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AMMONIAGENESIS IN THE RAT KIDNEY DURING RECOVERY FROM METABOLIC ACIDOSIS

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by

© David M. Parry, M.Sc.

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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June 1980

St. John's Newfoundland

TO MY WIFE

ABSTRACT

The regulation of renal glutamine metabolism in relation to acid-base status was investigated in rats. The model of NH_4Cl-induced acidosis was used and two new experimental situations introduced. Recovery from acidosis was investigated in rats allowed to drink H_2O *in lieu* of NH_4Cl and the process of recovery was accelerated by administration of $NaHCO_3$ (1.5 mmol/100g body wt). Animals allowed to recover from acidosis were again challenged with various acid loads and the responses of these rechallenged rats compared to the responses of naive-challenged rats.

Urinary ammonia excretion, total renal ammonia production and glutamine extraction returned to normal by about 24h in animals which were allowed to recover from metabolic acidosis by drinking H_2O. In comparison, in animals administered $NaHCO_3$ at the start of recovery, these parameters were back to normal by 8h. Rats which were permitted three days to recover from metabolic acidosis were able to excrete more acid as ammonium salt than animals challenged with NH_4Cl for the first time, when challenged with high acid loads. There was no difference in response to lower acid loads.

Decreases in the renal contents of glutamine, α-ketoglutarate and malate were found during metabolic acidosis and increases in glutamine, glutamate, α-ketoglutarate, malate, citrate, lactate, phosphoenolpyruvate and 3-phosphoglycerate occurred during recovery. No significant difference in the renal content of metabolites was observed between rats administered $NaHCO_3$ and rats administered the same amount of NaCl during recovery.

The renal activities of phosphoenolpyruvate carboxykinase (PEPCK), glutaminase (PDG) and glutamate dehydrogenase (GDH) in these animals were also investigated. PEPCK activity increased identically in naive-challenged and rechallenged rats. During recovery PEPCK activity returned to normal by about 16h and no significant difference in activity could be discerned between animals intubated with $NaHCO_3$ and animals intubated with NaCl. Immunotitration of PEPCK revealed that the increased activity during acidosis and the decreased activity during recovery are due to changes in the content of this enzyme. The activities of PDG and GDH increased during acidosis and remained elevated long after renal glutamine metabolism returned to normal, in vivo.

Ammonia formation by isolated mitochondria and the fluxes through PDG and GDH increased during metabolic acidosis. However, normal rates of ammonia production in vivo were attained 12-24h after the induction of acidosis while no acceleration of GDH flux in isolated mitochondria was evident at this time. In the most physiological medium used (1 mM glutamine, 3 mM glutamate, 4 mM phosphate) ammonia formation and GDH flux decreased coincidentally during recovery and preceeded the fall in PDG flux. The differences in renal ammoniagenesis between naive-challenged rats and rechallenged rats and between rats intubated with NaCl and $NaHCO_3$ during recovery could not be accounted for by metabolic differences in isolated mitochondria. In the most unphysiological medium used (1 mM glutamine, 20 mM phosphate) the mitochondrial capacity to metabolize glutamine remained elevated for at least 15 days of recovery.

These observations suggest that renal glutamine metabolism is not

controlled simply by changes in the contents of PDG or PEPCK or by the mitochondrial events which are responsible for the changes in the fluxes through PDG or GDH in isolated mitochondria. The changes observed in αketoglutarate are consistent with its purported regulatory role.

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LIST OF ABREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(β-amino-ethylether)N,N'-tetraacetic acid
GABA	γ-aminobutyric acid
GDH	Glutamate dehydrogenase
GLN	Glutamine
GOT	Glutamate oxaloacetate transaminase
GLUT	Glutamate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ITP	Inosine triphosphate
α-Kg	α-ketoglutarate
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
MOPS	Morpholinopropane sulfonic acid
OAA	Oxaloacetate
PDG	Phosphoenolpyruvate carboxykinase
Phe	Phenylalanine
ϕPyr	Phenylpyruvate
NAD^+	Nicotinamide-adenine dinucleotide, oxidized
NADH	Nicotinamide-adenine dinucleotide, reduced
TRIS	Tris (Hydroxymethyl) aminomethane

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

There is "no doubt that glutamine is a factor of general importance in cell metabolism but the nature of its function is not yet clear" (122). This remark aptly reflects the state of knowledge of glutamine metabolism in 1939. It was in this year that glutamine was first shown to exist in its free form in various mammalian tissues (7, 112, 122). However, the demonstration by Krebs (87) in 1935 of the presence of mammalian enzymes capable of synthesizing and hydrolyzing glutamine was the first indication that glutamine played a role in animal metabolism. These early observations essentially represent the beginning of the study of glutamine metabolism. Since then a great deal of attention has been focussed on the many aspects of glutamine metabolism and regulation.

PART I. Physiological Importance of Ammonia and Glutamine

1.1 Ammonia: Friend and Foe

A marked polarity is imparted to ammonia by its pyramidal shape and projecting hybrid orbital. The unpaired electrons occupying this projecting orbital are a potential source for electron-seeking atoms. Hence, protons will readily bind with ammonia to form ammonium ions. Because ammonia is a small molecule, gaseous and soluble in lipids (129), it can readily diffuse across membranes and hence is extremely mobile in biological systems.

These molecular properties of ammonia have important physiological consequences. Ammonia plays a crucial role in the maintenance of acid-

base balance by facilitating the excretion of protons. This attribute of ammonia was first recognized over 130 years ago with the observation that more ammonia is excreted by carnivores, which have acid urine, than by herbivores, which have alkaline urine (68). That ammonia plays a role in the maintenance of acid-base balance was corroborated by two other observations. One, the administration of alkali to carnivores reduced urinary ammonia (68) and two, the administration of mineral acid increased the excretion of ammonia (68). These observations have been repeatedly confirmed and the importance of ammonia to acid excretion unequivocally established.

Although ammonia is now recognized as an important cellular constituent, it is also a very toxic molecule (70, 107). Recognition of this characteristic of ammonia dates back to the 17th century, when toxic effects were observed in experiments with dogs which died under violent convulsion after having ammonium chloride injected into their jugular veins (47). Apart from the direct effects of a buildup of ammonium ions (107), an increase in pNH_3 will indirectly affect cellular metabolism by its participation in the equilibrium reaction catalyzed by glutamate dehydrogenase (16). Since ammonia is very mobile it can easily enter the brain (29) where the results of such effects threaten vital life-sustaining processes.

1.2 Role of Ammonia in Acid Excretion

Large quantities of hydrogen ions arise daily from the dietary intake of preformed acids, and from acids formed during intermediary metabolism (109, 152). A vast potential source of acid is CO_2 produced

during intermediary metabolism since the hydration of CO_2 forms carbonic acid. However, CO_2 can be rapidly removed by pulmonary ventilation since it is volatile and can freely diffuse across membranes. In this sense, the lungs play a major role in the elimination of acid. In comparison, only a very small fraction of the total daily acid produced is non-volatile and cannot be eliminated by the lungs. Nevertheless, since the concentration of H^+ ions in the body is very low (in the order of 40 nmoles/L) and since cellular processes are extremely sensitive to these reactive ions, it is vital that highly effective mechanisms exist to buffer and excrete non-volatile acid.

The buffering of non-volatile acid is accomplished by the concerted action of a number of chemical buffering systems. Phosphate compounds and proteins (particularly the imidazolium group of histidine residues) possess the necessary pK's to act as buffers at physiological pH. However, the key role is attributed to the bicarbonate-carbonic acid buffer system (109, 201). The importance of this system is not due to its buffering ability, per se, since a pK of 6.1 is too far removed from physiological pH. Rather the dominant role played by this buffer is attributable mainly to two other characteristics. First, it is the buffer of highest concentration in the extracellular fluid and second, the components of this system are under extremely effective control by the lungs and kidneys. Combination of a hydrogen ion with bicarbonate results in the formation of carbonic acid, which rapidly equilibrates with CO_2. The CO_2 is removed by pulmonary ventilation and hence, due to a constant pCO_2, carbonic acid is esentially a "fixed" component of this buffer system (thereby increasing its effective buffering capacity).

The excretion of non-volatile acid is accomplished by the kidney. The acid, brought to the kidney mainly as neutral sodium salts, is excreted primarily as titratable acid and ammonium salts (129). The elimination of acid in these forms involves a mechanism which exchanges tubular sodium for hydrogen ions in the adjacent tubular cells. In this exchange mechanism the sodium ions enter the tubular cells down an electro-chemical gradient (129), which essentially drives the carrier-linked (115) secretion of H^+ ions into the tubular lumen, against an electro-chemical gradient (101, 144). The secreted hydrogen ions are derived from intracellular carbonic acid which also provides the bicarbonate ions which are returned, along with the sodium ions, to the general circulation through the peritubular blood (129). The form in which the secreted hydrogen ions are excreted depends upon the pK of the acids formed in the tubular fluid. If the pK of the acid formed is higher than the pH of the urine, then the hydrogen ions will be excreted as titratable acid. However, if the acid formed has a pK lower than the pH of the urine than the acid will remain dissociated. The sodium-proton exchange pump can operate against a glomerular pH of down to 4.6 only (up to a $[H^+]$ gradient of about 800-1000 to 1 between tubular lumen and peritubular blood (133)). Consequently the kidney cannot excrete appreciable amounts of strong acid in its free form.

Ammonia plays a unique role in the excretion of strong acids. It would appear that with a pK of 9.1, the ammonia - ammonium pair would be unsuitable as a urinary buffer since only a very small fraction would exist as ammonia at the pH of urine. This is true for ammonia filtered at the glomerulus. However, the ammonia required for buffering strong

acid is produced in the adjacent tubular cells (129). By virtue of its gaseous nature, small size and lipid solubility, ammonia rapidly diffuses from the tubular cells into the tubular lumen where, by virtue of its basicity, it readily binds the hydrogen ions in the tubular fluid to form ammonium ions. Since membranes are much less permeable to ammonium ions and since protonation is greatly favoured at acid pH, the NH_4^+ is essentially trapped in the tubular lumen. The conversion of ammonia to ammonium also keeps the pNH_3 of the tubular fluid low, thus favouring continued diffusion of ammonia. Ammonia thus plays a crucial role in the excretion of strong acids by permitting the exchange of Na^+ ions for H^+ ions to continue without forming a limiting pH gradient.

In the rat about 20-30 percent of the total acid excreted is excreted as titrable acid, the remaining 70-80 percent is excreted in combination with ammonia (125). Hydrogen ion excretion is dramatically increased during ammonium chloride-induced metabolic acidosis. A four to five fold increase in total acid excretion in the rat reduces titratable acidity to about 10 percent and increases the fraction excreted as ammonia to about 90 percent (125). These relative changes occur because of a six to seven fold increase in ammonia excretion with only a two to three fold increase, at most, in the excretion of titratable acid. The increase in titratable acid is probably due, in part, to the greater participation of low pK urinary buffers such as creatinine and urate, which would contribute more fully at the lower urinary pH reached in metabolic acidosis. Part of the increased titratable acidity is also probably due to extra phosphate mobilized from the cells and bone to provide extra buffer (152). By far the most substantial contribution to

the increased acid excretion is due to the increased ammonia formation by the kidney.

1.3 Glutamine: An Active Form of Ammonia

Although by 1939 the role of ammonia in acid excretion was firmly established and the toxic effects of this substance recognized, the relevance of glutamine to ammonia metabolism was not well appreciated. Since the demonstration by Nash and Benedict (118) in 1921 that the concentration of preformed ammonia in the blood was insufficient to account for the ammonia excreted in the urine, it was recognized that ammonia must be produced in the kidney from nitrogenous precursors extracted from the arterial blood. Over the subsequent twenty years a number of nitrogenous substances (urea, amino acids, adenylic acid and proteins) were suggested as possible precursors (129). The first clear-cut demonstration of the importance of glutamine as a percursor of ammonia came from experiments by Van Slyke et al (188) in 1943. These workers demonstrated that the rate of glutamine extraction by the kidney during acidosis was sufficient to account for a major portion of the ammonia produced. Subsequent studies (129) utilizing more advanced surgical techniques and refined methods of amino acid analysis all confirmed the original findings of Van Slyke and his associates. Experiments with N^{15}-labelled glutamine by Pitts and his associates (137, 138, 177) proved definitively that both the amide and amino nitrogens of glutamine are the major precursors of renal ammonia.

Today, the central role of glutamine in nitrogen metabolism is well appreciated. Its importance is attested to by the fact that it is

usually the amino acid present in highest concentration in mammalian plasma (114) and also by its presence in high concentrations in most mammalian tissues (114). In ammonia metabolism specifically, glutamine acts as a storage form in the uptake, transport and formation of ammonia. In nitrogen metabolism in general, glutamine serves as a preferred nitrogen donor in a number of enzymatic reactions which require nitrogen for the formation of a variety of compounds (174). Presumably, the concentration of ammonia permitted in the cells is not suitable for ammonia to participate as the nitrogen donor in these reactions. Not only does glutamine play a role in nitrogen metabolism but it also serves as a transport form for glutamate. Glutamine crosses cell membranes more readily than glutamate. Glutamate and γ-aminobutyrate, both derived from glutamine, have synaptic functions (114). The physiological role of glutamine, of course, depends on the tissue or cell.

The demands on glutamine evidently require that the continuous withdrawals from the circulating glutamine pool be balanced by continuous contributions from other tissues. The actual mechanisms operating to maintain a constant supply of glutamine in the face of continual removal are unclear. Part of the confusion presumably derives from differences in species and experimental states which are used to investigate this phenomenon. Glutamine release has been variously demonstrated to occur by the brain (106, 186), heart (69), liver and peripheral tissues (69, 158, 186). Only the kidney (158, 186) and the gastrointestinal-tract plus liver (186, 202) have been shown to extract glutamine. Glutamine turnover in the rat was unchanged after ammonium chloride loading (172). This implies that increased synthesis does not play a

role in supplying the extra glutamine utilized by the kidney. This leads to the conclusion that the extra glutamine extracted occurs at the expense of glutamine normally utilized by other tissues. However, measurement of arterio-venous differences failed to reveal which tissues used less glutamine (172). Since the magnitude of the change in glutamine utilization by any one organ necessary to continue supplying the kidney should be sufficiently large to be detected by the methods used, then it must be assumed that no single tissue is responsible for the provision of extra glutamine to the kidney. The alternative is that a combination of extra-renal tissues all decrease utilization such that the sum is sufficient to supply the extra glutamine required by the kidney. Changes of this magnitude might escape detection because individual arterio-venous are too small to be measured experimentally.

PART II. Renal Glutamine Metabolism

1.4 Enzymology

A number of renal enzymes capable of generating ammonia from glutamine, *in vitro*, have been demonstrated.

A. γ-Glutamyltranspeptidase

Part of the confusion as to which enzymes are responsible for generating ammonia is attributable to the multiple activities of γ-glutamyltranspeptidase, sometimes referred to as γ-glutamyltransferase. This enzyme can release ammonia by transferring the γ-glutamyl radical of glutamine to a receptor molecule (transpeptidation). When the receptor molecule is glutamine, the reaction catalyzed is referred to as an

autotranspeptidation reaction. This enzyme can also convert glutamine to glutamate and ammonia by its glutaminase activity (182, 183). The supposed involvement of the transpeptidation and glutaminase reactions rests primarily on the stimulation of these activities by maleate (183). Thompson and Meister (184) suggest that the binding of maleate to γ-glutamyltranspeptidase causes a conformational change in the enzyme which promotes the utilization of glutamine as substrate. Although this activator is non-physiological, the possibility of a physiological activator with maleate capabilities has been entertained (178). It has been proposed (184) that the effect of maleate on the enzyme may be related to structural similarities between maleate and aminoacylglycine acceptor substrates, which also promote a conformation change in the enzyme. Both of these activities have been shown to be responsive to pH changes in a "physiological manner". At lower pH, the glutaminase activity predominates over the transpeptidase reaction (34). An increase in the activity of γ-glutamyltranspeptidase has been found in kidneys from acidotic rats (6).

Support for the involvement of the transferase activity comes from the studies of Welbourne et al (126, 197). Welbourne proposed that the transferase activity of this enzyme contributes a significant fraction of the ammonia formed under normal acid-base conditions. This conclusion is based largely on two types of experiments. First, analysis of glutamate and ammonia failed to account for all of the glutamine nitrogen utilized by the kidney perfused with supra-physiological concentrations of glutamine (197). The unanalyzed nitrogen was assumed to be retained within the kidney as glutamylpeptides, the presumed product of the

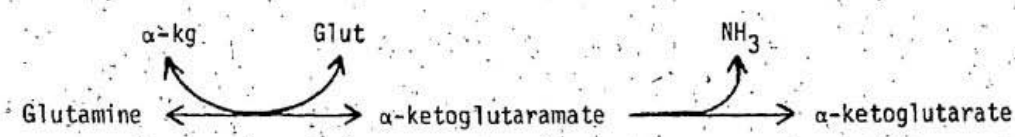
transferase reaction. Second, Welbourne showed that acetazolamide substantially reduced ammonia production by the intact functioning kidney and the perfused kidney when administered in vivo to the normal rat (126). Acetazolamide is a competitive inhibitor of transferase activity (126). Consequently, Welbourne concluded that the transferase reaction is a major contributor to renal ammoniagenesis in the normal rat.

On the other hand, Ross and Bullock (154) were able to completely account for nitrogen from glutamine left in the kidney after perfusion with high glutamine concentrations. Only eight percent of the glutamine metabolized was in a substance that liberated glutamate on acid hydrolysis and this was suggested to be 5-oxypyrolidine-2-carboxylate, a non-enzymatic breakdown product of glutamine. In addition, as Tannen points out (178), acetazolamide may not be a selective inhibitor of the transferase reaction. Acetazolomide has also been shown to inhibit gluconeogenesis (181) and hence a decrease in ammonia formation may be secondary to this effect.

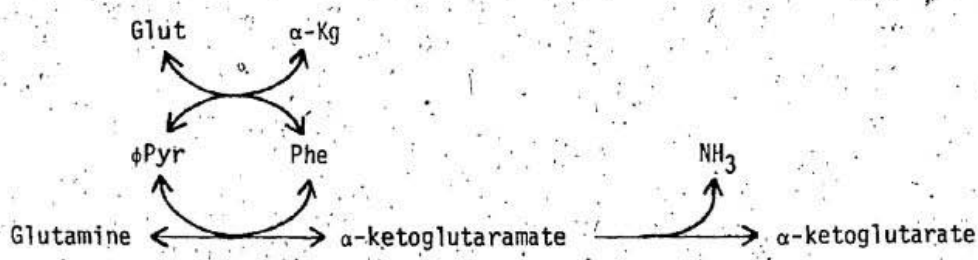
B. Glutaminase II Pathway

The cytosol of renal cells also contains an enzyme called ω-amidase (31). After transamination of glutamine with an α-keto-acid to form α-ketoglutaramate and the corresponding α-amino acid, ω-amidase is able to release the amide nitrogen of α-ketoglutaramate as ammonia (30). The concerted action of glutamine transaminase and ω-amidase is, for historical reasons, generally referred to as the glutaminase II pathway. If α-ketoglutarate is the α-keto acid substrate, then the corresponding

amino acid formed would be glutamate, the net result being the conversion of glutamine to glutamate and ammonia.



It has been demonstrated for the rat kidney enzyme that phenylpyruvate is the preferred α-keto acid (31) and that the renal tissue concentration of phenylpyruvate is well above the K_m of the enzyme for this substrate (31). In order to obtain net conversion of glutamine to glutamate and ammonia with phenylpyruvate as the α-keto acid substrate, an additional transamination reaction would have to be involved, coupling the formation of glutamate from α-ketoglutarate with the regeneration of phenylpyruvate from the product of the transamination reaction, phenylalanine.



Even though the activity of the glutaminase II pathway is apparently increased in the rat during metabolic acidosis (56, 81, 145, 176), the enzymatic activity expressed *in vitro* under "physiological conditions" is sufficient to contribute only a very small proportion of the renal ammonia production (51). However, all of these studies employed

pyruvic acid as the α-keto acid substrate for the glutamine transaminase reaction. Nevertheless, relatively low activity of the glutaminase II pathway was found when pyruvate was replaced with phenylpyruvate and this was only slightly increased by acidosis (81, 176). Tracer studies (177) using ^{15}N amino-labelled glutamine indicate appreciable transamination of the amino group of glutamine into amino acids other than glutamate, and this has been interpreted to suggest the involvement of the glutaminase II pathway in the renal metabolism of glutamine. In contrast, studies utilizing kidney cortex slices have shown that an inhibitor of glutamine transaminase (amino-oxyacetate) does not have any significant effect on the production of ammonia by these slices (147), whereas an analogue of glutamine (6-diazo-5-oxo-L-norleucine), which does not inhibit the glutaminase II pathway, markedly reduces ammonia production (53).

(C) Glutamine Synthetase

Another enzyme located in the cytosol of rat kidney is glutamine synthetase. This enzyme catalyzes the formation of glutamine from ammonia and glutamate, with the utilization of ATP as a source of energy. Although this reaction is reversible, its equilibrium constant greatly favours synthesis (129). Therefore, although glutamine synthetase cannot be directly involved in the release of ammonia from glutamine, its relative activity can potentially influence net ammonia production. It has been demonstrated that metabolic acidosis, induced in vivo, diminishes the flux through this enzyme in the intact functioning kidney (41), in the perfused kidney and in kidney tissue slices (66, 77). The

decreased flux is not attributable to reduced enzyme amount since the activity of glutamine synthetase, *in vitro*, is not affected (22, 75) or only minimally diminished (97, 145) by metabolic acidosis. Failure of methionine sulfoximine (an inhibitor of glutamine synthetase) to affect the increase in ammonia production during acidosis *in vivo* (197) does not necessarily rule out the involvement of glutamine synthetase in determining net ammonia production, especially since a decrease in glutamine synthetase activity was postulated as the mechanism.

(D) Phosphate-Dependent Glutaminase

Although the physiological importance of the above mentioned enzymes in renal ammoniagenesis cannot be discounted, the interpretation of their activities in terms of other functions or as phenomena observed *in vitro* has also been possible (178). However, no function, other than ammoniagenesis is known for phosphate-dependent glutaminase. This enzyme is associated with mitochondria (40, 76, 81) and since it is activated by phosphate, is called phosphate-dependent glutaminase (PDG). It is also referred to as glutaminase I to distinguish it from glutaminase II.

A large body of evidence now exists which suggests that PDG is the major enzyme responsible for generating ammonia from glutamine, *in vivo*. This evidence is primarily derived from measurements of phosphate-dependent glutaminase activity *in vitro*. Goldstein *et al* (51, 53) have shown that the activity of PDG under conditions designed to simulate those found *in vivo* (with physiological concentrations of substrates and inhibitors) quantitatively relates to the rate of ammonia excretion, *in*

vivo. Also, the activity of PDG in rat kidney is increased by acidosis (125, 146). All the available evidence is consistent with the currently favoured view that PDG is the major enzyme responsible for the release of ammonia from glutamine.

1.5 Metabolic Pathways for Glutamate Removal

The deamidation of glutamine by phosphate-dependent glutaminase results in the formation of glutamate. The physiological properties of glutamate make the fate of this amino acid of central importance to renal ammoniagenesis. First, glutamate is a potent inhibitor of phosphate-dependent glutaminase (54, 58, 148). It appears that the inhibition of glutaminase by glutamate is associated with reversal of dimer formation (49). Therefore, for renal ammoniagenesis to proceed, glutamate must be continuously removed from the site of PDG. Second, whereas glutamine is a neutral amino acid, glutamate is acidic and will be partially dissociated at physiological pH. As a result, deamidation of glutamine effectively results in the net formation of ammonium (NH_4^+) and not its basic form, ammonia (NH_3). In order for ammonia to become available to buffer urinary acid, glutamate must be metabolized to a neutral substance (176). Theoretically, glucose, CO_2 and fat are three possible neutral compounds. The most likely neutral end products are glucose and CO_2:

$$2(C_5H_{10}O_3N_2) + 2H_2O + 3O_2 \longrightarrow C_6H_{12}O_6 + 4CO_2 + 4NH_3$$

$$C_5H_{10}O_3N_2 \longrightarrow 5CO_2 + 2H_2O + 2NH_3$$

Glucose is essentially undissociated at physiological pH, CO_2 can be

respired off by the lungs and fat is uncharged. Third, the amino nitrogen of glutamine also contributes to urinary ammonia (138). Consequently, the conversion of glutamate to a neutral compound must provide for the release of ammonia.

The known enzymatic content of the rat kidney provides for four potential pathways for the further metabolism of glutamate.

A. Glutamate Dehydrogenase Pathway

One metabolic option considered likely is the intramitochondrial conversion of glutamate to α-ketoglutarate via glutamate dehydrogenase (GDH). Subsequent conversion of α-ketoglutarate to a neutral compound will permit the nitrogen released in this reaction to be available as ammonia. The likelihood of this possible route is augmented by the fact that GDH activity increases in the rat in response to metabolic acidosis (132, 145, 165). Also, acidosis has been shown to increase the flux through glutamate dehydrogenase in isolated mitochondria (161) and tissue slices (141) from rat kidney.

Conversion of α-ketoglutarate to either fat or glucose requires that the carbon skeleton leave the mitochondria and that reducing equivalents (NADPH and NADH for triglyceride synthesis and NADH for gluconeogensis) be generated in the cytosol. In order to meet these requirements, it is generally accepted that both the carbon skeleton of α-ketoglutarate and the mitochondrial reducing equivalents are transported out of the mitochondria as malate (150). Oxidation of malate to oxaloacetate via cytoplasmic malate dehydrogenase generates the required cytoplasmic NADH. Subsequent conversion of oxaloacetate to glucose or fat can then

proceed via phosphoenolpyruvate carboxykinase. The additional requirement in fat synthesis for NADPH may be met by the hexose monophosphate shunt which has been shown to be increased by acidosis (201). Malic enzyme may also play a role in the generation of cytosolic NADPH in the rat kidney (194, 196).

α-Ketoglutarate can not be removed solely through the reactions of the Krebs' cycle per se. This is because α-ketoglutarate is a Krebs' cycle intermediate and although two carbon atoms are removed as CO_2 for each turn of the cycle, two additional carbon atoms are added as acetate from acetyl-CoA. For complete oxidation of α-ketoglutarate, the carbon skeleton must leave the cycle and re-enter as acetyl-CoA. It is generally accepted that for complete oxidation of α-ketoglutarate, the carbon skeleton leaves the mitochondria as aspartate (150). Interconversion between oxaloacetate and aspartate occurs freely in both mitochondria and cytosol via the appropriate transaminase. Conversion of oxaloacetate to pyruvate via phosphoenolpyruvate carboxykinase does not generate NADH. Pyruvate is free to enter the mitochondria and be converted to acetyl-CoA via pyruvate dehydrogenase for its eventual dissipation as CO_2.

B. Glutamate Oxaloacetate Transaminase/Purine Nucleotide Cycle Pathway

The presence of glutamate-oxaloacetate transaminase (GOT) provides for another possible route for glutamate removal. Transamination of glutamate with oxaloacetate results in the formation of α-ketoglutarate and aspartate. Aspartate, so formed, can leave the mitochondria by an obligatory exchange for glutamate. Transamination of aspartate with

α-ketoglutarate in the cytosol would have the same net result as the previously described pathway for the transport of the α-ketoglutarate carbon skeleton out of the mitochondria as aspartate. However, owing to the existence of three other enzymes in the rat kidney, aspartate may also be metabolized by the so-called purine nucleotide cycle (PNC). The enzymes which catalyze the reactions of this cycle are adenylate deaminase, adenylsuccinate synthetase and adenylosuccinase. This cycle uses catalytic amounts of inosine monophosphate, adenylosuccinate and adenosine monophosphate as well as GTP as an energy source to convert aspartate to ammonia and fumarate (104). Coupling of this pathway with the glutamate-hydroxyl transporter will permit continued exit of aspartate from the mitochondria. It has also been demonstrated that reactions occur in the rat kidney which convert fumarate to malate (18). Subsequent conversion of malate must be to either glucose or fat via phosphoenolpyruvate carboxykinase since cytoplasmic NADH will be generated by malate dehydrogenase.

Support for the participation of this pathway in the removal of glutamate comes from the observed increases in adenylosuccinate synthetase and adenylosuccinase during metabolic acidosis (18). The lack of an effect of amino-oxyacetate (a potent transaminase inhibitor) on ammonia production by slices (147) and isolated mitochondria (92) has been interpreted to indicate that this metabolic pathway does not play a role in renal ammoniagenesis (178). However, Schoolwerth et al (160) have shown that in isolated rat kidney mitochondria, a decrease in glutamate transamination to aspartate in the presence of amino-oxyacetate is accompanied by a concomitant increase in glutamate deamination by glutamate dehydrogenase. Therefore, a reduction in the metabolism of

glutamine due to inhibition of glutamate-oxaloacetate transaminase may not be noticed by measuring ammonia formation, since in the presence of amino-oxyacetate more ammonia may be formed via glutamate dehydrogenase. Furthermore, Schoolwerth et al (161) have shown that glutamate utilization by rat kidney mitochondria occurs primarily by transamination. Therefore, it appears that the potential physiological importance of this pathway in renal ammoniagenesis cannot be ignored.

C. γ-Aminobytric Acid Shunt

Another metabolic option available to intramitochondrially-produced glutamate is decarboxylation to γ-aminobutyric acid (GABA). This so-called GABA shunt is catalyzed by the mitochondrial enzyme, glutamate decarboxylase and has been shown to be active in rat kidney (134). Transamination of GABA with co-substrate α-ketoglutarate results in the formation of glutamate and succinic semialdehyde. The carbon skeleton can then enter the Krebs cycle as succinate via the enzyme succinic semialdehyde dehydrogenase. Thus, when α-ketoglutarate is co-substrate in the transamination reaction, glutamate is regenerated and the net result of this pathway is the conversion of α-ketoglutarate to succinate. It has been shown in sonicates of rat renal mitochondria that GABA transamination also occurs with pyruvate and does so at a rate at least two-thirds of the rate with α-ketoglutarate (61). With pyruvate as co-substrate, the net result of the GABA shunt will be conversion of glutamate to alanine. In some species, a quantitatively significant amount of alanine is released by the kidney (123, 136, 166). In the rat, serine has consistently been shown to be added to the renal vein (2,

135, 173, 204). If hydroxypyruvate was used as co-substrate in the GABA transamination reaction, serine would be formed; hence this could be the possible origin of the serine released by the kidney.

It has also been shown that rat kidney slices are capable of forming ammonia from GABA (189). Regeneration of glutamate by transamination of GABA with α-ketoglutarate and subsequent deamination of glutamate by glutamate dehydrogenase has been ruled out as the mechanism (189). Therefore, potentially at least, glutamate can be removed by the GABA shunt and converted to either succinate or to another amino acid such as alanine or serine, or utilized to form ammonia by a route other than oxidative deamination via glutamate dehydrogenase. Glucose does not appear to be a possible end product of this pathway since it is not formed in slices incubated with GABA. The role of this pathway in ammoniagenesis is uncertain, especially since neither glutamate decarboxylase activity nor ammonia formation from GABA are increased in metabolic acidosis (95, 189). However, studies with kidney cortex slices have shown that semicarbazide (an inhibitor of glutamate decarboxylase) prevents the full expression of chronically acidotic rates of ammoniagenesis (61). Consequently the GABA shunt may be required for maximal ammoniagenesis.

D. Glutamate Exit

A final option for glutamine-derived glutamate is direct transport into the cytosol. Studies performed with rat liver and heart mitochondria have demonstrated the existence of a bidirectional glutamate-hydroxyl carrier (8, 96, 113). That such a transporter exists in rat

kidney mitochondria is apparent since mitochondria isolated from rat kidney can form ammonia (161) from glutamate and this requires participation of the glutamate-hydroxyl transporter.

Futile cycling of glutamate to glutamine probably does not occur since glutamine synthetase is not present in the same cells in which the adaptive changes in PDG occur (22). Transport of glutamate back into the mitochondria via the glutamate/aspartate carrier would serve the purpose of providing a continuous supply of glutamate for aspartate exit. Glutamate transported into the cytosol could also be transaminated to aspartate by cytoplasmic glutamate-oxaloacetate transaminase. However, the net result would be the same as the transport of aspartate into the cytosol after transamination from glutamate intramitochondrially. Consequently, although transport of glutamate into the cytosol may occur, it does not represent a distinct metabolic alternative for the removal of glutamate.

1.6 Metabolic Fate of Glutamine Carbon

Although it appears certain that both phosphate-dependent glutaminase and glutamate dehydrogenase play critical roles in renal ammoniagenesis, the metabolic fate of glutamine carbon remains less certain. The ability of the kidney to produce glucose from glutamine has been repeatedly confirmed in tissue slices (60, 77, 88) and perfused kidney (120, 153). An adaptive increase in this gluconeogenic capacity measured *in vitro*, in response to acidosis induced *in vivo*, has also been clearly demonstrated (3, 27, 66). Measurement of glucose enzymatically in the renal artery and vein fail to detect glucose release in normal animals

(26, 156) and detect only a very small, although significant, output in acidotic animals (175). However, this is not surprising since substantial glucose could be released by the kidney, but because of rapid blood flow would escape detection by the analytical method used. In addition, glucose A-V differences are difficult to interpret because the medulla is a potentially important user of glucose (110). Therefore, an increase in glucose output by the kidney may also be interpreted as a decrease in glucose utilization by the medulla. Application of an isotope-dilution method for the measurement of glucose release has shown addition of glucose to the blood by the kidneys of hepatectomized rats and also that this glucose release was increased by acidosis (78).

Total CO_2 production by the kidney is not affected by acid-base status (128, 131). This indicates that total oxidative metabolism remains the same and hence, the energy requirement of the kidney does not change in acidosis. Therefore, in order for acidosis to increase the net removal of glutamate to CO_2, oxidation of some other fuel must be reduced.

The kidney is also capable of storing fair amounts of neutral lipids (128). That fat may also represent a possible metabolic fate of the glutamine carbon has also received consideration. Dies and Lotspeich (43) have demonstrated that renal NADPH generation is markedly increased through the hexose monophosphate shunt during metabolic acidosis. As NADPH production is required for fatty acid synthesis, this may be interpreted as suggesting that fat synthesis is also increased in acidosis.

Experimental attempts to determine the metabolic fate of glutamine have involved the use of tissue slices, tubules and perfused and intact

kidneys. Conclusions from these studies vary. With the perfused kidney, Hems found that about 15 percent of the glutamine extracted by the normal kidney was converted to glucose (66). With kidneys taken from acidotic rats, glucose formation accounted for approximately 50 percent of the glutamine extracted. Since the extra glucose formed was equivalent to the extra glutamine extracted, Hems concluded that glucose is the major metabolic fate of the extra glutamine utilized during acidosis.

Kamm and Strope used kidney cortex slices (77). Results of their experiments using U-^{14}C-glutamine indicate that about 15 percent of the glutamine utilized by slices taken from normal rats was converted to glucose (whether determined by measurement of product or label). The remainder of the glutamine carbon could be completely accounted for by the formation of other products. Acidosis increased glutamine uptake and conversion to glucose, CO_2, pyruvate, lactate, glutamate and lipid without changing the percent of product formed. These results suggest that the removal of the extra glutamine utilized during acidosis does not occur by a single pathway, but by increased conversion to all end products. In studies using L-(1-^{14}C) glutamine, Baverel and Lund (9) also conclude that both CO_2 production and glucose formation are major metabolic fates of glutamine utilized by rat kidney cortex tubules. Attempts to determine the metabolic fate of glutamine, *in vivo*, were done in the dog by Pitts *et al* (139) by infusing tracer amounts of U-^{14}C-glutamine. It was concluded that both glucose formation and CO_2 production were increased by acidosis, but that CO_2 production represents the major metabolic fate of glutamine in the dog.

Conclusions as to the metabolic fate of glutamine, *in vivo*, based

on experiments of the type described above are inherently shaky for two reasons. First, it is true that when glutamine is the sole carbon source, the products formed are the metabolic fates of glutamine. However, glutamine is not the only substrate available to the kidney, *in vivo*. It has been shown that glutamine metabolism is dramatically altered in the presence of other substrates such as ketone bodies (99), fatty acids (192) and lactate (98, 157). Second, since the oxidation of other substrates occurs at the same time and in the same compartment as the degradation of glutamine, then the degradation products of glutamine will mix with those arising in the Krebs cycle from other substrates. Therefore, in experiments with labelled glutamine, the labelled molecules arising from glutamine will be diluted by unlabelled molecules arising from other substrates. This means that the yield of labelled product will not accurately represent the net flux of glutamine to that product.

Vinay *et al* (195) have taken into account the effect of isotope dilution on the formation of labelled glucose and CO_2 by rat kidney cortex tubules. The extent of isotope dilution was estimated from the relative fluxes through the Krebs cycle and gluconeogenesis, which were in turn, calculated from the oxygen consumed and the amounts of glucose and CO_2 formed. The results of their experiments indicate that in the presence of oleate, glutamine carbon is converted almost entirely to glucose. However, this approach still suffers the obvious criticism that isolated renal tubules are comprised of a number of different cell types and therefore the oxygen consumed by the whole tubule may not reflect what is happening inside the cells which metabolize glutamine. Hence, the overall applicability of such experiments to the situation *in*

vivo remains tenuous. The metabolic fate of glutamine still remains an open question, but it is probably reasonable to speculate that its metabolic fate varies according to the energy requirement of the kidney.

PART III. Present Concepts Concerning the Regulation of Renal Glutamine Metabolism

Although it is true that the elevation of plasma glutamine concentration by infusion of glutamine increases renal ammoniagenesis (24), other evidence indicates that the renal metabolism of glutamine is not regulated by the supply of this amino acid in the blood during chronic acidosis. The evidence for this is that large increases in glutamine extraction and ammonia excretion occur by the kidney during chronic acidosis, despite constant or decreased plasma glutamine levels (71, 125). Transport of glutamine into the kidney is also thought not to be of regulatory significance. Since in acidosis more glutamine is utilized by the kidney than is filtered and reabsorbed, then glutamine extraction occurs at both luminal and antiluminal sites (127). Transport of glutamine across the luminal surface can be ruled out as regulatory since essentially the same quantities are filtered and reabsorbed in acidosis and alkalosis, yet far greater quantities are utilized in acidosis (127). The possibility that cellular glutamine levels might be of regulatory importance is suggested by studies with isolated mitochondria. The results of Tannen and Kunin (177) show that rates of mitochondrial ammoniagenesis are very responsive to changes in glutamine concentration in the physiological range, being stimulated by elevated glutamine levels. However, it is well established that cellular glutamine levels

are decreased rather than increased during chronic metabolic acidosis (1, 33, 52, 83, 84, 85, 114).

In view of what has been said, it seems likely that renal ammoniagenesis is regulated at an intracellular step in the metabolism of glutamine. The complexity of renal glutamine metabolism provides for numerous potential regulatory steps. The more likely steps are, of course, those which execute irreversible reactions. According to our present understanding of glutamine metabolism, such events occur in both the mitochondria and cytosol.

1.7 Mitochondrial Events of Regulatory Potential

A. Glutamine Transporter

The first step in the intracellular metabolism of glutamine is its entry into mitochondria. This transport mechanism is not an active process since it takes place down a glutamine concentration gradient between cytosol and matrix. Hence, the entry of glutamine may be either by passive diffusion or facilitated diffusion. Although it has not been definitively proved, most evidence suggests a specific carrier-mediated transport system. The evidence for this rests on certain characteristics of glutamine transport which imply carrier mediation, namely, saturability (170) and susceptibility to inhibition (1, 52). However, as emphasized by Tannen, these properties can also be accounted for by alternative explanations (178).

The suggestion that renal ammoniagenesis is regulated by mitochondrial glutamine transport derives largely from the apparent adaptation of the transport process in metabolic acidosis. An adaptive increase in gluta-

mine transport is inferred from observed increases in the steady-state concentration of matrix label after incubation of rat (1, 52) and dog (170) kidney mitochondria with ^{14}C-glutamine. The accumulation of matrix ^{14}C is mainly in the form of ^{14}C-glutamate since metabolic inhibitors (rotenone, arsenite) were added to the medium to prevent conversion of glutamate to other products. The inference that an increase in the steady-state concentration of glutamate reflects an increase in glutamine transport assumes no alteration in the rate and affinity of the glutamate exit system. Accumulation of ^{14}C-glutamate could also be caused by either a decreased exit of glutamate from mitochondria or a decreased affinity of the exit mechanism. Adam and Simpson have already shown that the uptake of ^{14}C-glutamate is quite depressed in mitochondria isolated from rats given an acid load (1) and hence the activity of the glutamate transport system may be lowered.

Goldstein and Boylan (57) were able to measure the initial rates of accumulation of label by mitochondria incubated with ^{14}C-glutamine. The observed increase in initial rates of accumulation in response to metabolic acidosis indicates an adaptive increase in the uptake of glutamine into mitochondria. However, it was not possible to determine whether the increase in glutamine uptake is due to activation of glutamine transport or activiation of phosphate-dependent glutaminase. To help distinguish between these two possibilities other experimental approaches have been employed. Kidney mitochondria from both acidotic dogs (170) and acutely acidotic rats (1) do not exhibit increased PDG activity, yet ammoniagenesis and ^{14}C accumulation in the matrix were both accelerated (1, 170). However, the value of these observations is only to contribute

circumstantial evidence since PDG activity could be altered by changes in the intramitochondrial environment. In contrast, Goldstein (52) did not find an increase in the steady-state accumulation of ^{14}C-glutamate by rat kidney cortex mitochondria incubated with ^{14}C-glutamine after two days of acid challenge, even though ammonia excretion, in vivo, was significantly increased.

Another experimental approach taken by Goldstein (52) involves the use of 6-diazo-5-oxo-L leucine (DON), an analogue of glutamine. This analogue is apparently transported by the system that transports glutamine across the inner mitochondrial membrane but is not hydrolyzed by rat renal glutaminase (51). A 1.5-2.0 fold increase in the steady-state accumulation of this analogue occurred despite the fact that such accumulations cannot be due to either increased glutaminase activity or to an involvement of the glutamate transporter. However, Goldstein did not measure the initial rate of entry and hence an increased steady-state accumulation of DON does not necessarily imply an increased rate of entry. It is possible that alterations in the rate and affinity of DON efflux may be responsible.

The inability to demonstrate matrix accumulation of ^{14}C-glutamine by rat or dog kidney mitochondria incubated with as much as 10 mM glutamine has been taken to suggest that transport of glutamine across the inner mitochondrial membrane is slower than its subsequent deamidation inside the matrix (1, 170). Even in experiments using inhibitors of glutamine deamidation (low temperature, p-chloromercuribenzoate, mersalyl) glutamine was still not detectable (1, 170). However, three considerations must be kept in mind when interpreting these results. First, it is

possible that the transport process and deamidation are closely linked so that only glutamate is released into the matrix space. Second, the inhibitors used to reduce deamidation may also affect glutamine transport. Third, it has been shown that the brush border enzyme, γ-glutamyltranspeptidase, contaminates mitochondria prepared by differential centrifugation and that this enzyme is capable of hydrolyzing glutamine in vitro (40, 90). Consequently, from these considerations alone, it would be unwise to attach too much physiological significance to the above observations. Furthermore, Curthoys and Shapiro (38) can demonstrate the in vitro accumulation of glutamine by rat kidney mitochondria purified of γ-glutamyltranspeptidase. However, γ-glutamyltranspeptidase contamination is not a factor in the interpretation of results obtained with dog mitochondria since this enzyme is not present or only minimally so in dog kidney (38).

It has also been suggested that the activity of the mitochondrial glutamine transporter may be modulated by changes in the cytoplasmic content of α-ketoglutarate (50, 57). This suggestion is based upon the findings by Goldstein (57) and Goldstein and Boylan (50) that physiological concentrations of α-ketoglutarate inhibit glutamine deamidation and the accumulation of glutamine carbon in isolated rat kidney mitochondria. Since glutaminase does not appear to be inhibited at the concentrations of α-ketoglutarate used (54), it was concluded that α-ketoglutarate affected mitochondrial glutamine metabolism by acting on the glutamine transporter. Furthermore, mitochondrial glutamine carbon uptake and deamidation increased when α-ketoglutarate concentrations were decreased to the same extent that kidney α-ketoglutarate levels fall in metabolic

acidosis (57). Hence, even if the capacity of the mitochondrial glutamine transporter is not rate-limiting, in itself, this transport system may still be the focal point at which renal ammoniagenesis is regulated.

B. Phosphate-Dependent Glutaminase

The second obligatory step in the metabolism of glutamine is deamidation by phosphate-dependent glutaminase (PDG). Although PDG appears to have an important adaptive role in the rat kidney in response to acidosis, the original postulate by Pitts (134) that the amount of this enzyme is regulatory for renal ammoniagenesis is not reconcilable with all experimental phenomena. Discrepancies between changes in renal ammoniagenesis and changes in glutaminase activity are found in the early stages of acidosis and in experiments using inhibitors of protein synthesis. Studies employing kidney homogenates failed to detect an increase in PDG activity at times less than 24h of acid challenge despite increases in ammonia excretion (5, 101, 146). However, as demonstrated by Curthoys and Lowry (37), a twenty fold increase within the proximal convoluted tubules only appears as a 2-4 fold increase in kidney homogenates. Hence, it is certainly possible that changes in PDG activity do occur at times earlier than 24h after the start of acidosis, but are not detectable in kidney homogenates.

Actinomycin D inhibited the adaptive rise in PDG activity, but failed to inhibit the increase in ammonia excretion after 24h of acid challenge (55). However, actinomycin D is very toxic and cannot be administered at the dose used for periods longer than 24h. Consequently, conclusions drawn from this study can be applied only to the first

day of acidosis. Ethionine, another inhibitor of protein synthesis, failed to inhibit the adaptive rise in ammonia excretion (17). However, ethionine has been shown to be only partially effective in producing complete inhibition of PDG induction in the rat (121). Administration of actinomycin D at less toxic doses to infant (13) and subtotalnephrectomized rats (12) inhibited increases in both PDG activity and ammonium excretion, suggesting that the amount of PDG may be rate-limiting in these circumstances. Furthermore, during recovery from metabolic acidosis, PDG activity remains elevated whereas ammonia excretion returns to normal (125). This clearly demonstrates that at least during recovery, the amount of PDG is not the rate-limiting factor for renal ammoniagenesis. In addition to these findings in the rat, the induction of PDG in the dog is clearly not required for adaptation to acidosis since an increase in PDG activity is not an adaptive response in the dog (140, 145, 190).

