REGULATION OF RENAL GLUTAMINE METABOLISM IN THE RAT

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REGULATION OF RENAL GLUTAMINE METABOLISM IN THE RAT

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TO MY PARENTS

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ABSTRACT

One means whereby the mammalian kidney excretes strong acid is by the formation of neutral ammonium salts in the urine. In response to a continuous acid challenge, the rate of ammonia excretion increases to meet the increased acid load. In the present study a strong acid load was administered to rats for 7 days. Ammonia excretion increased slowly reaching plateau levels after about three days. After the acid challenge was discontinued, the rats were placed back on tap water and allowed to recover. Ammonia excretion abruptly fell to normal within 24 hours. Phosphate dependent glutaminase (PDG) activity, the penetration of glutamine into mitochondria (the site of PDG) and phosphoenolpyruvate carboxykinase (PEPCK) activity have all been considered as rate-determining for the renal production of ammonia. For this reason the activities of PDG and PEPCK were measured in vitro during adaptation and recovery. Also glutamine metabolism (ammonia production and oxygen consumption) and the metabolism (oxygen consumption) of related substrates (glutamate, α-ketoglutarate and succinate) by isolated kidney cortex mitochondria were followed. In addition, glutamine metabolism (glutamine utilization, ammonia production, glutamate formation and glucose production) and the metabolism (glucose production) of related substrates (oxaloacetate, malate, lactate and pyruvate) by kidney cortex slices were measured during recovery. Mitochondrial capacity (PDG activity, oxygen consumption and ammonia production from glutamine) appears to correlate with the excretion of ammonia in the urine during adaptation. This correlation is consistent with ammonia production being regulated by a mitochondrial event. PEPCK attained maximum activity in vitro 24 hours before ammonia excretion

reached its plateau. This suggests that the amount of PEPCK is not rate-determining during adaptation. During recovery mitochondrial capacity remained at high plateau levels whereas the activity of PEPCK *in vitro* fell to normal values within 24 hours. The lack of a correlation between mitochondrial capacity and urine ammonia excretion suggests that renal ammonia production is not regulated solely by a mitochondrial event during recovery. No change in the mitochondrial metabolism of glutamate, α-ketoglutarate or succinate was observed during either adaptation or recovery. The remarkable correlation found during recovery between PEPCK activity *in vitro*, the metabolism of glutamine and related substrates by kidney cortex slices, and urinary ammonia excretion is consistent with PEPCK playing a regulatory role in ammonia production during this phase. Glutamine arterio-renal venous differences were also measured during adaptation and recovery and at all times paralleled the rate of ammonia excretion in the urine. Blood pH, $[HCO_3^-]$, and pCO_2 were followed to help assess their possible regulatory significance. No simple correlation appears to exist between any of these parameters and urinary ammonia excretion.

Rats recovering from 7 days of acid challenge were also starved. During this starved recovery PEPCK decreased slightly but remained elevated, yet ammonia excretion returned to normal. This dissociation is not consistent with this enzyme alone being regulatory for ammonia production.

Rats were also returned to tap water after only 3 days of acid challenge. In comparison to recovery from 7 days of acid challenge urinary ammonia excretion persisted for an additional 24 hours. Since the

acid-base status of the animal was shown to improve only after ammonia excretion had been fully adapted for some time, then the persistence of elevated ammonia excretion supports the idea that the excretion of ammonia during recovery is related to restoration of acid-base balance.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-Diphosphate
a_{H}	Hydrogen Ion Activity
ATP	Adenosine 5'-Triphosphate
BSA	Bovine Serum Albumin
EDTA	Ethylenediamine Tetraacetic Acid
EGTA	Ethyleneglycol-bis-(β-Amino-ethyl ether)N,N'-Tetra Acetic Acid
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid
ITP	Inosine 5'-Triphosphate
β-NAD	β-Nicotinamide Adenine Dinucleotide
β-NADH	β-Nicotinamide Adenine Dinucleotide, Reduced Form
OAA	Oxaloacetate
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate Carboxykinase
PDG	Phosphate Dependent Glutaminase
Tris	Tris(hydroxymethyl) amino-methane

INTRODUCTION

Recently, Robert Pitts called attention to Claude Bernard's concept that "we have achieved a free and independent life, mentally and physically by becoming relatively independent of our external environment" (84). Since the kidneys play a prominent role in the regulation of the composition of the internal environment, Pitts feels justified to claim "that our present high station in the animal kingdom ultimately depends on our kidneys" (84).

