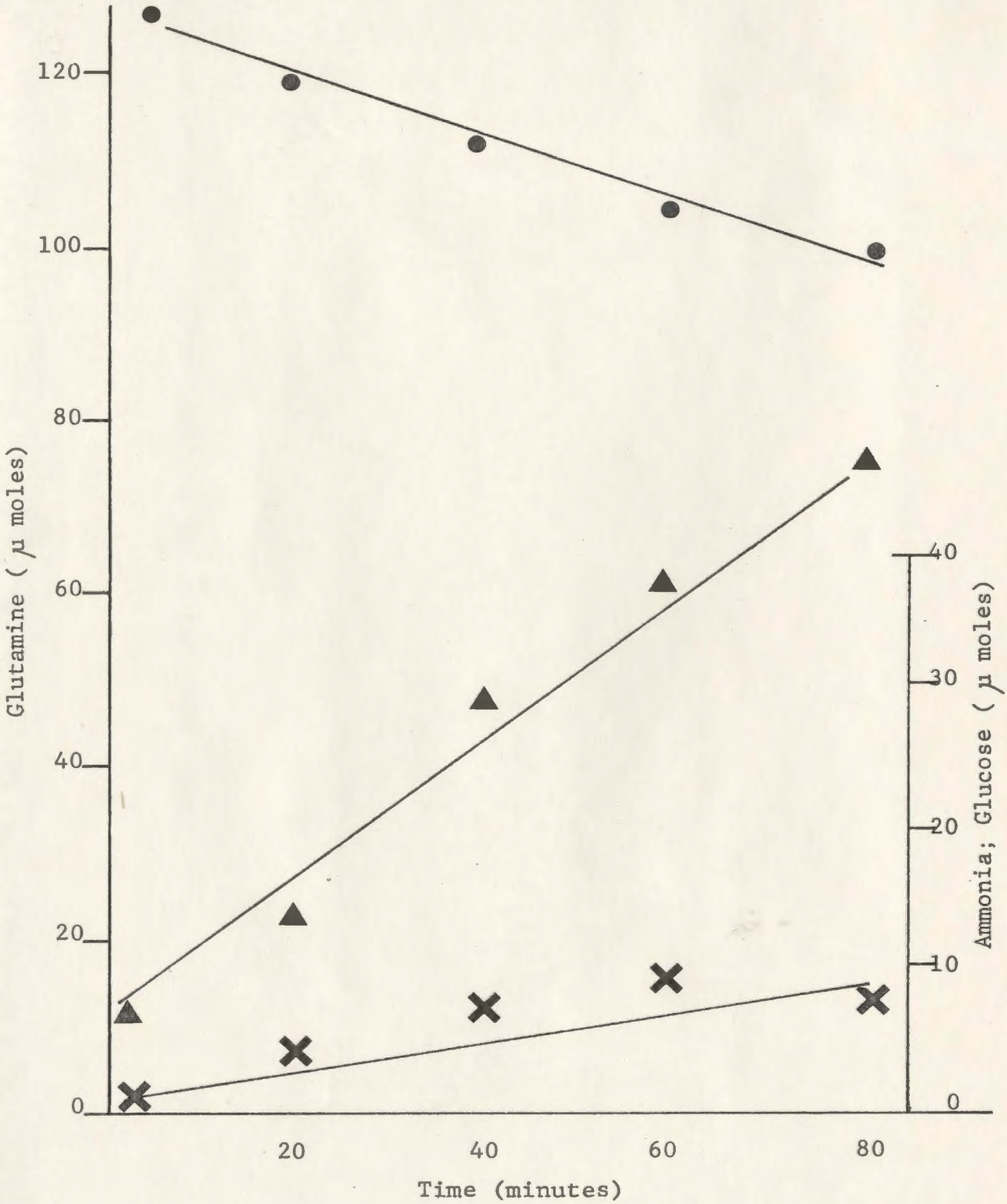

1200
1000
800
600
400
200
0
Pyruvate ( µ moles)
20
40
60
80
Time (minutes)
120
80
40
0
Glucose; lactate ( µ moles)

Fig. 3.2 Perfusion with 1 mM glutamine

1. glutamine ●
2. $NH_3$ ▲
3. glucose x



Glutamine ( μ moles)
120
100
80
60
40
20
0
Ammonia; Glucose ( μ moles)
40
30
20
10
0
20
40
60
80
Time (minutes)

fall below 160 mm Hg and that the $PCO_2$ rose slightly in the venous flow but did not exceed 36 mm Hg. The pH was maintained close to 7.4. Thus $O_2$ delivery to the perfused kidney was adequate. To test this presumption at the biochemical level adenine nucleotides were measured in a freeze-clamped kidney following 30 minutes of perfusion. The total nucleotide concentration was found to be 1.678 μmoles/gww (ATP = .960; ADP = .550; AMP = .163). This is a somewhat lower value (by 27%) than concentrations measured in our laboratory in _in vivo_ freeze clamped kidneys (ATP = 1.520; ADP = .670; AMP = .120; total 2.310). However, when one considers the higher wet/dry ratio in the perfused kidney compared to the _in vivo_ situation and corrects for this, the values for total adenine nucleotides are nearly identical (perfused = 8.76 μmole/gdw; _in vivo_ = 9.10 μmole/gdw).

Cortical mitochondria were isolated following 30 minutes of perfusion to test their biochemical integrity. This was thought to be a crucial parameter as the mitochondria play a central role in renal glutamine metabolism. A respiratory control ratio was determined using α-ketoglutarate as a substrate. Ratios obtained were high (RCR = 9.2 ± 1.05; n = 3) indicating good metabolic function.

The perfused kidney has been shown to retain fluid, resulting in elevated wet/dry weight ratios. Nishiitusutsuzi _et al_. (83) observed that the ratio increases from 3.94 in the unperfused kidney to 5.63 in the perfused. This change, also obtained in this study (5.22) is probably due to increased interstitial fluid volume because of the greater flow rate.

In addition to these physiological and biochemical parameters we examined the perfused kidney under the electron microscope to detect any abnormalities or changes in the microanatomy after perfusion. Because the metabolic process under consideration in this study occurs in the proximal convoluted tubule primarily, attention was focused on this part of the nephron.

As seen in Fig. 3.3 and 3.4 in normal, unperfused proximal tubules a regular brush border is obtained. The cells can be seen to contain numerous mitochondria with, as is better demonstrated in Fig. 3.4, an apparent alignment which is perpendicular to the antiluminal membrane. In Fig. 3.5, a high magnification of the proximal tubule in a 7-day chronically acidotic rat, no changes can be seen in the mitochondria as compared to the normal condition. In the perfused kidney, Fig. 3.6, a regular brush border is seen with open tubular lumens. The alignment of the mitochondria is lost, which could to be due to physical compression due to the greatly increased number of pinocytotic vesicles. These vesicles are possibly a result of the high concentration of albumin in the perfusate and function to transport the albumin across the cell. No evidence of hypoxic damage to the tissue is seen.

## 3.2 Ammonia production in the perfused kidney

### 3.2.1 General goals

The objective of the first series of experiments was to measure rates of ammoniagenesis from glutamine in kidneys from rats that had been experimentally manipulated in a number of ways. In all of these situations the rates of ammoniagenesis *in vivo* were already known and the activities of the key enzymes were also known. However, reliable information on ammoniagenesis in a good intact-cell *in vitro* situation

Fig. 3.3. EM of normal rat proximal convoluted tubule (11,628X).

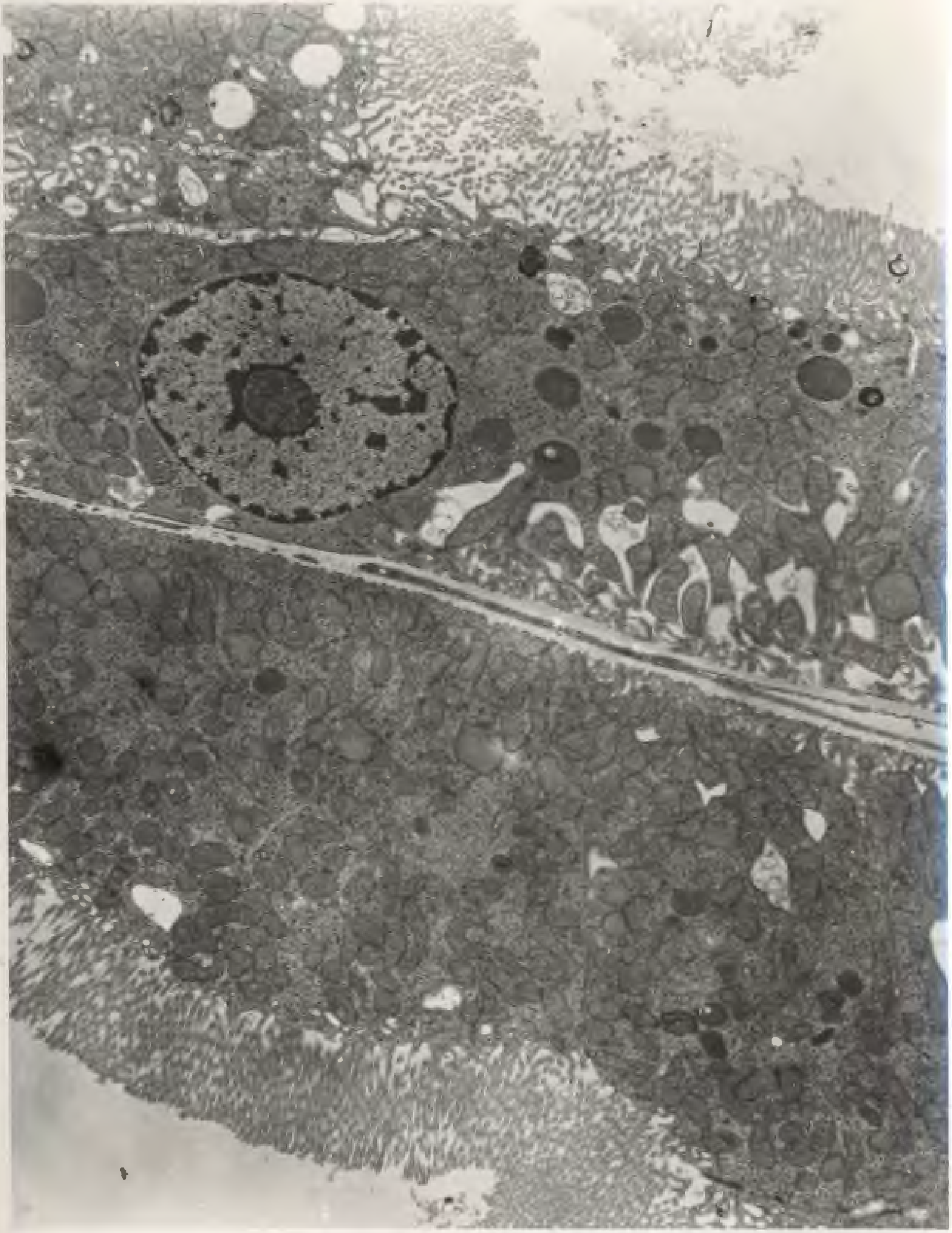


Fig. 3.4 EM of normal rat proximal convoluted tubule (20,520X).