When taken together, the above results strongly suggest that the amount of PDG in the normal adult rat or dog is not regulatory for renal ammonia production. However, since PDG activity is modified by a variety of substances (glutamate and phosphate included) in vitro (35, 58), this enzyme may well be the regulatory locus at which changes in mitochondrial composition regulate ammoniagenesis. In accord with this concept is the observation by Curthoys and Shapiro that the addition of phosphate to isolated mitochondria lowers matrix glutamine concentration and increases glutamate formation (38), suggesting activation of PDG activity. Since mitochondrial ammoniagenesis is also stimulated by phosphate (38), it follows that PDG activity was rate-limiting under these conditions and was the locus at which phosphate acted. Addition of a specific inhibitor

of PDG to isolated mitochondria prevented the increased uptake of glutamine in acidosis (39, 167), further attesting to the importance of changes in PDG activity in acidosis.

C. Glutamate Dehydrogenase

Another important enzyme in renal glutamine metabolism is glutamate dehydrogenase (GDH). Increased flux through glutamate dehydrogenase in response to acidosis has been demonstrated in tissue slices (141) and isolated mitochondria (161). Comparison of the mass action ratio of this reaction in the kidney with its equilibrium constant has led to the conclusion that the GDH reaction functions near equilibrium, in vivo (67, 86). If GDH catalyzes an equilibrium reaction, then changes in the amount of this enzyme cannot be of regulatory importance. In accord with this, GDH does not increase in the dog during acidosis (140, 145). However, if this reaction is at equilibrium, then alterations in the removal of each of its end-products, H^+, NH_3, NADH and α-ketoglutarate, will affect the flux through this enzyme.

On the other hand, studies with isolated mitochondria do not support the conclusion that the glutamate dehydrogenase reaction is at equilibrium. Schoolwerth et al (161) find that the mass action ratio of products to reactants present in mitochondrial incubations is too far removed from K_{eq} to assume an equilibrium condition. However, in both estimations (in vivo and in vitro) the NAD to NADH ratio was calculated using reactants of the β-hydroxybutyrate dehydrogenase reaction. This assumes that β-hydroxybutyrate dehydrogenase is also located in the mitochondria which undergo an adaptive increase in ammoniagenesis during acidosis. However,

this has not been demonstrated and because of great heterogeneity in the kidney, may be a hazardous assumption. Full appreciation of the role of GDH in renal ammoniagenesis will depend upon resolving this quandary.

D. Other Mitochondrial Parameters

Other mitochondrial parameters have also been regarded as potentially regulatory. These include α-ketoglutarate dehydrogenase (57, 93, 190), succinate dehydrogenase (93) and pyruvate dehydrogenase (149). The possibilities of their regulatory involvement are based, for the most part, on theoretical considerations and on mitochondrial responses to acid-base manipulations, in vitro. The mitochondrial malate transporter has also received consideration. Efflux of malate from the mitochondria is enhanced by a decrease in medium pH (81, 117). Elevated concentrations of extra-mitochondrial phosphate also stimulates the exit of malate via the malate/phosphate antiporter (205). That malate transport may play a regulatory role, in vivo, derives from the observation that infusion of neutral sodium phosphate elicits a large increase in ammonia excretion by the acidotic rabbit (205). However, this has been shown only in the rabbit where the activity of the malate/phosphate antiporter is very low.

1.8 Cytoplasmic Events of Regulatory Potential

A. Phosphoenolpyruvate Carboxykinase

Since the irreversible conversion of oxaloacetate to phosphoenolpyruvate is thought to be involved in the removal of the glutamine

carbon skeleton irrespective of its metabolic fate, then phosphoenolpyruvate carboxykinase (PEPCK) is a potential cytoplasmic regulatory parameter. The demonstration in the rat by Alleyne (4) that malate decreases and phosphoenolpyruvate increases during metabolic acidosis was the first indication that PEPCK may be of regulatory importance in renal ammoniagenesis. An increase in PEPCK activity was subsequently shown to occur in acidosis (5), thereby providing an explanation for the increased flux through this step.

In order to attribute a regulatory role to PEPCK it was postulated (60) that changes in the activity of this enzyme exerted a "pull" on matrix glutamate across the inner mitochondrial membrane and through all the intervening reactions. According to this hypothesis, the level of glutamate inside the mitochondria is regulated by this "pull" which in turn controls the activity of PDG by product inhibition. Consistent with this hypothesis is that an increase in PEPCK can be detected as early as 4-6h after the onset of acidosis (3), suggesting that it is an important primary event during adaptation. However, increases in ammonia formation can be detected within 30 minutes (44). This may indicate that PEPCK is not important during acute acidosis or it could also mean that the ability to detect small changes in PEPCK is limited in this heterogeneous organ. Certainly, increased synthesis of PEPCK is evident at 2h (73).

Most of the objections to this hypothesis are based upon apparent dissociations between ammonia formation and relevant features of this hypothesis. The repeated lack of correlations between glucose and ammonia formation in a variety of situations (10, 25, 27, 72, 80, 142,

147, 151) is not relevant to the present version of this hypothesis since it is recognized that PEPCK is also involved in the conversion of glutamine carbon to metabolic fates other than glucose. Criticisms based upon the observed tissue glutamate levels are also of dubious value. One such criticism is that the drop in renal cortical glutamate is not of sufficient magnitude to account for the adaptive increase in urine ammonia excretion (54) and that increases in ammonia excretion during prolonged acid challenge occur without corresponding decreases in glutamate concentration (58). Furthermore, administration of an acid load to rats in an amount inadequate to decrease cortical glutamate increased the capacity of cortex to produce ammonia from glutamine (124). However, it may be that the level of glutamate at the site of PDG in the mitochondria is not reflected in total glutamate concentrations and that changes may occur in matrix glutamate without being detected in total tissue content.

A more serious objection to this hypothesis is based upon theoretical considerations. In order for PEPCK to exert a "pull" on glutamate, it is necessary that all the intervening reactions be poised quite close to equilibrium, which has never been demonstrated and appears most unlikely. It is certainly true that this theoretical objection can not be ignored; however, it is still possible that PEPCK may play a regulatory role by a mechanism yet unknown.

That PEPCK is involved in the renal metabolism of glutamine is indicated by studies using mercaptopicolinic acid, a potent inhibitor of PEPCK. In both tissue slices (11) and perfused kidney (53) mercaptopicolinic acid had an inhibitory effect on ammoniagenesis from glutamine,

presumably as a result of inhibition at the level of PEPCK. However, it appears that PEPCK may not be absolutely essential for renal ammoniagenesis in either the rat or dog. It was shown by Vinay et al (191) that renal ammonia production can proceed through "non-PEPCK-dependent" pathway(s) in vivo when PEPCK is inhibited by mercaptopicolinic acid.

B. Other Cytoplasmic Parameters

The purine nucleotide cycle enzymes, adenylosuccinate synthetase and adenylosuccinase and the hexose monophosphate shunt dehydrogenases are other enzymes in the cytosol which also increase during adaptation to metabolic acidosis (18, 43). However, the relevance of these adaptive changes to renal ammoniagenesis is still obsure. The possibility of a regulatory function in a specialized situation cannot be discounted; however, it is more likely that these enzymes participate in non-regulatory functions essential to the overall adaptive process. Elucidation of the role of these enzymes will have to await further investigation.

PART IV. Purpose and Approach of this Study

Despite the widespread interest in the control of renal ammoniagenesis over the past thirty years, abundant enthusiasm has not yet been matched by corresponding progress in understanding the specific control mechanisms involved. In this regard a remark made by Pitts in 1973 that "no presently proposed theory of control of ammonia production is intellectually satisfying" (129) still holds true today.

1.9 Theoretical Aspects

Essentially all of the approaches taken to study the control of renal ammoniagenesis involve the study of events which occur during adaptation to acidosis. Correlative changes between ammonia formation and various renal parameters are used as evidence for hypotheses and dissociations are used as evidence against hypotheses. However, conclusions based on correlative and dissociative findings must be interpreted cautiously. Correlative relationships are not proof of causal relationships; such results are only suggestive. The lack of a correlation, on the other hand, may be taken as evidence of non-causality. However, most of the dissociations taken as evidence against one or another hypothesis occurred in situations where inhibitors were employed or where methods of detecting change are subject to question.

In this thesis, "physiologically-relevant" models in addition to the onset of acidosis were studied, namely recovery and rechallenge. The initial thinking for this approach had its origin in the studies of Dies and Lotspeich (43). These investigators observed that ammonia excretion abruptly fell to normal values within 24h after cessation of an acid challenge. This observation was later confirmed by Parry and Brosnan (125). It was also reported by Dies and Lotspeich (43) that resumption of the acid load resulted in a rapid return of ammonia excretion to acidotic levels, without the lag period observed during the initial onset.

Since changes in ammonia excretion occur rapidly in these experimental situations, then it was thought that these situations would be useful to establish dissociations and correlative relationships. It was antici-

pated that changes in ammonia excretion would be accompanied by corresponding changes in regulatory parameters, but not necessarily in non-regulatory parameters. The establishment of dissociations would indicate non-causality and the persistence of correlations would be highly suggestive of causal relationships.

1.10 Practical Aspects

In general, the practical approach taken was the utilization of a variety of techniques to examine the metabolic responses in renal glutamine metabolism to a number of experimentally-induced acid-base conditions. The main emphasis was the application of established techniques to new experimental situations. The value of this approach is that it is relatively simple, yet potentially fruitful, since recovery and rechallenge have not previously been subjected to detailed investigation. The techniques used were chosen in order to study glutamine metabolism at different levels of organization, from the intact functioning kidney, through tissue and organelle function, to the level of the enzyme. Techniques used for studies, *in vivo*, include clearance techniques and freeze-clamping and studies, *in vitro*, involve tissue slice and mitochondrial incubations and the measurement of enzyme activities.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

A. Animals

Male Sprague-Dawley rats, weighing 250 to 500 g, were used in all experiments. These rats were obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, La Prairie, Quebec. They were fed a commercial Purina Rat Chow (Ralston Purina of Canada Ltd., Don Mills, Ontario) which consisted of a minimum of 22.0% protein, a minimum of 4.0% fat and a maximum of 5% fibre. The remainder of the rat chow was mainly carbohydrate plus vitamin and mineral supplements.

B. Substrates and Enzymes

All substrates and enzymes were purchased from Sigma Chemical Company, St. Louis, Missouri.

C. Cofactors and Nucleotides

NAD, NADH, ADP, ATP and ITP were obtained, as sodium salts, from Sigma Chemical Company, St. Louis, Missouri.

D. Radioisotopes

[^{14}C]-inulin was obtained from New England Nuclear, Lachine, Quebec and [^{14}C]-acetyl-CoA purchased from P-L-Biochemicals Inc., Milwaukee, U.S.A.

E. Antibody

Antibody to phosphoenolpyruvate carboxykinase was a kind gift of Dr. F. John Ballard. It was prepared in goat against purified rat liver cytosolic PEPCK and has been treated with fetal liver cytosol to reduce non-specific precipitin lines. The antibody preparation was freeze-dried and stored at -20°C until used. The amount of antibody for each experiment was weighed and reconstituted in 0.15 N NaCl containing 5 mM Hepes buffer. A unit of antibody is defined as the amount needed to titrate one unit of enzyme.

F. Anaesthesia

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

G. Other Biochemicals

Heparin (1000 units/ml) was obtained from M.T.C. Pharmaceuticals, Hamilton, Ontario. All other biochemicals were purchased from Sigma Chemical Company, St. Louis, Missouri and were of the highest purity available.

H. Other Chemicals

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

or J.T. Baker Chemical Company, Phillipsburg, N.J. and were of the highest grade available.

2.2 Methods

A. Treatment of Animals

The animals were housed in standard rat-cages (not more than four rats to a cage) containing hardwood bedding chips. The animal room was continuously light from 9:00 a.m. to 9:00 p.m. and continuously dark from 9:00 p.m. to 9:00 a.m. Room temperature was continuously monitored and remained constant. The animals were allowed free access to food at all times and free access to tap water for a minimum of three days after arrival. This period was allowed for them to adjust to their new environment. Normal rats were permitted continued free access to tap water until used. Normal rats are also referred to as naive rats. Rats were made acidotic by replacing the tap water with 1.5% NH_4Cl for seven days. All other rats were apportioned to the following experimental groups. All experiments were timed so that animals were sacrificed or otherwise used at approximately 9.00 a.m.

(1) Normal Recovery Group - Acidotic rats were returned to tap water for 1, 2 and 3 days.

(2) Rapid Recovery Group - Rats were killed at 4h and 8h after the intragastric administration of $NaHCO_3$ (1.5 mmoles/100g body weight) to acidotic rats. For intubation, the animals were lightly anaesthetized with ether (i.e. immobilized for 1-2 min). A second group of acidotic rats were intubated with NaCl (1.5 mmoles/100g body weight) to serve as control. All rats were permitted free access to tap water and food until sacrificed.

(3) Long Recovery Group - Acidotic rats were returned to tap water for 5, 7, 9, 11 and 15 days.

(4) Rechallenge Group - The animals used in this group were made acidotic and then returned to tap water. After 3 days of recovery, the rats were administered either 0.5, 1.0, 1.5, 2.0 or 2.5 mmoles/100g body weight of NH_4Cl by intragastric tube (after light, ether anaesthesia) every 12h for up to 3 consecutive times. Continuous access to tap water was permitted. Rats were sacrificed 12h after each intubation. Similar treatment of naive rats served as controls.

Ammonium chloride has been used experimentally since the 17th century. Originally, it was injected directly into the blood of animals to produce ammonia toxicity and to stimulate urea formation. However, the acid producing character of this substance when ingested was recognized by Haldane (64) in 1921. Since then the administration of NH_4Cl by ingestion has become a commonly used method of inducing metabolic acidosis. The nitrogen from absorbed ammonium chloride is incorporated directly into urea for excretion. It is generally assumed that NH_3 and CO_2 are utilized in the formation of urea. Hence, the removal of NH_3 from NH_4Cl would leave behind hydrochloric acid to induce acidosis.

B. Urine Collection and Analysis

Metabolic cages were used in the manner described by Parry and Brosnan (125) to collect urine at intervals of 4h, 12h or 24h. To minimize variability with the 4h urine collections, the bladders were emptied by manual expression at the end of each interval. Care was taken when using this technique so as to not unduly upset the rat.

Frightened rats are less active and normal activities of eating and drinking greatly reduced. After appropriate dilutions, the ammonia content of the urines was measured by the method of Kirsten et al (79).

C. Blood Collection and Analysis

Blood samples were taken by two methods depending upon the parameters measured.

(1) pH, pCO_2 and $[HCO_3^-]$ - For the determination of acid-base parameters, blood samples were taken into 100 µl heparinized capillary tubes from a small incision along the tails of restrained rats which had been appropriately treated. Each capillary tube was plugged immediately after sampling and the blood mixed by use of a magnetic bar and magnet and then placed on ice. The pH and pCO_2 were measured as rapidly as possible (and always within one hour) with an Instrumentation Laboratories pH Blood Gas Analyzer, model 213. Bicarbonate concentrations were calculated from the pCO_2 and pH values using the Henderson-Hasselbach equation. Although this procedure obtains capillary blood, the method of sampling provides a constant type of blood sample (as evident by constant pO_2) and hence the relative changes in the acid-base parameters which occurred in this blood represent the relative changes which took place in the general circulation.

(2) Glutamine Arterio-Renal Venous Differences - For the determination of arterio-renal venous differences of glutamine, about 1 ml of blood was withdrawn into heparinized plastic syringes from the renal vein and the abdominal aorta of appropriately treated rats aneasthetized with sodium pentobarbital (6.5 mg/100g body wt., i.p.). The plasmas

obtained from these samples were deproteinized with perchloric acid and the supernatants then adjusted to pH 2.2 with 2 N LiOH in the presence of an equal volume of 0.3 N lithium citrate buffer (pH 2.2). Glutamine was measured in these buffered samples on a Beckman Model 121 Automatic Amino Acid Analyzer as described by Squires et al (173). The concentrations of glutamine from paired samples were used to calculate arterio-renal venous differences.

D. Preparation of Kidney Homogenates and Measurement of Phosphoenolpyruvate Carboxykinase Activity

Appropriately-treated rats were sacrificed by cervical dislocation following a blow on the head. Both kidneys were quickly removed, freed from fat and connective tissue and weighed. The entire right kidney was cut into small pieces with scissors and suspended in 50 volumes of an ice-cold homogenization medium consisting of 0.25 M sucrose, 0.15 M NaF, 3 mM Tris and 1 mM EGTA, adjusted to pH 7.4 with HCl. Homogenization was carried out in a smooth-glass Potter-Elvehjem homogenizer with a motor-driven Teflon pestle (clearance 0.15 mm). The assay used was that of Seubert and Huth (163) except that NaF was omitted from the incubation medium since it was present in the homogenization medium. Both the incubation time (15 min) and the amount of homogenate (0.1 ml) used were shown to be within the linear range of this assay for both normal and acidotic rats.

The activity of PEPCK is expressed in two ways: first, as mmoles/24h/g kidney and second, as mmoles/24h/100g body weight. The activity is expressed per g kidney to allow comparison with the results of other

investigators, since this is the usual mode of expression. However, because larger rats do not necessarily have correspondingly larger kidneys and because of the relatively wide weight range of rats used, it was felt that the activity should also be expressed as per 100g body weight. This mode of expression has the advantage of making comparisons with ammonia excretion (also expressed in these units) more meaningful.

E. Preparation of Mitochondria and Mitochondrial Studies

Appropriately-treated rats were sacrificed by cervical dislocation following a blow to the head. Both kidneys were quickly excised, demedullated, cut into small pieces with scissors and suspended in an ice-cold homogenization medium consisting of 0.25 M sucrose, 5 mM Hepes and 1 mM EGTA, adjusted to pH 7.4 with NaOH. Mitochondria were prepared by homogenization and centrifugation as previously described (76). All operations were carried out at 0-4°C. The protein content of the mitochondrial preparation was determined by the biuret method (62) after solubilization of lipid with deoxycholate (74). Bovine serum albumin was used as a standard. The final mitochondrial pellet was suspended in a volume of homogenization medium to yield a protein concentration of approximately 20 to 30 mg per ml. For some experiments the kidneys from 2 or 3 rats were pooled to provide enough mitochondria at this concentration. The physiological integrity of the mitochondrial preparation was assessed by measuring the respiratory control ratio in the presence of 10 mM α-ketoglutarate or succinate. The respiratory control ratio

was determined, after the addition of a limiting amount of ADP, by measuring oxygen consumption polarographically with a Clark type electrode at 28°C in a medium containing 0.14 M KCl, 4 mM KH_2PO_4, 2.5 mM $MgCl_2$, 1 mg/ml BSA, 1.5 mM EDTA and 20 mM Hepes buffer (pH 7.4). All mitochondria used in the experiments in this thesis were shown to have a respiratory control ratio of about 4 or higher. The mitochondrial preparations were used for the measurement of the activities of phosphate-dependent glutaminase and glutamate dehydrogenase and also for studies involving the incubation of mitochondria in different media.

(1) Phosphate-Dependent Glutaminase and Glutamate Dehydrogenase - The mitochondrial preparations were diluted with water 5 to 15 fold for the measurement of phosphate-dependent glutaminase and 100 to 200 fold for the measurement of glutamate dehydrogenase. The exact dilution was dependent upon the experimental condition under study. Sonication was shown to have no additional effect on the activity of these enzymes. This indicates that the changes in osmotic pressure upon dilution with water and after addition to the assay media were sufficient to disrupt the mitochondria and remove any permeability barriers to the substrates. Consequently the mitochondia were used without sonication. Aliquots of 100 µl were used for the assay of phosphate-dependent glutaminase by the method of Curthoys and Lowry (37). Glutamate dehydrogenase was measured by the method of Bridiczka et al (20) using 20 µl of the diluted samples. The conditions used were shown to be within the linear range of time and protein for both assays with mitochondria from both normal and acidotic rats.

(2) Mitochondrial Incubations - The incubation procedure is essentially that of Schoolwerth et al (161). The practical aspects of the procedure are as follows. The incubations were carried out in 3 ml glass vessels with water jackets and maintained at 28°C with circulating water from a constant temperature water bath. Mitochondria (5-6 mg/ml) were incubated at pH 7.4 with 120 mM KCl, 10 mM Tris-HCl, 20 mM MOPS, 5 mM $MgCl_2$, 0.1 mM malate, 0.2 mM ADP, 30 mM glucose, hexokinase (6 units/ml) and either 4 mM KH_2PO_4 (low-phosphate medium) or 20 mM KH_2PO_4 (high-phosphate medium). Each incubation also contained either 1 mM glutamine or 1 mM glutamine plus 3 mM glutamate. Incubations were initiated by addition of mitochondria to the temperature-equilibrated, oxygen-saturated and continuously stirred media. The vessels were stoppered with caps having inlet and outlet holes to permit the flow of oxygen over the media during incubation. Samples were taken at zero time and after 4 min of incubation and transferred into Eppendorf microcentrifuge tubes containing 70% perchloric acid. The acidified samples were then neutralized with 4 M K_3PO_4 in the presence of universal indicator, centrifuged and the supernatants used for the measurement of the following metabolites. Ammonia was measured with the pyridine nucleotide-linked glutamate dehydrogenase reaction by the method of Kun and Kearney (91). The presence of glutamine had little or no effect on this assay. Glutamate was measured as described by Bernt and Bergmeyer (15) and aspartate was measured by the method of Lowry and Passoneau (105). The sensitivity of these spectrophotometric assays was optimized by using a final assay volume of 1.0 ml.

Preliminary experiments were carried out to ensure that the rate of

mitochondrial metabolism was linear under the conditions of incubation. Figure 2.1 (a & b) shows that ammoniagenesis in all media remained linear for at least 8 min with mitochondria isolated from both normal and acidotic rats. This linear rate was observed even with acidotic mitochondria (which had the highest rates of ammoniagenesis) although, by calculation, up to about 90% of the glutamine in the medium was utilized after 8 min of incubation. In some experiments the rate of glutamine utilization was followed. Glutamine was measured on a Beckman Model 121 Automatic Amino Acid Analyzer (173). Figure 2.2 (a & b) shows the reciprocal linear relationship between ammonia formation and glutamine utilization in the high-phosphate media with normal mitochondria. Medium changes in glutamate and aspartate were found to be linear up to 8 min of incubation as shown in Figure 2.3 for normal mitochondria incubated in the high-phosphate media.

The measured changes in medium ammonia, glutamate and aspartate were used to calculate fluxes through phosphate-dependent glutaminase (PDG), glutamate dehydrogenase (GDH) and glutamate oxaloacetate transaminase (GOT) with the flux equations derived by Schoolwerth et al (161). A flow diagram illustrating the reactions catalyzed by these enzymes is depicted below (page 51).

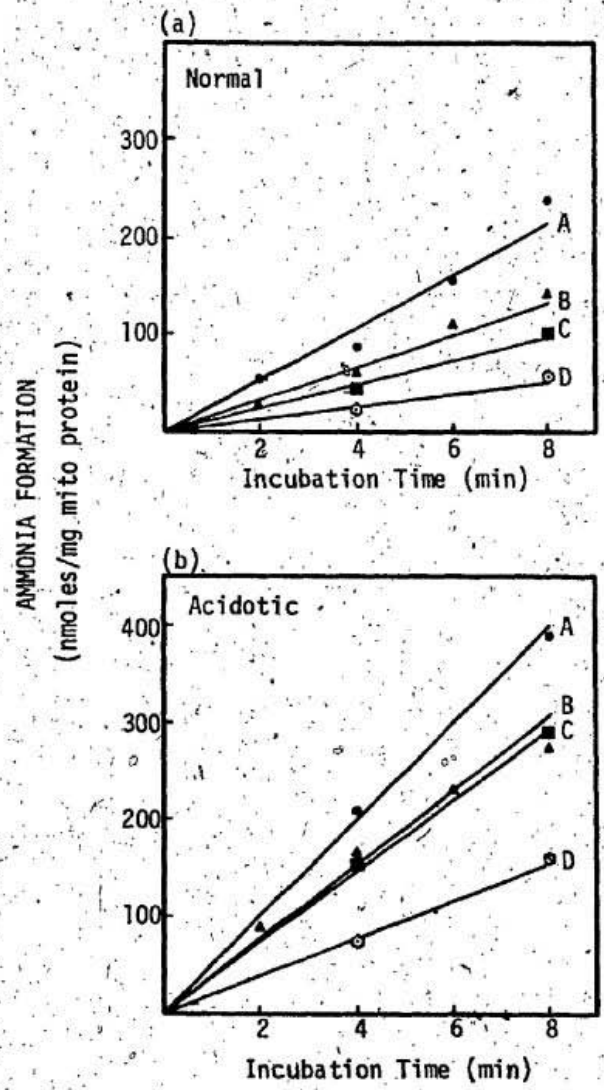


Figure 2.1 The formation of ammonia by mitochondria as a function of incubation time.

Kidney cortex mitochondria were isolated from normal (a) and acidotic (b) rats and incubated for up to 8 minutes in media containing:

A. 1 mM glutamine + 20 mM phosphate
B. 1 mM glutamine + 3 mM glutamate + 20 mM phosphate
C. 1 mM glutamine + 4 mM phosphate
D. 1 mM glutamine + 3 mM glutamate + 4 mM phosphate

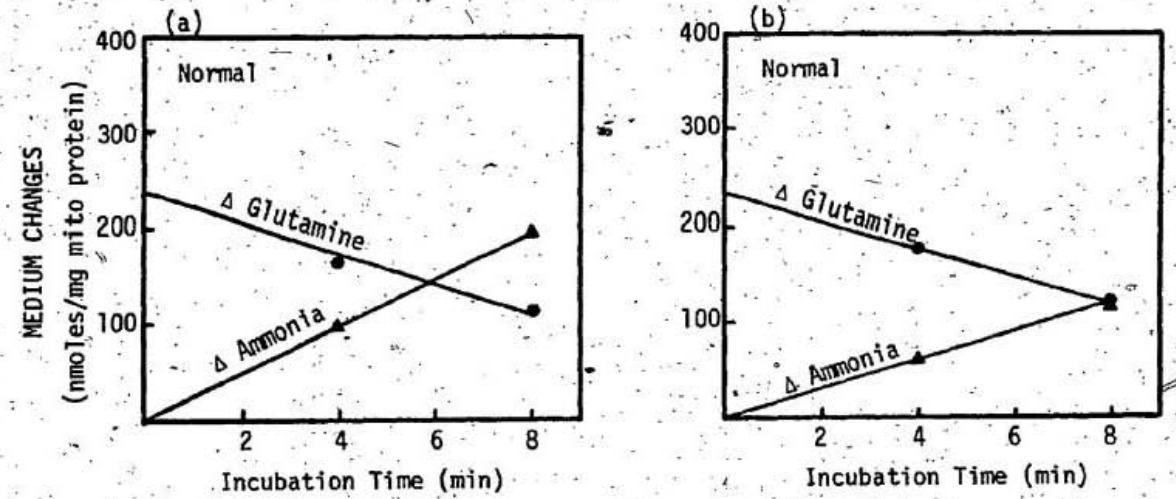


Figure 2.2 Relationship between ammonia formation and glutamine utilization.

Kidney cortex mitochondria were isolated from normal rats and incubated in the medium containing 1 mM glutamine + 20 mM phosphate (a) or in the medium containing 1 mM glutamine + 3 mM glutamate + 20 mM phosphate (b).

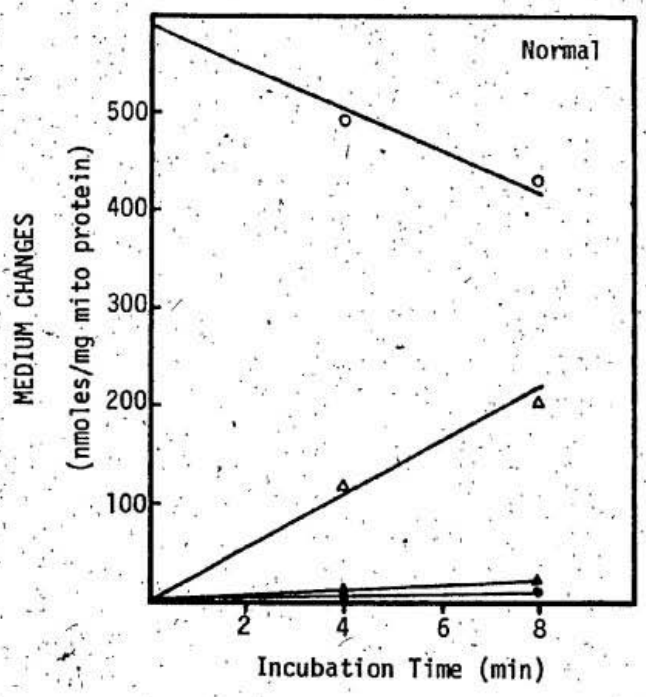
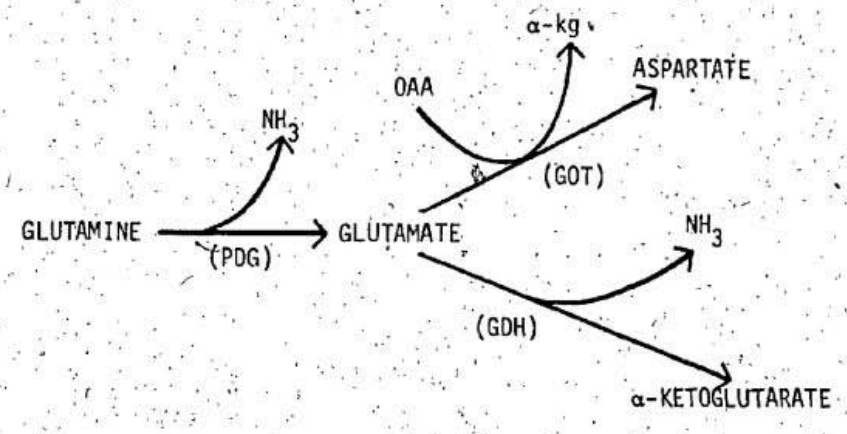


Figure 2.3 Medium changes in glutamate and aspartate as a function of incubation time.
Kidney cortex mitochondria were isolated from normal rats and incubated in media containing 1 mM glutamine + 20 mM phosphate ± 3 mM glutamate. The open symbols represent medium changes in glutamate (o) and aspartate (Δ) in the medium containing 3 mM glutamate. The closed symbols represent medium changes in glutamate (●) & aspartate (▲) in the medium without added glutamate.



Flow diagram of mitochondrial metabolism of glutamine and glutamate

Flux Equations:

$$\text{GOT flux} = \Delta \text{ aspartate}$$

$$\text{GDH flux} = \frac{\Delta \text{ ammonia} - \Delta \text{ aspartate} - \Delta \text{ glutamate}}{2}$$

$$\text{PDG flux} = \Delta \text{ ammonia} - \text{GDH flux}$$

(3) Assessment of the Contribution of Phosphate-Independent Glutaminase to Mitochondrial Ammoniagenesis - The brush border membrane enzyme, γ-glutamyltranspeptidase has been shown to contaminate mitochondria prepared by differential centrifugation (40, 90). Phosphate-independent glutaminase activity is a partial reaction of this enzyme (34) and has been shown by Curthoys et al (38) to contribute to the total glutaminase activity of mitochondria under the conditions of their incubation. Therefore, it was of interest to determine the extent of

contribution of phosphate-independent glutaminase to ammonia formation by mitochondria incubated under the conditions described above. For this purpose microsomes were prepared by the method of Kalra and Brosnan (76) and mitochondria were prepared in the usual manner. The specific activity of γ-glutamyltranspeptidase was determined in both preparations. Table 2.1 shows the specific activity of this enzyme in both normal and acidotic preparations. The microsomal measurements suggest an increase in the activity of this enzyme during acidosis. This agrees with the results of Anderson and Alleyne (6). The mitochondrial preparation had about 6% to 10% the specific activity of γ-glutamyltranspeptidase as the microsomal preparation.

Next, microsomes were incubated under the same conditions used for the mitochondrial incubations and rates of ammonia formation determined. Table 2.2 shows the rates of ammoniagenesis for microsomes isolated from both normal and acidotic rats. The percentage of maximum contribution of phosphate-independent glutaminase can now be calculated. This involves multiplying the fraction of contamination of the mitochondrial preparation with γ-glutamyltranspeptidase (derived from Table 2.1) times the rate of ammonia formation by phosphate-independent glutaminase (derived from Table 2.2) and comparing this with the rate of mitochondrial ammoniagenesis observed under the same conditions. Table 2.3 shows the results of these calculations and are interpreted as indicating that phosphate-independent glutaminase contributes only a minor and insignificant fraction to the total ammonia formed by mitochondria under the incubation conditions used.

Table 2.1 Specific activity of γ-glutamyltranspeptidase in microsomes and mitochondria from kidney cortex of normal and acidotic rats.

Experimental Condition	Specific Activity of γ-GTP (nmoles/mg protein/min)	
	Microsomal Fraction	Mitochondrial Fraction
Normal	3826.3	360.0
Acidotic	6559.0	439.6

Table 2.2 Rates of ammoniagenesis by microsomes from kidney cortex of normal and acidotic rats.

Experimental Condition	Rate of Microsomal Ammoniagenesis (nmoles/mg protein/min) High Phosphate Medium 20 mM KH_2PO_4	
	1 mM Glutamine	1 mM Glutamine + 3 mM Glutamate
Normal	7.0	6.1
Acidotic	10.3	7.0

Microsomes were incubated in media containing 20 mM phosphate and either 1 mM glutamine or 1 mM glutamine plus 3 mM glutamate.

Table 2.3 Percentage contribution of phosphate - independent glutaminase to mitochondrial ammoniagenesis.

Experimental Condition	Low Phosphate Medium 4 mM KH_2PO_4		High Phosphate Medium 20 mM KH_2PO_4	
	1 mM Glutamine	1 mM Glutamine + 3 mM Glutamate	1 mM Glutamine	1 mM Glutamine + 3 mM Glutamate
	Percentage Ammonia			
Normal	4.9	9.1	2.8	3.9
Acidotic	1.8	2.3	1.4	1.3

These results are calculated from the data reported in Tables 2.1 and 2.2.

F. Preparation and Incubation of Kidney Cortex Slices

Appropriately-treated rats were sacrificed by cervical dislocation following a blow to the head and the right kidney was immediately removed. Kidney cortex slices of approximately 20-30 mg wet weight were cut free-hand by the method of Deutch as described by DeLuca (42). The outside slice of kidney was discarded and the next 2 slices were incubated together with 2 mM glutamine for 90 min as described by Krebs et al (88), except that the concentration of Ca^{2+} was 1.25 mM. The incubations were stopped by the addition of 70% perchloric acid and the slices homogenized in the acidified media with a Potter-Elvehjem homogenizer and motor-driven teflon pestle. After neutralization with K_3PO_4, the percipitates were removed by centrifugation (2000 g for 10 min) and the resulting supernatants used for the measurement of glucose (Sigma Technical Bulletin #510), glutamate (15) and ammonia (91).

G. Freeze-Clamping Procedure and Measurement of Metabolites

Appropriately-treated rats were anaesthetized with sodium pentobarbital injected intraperitoneally at a dose of 6.5 mg/100g body weight. The abdominal cavity was opened by a mid-line incision and the right kidney gently cleared of fat and connective tissue. After allowing a few minutes for the rat to recover from this handling, the blood vessels were severed and the kidney immediately clamped with "Wollenberger tongs" whose jaws had been precooled in liquid N_2. The interval between removal of the kidney and the freeze-clamping was less than 3 seconds. The frozen tissue was then ground in the presence of liquid nitrogen with pestle and mortar and the resulting powder deproteinized with

perchloric acid and neutralized with KOH as described by Hems and Brosnan (67). After final centrifugation, the supernatant was used for determination of metabolites. Glutamine and glutamate were measured with a Beckman Model 121 Automatic Amino Acid Analyzer (173) after adjusting a portion of the supernatant to pH 2.2 with lithium citrate. Another portion of the neutralized supernatant was used for the determination of lactate by the method of Lowry and Passonneau (105) and for inorganic phosphate by the method of Martin and Doty (108). The remaining tissue extract was used for the determination of the following metabolites. The methods used are standard assays essentially the same as those described in "Methods of Enzymatic Analysis" edited by Bergmeyer (14). Pyruvate, phosphoenolpyruvate and 3-phosphoglycerate were measured with a combined assay. Fructose-6-phosphate and glucose-6-phosphate were measured in the same cuvette. ADP and AMP were assayed together. ATP, citrate, aspartate, α-ketoglutarate and malate were all measured separately. All the spectrophotometric assays were done using a Gilford Model 240 recording spectrophotometer which permits chart calibration to the sensitivity desired.

Oxaloacetate is difficult to measure because of its extreme lability and minute concentration. Because of this, oxaloacetate content was indirectly estimated from other data. Two independent methods of calculation were used. One method depends on the presumed equilibrium of lactate dehydrogenase and cytoplasmic malate dehydrogenase. Thus oxaloacetate was calculated from the renal content of lactate, pyruvate and malate according to the following equations as described by Williamson, Lund and Krebs (200):

$$\frac{[NAD^+]}{[NADH]} = \frac{[Pyruvate]}{[Lactate]} \times \frac{1}{K_{eq}(LDH)}$$

$$[Oxaloacetate] = [Malate] \times \frac{[NAD^+]}{[NADH]} \times K_{eq}(MDH)$$

where: $K_{eq}(LDH) = 1.11 \times 10^{-4}$

$K_{eq}(MDH) = 2.7 \times 10^{-5}$

The other method used for the calculation depends on the assumption that cytoplasmic glutamate oxaloacetate transaminase is at or near equilibrium. Thus oxaloacetate was calculated from the renal content of aspartate, α-ketoglutarate and glutamate according to the following equation as described by Hems and Brosnan (67):

$$[Oxaloacetate] = \frac{[Aspartate][\alpha\text{-Ketoglutarate}]}{[Glutamate]} \times \frac{1}{K_{eq}(GOT)}$$

where: $K_{eq}(GOT) = 6.7$

H. Assessment of Renal Function

Renal blood flow and glomerular filtration rate were estimated from renal clearance of ^{14}C-inulin as determined by the constant-infusion technique according to the following procedure. Appropriately-treated rats were anaesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 6.5 mg/100g body weight. The left jugular vein was exposed by a small incision in the skin and a heparinized catheter (polyethylene tubing PE50) was inserted into the vein towards the heart and

the right ureter cathetèterized with polyethylene tubing (PE 10) for urine collection. The rat was then placed in a temperature controlled cabinet at 38°C. After the injection of an initial priming dose of 0.4 ml of ^{14}C-inulin (~ 2μC$_i$) via the saphenous vein, a maintaining dose of ^{14}C-inulin in 5% mannitol (1.2 - 1.8 μCi/ml) was infused via the jugular venous catheter at a constant rate of about 0.03 ml/min using a constant infusion pump. Blood pH, pCO_2 and pO_2 measurements indicated that the acid-base status of the animal was not affected by the surgery and infusion. Urine was collected during a 10-20 min clearance period starting 30-40 min. after the start of infusion. At the end of the clearance period, arterial blood was sampled from the abdominal aorta and renal venous blood from the renal vein. A portion of each blood sample was centrifuged and the plasma (0.1 ml) treated with NCS tissue solubilizer for the subsequent determination of ^{14}C-inulin. The remaining portion of each blood sample was deproteinized with perchloric acid, one part was adjusted to pH 2.2 for the determination of glutamine as previously described (173) and another part was neutralized with 20% K_2CO_3 for the measurement of ammonia (79). Urine samples were used directly for the determination of ammonia (91) and ^{14}C-inulin. Radioactivity was measured in samples dissolved in Omniflor/Toluene with a Beckman Model LS-330 liquid scintillation counter.

The glomerular filtration rate (GFR) and renal blood flow (RBF) were calculated by the following equations:

$$GFR = \frac{UF}{A}$$

$$RBF = \frac{UF}{(A-V)(1-\text{hematocrit})}$$

where: U = the concentration of inulin in the urine in dpm/ml

F = the urinary flow rate in ml/min

A = the concentration of inulin in the renal arterial plasma in dpm/ml

V = the concentration of inulin in the renal venous plasma in dpm/ml

The hematocrit used in these calculations was 41%. The renal blood flow was used to calculate release of ammonia in the renal vein and extraction of glutamine by the kidney from the blood concentrations of ammonia and glutamine, respectively. The above described procedure was performed entirely by Dr. K.C. Man and the results are presented in this thesis with his kind permission.

I. Immunotitration of Phosphoenolpyruvate Carboxykinase

Kidneys from a set of appropriately-treated rats were separately homogenized in 20 volumes of homogenization medium and phosphoenolpyruvate carboxykinase assayed as described above. Each set was comprised of one rat from each experimental group under study. The homogenates were adjusted with homogenization medium so that the PEPCK activities were approximately equal to the lowest activity in the set. The immunotitration of PEPCK was carried out in microcentrifuge tubes by incubating 100 µl of each homogenate at varied dilutions with 100 µl of antibody preparation (~20-30 milliunits). Homogenate samples of 100 µl were also incubated with 100 µl of Hepes-buffered saline but without antibody for the measurement of PEPCK activity after incubation. Incubations were for 18h at 4°C after which the immunoprecipitates were then centrifuged at 12,000 g

for 5 min in an Eppendorf microcentrifuge and 0.15 ml aliquots of the supernatants removed for determination of PEPCK activity.

J. Presentation of Data and Statistical Treatment

Data are reported as mean ±S.D. (standard deviation) with sample size (n) in parenthesis. Differences between means were tested for statistical significance by t-test. A paired t-test was used for paired data. All computations were done with a Hewlett-Packard HP-65 programable calculator. P values of 0.05 or less were taken to indicate a significant difference between means. Bivariate correlation analysis was performed with a NLCS IBM 370/158 computer using SPSS subprograms (119) for the determination of Pearson product-moment correlation coefficient, regression intercept and slope and scatterplot diagrams.

CHAPTER 3

NORMAL RECOVERY

CHAPTER 3

NORMAL RECOVERY

The concentration of H^+ ions in biologic systems is exceedingly low. The blood pH of animals is maintained very precisely at about 7.4. Even taking into account the ionic strength of the blood, this pH only corresponds to approximately 50 nanomoles of H^+ ions per litre (23). For survival, the blood H^+ ion concentration must not exceed 150 to 200 nanomoles per litre and even at these concentrations animals can not function properly. Hence, death ensues at hydrogen ion concentrations which are negligible in comparison to the concentration of other important ions in plasma such as K^+, Na^+ and Ca^{++}.

To maintain such low H^+ ion concentrations in the face of the relatively large amounts of acid which animals normally encounter each day, a highly efficient system for the buffering and removal of H^+ ions must exist. Metabolic acidosis occurs in certain pathological states and other abnormal situations where the amount of acid formed is greater than the "normal capacity" of the animal to remove this extra acid. The clinical manifestations of metabolic acidosis are decreased blood pH, reduced bicarbonate and in some cases compensatory hyperventilation resulting in decreased blood pCO_2. Masoro and Siegal (109) define acidosis as "an abnormal condition or process which would produce a fall in the pH or a rise in the $[H^+]$ of the blood if there were no secondary changes". These authors further add that "since secondary changes which diminish the extent of pH change do occur it is possible to have acidosis with normal blood pH or H^+ concentration". In this

thesis, the definition of Masoro and Siegal is adhered to. For conditions in which there is a significantly lower than normal blood pH, the term "acidemia" is used.

The concept of recovery is not new. The re-establishment of normality is a commonly-used approach in nutritional studies and recovery from metabolic acidosis is essentially an extension of that approach. Pitts (134) described a human study in 1948 in which the renal excretion of ions was followed for five days after five days of ammonium chloride ingestion. Other human studies were reported in 1954 in which Wood (203) continued to follow plasma and urine parameters after termination of a grueling 44 days of ammonium chloride administration and in 1972 in which Welbourne et al (199) studied recovery in normal subjects and in patients with renal disease. Recovery from metabolic acidosis in rats was first investigated in 1967 by Dies and Lotspeich (43). Their study was primarily concerned with the relationship between the hexose monophosphate shunt and renal acid excretion. It was shown by these investigators that ammonia excretion in the rat abruptly fell to normal within 24h after cessation of the acid challenge. Another study concerning recovery from metabolic acidosis in rats was reported by Parry and Brosnan in 1978. The main purpose of their study was to investigate the control of renal ammonia production. It was found in their study that glutamine A-V differences across the kidney, in vivo, and glutamine metabolism by kidney cortex slices returned to normal along with renal ammonia excretion after termination of the acid challenge, whereas, the metabolism of glutamine by isolated mitochondria remained elevated. This was interpreted as suggesting the involvement of extramitochondrial

factors in the regulation of mitochondrial glutamine metabolism, *in vivo*. This chapter is concerned with studying the regulation of renal glutamine metabolism further by exploiting the recovery situation in greater detail.

Study of the regulation of renal glutamine metabolism requires knowledge of the relationship of this pathway to the animal as a whole. The response of the rat to ammonium chloride-induced acidosis has been well characterized. However, considerably less attention has been given to recovery from metabolic acidosis. For this reason the present study of renal glutamine metabolism during recovery was initiated at the level of the whole animal.

3.1 Blood Acid-Base Parameters

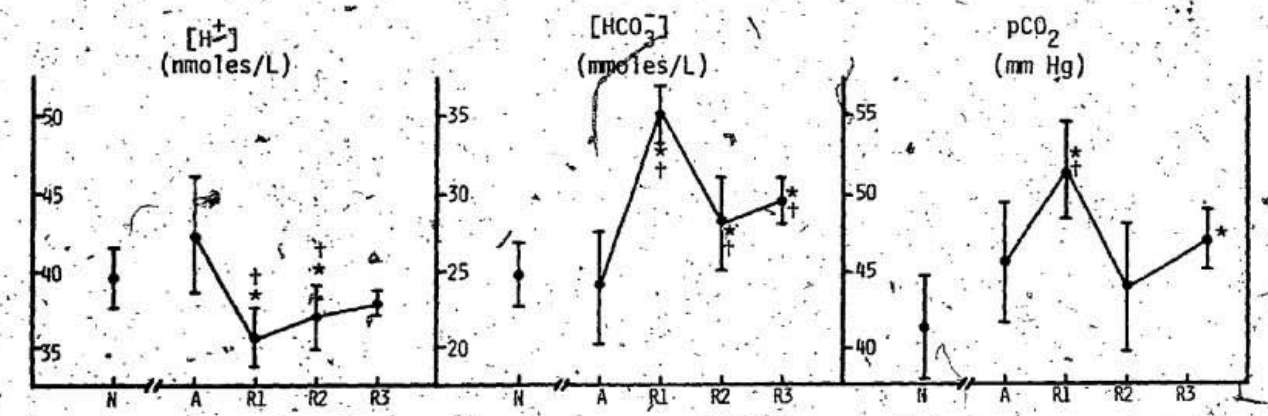
Blood $[H^+]$, $[HCO_3^-]$ and pCO_2 comprise a group of interrelated parameters that define the acid-base status of the animal. In any study concerned with acid-base homeostasis, it is important to define the experimental situation under investigation in terms of these parameters. Accordingly, blood $[H^+]$, $[HCO_3^-]$ and pCO_2 were measured before other studies were performed. For these determinations blood was sampled from animals without the use of an anesthetic. It was undesirable to use an anesthetic because even light anesthesia affects respiration and hence alters pCO_2.