A. Excretion of Acid

One of the most precisely regulated components of the internal environment is the concentration of hydrogen ions. Under normal circumstances, the pH of blood plasma of mammals is maintained remarkably constant within the limits of 7.35 and 7.45, in spite of the many processes occuring which might be expected to change it greatly. This precise regulation is necessary because of the highly reactive nature of hydrogen ions. Their small radius permits very strong interactions with negatively charged groups of macromolecules. Since the physiological activities of macromolecules depend upon charge and conformation, changes in the concentration of hydrogen ions will markedly affect their activities, and thus impair their biological roles. The minimum pH of blood which can be tolerated is generally stated to be 7.0.

Hydrogen ions are derived from the daily ingestion of acid in the diet and from acids produced in the normal metabolism of foodstuffs and from the catabolism of body proteins and phospholipids. The stabilization of hydrogen ions is accomplished by the chemical buffering systems of the

body. Some acids (lactic, β-hydroxybutyric and acetoacetic acids), under normal conditions, only transiently contribute hydrogen ions because they are metabolized further. Other acids (carbonic, sulfuric, hydrochloric and phosphoric acids) cannot be metabolized further and in order to prevent exhaustion of the chemical buffers, these acids must be eliminated from the body. Carbonic acid is in equilibrium with CO_2 and H_2O. The removal of CO_2 by the lungs effects the elimination of this acid. All other acids which cannot be metabolized further are transported, mainly as neutral sodium salts, from their source via the blood to the kidneys.

In the span of 24 hours, acid production for normal man on a normal mixed diet is such that the kidneys are required to excrete about 40-80 milliequivalents of hydrogen ions in the urine (83). The elimination of these hydrogen ions (Figure 1) involves the exchange of tubular sodium ions for hydrogen ions of the adjacent tubular cells (81). In this exchange, Na^+ ions enter the tubular cells down an electrochemical gradient, which essentially drives the carrier-linked (72) secretion of H^+ ions into the tubular lumen against an electrochemical gradient (68, 89). Although the favourable Na^+ electrochemical gradient is maintained by the pumping of sodium ions from the tubular cells into the peritubular blood (83), the exchange mechanism can operate only up until a H^+ ion concentration gradient of about 800-1000 to 1 between the tubular lumen and the peritubular blood (81). This means that the kidney cannot form urine of pH less than about 4.4-4.8.

In the elimination of H^+ ions, the exchange mechanism titrates the conjugate bases of the neutral sodium salts which were formed in the blood