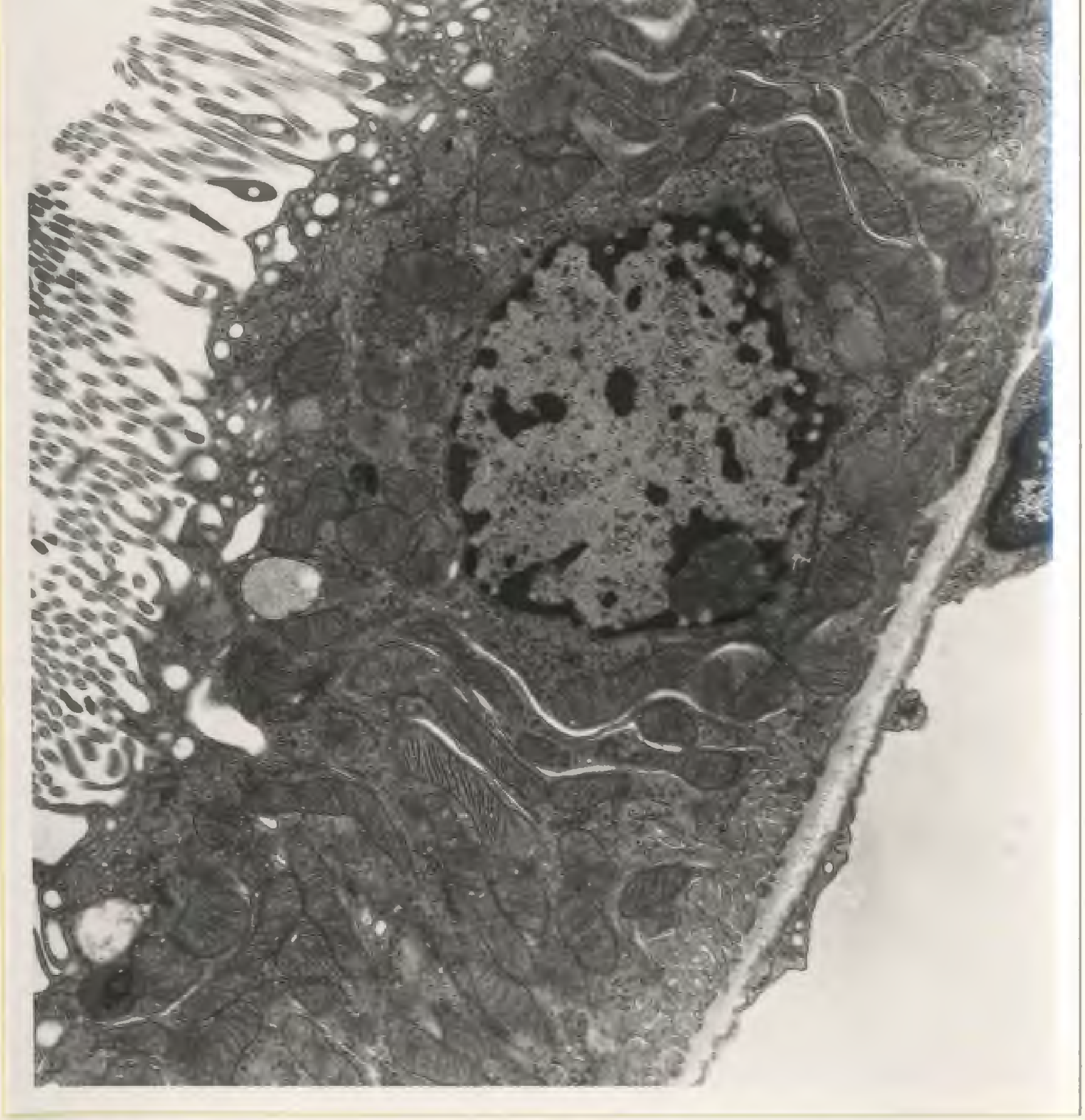


Fig. 3.5 EM of 7-day acidotic rat proximal convoluted tubule (20,520X).

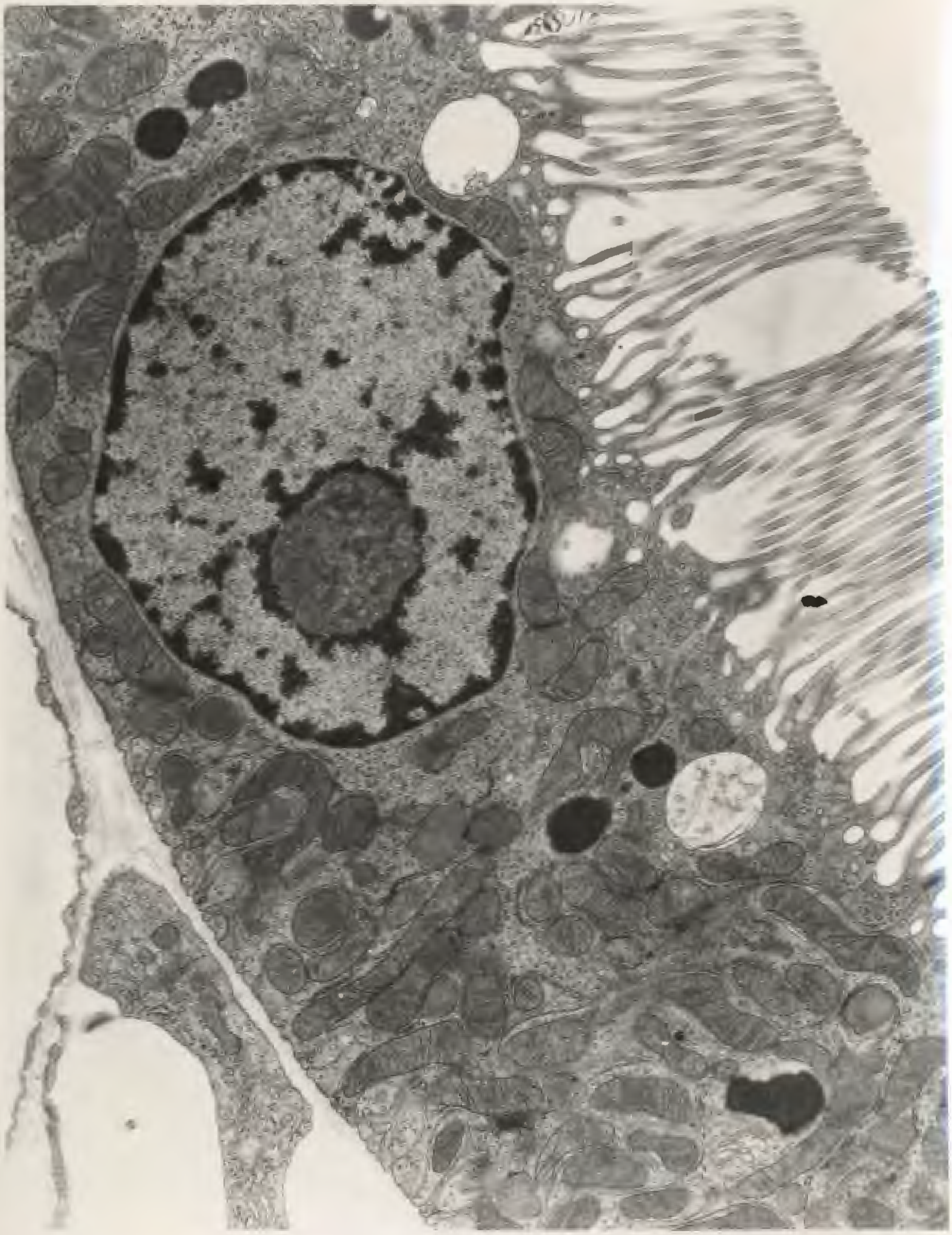
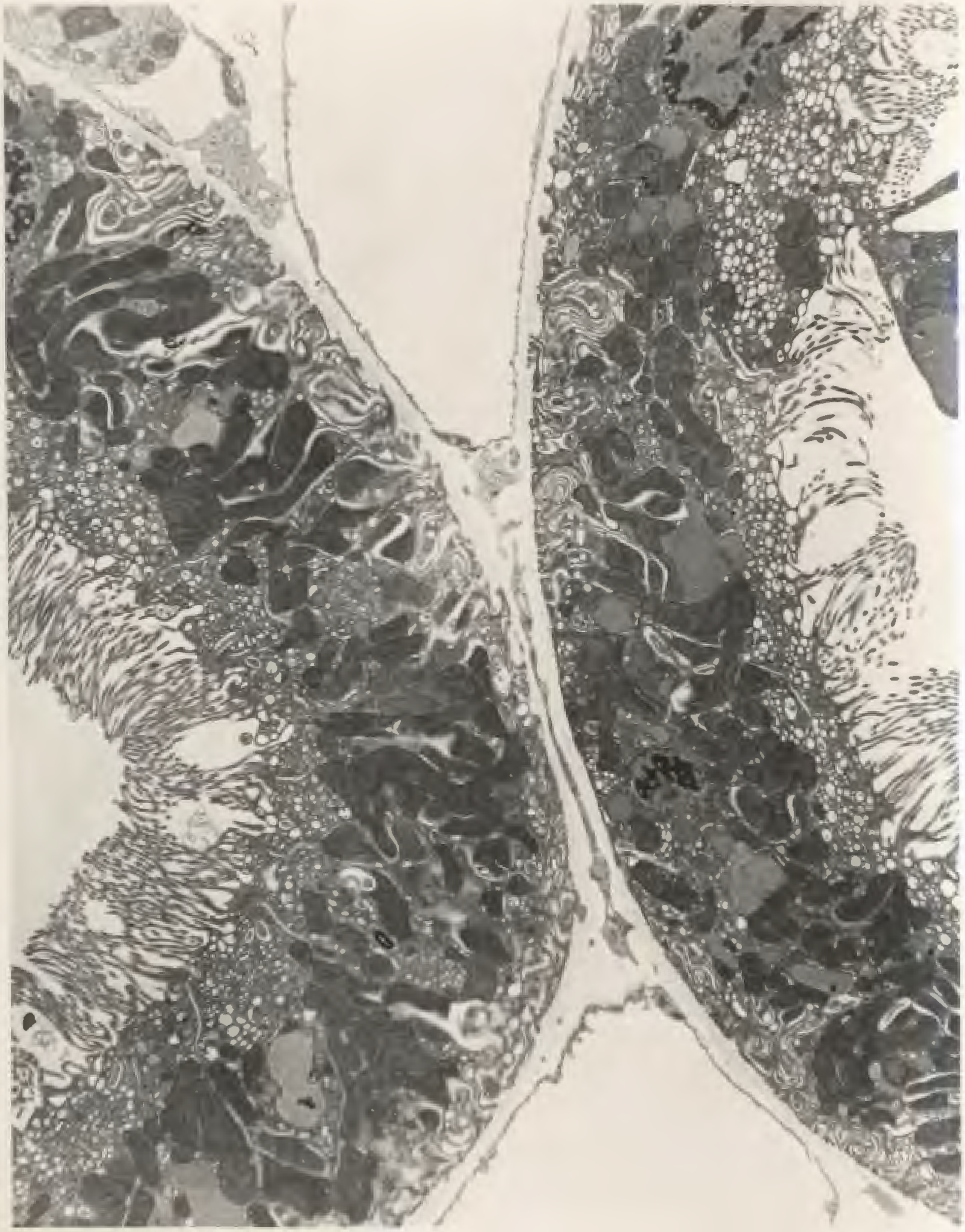


Fig. 3.6 EM of normal rat proximal convoluted tubule, perfused one hour (9,576X).



were missing. Some data with slices did exist (57, 79) but the limitations of kidney slices as an experimental model have recently been pointed out (80). While it is well appreciated that chronic metabolic acidosis in rats induces a set of metabolic adaptations which permit kidneys from acidotic animals to produce more ammonia from glutamine *in vitro*, data of similar quality are not available for the various recovery situations. The first set of experiments were designed to examine ammoniagenesis in kidneys from normal, chronically acidotic and 24 h- and 48 h-recovered rats.

### 3.2.2 Normal acid-base status

Kidneys from normal rats perfused with 1 mM glutamine demonstrated linear rates of ammonia production over the 80 min perfusion period as can be seen in a typical perfusion (Fig. 3.2). These rates of 106.2 moles/gdw/hr (Table 3.1) are close to rates of ammoniagenesis obtained by Ross and Bullock (13.0 moles/30'/gww or 135.2 moles/hr/gdw) (34). Glutamine utilization was also linear at 69.5 moles/gdw/hr as compared to Ross and Bullock's rate of 70.2. The ratio of $NH_3$ produced/glutamine uptake (1.59) indicates that there is a significant amount of GDH activity being expressed in the normal conditions. At least 35% of the ammonia produced must be attributed to the action of GDH.