In the strictest sense, the hydrogen ion concentration as calculated by the relationship $[H^+]$ = antilog (-pH) is in actual fact hydrogen ion activity (23). In the case of blood, the ionic strength is sufficiently great that it would not be correct to assume that the hydrogen ion

activity coefficient is equal to 1. Consequently, in order to convert pH to $[H^+]$, it would be necessary to determine the value of the activity coefficient which would in turn introduce further error. Instead, it is simply recognized that the hydrogen ion concentrations referred to in this thesis are actually hydrogen ion activities.

Figure 3.1, shows the response of blood $[H^+]$, $[HCO_3^-]$ and pCO_2 to recovery from metabolic acidosis. None was significantly different from normal on the last day of acidosis. Hence, the adaptations which took place during acidosis were sufficient to fully compensate for the acid load administered. All three parameters were altered during recovery. The concentration of H^+ ions in the blood fell and remained decreased for two days and the concentration of bicarbonate ions rose and remained elevated for at least three days. Increased pCO_2 values were evident on the first and third days of recovery. The same type of changes in the blood acid-base parameters during recovery has also been reported for humans (203) and for rats (125).

These changes in acid-base parameters during recovery probably represent a metabolic alkalosis with a substantial respiratory compensation. The increase in blood HCO_3^- concentration during recovery is probably due to the inappropriate excretion of acid as ammonium salt and to an increase in the renal reabsorption of HCO_3^-. The continued excretion of acid in the urine results in the equimolar generation of HCO_3^- in the blood. Since NH_4Cl was not being consumed then the sodium bicarbonate was no longer being utilized to buffer excess acid. However, the generation of bicarbonate, in itself, is not usually sufficient to cause metabolic alkalosis. The capacity of the kidney for HCO_3^- excretion is



	$[H^+]$ (nmoles/L)	$[HCO_3^-]$ (mmoles/L)	pCO_2 (mm Hg)
NORMAL (N)	39.6 ± 1.8 (8)	24.8 ± 2.2 (8)	41.2 ± 3.6 (8)
ACIDOTIC (A)	42.3 ± 3.7 (8)	23.8 ± 3.7 (8)	45.6 ± 4.1 (8)
RECOVERY DAY 1 (R1)	35.6 ± 2.2 (8)*†	35.2 ± 1.9 (8)*†	51.6 ± 3.0 (8)*†
RECOVERY DAY 2 (R2)	37.1 ± 2.0 (8)*†	28.2 ± 3.1 (8)*†	43.8 ± 4.4 (8)
RECOVERY DAY 3 (R3)	37.8 ± 0.9 (4)	29.7 ± 1.6 (4)*†	47.1 ± 1.9 (4)*

Figure 3.1 Blood H^+ and HCO_3^- concentrations and partial pressure of CO_2 during acidosis and recovery.

Blood was obtained from normal rats (N), from rats made acidotic for seven days (A) and from rats which were allowed one (R1), two (R2) and three (R3) days to recover. The results are expressed as means ±SD. The number of observations are given in parentheses.

* indicates significantly different from normal value ($P<0.05$)
† indicates a significant difference ($P<0.05$) from the acidotic value

quite high and therefore has the ability to correct for increased generation of HCO_3^- by excreting excess in the urine. Therefore, the development of metabolic alkalosis also requires an impairment in renal HCO_3^- excretion. This is probably caused by enhanced HCO_3^- reabsorption. It is known that HCO_3^- reabsorption is enhanced by K^+ depletion (94). K^+ depletion is common in chronic metabolic acidosis due to gastrointestinal and/or renal losses of K^+ (152). Hence, the increased HCO_3^- reabsorption observed during recovery may be due to hypokalemia which resulted from the previous metabolic acidosis.

3.2 Renal Function

Previous conclusions about renal function during recovery from metabolic acidosis were based on urinary ammonia excretions and glutamine A-V differences (125). However, ammonia excretion is determined not only by the rate at which it is produced but also by the relative pHs and flow rates of the blood and urine. Hence, the abrupt decrease in ammonia excretion observed during recovery can not be taken with assurance to indicate a similarily abrupt decrease in total ammonia production. Differences in glutamine concentrations between arterial and renal venous bloods provide only a qualitative measure of glutamine utilization. Renal blood flows are also necessary to quantitate the amount of glutamine extracted by the kidney. For these reasons, clearance studies were performed *in vivo* by Dr. K.C. Man to assess the function of the kidney during recovery from metabolic acidosis.

The results of these studies are contained in Table 3.1. These results indicate that in the normal rat 38 percent of the total ammonia

Table 3.1 Effect of acidosis and recovery on total ammonia production and glutamine extraction by the left kidney in the rat, in vivo.

Experimental Condition (No. of Animals)	Arterial NH_4^+ µmol/ml	Venous NH_4^+ µmol/ml	Urine NH_4^+ µmol/ml	Arterial Glutamine µmol/ml	Venous Glutamine µmol/ml	Renal Vein NH_4^+ Release µmol/min	Urinary NH_4^+ Release µmol/min	Total NH_3 Production µmol/min	Glutamine Extraction µmol/min	GFR ml/min	RBF ml/min
NORMAL (5)	0.04±0.01	0.06±0.02	74.6±6.5	0.46±0.02	0.39±0.01	0.27±0.06	0.44±0.05	0.71±0.05	0.50±0.09	1.04±0.04	7.37±0.92
ACIDOTIC (5)	0.06±0.02	*0.20±0.03	*155.3±49.9	*0.39±0.05	*0.21±0.02	*0.93±0.34	0.71±0.24	*1.64±0.29	*1.16±0.26	1.02±0.10	6.62±0.48
RECOVERY DAY 1 (5)	*0.07±0.02	*0.16±0.04	†56.9±23.0	0.48±0.09	†0.37±0.04	0.54±0.30	*†0.23±0.09	†0.77±0.28	0.70±0.39	*0.90±0.04	*6.24±0.22
RECOVERY DAY 2 (4)	0.05±0.01	†0.09±0.02	†78.8±12.7	0.46±0.03	†0.39±0.02	†0.20±0.06	0.45±0.05	†0.65±0.06	†0.43±0.08	0.95±0.10	*†5.83±0.18

Acidotic rats were administered NH_4Cl for seven days and other rats were allowed to recover by drinking water for one and two days. The number of experiments is given in parenthesis. Results are expressed as means ±SD. * indicates significantly different from normal value and † indicates significantly different from acidotic value ($P<0.05$).

produced was released in the renal vein. A 2.3 fold increase in total ammonia production occurred during acidosis, increasing the amount released into the renal vein to 57 percent of the total ammonia produced. After one day recovery, total ammonia production fell to normal and the release of ammonia into the renal vein increased to 70 percent. Hence, the fall in ammonia release into the urine to below normal was due to both a decrease in total ammonia production and a shift in the percentage release of ammonia from the urine into the blood. This probably reflects an increase in the pH of the urine to above normal values as a result of decreased acid excretion. After two days of recovery, the percent distribution of ammonia between blood and urine was essentially back to normal, although urinary release was slightly elevated compared with normal. Presumably the relationship between urine pH and blood pH was also back to normal.

In these studies, the release of urinary ammonia in acidotic rats is only about double that of normal rats. In contrast, a six to seven fold increase in ammonia excretion occurred in twenty-four hour urine collections in response to acidosis of the same design (125). The normal rate of ammonia release into the urine can account for the normal rate of ammonia excretion. However, the rate at which ammonia was released into the urine in acidotic rats as determined by in vivo clearance studies (Table 3.1) is appreciably lower than that which is required for the rates of ammonia excretion found in experiments in which urine was collected for 24h from acidotic rats in metabolic cages. The reason for this discrepancy is not known. A contributing factor may be the reduced rate of daytime ammonia excretion which was observed for acidotic

rats in a study presented later on in this thesis in which four hour urine collections were made for 24h.

Also shown in Table 3.1 are the rates of glutamine extraction by the kidney for each of the four experimental situations studied. A significant increase in renal glutamine extraction occurred during acidosis. After one day of recovery the rate at which glutamine was extracted by the kidney is not significantly different from normal. Figure 3.2 illustrates the relationship between glutamine A-V differences and glutamine extraction. As can be seen, glutamine A-V differences provide a good indication of the changes in glutamine extraction during recovery since the changes in renal blood flows were small. It is interesting to note that the ratio of ammonia formed to glutamine utilized decreased from 1.4 to 1.1 on the first day of recovery (Figure 3.2). This occurred because of a larger decrease in ammonia formation than in glutamine utilization and may represent a shift in the pattern of glutamine metabolism. That the rate at which glutamine is metabolized by the kidney returned to normal during the first day of recovery is clearly indicated by the changes in the rates of glutamine extraction and total ammonia production observed in the present study.

3.3 In vivo, Freeze-Clamp Studies

Freeze-clamping has been used extensively to study the changes in renal glutamine metabolism, in vivo, during metabolic acidosis (3, 4, 19, 67, 117, 119). Similar studies have not been reported for recovery. Therefore, freeze-clamping was performed as an attempt to gain some insight into the metabolic changes in renal glutamine metabolism, in

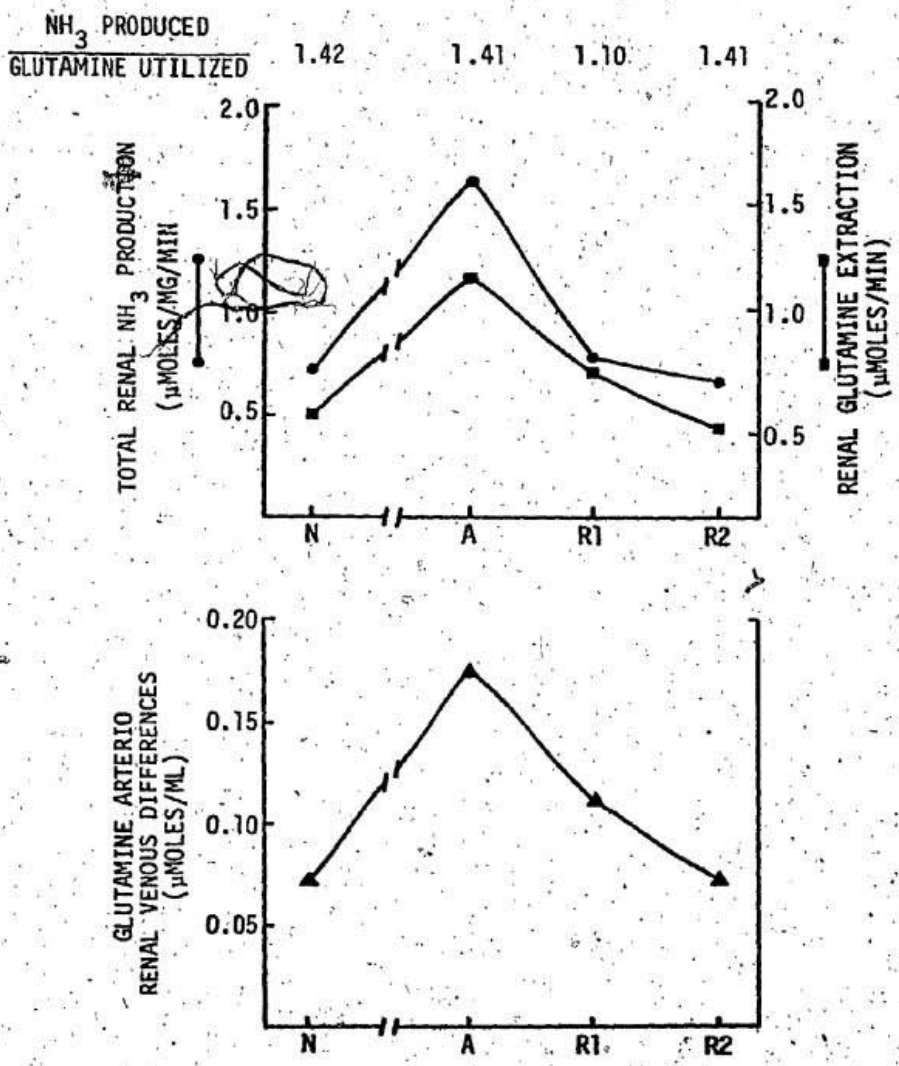


Figure 3.2 Changes in renal NH_3 production, glutamine extraction and glutamine A-V differences during metabolic acidosis and recovery from acidosis.

The points plotted represent mean values for normal (N), acidotic (A) and one- (R1) and two- (R2) day recovered rats and are derived from the data reported in Table 3.1.

vivo, during recovery from metabolic acidosis.

Although freeze-clamping is a useful technique, the results obtained are subject to certain limitations. Although changes in the metabolite profile may reflect changes in metabolism, the immense heterogeneity of the kidney makes interpretation difficult. The lack of an observed change in metabolite content can not be taken to rule out the possibility that a change did not occur in specific cells or compartments within the kidney. Certainly a change from one compartment to another would go unnoticed in whole tissue content as well as would the utilization by one cell type and the production by another of the same metabolite. Consequently, the results obtained by freeze-clamping must be interpreted cautiously.

A. Measurement of Adenine Nucleotides

Metabolic turnover is extremely rapid and hence the potential for rapid changes in the content of metabolites when conditions are altered is therefore very great. Useful data will only be derived from accurate steady-state determinations, so it is very important that the kidney be removed and frozen as rapidly as possible to prevent any alterations being introduced by the experimental technique. Killing, opening the abdominal cavity and then removing the kidney for clamping requires at least seven seconds in the present writer's hands and can only be done with relatively small rats. Exposing the kidney first by opening the abdominal cavity of anaesthetized rats permits its removal and subsequent clamping at times less than two seconds.

The relative amounts of adenine nucleotides in the kidney is con-

trolled by the adenylate kinase equilibrium (21). Between removal of the kidney and freeze-clamping, interconversion of ATP, ADP and AMP can still occur via this enzyme, the longer the interval between removal and clamping, the greater the opportunity for interconversion. Hence, the measurement of adenine nucleotides serves to evaluate the energy state of the kidney and is also an important assessment of experimental technique.

Table 3.2 shows the content of adenine nucleotides in the renal tissue of normal, acidotic and one-day recovered rats. The content of ATP, ADP and AMP are similar to that reported by others (21, 67, 190) for the kidneys of normal animals. This gives assurance of satisfactory technique. There is no significant difference in their content between groups. This indicates that there was no gross derangement of energy metabolism in the kidney during either acidosis or recovery from acidosis. Since the adenine nucleotide contents are similar in all groups of animals, any changes in other metabolites cannot be attributed to altered energy metabolism.

B. Measurement of Metabolites Relevant to Glutamine Metabolism

Table 3.3 shows the data obtained by freeze-clamping kidneys taken from normal, acidotic and one-day recovered rats. The contents of all metabolites, except lactate, are essentially of the same order of magnitude as those reported by others (19, 67, 190). The reason for the appreciably lower content of lactate in the present study is not known. As a result, the lactate to pyruvate ratios are substantially lower and the calculated NAD/NADH ratios are substantially higher than those

Table 3.2 Renal content of adenine nucleotides in normal, acidotic and one-day recovered rats.

Experimental Condition:	CONTENT OF METABOLITES µmoles/g kidney ±SD (No. of Animals=6) Normal	Acidotic	Recovery Day 1
ATP	1.52±0.39	1.25±0.35	1.34±0.05
ADP	0.67±0.08	0.64±0.04	0.61±0.08
AMP	0.12±0.02	0.14±0.03	0.14±0.03
Total Adenine Nucleotides	2.31	2.03	2.09
$\frac{ATP}{ADP}$	2.27	1.95	2.20

Rats were made acidotic by administering NH_4Cl, ad libitum for seven days. Other rats were permitted to recover by drinking water for one day.

Table 3.3 Effect of acidosis and recovery on metabolite levels in rat kidney

Experimental Condition:	CONTENT OF METABOLITES nmoles/g kidney ±SD (No. of Animals) Normal	Acidotic	Recovery Day 1
Glutamine	950±330(6)	380±400(6)*	930±470(6)†
Glutamate	2350±300(6)	1730±420(6)	3590±920(6)†*
α-Ketoglutarate	210±60(6)	70±40(6)*	230±110(6)†
Aspartate	400±50(8)	350±110(8)*	370±70(8)
Malate	100±40(8)	50±20(8)*	100±40(8)†
Citrate	160±40(8)	120±30(8)	240±60(8)†*
Oxaloacetate (calculated from MDH)	6.9	3.5	5.0
Oxaloacetate (calculated from GOT)	5.3	2.1	3.5
Phosphoenolpyruvate	60±10(8)	80±20(8)	50±10(8)†
Pyruvate	100±20(8)	90±20(8)	100±20(8)
Lactate	350±80(8)	280±90(8)	490±140(8)†*
3-Phosphoglycerate	130±40(14)	150±50(14)	110±30(14)†
Fructose-6-Phosphate	12±4(8)	14±10(8)	12±3(8)
Glucose-6-Phosphate	33±7(8)	34±13(8)	40±18(8)
Inorganic Phosphate	3730±600(8)	4300±900(8)	3500±600(8)
	Metabolites Ratios		
$\frac{\text{Lactate}}{\text{Pyruvate}}$	3.5	3.5	4.9
Cytoplasmic $\frac{NAD^+}{NADH}$ (calculated from LDH)	2570	2570	1840

Acidotic rats were administered NH_4Cl for seven days and other rats were allowed to recover from metabolic acidosis by drinking water for one day. * indicates significantly different from normal value and † indicates a significant difference from acidotic value ($P<0.05$).

reported elsewhere (67, 190, 193). Despite the higher NAD/NADH ratio reported here, the oxaloacetate content calculated from the malate dehydrogenase equilibrium agrees with other reported values (67, 193). This is probably because of the slightly lower malate content in the present study. Remarkable concordance between both estimates of oxaloacetate content lends credibility to these calculated values.

Percent changes in the renal content of metabolites relevant to glutamine metabolism during acidosis and recovery are illustrated in Figure 3.3. The upper histogram plot represents acidotic versus normal. In agreement with the results obtained by others (67, 190), significant decreases occurred in the contents of glutamine, α-ketoglutarate and malate, and as well the oxaloacetate content was calculated to be lower in acidosis. Decreases in glutamate, aspartate and citrate and increases in phosphoenolpyruvate and 3-phosphoglycerate, as found by others (4, 67, 190), were not statistically proven in the present study, but the same trends are evident. No change in the contents of pyruvate, lactate, fructose-6-phosphate, glucose-6-phosphate and phosphate was found, in agreement with studies elsewhere (67, 190).

The percent change in the metabolite profile during recovery is illustrated in the middle histogram plot (Figure 3.3) and the result of these changes in relation to normal is presented in the lower histogram plot (Figure 3.3). Glutamine, glutamate, α-ketoglutarate, malate, citrate and lactate increased significantly while phosphoenolpyruvate and 3-phosphoglycerate decreased significantly. The increases in glutamate, citrate and lactate elevated the levels of these metabolites significantly above normal. The contents of all other metabolites after one day of recovery were the same as those in the kidneys of normal rats.

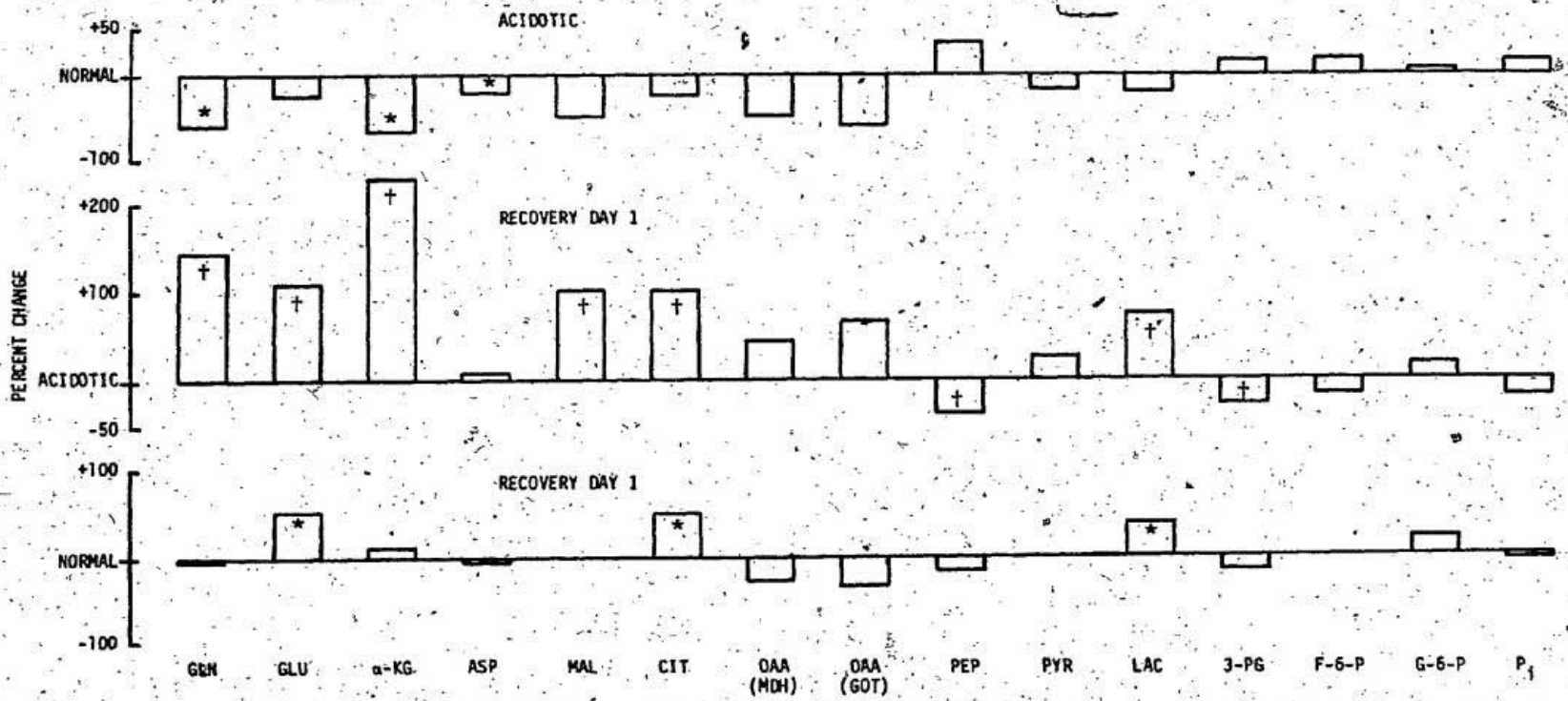


Figure 3.3 Changes in mean kidney metabolite levels expressed as percentage change from acidotic or normal values.

* indicates a significant change from normal value and + indicates a significant change from acidotic value.

C. Comments on the Results of the *in vivo*, Freeze-Clamp Studies

The decreased tissue glutamine observed during acidosis corresponds with the decreased plasma glutamine and probably reflects an increased rate of glutamine removal. Consequently, the supply of glutamine can not be a decisive factor in promoting its increased utilization in chronic metabolic acidosis. The return of tissue glutamine to normal during recovery also coincides with the return of plasma glutamine to normal and probably reflects the re-establishment of normal rates of glutamine utilization.

The alterations in the renal content of α-ketoglutarate and of glutamate are particularly important in view of their potential regulatory roles (50, 57, 130, 202). The increase in glutamate content during recovery is suggestive of it playing a regulatory role in turning off ammonia formation and the above normal levels obtained may be required because of the higher amounts of PDG present in one-day recovered rats compared with normal (125). The decrease in α-ketoglutarate during metabolic acidosis is consistent with its purported regulatory properties (50, 57) and with its proposed involvement in the removal of glutamate via glutamate dehydrogenase (59). Unlike glutamate, α-ketoglutarate does not attain above-normal levels during recovery which presumably would be necessary to inhibit the elevated mitochondrial capacity still evident (125).

It has been suggested by Hems and Brosnan (67) that the decreases in α-ketoglutarate, malate, oxaloacetate and citrate during metabolic acidosis result from increased glutaminase activity. According to this suggestion, increased glutaminase activity results in increased concen-

tration of ammonia and this is thought to cause a fall in α-ketoglutarate if glutamate dehydrogenase is assumed to be close to equilibrium and if there is no major change in the redox state of the mitochondrial nicotinamide nucleotide couple. The decreases in malate, oxaloacetate and citrate were thought to follow from the fall in α-ketoglutarate on the assumptions that aconitase, isocitrate dehydrogenase, aspartate aminotransferase and malate dehydrogenase are all close to equilibrium and also that the cytoplasmic NAD/NADH ratio is not altered. However, this explanation now appears unlikely in view of the demonstration by Vinay et al (193) that a rapid increase in renal ammonia concentration does not result in a rapid fall in the concentration of α-ketoglutarate. In addition, alterations in cellular equilibria according to this explanation would not account for the fall observed in glutamate content (53, 58, 67).

The decreased renal contents of α-ketoglutarate, malate, oxaloacetate and citrate during metabolic acidosis have also been attributed to the acceleration of the phosphoenolpyruvate carboxykinase reaction. According to this explanation increased flux through PEPCK causes a fall in oxaloacetate which, in turn, causes a fall in malate via the equilibrium reaction catalyzed by malate dehydrogenase. The falls in α-ketoglutarate and citrate arise by simple mass action as a result of the removal of Krebs' cycle intermediates subsequent to the fall in malate. This explanation also accounts for the decrease in glutamate if glutamate dehydrogenase is assumed to be close-to-equilibrium. For such a mechanism to operate one must assume that all the intervening reactions are held close to thermodynamic equilibrium. This is certainly not the case for α-ketoglutarate dehydrogenase. However, this hypothesis could still

be valid provided that renal α-ketoglutarate dehydrogenase is inhibited by succinyl CoA as it is in heart and liver (45, 100). If so, the fall in α-ketoglutarate could result from the de-inhibition of α-ketoglutarate dehydrogenase as a result of a decrease in succinyl CoA.

If this hypothesis is correct then the increases in α-ketoglutarate, malate, oxaloacetate, citrate and glutamate observed during recovery may be due to a decrease in the flux through PEPCK and a reversal of the events which are presumed to have occurred during metabolic acidosis. The increase in citrate to above normal levels may be due to a direct effect of bicarbonate on the mitochondria. It is known that renal citrate levels are increased in metabolic alkalosis (32, 169) and it is apparent that this is due to an effect of bicarbonate on citrate transport across the mitochondrial membrane such that citrate accumulates extramitochondrially (149, 171). Since plasma bicarbonate levels are elevated during recovery then the elevated citrate levels also observed may be due to a direct effect of bicarbonate on the citrate transporter.

The observed falls in phosphoenolpyruvate and 3-phosphoglycerate during recovery are also consistent with decreased flux through PEPCK. The fall in the content of 3-phosphoglycerate is due to the fact that enolase catalyzes a freely reversible, close-to-equilibrium reaction and hence a change in phosphoenolpyruvate will be accompanied by a change in 3-phosphoglycerate. The increase in lactate to above normal levels is not accounted for by a similar change in pyruvate. It must be assumed therefore, that in order to maintain the lactate dehydrogenase equilibrium, the cytoplasmic NAD/NADH ratio is also reduced in recovery.

3.4 Immunochemical Studies of Phosphoenolpyruvate Carboxykinase

In the rat, the increase in renal PEPCK activity during metabolic acidosis occurs in the cytoplasm of the proximal convoluted tubular cells (46, 63, 159). Immunochemical studies indicate that this increased activity is due to an increase in the amount of enzyme (103) and that this, in turn, to an increase in the rate of enzyme synthesis (73). Increased synthesis was detected as early as 2h after the induction of acidosis (73) and by 13h the relative rate of PEPCK synthesis increased from 2-3% to 7% of the synthesis of all soluble protein in the kidney cortex (73). During recovery from metabolic acidosis the activity of renal PEPCK returned to control levels within 24h (125). Since glutamine extraction and renal ammonia production increase during metabolic acidosis and are also back to normal after one day recovery, then the similar response of PEPCK activity to acidosis and recovery is consistent with it being of regulatory importance in renal ammoniagenesis. In the previous section of this chapter it was shown that the metabolite changes which occurred during acidosis and recovery are consistent with an increase in PEPCK flux, in vivo during acidosis and a decrease in PEPCK flux, in vivo during recovery. Because of the potential regulatory importance of this enzyme, it was of interest to know whether or not the fall in PEPCK activity during recovery was also due to a fall in the amount of PEPCK present or due to a decrease in the catalytic activity of a constant amount of enzyme protein. The low values (a matter of hours) reported for the half-life of PEPCK (73, 102) suggest that rapid decreases in PEPCK content can occur as a result of decreases in the rate of synthesis of this enzyme. That a decrease in PEPCK synthesis

occurs upon cessation of an acid challenge was shown by Iynedjian et al (73). Hence, it is plausible that the decrease in PEPCK activity during recovery is due to a decrease in the amount of this enzyme. This possibility is supported by the fact that the circadian increase in phosphoenolpyruvate carboxykinase activity is due to increased synthesis (116).

Immunochemical titration of PEPCK was used to investigate the cause of the decrease in PEPCK activity during recovery. This technique is a means of relating catalytic activity to immunochemical reactivity. In these experiments, the titration of PEPCK was carried out by varying the amount of enzyme incubated with a constant amount of PEPCK antibody. Typical titration curves are shown in Figure 3.4. The slope of the immunotitration curve reflects the stoichiometry of the antigen-antibody reaction and hence the affinity of the antibody for the antigen. A slope of 1.0 reflects a ratio of antigen to antibody of unity in the immunoprecipitate. Slopes of less than 1.0 arise as a result of an increase in the ratio of antigen to antibody whereas slopes of greater than 1.0 are seen when this ratio decreases. Since the stoichiometry of the antigen-antibody complex is not invariant then the equivalence points obtained by immunotitration may be used to indicate the relative amounts of enzyme protein present in different enzyme preparations only if the slopes of immunotitration curves are identical.

Immunochemical titrations of PEPCK were performed with cytosols obtained from normal and acidotic rats and from rats which were allowed 12h and 24h to recover from metabolic acidosis. The titrations were performed a number of times for each experimental condition and the means of the slopes and equivalence points obtained appear in Table 3.4.

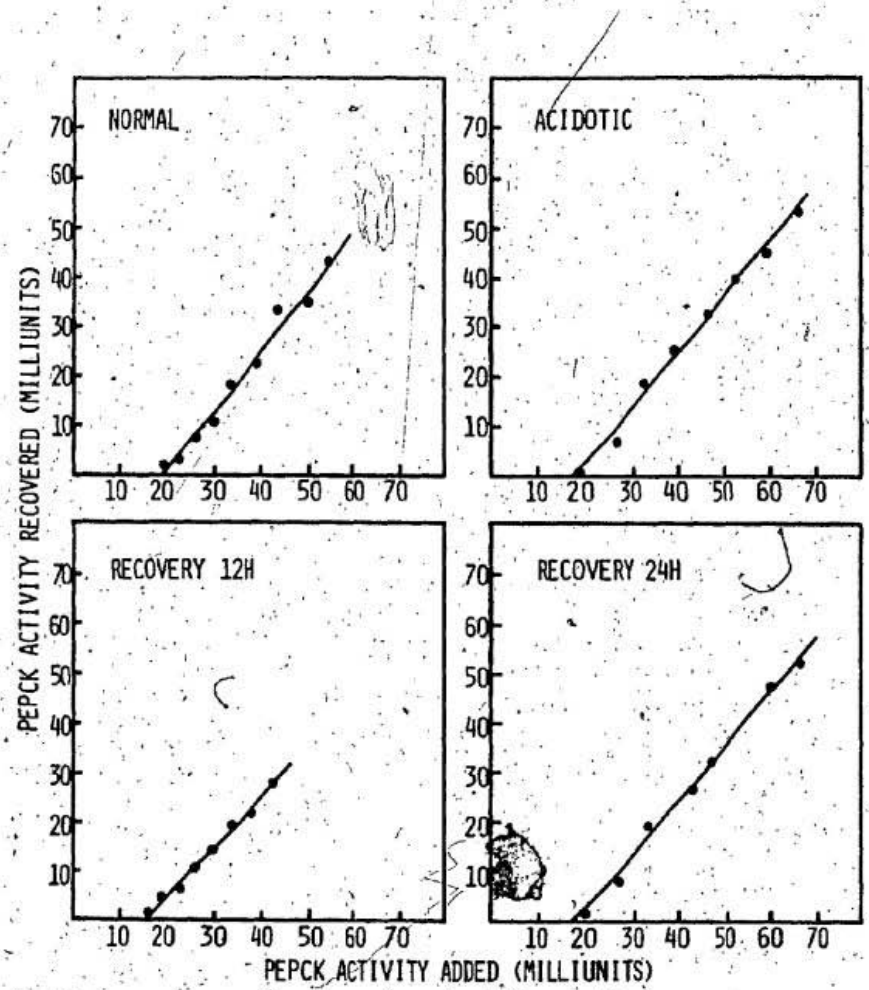


Figure 3.4 Typical immunotitration curves obtained with kidney extracts from normal, acidotic and recovered rats.

Rats were made acidotic by administering NH_4Cl, ad libitum for seven days and other rats were allowed to recover from metabolic acidosis by drinking water for 12h and 24h.

Table 3.4 Equivalence points obtained by immunotitration of renal phosphoenolpyruvate carboxykinase.

Experimental Condition	Equivalence Point (milliunits)	Slope	Phosphoenolpyruvate Carboxykinase Activity (milliunits/mg kidney ±SD)
Normal	18.2±2.9 (7)	1.05±0.25 (7)	7.0±0.5 (4)
Acidotic	18.0±6.2 (7)	0.97±0.22 (7)	20.8±2.6 (4)
Recovery (H_2O) 12h	16.6±4.9 (7)	0.92±0.12 (7)	14.2±5.3 (4)
Recovery (H_2O) 24h	16.8±4.6 (6)	1.04±0.33 (6)	8.8±3.7 (4)

Results are expressed as means ±SD.
Number in parentheses indicates number of animals used.

It is important to note that no significant change in the slope occurred and hence the affinity of the antibody for PEPCK is not altered. Since the slopes are all near 1.0, then it may be assumed that excess PEPCK does not affect the interaction between PEPCK and antibody. Also contained in Table 3.4 are the activities of PEPCK for each condition studied. These activities confirm that PEPCK activity returns to normal during the first day of recovery. The activity was also reduced after 12h of recovery although it was still higher than in normal animals. Despite this fall in PEPCK activity, no significant difference in the equivalence points obtained by the immunotitration of the different PEPCK activities is observed. Thus the decrease in catalytic activity is accompanied by a decrease in immunochemical reactivity. Hence, it can be concluded that the increased activity of PEPCK observed in acidosis and the fall in activity during recovery from acidosis are due to changes in the total amount of enzyme protein rather than to changes in the catalytic activity of the enzyme.

Although the simultaneous loss of catalytic activity and immunological reactivity is most probably due to the decreases in PEPCK activity being the result of decreases in the amount of enzyme, it does not rule out other possibilities. PEPCK may be reversibly converted from an immunochemically and catalytically-active form to an immunochemically and catalytically-inactive form. This requires that the inactivation of PEPCK involves a molecular change which affects its immunochemical activity. However, it can be concluded that the inactivitaion of PEPCK during recovery does not involve conversion to an immunochemically-active but catalytically-inactive form.

3.5 Mitochondrial Studies

The renal enzymes responsible for generating ammonia from glutamine, namely phosphate-dependent glutaminase and glutamate dehydrogenase are both mitochondrial. Glutamate dehydrogenase is a soluble enzyme located in the matrix space (76). The exact location of glutaminase is less certain. The results of submitochondrial fractionation studies are conflicting, suggesting both a matrix location (76) and an inner mitochondrial membrane location (40, 83). It has been suggested that these conflicting results indicate that PDG is present in both locations (82). That is, PDG is bound or embedded in the inner membrane such that part of the molecule projects out into the matrix space. As a consequence, PDG is easily solubilized and hence the results obtained by submitochondrial fractionation vary depending upon the vigor of the fractionation procedure. Support for the suggestion that PDG is membrane-bound comes from studies by Kovacevic (82). Kovacevic reports that a discontinuity or break in the Arrhenius plot of glutaminase occurs if the activation energy of the enzyme is determined with frozen mitochondrial preparations. This agrees with the fact that some membranous and membrane-bound enzymes have such breaks in their Arrhenius plots (143). Furthermore, the addition of a detergent completely abolished the break in the Arrhenius plot of PDG. Highly purified PDG does not contain a hydrophobic region (28) which might account for its apparent association with the inner mitochondrial membrane. However, analysis of subunit structure indicates that a hydrophobic portion of PDG may be split off during the purification procedure (28).

Since phosphate-dependent glutaminase and glutamate dehydrogenase

are the enzymes responsible for generating ammonia from glutamine, then any theory for the regulation of renal ammoniagenesis must account for the control of the flux through these enzymes. The only known modulators of physiological importance are α-ketoglutarate, phosphate and glutamate. It has been postulated that α-ketoglutarate indirectly controls the flux through these enzymes by regulating the entry of glutamine into the mitochondria (50, 57). Phosphate is required for PDG activity *in vitro*. It has been demonstrated that this phosphate-activation is associated with a phosphate-induced dimerization (49). Phosphate activation was also observed with intact mitochondria (38, 181). Kovacevic has argued that the effect of phosphate on glutaminase activity in intact mitochondria is more determined by the flux of phosphate across the inner membrane than by the intramitochondrial concentration of phosphate (82). PDG activity is also affected by glutamate. It has been demonstrated that PDG is significantly inhibited by physiological concentrations of glutamate and that this inhibition is not competitive with glutamine, but is competitive with phosphate (54, 187). Glutamate reverses dimer formation (49). The inhibitory effect of glutamate was also observed with isolated mitochondria (161). Accordingly, isolated mitochondria provide a unique system for studying a portion of the metabolic pathway of renal glutamine metabolism with the microenvironment still intact. (that is, as it may be affected by mitochondrial activators and inhibitors, transport processes and competing pathways) and unaffected by the influence of PEPCK.

That isolated intact mitochondria exhibit an adaptive increase in glutamine metabolism in response to acidosis induced *in vivo* is well

recognized (1, 51, 57, 125, 161). However, the metabolic basis for this adaptive change and the relevance of a mitochondrial adaptation to the regulation of renal glutamine metabolism, in vivo, remain unresolved. Several mitochondrial events have been implicated as responsible for the augmented metabolism of glutamine observed in mitochondria isolated from acidotic rats. The apparent increase in the mitochondrial transport of glutamine (57) and the increase in phosphate-dependent glutaminase (125) are two events which have received consideration as potentially rate-limiting adaptive phenomena.

A third possibility derives from the mitochondrial studies of Schoolwerth et al (161). Schoolwerth et al incubated mitochondria from normal and acidotic rats with physiological concentrations of the substrates glutamine and glutamate. By measuring the changes in ammonia, glutamate and aspartate, these investigators were able to calculate the fluxes through glutamate dehydrogenase (GDH), glutaminase (PDG) and glutamate-oxaloacetate transaminase (GOT). The results of their studies indicate that in the presence of both substrates, the flux through glutamate dehydrogenase is markedly enhanced in mitochondria isolated from acidotic rats. The augmented flux through glutamate dehydrogenase could not be explained by enhanced provision of medium glutamate to the site of glutamate dehdrogenase via the glutamate-hydroxyl transporter. Nor could the enhanced flux through GDH be accounted for on the basis of equilibrium considerations. In fact, measurement of the ratio of matrix concentrations of GDH reactants indicated that GDH was far from equilibrium. Since the increase in total GDH enzyme protein was also not able to account for the much larger change observed in GDH flux, then Schoolwerth et al concluded

that an unknown alteration occurred in the environment of the mitochondria during metabolic acidosis. The result of this change in the environment was hypothesized to increase the availability of glutamate derived from glutamine to glutamate dehydrogenase. This profound change in GDH flux during metabolic acidosis may, in turn, regulate glutaminase activity and glutamine entry and hence represents a possible rate-determining adaptive mechanism responsible for the increased rate of glutamine metabolism in mitochondria isolated from acidotic rats.

Previous studies on mitochondrial glutamine metabolism during recovery from metabolic acidosis were performed with isolated mitochondria incubated with glutamine as sole substrate (125). No attempt was made to differentiate between the individual fluxes associated with the metabolism of glutamine. By measuring ammonia formation; it was found that the mitochondrial metabolism of glutamine remained elevated for at least two days of recovery. This was taken to indicate that regardless of which mitochondrial step is rate-limiting for glutamine metabolism, *in vitro*, it is not the sole factor which determines the rate of glutamine metabolism, *in vivo*. This conclusion is based upon the observation that, unlike glutamine metabolism *in vitro*, the metabolism of glutamine *in vivo* returns to normal by the end of the first recovery day.

Schoolwerth *et al* did not find the same marked enhancement of GDH flux in mitochondria from acidotic rats when the mitochondria were incubated with glutamine as sole substrate. Since the presence of both glutamine and glutamate in the incubation medium represents a more physiological situation than glutamine alone, then it was of interest to study the metabolic response of isolated mitochondria to recovery from

metabolic acidosis in the presence of both substrates. If the increase in GDH flux observed by Schoolwerth et al represents the rate-limiting adaptation, in vitro, then it is important to know the response of GDH flux to recovery in the same incubation medium in order to better assess the role of mitochondria in the regulation of renal glutamine metabolism, in vivo.

The phosphate concentrations of the mitochondrial incubation media used in the previous recovery studies (125) and in the studies performed by Schoolwerth et al (161) were about 4 to 5 fold greater than that in vivo. Since phosphate also affects mitochondrial glutamine metabolism (38, 82), it was of interest to compare the metabolic response of isolated mitochondria to recovery at the physiological concentration of phosphate with that obtained at the higher phosphate concentration.

The mitochondrial studies reported in this chapter were performed with the above-mentioned interests in mind. As in the studies of Schoolwerth et al (161), the medium changes in ammonia, glutamate and aspartate were measured for each mitochondrial incubation so that the fluxes through PDG, GDH and GOT could be calculated. The results of these measurements and calculations are contained in Tables 3.5 and 3.6, respectively and are discussed below.

A. Ammonia Formation

In these studies, mitochondria were incubated with glutamine (1 mM) in a low-phosphate medium (4 mM) and a high-phosphate medium (20 mM). These incubations were carried out both in the absence and presence of glutamate (3 mM). Hence, for each experimental condition examined, four

Table 3.5 Medium Changes in different media during the incubation of mitochondria from rats during acidosis and recovery.

	LOW PHOSPHATE MEDIUM 4 mM KH_2PO_4						HIGH PHOSPHATE MEDIUM 20 mM KH_2PO_4					
	1 mM GLUTAMINE			1 mM GLUTAMINE + 3 mM GLUTAMATE			1 mM GLUTAMINE			1 mM GLUTAMINE + 3 mM GLUTAMATE		
MEDIUM CHANGES:	AMMONIA	GLUTAMATE	ASPARTATE	AMMONIA	GLUTAMATE	ASPARTATE	AMMONIA	GLUTAMATE	ASPARTATE	AMMONIA	GLUTAMATE	ASPARTATE
EXPERIMENTAL CONDITION	Δ nmoles / mg mito protein / min ± SD (No. of Animals)											
NORMAL	14.4±3.7 (12)	1.6±1.2 (12)	4.2±2.0 (12)	6.6±2.6 (12)	-15.7±4.0 (8)	20.9±5.5 (12)	24.8±6.8 (10)	3.8±2.3 (10)	5.1±1.9 (10)	15.4±4.4 (10)	-19.8±4.3 (8)	30.5±4.5 (7)
ACIDOTIC	38.2±5.3 (9)	1.6±0.7 (9)	2.9±0.9 (8)	21.9±3.9 (9)	-12.4±5.4 (7)	20.9±2.8 (9)	51.1±6.2 (9)	2.2±1.1 (9)	3.2±1.1 (9)	38.0±6.1 (9)	-16.4±4.6 (6)	25.4±6.6 (9)
RECOVERY DAY 1	21.9±4.1 (9)	1.5±0.8 (9)	3.9±1.1 (9)	10.5±2.5 (9)	-8.7±5.5 (8)	23.6±7.7 (9)	42.0±7.6 (9)	3.2±1.0 (9)	4.8±1.8 (9)	24.7±5.7 (9)	-10.3±5.5 (8)	27.5±6.4 (9)
RECOVERY DAY 3	20.7±4.1 (8)	2.1±1.3 (8)	3.8±1.2 (8)	8.0±2.1 (8)	-9.4±4.2 (8)	28.5±7.6 (8)	39.8±4.8 (7)	3.8±1.7 (7)	6.7±2.2 (7)	21.6±3.6 (8)	-16.1±4.5 (8)	36.8±6.6 (8)

Kidney cortex mitochondria from normal, acidotic and recovered-rats (after one and three days recovery) were incubated for 4 minutes with 1 mM glutamine ±3 mM glutamate and either 4 mM phosphate or 20 mM phosphate.

Table 3.6 Enzyme fluxes in mitochondria incubated in different media and isolated from rats during acidosis and recovery.

	LOW PHOSPHATE MEDIUM 4 mM KH_2PO_4						HIGH PHOSPHATE MEDIUM 20 mM KH_2PO_4					
	1 mM GLUTAMINE			1 mM GLUTAMINE + 3 mM GLUTAMATE			1 mM GLUTAMINE			1 mM GLUTAMINE + 3 mM GLUTAMATE		
FLUX:	PDG	GDH	GOT	PDG	GDH	GOT	PDG	GDH	GOT	PDG	GDH	GOT
EXPERIMENTAL CONDITION	nmoles / mg mito protein / min ± SD (No. of Animals)											
NORMAL	10.1±2.4 (12)	4.3±1.9 (12)	4.2±2.0 (12)	7.9±3.8 (10)	0.6±5.3 (12)	20.9±5.5 (12)	16.8±4.0 (10)	8.0±3.2 (10)	5.1±1.9 (10)	14.4±4.1 (9)	1.6±4.9 (9)	30.5±4.5 (7)
ACIDOTIC	20.8±3.1 (8)	16.6±2.2 (8)	2.9±0.9 (8)	15.1±4.5 (8)	6.8±3.5 (9)	20.9±2.8 (9)	28.0±3.0 (8)	22.9±3.4 (9)	3.2±1.1 (9)	23.8±3.8 (8)	12.2±3.8 (8)	25.4±6.6 (9)
RECOVERY DAY 1	13.7±2.4 (9)	8.3±2.0 (9)	3.9±1.1 (9)	11.8±5.7 (8)	0.1±6.3 (9)	23.6±7.7 (9)	24.9±4.2 (9)	17.1±3.7 (9)	4.8±1.8 (9)	21.6±5.3 (8)	3.2±5.1 (8)	27.5±6.4 (8)
RECOVERY DAY 3	13.3±2.2 (8)	7.4±2.3 (8)	3.8±1.2 (8)	13.6±3.3 (8)	-5.6±3.4 (8)	28.5±7.6 (8)	25.1±2.1 (7)	14.6±3.7 (7)	6.7±2.2 (7)	20.6±5.6 (8)	1.1±4.3 (8)	36.8±6.6 (8)

Kidney cortex mitochondria from normal, acidotic and recovered rats (after one and three days recovery) were incubated for 4 minutes with 1 mM glutamine ±3 mM glutamate and either 4 mM phosphate or 20 mM phosphate.

separate mitochondrial incubations were performed. The different media were designed to study the effects of phosphate and glutamate on the metabolic response of mitochondria to metabolic acidosis and recovery from acidosis. The medium containing 1 mM glutamine, 3 mM glutamate and 4 mM phosphate is considered the more physiologically-relevant medium since these concentrations approximate those obtained in vivo.