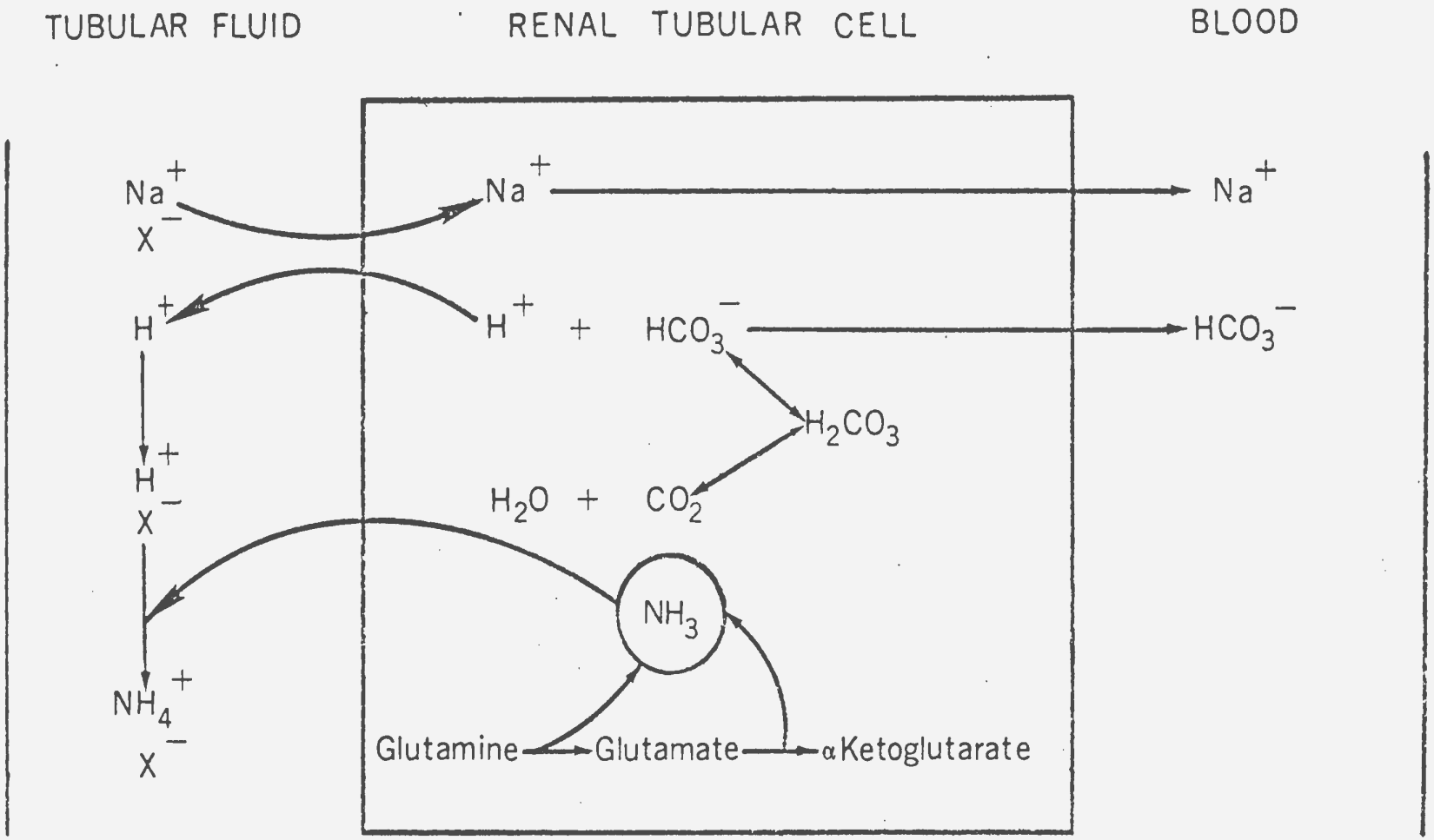


Figure 1 Excretion of Acid

for their transportation to the kidney (Figure 1). The H^+ ions, which are secreted into the tubular fluid, and the HCO_3^- ions, which are returned to the peritubular blood in association with Na^+ ions, are derived from cellular carbonic acid. This H_2CO_3 is derived in part from CO_2 produced in cellular metabolism and in part from CO_2 brought to the cell in peritubular blood. The amount of free acid which can be formed without achieving a limiting pH gradient depends upon the pK of the acid. If the pK is high, in comparison to the pH of the urine, then a relatively large amount of H^+ ions can be excreted as the free form of the acid. If the pK is low, then the acid being formed will largely be dissociated and the H^+ ions may easily accumulate so as to prevent continued exchange.

In normal man some 10 to 30 mEq of the total 40 to 80 mEq of H^+ ions excreted each day are excreted as free or titratable acid (83). The major fraction of this titratable acid is dihydrogen phosphate. The pK of this acid is 6.8 and therefore the monohydrogen form of the acid presented to the kidney is largely titrated to the dihydrogen form without appreciably lowering the urinary pH. The remaining 30-50 mEq of H^+ ions cannot be excreted as titratable acid because the pKs of the acids that would be formed are very low and the necessary fall in urinary pH would prevent continued secretion of H^+ ions.

Ammonia plays a unique role in overcoming this potential limitation in the excretion of stronger acids. Its importance is dependent on the fact that ammonia is both a gas and a base. By virtue of its gaseous nature, small size and lipid solubility, ammonia can rapidly diffuse from the renal tubular cells, its site of production, into the tubular lumen.