In order to assess the effects of a more physiological perfusion medium on ammoniagenesis from glutamine the addition of 5 mM glucose and 1.2 mM oleate to the perfusion was undertaken. Presumably the provision of normal substrates to the kidney would permit glutamine utilization to be primarily regulated by the need for ammoniagenesis rather than for the production of ATP. It is interesting to note that

TABLE 3.1 Kidney perfusions with 1 mM glutamine.

| | $NH_4$ | glutamine | glucose |
|---|---|---|---|
| Normal | +106.1±29.2(5) | -69.5±26.0(5) | +14.1±5.9 (4) |
| Acidotic | +214.4±51.9(5)* | -168.2±56.5(5)** | +43.0±23.8(5)** |
| 24 hour recovery | +123.3±28.9(4)$^+$ | -94.0±28.3(4)$^{++}$ | +11.1 (2) |
| 48 hour recovery | +124.3±26.9(6) | -90.5±22.9(4) | +16.2 (2) |

$\mu$ moles.hr$^{-1}$.gdw$^{-1}$

mean ± S.D.

* $P < .005$
** $P < .010$ different than normal
*** $P < .050$

+ $P < .01$ different than acidotic
++ $P < .05$

the rate of ammoniagenesis under these conditions remained unchanged (116.6 moles hr$^{-1}$gdw$^{-1}$) (Table 3.2) as did the glutamine uptake (71.6 moles hr$^{-1}$gdw$^{-1}$).

### 3.2.3 Chronic acidosis

Perfusion of kidneys from seven day $NH_4Cl$-acidotic animals with 1 mM glutamine also resulted in linear rates of ammonia production and glutamine utilization (Fig. 3.7). Following 80 minutes of perfusion, glutamine was not depleted and the rate of utilization remained linear. Ammonia production was increased 2-fold over the normal kidneys to 214.4 moles gdw$^{-1}$hr$^{-1}$ (Table 3.1). Glutamine uptake was found to be 168.2 moles/gdw/hr, a 2.4-fold stimulation over the normal condition. The ammonia production/glutamine utilization ratio in the acidotic kidney is 1.30 which is not significantly different from the ratio obtained in perfusions of normal kidneys. This is unlike the results of Welbourne (82) who demonstrated an increase in the $NH_3$/gln ratio in acidosis.

Acidosis, in the presence of oleate and glucose in addition to glutamine, stimulated $NH_3$ production 2.7-fold (Table 3.2) (Fig. 3.8). This rate (309 moles.hr$^{-1}$gdw$^{-1}$) is significantly greater than the comparable rate of ammoniagenesis from glutamine alone. Glutamine uptake was 190.5 moles hr$^{-1}$gdw$^{-1}$. The addition of these metabolic fuels significantly increased the $NH_3$/glutamine ratio to 1.64.

It appears therefore, that the more physiological perfusion medium affects glutamine metabolism by stimulating ammoniagenesis, but only in the acidotic condition. From the $NH_3$/glutamine ratios the site of this alteration appears to be at GDH. This is in keeping with

TABLE 3.2 Kidney perfusions with 1 mM glutamine.
1.2 mM oleate
5 mM glucose

| | $NH_3$ Production | glutamine uptake |
|---|---|---|
| Normal | 116.6±27.1(5) | 71.6±26.3(4) |
| Acidotic | 309.6±54.4(7)* | 190.5±39.8(5)** |
| 24 hour recovery | 195.7±63.7(6)***$^{+}$ | 120.8±37.4(4)$^{++}$ |
| 48 hour recovery | 135.5±40.2(4) | 93.4±31.5(3) |
| 8 hour recovery (NaCl) | 331.6±74.0(3) | 175.6±50.6(3) |
| 8 hour recovery ($NaHCO_3$) | 307.2±63.0(5) | 160.4±14.9(3) |

$\mu$moles.$hr^{-1}$.$gdw^{-1}$

mean ± S.D.

$* \quad P < .001$
$* \quad P < .005$ different than normal
$*** \quad P < .050$

$+ \quad P < .01$ different than acidotic
$++ \quad P < .05$

Fig. 3.7 Acidotic rat kidney - perfusion with 1 mM gln.

● glutamine

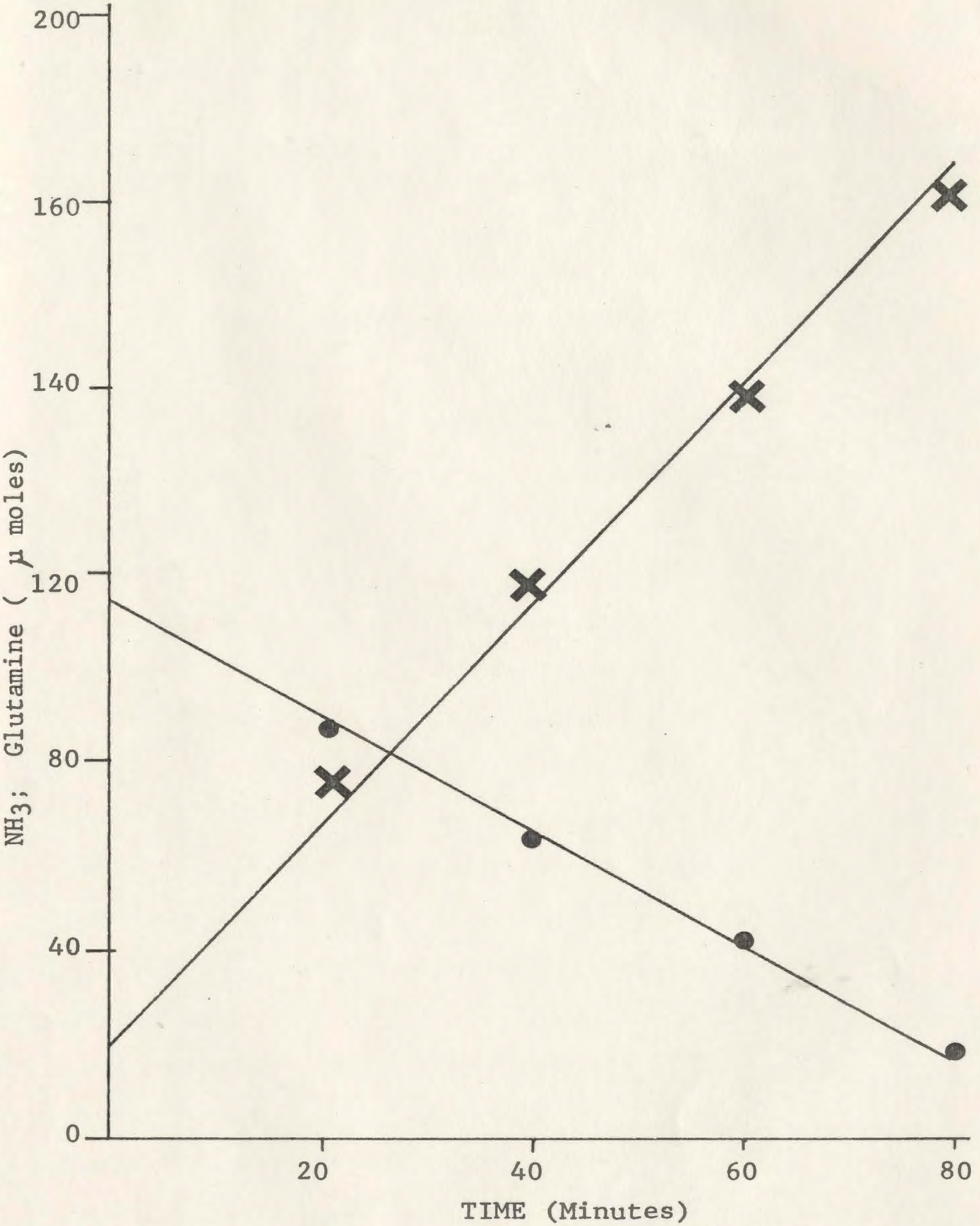
▲ $NH_3$

x glucose

Fig. 3.8 Acidotic rat kidney perfused with 1 mM gln.
5 mM glucose
1.2 mM oleate

● glutamine

x $NH_3$


200
160
140
120
80
40
0
NH3; Glutamine ( μ moles)
20
40
60
80
TIME (Minutes)

work by Preuss *et al*. (94) who have shown data which indicate that GDH may be influenced by certain physiological fuels. They have demonstrated that glucose, lactate and citrate inhibit flux through this enzyme whereas other fuels such as palmitate and hydroxybutyrate stimulate GDH flux.

Glucose production from 1 mM glutamine is stimulated 3-fold when the acidotic kidneys are compared to control perfusions (Table 3.1). This expected phenomenon is in agreement with other studies (66, 70, 75). Because of the high initial concentration of glucose (5.0 mM) changes were not detectable in the perfusions with oleate, glutamine and glucose.

### 3.2.4 Recovery from acidosis

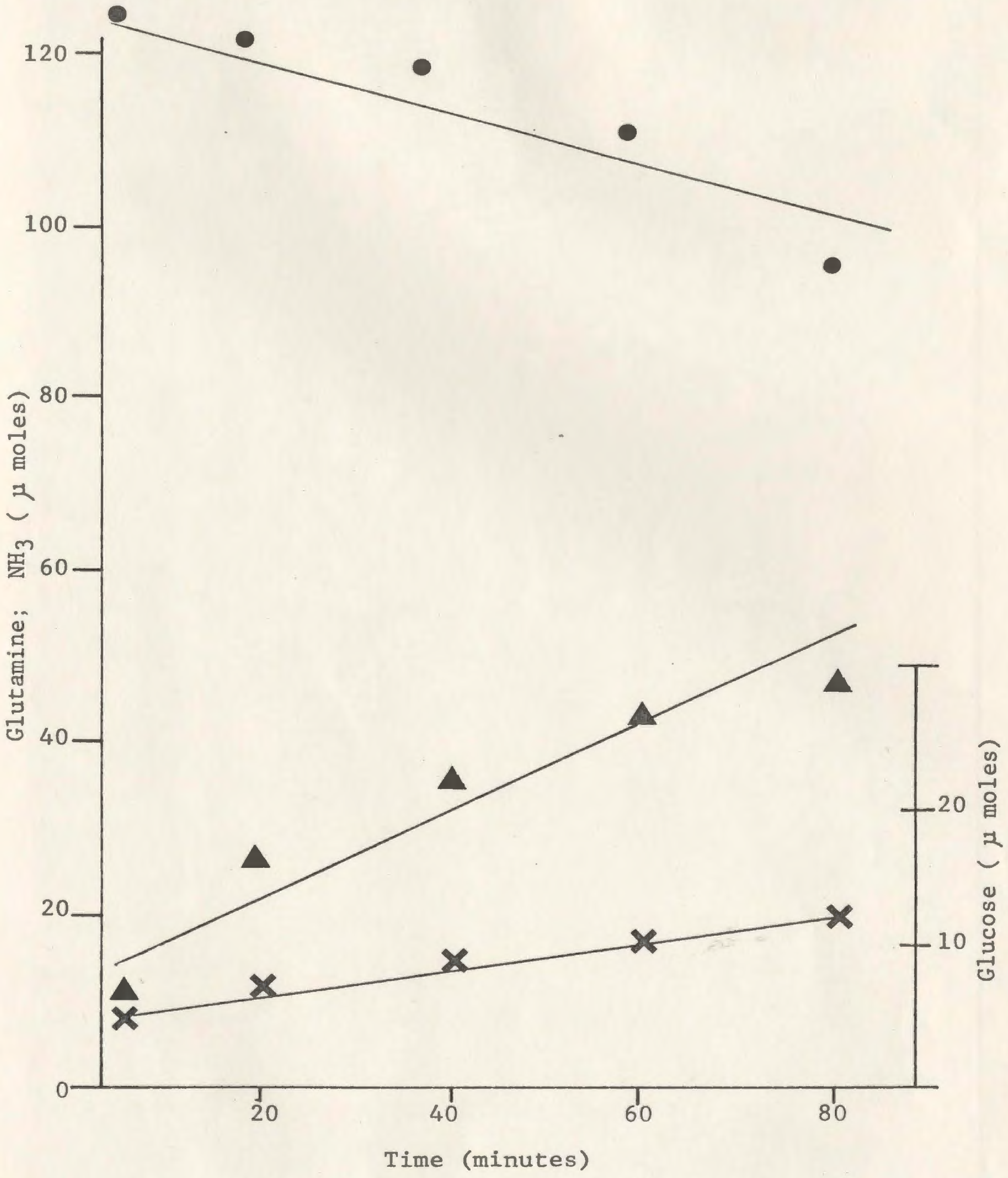
Acidotic rats were allowed to recover by replacing their $NH_4Cl$ solution with tap water. Upon perfusion of their kidneys it was found that rates of $NH_3$ production from 1 mM glutamine alone dropped to normal after 24 hours (123 moles $gdw^{-1}hr^{-1}$) (Table 3.1, Fig. 3.9). Glutamine uptake remained slightly higher at 94.0 moles $gdw^{-1}hr^{-1}$ and even after 48 hours (90.5) but this was not significant. In the perfusions with glutamine, oleate and glucose, ammonia production in the 24-h recovered rat is down significantly from the acidotic but was still significantly elevated compared with the normal condition (1.7 fold). By 48 hours ammoniagenesis is back to normal ($135.5 \pm 40.2$ moles $gdw^{-1}hr^{-1}$). Glutamine uptake in the 24 hour recovered rat is down significantly from the acidotic condition and is somewhat elevated from the normal rate. This difference is not statistically significant however. It is apparent that in some way the more physiological perfusion

Fig. 3.9 24 hr recovered kidney perfusion with 1mM gln.