(1) Effect of Phosphate - Figure 3.5 shows the plots of ammonia formation by mitochondria incubated in the low-phosphate media versus ammonia formation by the same mitochondrial preparations in the corresponding high-phosphate media. These plots represent the effect of phosphate on mitochondrial ammoniagenesis in the absence and presence of glutamate. Phosphate activation is apparent in both situations, however slightly greater activation is observed when glutamate is present.

(2) Effect of Glutamate - Figure 3.6 shows plots of ammonia formation by mitochondria incubated with glutamine versus ammonia formation by mitochondria incubated with glutamine plus glutamate for the low-phosphate media and the high-phosphate media. These plots represent the effects of glutamate on ammonia formation in the presence of 4 mM phosphate and 20 mM phosphate. As can be observed, glutamate inhibits ammonia formation at both phosphate concentrations. However, the inhibition by glutamate in the low phosphate medium is slightly more than that observed in the high-phosphate medium, again reflecting the competitive nature of phosphate activation and glutamate inhibition.

(3) Effect of Acidosis and Recovery from Acidosis - Figure 3.7 illustrates the effect of acidosis and recovery on ammonia formation by isolated mitochondria. In the high-phosphate media, the rates of ammonia-

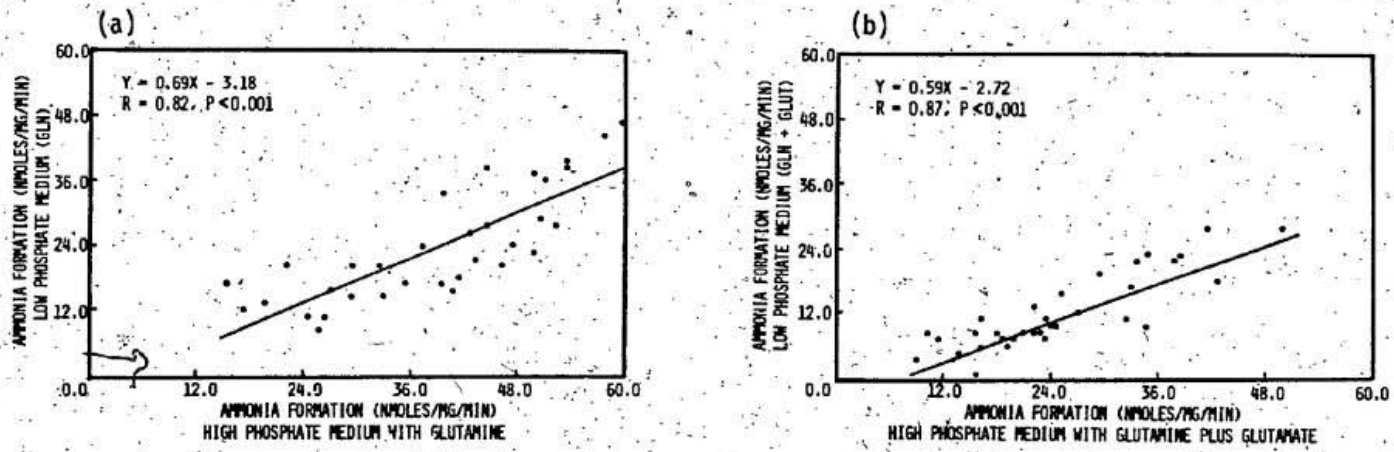


Figure 3.5 Effect of phosphate on ammonia formation by isolated mitochondria.

The data for these correlations are derived from incubations of mitochondria from normal, acidotic and one and three-day recovered rats. Plot (a) represents the effect of phosphate on ammonia formation in the absence of glutamate and plot (b) the effect of phosphate on ammonia formation in the presence of glutamate.

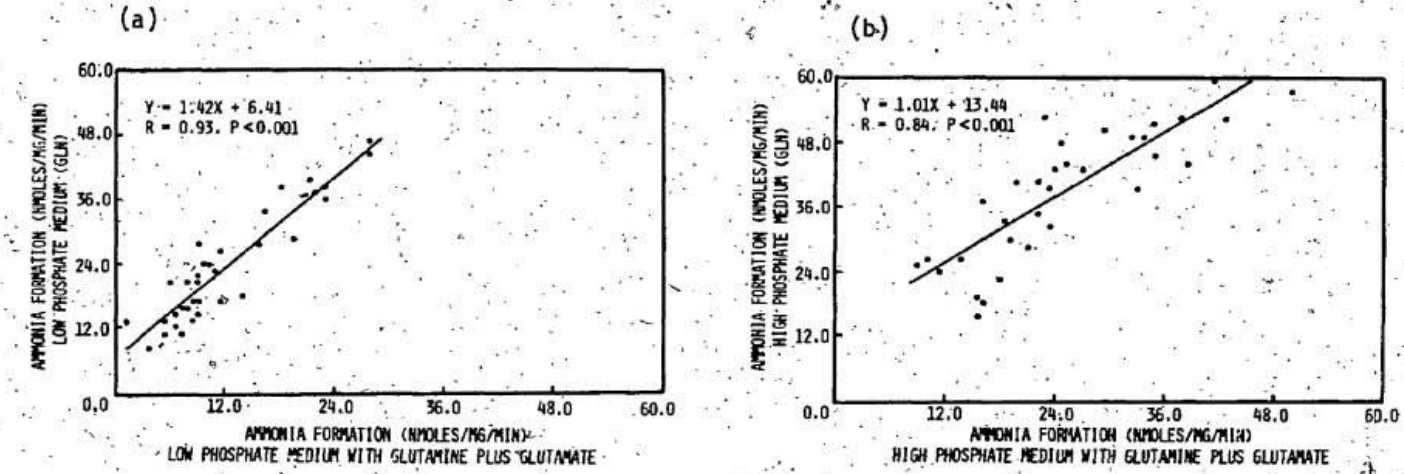


Figure 3.6 Effect of glutamate on ammonia formation by isolated mitochondria.

The data for these correlations are derived from incubations of mitochondria from normal, acidotic and one and three-day recovered rats. Plot (a) represents the effect of glutamate on ammonia formation in the presence of 4 mM phosphate and plot (b) the effect of glutamate on ammonia formation in the presence of 20 mM phosphate.

Legend for Figure 3.7

The data presented in this figure are contained in Tables 3.5 and 3.6. Kidney cortex mitochondria were isolated from seven-day acidotic rats (A) and rats which were allowed one (R1) and three (R3) days to recover from metabolic acidosis. Incubations were for 4 minutes in media containing 1 mM glutamine ± 3 mM glutamate and either 4 mM phosphate or 20 mM phosphate. Dashed lines represent normal values, * indicates significantly different from normal value and † indicates significantly different from acidotic value ($P < 0.05$).

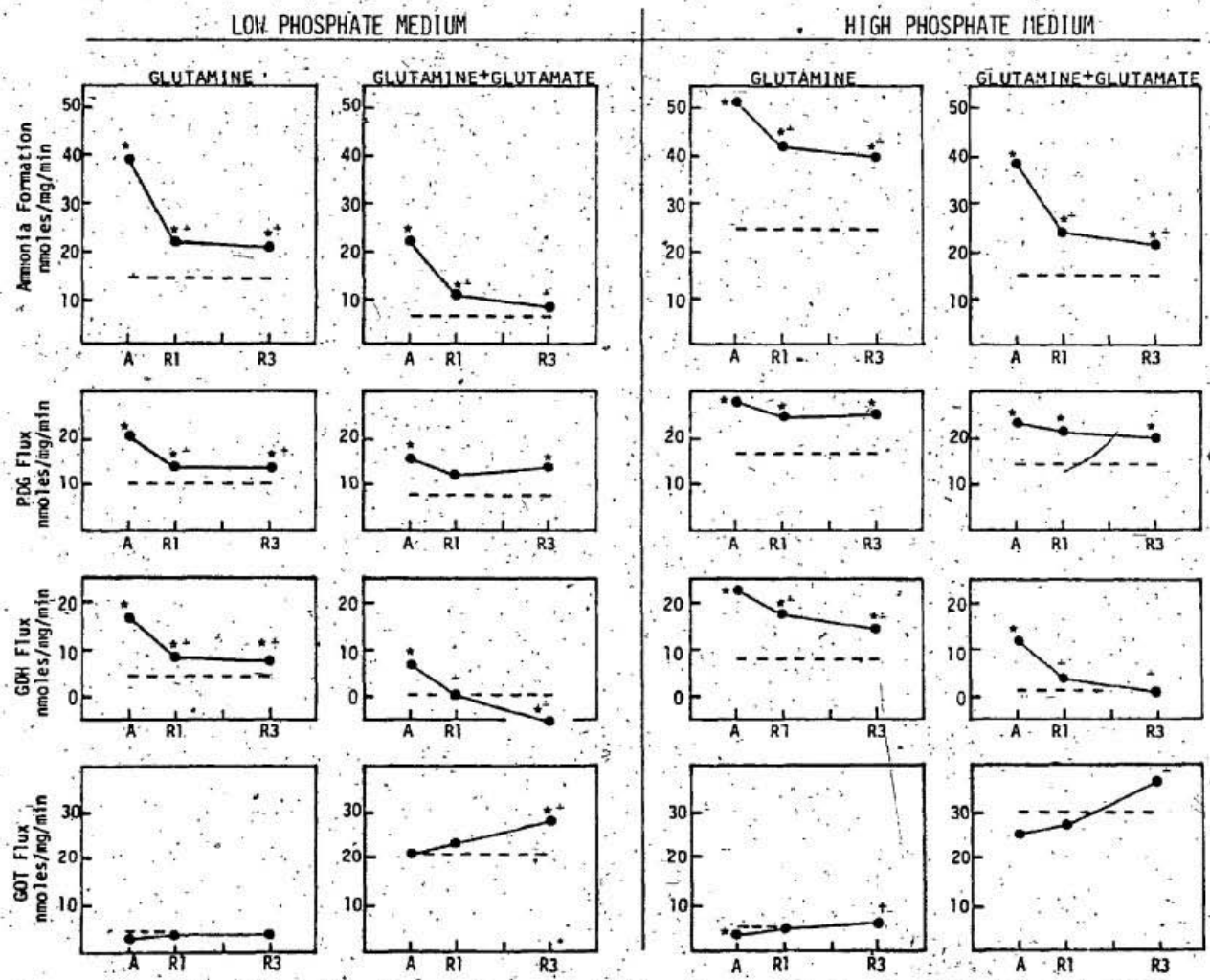


Figure 3.7 Effect of acidosis and recovery on the mitochondrial metabolism of glutamine ± glutamate at different phosphate concentrations.

genesis by mitochondria from normal rats agree with those obtained in identical media by Schoolwerth et al (161). The normal rates of ammoniagenesis in the low-phosphate media are about one-half the rates obtained in the corresponding high-phosphate media. Significant increases in ammonia formation occurred during metabolic acidosis in all four media. The increases observed in the high-phosphate media are comparable to those reported by Schoolwerth et al (161).

The exact response of isolated mitochondria to recovery was dependent upon the medium in which the mitochondria were incubated. Significant decreases in ammonia formation occurred in all media during the first recovery day. However, these decreases on the first day of recovery were not sufficient to achieve normal rates of ammoniagenesis in any of the media studied.

Although a significant decrease in ammonia formation occurred in the high-phosphate medium containing glutamine as sole substrate, the obvious trend during recovery for mitochondrial ammoniagenesis in this medium was to remain elevated. This confirms previous results obtained with a similar incubation medium (125). On the other hand, in the high-phosphate medium containing both substrates a pronounced fall in ammonia formation is the obvious response to recovery. In contrast, at the lower phosphate concentration, a fall in ammonia formation was apparent in both the absence and presence of glutamate, although normal rates were achieved by the third day of recovery only in the medium containing both substrates.

Obviously, none of these responses to recovery coincide exactly with the return of ammonia production to normal by the kidney, in vivo.

However, since the response of isolated mitochondria to recovery varied depending upon the medium in which they were incubated and since the results obtained in the most physiological medium "approach" the response, *in vivo*, then it is possible that isolated mitochondria will exhibit a response like that *in vivo* in an appropriately-devised medium. Although changes in ammonia formation indicate the overall responses of isolated mitochondria to acidosis and recovery from acidosis, it does not by itself provide any indication as to the metabolic basis for these responses. For this reason, medium changes in glutamate and aspartate were also measured in addition to ammonia formation, in order to calculate the fluxes through PDG, GDH and GOT.

B. Phosphate-Dependent Glutaminase Flux

The responses to acidosis and recovery of the flux through phosphate-dependent glutaminase in mitochondria incubated in the four different media are also illustrated in Figure 3.7. The normal rates of PDG flux obtained in the high-phosphate media are in good agreement with those obtained by Schoolwerth *et al* (161). In all four incubation media PDG flux increased during metabolic acidosis. The increases observed in the high-phosphate media compare well with those obtained by Schoolwerth *et al*, at the same phosphate concentration.

The response of PDG flux to recovery varied depending upon the medium in which the mitochondria were incubated. In the high-phosphate media, the fluxes through PDG did not decrease significantly during recovery. Hence, the decrease observed in ammonia formation in the high-phosphate medium containing both substrates is not attributable to

a change in PDG flux. However, the persistence of elevated rates of PDG flux in the medium containing glutamine as sole substrate at the high phosphate concentration coincides with the response of ammonia formation in this medium.

At the lower phosphate concentration PDG flux decreased during recovery in mitochondria incubated with glutamine alone. This decrease in PDG flux coincides with the decrease in ammonia formation also observed in this medium. In contrast, in the presence of both substrates at the lower phosphate concentration, a change in PDG flux is not evident. Hence the fall in ammonia formation observed in this medium can not be attributed to a fall in PDG flux.

In Figure 3.8 are shown plots of ammonia formation versus PDG flux for each medium. Most striking are the excellent correlations found in the media without glutamate. The correlations between ammonia formation and PDG flux obtained in the media containing both substrates have markedly reduced correlation coefficients. This may be explained if glutamate differentially affects PDG and GDH fluxes.

C. Glutamate Dehydrogenase Flux

The effect of acidosis and recovery on the flux through glutamate dehydrogenase in mitochondria incubated in the four different media is also illustrated in Figure 3.7. The rates of GDH flux in control mitochondria incubated in the high-phosphate media are similar to those reported by Schoolwerth et al (161). In agreement with the results of Schoolwerth et al, the flux through GDH obtained in the high phosphate-medium containing both substrates is neglible in mitochondria isolated

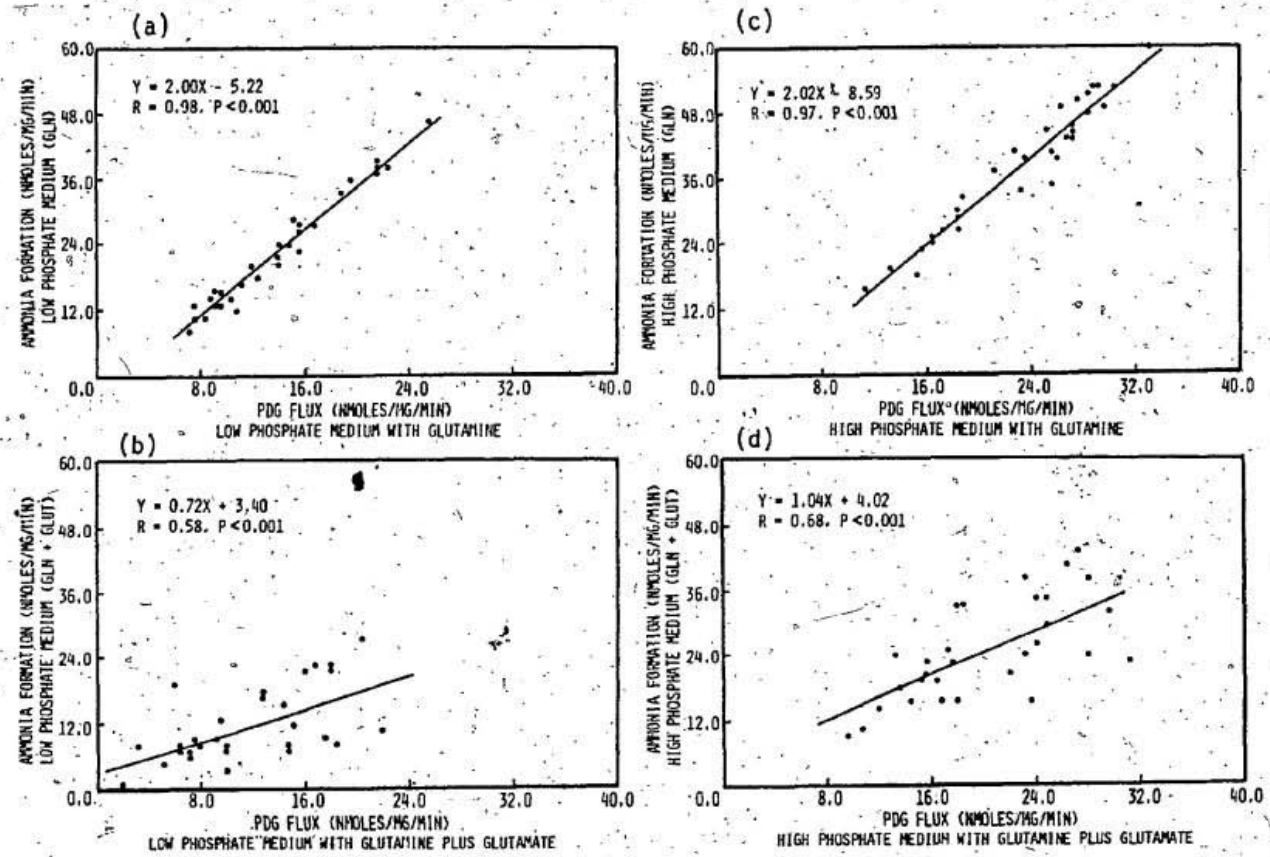


Figure 3.8 Relationship between ammonia formation and the flux through phosphate-dependent glutaminase in isolated mitochondria.

Data for these correlations are derived from incubations of mitochondria from normal, acidotic and one and three-day recovered rats. Plots (a) and (b) represent the relationship between ammonia formation and PDG flux in the low-phosphate (4 mM) media in the absence and presence of 3 mM glutamate, respectively. Plots (c) and (d) represent this relationship in the high-phosphate (20 mM) media in the absence and presence of 3 mM glutamate, respectively.

from normal rats, whereas in the high-phosphate medium containing glutamine as sole substrate, the GDH flux contributed about one-third of the ammonia formed. At the lower phosphate concentration, GDH flux in control mitochondria is also negligible in the presence of glutamate and contributed between one-third to one-half of the ammonia formed in the medium without added glutamate.

Acidosis increased the flux through GDH in all media. In agreement with the results of Schoolwerth et al (161), the percent enhancement of GDH flux was substantially greater in the presence of both glutamine and glutamate than in the presence of glutamine alone. This effect of glutamate on the response of GDH flux to acidosis is observed at both phosphate concentrations.

The response of GDH flux to recovery depended upon the medium in which the mitochondria were incubated. GDH flux decreased significantly in all four media on the first day of recovery. Although GDH flux fell during recovery in the high-phosphate medium containing glutamine as sole substrate, it tended to remain well elevated above normal as did ammonia formation and PDG flux. In the corresponding medium at the lower phosphate concentration the fall in GDH flux was more pronounced as were the falls in ammonia formation and PDG flux. Only in the media containing both glutamine and glutamate were normal rates of GDH flux attained. Obviously, the falls in GDH flux in these media do not depend upon decreases in PDG flux. Presumably PDG flux would be inhibited if the matrix concentration of glutamate rose as a result of the decrease in GDH flux. Since this does not appear to happen then the efflux of glutamate must be sufficient to prevent the intramitochondrial accumulation of glutamate.

The negative value for GDH flux obtained in the low-phosphate medium suggests the formation of glutamate via glutamate dehydrogenase. This is conceivable since NADH and α-ketoglutarate may be generated from added malate via malate dehydrogenase and glutamate oxaloacetate transaminase, respectively (Figure 3.9). Calculations indicate that there is sufficient malate in the incubation medium (0.1 mM) to fully account for the glutamate formed via glutamate dehydrogenase.

Shown in Figure 3.10 are plots of ammonia formation versus GDH flux. Excellent correlations are observed with the data derived from the mitochondrial incubations in the media without glutamate. However, when glutamate is present the correlation between ammonia formation and GDH flux is markedly less. This gives further support to the suggestion made in Section 3.5 (B) that glutamate differentially affects PDG and GDH fluxes.

D. Relationship between Phosphate-Dependent Glutaminase Flux and Glutamate Dehydrogenase Flux

Figure 3.11 contains plots of PDG flux versus GDH flux for all four media. As can be seen, in the media without glutamate excellent correlations exist between these two parameters. Hence, it may be concluded that in these media the fluxes through these enzymes are under control of the same rate-limiting step, presumably PDG activity. However, it is apparent that PDG flux and GDH flux do not correlate in the media containing glutamate. It may therefore be concluded that PDG flux and GDH flux are controlled by different mechanisms. Hence, glutamate must do more than just inhibit PDG activity.

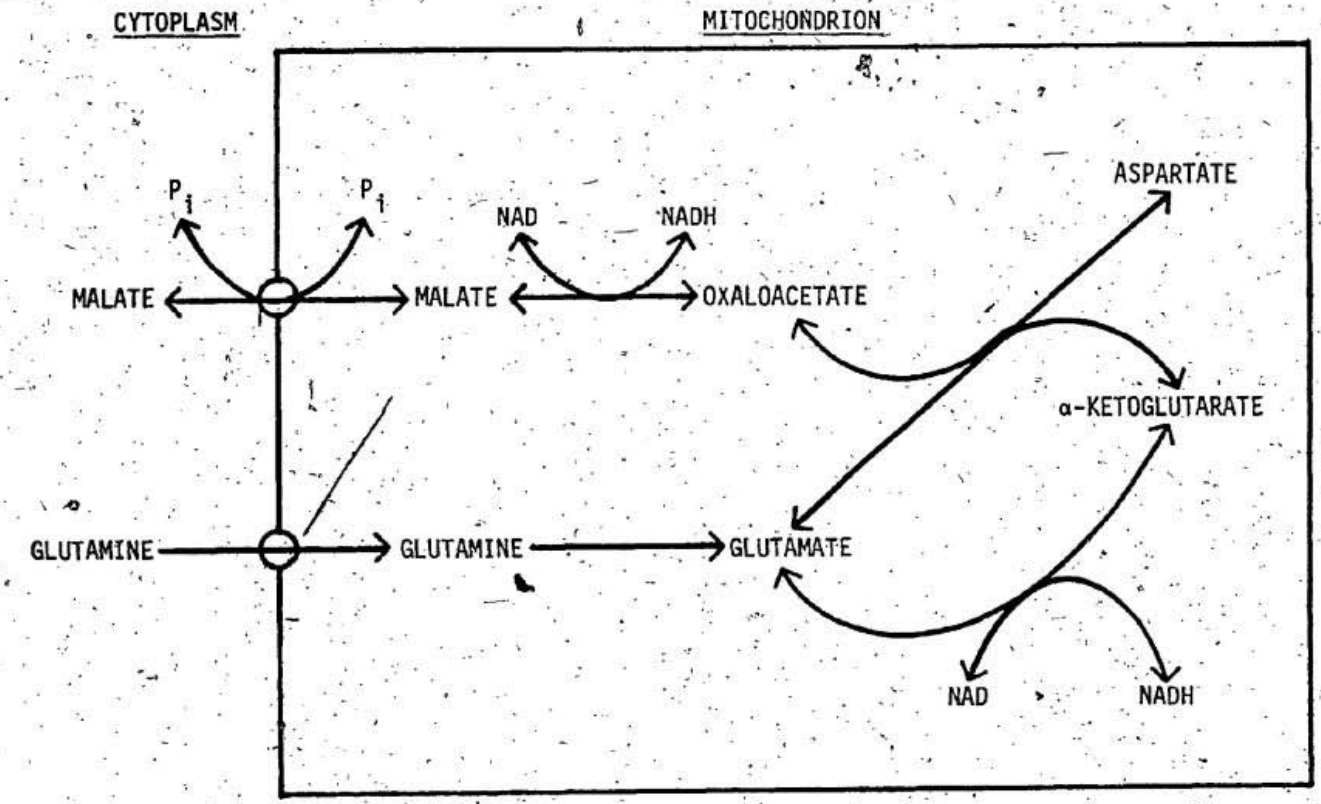


Figure 3.9 Scheme for the generation of α-ketoglutarate and NADH from medium malate by isolated mitochondria.

Double-headed arrows represent reversible reactions and single-headed arrows, irreversible reactions.

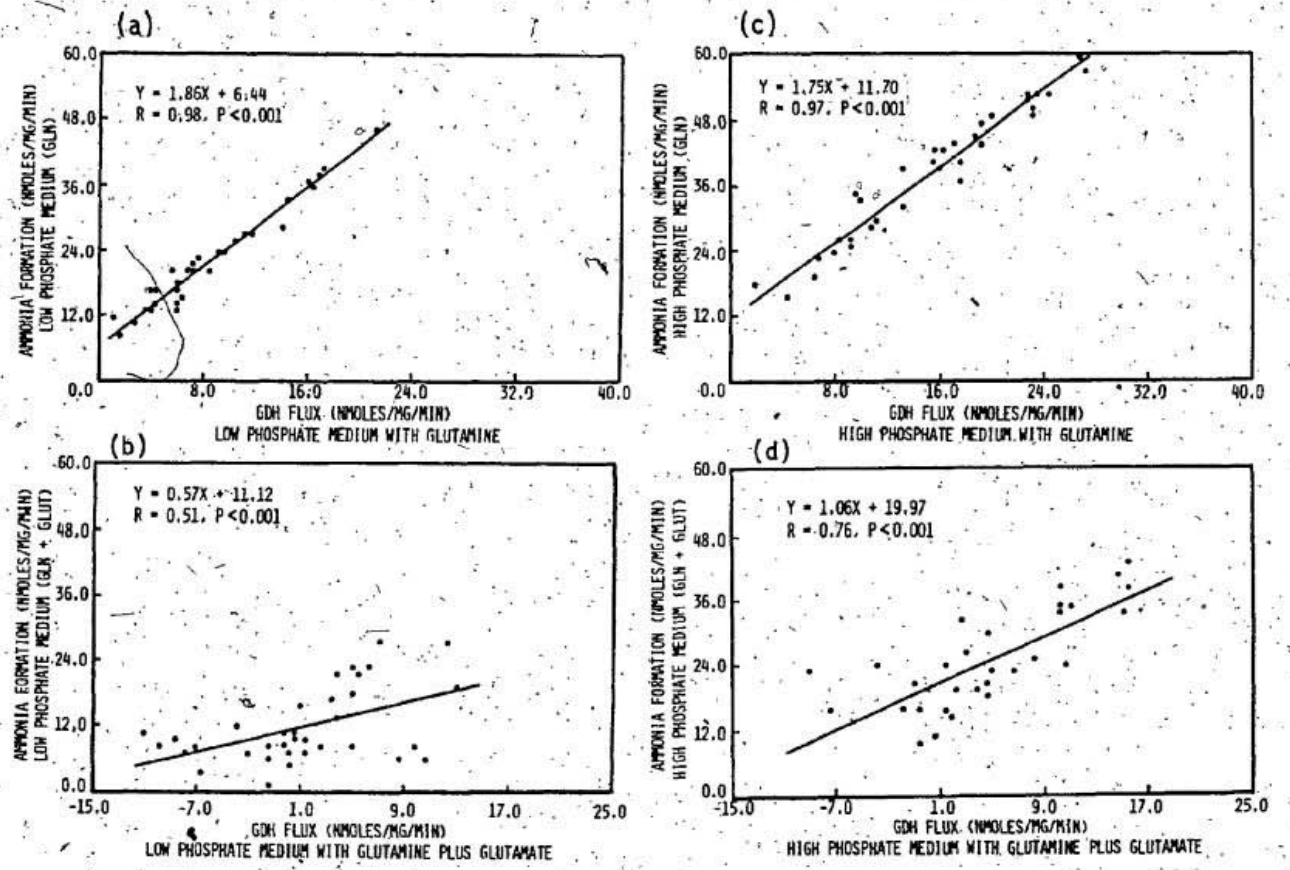


Figure 3.10 Relationship between ammonia formation and the flux through glutamate dehydrogenase in isolated mitochondria.

Data for these correlations are derived from incubations of mitochondria from normal, acidotic and one and three-day recovered rats. Plots (a) and (b) represent the relationship between ammonia formation and GDH flux in the low-phosphate (4 mM) media in the absence and presence of 3 mM glutamate, respectively and plots (c) and (d) represent this relationship in the high-phosphate (20 mM) media in the absence and presence of 3 mM glutamate, respectively.

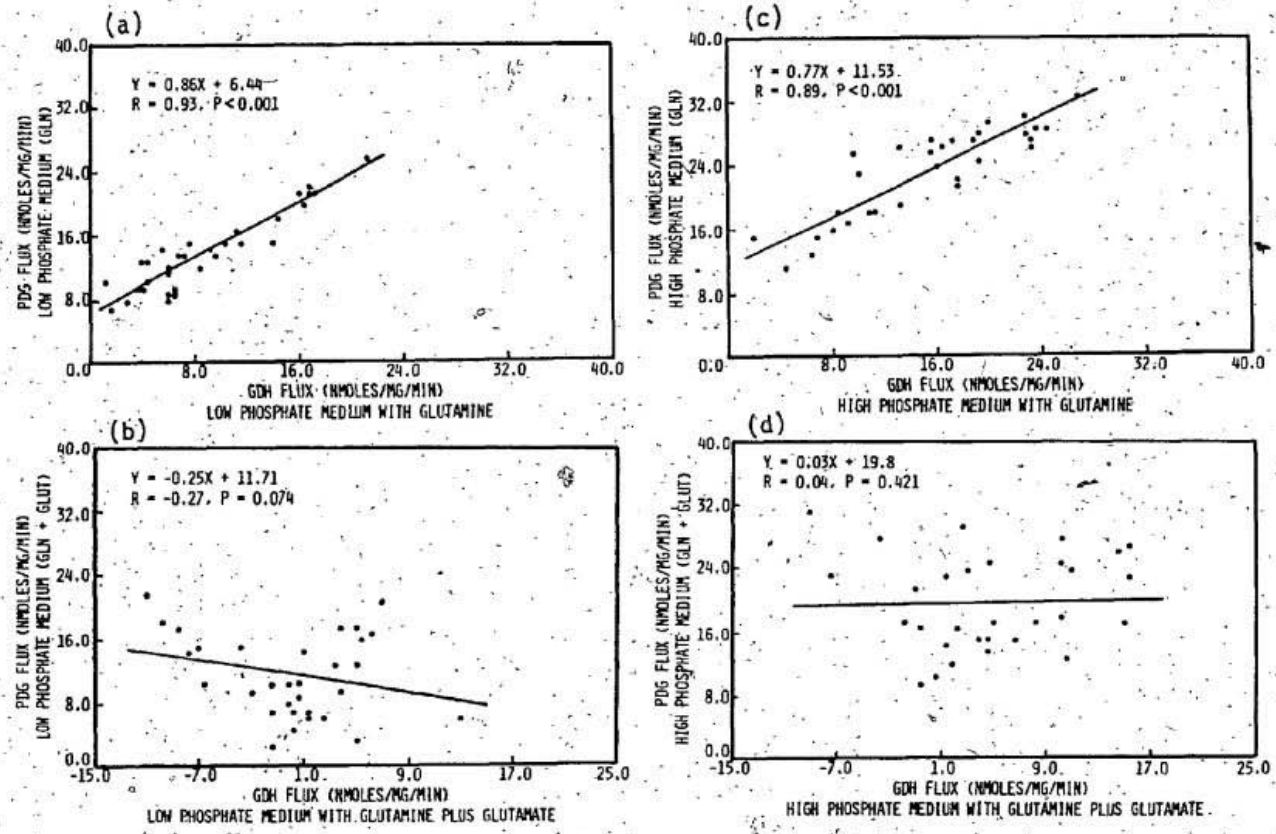


Figure 3.11 Relationship between PDG flux and GDH flux in isolated mitochondria.

Data for these correlations are derived from incubations of mitochondria from normal, acidotic and one and three-day recovered rats. Plots (a) and (b) represent the relationship between PDG flux and GDH flux at 4 mM phosphate in the absence and presence of 3 mM glutamate, respectively and plots (c) and (d) represent this relationship at 20 mM phosphate in the absence and presence of 3 mM glutamate, respectively.

E. Glutamate Oxaloacetate Transaminase Flux

Figure 3.7 also illustrates the effect of acidosis and recovery on the flux through glutamate oxaloacetate transaminase in mitochondria incubated in the four different media. In the media without glutamate GOT flux is low and apparently unaffected by the phosphate concentration. In the absence of glutamate, aspartate formation did not change during acidosis or recovery (low-phosphate medium) or was slightly depressed in acidosis with a return to normal during recovery (high-phosphate medium). The normal rate of GOT flux in the high-phosphate medium containing glutamine as sole substrate agrees well with that reported by Schoolwerth et al (161) and the slight decrease observed during acidosis in the present study reflects the same trend evident in their results.

In comparison to that obtained in the media without added glutamate, GOT flux was substantially greater in the media containing both substrates. In the presence of both glutamine and glutamate, aspartate formation was slightly higher in the high-phosphate medium with significant differences for mitochondria from normal and three-day recovered rats. This may be related to higher matrix glutamate concentrations at the higher phosphate concentration due to activation of PDG by phosphate. No significant change is observed in GOT flux during acidosis in the presence of glutamate at either phosphate concentration. During recovery GOT flux increased on the third day to above normal or acidotic rates in the low-phosphate medium and to above acidotic rates in the high-phosphate medium. The fluxes obtained in the high-phosphate medium containing both substrates differ from those reported by Schoolwerth et al (161) in

that the GOT flux in the present study is slightly higher for mitochondria from normal rats and it does not change during acidosis.

F. Comments on the Results of the Mitochondrial Studies

(1) Mitochondrial Incubations with Glutamine as Sole Substrate - In mitochondria isolated from the kidneys of normal rats and incubated in the media containing glutamine as sole substrate, the flux through GDH contributes substantially to the formation of ammonia. About one-half of the glutamate derived from glutamine was metabolized via GDH. The remainder either accumulated as glutamate or was eventually converted to aspartate via GOT. During metabolic acidosis increases of comparable magnitude occurred in the fluxes through PDG and GDH. Although the actual increases were of similar magnitude, the change which occurred in GDH flux represented a slightly greater proportional activation compared with the change observed in the flux through PDG. Since the increases in glutamate formation via PDG in these media were accompanied by comparable increases in glutamate removal via GDH then the net formation of glutamate did not change. As a result, aspartate formation was not significantly altered during acidosis either.

The responses to recovery of PDG flux and GDH flux also generally coincided with one another. In the low-phosphate medium decreases of comparable magnitude occurred in the fluxes through both PDG and GDH. Consequently, net glutamate and aspartate formation were not altered. In the high-phosphate medium, the general trend for both PDG flux and GDH flux was to remain elevated although a significant fall in GDH flux was evident on the first day of recovery. However, the fall in GDH flux

was not sufficient to alter the net formation of either glutamate or aspartate to any detectable extent.

The observation that the fluxes through PDG and GDH fell during recovery at the physiological concentration of phosphate but tended to remain elevated at the higher phosphate concentration is interesting. This suggests that a change occurred in the mitochondria during recovery which decreased the activity of the rate-limiting step at the lower phosphate concentration in the presence of glutamine alone but that this change was either masked or reversed by the higher phosphate concentration. Masking could have occurred by activating the responsible step such that it was no longer rate-limiting or by activating the transport of inhibitory agents (such as glutamate) out of the mitochondria. However, in light of the known effect of phosphate on PDG activity it is possible to provide a tenative explanation for this observation. According to this speculation the dissociation of PDG dimers increased during recovery such that, although the amount of enzyme did not change, its activity decreased. This change in PDG was preserved in mitochondria incubated at the physiological concentration of phosphate and as a result the fluxes through PDG and GDH decreased. Since phosphate activates PDG by dimerization then the effect of recovery on PDG was presumably reversed at the elevated phosphate concentration and consequently the fluxes through PDG and GDH remained elevated. However, a fall in PDG flux is not observed at the physiological phosphate concentration in the presence of glutamate and therefore raises some doubt as to the physiological relevance of the speculated effect of recovery on PDG.

(2) Mitochondrial Incubations with Glutamine and Glutamate as Substrates - In the media containing both substrates, the flux through GDH in kidney mitochondria from normal rats was negligible in comparison with the flux through PDG. This means, of course, that nearly all of the glutamine metabolized accumulated as glutamate or was eventually converted to aspartate via GOT. Consequently, the flux through GOT in mitochondria from normal rats is approximately equal to the rate of glutamate utilization plus the flux through PDG.

During acidosis increases of comparable magnitude occurred in the fluxes through both PDG and GDH. However, since in these media there was virtually no GDH flux in mitochondria from normal rats, then the percent change which occurred in GDH flux during acidosis is considerable in comparison to the percent change which occurred in PDG flux. This is interpreted as reflecting a marked activation of GDH flux during acidosis which is not accountable for simply by the change in the flux through PDG.

The apparent reversal in GDH flux observed in the low-phosphate medium during recovery is accountable for by the following speculation. Adam and Simpson (1) have shown that the uptake of glutamate (in the presence of inhibitors of glutamate metabolism) is quite depressed in mitochondria isolated from acidotic rats and hence the activity of the glutamate transport mechanism may be lowered in acidosis. Assuming that the glutamate transporter affected is the bidirectional glutamate/hydroxyl transporter, then the efflux of glutamate would also probably be depressed during acidosis. If the activity of this transporter increased during recovery such that by the first day it was back to

normal but by the third day it had overshot normal, then mitochondria from three-day recovered rats would have an increased capacity to transport glutamate. Increased transporter capacity would permit the increased efflux of glutamate, thereby tending to lower matrix glutamate concentration. In the low-phosphate medium where the supply of glutamate via PDG is low then the increased efflux of glutamate would tend to pull the glutamate dehydrogenase reaction in favour of glutamate formation. However, at the higher phosphate concentration this is not observed since glutamate formation via PDG is greater and presumably sufficient to prevent the matrix concentration of glutamate from falling.

3.6 Discussion

It was important, a priori, to establish the function of the kidney in terms of glutamine metabolism and ammoniagenesis before proceeding with the metabolic studies presented in this chapter. It can now be stated with assurance that normal rates of glutamine metabolism, in vivo, are re-established within one-day of recovery from metabolic acidosis. This conclusion is based upon measurements of glutamine extraction and total ammonia production by the kidney. Both parameters were back to normal within 24h of recovery.

Although renal ammonia production was back to normal by 24h of recovery, previous studies indicate that the amount of ammonia excreted in the urine during the first 24h of recovery was significantly greater than that excreted by normal rats in the same amount of time (125). This indicates that above normal rates of ammonia excretion persisted for a while after the acid challenge was removed. It is suggested that

the continued excretion of acid as ammonium salt for the first few hours of recovery resulted in the inappropriate regeneration of bicarbonate. This continued regeneration of bicarbonate associated with the continued excretion of acid as ammonium salt is considered inappropriate since normal bicarbonate levels had already been established by the time recovery was initiated and bicarbonate was no longer being utilized to buffer excess acid. To explain why the bicarbonate levels in the blood rose as a result, it was hypothesized that the renal threshold for bicarbonate reabsorption was increased due to potassium depletion during the previous acid challenge.

The abrupt fall in renal glutamine metabolism observed in Figure 3.2 differs from the response in renal glutamine metabolism to recovery in experiments with humans. In the studies with humans (134), high levels of ammonia were excreted in the urine for several days after the NH_4Cl regimen was discontinued. This contrasting result seen with rats and humans may be due to species differences. However, another explanation seems more appropriate. It appears that in the experiments with humans, the urine ammonia excretion was continually increasing over the duration of NH_4Cl ingestion (4 or 5 days). It is, therefore, suspected that the fully adapted rates of ammonia formation had not yet been achieved or at best had just been reached. Since during the adaptation period there is an excess of acid ingestion over acid excretion, then there would be an excess of acid in the body fluids and tissues. Consequently, when the NH_4Cl regimen was terminated in the human studies, continued excretion of acid as ammonium salt was required to restore acid-base balance. In contrast, in the studies with rats, fully adapted rates of ammonia

excretion were established for about five days before recovery was initiated (125). This period provided the opportunity for normal acid-base status of the animal to be restored while still consuming NH_4Cl and therefore continued excretion of acid as ammonium salt and the regeneration of buffer was not required during recovery. The results of an experiment by Wood (203) support this later explanation. In his experiment NH_4Cl was administered to a human for 44 days. During recovery ammonia excretion fell much more abruptly than it did during recovery in experiments in which NH_4Cl was administered to humans for only 4 or 5 days (134). The assumption here is that the longer period of acidosis permitted restoration of the buffer lost during the period of adaptation.

A. Metabolite Changes in the Kidney in Response to Recovery from Metabolic Acidosis

From the results of the freeze-clamp studies presented in this chapter, it is noted that during recovery increases occurred in the renal contents of glutamate, α-ketoglutarate, malate, citrate, lactate and calculated oxaloacetate and decreases occurred in the renal contents of phosphoenolpyruvate, and 3-phosphoglycerate and as well the cytoplasmic ratio of NAD to NADH decreased. Certainly the changes in phosphoenolpyruvate, 3-phosphoglycerate, lactate and the calculated NAD/NADH ratio may be assumed to have occurred in the cytoplasm. The increase in citrate also probably occurred in the cytoplasm if it is caused, as hypothesized, by a direct effect of bicarbonate on the mitochondrial citrate transporter.

The metabolites glutamate, α-ketoglutarate, malate and oxaloacetate, of course, exist in both the cytoplasm and the mitochondria. Obviously, the contents of these metabolites in these compartments can not be

distinguished by measuring their contents in the extracts obtained by freeze-clamping the entire kidney. However, based upon the assumption that a steady-state situation existed in the renal cells metabolizing glutamine at the time of freeze-clamping then certain reasonable inferences can be made. The presence of highly active and freely reversible mitochondrial antiporters for exchanging malate with phosphate and malate with α-ketoglutarate provide a basis for assuming that the concentrations of α-ketoglutarate and malate in the mitochondria and cytoplasm are in equilibrium though not necessarily at the same concentration. Hence, changes in the levels of these metabolites can be rapidly transmitted from one compartment to the other. The same situation may exist for glutamate since kidney mitochondria also contain a bidirectional glutamate-hydroxyl antiporter. However, the activity of this antiporter is low in the kidney and therefore equilibrium between cytoplasmic and mitochondrial glutamate may not be achieved as rapidly.

B. Metabolic Responses in the Cytoplasm to Recovery from Metabolic Acidosis

Phosphoenolpyruvate carboxykinase is primarily, if not exclusively a cytoplasmic enzyme in the rat kidney (46). The increases in the renal contents of malate and calculated oxaloacetate and the decrease in phosphoenolpyruvate during recovery probably reflect the re-attainment of normal PEPCK flux. This is consistent with the normal rates of PEPCK activity evident in vitro in homogenates from the kidneys of one-day recovered rats (125). Presumably the fall in 3-phosphoglycerate followed the fall in phosphoenolpyruvate since enolase catalyses a freely reversible close-to-equilibrium reaction. The significance of the increase

in lactate and the decrease in cytoplasmic NAD/NADH is not fully appreciated. An increase in cytoplasmic α-ketoglutarate could serve a regulatory role by acting from the cytoplasmic side to inhibit the glutamine transporter. Whether or not the increase in citrate plays a regulatory role has not been investigated.

The decrease which occurred in the content of renal PEPCK during recovery was shown to be most probably due to the loss of enzyme protein. Although there is some controversy regarding the half-life of renal PEPCK (3.4h according to Longshaw et al (102) and 13h according to Iynedjian et al (73)) the reported half-lives are short and thus it is probable that the fall in enzyme amount is due to the normal turnover of PEPCK in the face of a decreased rate of synthesis.

C: Metabolic Responses in the Mitochondria to Recovery from Metabolic Acidosis

From the mitochondrial studies reported in this chapter it is apparent that a mitochondrial event occurred during recovery which decreased the flux through glutamate dehydrogenase. This event was evident only in the presence of both glutamine and glutamate and the metabolic basis responsible is unknown. It is reasonable to assume that an increase in mitochondrial glutamate may have occurred as a result of the decrease in GDH flux. Changes in the concentration of glutamate in mitochondria have been considered of especial importance in the control of renal glutamine metabolism because of its inhibitory effect on phosphate-dependent glutaminase.

D. Cytoplasmic Versus Mitochondrial Control of Renal Glutamine Metabolism during Recovery from Metabolic Acidosis

The results of the studies presented in this chapter indicate that events occur in both the cytoplasm and mitochondria which could be responsible for the return of renal glutamine metabolism to normal during recovery. The fall in PEPCK activity is a cytoplasmic event which could be of regulatory significance and a mitochondrial event of regulatory potential is that which causes the fall in the flux through glutamate dehydrogenase. Since both parameters were back to normal at the time of freeze-clamping then the metabolite picture obtained does not provide any indication as to which event is primary. Presumably, a decrease in the flux through the rate-limiting step would be the primary event.

It is, of course, true that only one rate-limiting step can exist. However, it is nevertheless possible for the locus of the rate-limiting step to change as conditions change. The following models are proposed as working hypotheses for the control of renal glutamine metabolism during recovery from metabolic acidosis. They are meant to serve only as tenative models and are described with reference to Figure 3.12.

(1) PEPCK Model - This model is based upon the premise that the regulatory event during recovery is a fall in the flux through PEPCK. As a result of this fall the cytoplasmic concentration of oxaloacetate is increased. It is presumed that this would affect α-ketoglutarate levels in two ways. Firstly, it could increase α-ketoglutarate via re-establishment of equilibrium by the cytoplasmic glutamate oxaloacetate transaminase. Secondly, and more important, the increased cytoplasmic concentration of oxaloacetate would cause an increase in the mitochond-

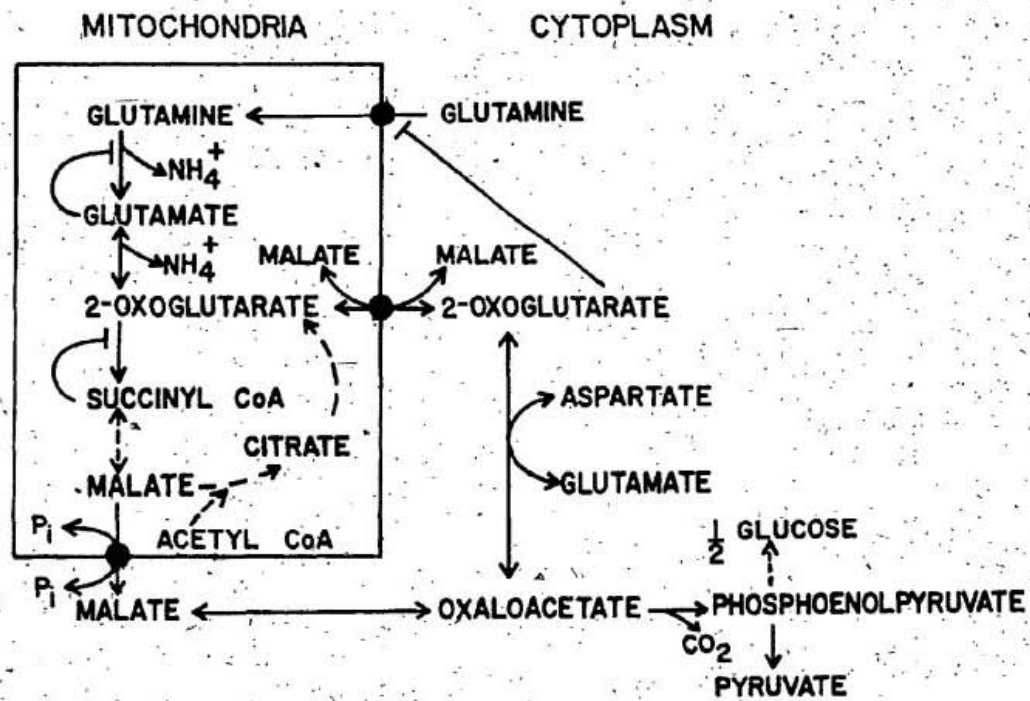


Figure 3.12 Hypothetical scheme for the control of renal glutamine metabolism.

The double-headed arrows represent reversible reactions and the single-headed arrows, irreversible reactions. The solid lines represent single reactions and the dashed lines, a sequence of reactions. An inhibition is represented by ——|.

rial succinyl CoA concentration since all the intervening reactions including the malate/phosphate antiporter are freely reversible. The rise in mitochondrial succinyl CoA concentration could inhibit α-ketoglutarate dehydrogenase and this, in turn, would cause an increase in the mitochondrial concentration of α-ketoglutarate. Firstly, it could result in an increased intramitochondrial glutamate concentration, since glutamate dehydrogenase is freely reversible, and an inhibition of glutaminase would ensue. Secondly, the increased mitochondrial concentration of α-ketoglutarate would result in an increased cytoplasmic concentration of α-ketoglutarate via the operation of the reversible malate/α-ketoglutarate antiporter and this would result in an inhibition of the mitochondrial glutamine transporter (50, 57). Thus a decrease in the activity of phosphoenolpyruvate carboxykinase in the cytoplasm can inhibit the glutamine transporter and the mitochondrial glutaminase.

(2) GDH Model - According to this model the regulatory event in renal glutamine metabolism during recovery is that which causes the fall in glutamate dehydrogenase flux. A decrease in GDH flux would increase the mitochondrial concentration of glutamate resulting in the inhibition of glutaminase. This model depends upon the view by Schoolwerth et al (161) that glutamate dehydrogenase does not catalyze an equilibrium reaction. The subsequent fall in PEPCK flux would result in the re-establishment of normal malate and oxalacetate levels as well as increased α-ketoglutarate which would presumably act to inhibit the mitochondrial glutamine transporter.

CHAPTER 4

LONG RECOVERY

CHAPTER 4

LONG RECOVERY

Mitochondrial adaptation is an important part of the adaptive response of the kidney to metabolic acidosis. Most important are the adaptations in glutamine transport, phosphate-dependent glutaminase and glutamate dehydrogenase.

Kinetic data for the mitochondrial glutamine transporter has been published by Goldstein and Boylan (57). These workers developed a rapid-mixing and rapid-filtering system to obtain data on the uptake of radioactive glutamine. By measuring the initial rates of accumulation of glutamine label, they attempted to avoid the potential problem of confusing glutamine carrier activity with glutaminase activity and the rate of glutamate efflux. Their results indicate that at pH 7.4 and 23°C, the Km of the transporter for glutamine is 2.7 mM and the V_{max} between 23 and 32 nmoles/mg protein per min. Compared to the higher rates of glutaminase activity in mitochondrial preparations (161), the glutamine transporter could be rate-limiting for the metabolism of glutamine. The uptake of radioactive glutamine by rat kidney mitochondria was demonstrated to increase in acute and chronic metabolic acidosis (1, 57). This has been interpreted to indicate that the capacity of the mitochondria to transport glutamine across the inner mitochondrial membrane is increased by metabolic acidosis.

A 20-fold increase in the activity of PDG has been shown to occur in the proximal convoluted tubules during metabolic acidosis (37). Immunochemical studies indicate that this increased activity is due to

an increase in the amount of this enzyme (36). Since actinomycin D prevented the increase in PDG activity (55), it is likely that increased synthesis is the mechanism of PDG adaptation in metabolic acidosis. It has also been shown that metabolic acidosis increases the activity of GDH in the proximal convoluted tubules and that this increase in enzyme activity is due to an increase in the amount of enzyme (164, 165).

In the previous chapter it was shown that renal glutamine metabolism, *in vivo* returned to normal by one day of recovery. It is also apparent from the results of Chapter 3 that the mitochondrial capacity to metabolize glutamine remained elevated for at least 3 days of recovery while the expression of this capacity depended upon the medium in which the mitochondria were incubated. The results of the previous chapter also show that in the "physiological" incubation medium the flux through GDH reversed during recovery such that glutamate was formed via glutamate dehydrogenase in mitochondria isolated from three-day recovered rats. So as to better assess these trends, it was of interest to follow mitochondrial metabolism in the different media for a longer period of recovery. It was also of interest to relate the activities of PDG and GDH with the metabolic responses to recovery, both *in vivo* and in isolated mitochondria.

4.1 Blood Acid-Base Parameters

Blood acid-base parameters were routinely measured in all the experimental situations studied. The $[H^+]$, $[HCO_3^-]$ and pCO_2 in the blood of rats which were permitted up to 15 days to recover from a previous acid challenge (i.e. 7 days of 1.5% NH_4Cl *ad libitum*) are shown in Table

4.1. The results indicate that there are increases in $[H^+]$ and pCO_2 on day 8 of recovery. However, the period from 5 to 12 days of recovery is generally a stable period in terms of acid-base homeostasis. This is in contrast to the first few days of recovery in which a distinct metabolic alkalosis is apparent (Figure 3.1). Presumably, by the fifth day of recovery the kidney had re-adjusted its function to meet the demands of normal acid ingestion.

4.2 Mitochondrial Enzymes

Assay of enzyme activities, in vitro under optimal conditions is, in effect, a measure of maximum activities. This does not necessarily bear any relation to the activities expressed, in vivo. However, measuring maximum activities provides a means of detecting changes in the amount of enzyme which is present and presumably capable of catalyzing a particular reaction.

Because of the importance of the reactions catalyzed by PDG and GDH to the metabolism of glutamine, these enzymes were measured during recovery from metabolic acidosis. These activities were measured in the same mitochondrial preparations that were used for the incubation studies reported below. The results of these determinations appear in Table 4.2 and Figure 4.1.

A. Phosphate-Dependent Glutaminase Activity

No significant change in PDG activity occurred during the first three days of recovery. This confirms previous findings with entire kidney homogenates (125). After the third day of recovery, a fall in

Table 4.1 Blood H^+ and HCO_3^- concentration and partial pressure of CO_2 during long recovery.

Experimental Condition:	Normal	Day 5	Day 6	Day 7	Recovery Day 8	Day 9	Day 10	Day 11	Day 12
H^+ (nmoles/L)±SD	37.2±1.9 (5)	38.2±1.4 (4)	38.6±0.8 (4)	36.6±1.1 (4)	43.6±2.5* (4)	41.2±3.0 (4)	38.7±0.7 (4)	35.8±1.5 (4)	38.0±1.3 (3)
HCO_3^- (mmoles/L)±SD	29.0±0.7 (5)	28.7±3.4 (4)	29.5±0.7 (4)	29.1±1.3 (4)	29.7±1.6 (4)	28.9±1.6 (4)	28.0±0.4 (4)	29.1±0.9 (4)	29.0±0.6 (3)
pCO_2 (mmHg)±SD	45.3±2.8 (5)	46.2±6.8 (4)	47.8±1.9 (4)	44.7±3.2 (4)	54.3±3.8* (4)	50.1±4.9 (4)	45.8±1.3 (4)	43.5±3.0 (4)	46.3±1.3 (3)

Blood was sampled from normal rats and rats which were allowed five to twelve days to recover from metabolic acidosis.

* indicates significantly different from normal value. Number in parentheses indicates number of animals used.

Table 4.2 Activities of phosphate-dependent glutaminase and glutamate dehydrogenase during recovery from metabolic acidosis.

Experimental Condition	Phosphate-Dependent Glutaminase Activity	Glutamate Dehydrogenase Activity
	nmoles/mg mito. protein/min ±SD (No. of Animals)	
Normal	393.9±76.5 (12)	857.5±161.6 (9)
Acidotic	1175.0±259.2 (13)*	2142.8±348.7 (9)*
Recovery Day 1	1210.8±258.4 (11)*	1789.7±422.0 (9)*
Recovery Day 3	1235.4±420.2 (6)*	1357.1±169.9 (10)*†
Recovery Day 5	747.2±261.8 (7)*†	1594.0±295.6 (4)*†
Recovery Day 7	939.5±199.2 (8)*†	1805.4±356.1 (12)*
Recovery Day 9	781.8±64.5 (4)*†	1672.7±148.4 (4)*†
Recovery Day 11	675.9±46.8 (3)*†	1147.7±93.3 (3)*†
Recovery Day 15	554.5±54.2 (3)*†	1075.9±163.4 (3)†

Acidotic rats were administered NH_4Cl for seven days. Other rats were allowed to recover from metabolic acidosis by drinking water for up to 15 days. * indicates significantly different from normal value and † indicates significantly different from acidotic value ($P < 0.05$).

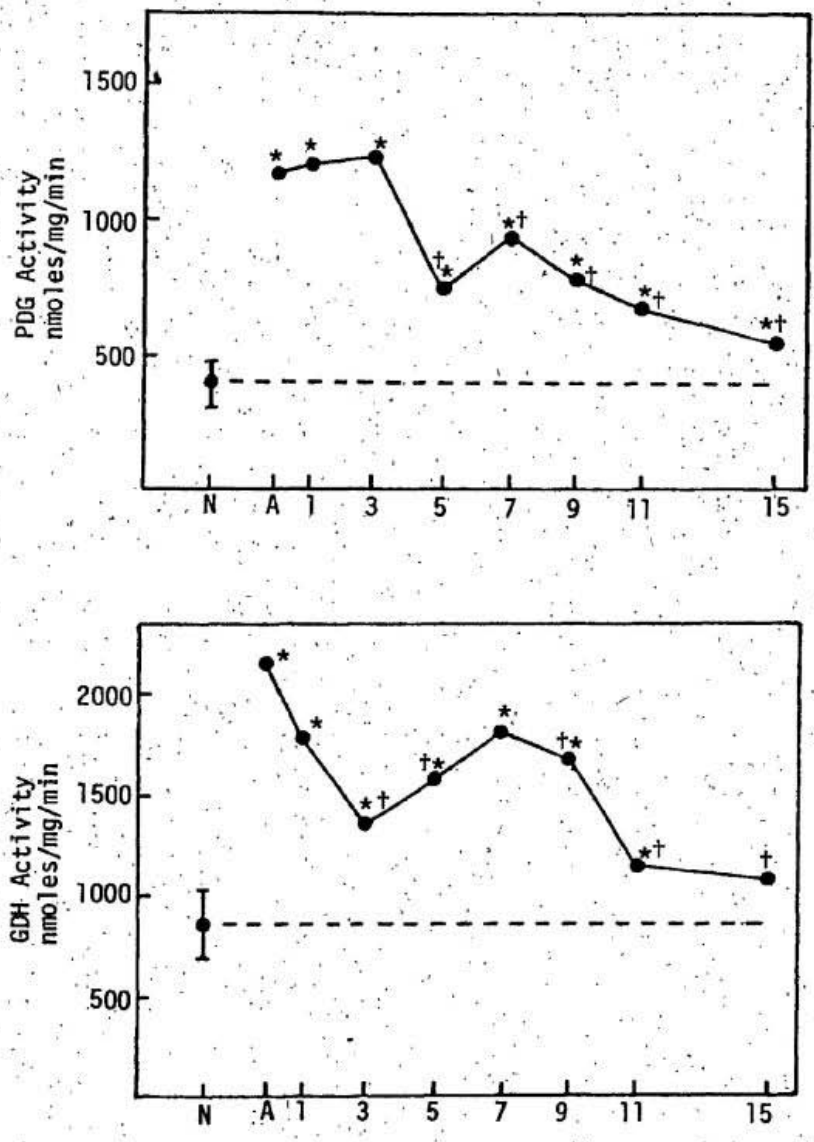


Figure 4.1 Response of the activities of phosphate-dependent glutaminase and glutamate dehydrogenase to recovery from metabolic acidosis.

The data presented in this figure are taken from Table 4.2. Metabolic acidosis (A) was induced by administering NH_4Cl for seven days. The dashed line represents normal activity. * indicates significantly different from normal value and † indicates a significant difference from acidotic value ($P < 0.05$).

PDG activity occurred but no further significant decrease is observed. However, the general trend after the third recovery day is a gradual fall in the activity of this enzyme. Despite this downward trend, the activity of PDG still remained significantly elevated above normal after 15 days of recovery. This delayed fall and then a gradual decrease in enzyme activity does not coincide with the relatively abrupt return of glutamine extraction and ammonia production by the kidney, *in vivo* during recovery (Table 3.1). Hence, the activity of PDG (as determined by its maximal activity, *in vitro*) can not be rate-limiting for renal glutamine metabolism, *in vivo*, during this period.

B. Glutamate Dehydrogenase Activity

The response of GDH activity to recovery differs from that for PDG activity. A trend for decreased GDH activity appears after 24h of recovery with a significant fall evident on the third recovery day. After this, the activity of GDH appears to rebound such that by the seventh recovery day it is not significantly different from acidotic values. After the seventh day, GDH activity fell and by the fifteenth day of recovery, normal values were attained. It is obvious that the response of GDH activity to recovery does not resemble the return of renal glutamine metabolism, *in vivo*, to normal and hence the activity of this enzyme can not be rate determining for this metabolic pathway during this period.

4.3 Mitochondrial Incubations

The studies presented in this section are essentially an extension of the mitochondrial studies presented in Chapter 3 and include data

from that chapter for comparison. The intent of these studies was to follow the mitochondrial response to recovery past the third day and to relate these to the activities of PDG and GDH. The results are contained in Tables 4.3 and 4.4. Figure 4.2 illustrates the effect of recovery on ammonia formation, PDG flux and GDH flux.

A. Ammonia Formation

As can be observed in Figure 4.2 ammonia formation by isolated mitchondria returned to normal in all but one of the media used. In general, the return of ammonia formation to normal was complete by 5 days of recovery with some fluctuations occurring after that. In the high-phosphate medium with glutamine as sole substrate, ammonia formation remained elevated for the entire 15 days although a fall from acidotic values is apparent. It, therefore, appears that mitochondria from recovered rats retain the necessary metabolic adaptations for at least 15 days after the initiation of recovery to metabolize glutamine at above-normal and near-acidotic rates. Hence, the capacity to transport glutamine and the capacity to release ammonia from it must still be elevated. The decreased formation which occurred in the other media are presumably due to alternations in the mitochondria during recovery which affect mitochondrial metabolism without affecting metabolic potential.

B. Phosphate-Dependent Glutaminase Flux

PDG flux also fell to normal in all media except in the high-phosphate medium containing glutamine as sole substrate. The return of PDG flux to normal was essentially complete by day seven of recovery and

Table 4.3 Medium changes in different media during the incubation of mitochondria from rats during long recovery.

	LOW PHOSPHATE MEDIUM 4 mM KH_2PO_4						HIGH PHOSPHATE MEDIUM 20 mM KH_2PO_4					
	1 mM GLUTAMINE			1 mM GLUTAMINE + 3 mM GLUTAMATE			1 mM GLUTAMINE			1 mM GLUTAMINE + 3 MM GLUTAMATE		
MEDIUM CHANGES:	AMMONIA	GLUTAMATE	ASPARTATE	AMMONIA	GLUTAMATE	ASPARTATE	AMMONIA	GLUTAMATE	ASPARTATE	AMMONIA	GLUTAMATE	ASPARTATE
EXPERIMENTAL CONDITION	Δ nmoles / mg mito protein / min ± SD (No. of Animals)											
NORMAL	14.4±3.7 (12)	1.6±1.2 (12)	4.2±2.0 (12)	6.6±2.6 (12)	-15.7±4.0 (8)	20.9±5.5 (12)	24.8±6.8 (10)	3.8±2.3 (10)	5.1±1.9 (10)	15.4±4.4 (10)	-19.8±4.3 (8)	30.5±4.5 (7)
ACIDOTIC	38.2±5.3 (9)	1.6±0.7 (9)	2.9±0.9 (8)	21.9±3.9 (9)	-12.4±5.4 (7)	20.9±2.8 (9)	51.1±6.2 (9)	2.2±1.1 (9)	3.2±1.1 (9)	38.0±6.1 (9)	-16.4±4.6 (6)	25.4±6.6 (9)
RECOVERY DAY 5	14.1±1.4 (3)	1.5±0.2 (3)	2.6±0.4 (3)	10.5±4.2 (7)	-3.8±3.9 (7)	24.3±5.7 (7)	35.9±9.9 (7)	3.7±0.4 (7)	5.5±1.4 (7)	20.9±5.7 (7)	-15.8±5.4 (7)	29.1±6.9 (7)
RECOVERY DAY 7	15.5±4.4 (4)	1.8±0.4 (4)	2.7±0.3 (4)	6.9±1.4 (4)	-9.2±4.6 (4)	20.0±2.2 (4)	44.6±5.3 (8)	3.7±0.6 (8)	5.6±1.0 (8)	20.6±9.3 (4)	-19.8±4.2 (4)	34.1±2.3 (4)
RECOVERY DAY 9	18.2±2.9 (4)	2.3±0.5 (4)	4.0±0.4 (4)	8.3±6.7 (4)	-18.0±5.7 (4)	23.6±2.7 (4)	40.5±4.8 (4)	4.1±0.5 (4)	6.8±0.7 (4)	22.2±3.7 (4)	-25.7±5.1 (4)	35.7±4.5 (4)
RECOVERY DAY 11	16.1±4.8 (3)	1.9±0.9 (3)	4.5±0.4 (3)	4.2±3.0 (3)	-17.0±2.6 (3)	27.6±1.0 (3)	35.0±5.7 (3)	4.1±0.8 (3)	6.7±0.4 (3)	12.9±1.7 (3)	-23.5±3.3 (3)	35.7±2.1 (3)
RECOVERY DAY 15	14.2±5.1 (3)	2.6±0.7 (3)	4.4±1.2 (3)	8.9±1.5 (3)	-14.4±3.3 (3)	21.7±0.6 (3)	40.0±11.2 (3)	4.7±0.1 (3)	6.3±0.6 (3)	21.7±5.0 (3)	-23.3±4.5 (3)	32.8±2.7 (3)

Kidney cortex mitochondria were incubated for 4 minutes with 1 mM glutamine ±3 mM glutamate and with either 4 mM phosphate or 20 mM phosphate. Normal and acidotic values are the same as those reported in Table 3.5.

Table 4.4 Enzyme fluxes in mitochondria incubated in different media and isolated from rats during long recovery.

	LOW PHOSPHATE MEDIUM 4 mM KH_2PO_4						HIGH PHOSPHATE MEDIUM 20 mM KH_2PO_4					
	1 mM GLUTAMINE			1 mM GLUTAMINE + 3 mM GLUTAMATE			1 mM GLUTAMINE			1 mM GLUTAMINE + 3 mM GLUTAMATE		
FLUX:	PDG	GDH	GOT	PDG	GDH	GOT	PDG	GDH	GOT	PDG	GDH	GOT
EXPERIMENTAL CONDITION	nmoles / mg mito protein / min ± SD (No. of Animals)											
NORMAL	10.1±2.4 (12)	4.3±1.9 (12)	4.2±2.0 (12)	7.9±3.8 (10)	0.6±5.3 (12)	20.9±5.5 (12)	16.8±4.0 (10)	8.0±3.2 (10)	5.1±1.9 (10)	14.4±4.1 (9)	1.6±4.9 (9)	30.5±4.5 (7)
ACIDOTIC	20.8±3.1 (8)	16.6±2.2 (8)	2.9±0.9 (8)	15.1±4.5 (8)	6.8±3.5 (9)	20.9±2.8 (9)	28.0±3.0 (8)	22.9±3.4 (9)	3.2±1.1 (9)	23.8±3.8 (8)	12.2±3.8 (8)	25.4±6.6 (9)
RECOVERY DAY 5	9.1±0.9 (3)	5.0±0.5 (3)	2.6±0.4 (3)	15.5±4.3 (7)	-5.0±5.2 (7)	24.3±5.7 (7)	21.3±7.0 (7)	13.4±4.5 (7)	5.5±1.4 (7)	15.7±5.3 (7)	3.8±2.5 (7)	29.1±6.9 (7)
RECOVERY DAY 7	10.1±2.2 (4)	5.6±2.3 (4)	2.7±0.3 (4)	8.8±2.5 (4)	-2.0±1.8 (4)	20.0±2.2 (4)	22.4±7.0 (8)	17.7±2.5 (8)	5.6±1.0 (8)	17.4±7.7 (4)	3.2±2.3 (4)	34.1±2.3 (4)
RECOVERY DAY 9	10.6±4.4 (4)	7.6±2.4 (4)	4.0±0.4 (4)	6.9±4.7 (4)	1.4±3.5 (4)	23.6±2.7 (4)	25.7±2.5 (4)	14.8±2.4 (4)	6.8±0.7 (4)	16.1±2.1 (4)	6.1±1.9 (4)	35.7±4.5 (4)
RECOVERY DAY 11	11.3±2.3 (3)	4.9±2.5 (3)	4.5±0.4 (3)	7.4±1.0 (3)	-3.2±3.1 (3)	27.6±1.0 (3)	22.9±3.4 (3)	12.1±2.3 (3)	6.7±0.4 (3)	12.3±0.6 (3)	0.6±1.2 (3)	35.7±2.1 (3)
RECOVERY DAY 15	10.5±3.2 (3)	3.6±1.9 (3)	4.4±1.2 (3)	8.1±1.5 (3)	0.7±2.5 (3)	21.8±0.6 (3)	25.5±5.6 (3)	14.5±5.7 (3)	6.3±0.6 (3)	15.6±2.8 (3)	6.1±2.6 (3)	32.8±2.7 (3)

Kidney cortex mitochondria were incubated for 4 minutes with 1 mM glutamine ±3 mM glutamate and either 4 mM phosphate or 20 mM phosphate. Normal and acidotic values are the same as those reported in Table 3.6.

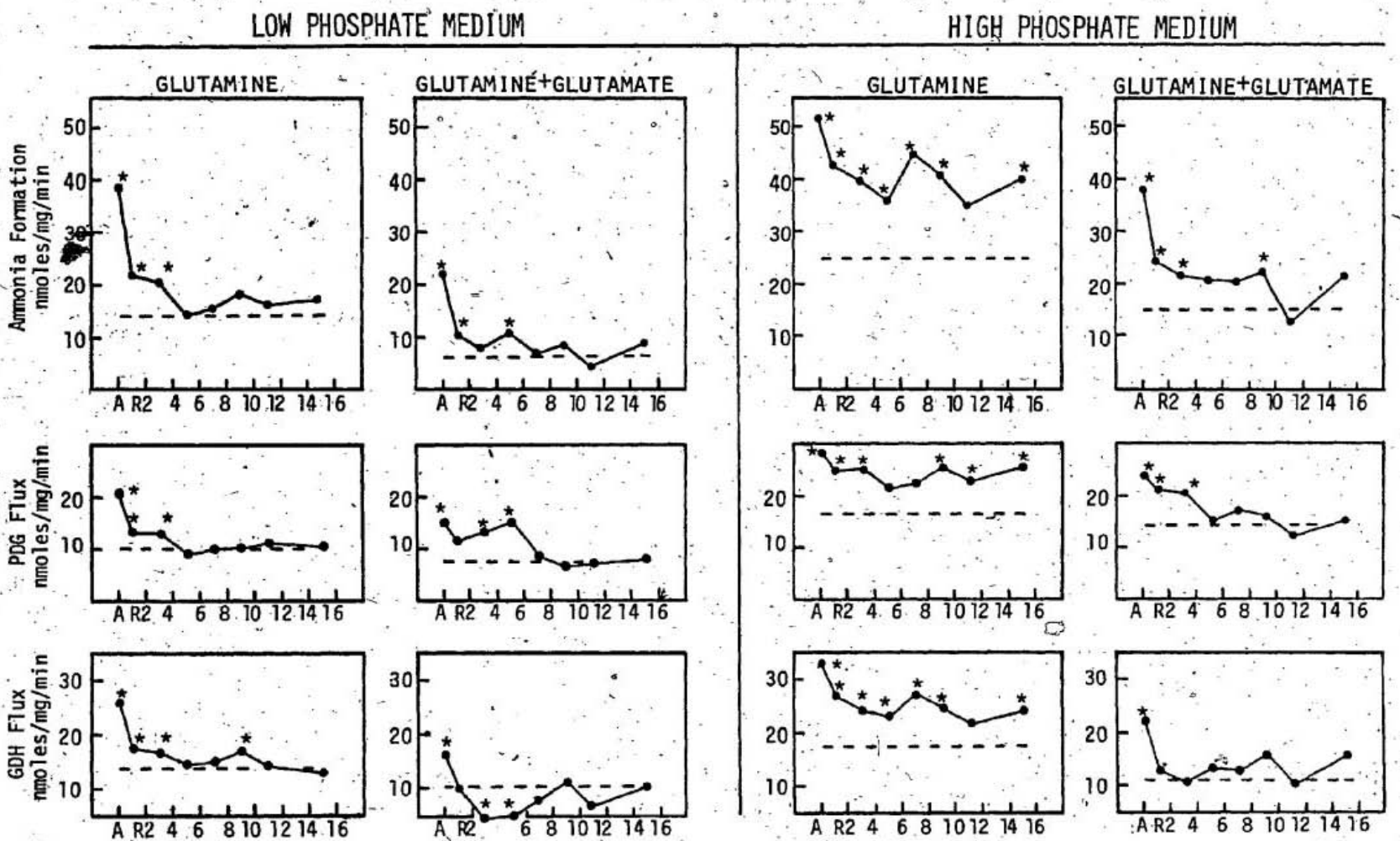


Figure 4.2 Effect of long recovery on the mitochondrial metabolism of glutamine ± glutamate at different phosphate concentrations.

Data presented in this figure are contained in Tables 3.5, 3.6, 4.3 and 4.4. Kidney cortex mitochondria from rats during long recovery were incubated for 4 minutes in media containing 1 mM glutamine + 3 mM glutamate and either 4 mM or 20 mM phosphate. Dashed lines represent normal values. * indicates significantly different from normal value.

did not coincide well with the return of ammonia formation to normal except in the low-phosphate medium containing glutamine as sole substrate. In the high-phosphate medium with glutamine alone, it is evident that PDG flux remained well above normal for the entire recovery period examined although the values for days 5 and 7 are not significantly different from normal.

Comparison of these responses in PDG flux to recovery with that of PDG activity clearly establishes that the decreases in flux which are observed do not depend upon decreases in the activity of this enzyme. The relationship between enzyme flux and activity in the high-phosphate medium with glutamine alone is less certain. A definite fall in PDG activity is evident during recovery and by the 15th day, PDG activity was only marginally above that in normal rats. However, the flux through PDG in the high-phosphate medium containing glutamine alone was not even significantly different from the acidotic rate on the 15th day of recovery and the trend is to remain elevated. This comparison would therefore appear to indicate that the elevated flux through PDG may not depend upon the activity of PDG present. If this is correct then the elevated flux through PDG maybe related to another phenomenon such as glutamine transport.

C. Glutamate Dehydrogenase Flux

The response of GDH flux to recovery is also variable depending upon the medium in which the mitochondria were incubated. The abrupt decreases in GDH flux observed on the first day of recovery in the media containing both glutamine and glutamate are obviously not caused by

changes in the mitochondrial activity of glutamate dehydrogenase. Certainly the reversal of GDH flux observed at the lower phosphate concentration is not related to changes in enzyme activity.

The fall in GDH flux in the low-phosphate medium containing glutamine as sole substrate appears to better resemble the changes in PDG flux than the changes in the activity of glutamate dehydrogenase. In the corresponding high-phosphate medium, the relationship between flux and enzyme activity is not clear. There is a remarkable resemblance between the trend in GDH flux in this medium and the response of GDH activity to recovery in that the peak of the apparent rebound occurred on the seventh day for both flux and enzyme activity. However, this may be coincidental, especially since GDH flux was still elevated on the 15th day of recovery whereas GDH activity is not significantly different from normal. It is probable that the flux through GDH in mitochondria incubated in this medium is more dependent upon the flux through PDG than upon changes in the activity of glutamate dehydrogenase.

4.4 Discussion

As a result of the sustained metabolic demand placed upon renal glutamine metabolism during chronic metabolic acidosis, the mitochondrial capacity to metabolize glutamine increases. However, only an increase in the activity of the rate-limiting step will increase the flux through this pathway. Increases which occur in non rate-limiting steps are a consequence, not a cause of the increased flux and presumably occur to provide a comfortable margin between maximum capacity and flux. Adaptations of this type make it difficult to distinguish between

rate-limiting and non rate-limiting events.

During recovery it was shown that the activities of PDG and GDH remained elevated long after renal glutamine metabolism, *in vivo* returned to normal. Certainly then, at least for recovery, the activities of these mitochondrial enzymes were well in excess of the amounts required to metabolize glutamine. Therefore, the actual amounts of these enzymes can not be rate-limiting and hence renal glutamine metabolism is not controlled by changes in the contents of these enzymes during recovery. The relatively long duration of elevated PDG and GDH activities presumably reflect either long half-lives of these enzymes or that the mechanism responsible (i.e. increased synthesis and/or decreased degradation) is sustained for some time during recovery. Since glutamine must be transported into the mitochondria to maintain the elevated flux through PDG observed in the high-phosphate medium with glutamine alone, then obviously the capacity of the glutamine transporter did not decrease appreciably during the 15 days of recovery. Hence, the capacity of mitochondria to transport glutamine can not be rate-limiting during recovery.

In order for the glutamine transporter, phosphate-dependent glutaminase or glutamate dehydrogenase to be rate-determining during recovery it must be assumed that alterations occur which affect the activity of these parameters, rather than changes in their contents which would presumably affect the capacity of the mitochondria to metabolize glutamine. That such alterations may occur is reflected by the metabolic responses of isolated mitochondria to recovery. A possible alteration in either the glutamine transporter or phosphate-dependent glutaminase

is indicated by the decrease in PDG flux observed in the low-phosphate medium containing glutamine as sole substrate. That some alteration occurred which affects glutamate dehydrogenase is indicated by the abrupt decreases in GDH flux in the media containing glutamate. However, the metabolic basis and the physiological significance of these alterations remain obscure.

CHAPTER 5

RAPID RECOVERY

CHAPTER 5

RAPID RECOVERY

The increase in renal ammonia production during metabolic acidosis is a well-recognized phenomenon in acid-base homeostasis. Increased formation of ammonia by the kidney permits the increased excretion of fixed acid as neutral ammonium salts. To meet the demands of acid excretion, the kidney must produce more ammonia than it actually excretes. This is because the ammonia secreted into the urine is derived from an "intracellular pool" of ammonia which is in diffusion equilibrium with all the cortical phases of the kidney, including peritubular blood. Hence, not all of the ammonia produced in the renal tubular cells is secreted into the urine. The factors influencing the release of ammonia into these phases include concentration gradients of ammonia between cells, tubular fluid and peritubular blood, hydrogen ion concentrations in these phases and rates of urine flow and renal blood flow.

The major determinant of the release of ammonia into the urine is urinary pH, whereas the release of ammonia into the blood is primarily dependent upon blood flow. Accordingly, the higher the pH of the urine the more competitive the blood is in removing the ammonia produced by the kidney. Since ammonia is very toxic (107) and since ammonia which is not trapped in the urine will be released into the blood, then it is reasonable to assume that the production of ammonia closely coincides with its requirement for acid excretion. Presumably then, during recovery from metabolic acidosis the return of ammonia production to normal is carefully tuned to coincide with the return of acid excretion to

normal. Otherwise, the continued production of ammonia during recovery without its excretion in the urine would result in the increased release of ammonia into the blood. Therefore, although the decrease in ammonia production during recovery from metabolic acidosis is related to acid-base requirements, it is also important in terms of "ammonia homeostasis".

This chapter is concerned with investigating the recovery situation in greater detail. It was established in Chapter 3 that renal ammonia production and glutamine extraction by the kidney, in vivo, were back to normal by 24h of recovery. However, the time course for the return of renal glutamine metabolism to normal during the first day of recovery and how this relates to the return of the potential regulatory parameters is not known. Therefore, studies were performed at recovery times of less than 24h.

In addition, since the return of renal ammonia production to normal is presumed to closely coincide with the return in the requirement for acid excretion to normal, it was thought possible to manipulate the response of ammonia production to recovery by altering the need for acid excretion. Presumably, the administration of $NaHCO_3$ at the outset of recovery would remove the residual acid present at the beginning of recovery and would therefore minimize the need for excreting acid as ammonium salts. The objective of this approach was to determine if the potential regulatory events correlated with the return of renal ammonia production under these circumstances.

5.1 Blood Acid-Base Parameters

It was shown in Chapter 3 that pH, $[HCO_3^-]$ and pCO_2 all overshoot

normal during recovery from metabolic acidosis. The overshoot in $[HCO_3^-]$ is considered the result of an increase in bicarbonate reabsorption which thereby elevated blood pH above normal. The increase in pCO_2 probably occurred as a result of a respiratory compensation to this metabolic alkalosis. The purpose of measuring these parameters in the present study was to determine to what extent the development of metabolic alkalosis during recovery was affected by the intubation of 1.5 mmoles of $NaHCO_3$ per 100g body weight control. As control, rats were also intubated with an equal amount of NaCl. Presumably the ensuing metabolic alkalosis would be achieved sooner in rats intubated with $NaHCO_3$ as compared with rats intubated with NaCl.

The results of this study are presented in Table 5.1 and Figure 5.1. In rats intubated with NaCl, the blood hydrogen ion concentration fell to normal by 8h of recovery. By 16h of recovery the minimum $[H^+]$ was attained. During recovery after intubation with $NaHCO_3$ the fall in $[H^+]$ was complete by 8h. Although at 8h of recovery the $[H^+]$ in the $NaHCO_3$ group is not significantly different from that in the NaCl group, it is apparent that the fall was more precipitous after intubation with $NaHCO_3$. The minimum level to which the concentration of H^+ ions fell was identical in both groups.

A similar difference between the two groups is also observed in the blood bicarbonate concentrations. The rise in blood bicarbonate occurred gradually in rats intubated with NaCl such that by 8h of recovery normal values are evident and by 16h the maximum increase had occurred. In rats intubated with $NaHCO_3$ the blood bicarbonate was maximum by 8h of recovery. At 8h of recovery the $[HCO_3^-]$ is significantly higher in the

Table 5.1 Blood acid-base parameters in normal and acidotic rats and during the first day of NaCl and $NaHCO_3$ - induced recovery from metabolic acidosis.

	[H+] (nmoles/L)	$[HCO_3]$ (mmoles/L)	pCO_2 (mmHg)
Normal	41.3±2.2	24.1±2.1	45.5±1.8
Acidotic	49.2±5.2	20.6±2.4	43.2±2.8
Recovery		NaCl	
8h	39.8±4.0†	27.7±2.9†	45.9±2.3
16h	34.3±2.7*†	32.3±1.6*†	46.4±2.1
24h	33.5±1.2*†	33.8±2.4*†	47.5±2.8
Recovery		$NaHCO_3$	
8h	32.0±4.0*†	35.4±1.4*†	47.0±4.4
16h	34.0±3.0*†	35.0±3.7*†	49.4±8.9
24h	34.0±3.0*†	30.8±2.1*†	43.6±4.9

Rats were made acidotic by administering NH_4Cl ad libitum for seven days. Other rats were intubated with 1.5 mmoles per 100g body weight of NaCl or $NaHCO_3$ at the start of recovery. Blood was sampled at 8h, 16h and 24h of recovery from metabolic acidosis. Results are expressed as mean ±SD. Each mean consists of 4 observations.

* indicates significantly different from normal value and † indicates significantly different from acidotic value.

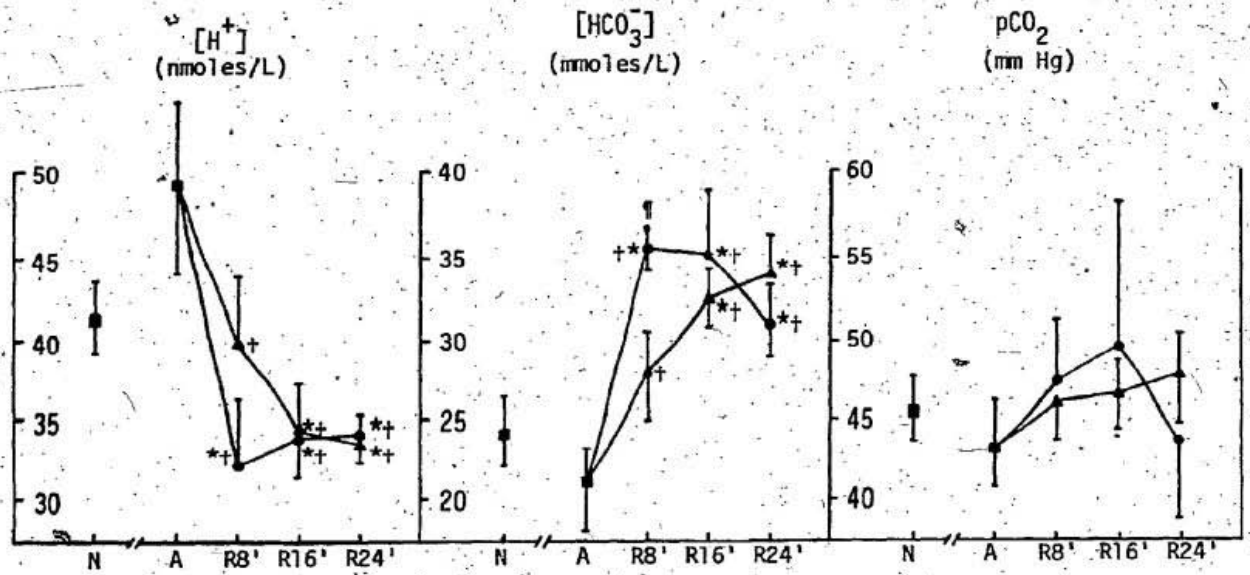


Figure 5.1 Blood acid-base parameters during NaCl and $NaHCO_3$-induced recovery from metabolic acidosis.

Acidotic rats were intubated with 1.5 mmoles per 100g body wt of NaCl or $NaHCO_3$ and blood sampled 8, 16 and 24 hours later for the determination of acid-base parameters. The data for this figure is contained in Table 5.1. Closed circles represent the $NaHCO_3$ group and closed triangles represent the NaCl group. Vertical error bars represent 1 SD, * indicates significantly different from normal value, † indicates significantly different from acidotic value and ¶ indicates a significant difference between the NaCl group and the $NaHCO_3$ group ($P<0.05$).

$NaHCO_3$ group compared with that in rats intubated with NaCl. The maximum concentration of bicarbonate reached was the same in both groups suggesting that the determining factor was the presumed altered renal threshold for bicarbonate reabsorption.

No significant change in blood pCO_2 was apparent in this study suggesting that respiratory compensation did not occur or that it was not as pronounced as in the study reported in Chapter 3. The large standard deviations associated with the pCO_2 values make it difficult to draw firm conclusions from this data.

The results of this study indicate that metabolic alkalosis was achieved earlier during recovery in rats intubated with $NaHCO_3$. These results are not unexpected. Since the acid-base status of the animal could be manipulated during recovery by the intubation of $NaHCO_3$, then the effect of this manipulation on the response of the kidney to recovery was investigated.

5.2 Urinary Ammonia Excretion

The first approach to establish the response of the kidney to NaCl and $NaHCO_3$ intubation during recovery involved measuring urinary ammonia excretions. To obtain as detailed a picture as possible, urine was collected for 4h intervals. Intervals of less than 4h did not prove practical since urine excretions were too variable. Urine was also collected every 4h for 24h in both normal and acidotic rats.

The data collected from this study is contained in Table 5.2. Figure 5.2 illustrates the same data in the form of histograms for easier comparison. A pattern is evident in both normal and acidotic

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Table 5.2 Urinary ammonia excretion for normal and acidotic rats and for rats intubated with H_2O, NaCl or $NaHCO_3$ at the start of recovery.

URINARY AMMONIA EXCRETION (μmoles/4h/100gbw ±SD (No. of Animals))

Experimental Condition:		Normal	Acidotic	Recovery H_2O	Recovery NaCl	Recovery $NaHCO_3$
Collection Period	Duration					
9am→1pm	4 h	6.3±3.3(4)	358.0±99.8(4)*	259.8±120.5(4)*	282.4±101.2(7)*	98.5±72.9(7)*†
1pm→5pm	8 h	15.3±10.0(4)	186.7±96.2(7)*	229.5±30.9(3)*	182.5±53.7(8)*	53.2±46.6(8)†
5pm→9pm	12 h	18.7±13.2(4)	258.2±171.1(12)*	151.8±58.9(4)*	144.0±88.3(7)*	19.6±19.4(8)†
9pm→1am	16 h	21.8±13.2(4)	336.0±127.1(12)*	141.2±135.3(4)†	85.2±19.7(3)*†	16.0±7.6(4)†
1am→5am	20 h	23.8±14.4(4)	363.9±72.7(11)*	67.1±35.5(4)†	74.7±25.8(3)*†	33.0±22.1(4)†
5am→9am	24 h	26.6±11.6(4)	348.7±111.7(19)*	46.3±31.0(4)†	32.5±24.2(3)†	----

Urine was collected at 4h intervals for 24h from normal and acidotic rats and from rats which were made to recover from metabolic acidosis by intubating H_2O (2 ml/100g body wt), NaCl (1.5 mmoles/100g body wt) or $NaHCO_3$ (1.4 mmoles/100g body wt). Urine was collected from acidotic rats on the seventh day of NH_4Cl treatment.

* *indicates significantly different from corresponding normal value and † indicates significantly different from corresponding acidotic value (P<0.05).*

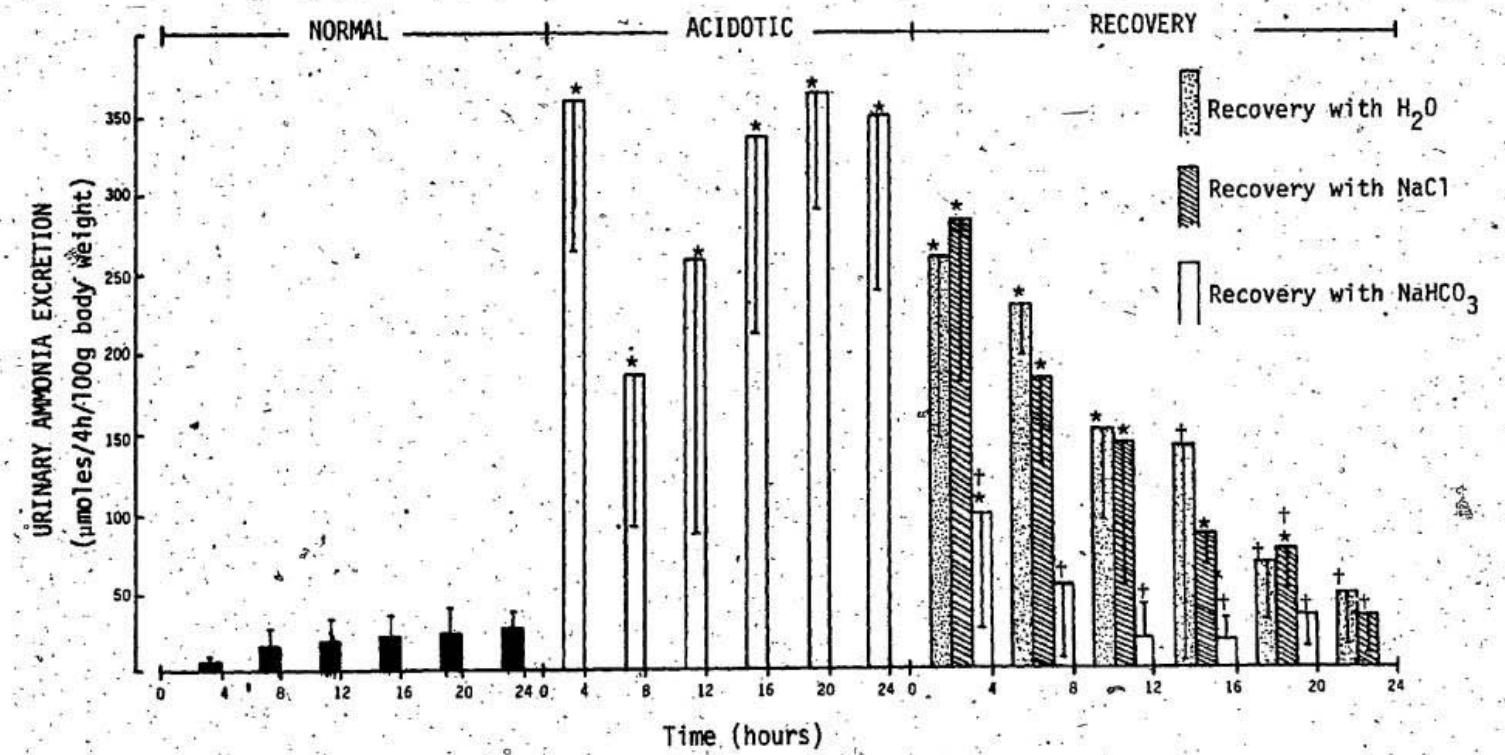


Figure 5.2 The effect of H_2O, NaCl and $NaHCO_3$ on urinary ammonia excretion during recovery from metabolic acidosis.