● glutamine

▲ $NH_3$

x glucose



120
100
80
60
40
20
0
Glutamine; $NH_3$ ( µ moles)
20
40
60
80
Time (minutes)
20
10
Glucose ( µ moles)

medium has an ammoniagenic action which possibly has an effect at GDH. This action does not necessarily increase the net ammonia production, as in the normal condition, but it changes the relative activities of PDG and GDH which determines whether both the amide and amino groups of glutamine are cleaved. This is evident in the $NH_3$/gln ratios as summarized in Table 3.3. Glucose production in the 24-h recovered animals fell to normal rates (11.1$\mu$ moles.gdw$^{-1}$.hr$^{-1}$). Thus no apparent discrepancy is detected between the return of ammonia production and gluconeogenesis to normal in these perfusions.

### 3.2.5 Accelerated recovery

The intubation of acidotic animals with $NaHCO_3$, 1.5 mMoles/100 g body weight has been shown to decrease ammonia excretion to normal levels within 8 hours (57). This procedure induces an alkalosis (pH 7.5, $HCO_3$ = 35 mmoles/l) within the 8-h period. Measurement of PDG and GDH activities during this period has shown that there is no significant decrease in activity from the acidotic condition. To assess whether the mechanism of suppression of ammonia excretion over this time can be demonstrated in the perfused kidney, perfusions were performed following 8 hours of recovery in rats that had been intubated with $NaHCO_3$. I also perfused, as a control group, kidneys from acidotic rats that had been intubated with an equivalent dose of NaCl. As seen in Table 3.2 there is no decrease in ammonia production in the control or $NaHCO_3$ intubated rats (331 and 307$\mu$ moles.hr$^{-1}$.gdw$^{-1}$) from the perfused rat kidneys. Glutamine uptake in the $NaHCO_3$ intubated rats was 160.4, 2.2 times normal glutamine uptake. The $NH_3$/gln ratio in these intubated

TABLE 3.3 $NH_3$ Production/glutamine uptake ratios.

(a) Perfusions with 1 mM glutamine

(b) Perfusions with 1 mM glutamine, 1.2 mM oleate and 5 mM glucose.

(a)

| | $NH_3$/gln | n | SEM | P different from N |
|---|---|---|---|---|
| Normal | 1.59 | 5 | .13 | |
| Acidotic | 1.30 | 5 | .09 | NS |
| 24 hr R. | 1.34 | 4 | .07 | NS |
| 48 hr R. | 1.58 | 4 | .12 | NS |

(b)

| | $NH_3$/gln | n | SEM | P different from 1 mM gln |
|---|---|---|---|---|
| Normal | 1.53 | 4 | .17 | NS |
| Acidotic | 1.64 | 5 | .12 | $p < .05$ (ACIDOTIC) |
| 24 hr R. | 1.66 | 4 | .28 | NS |
| 48 hr R. | 1.37 | 3 | .02 | NS |
| 8 hr R. (NaCl) | 1.82 | 3 | .18 | NS |
| 8 hr R. ($HCO_3$) | 1.91 | 3 | .08 | NS |

R = recovery

NS = not significant

= number

SEM = standard error of the mean

animals (19) was the highest I observed indicating a brisk flux through GDH. The ratio was not significantly different from normal rat kidney ratios however. The observed trend, is nonetheless contrary to what Parry and Brosnan reported in mitochondria isolated from similarly treated rats (57). Thus an apparent discrepancy exists between isolated mitochondria and the perfused kidney under these conditions. It is evident, from these studies that recovery from acidosis is not a simple process. The early cessation of ammoniagenesis *in vivo* is not paralleled in the isolated perfused kidney. Thus we see rapid ammoniagenesis in the perfused kidneys from 8-h recovered rats whereas ammoniagenesis *in vivo* has greatly decreased. In the 24-h recovered, and more clearly in the 48-h recovered animals ammoniagenesis has returned to normal in the perfused kidney. Throughout this time the activities of PDG and GDH remain elevated. From these studies two possible conclusions are possible. The first is that recovery from acidosis is dependent upon *in vivo* factors which were not present in the perfused kidney. The most likely is the alkalosis which has been observed in 24-h recovered rats and 8-h recovered rats which have been intubated with $NaHCO_3$. Secondly, the rate of ammoniagenesis is closely coupled to PEPCK activity and therefore the activity of PEPCK controls the rates of ammonia production. This latter hypothesis was further studied in the perfused kidney. Because of the availability of 3-AP and an alleged activation of PEPCK by this compound I decided to test the second hypothesis.

### 3.2.6 Effect of 3-aminopicolinic acid in the perfused kidney

Phosphoenolpyruvatecarboxykinase (PEPCK) is an important rate limiting enzyme of gluconeogenesis. Its activity can be increased in certain experimental conditions (i.e. starvation, acidosis, chronic steroid therapy). 3-Aminopicolinic acid (3-AP) is a hyperglycemic agent (93) which is believed to exert its effects by stimulating hepatic PEPCK (81). This effect is thought to be an indirect action, that is, 3-AP permits metal ions (Fe & Mn) to activate PEPCK through an unknown mechanism. There has been isolated, however, a protein named ferroactivator that seems to regulate liver PEPCK activity *in vivo* (91). 3-AP seems to have the same mechanism of action as ferroactivator. An activator of an enzyme will only activate an overall pathway if that enzyme is rate limiting for flux through the pathway. On the other hand an inhibitor, provided it is sufficiently powerful, could inhibit a pathway even if the inhibited enzyme were not normally the rate-limiting step. Thus the potential information available from the use of an activator is very valuable. For this reason I employed 3-AP in the perfused kidney.

In 24-hour recovered rats, ammonia excretion *in vivo* and in the perfused kidney is back to normal even though the activity of PDG and mitochondrial ammonia production is still elevated (57). PEPCK activity, however, follows *in vivo* $NH_3$ excretion. To directly test the importance of PEPCK on renal ammoniagenesis, kidneys from 24-hour recovered rats were perfused with 1mM gln for 35 minutes and then challenged with 0.05 mM 3-AP for a further 30 minutes. Because the *in vitro* measured activities of the ammoniagenic enzymes are still elevated at this time but $NH_3$ production is reduced to normal, a large reserve capacity exists. If PEPCK is directly linked with the regulation of ammoniagenesis, a stimulation of the enzyme should stimulate ammoniagenesis under these optimal conditions. As seen in Fig. 3.10 however, no stimulation was seen with .05 mM 3-AP. Furthermore, no effect on gluconeogenesis was observed. In further studies, kidneys were perfused with 10 mM pyruvate and the rate of gluconeogenesis was measured before and after the addition of various concentrations of 3-AP (Fig. 3.11). No effect was detected at 0.01 mM whereas at 0.10 mM there was slight inhibition of glucose production. At 0.5 and 1.0 mM concentrations total inhibition of gluconeogenesis was obtained. Subsequent work has also cast doubt on the reality of 3-aminopicolinate as an activator of PEPCK. When the effect of intravenous injection of 3-AP on _in vivo_ metabolite concentrations in kidneys of normal and recovered rats was examined the data were not consistent with an activation of PEPCK (103). In fact, the concentrations of PEP, 3-PG, 2-PG either remained the same or decreased, which is more compatible with an inhibition of the enzyme. Malate and aspartate concentrations increased which also supports inhibition. A recent study (92) in isolated kidney tubules indicated that 3-AP inhibits PEPCK. Reynolds (95) has demonstrated that at low $Fe^{2+}$ or $Co^{2+}$ concentrations, 3-AP inhibited rat liver PEPCK when assayed spectrophotometrically. Thus 3-aminopicolinate has not been shown to activate PEPCK and therefore is not useful in studies on the role of this enzyme in ammoniagenesis and gluconeogenesis.

### 3.3 Isolated cortical tubules

The present experiments were designed so as to examine the acute effects of increased acidity on the stimulation of ammoniagenesis from glutamine. In particular I wished to test the hypothesis that

Fig. 3.10 Kidney perfusion with 1 mM gln; 24 hr recovery. Aminopicolinic acid at 30 min. (.05 mM).