Urine was collected at 4h intervals for 24h from normal and acidotic rats and rats which were allowed to recovery from metabolic acidosis. Urine was collected from acidotic rats on the seventh day of NH_4Cl administration. Recovery was initiated in other acidotic rats by intubating H_2O (2 ml/100g body wt), NaCl (1.5 mmoles/100g body wt) or $NaHCO_3$ (1.5 mmoles/100g body wt). The data for this figure is from Table 5.2.

Vertical error bars equal 1 SD, * indicates significantly different from normal value and † indicates significantly different from acidotic values. ($P < 0.05$).

urine collections. Normal rats excreted less ammonia between 9 a.m. and 1 p.m., whereas ammonia excretion by acidotic rats was less between 1 p.m. and 5 p.m. Since rats are nocturnal, then these low rates of ammonia excretion may be related to reduced food and fluid intake during the daytime.

To ensure that the intubation of NaCl did not have an effect on ammonia excretion, another group of rats was intubated with water (2 ml per 100g body weight) at the start of recovery. As can be seen in Figure 5.2, there is no difference in ammonia excretion between the NaCl group and the group intubated with water. In the NaCl group, a gradual decrease in ammonia excretion is observed and normal rates were attained between 16 and 20h of recovery. Ammonia excretion had returned to normal within 4h of recovery in the group intubated with $NaHCO_3$ since the amount of ammonia excreted in the 4h to 8h urine collection is not significantly different from normal.

It is concluded that ammonia excretion in animals intubated with $NaHCO_3$ during recovery returned to normal 12h before it did in animals intubated with NaCl. The difference observed between these two recovery groups provides for a useful experimental situation to study renal glutamine metabolism.

5.3 Renal Function

To further establish the response of renal glutamine metabolism to recovery in rats intubated with $NaHCO_3$ and NaCl, clearance studies were performed by Dr. K.C. Man. This would permit definitive conclusions to be made about renal ammonia production and glutamine extraction. Based

upon the results obtained by measuring urinary ammonia excretions, these studies were made at 8h of recovery. This recovery time was selected since at 8h of recovery normal rates of ammonia excretion had been established for about 4h in the $NaHCO_3$ group while in the NaCl group the rate of ammonia excretion was still well elevated above normal and did not return to normal for at least another 8h. Hence, the clearance studies would be expected to reveal a difference between the two groups at 8h of recovery.

The results of these studies are contained in Table 5.3. No change in GFR or RBF is observed. Taking into account the release of ammonia into both the blood and urine, it is seen that total ammonia production returned to normal in the $NaHCO_3$ group but is still significantly elevated above normal and not significantly different from acidotic values in the NaCl group. In the NaCl group, the release of ammonia into the urine is significantly elevated above normal although the release of ammonia into the blood is not significantly different from normal or acidotic values. In the $NaHCO_3$ group, the release of ammonia into the urine and blood are both significantly different from acidotic values but not signficantly different from normal.

The pattern observed with glutamine extraction in general supports the results obtained for total ammonia production. In the $NaHCO_3$ group, glutamine extraction returned to normal by 8h of recovery. Although at 8h of recovery glutamine extraction is significantly less than acidotic values in the NaCl group, it is significantly elevated compared with normal.

It can be concluded from these studies that compared with the

Table 5.3 Effect of $NaHCO_3$ intubation at the start of recovery on total ammonia production and glutamine extraction by the left kidney in the rat, in vivo.

Experimental Condition (No. of Animals)	Arterial NH_4^+ µmol/ml	Venous NH_4^+ µmol/ml	Urine NH_4^+ µmol/ml	Arterial Glutamine µmol/ml	Venous Glutamine µmol/ml	Renal Vein NH_4^+ Release µmol/min	Urinary NH_4^+ Release µmol/min	Total NH_3 Production µmol/min	Glutamine Extraction µmol/min	GFR ml/min	RBF ml/min
NORMAL (5)	0.04±0.01	0.06±0.02	74.6±6.5	0.46±0.02	0.39±0.01	0.27±0.06	0.44±0.05	0.71±0.05	0.50±0.09	1.04±0.04	7.37±0.92
ACIDOTIC (5)	0.06±0.02	0.02±0.03*	155.3±49.9*	0.39±0.05*	0.21±0.02*	0.93±0.34*	0.71±0.24	1.64±0.29*	1.16±0.26*	1.02±0.10	6.62±0.48
RECOVERY 8h (NaCl) (4)	0.05±0.02	0.13±0.02*†	195.7±23.9*	0.39±0.03*	0.28±0.04	0.54±0.07	0.74±0.07*	1.27±0.09*	0.70±0.12*†	1.11±0.11	6.82±0.36
RECOVERY 8h ($NaHCO_3$) (4)	0.06±0.01*	0.13±0.02*†	86.0±20.7	0.48±0.06	0.42±0.06	0.43±0.14†	0.34±0.08†	0.77±0.13†	0.41±0.08†	1.07±0.10	6.88±0.62

Normal and acidotic values are the same as those reported in Table 3.1. Other acidotic rats were intubated with 1.5 mmoles per 100g body wt of NaCl or $NaHCO_3$ and used 8h later. Results are expressed as means ±SD. The number of experiments is given in parenthesis. * indicates significantly different from normal value and † indicates significantly different from acidotic value ($P<0.05$).

$NaHCO_3$ group more glutamine was utilized by the NaCl group at 8h of recovery and that more ammonia was produced and excreted in the urine. With these results it is now clear that a definite difference in renal glutamine metabolism exists between the two recovery groups and the remaining studies in this chapter are concerned with discerning a metabolic basis for this difference.

5.4 In vivo Freeze-Clamp Studies

As in Chapter 3 the renal contents of the adenine nucleotides were measured in the recovery groups and compared with normal and acidotic values. These results appear in Table 5.4. The only significant difference is observed in the NaCl recovery group at 8h in which the renal content of ADP is less than both normal and acidotic values. There is no difference in the ADP content between the NaCl group and the $NaHCO_3$ group. Most important, the ATP content is not significantly different between any of the groups and hence it would appear that the difference observed in ADP content is not related to a change in the energy state of the kidney. No significant difference between the two recovery groups is apparent and hence the two groups are directly comparable.

A. Measurement of Metabolites Relevant to Glutamine Metabolism

Table 5.5 shows the data obtained by freeze-clamping kidneys taken from rats at 8h of recovery after intubation with NaCl and $NaHCO_3$. There is no significant difference between the NaCl group and the $NaHCO_3$ group in the renal content of any of the metabolites measured. The same

Table 5.4 Renal content of adenine nucleotides in normal and acidotic rats and in rats intubated with NaCl or $NaHCO_3$ at the start of recovery.

Experimental Condition:	CONTENT OF METABOLITES µmoles/g kidney ±SD (No. of Animals) Normal	Acidotic	Recovery (NaCl) 8h	Recovery ($NaHCO_3$) 8h
ATP	1.52±0.39(6)	1.25±0.35(6)	1.37±0.40(4)	1.11±0.12(4)
ADP	0.67±0.08(6)	0.64±0.04(6)	0.49±0.10(8)*†	0.57±0.08(4)
AMP	0.12±0.02(6)	0.14±0.03(6)	0.11±0.20(8)	0.14±0.03(8)
Total Adenine Nucleotides	2.31	2.03	1.97	1.82
$\frac{ATP}{ADP}$	2.27	1.95	2.80	1.95

Normal and acidotic values are the same as those reported in Table 3.2. Adenine nucleotides were also measured in the kidneys of rats 8h after the intubation of 1.5 moles per 100g body wt of NaCl or $NaHCO_3$ to acidotic rats.

* indicates significantly different from normal value and † indicates significantly different from acidotic value ($P<0.05$).

Table 5.5 Effect of $NaHCO_3$ intubation on metabolite content in rat kidney during recovery.

Experimental Condition:	Normal	Acidotic	Recovery (NaCl) 8h	Recovery ($NaHCO_3$) 8h
	CONTENT OF METABOLITES nmoles/g kidney ±SD (No. of animals)			
Glutamine	950±330(6)	380±400(6)*	597±356(4)	957±164(4)†
Glutamate	2350±300(6)	1730±420(6)	1516±754(4)	2166±297(4)
α-Ketoglutarate	210±60(6)	70±40(6)*	221±114(8)†	218±80(8)†
Aspartate	400±50(8)	350±110(8)	394±72(8)	391±98(8)
Malate	100±40(8)	50±20(8)*	122±90(8)	105±27(8)†
Citrate	160±40(8)	120±30(8)	179±65(7)	231±74(8)*†
Oxaloacetate (calculated from MDH)	6.9	3.5	5.1	5.5
Oxaloacetate (calculated from GOT)	5.3	2.1	8.6	5.9
Phosphoenolpyruvate	60±10(8)	80±20(8)	60±11(7)	68±6(4)
Pyruvate	100±20(8)	80±20(8)	88±20(8)	88±28(8)
Lactate	350±80(8)	280±90(8)	516±231(8)†	407±168(8)
3-Phosphoglycerate	130±40(14)	150±50(14)	134±21(4)	146±32(4)
Fructose-6-phosphate	12±4(8)	14±10(8)	14±5(7)	12±7(8)
Glucose-6-phosphate	33±7(8)	34±13(8)	42±9(6)	42±6(7)
Inorganic Phosphate	3730±600(8)	4300±900(8)	4750±1112(4)	3740±520(4)
	Metabolites Ratios			
$\frac{\text{Lactate}}{\text{Pyruvate}}$	3.5	3.5	5.9	4.6
Cytoplasmic $\frac{NAD^+}{NADH}$ (calculated from LDH)	2570	2570	1540	1950

Normal and acidotic values are the same as those reported in Table 3.3. Acidotic rats were also intubated with 1.5 mmoles per 100g body wt of NaCl or $NaHCO_3$ and the kidneys freeze-clamped 8h later.

* indicates significantly different from normal value and † indicates significantly different from acidotic value.

normal and acidotic values presented in Chapter 3 are also included for comparison. These data are also presented as percent changes in Figure 5.3. The upper histogram plot represents the percent changes which occurred during recovery after intubation with NaCl and the result of these changes in relation to normal is presented in the histogram plot next to the top. The lower two histogram plots represent the percent changes in the $NaHCO_3$ group versus the acidotic and normal groups, respectively.

In the NaCl group, α-ketoglutarate and lactate increased significantly during recovery increasing the contents of these metabolites to values not significantly different from normal. Although none of the other metabolites changed significantly, the following trends are apparent. Glutamine tended to be higher than acidotic values and lower than normal values. The mean for glutamate is lower than that for acidotic rats and hence any tendency to increase in this group by 8h of recovery can be ruled out. The trend for malate, citrate and calculated oxaloacetate to be decreased in acidosis was reversed in the NaCl group. The trends for phosphoenolpyruvate and 3-phosphoglycerate to be increased during acidosis were also reversed during recovery after intubation with NaCl.

In the $NaHCO_3$ group significant increases occurred in glutamine, α-ketoglutarate, malate and citrate by 8h of recovery. The increase in citrate elevated the content of this metabolite above normal. The increases in glutamine, α-ketoglutarate and malate returned the contents of these metabolites to normal. Although not significant, the following trends are apparent. Glutamate tends to be elevated in comparison with that in the NaCl and acidotic groups. The calculated content of oxaloacetate suggest an increase in the content of this metabolite.

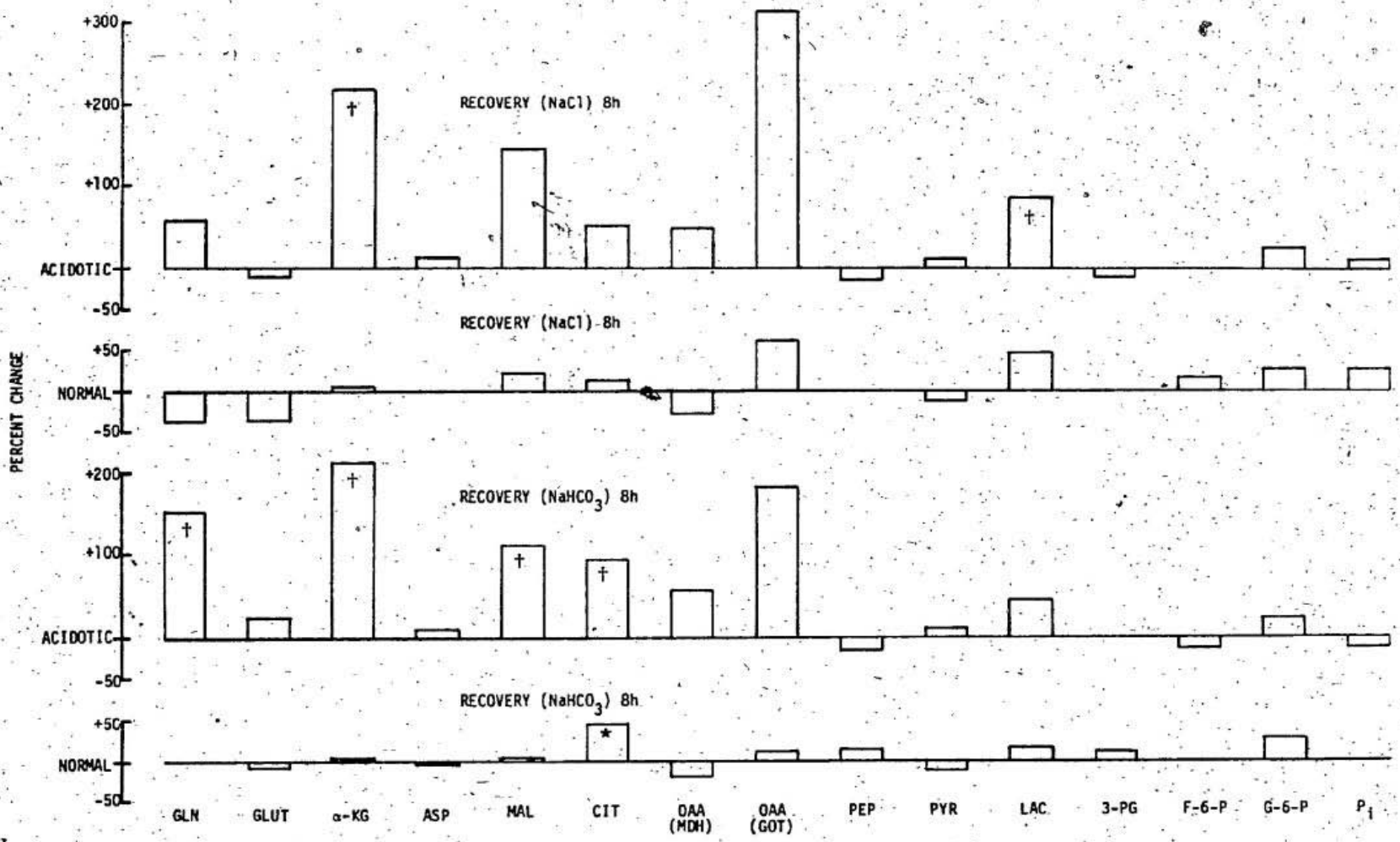


Figure 5.3 Percentage change in mean kidney metabolite levels from normal or acidotic values during NaCl and $NaHCO_3$-induced recovery.

The percent changes in metabolite levels 8h after the intubation of 1.5 mmoles per 100g body wt of NaCl or $NaHCO_3$ to acidotic rats are derived from the data in Table 5.5.

* indicates a significant change from normal value and † indicates a significant change from acidotic value ($P<0.05$).

B. Comments on the Results of the in vivo Freeze Clamp Studies

The renal contents of glutamine in the NaCl group and $NaHCO_3$ group agree well with the physiological observations made thus far. The increase in glutamine content to normal in the $NaHCO_3$ group probably reflects the re-establishment of normal rates of glutamine utilization and agrees well with the return of glutamine extraction and ammonia production to normal in the same animals. The tendency for glutamine to remain lower than normal in the NaCl group also fits well with the continued utilization of glutamine and the production of ammonia at the above-normal rates observed in this group.

Although not statistically significant, it is interesting to note that the trend for glutamate in the $NaHCO_3$ group is to be elevated compared to acidotic levels whereas in the NaCl group it tended to remain decreased compared to normal levels. This may be suggestive of an inhibitory role for glutamate in turning off renal ammoniagenesis. α-Ketoglutarate increased almost identically in both groups during recovery. The increases in α-ketoglutarate are consistent with it playing a regulatory role in renal glutamine metabolism. However, since the content of α-ketoglutarate is the same in both groups then changes in the content of this metabolite cannot be the sole means whereby regulation is effected.

The increase in citrate to above normal values in the $NaHCO_3$ group but not in the NaCl group is noteworthy. It was hypothesized in Chapter 3 that the increase in citrate is due to a direct effect of bicarbonate on the citrate transporter causing citrate to accumulate extramitochondrially. This fits well with the observations made here. At 8h of

recovery blood bicarbonate was significantly elevated above normal in the $NaHCO_3$ group whereas in the NaCl group only normal bicarbonate levels were attained.

Increased lactate is also observed during recovery in the NaCl group. This agrees with the increase found in lactate content in kidneys of one-day recovered rats reported in Chapter 3. Since pyruvate did not increase along with this increase in lactate then presumably the cytoplasmic NAD^+/NADH ratio decreased, as was observed previously.

5.5 Tissue Slices

The next level at which the metabolism of glutamine was studied is the kidney cortex slice. Although it has been demonstrated that liver slices are irreparably damaged and exhibit metabolic rates much lower than expected (89), the same is not true for tissue slices from the kidney. Therefore, the slice remains a useful preparation for studying the metabolism of kidney cortex. An obvious advantage of the slice technique is that it is technically much simpler than kidney perfusion and thereby does not require the time and expense required for successful perfusion.

The metabolism of glutamine by kidney cortex slices was followed by measuring the production of ammonia, glutamate and glucose. The results of this study are contained in Table 5.6. The formation of all products increased during metabolic acidosis. Ammonia formation by kidney cortex slices increased approximately two-fold during metabolic acidosis confirming previous results (125). At 8h of recovery ammonia formation by kidney slices from either the NaCl group or the $NaHCO_3$ group is not

Table 5.6 Metabolism of glutamine by kidney cortex slices from normal and acidotic rats and from rats intubated with NaCl or $NaHCO_3$ at the start of recovery.

Experimental Condition	Formation of Product (µmoles/90 min/g wet wt ±SD, n=4)		
	Ammonia	Glutamate	Glucose
Normal	81.5±29.2	3.2±1.1	5.0±2.3
Acidotic	157.9±19.4*	10.4±2.7*	22.3±2.1*
Recovery (NaCl) 8h	134.9±13.7*	16.5±3.6*	15.5±1.9*†
Recovery ($NaHCO_3$) 8h	123.7±20.3	13.3±6.2*	12.9±1.3*†

Rats were made acidotic by administering NH_4Cl ad libitum for seven days. Tissue slices were also obtained from kidneys 8h after the intubation of 1.5 mmoles per 100g body wt of NaCl or $NaHCO_3$ to acidotic rats. Slices were incubated with 2mm glutamine.

* indicates significantly different from normal value and † indicates significantly different from acidotic value ($P<0.05$).

significantly different from that in the acidotic group. However, only in the NaCl group is the formation of ammonia significantly elevated compared to normal. In agreement with previous results, an increase in glutamate formation of about three-fold was also evident in the slices taken from acidotic rats. During recovery the formation of glutamate remained elevated at acidotic rates in both the NaCl group and the $NaHCO_3$ group. Gluconeogenesis increased approximately five-fold in kidney slices taken from acidotic rats. This confirms results previously reported (125). By 8h of recovery glucose production fell comparably in both recovery groups but remained significantly elevated compared to normal.

The results obtained in the present study do not prove a difference between the NaCl group and the $NaHCO_3$ group in the metabolism of glutamine by kidney cortex slices. The formation of products tended to be lower in the $NaHCO_3$ group compared to the NaCl group. The increased accumulation of glutamate in acidosis indicates that the removal of glutamate does not keep pace with its formation. However, since glutamate is not released by the normal kidney or the chronically-acidotic kidney in vivo (71, 173) this glutamate accumulation may indicate the unphysiological handling of glutamine by slices. Glucose production decreased by 8h of recovery in both groups but glutamate formation remained at least as elevated as acidotic rates. Since glucose production decreased during recovery without a fall in glutamate formation, it would appear that a step in the metabolism of glutamine between glutamate and glucose was inhibited. Perhaps glutamate is continually removed from the mitochondria and thereby does not accumulate intramitochondrially where it would presumably inhibit glutaminase.

5.6 Phosphoenolpyruvate Carboxykinase Activity

In this study, rats were intubated with either water, NaCl or $NaHCO_3$ at the initiation of recovery. The results obtained appear in Table 5.7. The activities are expressed as mmoles/24h/g kidney and as mmoles/24h/100g body weight.

At 24h of recovery PEPCK activity was back to normal in all three groups. This confirms previous findings obtained in Chapter 3 and reported elsewhere (125). The animals intubated with water served as controls for the NaCl group. There was no significant difference in PEPCK activity between these two groups at any of the times measured. This indicates that NaCl did not alter the response of PEPCK activity to recovery. Comparison between the NaCl group and the $NaHCO_3$ group reveals that there is no significant difference in PEPCK activity between these two groups. Hence, $NaHCO_3$ also did not alter the response of PEPCK activity to recovery. In all three groups, PEPCK activity remained significantly elevated above normal at 16h of recovery except in the $NaHCO_3$ group when the enzyme activity is expressed as mmoles/24h/g kidney. However, when expressed as mmoles/24h/100g body weight, the activity of PEPCK was still significantly elevated at 16h of recovery.

The response of PEPCK activity to recovery in the $NaHCO_3$ group is not consistent with changes in the content of this enzyme being responsible for the return of renal ammoniagenesis to normal by 8h of recovery. In animals intubated with NaCl or water, the return of urinary ammonia excretion to normal coincides with the return of PEPCK activity to normal. However, this does not prove a causal relationship between PEPCK activity and renal ammoniagenesis. On the other hand, the disso-

Table 5.7 Phosphoenolpyruvate carboxykinase activity in rat kidney during recovery from metabolic acidosis.

	PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY					
	mmoles/24h/g kidney ±SD (No. of Animals)			mmoles/24h/100gbw ±SD (No. of Animals)		
Normal	10.1±0.7(8)			7.3±0.9(8)		
Acidotic	30.0±3.7(8)			25.4±3.8(8)		
Recovery	H_2O	NaCl	$NaHCO_3$	H_2O	NaCl	$NaHCO_3$
8h	38.8±1.8(4)*†	38.6±5.1(4)*†	32.7±3.3(4)*	32.4±2.2(4)*†	28.7±2.2(3)*	26.0±3.0(4)*
12h	20.4±7.6(5)*†	19.2±8.0(4)*†	18.0±4.1(4)*†	17.6±7.6(5)*†	16.9±5.2(4)*†	16.0±4.2(4)*†
16h	15.7±6.3(5)*†	13.0±2.5(4)*†	12.4±3.5(8)†	13.9±5.9(5)*†	10.9±2.0(4)*†	10.8±3.0(8)*†
24h	12.7±5.3(5)†	11.2±2.5(4)†	8.8±2.2(4)†	9.2±4.0(5)†	8.3±1.8(4)†	6.9±2.0(4)†

Values are reported for normal and 7-day acidotic rats and for different times after the intubation of acidotic rats with H_2O (2 ml/100g body wt), NaCl (1.5 mmoles/100g body wt) or $NaHCO_3$ (1.5 mmoles/100g body wt). Activity is expressed as mmoles/24h/g kidney and as mmoles/24h/100g body wt.

* indicates significantly different from normal value and † indicates significantly different from acidotic value ($P<0.05$).

ciation between these parameters indicates that a change in PEPCK activity is not required for recovery from metabolic acidosis in rats intubated with $NaHCO_3$.

5.7 Immunochemical Studies of Phosphoenolpyruvate Carboxykinase

From the results of immunochemical titrations of PEPCK reported in Chapter 3, it was concluded that the fall in PEPCK activity observed during recovery is due to a fall in the content of immunochemically-reactive enzyme. Hence, the existence of an inactive form of PEPCK could not be demonstrated. The immunochemical studies reported in Chapter 3 were designed without prior knowledge of the time course for the return of PEPCK activity to normal. The recovery times studied were arbitrarily chosen as 12h and 24h. According to the results obtained in the previous section of this chapter PEPCK activity returned to normal between 16h and 24h of recovery. If a catalytically-inactive form of PEPCK existed during recovery, then presumably its demonstration by immunotitration would depend upon the time allowed for the inactive form to be degraded. Perhaps a catalytically-inactive form of PEPCK was formed during recovery but by 24h it was completely degraded and by 12h not enough of it was present to detect experimentally. Accordingly, immunochemical titrations were performed with kidney cytosols obtained from rats at 12h and 16h of recovery after intubation with $NaHCO_3$ at the start of recovery. Cytosols from normal and acidotic rats were also used for immunotitration of PEPCK.

Sample titration curves appear in Figure 5.4 for each of the experimental situations studied. Table 5.8 contains the means of the

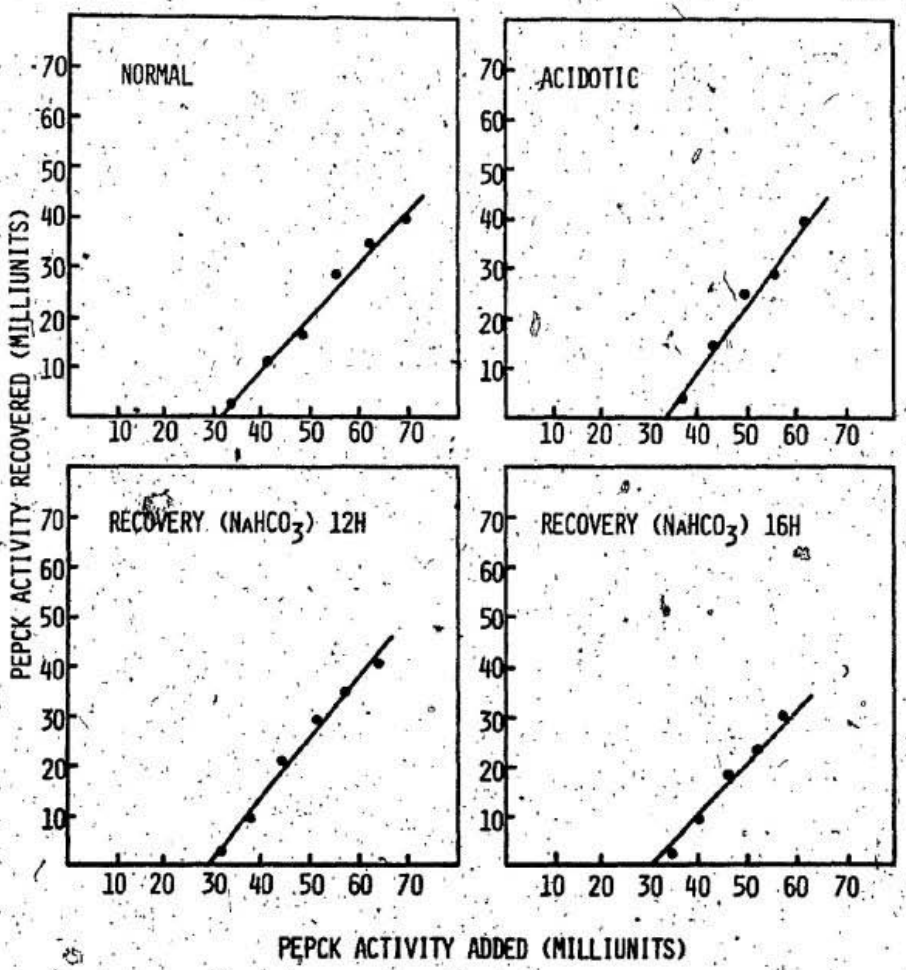


Figure 5.4 Typical immunotitration curves of PEPCK in kidney homogenates from normal and acidotic rats and rats administered $NaHCO_3$ at the start of recovery.

Acidotic rats were administered NH_4Cl ad libitum for seven days. Other rats were used at 12h and 16h after the intubation of $NaHCO_3$ (1.5 mmoles/100g body wt) at the start of recovery from metabolic acidosis.

Table 5.8 Equivalence points and slopes obtained by immunotitration of renal phosphoenolpyruvate carboxykinase in normal and acidotic rats and in rats during $NaHCO_3$-induced recovery.

Experimental Condition	Equivalence Point (milliunits)	Slope	Phosphoenolpyruvate Carboxykinase Activity (milliunits/mg kidney ±SD)
Normal	31.0±2.6 (4)	1.19±0.09 (4)	7.4±0.4 (4)
Acidotic	35.2±3.1 (4)	1.26±0.12 (4)	20.3±3.5 (4)*
Recovery ($NaHCO_3$) 12h	32.5±2.4 (4)	1.25±0.06 (4)	12.5±2.8 (4)*+
Recovery ($NaHCO_3$) 16h	25.8±4.2 (4)+	1.12±0.13 (4)	8.0±1.7 (4)+

Rats were made acidotic by administering NH_4Cl ad libitum for seven days. Recovery from metabolic acidosis was induced by intubating 1.5 mmoles $NaHCO_3$ per 100g body wt to 7-day acidotic animals.

* indicates significantly different from normal value and + indicates significantly different from acidotic value ($P<0.05$).

slopes and equivalence points obtained. There is no significant difference in slope between groups; hence, these results are directly comparable. The higher equivalence points obtained in the present study compared with the previous immunochemical titration study are due to the use of different amounts of different batches of antibody.

In agreement with the results in Chapter 3, the equivalence points obtained with the kidney extracts from acidotic rats and rats which were permitted 12h to recover are not significantly different from that obtained with the extracts from normal rats. In addition, the equivalence point obtained after 16h of recovery was also not significantly different from normal. However, a statistical decrease in the equivalence point was evident in extracts from animals intubated 16h earlier compared with those from acidotic rats. Although not definitive, it is possible that the slightly lower equivalence point obtained at 16h of recovery is a reflection of the presence of a catalytically-inactive and immunochemically-reactive form of PEPCK. If such a form of PEPCK exists, it could represent an early product in the degradation of this enzyme.

5.8 Mitochondrial Studies

It was shown in Chapter 3 that a mitochondrial event occurred during recovery from metabolic acidosis such that the flux through GDH returned to normal by 24h of recovery in mitochondria incubated in the presence of both glutamine and glutamate. Since renal ammonia production, *in vivo* was also back to normal by 24h of recovery then the change in GDH flux observed in isolated mitochondria represents a possible regulatory event. Therefore, it was of interest to investigate the response

of isolated mitochondria to recovery at times less than 24h and to determine if the intubation of $NaHCO_3$ *in vivo* affected the mitochondrial response to recovery.

In these studies mitochondria were incubated in the low-phosphate media only since at this phosphate concentration the return of mitochondrial glutamine metabolism to normal was evident in both the absence and presence of glutamate. At the higher concentration of phosphate the metabolism of glutamine remained well elevated for at least 15 days of recovery in the medium containing glutamine as sole substrate. It appears that the elevated concentration of phosphate may affect the mitochondrial response to recovery and therefore only the physiological concentration of phosphate (i.e. 4 mM) was used.

(A) Mitochondrial Incubations with 1 mM Glutamine

Table 5.9 shows the medium changes in ammonia, glutamate and aspartate and the fluxes through PDG, GDH and GOT in mitochondria incubated in the low-phosphate medium containing glutamine as sole substrate. The mitochondria were isolated from rats at 4h and 8h after the intubation of either NaCl or $NaHCO_3$ at the beginning of recovery. Also included are normal and acidotic values for comparison.

Ammonia formation (also illustrated in Figure 5.5) decreased in the NaCl group by 4h of recovery but remained significantly elevated in the $NaHCO_3$ group. At 8h of recovery the formation of ammonia by mitochondria from both groups were decreased compared with acidotic rates of ammoniagenesis but were still significantly elevated above normal. No significant difference in ammonia formation between the NaCl group and the

Table 5.9 Medium changes and enzyme fluxes in mitochondrial incubations with glutamine as sole substrate during NaCl and $NaHCO_3$-induced recovery.

LOW PHOSPHATE MEDIUM
(4 mM KH_2PO_4)

	1 mM Glutamine					
	Medium Changes:			Flux:		
Experimental Condition	Ammonia	Glutamate	Aspartate	PDG	GDH	GOT
	Δnmoles/mg/min±SD			nmoles/mg/min±SD		
NORMAL	14.4±3.7 (12)	1.6±1.2 (12)	4.2±2.0 (12)	10.1±2.4 (12)	4.3±1.9 (12)	4.2±2.0 (12)
ACIDOTIC	38.2±5.3 (9)	1.6±0.7 (9)	2.9±0.9 (8)	20.8±3.1 (8)	16.6±2.2 (8)	2.9±0.9 (8)
RECOVERY 4h (NaCl)	29.9±5.5 (4)	1.1±0.9 (4)	2.6±0.3 (4)	16.8±3.2 (4)	13.1±2.4 (4)	2.6±0.3 (4)
RECOVERY 4h ($NaHCO_3$)	38.2±6.7 (8)	2.4±0.5 (8)	3.2±0.8 (8)	21.9±3.5 (8)	16.3±3.2 (8)	3.2±0.8 (8)
RECOVERY 8h (NaCl)	21.3±1.4 (4)	1.3±0.4 (4)	2.9±1.3 (4)	12.7±1.1 (4)	8.6±0.6 (4)	2.9±1.3 (4)
RECOVERY 8h ($NaHCO_3$)	25.3±4.9 (5)	1.5±0.4 (5)	3.4±1.6 (5)	15.1±2.8 (5)	10.2±2.5 (5)	3.4±1.6 (5)

Kidney cortex mitochondria were isolated from rats 4h and 8h after the intubation of 1.5 mmoles/100g body wt of NaCl or $NaHCO_3$ at the start of recovery. Incubations were for 4 minutes in the presence of 1 mM glutamine and 4 mM phosphate. Normal and acidotic values are the same as those reported in Tables 3.5 and 3.6.

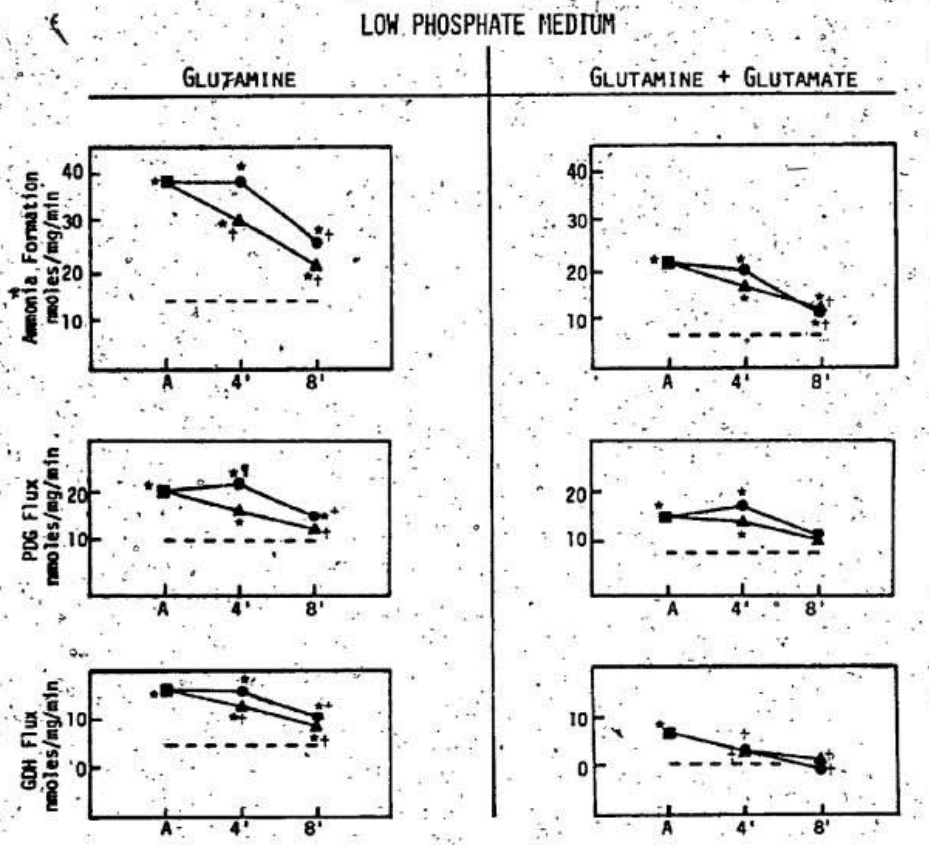


Figure 5.5 Effect of $NaHCO_3$ intubation on the mitochondrial metabolism of glutamine ± glutamate during recovery from metabolic acidosis.

Data presented in this figure are contained in Tables 5.9 and 5.10. Kidney cortex mitochondria were isolated from rats 4h and 8h after the intubation of NaCl or $NaHCO_3$ at the start of recovery. Incubations were for 4 minutes in the presence of 4 mM phosphate, 1 mM glutamine ± 3 mM glutamate. Closed circles represent the $NaHCO_3$-treated group and closed triangles represent the NaCl-treated group. Dashed lines represent normal values. * indicates significantly different from normal value, † indicates significantly different from acidotic value and ¶ indicates a significant difference between the NaCl group and the $NaHCO_3$ group ($P < 0.05$).

$NaHCO_3^-$ group was apparent at either 4h or 8h of recovery. Glutamate formation changed only at 4h of recovery in the $NaHCO_3$ group in which a slight increase above normal is observed. No significant change was found for aspartate formation and GOT flux.

Also illustrated in Figure 5.5 are the mitochondrial fluxes through PDG and GDH. The flux through PDG did not decrease significantly in either group by 4h of recovery but by 8h significant decreases in PDG flux are observed in mitochondria from both groups of rats. Only in the NaCl group did PDG flux attain a value not significantly different from normal. No significant difference between the two groups is apparent at 8h of recovery, but at 4h of recovery the flux through PDG is significantly higher in the $NaHCO_3$ group. GDH flux fell in the NaCl group by 4h of recovery and was decreased in both groups by 8h of recovery but remained significantly elevated above normal. No significant difference in GDH flux is noted between the two recovery groups. The changes in PDG and GDH fluxes in this medium tend to reflect the responses of mitochondrial ammoniagenesis to recovery and do not reveal a difference between the two recovery groups reconcilable with the difference in renal ammonia production, *in vivo*.

B. Mitochondrial Incubations with 1 mM Glutamine plus 3 mM Glutamate

Table 5.10 shows the medium changes in ammonia, glutamate and aspartate as well as the fluxes through PDG, GDH and GOT in mitochondria incubated in the low-phosphate medium containing both glutamine and glutamate as substrates. The mitochondria were isolated from rats at 4h and 8h of recovery after intubation with NaCl or $NaHCO_3$ at the beginning

Table 5.10 Medium changes and enzyme fluxes in mitochondrial incubations with glutamine and glutamate as substrates during NaCl and $NaHCO_3$-induced recovery.

LOW PHOSPHATE MEDIUM
4 mM KH_2PO_4

	1 mM Glutamine + Glutamate					
	Medium Changes:			Flux:		
Experimental Condition	Ammonia	Glutamate	Aspartate	PDG	GDH	GOT
	Δnmoles/mg/min±SD			nmoles/mg/min±SD		
NORMAL	6.6±2.6 (12)	-15.7±4.0 (8)	20.9±5.5 (12)	7.9±3.8 (10)	0.6±5.3 (12)	20.9±5.5 (12)
ACIDOTIC	21.9±3.9 (9)	-12.4±5.4 (7)	20.9±2.8 (9)	15.1±4.5 (8)	6.8±3.5 (9)	20.9±2.8 (9)
RECOVERY 4h (H_2O)	19.2±6.4 (4)	-5.8±3.3 (4)	21.9±4.0 (4)	20.1±5.6 (4)	1.6±3.6 (4)	21.9±4.0 (4)
RECOVERY 4h (NaCl)	17.2±4.0 (4)	-12.6±3.7 (4)	25.1±0.5 (4)	14.8±2.5 (4)	2.4±2.7 (4)	25.1±0.5 (4)
RECOVERY 4h ($NaHCO_3$)	20.4±4.4 (8)	-10.3±3.8 (8)	25.7±3.1 (8)	17.9±2.5 (8)	2.6±2.7 (8)	25.7±3.1 (8)
RECOVERY 8h (NaCl)	11.7±3.0 (4)	-16.5±1.6 (4)	25.6±1.5 (4)	10.4±2.1 (4)	1.3±1.2 (4)	25.6±1.5 (4)
RECOVERY 8h ($NaHCO_3$)	11.1±2.3 (5)	-15.1±3.7 (5)	26.6±3.1 (5)	11.3±1.8 (5)	-0.2±2.7 (5)	26.6±3.1 (5)

Kidney cortex mitochondria were isolated from rats 4h and 8h after the intubation of 1.5 mmoles/100g body wt of NaCl or $NaHCO_3$ at the start of recovery. Incubations were for 4 minutes in the presence of 1 mM glutamine, 3mM glutamate and 4 mM phosphate. Normal and acidotic values are the same as those reported in Tables 3.5 and 3.6.

of recovery. Also included are normal and acidotic values for comparison.

Ammonia formation (also illustrated in Figure 5.5) decreased comparably in both recovery groups by 8h of recovery but remained significantly elevated above normal. No significant decrease in ammonia formation is observed at 4h of recovery and no difference between the two groups is apparent at either 4h or 8h.

The fluxes through PDG and GDH are also illustrated in Figure 5.5. No significant fall in PDG flux is apparent in this medium although the values at 8h of recovery are not significantly different from either normal or acidotic rates. No difference in PDG flux between the two recovery groups is apparent. The flux through GDH fell to normal by 4h of recovery in both the NaCl group and the $NaHCO_3$ group and were still at normal values at 8h of recovery. No significant difference in GDH flux between the two groups is apparent. The results obtained in this medium do not reveal a mitochondrial difference between the NaCl group and the $NaHCO_3$ group in the metabolism of glutamine and glutamate which would account for the difference in renal ammoniagenesis, in vivo.

C. Mitochondrial Enzymes: Phosphate-Dependent Glutaminase and Glutamate Dehydrogenase

As in Chapter 4, the activity of PDG and GDH were measured. These activities (Table 5.11) were determined in the same mitochondrial preparations used for the incubation studies reported above. Also included for comparison are the activities of these enzymes in mitochondria from both normal and acidotic rats.

PDG activity did not decrease within 8h of recovery in either the NaCl group or in the $NaHCO_3$ group and in fact was slightly increased

Table 5.11 Phosphate-dependent glutaminase and glutamate dehydrogenase activities in rat kidneys during NaCl and $NaHCO_3$-induced recovery from metabolic acidosis.

Experimental Condition	Phosphate-Dependent Glutaminase	Glutamate Dehydrogenase
	nmoles/mg/min ± SD (No. of Animals)	
Normal	393.9±76.5 (12)	857.5±161.6 (9)
Acidotic	1175.0±259.2* (13)	2142.8±348.7* (9)
Recovery (NaCl) 4h	1479.6±326.6*† (8)	1797.7±355.6* (8)
Recovery ($NaHCO_3$) 4h	1679.5±283.0*† (8)	1779.6±620.0* (8)
Recovery (NaCl) 8h	1425.4±249.0* (8)	1818.5±788.3* (8)
Recovery ($NaHCO_3$) 8h	1365.3±408.2* (9)	1926.9±335.5* (9)

Mitochondrial preparations were used for the assay of phosphate-dependent glutaminase and glutamate dehydrogenase. Acidotic rats were intubated with 1.5 mmoles per 100g body wt of NaCl or $NaHCO_3$ and 4 and 8 hours later the kidneys removed for the measurement of enzyme activities.

* indicates significantly different from normal value and † indicates significantly different from acidotic value.

in both groups at 4h of recovery. These results are consistent with those reported in Chapter 4. Hence, the changes which occurred in PDG flux in the low-phosphate medium containing glutamine as sole substrate can not be attributed to changes in the mitochondrial content of glutaminase.

GDH activity also remained significantly elevated above normal as expected and did not change significantly from acidotic values. Hence, a similar conclusion can be made regarding the relationship of the activity of GDH and the flux through this enzyme. That is, the decreases observed in GDH flux during the first 8h of recovery in the low-phosphate media can not be attributed to a change in the content of this enzyme.

5.9 Discussion

The results reported in Table 5.3 show that the return of renal glutamine metabolism *in vivo* to normal during recovery could be accelerated by the intubation of $NaHCO_3$. This was established by measurements of urinary ammonia excretion, renal ammonia production and glutamine extraction. All parameters were back to normal by 8h of recovery in animals intubated with $NaHCO_3$, whereas in animals intubated with NaCl these parameters were still substantially elevated.

In the $NaHCO_3$-treated group, the renal content of glutamine returned to normal levels by 8h of recovery. This probably reflects the re-establishment of normal rates of glutamine utilization. The increase in malate content to normal levels in these same animals is consistent with an inhibition at the level of PEPCK. In these studies, the renal content of phosphoenolpyruvate was not significantly increased. An increase in

phosphoenolpyruvate might be expected if the flux through PEPCK was inhibited. However, as pointed out by Boyd and Goldstein (19) changes in malate need not necessarily be accompanied by changes in phosphoenolpyruvate if the rate of utilization of phosphoenolpyruvate remained equal to its rate of formation. Since the activity of PEPCK at 8h of recovery in the $NaHCO_3$-treated group is not significantly different from acidotic values, then a decrease in PEPCK flux *in vivo* can not be attributed to a change in the content of this enzyme. The increase of α-ketoglutarate content and the trend for the content of glutamate to increase are consistent with these metabolites playing regulatory roles in turning off renal ammoniagenesis in the manner described in Chapter 3.

Ammonia excretions indicate that renal ammonia production remained elevated for at least 16h in animals intubated with NaCl at the start of recovery. However, by 8h of recovery in the NaCl-treated group, renal glutamine extraction was significantly decreased as was urinary ammonia excretion. Total ammonia production also tended to be lower than acidotic values and in addition, the renal content of glutamine was intermediate between normal and acidotic values. These observations probably reflect a decrease (although not to normal) in the rate of renal glutamine metabolism in the NaCl-treated group at 8h of recovery. Although malate content tends to be increased by 8h of recovery in these same animals, this trend does not reach statistical significance. This may be due to greater variation associated with a situation which is not at steady-state but undergoing change. Since PEPCK inhibition was suggested by the changes in the contents of malate and phosphoenolpyruvate at 24h of recovery (Table 3.3), then the trend for increased malate content at

8h of recovery in the NaCl-treated group may also reflect an inhibition in PEPCK flux. Again, the increase in α-ketoglutarate content agrees well with its purported regulatory role in renal ammoniagenesis.

The flux through GDH in isolated mitochondria incubated in the most physiological medium (4 mM phosphate, 1 mM glutamine and 3 mM glutamate) also returned to normal by 8h of recovery in the $NaHCO_3$-treated group. Although this is consistent with the event responsible for this change playing a regulatory role in renal ammoniagenesis during recovery as was hypothesized in Chapter 3, it is obvious that this can not be the sole regulatory event. This is because GDH flux also returned to normal by 8h in animals intubated with NaCl at the start of recovery, although renal ammoniagenesis in vivo remained significantly elevated above normal.

CHAPTER 6

RECHALLENGE

CHAPTER 6

RECHALLENGE

The main emphasis of this thesis has been to characterize events which occur during recovery from metabolic acidosis. It is evident from these studies that not all of the adaptations which took place during metabolic acidosis accompanied the return of renal glutamine metabolism to normal during recovery. Some adaptations, notably the increases in PDG and GDH activities, remained evident long after normal glutamine metabolism was re-established. The response of isolated mitochondria during recovery depended upon the medium in which they were incubated. Under some conditions, PDG flux and GDH flux were back to normal by one to five days of recovery, whereas under other conditions, these parameters were still elevated after fifteen days recovery. These observations were interpreted to indicate that although mitochondrial changes occur which return mitochondrial glutamine metabolism to normal during recovery, the metabolic machinery to metabolize glutamine at above-normal rates was still present in mitochondria from recovered rats. Therefore, in terms of renal glutamine metabolism, recovery is a unique situation in the sense that mitochondrial potential is elevated and cytoplasmic potential (namely PEPCK) is normal. This situation was exploited by the studies presented in this chapter.

It has been proposed that in order to achieve maximum renal ammonia excretion during metabolic acidosis, an adaptation period is required for the kidney to develop the necessary metabolic capacity. It is not known which metabolic adaptation is responsible for the length of this

adaptation period. To help distinguish the importance of cytoplasmic versus mitochondrial adaptations for the increase in renal glutamine metabolism, the response of recovered rats to a second acid challenge was studied. It was anticipated that if mitochondrial adaptations delayed the original increase in renal ammoniagenesis then recovered rats may not experience the same delay. If the adaptive increase in the cytoplasmic parameter (PEPCK) was responsible for the length of the original adaptation period, then unless the induction of PEPCK was more responsive, the increase in ammonia excretion by recovered rats would presumably not differ from that of naive rats.

In these studies recovered rats with the required metabolic adaptations were obtained by allowing three days for rats to recover from a previous acid challenge. The previous challenge was administered by replacing tap water with 1.5% NH_4Cl as the sole drinking solution for seven days. It was determined that rats on this protocol received approximately 2 mmoles NH_4Cl per 100g body weight each day. At three days recovery, PEPCK activity was normal and GDH flux in isolated mitochondria metabolizing glutamine in the media containing glutamate was maximally decreased. The activities of PDG and GDH were still elevated on the third recovery day as was the capacity of mitochondria to metabolize glutamine. Both recovered rats and naive rats were administered NH_4Cl by stomach tube. This allowed exact quantitation of the acid load administered so that any variation in the response of naive and recovered rats would not be attributable to differences in adjusting to the taste of NH_4Cl. It is possible that rats challenged for a second time might have less aversion to drinking NH_4Cl than naive-challenged rats.

Before this study could be pursued at the metabolic level, it was important to establish whether recovered rats did, in fact, benefit from the previous acid challenge such that they were able to cope with an acid load more effectively than naive rats. To determine this, the response of the whole animal to acid loading was studied in naive-challenged and rechallenged rats. This involved measuring rates of urinary ammonia excretion, comparing glutamine arterial renal venous differences and following the response of acid-base parameters. The results of these studies are presented below.

6.1 Urinary Ammonia Excretion

Naive rats and three-day recovered rats were intubated with 0.5, 1.0, 1.5, 2.0 and 2.5 mmoles NH_4Cl per 100g body weight. This involved three successive intubations. After each intubation, the animals were placed in individual metabolic cages and the urines collected for 12h. Because of the apparent variations in the response of rats to acid loading in the results of other workers (43, 146, 198), it was considered possible that the relative responses of naive and recovered rats might differ depending upon the dose of NH_4Cl administered. Hence, five different doses of NH_4Cl were employed.

Table 6.1 contains the results of this study. The rates of ammonia excretion are expressed per 12h and hence must be multiplied by 2 to compare with 24h urine collections. The rates of ammonia excretion of naive and three-day recovered rats are not significantly different. Ammonia excretion rates achieved during naive challenge with 1.5 mmoles NH_4Cl per 100g body weight are approximately equivalent to the maximum

Table 6.1 Urinary ammonia excretion during naive challenge and rechallenge at different doses of NH_4Cl.

	URINARY AMMONIA EXCRETION mmoles/12h/100g body wt. ±SD (No. of Animals)				
NORMAL	0.08 ± 0.05 (30)				
NH_4Cl load (mmoles/100gbw)	0.5	1.0	1.5	2.0	2.5
Naive Challenge:					
0 → 12h	0.10±0.06(4)	0.39±0.09(4)	0.66±0.09(7)	0.44±0.10(7)	0.67±0.08(3)
12 → 24h	0.20±0.04(4)	0.73±0.24(4)	0.99±0.09(7)	0.81±0.15(7)	1.11±0.09(2)
24 → 36h	0.44±0.06(4)	0.69±0.11(3)	1.04±0.12(7)	0.97±0.27(7)	---
RECOVERY DAY 3	0.09 ± 0.03 (24)				
NH_4Cl load (mmoles/100gbw)	0.5	1.0	1.5	2.0	2.5
Rechallenge:					
0 → 12h	0.17±0.03(4)	0.49±0.20(4)	0.58±0.13(6)	0.84±0.13(7)¶	0.87±0.09(3)
12 → 24h	0.22±0.05(4)	0.70±0.09(4)	1.09±0.16(6)	1.34±0.34(7)¶	1.54±0.04(2)¶
24 → 36h	0.47±0.16(4)	0.78±0.10(4)	0.96±0.14(6)	1.31±0.16(7)¶	1.26 (1)

Naive rats and three-day recovered rats were administered different doses of NH_4Cl every 12h for 3 consecutive intubations and urines collected at 12h intervals for the analysis of ammonia.

¶ indicates significantly different from the corresponding value in the naive-challenge group ($P < 0.05$).

response of rats receiving 1.5% NH_4Cl, ad libitum (125). However, the dose administered (3 mmoles NH_4Cl/day per 100g body weight) to attain these rates is greater than the estimated amount of NH_4Cl consumed (2 mmoles/day per 100g body weight) during the 1.5% NH_4Cl challenge, ad libitum. This apparent discrepancy may be due to the different methods of inducing metabolic acidosis. Intubation of NH_4Cl would presumably place a more immediate demand on the animal for acid removal since the daily acid load is administered in two boluses, whereas drinking NH_4Cl may not require such urgent removal since its consumption is spread out over 24 hours. Consequently, the elimination of acid in forms other than ammonium salts may play a larger role in acidosis induced by NH_4Cl intubation.. Increased bone dissolution may be an important factor in this regard.

Figure 6.1 illustrates the response of ammonia excretion by naive and recovered rats to the different doses of NH_4Cl. At all doses except the lowest, both naive-challenged and rechallenged rats achieved maximum rates of ammonia excretion in the second collection period (12-24h), indicating that maximum rates were attained by 12 hours after the first intubation. Hence, the adaptation period is less than 12 hours in both groups. This suggests that the longer adaptation periods found for rats drinking NH_4Cl were a function of experimental protocol. At the lowest dose, presumably the acid-challenge was not sufficiently threatening to induce maximum rates of ammonia excretion within 12h of the first gavage.

In both naive-challenged rats and rechallenged rats, the rate of ammonia excretion increased with increasing NH_4Cl load up to 2.0 mmoles per 100g body weight. At this dose only the rechallenged animals showed

Legend for Figure 6.1

The data presented in this figure are contained in Table 6.1. Rats were permitted three days to recover from a previous acid challenge (7 days of NH_4Cl, ad libitum) and then rechallenged with different doses of NH_4Cl. Vertical error bars represent 1 SD. * indicates significantly different from normal or three-day recovery values and †, indicates significantly different from immediately preceeding value ($P < 0.05$).

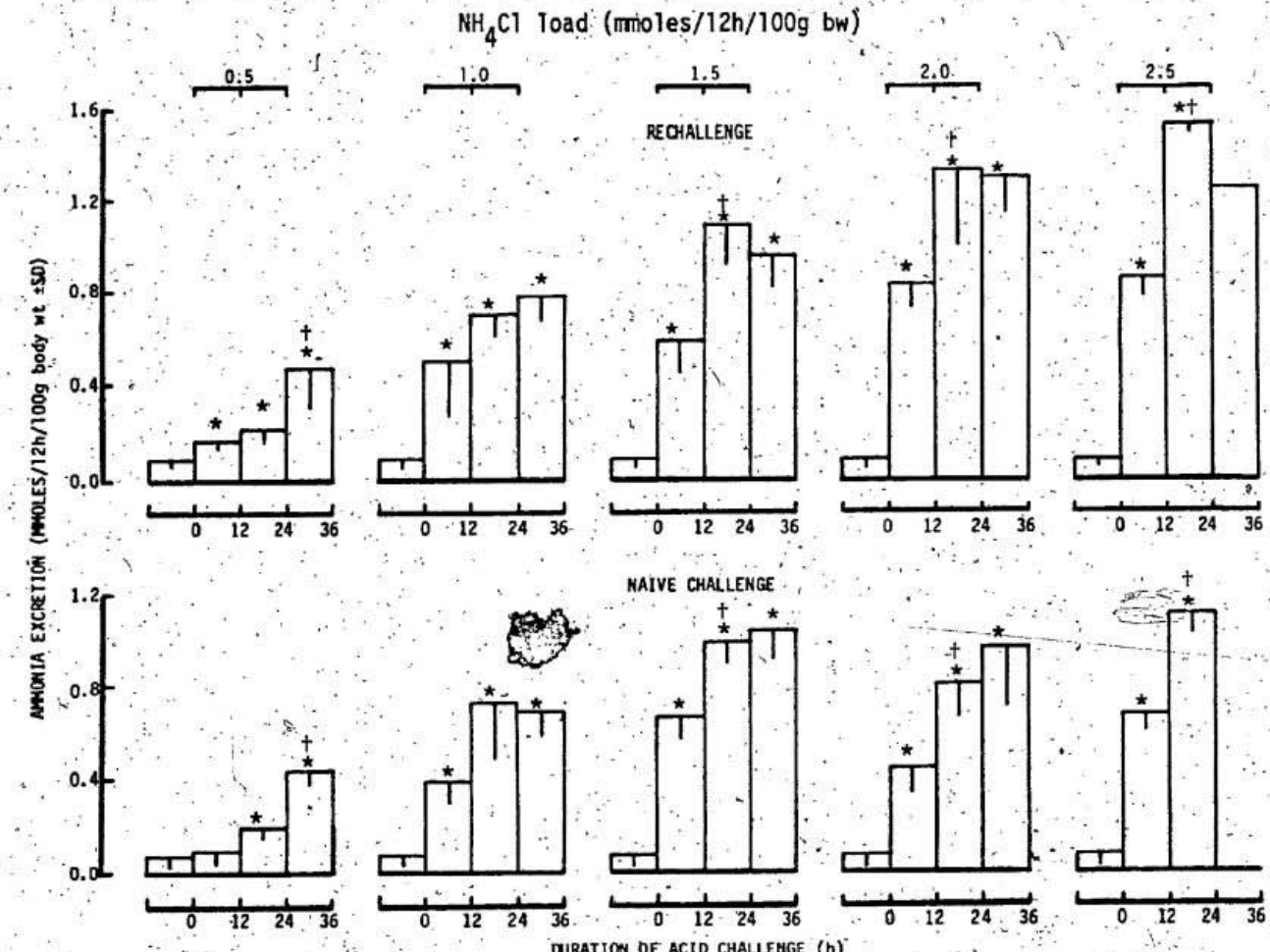


Figure 6.1 Effect of rechallenge on the response of urinary ammonia excretion to different doses of NH_4Cl.

a further increase in ammonia excretion. The naive-challenge group failed to increase their rate of ammonia excretion above that achieved at the dose of 1.5 mmoles NH_4Cl per 100g body weight. This lack of an increase does not agree with the remarkably steep increase in ammonia excretion found by Dies and Lotspeich (43) between the doses of 3 and 4 mmoles NH_4Cl per 100g body weight. However, these results are not directly comparable since the response obtained by Dies and Lotspeich was with rats which were intubated with NH_4Cl for three days. At the highest dose employed, 2.5 mmoles NH_4Cl per 100g body weight, both groups of rats became sick, hemoglobinuria was evident and none survived beyond 36 hours.

The results of this study indicate that recovered rats have an advantage over naive rats in being able to increase urinary ammonia excretion. However, this advantage is evident only when the animals are pushed to their extreme limit. This suggests that although the kidneys of naive rats have considerable ability to increase ammonia production, their maximum response is limited by some metabolic adaptation which is still present in recovered rats. All further studies were performed comparing the responses of naive and recovered rats to 1.5 and 2.0 mmoles NH_4Cl per 100g body weight only.

6.2 Glutamine Arterial Levels and Renal Arteriovenous Differences

Since glutamine is the major precursor for urinary ammonia, then the measurement of plasma glutamine levels should give some insight into the disposition of renal glutamine metabolism. For this reason, the concentrations of glutamine in the abdominal artery and renal vein were

determined and arteriovenous differences calculated. Comparison of the alterations in plasma glutamine levels in naive-challenged and rechallenged rats was used to gain some indication of the response of renal function of naive and recovered rats to acid challenge.

The results of this study are contained in Table 6.2. No significant difference is observed between normal and three-day recovered rats in either the arterial level of glutamine or the arterial renal venous difference. These low glutamine A-V differences are in agreement with the low rates of ammonia excretion in normal and recovered rats.

Figure 6.2 illustrates the response of arterial glutamine and glutamine A-V differences during acid challenge. Arterial glutamine levels fell significantly during the first intubation period. No further significant change occurred after 12 hours of acidosis. This decrease is consistent with previous observations (125). A significant difference between the naive-challenge group and the rechallenge group is observed at 12 hours with the lower dose of ammonium chloride. However, this finding may be artifactual since no difference is observed at the higher dose. The level to which arterial glutamine fell is the same in all four groups and may represent a minimum concentration which signals the release of additional glutamine by extra-renal tissues.

Glutamine A-V differences were increased at 12h in all four groups. Further increases are evident at 36h in the rechallenge group at the lower dose and in the naive-challenge group at the higher dose. The A-V differences observed at 12 hours after the first gavage are statistically identical for all groups except for the rechallenged animals given 2 mmoles NH_4Cl per 100g body weight, where glutamine extraction

Legend for Figure 6.2

The data presented in this figure are reported in Table 5.2. Naive rats and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl/12h per 100g body wt. Closed circles represent re-challenge group and closed triangles represent naive-challenge group. * indicates significantly different from normal or three-day recovery values and ¶ indicates a significant difference between groups ($P < 0.05$).

Table 6.2 Arterial glutamine levels and glutamine arterio renal-venous differences during naive challenge and rechallenge.

	ARTERIAL GLUTAMINE (μmoles/ml plasma ±SD)		GLUTAMINE A-V (μmoles/ml plasma ±SD)	
Normal	0.596±0.035 (4)		0.015±0.022 (4)	
NH_4Cl load (mmoles/100gbw)	1.5	2.0	1.5	2.0
Naive Challenge:				
12h	0.462±0.076 (4)	0.427±0.033 (5)	0.140±0.025 (4)	0.156±0.017 (5)
24h	0.367±0.039 (4)	0.496±0.119 (4)	0.173±0.041 (4)	0.206±0.060 (4)
36h	0.389±0.076 (4)	0.502±0.095 (4)	0.192±0.080 (4)	0.290±0.063 (4)
Recovery Day 3	0.677±0.085 (4)		0.032±0.038 (4)	
NH_4Cl load (mmoles/100gbw)	1.5	2.0	1.5	2.0
Rechallenge:				
12h	0.392±0.008 (4)	0.412±0.040 (5)	0.138±0.038 (4)	0.237±0.024 (4)
24h	0.405±0.087 (4)	0.392±0.069 (4)	0.220±0.046 (4)	0.245±0.036 (4)
36h	0.450±0.038 (4)	0.469±0.096 (7)	0.271±0.060 (4)	0.226±0.101 (7)

Naive rats and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl/12h per 100g body wt. Arterial and renal-venous bloods were sampled from animals at 12, 24 and 36 hours after starting treatment. Number in parenthesis indicates number of animals used.

was significantly elevated. This observation may reflect increased utilization of glutamine in these animals. Consequently, the advantage of recovered animals over naive animals in coping with 2.0 mmoles NH_4Cl per 100g body weight could be due to an increased ability of their kidneys to extract glutamine and produce ammonia from it.

6.3 Blood Acid-Base Parameters

The blood acid-base parameters were studied to obtain an indication of the effectiveness of naive and recovered rats to cope with an acid challenge. Since rechallenged animals excreted more ammonia than naive-challenged animals when confronted with a severe acid challenge, then it was of interest to see if this was advantageous to the animal in terms of acid-base homeostasis. Presumably, at the higher dose of NH_4Cl, the acid-base parameters of rechallenged rats would not be altered to the same extent as those in the naive-challenged rats. To test this hypothesis, blood was sampled from rats in all of the experimental situations under study. The results appear in Table 6.3. In this study, a slightly depressed H^+ ion concentration is observed in recovered rats compared with normal rats. However, no significant difference in the H^+ ion concentration between these animals is evident in the results from Chapter 3 of this thesis. Blood bicarbonate and pCO_2 are not significantly different and hence, the acid-base status of normal and three-day recovered rats is essentially the same. Figure 6.3 shows the response of these parameters during naive challenge and rechallenge with 1.5 and 2.0 mmoles NH_4Cl per 100g body weight.

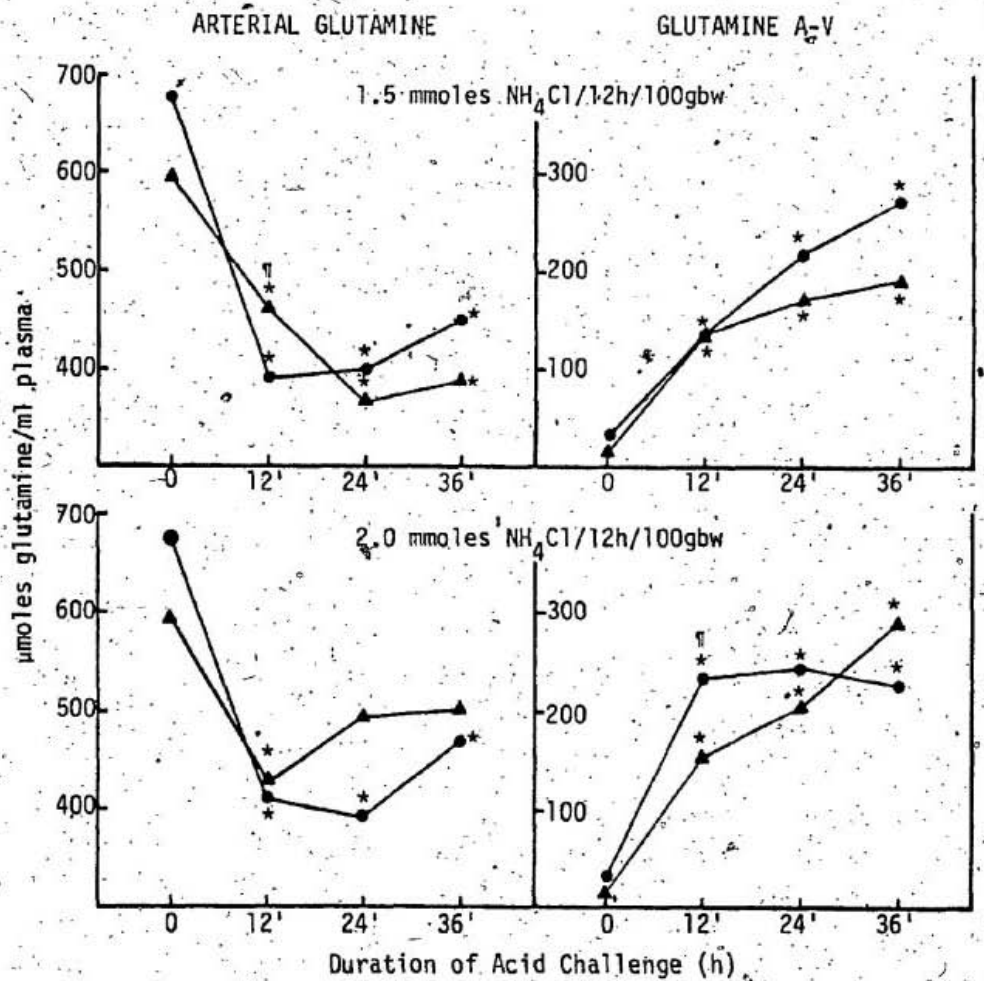


Figure 6.2 Effect of rechallenge on the response of glutamine arterial levels and glutamine arterio renal-venous differences to NH_4Cl intubation.

Table 6.3 Blood acid-base parameters during naive challenge and rechallenge.

	$[H^+]$ (mmoles/L)		$[HCO_3^-]$ (mmoles/L)		PCO_2 (mm Hg)	
Normal	43.8±2.3(4)		26.4±0.6(4)		48.6±2.3(4)	
NH_4Cl load (mmoles/100gbw)	1.5	2.0	1.5	2.0	1.5	2.0
Naive Challenge:						
12h	40.7±3.7(4)	48.9±4.1(3)	21.6±0.7(4)*	19.1±1.4(3)*	37.0±4.1(4)*	39.3±5.6(3)*
24h	52.6±3.7(4)*	70.3±10.1(3)*	18.4±2.0(4)*	13.6±2.3(3)*	40.3±1.5(4)*	39.4±1.6(3)*
36h	48.7±6.7(4)	65.1±7.0(4)*	19.7±4.8(4)*	15.4±2.8(4)*	39.3±4.7(4)*	41.5±3.6(4)*
Recovery Day 3	38.1±3.0(4)		26.0±2.3(4)		41.7±6.9(4)	
NH_4Cl load (mmoles/100gbw)	1.5	2.0	1.5	2.0	1.5	2.0
Rechallenge:						
12h	41.8±3.2(4)	47.7±3.0(3)*	21.8±1.7(4)*	23.4±0.9(3)	38.1±1.3(4)	46.8±1.3(3)
24h	41.6±2.6(4)	45.2±2.9(4)*	23.9±2.6(4)	24.1±1.4(4)	41.5±2.7(4)	45.8±4.5(4)
36h	54.2±6.0(4)*	50.0±5.4(6)*	15.6±1.5(4)*	21.4±2.9(6)*	35.2±0.5(4)	44.3±2.7(6)

Naive rats and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl/12h per 100g body wt and blood sampled at 12, 24 and 36 hours after the start of treatment for determination of acid-base parameters. Results are expressed as means ±SD. Number in parenthesis indicates the number of animals used. * indicates significantly different from normal or three-day recovery values ($P < 0.05$).

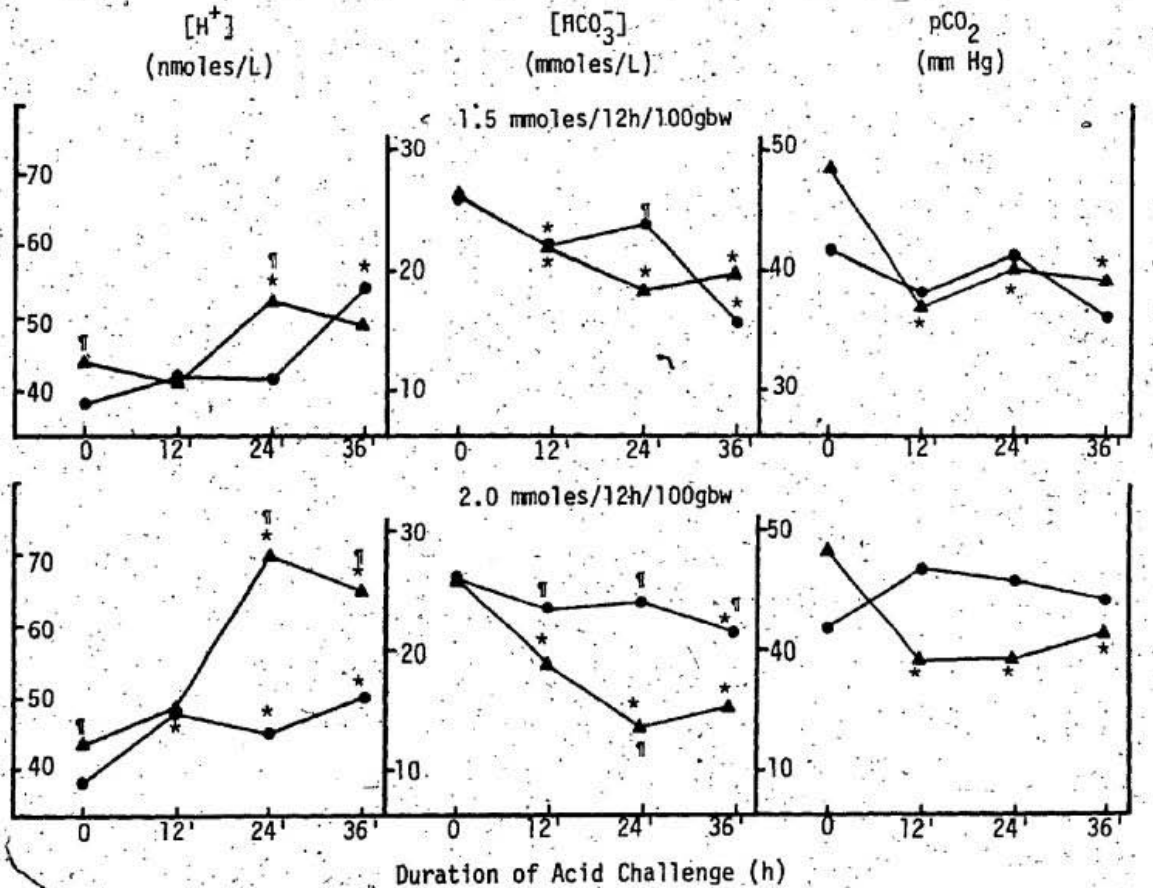


Figure 6.3 Effect of rechallenge on the response of blood acid-base parameters to NH_4Cl intubation.

The data presented in this figure are contained in Table 6.3. Naive and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl/12h per 100g body wt. Closed circles represent rechallenge and closed triangles represent naive challenge. * indicates significantly different from normal or three-day recovery value and ¶ indicates a significant difference between groups ($P<0.05$).

A. Response to 1.5 mmoles NH_4Cl per 100g body weight

In rats given the lower dose of NH_4Cl, there was no significant change in H^+ ion concentration at 12 hours. This does not imply that no change took place over this period, in fact, H^+ ion concentrations were acutely elevated but had returned to starting values by 12 hours (data not shown). Thereafter, a definite acidemia was evident at 24h in naive-challenged animals and at 36h in rechallenged animals. The bicarbonate concentration was significantly reduced after 12h in both groups of animals. A further decrease occurred in the naive-challenge group by 24h, which remained evident at 36 hours. The rechallenge group also showed a further decrease at 36h compared with the bicarbonate level at 12h. Significant decreases in pCO_2 are observed in the naive-challenge group only. The pCO_2 of normal rats is on the high side in this study although not significantly different from that of recovered rats. Although the pCO_2 values for the naive-challenged rats are significantly depressed compared with this high normal pCO_2, they are not significantly different from the corresppnding values in the rechallenge group.

The above results indicate that alterations in acid-base parameters occur in both naive-challenged and rechallenged rats intubated with 1.5 mmoles NH_4Cl per 100g body weight. H^+ ion and HCO_3^- concentrations are significantly different between the naive-challenged and rechallenged animals only at 24h of acid challenge. No other consistently distinguishable difference between the two groups is apparent. This is in keeping with the similar response of ammonia excretion and glutamine utilization by the kidneys of naive-challenged and rechallenged rats at this dose.

B. Response to 2.0 mmoles NH_4Cl per 100g body weight

Also illustrated in Figure 6.3 are the response of the acid-base parameters during intubation with 2.0 mmoles NH_4Cl per 100g body weight. A distinct difference between naive-challenged and rechallenged rats is noted. In the naive-challenge group, blood H^+ ion concentrations are markedly elevated at 24 and 36 hours. The concentrations reached in this group are much greater than those experienced by the rechallenge group. Although the H^+ ion concentrations at 12, 24 and 36 hours during rechallenge are significantly greater than that of three-day recovered rats, only the 36h value is significantly above normal. The changes in blood bicarbonate confirm the pattern observed with H^+ ion concentrations. In naive-challenged rats, bicarbonate was reduced after 12h and fell to dangerously low values at 24 and 36 hours of acid challenge. In rechallenged rats bicarbonate was reduced only in the 36h blood sample. At all times the bicarbonate concentrations were significantly higher in the rechallenge group. The pCO_2 values also appear to be affected in naive-challenged rats although no more so than at the lower dose. Again, these changes may be accentuated by the high normal pCO_2. No significant change in pCO_2 occurred during rechallenge. The results of this study clearly indicate a distinctly greater ability of recovered rats to cope with 2.0 mmoles of NH_4Cl per 100g body weight than naive rats.

These studies with the whole animal have provided the necessary groundwork for further, more detailed, metabolic studies. It was shown that recovered animals are more effective in coping with an acid challenge. This greater effectiveness is only evident at 2.0 mmoles NH_4Cl per 100g body weight and is due to the greater ability of the kidney of

these animals to extract glutamine and generate ammonia for the excretion of greater amounts of acid as ammonium salts. Further studies in this chapter are concerned with determining the reason for this difference and involve following the responses of PEPCK, PDG and GDH; as well as studying the response of isolated mitochondria incubated in different media.

6.4 Enzymes

The mitochondrial enzyme PDG and the cytoplasmic enzyme PEPCK catalyze two obligatory steps in the renal metabolism of glutamine. Both reactions are non-equilibrium steps and the contents of both enzymes increase during metabolic acidosis. These enzymes have received considerable attention because of their potential regulatory roles. The results reported in this thesis show that the activity of PDG remained elevated after renal ammonia production had returned to normal during recovery. This is taken to indicate that the capacity of this enzyme is not rate-limiting during recovery and hence renal ammoniagenesis is not governed by changes in the activity of PDG during this period. It was also demonstrated that the decrease in PEPCK activity and content does not necessarily coincide with the return of renal ammonia production during recovery. This indicates that changes in the amount of this enzyme are not rate-determining during recovery and therefore, the content of PEPCK also does not appear to be regulatory during this period.

Although the observed changes in the renal contents of these enzymes (as determined by the measurement of enzyme activity, in vitro) do not

appear to be regulatory for the metabolism of glutamine during recovery from metabolic acidosis, this does not necessarily imply that the same holds true for onset of metabolic acidosis. The increases in PDG and PEPCK activities during adaptation to metabolic acidosis in naive rats coincide with increases in renal ammonia excretion. The metabolic response of naive and recovered rats to acid challenge was first investigated by measuring the activities of PDG and PEPCK. It was thought that comparison of the responses of these enzymes to naive challenge and rechallenge may give some insight into the regulatory importance of these parameters during metabolic acidosis.

A. Phosphate-Dependent Glutaminase

Table 6.4 shows the activity of PDG during naive challenge and rechallenge. The activity is expressed in mmoles/24h/g kidney and mmoles/24h/100g body weight. PDG activity in the kidneys of three-day recovered rats was approximately twice that of naive rats. Figure 6.4 illustrates the response of PDG activity to 1.5 and 2.0 mmoles NH_4Cl per 100g body weight during naive challenge and rechallenge. The same trends are evident in both units.

At the lower dose of NH_4Cl, PDG activity increased in the naive-challenge group by 12h after the induction of acidosis and an additional increase occurred by 24h. No significant change occurred in PDG activity in the rechallenge group intubated with 1.5 mmoles NH_4Cl per 100g body weight. However, at this dose of NH_4Cl, the activity of PDG in rechallenged animals is significantly greater than that in naive-challenged rats at all times measured. Since ammonia excretion between these two

Table 6.4 Phosphate-dependent glutaminase activity in kidney homogenates during naive challenge and rechallenge.

	PHOSPHATE-DEPENDENT GLUTAMINASE ACTIVITY			
	mmoles/24h/g kidney ±SD, (n=4)		mmoles/24h/100g body wt ±SD, (n=4)	
Normal	25.9±1.2		20.9±0.9	
NH_4Cl load (mmoles/100gbw)	1.5	2.0	1.5	2.0
Naive Challenge:				
12h	32.2±3.8*	40.6±3.2*	24.0±3.2	29.2±2.2*
24h	43.8±5.5*	42.7±4.4*	33.7±5.4*	30.9±3.3*
36h	50.6±6.5*	54.2±11.4*	40.9±9.7*	42.1±8.6*
Recovery Day 3	56.7±16.3		43.9±9.1	
NH_4Cl load (mmoles/100gbw)	1.5	2.0	1.5	2.0
Rechallenge:				
12h	48.9±7.0	76.8±11.5	38.3±4.9	56.1±7.1
24h	60.5±13.4	89.8±10.9*	54.9±18.3	69.2±3.5*
36h	70.5±5.2	106.2±13.7*	58.9±7.3	84.5±9.9*

Naive rats and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl/12h per 100g body wt. Renal glutaminase activity was measured in homogenates of the entire kidny at 12, 24 and 36 hours after starting treatment. Activity is expressed as mmoles/24h/g kidney and as mmoles/24h/100g body wt. * indicates significantly different from normal or three-day recovery values ($P < 0.05$).

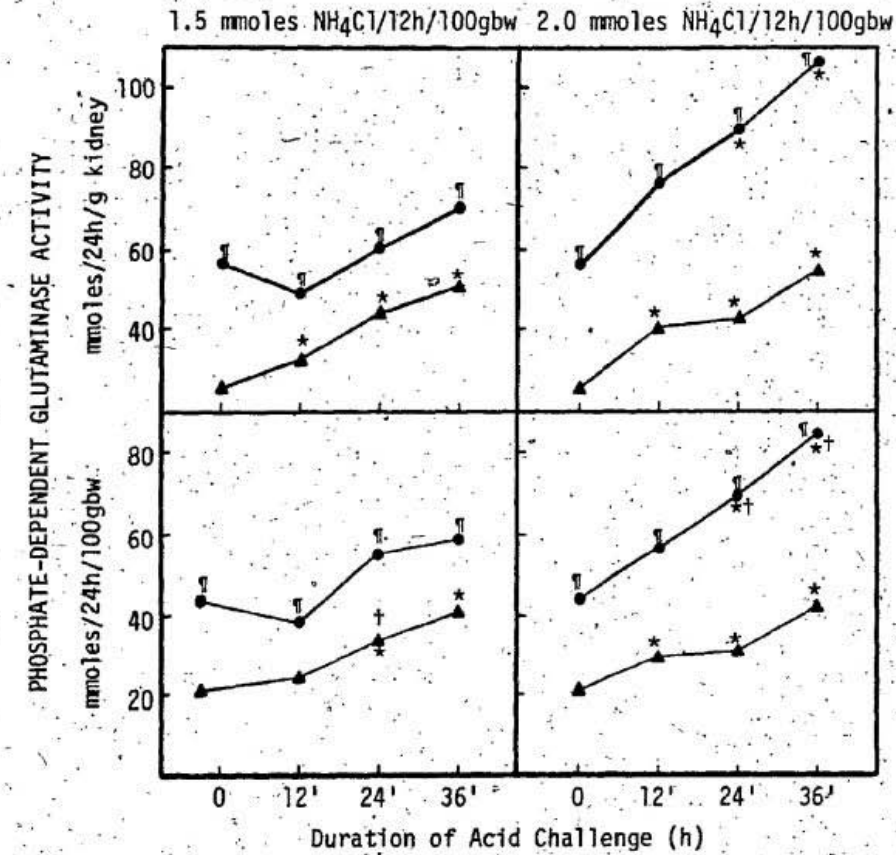


Figure 6.4 Effect of rechallenge on the response of phosphate-dependent glutaminase to NH_4Cl intubation.

Data presented in this figure are contained in Table 6.4. Naive and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl/12h per 100g body wt. Entire-kidney homogenates were used for the assay of glutaminase. Activity is expressed as mmoles/24h/g kidney and as mmoles/24h/100g body wt. Closed circles represent rechallenge and closed triangles represent naive challenge. * indicates significantly different from normal or three-day recovery values, † indicates significantly different from immediately preceeding value and ¶ indicates a significant difference between rechallenge and naive-challenge groups ($P < 0.05$).

groups was not different, then the extra amount of PDG in the rechallenge group can not be a decisive factor in governing the rate at which ammonia is formed during rechallenge with 1.5 mmoles NH_4Cl per 100g body weight. Obviously, the amount of PDG present in naive-challenged rats was sufficient for the rate of ammonia excretion obtained in these animals at this dose of NH_4Cl.

At the higher dose of NH_4Cl, PDG activity increased by 12h and again by 36h during naive challenge and by 24h and again by 36h in rechallenged animals. At all times, the activity of this enzyme was significantly greater in rats rechallenged with 2.0 mmoles NH_4Cl per 100g body weight than in the naive-challenge group intubated at the same dose. The presence of greater amounts of PDG may explain why rechallenged animals were able to excrete more ammonia than naive-challenged animals at this dose of NH_4Cl.

B. Phosphoenolpyruvate Carboxykinase

PEPCK activity was measured in supernatants prepared by high speed centrifugation of entire-kidney homogenates. Table 6.5 shows the activity of PEPCK during naive challenge and rechallenge with both doses of ammonium chloride. Again, enzyme activity is expressed in two ways. The activity of PEPCK in naive rats is not significantly different from that in three-day recovered rats. Figure 6.5 shows the response of PEPCK to acid challenge. PEPCK activity increased identically during the first 12h of acid challenge and again over the next 12h regardless of the experimental group or dose of NH_4Cl. PEPCK activity increased further only during naive challenge with 2.0 mmoles NH_4Cl per 100g body weight.

Table 6.5 Phosphoenolpyruvate carboxykinase activity during naive challenge and rechallenge.

	PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY			
	mmoles/24h/g kidney ±SD, (n=4)		mmoles/24h/100g body wt ±SD, (n=4)	
Normal	5.8±0.5		4.7±0.3	
NH_4Cl load (mmoles/100gbw)	1.5	2.0	1.5	2.0
Naive Challenge:				
12h	18.8±1.9	22.1±1.4	14.0±1.7	15.9±0.8
24h	31.7±4.3	30.8±2.9	24.3±3.2	22.3±2.3
36h	37.2±5.1	54.2±11.4	29.5±3.1	42.1±8.6
Recovery Day 3	4.8±1.0		3.8±1.1	
NH_4Cl load (mmoles/100gbw)	1.5	2.0	1.5	2.0
Naive Challenge:				
12h	19.8±1.1	20.9±1.4	15.5±0.8	15.4±2.2
24h	26.7±4.1	30.4±1.3	22.2±3.6	23.6±1.7
36h	25.3±2.1	31.2±2.6	21.1±1.9	24.9±2.7

Naive rats and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl/12h per 100g body wt. PEPCK activity was measured at 12, 24 and 36 hours after the start of treatment. All values are significantly different from normal or three-day recovered values ($P < 0.05$).

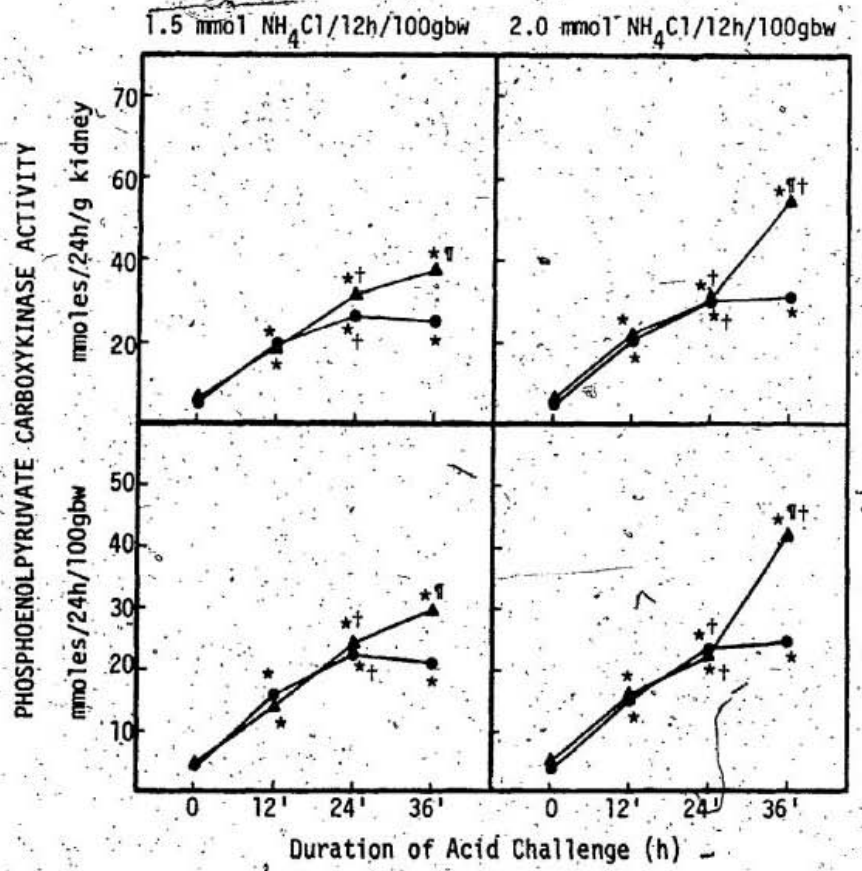


Figure 6.5 Effect of rechallenge on the response of phosphoenolpyruvate carboxykinase to NH_4Cl intubation.

Data presented in this figure are contained in Table 6.5. Naive and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl/12h per 100g body wt. Closed circles represent rechallenge and closed triangles represent naive challenge. * indicates significantly different from normal or three-day recovery values, † indicates significantly different from immediately preceeding value and ¶ indicates a significant difference between the naive-challenge and rechallenge groups ($P < 0.05$).

At 36h of acidosis, PEPCK activity is significantly higher in the naive-challenge groups compared with the rechallenge groups at the same dose of NH_4Cl.

The difference in ammonia excretions between naive-challenged and rechallenged rats are clearly not manifest in their contents of PEPCK, as reflected by PEPCK activities, in vitro. Although PEPCK may be essential for renal ammoniagenesis to proceed, changes in its activity can not account for the differences in ammonia excretion between rats rechallenged with 2.0 mmoles NH_4Cl per 100g body weight and the naive-challenge group at the same dose. Recovered rats do not appear to have a greater ability to increase the content of PEPCK compared with naive rats when challenged with an acid load. This is consistent with a permanent decrease in PEPCK protein and enzyme synthesis during recovery, rather than conversion of active enzyme to an inactive form which would be expected to be activated faster during rechallenge. Consequently, it appears that the content of PEPCK is not necessarily a decisive factor in regulating renal ammoniagenesis.

6.5 Mitochondrial Studies

The response of naive and recovered rats to acid challenge was also studied using isolated mitochondria. As in the studies reported in the previous chapter, mitochondria were incubated with 1 mM glutamine $\pm$3 mM glutamate at the lower phosphate concentration only. At this phosphate concentration, mitochondrial glutamine metabolism returned to normal during recovery in both the absence and presence of glutamate. Since maximum rates of ammonia excretion were attained by 12h after the start

Table 6.6 Medium changes and enzyme fluxes in mitochondrial incubations with glutamine as sole substrate during naive challenge and rechallenge.

	LOW PHOSPHATE MEDIUM ($4mM\ KH_2PO_4$)					
	1mM Glutamine					
	Medium Changes:			Flux:		
Experimental Condition	Ammonia	Glutamate	Aspartate	PDG	GDH	GOT
	Δnmoles/mg/min±SD			nmoles/mg/min±SD		
Normal	14.4±3.7 (12)	1.6±1.2 (12)	4.2±2.0 (12)	10.1±2.4 (12)	4.3±1.9 (12)	4.2±2.0 (12)
Acidotic	38.2±5.3 (9)	1.6±0.7 (9)	2.9±0.9 (8)	20.8±3.1 (8)	16.6±2.2 (8)	2.9±0.9 (8)
Recovery Day 3	20.7±4.1 (8)	2.1±1.3 (8)	3.8±1.2 (8)	13.3±2.2 (8)	7.4±2.3 (8)	3.8±1.2 (8)
Naive Challenge 12h (1.5 mmol NH_4Cl/100gbw)	18.7±0.6 (4)	2.2±0.5 (4)	3.5±0.1 (4)	12.2±0.3 (4)	6.6±0.5 (4)	3.5±0.1 (4)
Rechallenge 12h (1.5 mmol NH_4Cl/100gbw)	26.6±3.1 (4)	2.0±0.5 (4)	3.8±0.9 (4)	16.2±1.3 (4)	10.4±2.0 (4)	3.8±0.9 (4)
Naive Challenge 12h (2.0 mmol NH_4Cl/100gbw)	21.1±3.9 (4)	1.7±0.6 (4)	3.8±0.9 (4)	13.3±2.5 (4)	7.9±1.5 (4)	3.8±0.9 (4)
Rechallenge 12h (2.0 mmol NH_4Cl/100gbw)	22.8±4.1 (4)	2.5±0.3 (4)	3.3±0.3 (4)	14.3±2.0 (4)	8.6±2.2 (4)	3.3±0.3 (4)

Normal rats and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl per 100g body weight. Kidney-cortex mitochondria were prepared from these rats 12h later and incubated for 4 minutes in the presence of 4 mM phosphate and 1 mM glutamine. Normal, acidotic and three-day recovery values are the same as those reported in Tables 3.5 and 3.6.

of acid challenge, then the necessary adaptations had obviously taken place by then. Consequently, only mitochondria isolated from rats 12h after the first gavage were studied. Changes in medium ammonia, glutamate and aspartate were followed and these results were used to calculate fluxes through PDG, GDH and GOT.

The results of these studies represent the response *in vitro* of glutamine metabolism by isolated mitochondria to NH_4Cl administered *in vivo* to naive and three-day recovered rats. For discussion purposes, the experimental condition of the rats from which the mitochondria were obtained will be used to describe the type of mitochondria referred to. For example, "normal mitochondria" will be used to refer to mitochondria isolated from normal rats and "three-day recovery mitochondria" will mean mitochondria prepared from three-day recovered rats. The terms "naive challenge" and "rechallenge" always refer to the experimental situation induced *in vivo*, and when these terms are used in the discussion of the mitochondrial results it is assumed to be understood that the results represent the response of isolated mitochondria to acidosis, induced *in vivo*.