● glutamine

▲ $NH_3$

x glucose

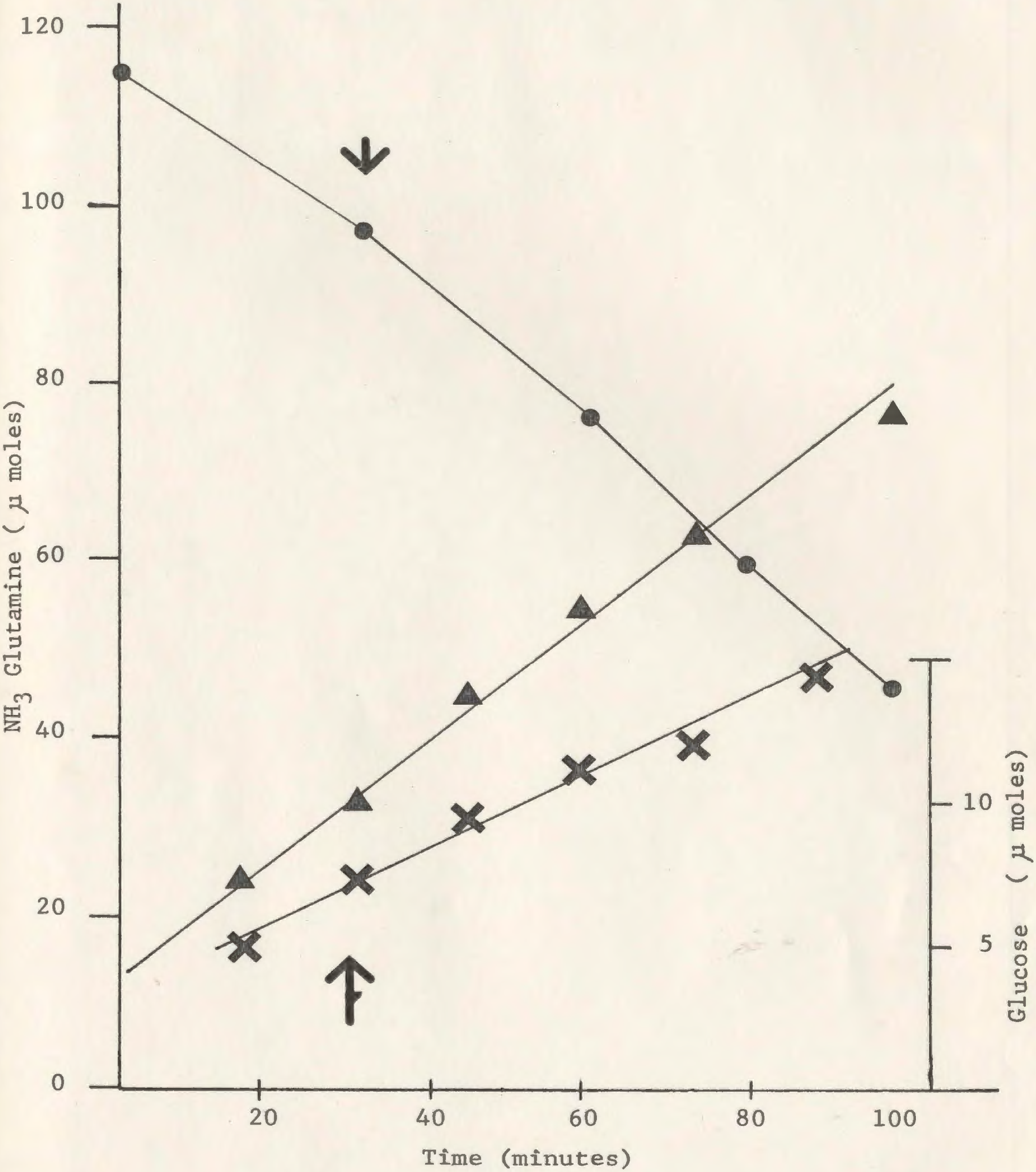
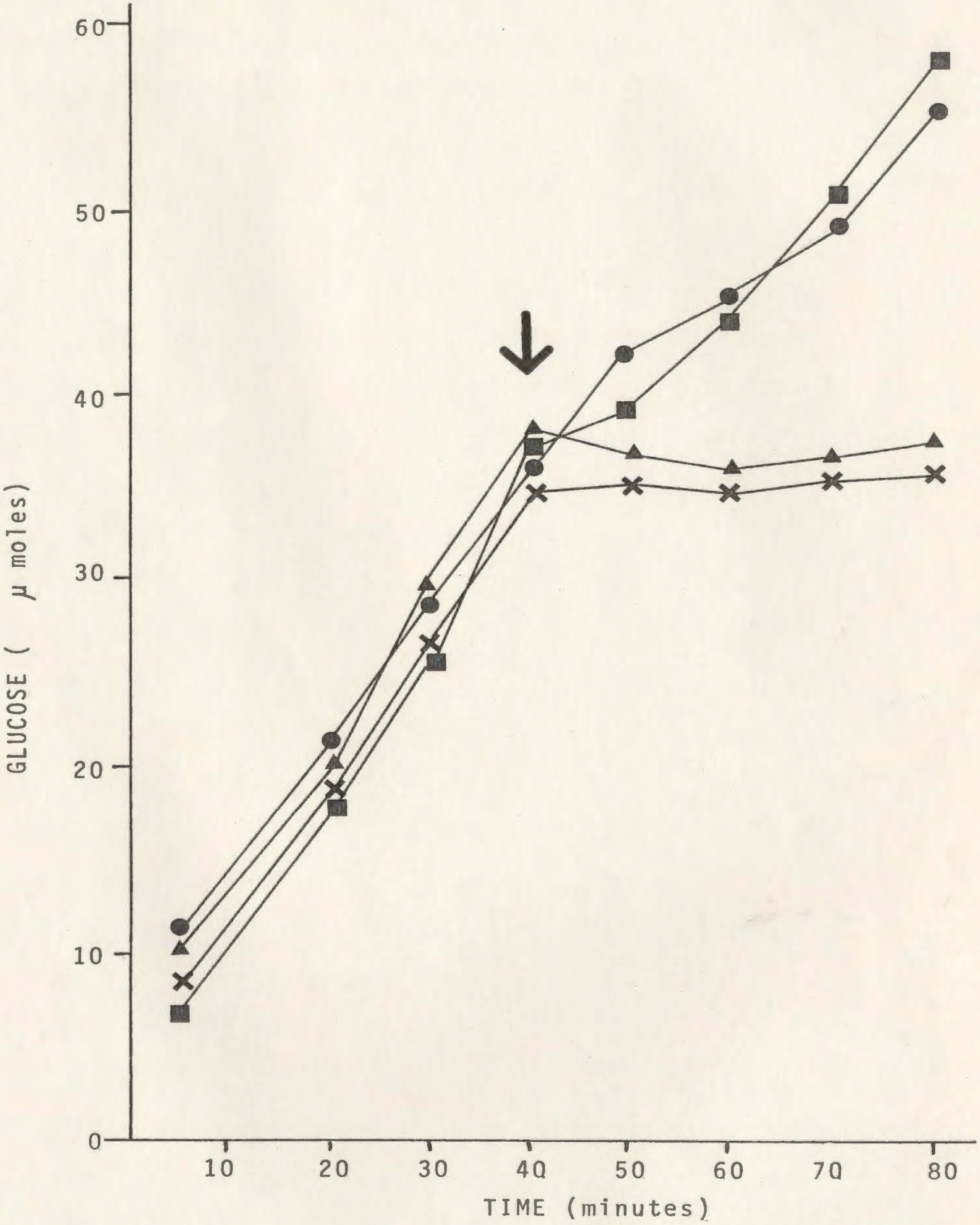

120
100
80
60
40
20
0
$NH_3$ Glutamine ( µ moles)
20
40
60
80
100
Time (minutes)
10
5
Glucose ( µ moles)

Fig. 3.11 Normal kidney perfused with 10 mM pyruvate

3-Aminopicolinate added at 35 min.

Concentration: .01 mM; .10 mM; + .50 mM; 1.0 mM


GLUCOSE ( µ moles)
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
TIME (minutes)

glutamine synthetase may play a role in this phenomenon. Since many studies (75, 90, 96) have readily demonstrated this effect in isolated renal tubules I elected to use this system. In addition it has the potential of performing many paired experiments with each batch of tubules.

### 3.3.1 Assessment of isolated tubules

The isolated tubule preparation was tested for biochemical and physiological stability by measuring the rate of gluconeogenesis from 1 mM glutamine at 15 min intervals for 45 minutes (Fig. 3.12). Although this rate was not linear, glucose accumulation was similar to that reported by others (90,96). A thirty minute incubation period was chosen and over this time period it was felt that the metabolic changes would be large enough to be easily detected. When tubules were incubated without additional substrates a net production of glucose, probably from endogenous precursors can be demonstrated (12.0 mole/30'/gdw). As can be seen from Fig. 3.13 a very small amount of $NH_3$ is produced when no precursor is supplied. No correction was felt necessary, therefore, to account for endogenous rates of ammoniagenesis. With 1 mM glutamine added to incubation medium as a substrate the rate of gluconeogenesis increased to 33.0 moles/30'/gdw. This compares well with rates obtained in other studies (75).

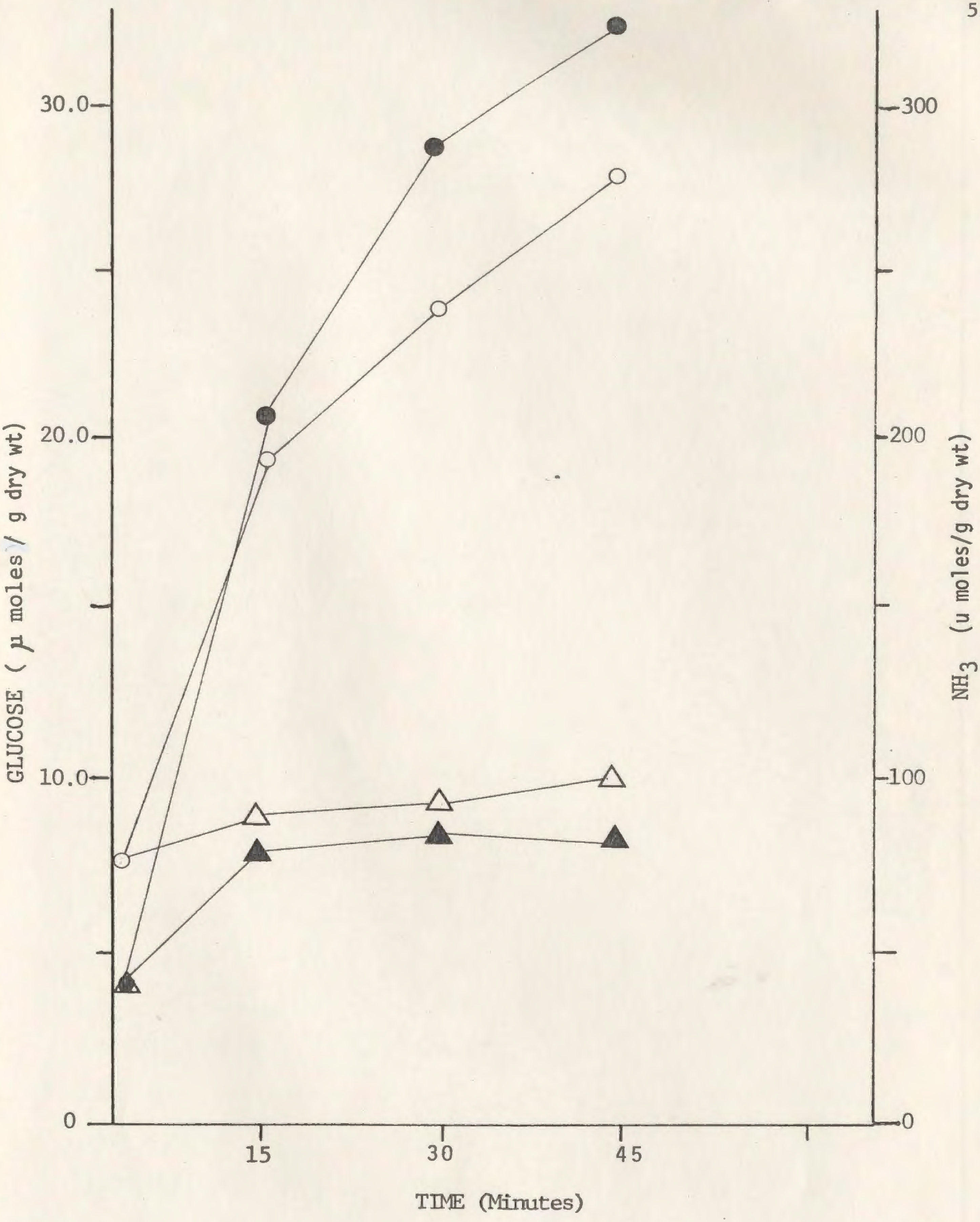
### 3.3.2 Acute effect of $H^+$ in isolated tubules

Although the effects of chronic acidosis on $NH_3$ excretion and production have been well documented, a direct effect of acute changes in $H^+$ concentration on ammoniagenesis has been, until the introduction of the tubule preparation, an elusive parameter to characterize. Many attempts at stimulating $NH_3$ production in

Fig. 3.12. Ammoniagenesis + gluconeogenesis in rat tubules with 1 mM glutamine and no substrate.

● glucose
o $NH_3$
with 1mM glutamine

▲ glucose
△ $NH_3$
with no substrate


GLUCOSE ( μ moles / g dry wt)
30.0
20.0
10.0
0
NH3 (u moles/g dry wt)
300
200
100
0
15
30
45
TIME (Minutes)

cortical slices by altering pH have failed (66, 94, 96, 97, 108). Often a decrease was observed with increased acidity as was seen in isolated mitochondria (51, 99, 100). Recently the physiological integrity of the cortical tissue slice preparation has been questioned (80). Comparison of the ATP content, oxygen consumption kinetics and the cytochrome redox state in rabbit cortical slices and isolated tubules have provided direct evidence for the existence of anoxic zones within the slices. This renders them questionable in usefulness in the investigations of renal cortical metabolism. Tubule preparations, however, were found to be well oxygenated and demonstrated a similar response of the redox state of the cytochromes to decreasing $O_2$ tension as that found in isolated mitochondria. The difference in the two preparations probably accounts for the ability of the tubules but not cortical slices to respond to acute acidosis.

Tannen and Ross (67) have shown an acute stimulation of ammoniagenesis from 2.0 mM glutamine in perfused kidney by changing the pH of the recirculating medium from pH 7.4 to 7.0. This stimulation was decreased in the non-filtering kidney model, a preparation which is incapable of urine acidification. Thus they concluded that urinary acidification in the functioning kidney is an important factor in the acute response. The preparation Hems (55) employed was incapable of acidifying the urine below pH 6.0 and this combined with the failure to acidify the perfusate below pH 7.1 may account for his inability to acutely stimulate ammoniagenesis from glutamine. In the same study however, Hems demonstrated an inhibition of glutamine synthesis from glutamate at pH 7.1. He observed a concomitant increase in $NH_3$ production and in the discussion of his results he postulated that the stimulation of ammoniagenesis may be a result of an inhibition of glutamine synthetase. In light of some recent work in our laboratory which demonstrates significant rates of glutamate- glutamine recycling (Dr. J.T. Brosnan, unpublished data) the effects of acute acidosis in tubules was studied with particular attention to the role of G.S.