A. Medium Changes in Ammonia, Glutamate and Aspartate

Tables 6.6 and 6.7 contain the medium changes in ammonia, glutamate and aspartate during the mitochondrial incubations in the low-phosphate media with glutamine in absence and presence of glutamate, respectively. These medium changes are also illustrated in Figures 6.6 and 6.7. Although maximum rates of ammonia excretion were evident by 12h of acid challenge, mitochondrial ammoniagenesis did not achieve acidotic levels

Table 6.7 Medium changes and enzyme fluxes in mitochondrial incubations with glutamine and glutamate as substrates during naïve challenge and rechallenge.

	LOW PHOSPHATE MEDIUM - (4mM KH_2PO_4)					
	1mM Glutamine + 3mM Glutamate					
	Medium Changes:			Flux:		
Experimental Condition	Ammonia	Glutamate	Aspartate	PDG	GDH	GOT
	Δnmoles/mg/min±SD			nmoles/mg/min±SD		
Normal	6.6±2.6 (12)	-15.7±4.0 (8)	20.9±5.5 (12)	7.9±3.8 (10)	0.6±5.3 (12)	20.9±5.5 (12)
Acidotic	21.9±3.9 (9)	-12.4±5.4 (7)	20.9±2.8 (9)	15.1±4.5 (8)	6.8±3.5 (9)	20.9±2.8 (9)
Recovery Day 3	8.0±2.1 (8)	-9.4±4.2 (8)	28.5±7.6 (8)	13.6±3.3 (8)	-5.6±3.4 (8)	28.5±7.6 (8)
Naive Challenge 12h (1.5 mmoles NH_4Cl/100gbw)	7.8±0.7 (4)	-7.4±4.0 (4)	21.1±3.0 (4)	10.8±2.1 (4)	-3.0±1.0 (4)	21.1±3.0 (4)
Rechallenge 12h (1.5 mmoles NH_4Cl/100gbw)	11.6±1.6 (4)	-15.7±6.1 (4)	27.7±1.2 (4)	11.4±2.8 (4)	-0.2±3.5 (4)	27.7±1.2 (4)
Naive Challenge 12h (2.0 mmoles NH_4Cl/100gbw)	10.4±3.4 (8)	-4.6±7.5 (8)	23.9±3.5 (8)	14.8±5.7 (8)	-4.5±3.9 (8)	23.9±3.5 (8)
Rechallenge 12h (2.0 mmoles NH_4Cl/100gbw)	14.5±3.4 (4)	-9.6±0.7 (4)	22.9±2.6 (4)	13.9±2.0 (4)	0.6±2.5 (4)	22.9±2.6 (4)

Normal rats and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl per 100g body wt. Kidney-cortex mitochondria were prepared from these rats 12h later and incubated for 4 minutes in the presence of 4 mM phosphate, 1 mM glutamine and 3 mM glutamate. Normal, acidotic and three-day recovery values are the same as those reported in Tables 3.5 and 3.6.

in the same amount of time. Obviously, the maximum mitochondrial adaptations observed during metabolic acidosis are not essential for acidotic rates of ammonia excretion.

(1) Incubations with 1 mM Glutamine - In the incubations with glutamine as sole substrate, ammonia formation by "three-day recovery mitochondria" was still significantly higher than that of "naive mitochondria" (Figure 6.6). Significant increases occurred in this medium in all cases except during rechallenge with 2.0 mmoles NH_4Cl per 100g body weight. At the lower dose of NH_4Cl, comparable increases in ammonia formation occurred in both groups such that at 12h after the induction of acidosis mitochondrial ammoniagenesis in the rechallenge group was still significantly higher than that in the naive-challenge group. However, at this dose of NH_4Cl, ammonia excretion *in vivo* during rechallenge is not significantly different from that during naive challenge (Table 6.1). Therefore, the higher mitochondrial capacity evident in this medium during rechallenge must not be expressed *in vivo*.

At the higher dose of NH_4Cl, mitochondrial ammoniagenesis increased during naive challenge such that the two groups were not significantly different at 12h. At this dose of NH_4Cl, rechallenged rats excreted more ammonia than naive-challenged rats (Table 6.1). Obviously, the responses of mitochondrial ammoniagenesis in this medium do not reflect the different responses in ammonia excretion *in vivo* during naive challenge and rechallenge with 2.0 mmoles NH_4Cl per 100g body weight. If a mitochondrial adaptation is responsible for the different rates of urinary ammonia excretion observed during intubation with 2.0 mmoles NH_4Cl per 100g body weight, then it is not evident in this medium.

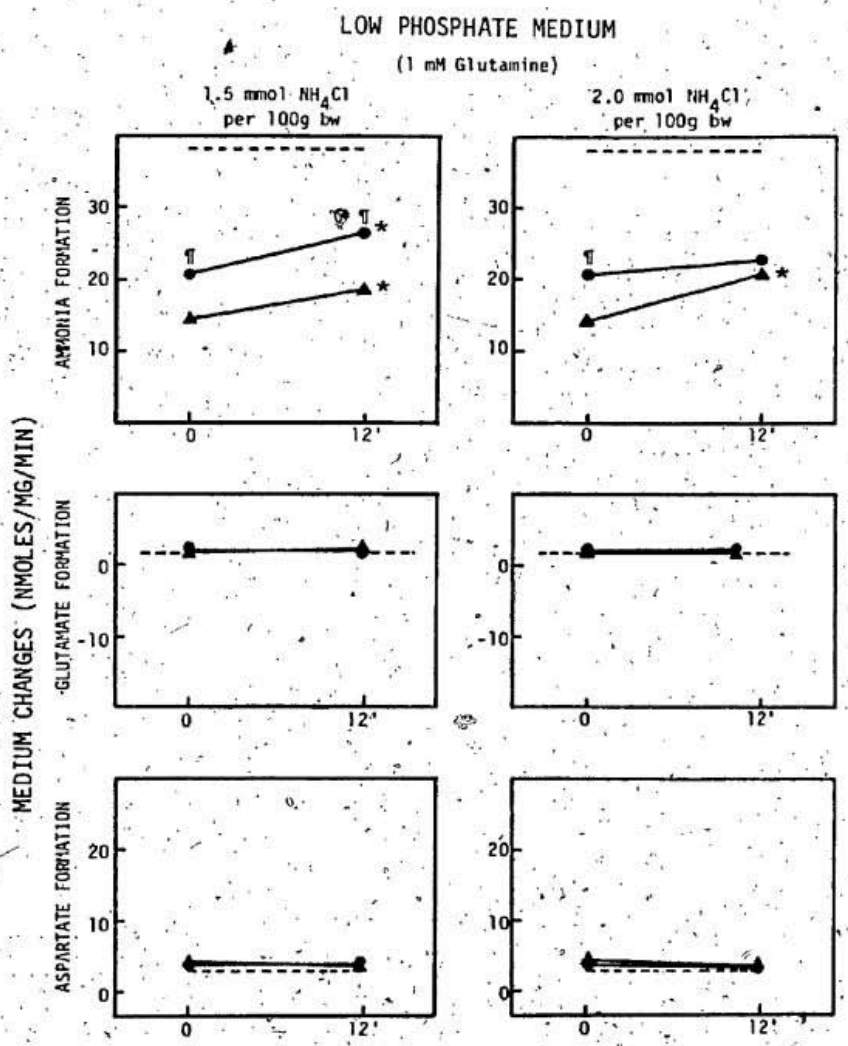


Figure 6.6 Effect of rechallenge on medium changes in mitochondrial incubations with glutamine as sole substrate.

Data presented in this figure are contained in Table 6.6. Normal and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl per 100g body wt. Kidney-cortex mitochondria were isolated from these rats 12h later and incubated for 4 minutes in the presence of 4 mM phosphate and 1 mM glutamine. Closed circles represent the rechallenge group and closed triangles represent the naive-challenge group. Dashed lines represent acidotic values. * indicates significantly different from normal or three-day recovery values and ¶ indicates a significant difference between the naive-challenge and rechallenge groups ($P < 0.05$).

Also shown in Figure 6.6 are the medium changes in glutamate and aspartate during the incubations in the low-phosphate medium without added glutamate. Both glutamate formation and aspartate formation were low in this medium and did not change significantly during naive challenge or rechallenge at either dose of NH_4Cl.

(2) Incubations with 1 mM Glutamine plus 3 mM Glutamate - In the low-phosphate medium containing glutamate, rates of ammonia formation by "naive" and "three-day recovery mitochondria" are not significantly different (Figure 6.7). This is consistent with the similar rates of urinary ammonia excretion for these same animals (Table 6.1). Significant increases in mitochondrial ammoniagenesis occurred in all cases except during naive challenge with 1.5 mmoles NH_4Cl. The increase during rechallenge at the lower dose of NH_4Cl was such that at 12h of acidosis mitochondrial ammoniagenesis in the rechallenge group is significantly higher than that in the naive-challenge group. Despite this difference, rates of urinary ammonia excretion at this dose of NH_4Cl are not significantly different between naive-challenged and rechallenged rats (Table 6.1). At the higher dose of NH_4Cl, mitochondrial ammoniagenesis increased in both the naive-challenge and rechallenge groups. After 12h of acidosis at this dose, ammonia formation in the rechallenge group is not significantly different from that in the naive-challenge group, despite the fact that urinary ammonia excretion is (Table 6.1). Also illustrated in Figure 6.7 are the medium changes in glutamate and aspartate.

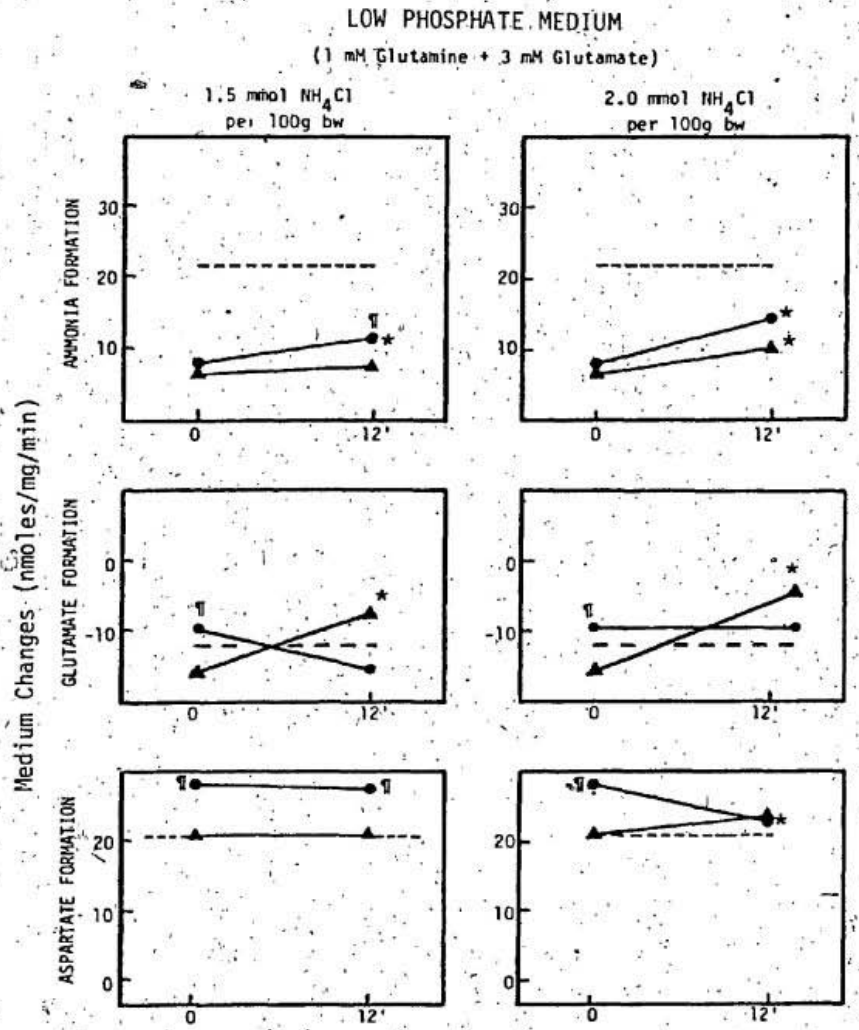


Figure 6.7 Effect of rechallenge on medium changes in mitochondrial incubations with glutamine and glutamate as substrates.

Data presented in this figure are contained in Table 6.7. Normal and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl per 100g body wt. Kidney-cortex mitochondria were isolated from these rats 12h later and incubated for 4 minutes in the presence of 4 mM phosphate, 1 mM glutamine and 3 mM glutamate. Closed circles represent the rechallenge group and closed triangles represent the naive-challenge group. Dashed lines represent acidotic values. * indicates significantly different from normal or three-day recovery values and ¶ indicates a significant difference between the naive-challenge and rechallenge groups ($P < 0.05$).

B. Mitochondrial Fluxes through Phosphate-Dependent Glutaminase, Glutamate Dehydrogenase and Glutamate Oxaloacetate Transaminase

Tables 6.6 and 6.7 also contain the fluxes through PDG, GDH and GOT during the mitochondrial incubations with glutamine in the two low-phosphate media. These fluxes are also illustrated in Figures 6.8 and 6.9. Although a mitochondrial basis responsible for the enhanced renal ammonia excretion in rats rechallenged with 2.0 mmoles NH_4Cl per 100g body weight was not demonstrated in these media, it is apparent from the medium changes in glutamate and aspartate that "naive-challenge mitochondria" and "rechallenge mitochondria" metabolize glutamine similarly in the medium containing glutamine as sole substrate, but do so differently in the presence of 3 mM glutamate. Examination of the mitochondrial fluxes involved in the metabolism of glutamine helps to explain these observations.

(1) Incubations with 1 mM Glutamine - In this medium, the mitochondrial fluxes through PDG and GDH in mitochondria from three-day recovered rats are still significantly elevated above normal (Figure 6.8). This accounts for the difference in the rates of mitochondrial ammoniagenesis between "naive" and "three-day recovery mitochondria". The fluxes through GOT are not significantly different between these same groups. A significant increase in PDG flux occurred only during naive challenge with 2.0 mmoles NH_4Cl per 100g body weight. GDH flux increased during naive challenge at both doses of NH_4Cl, but did not change significantly during rechallenge. At the lower dose of NH_4Cl, both PDG flux and GDH flux remained significantly elevated in the rechallenge group compared to the naive-challenge group. After 12h of acidosis at the higher dose of NH_4Cl, the increases which occurred during naive challenge were

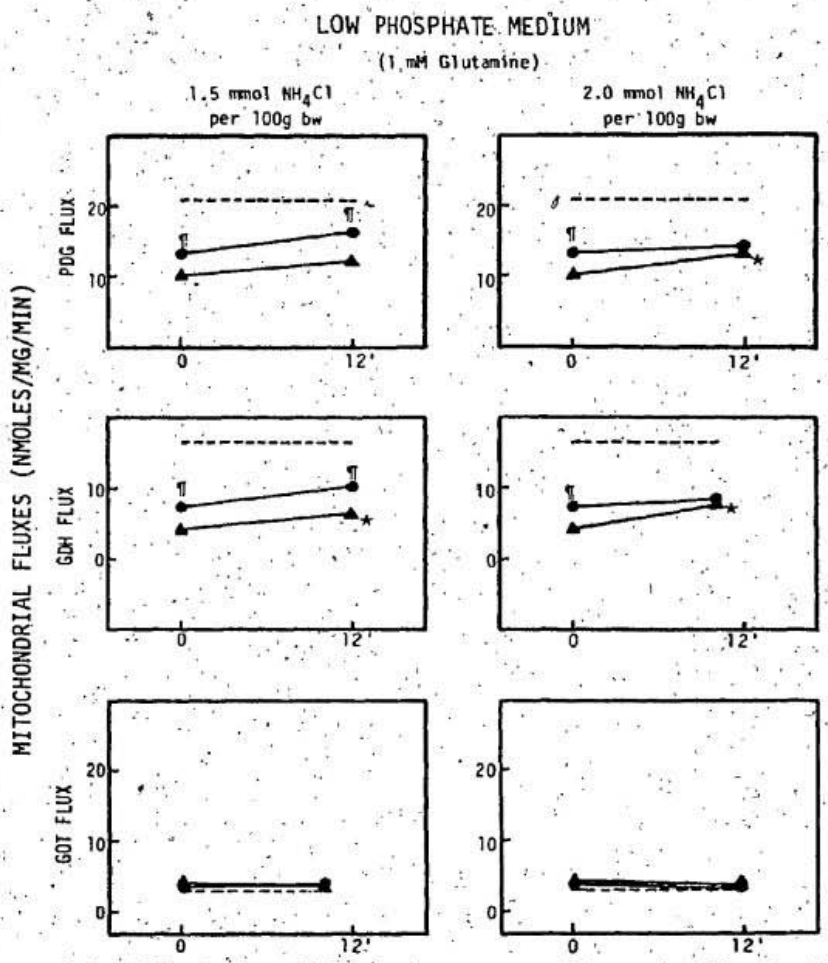


Figure 6.8 Effect of rechallenge on enzyme fluxes in mitochondrial incubations with glutamine as sole substrate.

Data presented in this figure are contained in Table 6.6. Normal and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl per 100g body wt. Kidney-cortex mitochondria were isolated from these rats 12h later and incubated for 4 minutes in the presence of 4 mM phosphate and 1 mM glutamine. Closed circles represent the rechallenge group and closed triangles represent the naive challenge group. Dashed lines represent acidotic values. * indicates significantly different from normal or three-day recovery values and ¶ indicates a significant difference between the naive-challenge and rechallenge groups ($P < 0.05$).

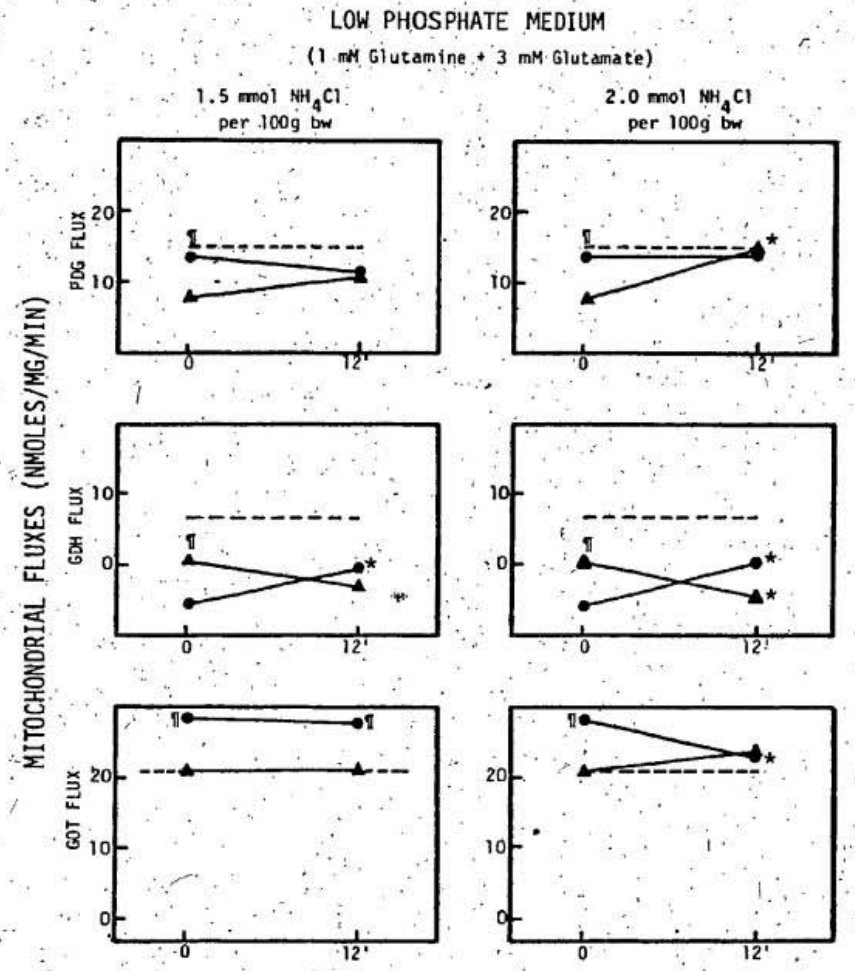


Figure 6.9 Effect of rechallenge on enzyme fluxes in mitochondrial incubations with glutamine and glutamate as substrates.

Data presented in this figure are contained in Table 6.6. Normal and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl per 100g body wt. Kidney-cortex mitochondria were isolated from these rats 12h later and incubated for 4 minutes in the presence of 4 mM phosphate, 1 mM glutamine and 3 mM glutamate. Closed circles represent the rechallenge group and closed triangles represent the naive challenge group. Dashed lines represent acidotic values. * indicates significantly different from normal or three-day recovery values and ¶ indicates a significant difference between the naive-challenge and rechallenge groups ($P < 0.05$).

such that the fluxes through PDG and GDH were not significantly different in "rechallenge mitochondria" compared to "naive-challenge mitochondria". The fluxes through GOT in the mitochondria incubated in this medium did not change significantly during the acid challenges.

These fluxes indicate that in mitochondria incubated in the low-phosphate medium with glutamine as sole substrate, the rate of mitochondrial ammoniagenesis is determined by the fluxes through both PDG and GDH. The trends observed in these fluxes during naive challenge and rechallenge indicate that the increases in mitochondrial ammonia formation were due to increases of similar magnitude in the fluxes through both of these enzymes. The amount of glutamate formed was not affected since glutamate formation and removal were equally altered.

(2) Incubations with 1 mM Glutamine plus 3 mM Glutamate - In the low phosphate medium containing both glutamine and glutamate, PDG flux in "three-day recovery mitochondria" was still significantly elevated compared to normal (Figure 6.9). In these same mitochondria, the net flux through GDH was in the direction of glutamate formation. Hence, the "extra" ammonia formed via PDG was utilized to form glutamate via GDH. Consequently, in this medium, mitochondrial ammoniagenesis in "three-day recovery mitochondria" is not significantly different from normal.

PDG flux increased significantly only during naive challenge with 2.0 mmoles NH_4Cl per 100g body weight. After 12h of acidosis at both doses of NH_4Cl, the fluxes through this enzyme were not significantly different between the naive-challenge group and the rechallenge group. During naive challenge, GDH flux changed significantly only at the

higher dose of NH_4Cl and this change was such that after 12h of acidosis the net flux through GDH was in the direction of glutamate formation. During rechallenge, glutamate formation via GDH decreased such that after 12h of acidosis at both doses of NH_4Cl, the net fluxes through GDH were essentially zero. GOT flux did not change significantly during naive challenge at either dose of NH_4Cl and remained significantly elevated during rechallenge at the lower dose compared to that in the naive-challenge group. At the higher dose of NH_4Cl, GOT flux fell during rechallenge such that the fluxes through this enzyme in "naive-challenge" and "rechallenge mitochondria" were not significantly different.

These fluxes indicate that when 3 mM glutamate is present in the incubation medium "naive mitochondria" and "three-day recovery mitochondria" metabolize glutamine differently and respond differently to an acid load, administered in vivo. In "naive mitochondria", ammonia formation is determined by the flux through PDG since GDH flux is essentially zero. The increase in mitochondrial ammoniagenesis observed during naive challenge with 2.0 mmoles NH_4Cl per 100g body weight was the result of an increase in the flux through PDG. This increase in ammonia formation occurred despite an increase in the flux through GDH in the direction of glutamate formation since the change in PDG flux was greater than the change in GDH flux.

In "three-day recovery mitochondria", the amount of ammonia formed was determined by the fluxes through both PDG and GDH. In these mitochondria the flux through GDH utilized a portion of the ammonia formed via PDG. The difference in these fluxes determined the rate of mito-

chondrial ammoniagenesis for "three-day recovery mitochondria". During rechallenge the increases in ammonia formation observed in this medium were the result of decreases in the rate of ammonia utilization for glutamate formation via GDH.

C. Mitochondrial Enzymes: Phosphate Dependent Glutaminase and Glutamate Dehydrogenase

The activities of PDG and GDH were determined in the same mitochondrial preparations that were used in the incubation studies reported above. These activities are contained in Table 6.8 and are also illustrated in Figure 6.10.

Phosphate-dependent glutaminase activity rose 70% and 68% after 12h of acidosis during naive challenge with 1.5 and 2.0 mmoles NH_4Cl per 100g body weight, respectively. During naive challenge, PDG flux in isolated mitochondria increased significantly only at the higher dose of NH_4Cl. An increase in PDG flux of 32% occurred in mitochondria incubated with glutamine as sole substrate and in the mitochondrial incubations with both substrates present, PDG flux increased 87 percent. Hence, the increase in PDG activity during naive-challenge with 2.0 mmoles NH_4Cl per 100g body weight is sufficient to account for these increases in PDG flux.

Although the difference in the means of PDG activity between zero time and 12h of acidosis is of the same order of magnitude during rechallenge as during naive-challenge, the activity of PDG after 12h of acidosis during rechallenge is not significantly different from that in three-day recovered rats. This is probably because of the greater variation associated with the higher PDG activities during rechallenge compared

Table 6.8 Phosphate-dependent glutaminase and glutamate dehydrogenase activities in mitochondrial preparations during naive challenge and rechallenge.

Experimental Condition	Phosphate-Dependent Glutaminase	Glutamate Dehydrogenase
	nmoles/mg/min ± SD (No. of Animals)	
Normal	393.9±76.5 (12)	857.5±161.6 (9)
Acidotic	1175.0±259.2 (13)	2142.8±348.7 (9)
Recovery Day 3	1235.4±420.2 (6)	1357.1±169.9 (10)
Naive Challenge 12h (1.5 mmoles NH_4Cl/100gbw)	667.9±80.9* (4)	1098.8±63.1 (4)
Rechallenge 12h (1.5 mmoles NH_4Cl/100gbw)	1422.1±200.6 (4)	2157.2±206.1* (4)
Naive Challenge 12h (2.0 mmoles NH_4Cl/100gbw)	661.2±72.8* (8)	959.0±157.6 (8)
Rechallenge 12h (2.0 mmoles NH_4Cl/100gbw)	1502.3±433.0 (8)	2135.0±172.0* (4)

Naive and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl per 100g body wt and 12h later mitochondria prepared from kidney cortex for the assay of glutaminase and glutamate dehydrogenase.
* indicates significantly different from normal or three-day recovery values ($P < 0.05$).

to those during naive-challenge. This lack of a significant change in PDG activity during rechallenge is consistent with a similar absence of a significant change in PDG flux in isolated mitochondria during rechallenge.

Glutamate dehydrogenase activity was not significantly altered by 12h of acidosis during naive-challenge at either dose of NH_4Cl. Despite this, the flux through GDH increased significantly in mitochondria incubated with glutamine as sole substrate during naive-challenge at both doses of NH_4Cl. This may be taken to suggest that changes in the flux through GDH are not governed by changes in the total mitochondrial activity of this enzyme. A lack of a relationship between GDH activity and GDH flux is also seen during the mitochondrial incubations containing both glutamine and glutamate as substrates. In this medium GDH flux changed significantly (i.e. the flux in the direction of glutamate formation increased) during naive-challenge with 2.0 mmoles NH_4Cl per 100g body weight despite the fact that enzyme activity was not altered.

Glutamate dehydrogenase activity rose 59% and 57% after 12h of acidosis during rechallenge with 1.5 and 2.0 mmoles NH_4Cl per 100g body weight, respectively. Despite these increases, GDH flux did not change significantly during rechallenge in mitochondria incubated in the medium containing glutamine as sole substrate and decreased (i.e. in the direction of glutamate formation) in the medium containing both substrates. These observations further support the conclusion that changes in GDH flux are not dependent upon changes in the activity of this enzyme.

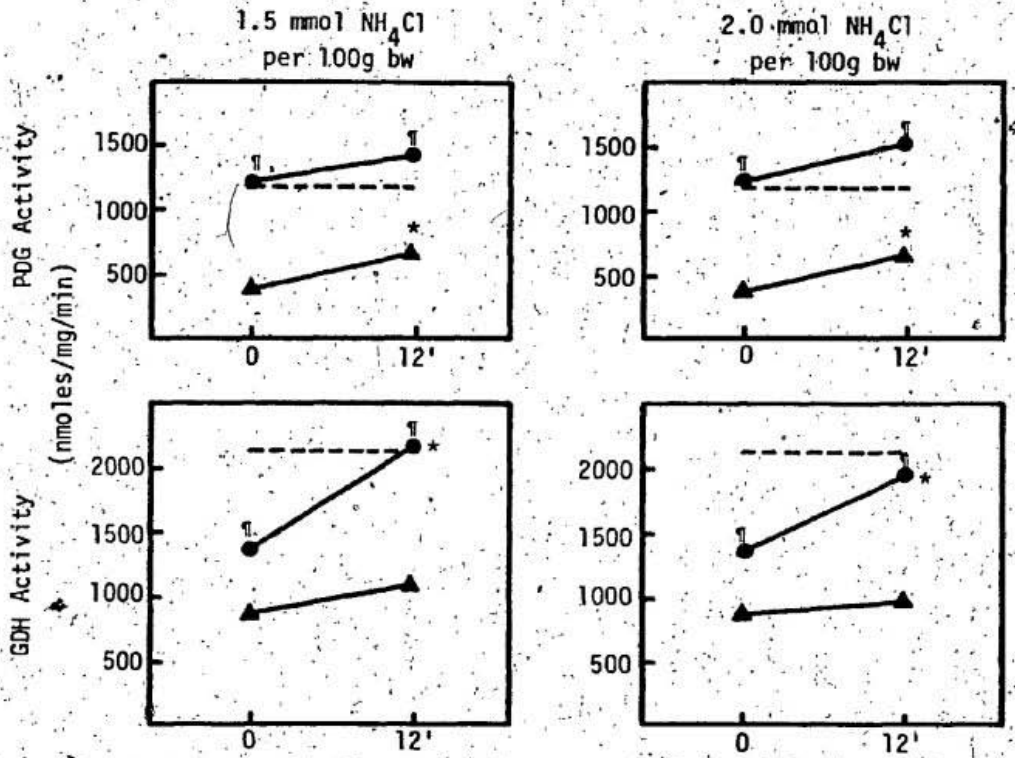


Figure 6.10 Effect of rechallenge on the response of phosphate-dependent glutaminase and glutamate dehydrogenase to NH_4Cl intubation.

Data presented in this figure are contained in Table 6.8. Naive and three-day recovered rats were intubated with 1.5 or 2.0 mmoles NH_4Cl per 100g body wt and 12h later mitochondria prepared from kidney cortex for the assay of glutaminase and glutamate dehydrogenase. Closed circles represent rechallenge and closed traingles represent naive challenge. Dashed lines represent enzyme activity in acidotic rats. * indicates significantly different from normal or three-day recovery values and ¶ indicates a significant difference between naive-challenge and rechallenge groups ($P < 0.05$).

6.6 Discussion

The results of this chapter clearly indicate that three-day recovered rats are better able to cope with a large acid load than naive rats. This is indicated by the lesser perturbation of blood acid-base parameters in rechallenged rats compared to that in naive-challenged rats. That this advantage in coping with a large acid load was due to an enhanced renal ability of rechallenged rats to produce ammonia is suggested by the observation that rechallenged rats excrete more ammonia than naive-challenged rats when intubated with 2.0 mmoles NH_4Cl per 100g body weight. Furthermore, the glutamine A-V difference at the end of the first urine collection period (during which the difference in urinary ammonia excretions between the naive-challenge and rechallenge groups was greatest) was significantly elevated in the rechallenge group compared with that in the naive-challenge group. This suggests that more glutamine was extracted by the kidneys during rechallenge than during naive-challenge. Taken together these observations may reflect that more ammonia was produced by the kidneys of rechallenged rats intubated with 2.0 mmoles NH_4Cl per 100g body weight than by the kidneys of naive-challenged rats administered the same dose.

This apparent difference in renal ammoniagenesis between naive-challenged and rechallenged rats can not be accounted for by differences in PEPCK activity. However, the identical increases in PEPCK activity during the first 24h of naive challenge and rechallenge can not be taken to indicate that the flux through this enzyme was identically changed in both groups. It was shown in Chapter 5 of this thesis that changes in PEPCK flux are not necessarily accompanied by changes in PEPCK activity.

There was no significant difference in the abilities of isolated mitochondria from naive-challenged and rechallenged rats (intubated with 2.0 mmoles NH_4Cl per 100g body weight) to generate ammonia when incubated in the most physiological medium employed (1 mM glutamine, 3 mM glutamate and 4 mM phosphate) as might be expected if mitochondrial adaptations were responsible for the apparent difference in renal ammoniagenesis. However, the results of the mitochondrial studies do show that isolated mitochondria respond differently to naive challenge and rechallenge when incubated in the presence of physiological concentrations of phosphate (4 mM), glutamine (1 mM) and glutamate (3 mM). The increase observed in mitochondrial ammoniagenesis during naive challenge was shown to be due to an increase in the flux through PDG. On the other hand, PDG flux was not significantly altered in mitochondria during rechallenge. The increase in mitochondrial ammoniagenesis during rechallenge occurred as a result of a change in GDH flux, such that the formation of glutamate from ammonia and α-ketoglutarate was reduced. It is also apparent from the mitochondrial studies that a change in GDH flux in isolated mitochondria is not essential for increased renal ammoniagenesis since during naive challenge, maximum rates of ammonia excretion were attained while the flux through GDH was not significantly changed or was slightly decreased. Furthermore, it appears that the glutamate dehydrogenase reaction is at or near equilibrium since the flux through this enzyme can proceed in either direction. The factors responsible for governing the direction of GDH flux are not known, but obviously involve mitochondrial events which remain evident in isolated mitochondria.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

7.1 Summary

The experiments reported in this thesis show the following:

1. An increase in malate content and a decrease in phosphoenolpyruvate content indicate that the conversion of malate to phosphoenolpyruvate via PEPCK was inhibited at one day of recovery. The renal contents of glutamine and α-ketoglutarate returned to normal and the contents of glutamate, citrate and lactate increased to above-normal levels by one day of recovery.

2. PEPCK activity returned to control values by 24h of recovery.

3. Immunotitration of PEPCK revealed that the decrease in PEPCK activity during recovery is due to a decrease in the renal content of this enzyme.

4. The activities of phosphate-dependent glutaminase and glutamate dehydrogenase were not different from acidotic values at one day of recovery and remained significantly elevated above normal for at least eleven days.

5. Mitochondrial ammoniagenesis and the flux through glutaminase in isolated mitochondrial increased during metabolic acidosis. During recovery, these parameters returned to normal between one and seven days of recovery in all media except in that containing 1 mM glutamine and 20 mM phosphate. In this medium, both ammonia

formation and PDG flux remained elevated for at least 14 days of recovery.

6. The flux through glutamate dehydrogenase in isolated mitochondria incubated in the presence of 1 mM glutamine and 3 mM glutamate was markedly enhanced in acidosis and decreased to normal by one day of recovery.

7. Changes in the fluxes through phosphate-dependent glutaminase and glutamate dehydrogenase in isolated mitochondria can not be attributed to changes in enzyme activities alone. In the medium containing physiological concentrations of phosphate (4 mM), glutamine (1 mM) and glutamate (3 mM), the fluxes through PDG and GDH decreased to normal even though the activities of these enzymes were still elevated.

8. Recovery from metabolic acidosis was accelerated by the administration of $NaHCO_3$. Urinary ammonia excretion was back to normal within 8h of recovery in rats intubated with $NaHCO_3$ whereas in rats intubated with NaCl ammonia excretion was still significantly elevated above normal at 8h of recovery.

9. The renal content of glutamine returned to normal by 8h of recovery in the $NaHCO_3$-treated group while that in the NaCl-treated group tended to remain depressed. The renal content of α-ketoglutarate returned to normal by 8h of recovery in both groups.

10. PEPCK activity remained elevated at acidotic levels at 8h of recovery in animals intubated with $NaHCO_3$ and returned to normal on

the same time course as that in animals intubated with NaCl.

11. The flux through GDH in isolated mitochondria incubated with physiological concentrations of phosphate (4 mM), glutamine (1 mM) and glutamate (3 mM) returned to normal by 8h of recovery in both the NaCl-treated group and the $NaHCO_3$-treated group.

12. Three-day recovered rats excreted more acid as ammonium salt than naive animals when intubated with 2.0 mmoles NH_4Cl/12h per 100g body weight. No difference in ammonia excretion between naive-challenged and rechallenged animals was found at lower doses.

13. Maximum rates of ammonia excretion in naive-challenged rats were achieved by 12h after the initiation of metabolic acidosis despite the fact that GDH flux in isolated mitochondria incubated in the presence of physiological concentrations of phosphate (4 mM), glutamine (1 mM) and glutamate (3 mM) was not increased.

14. Isolated mitochondria responded differently to naive-challenge and rechallenge. The increase in ammoniagenesis by mitochondria incubated with 1mM glutamine and 3 mM glutamate was due to an increase in the flux through PDG during naive-challenge, whereas during rechallenge the increase in mitochondrial ammoniagenesis was due to a change in GDH flux.

7.2 General Discussion

The complexity of the control of renal ammoniagenesis is attested to by the numerous hypotheses which have been advanced to explain this

account for the differences in renal ammoniagenesis in the experimental situations studied. However, the results of this thesis permit certain conclusions to be made regarding specific features of the regulatory mechanisms which have been proposed.

(1) Mitochondrial Glutamine Transporter

The transport of glutamine into mitochondria was not measured in the present studies. However, the ability of isolated mitochondria to generate ammonia from glutamine provides a minimum measure of glutamine transport. During recovery from metabolic acidosis, isolated mitochondria produced ammonia at near-acidotic rates for at least 15 days when incubated with glutamine as sole substrate and 20 mM phosphate. Since renal ammoniagenesis *in vivo* had returned to normal by day one of recovery then the capacity of the glutamine transporter (i.e. number of carrier molecules in the inner mitochondrial membrane) can not be rate-limiting during recovery from metabolic acidosis. Furthermore, the importance of a full adaptation in glutamine transport to the regulation of renal ammoniagenesis during the onset of acidosis is undermined by the fact that acidotic rates of ammonia excretion were achieved during naive-challenge despite the fact that mitochondrial ammoniagenesis from glutamine did not increase maximally. However, the glutamine transporter could well be a regulatory locus since changes in α-ketoglutarate affect glutamine transport by altering the K_m of the transport system (20). The changes observed in the renal content of α-ketoglutarate during acidosis and recovery found in the present studies are consistent with this idea.

phenomenon. Despite this apparent complexity, the proposed hypotheses are, in principal, relatively simple. It should be recognized that although postulating simple mechanisms of control is initially appropriate and essential from an experimental point of view, it may not be appropriate from a theoretical point of view. It is easier to test simpler hypotheses than more complex ones. Theoretically, however, it may not be appropriate because living organisms, through the evolutionary process, often deliberately favoured the more complex systems of control since the flexibility of these systems made the organism more adaptable. Hence, the control of renal ammonia production is probably multifactorial, involving a variety of mechanisms at multiple sites.

The approach taken in this thesis involved studying the renal metabolic response to changes in the requirement for acid excretion. Requirements for acid excretion change during the onset of metabolic acidosis and during recovery from metabolic acidosis. It was shown that the excretion of ammonia in both situations was subject to experimental manipulation. Animals which were allowed three days to recover from a previous acid challenge were able to excrete more acid as ammonium salt during rechallenge compared with naive-challenged animals when intubated with a large acid load. The intubation of $NaHCO_3$ at the start of recovery accelerated the return of renal ammonia excretion and production to normal. Determining the relationship of purported regulatory parameters to renal ammoniagenesis in these situations was an experimental necessity and not meant to imply that the regulation of this important process may be entrusted to a single mechanism. Although a number of different metabolic parameters were studied, no single event could be found to

(2) Phosphate-Dependent Glutaminase

The original form of the hypothesis proposed by Pitts (23) was that renal ammoniagenesis is regulated by changes in the concentration or amount of glutaminase within the renal tubular cells. However, it is now apparent that changes in the content of PDG are not necessarily of regulatory importance since renal ammoniagenesis increases during metabolic acidosis in dogs without a change in renal glutaminase activity (181) and in rats when the increase in PDG activity was inhibited by the administration of actinomycin D (91). A similar conclusion can be drawn from the results of the present studies. The activity of PDG remained elevated long after renal ammoniagenesis returned to normal during recovery.

However, it is now known that renal glutaminase exists in two forms, as a catalytically-active dimer and as an inactive monomer (154). Phosphate has been shown to cause dimerization and glutamate competes with phosphate and causes dissociation of PDG into monomers (154). Accordingly, a change from one form to another could serve to regulate renal glutamine metabolism <u>in vivo</u> without changing assayable PDG activity. In the present studies, deamidation of glutamine by isolated mitochondria incubated at the physiological concentration of phosphate (glutamine as sole substrate) decreased during recovery whereas at the higher phosphate concentration the flux through PDG remained elevated for at least 15 days of recovery. Although speculative, these results may reflect the conversion of PDG dimers to monomers during recovery, an event which presumably was reversed by the higher phosphate concentration.

(3) Glutamate Dehydrogenase

It has been suggested that alterations in the metabolism of intramitochondrial glutamate may be responsible for the regulation of renal glutamine metabolism (28). According to Schoolwerth et al (28) glutamate dehydrogenase does not catalyze a reaction close to thermodynamic equilibrium and hence changes in the flux through this enzyme can not be accounted for by equilibrium considerations. This conclusion was based on the value of the mass action ratio for the glutamate dehydrogenase reaction in isolated mitochondria which was calculated using the NAD/NADH ratio determined from the β-hydroxybutyrate dehydrogenase equilibrium. However, it has not been shown that glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase are located in the same part of the nephron. Studies by Vinay et al (112) using isolated kidney tubules indicate that the GDH reaction is reversible and that the flux through this system is dependent upon the concentration of the reactants involved. Furthermore, reversibility of glutamate dehydrogenase is also indicated in the present studies since the direction of the flux through GDH changes in different experimental situations. These findings are suggestive of an equilibrium situation for the glutamate dehydrogenase system in vitro.

Regardless of whether or not glutamate dehydrogenase catalyzes an equilibrium reaction, the flux through this enzyme is markedly altered in isolated mitochondria during acid-base manipulations in vivo. What events are responsible for these changes is not known. However, the event which is responsible for the increased flux through GDH during metabolic acidosis is probably not of regulatory importance since maximum rates of renal ammoniagenesis were achieved during naive challenge.

without an increase in GDH flux in isolated mitochondria when incubated in the presence of physiological concentrations of phosphate (4 mM), glutamine (1 mM) and glutamate (3 mM). The change in GDH flux during recovery indicates that the event responsible for this change could be of regulatory potential. However, since GDH flux in isolated mitochondria also returned to normal during recovery in the NaCl group while renal ammoniagenesis remained elevated, then presumably other factors must also be involved in the regulation of renal glutamine metabolism during recovery.

(4) Phosphoenolpyruvate Carboxykinase

It is clear from the results in this thesis that changes in renal ammoniagenesis can occur without changes in PEPCK activity.. This is evident at 8h of recovery in the $NaHCO_3$ group when PEPCK activity was not significantly different from acidotic values while ammonia production by the kidney was back to normal. Furthermore, PEPCK activity increased identically during the first 24h of acidosis in both naive-challenged and rechallenged rats intubated with 2.0 mmoles NH_4Cl/12h per 100g body weight although the ammonia excretions are significantly different between these two groups. Therefore, it appears that the content of PEPCK is not necessarily important in the regulation of renal ammoniagenesis.

(5) α-Ketoglutarate Dehydrogenase

One potential regulatory mechanism not considered in the course of this work involves α-ketoglutarate dehydrogenase. Boyd and Goldstein

(93) report a fall in α-ketoglutarate with no significant change in malate in the kidneys of rats one hour after the oral administration of 0.5 M NH_4Cl (1% body wt). Vinay et al (181) found decreases in the renal contents of glutamine, glutamate and α-ketoglutarate in rat and dog during the first 2h of HCl-induced acidosis, while malate content remained unchanged. Furthermore, α-ketoglutarate content remained depressed in dog kidney during chronic acidosis despite a five-fold increase in the content of malate (181). Ross and Tannen (182) found that inhibition of PEPCK increased the malate content in perfused kidneys but did not prevent the fall in glutamate and α-ketoglutarate contents upon acidification of the perfusion medium. These findings suggest a direct effect of acidosis on a metabolic step situated between α-ketoglutarate and malate. One potential step which might be affected is the irreversible reaction catalyzed by α-ketoglutarate dehydrogenase. Studies with pig-heart α-ketoglutarate dehydrogenase indicate that changes in Ca^{++} and H^{+} concentrations alter the K_m of this enzyme for α-ketoglutarate (183). Considering this, it is possible that changes in the content of α-ketoglutarate in the kidney may occur as a result of alterations in the K_m of renal α-ketoglutarate dehydrogenase for α-ketoglutarate. Changes in the removal of α-ketoglutarate would presumably affect the removal of glutamate via glutamate dehydrogenase and thereby could regulate the activity of phosphate-dependent glutaminase. In addition, alterations in the mitochondrial content of α-ketoglutarate may affect the concentration of this metabolite in the cytoplasm via the operation of the reversible malate/α-ketoglutarate antiporter and in turn alter the transport of glutamine into mitochondria (20). Potentially,

(190, 191, 192, 193). The application of similar methods to kidney cells may well be required in order to elucidate the regulatory mechanisms operating in renal glutamine metabolism.

at least, α-ketoglutarate dehydrogenase may figure prominently in the regulation of renal ammoniagenesis but its involvement and exact role remain to be demonstrated.

(6) Heterogeneity and Compartmentation

One of the major problems associated with studying renal glutamine metabolism is tissue heterogeneity. The presence of numerous cell types in the kidney hampers the study of events which occur in a specific type of cell. The application of sophisticated methods of analyzing enzyme activities in selected portions of the nephron has been one approach which has been successful in dealing with the problem of heterogeneity. However, enzyme activities can only be used as a measure of the maximal metabolic capacities present and do not provide any indication of metabolic fluxes. The use of isolated cells and tubules reduces the complexity of the kidney preparation under consideration. However, such *in vivo* preparations not only lose intact renal function but also suffer from the obvious criticism that the conditions in which these preparations are studied do not entirely mimic *in vivo* conditions. Renal cells in culture offer the greatest potential for solving the problems associated with tissue heterogeneity, however, cells in culture may not manifest all the same characteristics they have *in situ* (185).

Compartmentation within the cell, especially between cytoplasm and mitochondria also complicates the interpretation of metabolic studies in the kidney. Recent attempts have been made to determine the concentrations of metabolites in the cytoplasm and mitochondria of rat liver cells after separation of these compartments following cell disruption

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