### 3.3.3 Cortical tubule incubations with 1.0 mM glutamine

The rate of $NH_3$ production from 1 mM glutamine at pH 7.4 was 292.1 moles 30 $min^{-1}gdw^{-1}$ (Table 3.4). This value is close to that obtained by Lowry and Ross (75) under the same conditions (574.1 moles/hr/gdw). At pH 7.06 the rate of ammoniagenesis increased to 326.9 (significant by paired t-test). This represents a 12% stimulation, somewhat less than the 33% stimulation obtained by Lowry and Ross. The stimulation in their study however was magnified by subtracting the rates of endogenous ammoniagenesis from the rates of ammoniagenesis from glutamine. Glucose production at pH 7.4 was found to be 54.2 moles/30'/gdw and was stimulated 28% to 69.5 at pH 7.0.

### 3.3.4 Tubule incubation with L-methionine-D.L.-sulfoximine

M.S.O., the inhibitor of GS, stimulated ammonia production from 1 mM glutamine to 369.3 moles 30 $min^{-1}gdw^{-1}$. No additional effect was observed when MSO was added to incubations at pH 7.0. The interpretation of these results support the observation made by Hems in 1972 (55) that an effect of acutely lowered pH is a decreased rate of glutamine synthesis. An interesting new observation in this study was the 20% stimulation of gluconeogenesis with MSO (to 64.9

TABLE 3.4 Rat kidney tubules incubated with 1 mM glutamine.

| | pH 7.40 | pH 7.06 | pH 7.40 + M.S.O. | pH 7.06 + M.S.O. |
|---|---|---|---|---|
| Ammonia production: | 292.1±81.6 | 326.9±72.6 | 369.3±93.8 | 371.1±124.3 |
| Glucose production: | 54.2±19.2 | 69.5±15.8 | 64.9±14.0 | 73.4±18.8 |

$\bar{x} \pm$ S.D.

n = 5. $\mu$moles.gdw$^{-1}$.30 min$^{-1}$.

$\mu$moles.30 $min^{-1}.gdw^{-1}$).

### 3.3.5 Incubations with 1.0 mM glutamine and 4.0 mM lactate

Because of the high rates of ammoniagenesis, large degree of variation from incubation to incubation and a relatively small stimulation with lowered pH, the conditions were modified to accentuate the changes and thus more vigorously test our hypothesis. 5.0 mM Lactate was added to the incubation medium. Lactate suppressed the rate of ammoniagenesis from 1.0 mM glutamine at pH 7.40 by 77% to 67.3$\mu$moles.30 $min^{-1}.gdw^{-1}$ (Table 3.5). Thus it seems that when glutamine is used as a sole exogenous metabolite a large amount is used for normal respiratory purposes and when lactate, which appears to be a preferential fuel is supplied a truer reflection of in vivo $NH_3$ metabolism is seen. The actual mechanism of suppression of ammoniagenesis by other oxidizable substrates has been examined by Preuss et al (94). From their studies the site of action seems to be GDH.

In the presence of 1 mM glutamine and 5.0 mM lactate, $NH_3$ production increased 35% when incubated at pH 7.06. The inclusion of lactate in the incubation medium was a successful stratagem since by lowering the basal rate of ammoniagenesis from glutamine the effects of altered pH became more apparent. When M.S.O. was added to the incubation medium, with lactate and glutamine as substrate, $NH_3$ production doubled to 140.2 $\mu$moles.30 $min^{-1}.gdw^{-1}$. The absolute magnitude of the increase (72.9) obtained under these conditions is the same as the stimulation seen with glutamine alone (77.2). As in incubations with glutamine alone, M.S.O. addition at pH

TABLE 3.5 Kidney tubule incubations with 1.0 mM gln and 5.0 mM lactate.

| | pH 7.40 | pH 7.06 | pH 7.40<br>+ M.S.O. | pH 7.06<br>+ M.S.O. |
|---|---|---|---|---|
| $NH_3$ | 67.3±10.5 | 91.0±14.9* | 140.2±20.4**$^{+}$ | 143.3±23.8 |
| Glucose | 102.2±19.9 | 141.8±18.7* | 138.8±16.4* | 154.4±19.2 |

$\bar{x} \pm$ S.D.

* $P < .02$ different from pH 7.40

** $P < .001$

$^{+}$ $P < .005$ different from pH 7.06

($\mu$moles.30 $min^{-1}.gdw^{-1}$)

n = 5

7.06 has no additional effect on ammoniagenesis (143.3).

Glucose production in the presence of lactate (102.2) was double the rate obtained with glutamine alone at pH 7.4. This rate was stimulated to 141.8 and 138.8 by pH 7.0 and M.S.O. respectively.

### 3.3.6 Effects of varying pH on $NH_3$ utilization (with 2.0 mM glutamate; 0.5 mM $NH_4Cl$ and 5.0 mM lactate

The above experiments suggest that increased acidity inhibits glutamine synthesis and by this mechanism an apparent stimulation of a ammoniagenesis is observed. To test this hypothesis in a more direct fashion, incubations were performed under conditions which favored G.S. activity. Glutamate, 2.0 mM; $NH_4Cl$, 0.5 mM and lactate 5.0 mM were added to the incubation medium. At pH 7.4 a net uptake of 59 $\mu$moles.30 $min^{-1}.gdw^{-1}$ of $NH_3$ was observed (Table 3.6). At pH 7.06 the uptake was inhibited to 18 $\mu$moles.30 $min^{-1}.gdw^{-1}$. Thus ammonia removal is pH regulated. In an attempt to find the optimum pH for observing the effect in further studies two further pH's were tested. At pH 7.16 a net uptake of 32.4 $\mu$moles. 30 $min^{-1}.gdw^{-1}$ was seen and at pH 6.96 the ammonia uptake turned into a production of 25.9 moles 30 $min^{-1}gdw^{-1}$ (Table 3.7). From this study it was felt that pH 7.06 is optimum, that is, a large effect is obtained without the risks of tissue destruction associated with higher acidity.

### 3.3.7 Incubations with 2.0 mM glutamate; 0.5 mM $NH_4Cl$ and 5.0 mM lactate

At pH 7.4 a net uptake of ammonium of 59.4 $\mu$moles.30 $min^{-1}.gdw^{-1}$ was demonstrated (Table 3.6). When glutamine synthetase activity was blocked with M.S.O. this uptake was inhibited and a net production of

TABLE 3.6 Kidney tubule incubations with 2.0 mM glutamate, 0.5 mM $NH_4$ and 5.0 mM lactate.

| | $NH_3$ |
|---|---|
| pH 7.40 | $-59.4 \pm 7.2$ |
| pH 7.06 | $-18.5 \pm 6.8$* |
| pH 7.40 + MSO | $+41.8 \pm 16.6$** |
| pH 7.06 + MSO | $+48.3 \pm 23.4$ |

$\overline{x} \pm$ S.D.

* $P \leqslant .001$ P different from pH 7.40

** $P \leqslant .001$ P different from pH 7.06

n = 5. $\mu$moles.30 $min^{-1}.gdw^{-1}$.

TABLE 3.7 Kidney tubule incubations with 1.0 mM gln, 0.5 mM $NH_4Cl$ and 5.0 mM lactate at various degrees of acidity.

| | $NH_3$ |
|---|---|
| pH 7.40 | −56.6±6.8 |
| pH 7.16 | −32.4±9.9 |
| pH 7.06 | −16.1±4.6 |
| pH 6.96 | +25.9±21.5 |

n = 3. $\mu$ moles.30 $min^{-1}.gdw^{-1}$

mean ± S.D.

41.8 μmoles.30 $min^{-1}.gdw^{-1}$ was observed. Thus when glutamate and ammonia are present, the activity of glutamine synthetase is increased from about 75 μmoles.30 $min^{-1}.gdw^{-1}$ (as calculated from incubations with gln alone and gln + lactate) to 101.2 μmoles [illegible] (59.4 + 41.8) in the present incubations. Of course this is a minimal rate as it does not account for PDG activity on the glutamine formed in the incubation without MSO. This calculated flux through GS is well below the measured *in vitro* activity of GS in rat renal cortex (216.7μmoles.30 $min^{-1}.gdw^{-1}$) as measured by Lemieux *et al* (30), and 2.3 μmole.$min^{-1}.gww^{-1}$ by Lund (101). The estimated rate of G.S. activity in this study corresponds to about 0.60 μmoles.$min^{-1}.gww^{-1}$ and compares with a rate of about 0.55 μmole.$min^{-1}.gww^{-1}$ by Hems (55) in kidneys perfused with glutamate and a rate of 0.408 mole $min^{-1} gww^{-1}$ reported by Damian & Pitts for the normal kidney *in vivo* (102).

Incubations at pH 7.06 decreased the rate of $NH_3$ uptake to 18.5 μmoles.30 $min^{-1}.gdw^{-1}$ which I interpreted as indicating that the increased $H^+$ concentration inhibits the GS activity. Addition of MSO to incubations at pH 7.06 resulted in the same stimulation of ammoniagenesis as was seen in incubations at pH 7.4 with MSO.

The sum total of experiments suggest to me the hypothesis that the acute stimulation of ammoniagenesis by increased medium acidity is due to an inhibition of glutamine synthetase activity in the isolated tubules.

### 3.3.8 Measurement of glutamine formation from glutamate

The evidence that MSO and acidotic conditions lead to inhibition of GS activity by merely measuring ammonia production and utilization is indirect. A further study was undertaken to measure glutamine production, glutamate removal and $NH_3$ utilization in incubations with 2.0 mM glutamate, 0.5 mM $NH_4Cl$ and 5.0 mM lactate (Table 3.8). $NH_3$ utilization was markedly inhibited with MSO as expected but the absolute magnitude of the rates of utilization were quite different from the studies that were done previously (in Oxford). One likely explanation for this discrepency is that different breeds of rats were used in the two laboratories. At pH 7.06 $NH_3$ utilization was inhibited. There was no additional effect of pH 7.06 in addition to MSO.

Glutamate utilization was 181.0 moles 30 $min^{-1}gdw^{-1}$ at pH 7.40 and decreased to 129.0 with MSO. At pH 7.06 glutamate utilization was not shown to be significantly reduced (174.3). At pH 7.06 with MSO an unexplained 161.0 umoles 30 $min^{-1}gdw^{-1}$ uptake was noted.

The rate of glutamine production at pH 7.4 was found to be 112.5 moles 30 $min^{-1}gdw^{-1}$ and this was abolished with MSO. Acute acidosis reduced the rate of glutamine production to 74.3 which was found to be significantly different from the rate at pH 7.40. Thus this study provided more definitive evidence by showing that net glutamine synthesis is markedly reduced in isolated tubules incubated under acidotic conditions.

### 3.3.9 Effects of acute on chronic acidosis on ammoniagenesis and gluconeogenesis

The above observed effects on the metabolism of renal tubules incubated at low pH were next compared to the effects of acute acid challenge on tubules from 7-day chronically acidotic rats. In the acidotic state, rates of ammonia production are already enhanced and

TABLE 3.8 Incubation of rat tubules with 2.0 mM glutamate, 0.5 mM $NH_4CL$ and 5.0 mM lactate - glutamine production, $NH_3$ and glutamate uptake.

| | pH 7.40 | pH 7.06 | pH 7.40 + M.S.O. | pH 7.06 + M.S.O. |
|---|---|---|---|---|
| Glutamate | -181.0±25.6 | -174.3±12.7 | -129.0±13.1$^{++}$ | -161.0±26.9 |
| Glutamine | +112.5±33.7 | +74.3±22.6* | +4.3±1.5$^{+}$ | +2.8±1.4$^{\circ}$ |
| NH3 | -129.1±62.2 | -49.7±95.5* | -14.6±72.9$^{\circ\circ}$ | 10.4±76.7$^{\circ}$ |

* significant from pH 7.4 $p < .05$

+ significant from pH 7.4 $p < .05$

++ significant from pH 7.4 $p < .025$

° significant from pH 7.06 $p < .05$

°° significant from pH 7.06 $p < .005$

$\bar{x}$ ± S.D.
n = 5. $\mu$ Mol/g dry wt$^{-1}$.30 min$^{-1}$

whether or not further enhancement is possible with an acute challenge was examined. In addition I examined tubules from rats that had recovered for 1 day from acidosis as these animals have elevated activities of glutaminase in the face of low rates of ammonia production and it is possible that enhanced activity of glutamine synthase is responsible for the inhibition of ammoniagenesis during recovery from acidosis. As seen in Table 3.9, the tubules from acidotic rats at pH 7.4 produced $NH_3$ at a rate 1.6 times tubules from normal rats at pH 7.4. This enhanced rate of ammoniagenesis was stimulated by the addition of MSO at pH 7.40. There was no demonstratable effect at pH 7.06. In the recovered condition, rates of ammoniagenesis were back to normal and, again, a stimulation was achieved with MSO at pH 7.4. This stimulation, however, was not significantly greater in magnitude than that observed in the normal rat kidneys (84 vs 78).

Rates of gluconeogenesis were found to be 2.5 times normal in the acidotic rat tubules. These were down to normal in the 24-hour recovered incubations. No significant differences were observed in rates of glucose production between pH 7.4 and 7.06 or with MSO.

Because the rate of ammoniagenesis is so high with glutamine alone (as alluded to above) the above experiment was repeated with lactate added to the glutamine in the incubation flasks. As seen in Table 3.10 this resulted in a 2.0-fold difference in ammonia production in the tubules from the acidotic rats compared with those from normal rats. Ammoniagenesis was again back to normal rates in the 24-hour recovered tubules. No differences from the 1 mM glutamine incubations were seen in the absolute magnitude of $NH_3$ stimulation whether or not further enhancement is possible with an acute challenge was examined. In addition I examined tubules from rats that had recovered for 1 day from acidosis as these animals have elevated activities of glutaminase in the face of low rates of ammonia production and it is possible that enhanced activity of glutamine synthetase is responsible for the inhibition of ammoniagenesis during recovery from acidosis. As seen in Table 3.9, the tubules from acidotic rats at pH 7.4 produced $NH_3$ at a rate 1.6 times tubules from normal rats at pH 7.4. This enhanced rate of ammoniagenesis was stimulated by the addition of MSO at pH 7.40. There was no demonstrable effect at pH 7.06. In the recovered condition, rates of ammoniagenesis were back to normal and, again, a stimulation was achieved with MSO at pH 7.4. This stimulation, however, was not significantly greater in magnitude than that observed in the normal rat kidneys (84 vs 78).

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TABLE 3.9 Incubation of normal, acidotic and 24-hr recovered rat kidney tubules with 1 mM gln.

$NH_3$ PRODUCTION:

| | pH 7.40 | pH 7.06 | pH 7.40 + M.S.O. | pH 7.06 + M.S.O. |
|---|---|---|---|---|
| Normal | 294.3±76.3 | 327.4±76.5 | 372.3±97.3 | 371.2±107.3 |
| Acidotic | 464.8±46.9* | 446.4±56.1 | 515.3±69.0 | 482.7±92.8 |
| Recovery (24 hr) | 278.0±50.1 | 327.8±38.7 | 364.5±66.0 | 367.1±50.9 |

GLUCOSE PRODUCTION:

| | pH 7.40 | pH 7.06 | pH 7.40 + M.S.O. | pH 7.06 + M.S.O. |
|---|---|---|---|---|
| Normal | 31.0±5.2 | 41.8±15.0 | 38.3±15.2 | 47.2±18.0 |
| Acidotic | 79.1±5.0** | 77.7±6.3* | 77.3±8.7* | 84.3±10.8*** |
| Recovery | 39.9±6.7 | 48.3±6.8 | 51.5±6.8 | 58.1±12.4 |

$\bar{x} \pm$ S.D.

n = 3. Results as expressed as $\mu$moles $30min^{-1} gdw^{-1}$.

$*P<0.05$, $**P<0.02$, $***P<0.001$

TABLE 3.10 Incubation of normal, acidotic and 24-hr recovered rat kidney tubules with 1.0 mM gln + 5.0 mM lactate.

$NH_3$ PRODUCTION:

| | pH 7.40 | pH 7.06 | pH 7.40 + M.S.O. | pH 7.06 + M.S.O. |
|---|---|---|---|---|
| Normal | 124.9±5.9 | 172±16.2 | 200.3±12.4 | 204±22 |
| Acidotic | 259.6±7.9 | 313.9±35.0 | 309.7±33.6 | 365.2±34.5 |
| 24 hr recovery | 101.5±10.9 | 145.0±11.4 | 182.0±27.1 | 187.3±35.0 |

GLUCOSE PRODUCTION:

| | pH 7.40 | pH 7.06 | pH 7.40 + M.S.O. | pH 7.06 + M.S.O. |
|---|---|---|---|---|
| Normal | 68.4±34.9 | 80.5±43.4 | 77.7±45.0 | 76.1±50.6 |
| Acidotic | 141.2±21.5 | 141.9±21.9 | 146.4±23.7 | 148.9±18.4 |
| 24 hr recovery | 71.0±11.7 | 79.1±26.6 | 81.8±17.0 | 80.9±26.4 |

n = 3. $\mu$moles.30 $min^{-1}.gdw^{-1}$.

when MSO was added at pH 7.4.

As in the incubations with glutamine alone, the stimulation in ammoniagenesis in the presence of MSO in the cortical tubules from 24-h recovered rats was similar in magnitude to that seen in the normal and acidotic conditions. A stimulation in ammoniagenesis was clearly demonstrated, however, at pH 7.06 in the acidotic condition.

In the chronically acidotic rat kidney tubules, gluconeogenesis was stimulated 2-fold. This accelerated rate was back to normal in the 24-hour incubations. No significant changes were detected between pH 7.40, pH 7.06 and with MSO however.

This set of experiments demonstrates quite clearly that the renal responses to acute acidosis and chronic acidosis operate independently of each other. Thus an acute response was elicited in chronically adapted kidney tubules. Additionally, in tubules from recovered rats the response to acid incubation or to MSO was similar in magnitude to that obtained in tubules from either normal or acidotic rats. This eliminates the possibility that the inhibition of ammoniagenesis from glutamine that occurs during recovery in rats that maintain greatly elevated activities of PDG and GDH is due to increased activity of GS.

# 4. GENERAL DISCUSSION

## 4.1 Summary

Research in the area of renal ammoniagenesis has been hampered by several factors. The search for a single mechanism to emerge from the growing literature is undoubtedly a misleading approach. As data accumulate the regulation is beginning to appear multifactorial. This is probably one reason why a complete intellectually satisfying theory has yet to be proposed. The problem is compounded by the interpretation difficulties and technical barriers which are a result of anatomical interrelationships of the various renal cell types. In addition, cellular and subcellular heterogeneity exists. A third confounding factor is the various forms of acidosis. That is, respiratory versus metabolic and acute versus chronic. The triggering mechanism responsible for a response to an acute acid challenge probably differs from that seen in the chronic situation. This is evident by the observation that stimulation of ammoniagenesis occurs in acute acidosis before the enzymatic changes which are seen in chronic acidosis occur.

With the current state of knowledge *in vitro* enzyme activity studies would contribute little to the understanding of renal glutamine metabolism. *In vivo* studies may well be rewarding but experimental control is difficult. In this thesis several unanswered questions have been examined by using *in vitro* preparations which have the advantage of experimental control and ease of manipulating conditions. The perfused kidney is thought to be the best *in vitro* preparation for mimicking *in vivo* renal function and in addition suffers less from the biological variability associated with intact animals. The preparation is technically very demanding and this limits its usefulness. The cortical tubule preparation is a less demanding tool than the perfused kidney, however one cannot say with certainty that one is working with pure cortical tubules due to the anatomical interrelationship alluded to above. The technique is an ideal screening tool, however, as it is amenable to the collection of large quantities of data in a short time period. The two techniques were deemed to be well suited for studying the two main thrusts of this thesis, that is:

(1) The study of the relationship between ammoniagenesis and gluconeogenesis in recovery from metabolic acidosis in a good _in vitro_ preparation (perfusion studies).

(2) The testing of an hypothesis regarding the nature of the regulatory mechanism involved in acute acidosis (cortical tubules).

From these studies the following contributions were made:

(1) It was demonstrated that in 24-h recovery, rates of ammoniagenesis and gluconeogenesis from glutamine were back to normal in the perfused kidney. At 8-h recovery no decrease from acidotic rates of ammoniagenesis was obtained in the perfused kidney even when recovery _in vivo_ was initiated by an induced systemic alkalosis.

(2) Aminopicolinic acid previously thought to be an activator of PEPCK was shown to inhibit gluconeogenesis in the perfused kidney.

(3) Good evidence is presented for the hypothesis that inhibition of

glutamine synthetase is responsible for the stimulation of ammoniagenesis from glutamine in acute acidosis. This apparent stimulation of ammoniagenesis is a result of an inhibition of $NH_3$ utilization. This response can also be achieved in the chronic acidotic condition and is of the same magnitude as in isolated tubules from normal rats and rats that had been allowed to recover from metabolic acidosis for 24-h.

## 4.2 Kidney perfusion technique

The perfused kidney preparation was set up and defined as being functionally intact. Physiological parameters such as GFR and fractional sodium reabsorption were within acceptable limits for the perfusion system. Rates of gluconeogenesis from various substrates were determined as being linear over the time course of the perfusion. Adenine nucleotide concentrations were found to be maintained at a high tissue concentration at the end of a perfusion period and isolated mitochondria were demonstrated to be well coupled following a perfusion period. Finally EM studies failed to demonstrate structural abnormalities which would be caused by hypoxia or other unphysiological insults from the perfusion. The system was therefore deemed valid for the present study and met the criteria for being a functionally intact system.

## 4.3 Recovery from chronic acidosis

This model has been useful in studying renal metabolic adaptations to acid-base alterations. Parry and Brosnan (57) have demonstrated a dissociation between *in vivo* $NH_3$ excretion and in vitro PDG activity. PDG remains significantly elevated throughout 11 days of recovery and $NH_3$ excretion returns to normal limits by 24 hours. This in vivo response has been obtained by mitochondrial preparations in some studies, however the mitochondrial response is highly variable and depends on factors such as the extra mitochondrial concentration of phosphate (79). This is probably due to the PDG conformational equilibrium which exists between the monomeric and dimeric states (43). Phosphate shifts the equilibrium towards dimerization which is the active form of the enzyme and thus higher phosphate concentrations stimulate ammoniagenesis. Incubated cortical slices indicate that ammoniagenesis from glutamine is back to normal by 24 hours of recovery. However the integrity of this preparation has been questioned (80) as discussed previously. The results of the perfused kidney agrees with the slice work with respect to rates of ammoniagenesis. Rates of gluconeogenesis however were back to normal in the perfused kidney after 24 hours of recovery whereas in the slices they were still elevated. The rate of gluconeogenesis in the perfusion studies is more in keeping with measured PEPCK activity under these conditions.

Data in the perfused kidney which shows a sustained elevation of ammoniagenesis following an 8-hour accelerated recovery suggests that in vivo factors which are not present in the perfused kidney are required for a suppression of ammoniagenesis in the early stages of recovery. The alkalosis which has been shown in 24 hr-recovery and 8-hr accelerated (47) recovery is one possible regulator. This change in pH is not seen in the 8-hour recovered rats incubated with NaCl and no suppression of ammonia excretion is obtained in these animals.

Although blood pH may be important in the recovery from acidosis a dissociation from ammoniagenesis has been demonstrated in chronic acidosis. In the induction of acidosis a marked drop of blood pH is seen which coincides with an increase in $NH_3$ excretion (57). This lasts for about 4-5 days after which the pH is returned towards normal. This return occurs without a simultaneous drop in ammonia excretion. Thus a simple relationship between blood pH and ammoniagenesis can not be demonstrated. The results obtained in this study of recovery from acidosis indicate that ammoniagenesis in the perfused kidney follows PEPCK activity despite the dissociation seen by Brosnan and Parry in the intact animals (79).

### 4.4 Effect of 3-aminopicolinic acid on gluconeogenesis in the perfused kidney

Earlier studies demonstrated that 3-AP is a hyperglycemic agent in rats (94), caused apparent *in vivo* stimulation of hepatic PEPCK (91) and activates isolated hepatic PEPCK in the presence of $Fe^{2+}$ (94). The data obtained in this thesis demonstrate that in the perfused kidney an inhibition of gluconeogenesis from pyruvate is obtained. This is in agreement with subsequent studies which have demonstrated an inhibition of renal PEPCK (92, 95, 103). 3-AP therefore should be considered an inhibitor of renal PEPCK and not considered useful in the study of renal ammoniagenesis and gluconeogenesis.

### 4.5 Role of glutamine synthetase in acute acidosis

Data are presented in this thesis which strongly suggest that glutamine synthetase inhibition in acute acidosis is responsible for the acute stimulation of ammoniagenesis. G.S. inhibition by acidosis is not a new observation, and decreased *in vivo* renal glutamine synthesis has been shown to occur in acute acidosis in the rat by Damian and Pitts (102), and in acute acidosis in perfused kidneys by Hems (55). In addition, Lemieux *et al*. have shown a small, but significant, decrease in renal G.S. activity in chronically acidotic rats (30). Despite these observations the increase in $NH_3$ observed in acidosis has always been generally attributed to increased PDG and/or GDH flux with most attention being focused on the regulation of these enzymes. This thesis has tested the possibility that the stimulation of ammoniagenesis seen in acute acidosis in cortical tubules is a result of decreased flux through G.S. The data with isolated tubules were entirely consistent with this view.

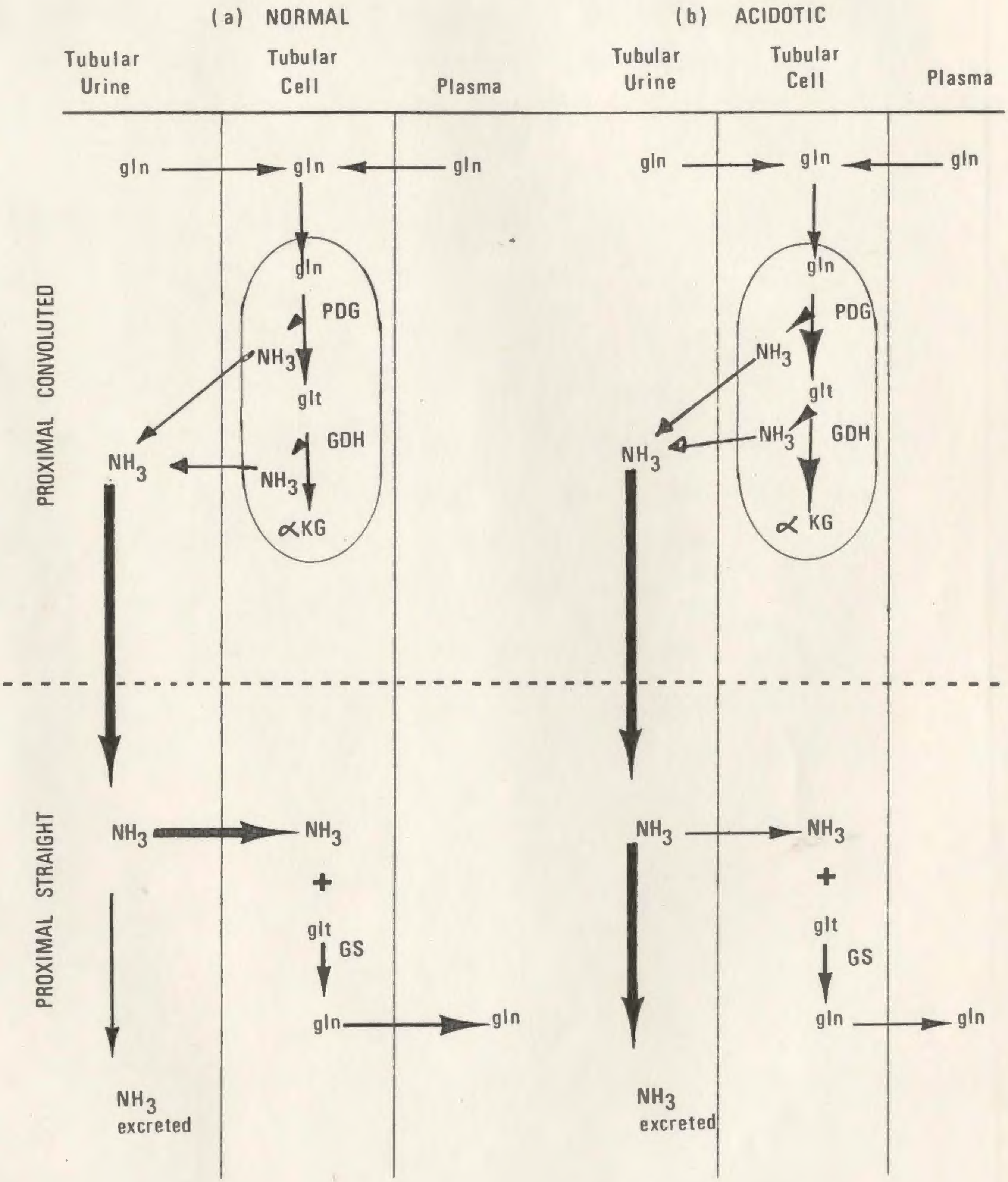
Decreased rates of $NH_3$ utilization and glutamine synthesis from glutamate have been observed with the order of magnitude of G.S. flux being compatible with observed G.S. activity from other studies. Thus the estimated rate of G.S. activity in this study (0.60 moles. $min^{-1}.gww^{-1}$) corresponds well with that estimated by Hems (55) in the perfused kidney (0.55 moles $min^{-1}.gww^{-1}$) and is well below measured *in vitro* activity (2.3 moles $min^{-1}.gww^{-1}$) by Lund (101). This leads to the formation of a new hypothesis, the glutamine synthetase inhibition hypothesis, to explain the acute response to acidosis.

The feasibility of this hypothesis becomes increasingly clear when the sites of the key enzymes are considered. PDG is located all along the nephron but most $NH_3$ production occurs in the proximal convoluted tubule. In fact, it is only in this portion of the nephron in which an adaptive increase in PDG activity in response to chronic acidosis occurs (24). Glutamine synthetase on the other hand is located primarily in the proximal straight portion of the nephron and it is only here in which an adaptive decrease in activity occurs in acidosis (30).

Fig. 4.1 illustrates my hypothesis concerning the role of G.S. in the control of ammoniagenesis. Clearly a classical substrate cycle does not exist as the main enzymes involved are located in different cells. $NH_3$ produced in the proximal convoluted tubule must traverse the proximal straight tubule in which it is subject to the action of glutamine synthetase. If the activity is high, as in normal acid-base conditions, most $NH_3$ is converted back into glutamine thus completing a functional cycle. Because of this $NH_3$ reutilization, little is excreted into the urine and arteriovenous glutamine differences are low (57). In acute acidosis however glutamine synthetase activity is postulated to be markedly decreased as demonstrated in this thesis and as indicated in scattered observations throughout the literature. This inhibits the uptake of $NH_3$ and promotes excretion into the urine. Large A-V glutamine differences are thus seen (102) as observed by Damian and Pitts. The diffusion properties of $NH_3$ are a key element in this model. In the normal condition a rapid utilization of $NH_3$ within the proximal straight tubule causes a diffusion gradient with subsequent influx of $NH_3$ from the tubular lumen. The converse happens in the acidotic condition.

An interesting observation which lacks a satisfying explanation

Fig. 4.1 Glutamine synthetase inhibition hypothesis for acute stimulation of $NH_3$ excretion.


(a) NORMAL
(b) ACIDOTIC
Tubular Urine
Tubular Cell
Plasma
PROXIMAL CONVOLUTED
PROXIMAL STRAIGHT
gln
PDG
$NH_3$
glt
GDH
αKG
+
GS
$NH_3$ excreted

is the stimulation of gluconeogenesis from glutamine in tubules incubated with M.S.O. at pH 7.4. One can only suggest a mechanism until further research is carried out. One could hypothesize that since the synthesis of both glutamine and glucose draw on Krebs cycle intermediates a competition for these intermediates may occur in the proximal straight tubule. In this way inhibition of glutamine synthetase could result in a stimulation of gluconeogenesis.

### 4.6 Mechanism of inhibition of glutamine synthetase

G.S. is a cytosolic enzyme which is thought to be loosely associated with the endoplasmic reticulum (104). It is found in most tissues of the body and, because inhibition of brain G.S. is related to convulsions, most work has been on G.S. isolated from this organ. Inhibition of G.S. by M.S.O. is a two-step process. The first is a competitive binding to the glutamate site of the enzyme. This is followed by a phosphorylation of M.S.O. and leads to an irreversible inhibition (105). M.S.O. also inhibits $\gamma$-glutamylcysteine synthetase, an enzyme in the pathway of glutathione synthesis (105). This effect can hardly be responsible for the findings in this thesis in view of the observation of Moldeus *et al*. (106) that glutathione is not synthesized in isolated cells unless an exogenous supply of cystine is added.

The mechanism of inhibition of G.S. in acute acidosis is merely speculative based on our current knowledge of the kinetic properties of the enzyme. The enzyme is an octomer which in ovine brain has been shown to be influenced by metal ions (108). The pH optimum can range from 4.8 to 8.5 depending on the concentration of the divalent cations. In addition rat liver G.S. has been shown to be inhibited by inorganic phosphate (109). The Km value for ovine brain G.S. for ammonia is .18 mM (105) a near physiological concentration. The substrate for G.S. reaction has been shown to be $NH_3$ rather than $NH4^+$ (105). From the Km of the enzyme for $NH_3$ (assuming kidney G.S. doesn't vary greatly from brain G.S.) and the physiological concentration of ammonia, one could envisage how the rate of G.S. activity could be limited by substrate availability ($NH_3$). This is apparent when one considers that the pK of the $NH_3 + H^+ \quad NH4^+$ reaction is 9.2. A drop in pH from 7.4 to 7.0 would dramatically alter $NH_3$ concentration.

A further possible control mechanism exists in the observation that liver G.S. has been shown to be activated by 20 mM -ketoglutarate (109). The concentrations of -ketoglutarate necessary for stimulation are rather high. Many studies have shown that -ketoglutarate is profoundly influenced by acid-base alterations (65, 73, 74, 75). Thus a decrease in -ketoglutarate concentration which occurs in acidosis may well deactivate glutamine synthetase leading to the stimulation of ammoniagenesis.

## 4.7 Further studies

Further studies should involve confirmation of these observations in another species. The guinea pig would be an ideal model as glutamine synthetase activity is extremely high and PDG activity low in the kidneys of these animals. This work has, in fact, been subsequently performed by a graduate student in our laboratory and confirms the present study. A 50% inhibition of glutamine synthesis has been observed in cortical tubules incubated at pH 7.1 compared with 7.4 (Ezekiel Quarcoo, unpublished information).

The inhibitor M.S.O. is not entirely specific for G.S. as alluded to above and therefore another inhibitor, α-ethylmethionine sulfoximine, which has been shown to be specific (111), but is not commercially available, should be synthesized and employed to confirm the M.S.O. studies.

The mechanism of action of the inhibition needs to be resolved. If $NH_3$ is limiting at low pH, as hypothesized previously, the inhibition of glutamine synthetase should be abolished at high ammonia concentrations.

The ultimate work however which needs to be completed is the *in vivo* studies. These could be done employing an infusion technique in which M.S.O. (or α-ethyl M.S.O.) (112) is infused at low concentrations into a single kidney. By infusing the proper concentration, the irreversible binding to M.S.O. will result in no effect in the contralateral kidney. The function of each kidney could be simply monitored by bilaterally cannulating the ureters. When an adequate G.S. block has been achieved in one kidney with no effect on the other, an acute systemic acidosis could be induced. The result, if the hypothesis is correct, would be no further stimulation in ammonia excretion by the blocked kidney and a stimulation in the contralateral.

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