By virtue of its basicity, ammonia can bind hydrogen ions in the tubular fluid to form ammonium ions. A pK of 9.2 - 9.3 for ammonia means that at urinary pH the formation of ammonium ions is greatly favoured. Ammonium ions are not lipid-soluble and as a result are essentially trapped in the tubular fluid.

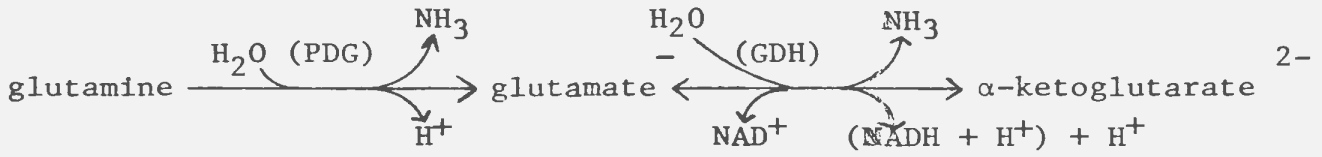
The diffusion of ammonia into the acid urine and the formation of impermeant ammonium ions, therefore, prevents the accumulation of tubular H^+ ions and allows for the excretion of strong acids as neutral ammonium salts. The conversion of ammonia to ammonium tends to lower the pNH_3 of the tubular fluid and thus favours continued ammonia diffusion. This mechanism therefore allows for the continued exchange of Na^+ ions for H^+ ions, effecting the elimination of H^+ ions from the body and the replenishment of blood buffer.

B. Production of Ammonia

1. Metabolic Pathways

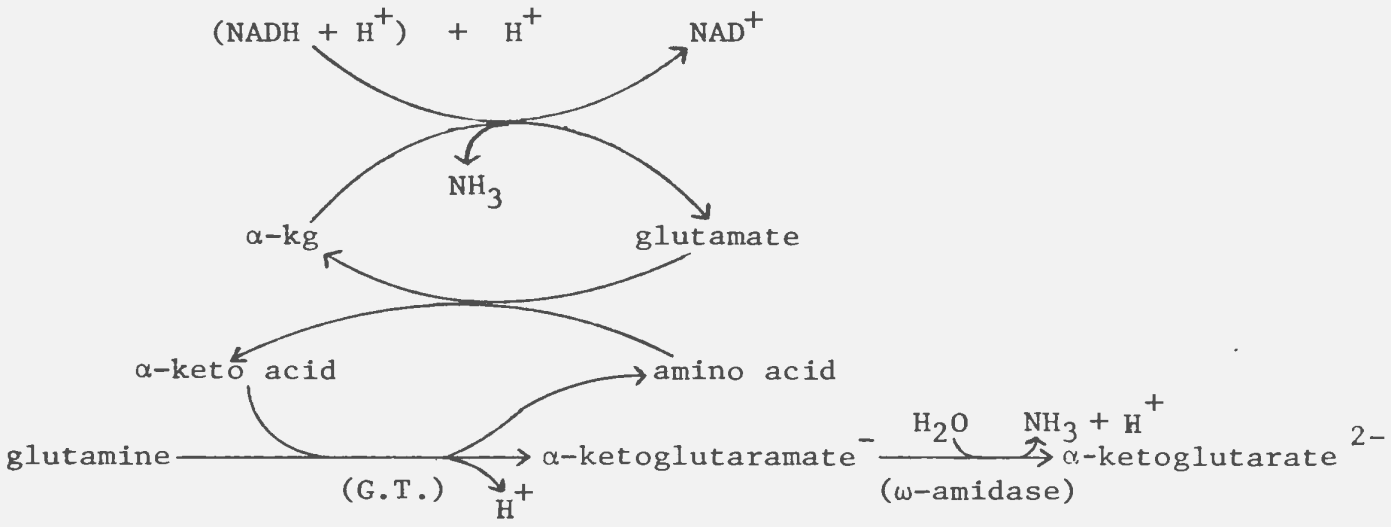
In the mammalian kidney, ammonia is produced in the renal tubular cells along the entire length of the nephron (83). The principal source of this urinary ammonia in man (76), dog (85) and rat (106) is glutamine. Glutamine is supplied to the kidney in the arterial blood and enters the tubular cells, from the filtered fluid, across the luminal surface and, from the peritubular blood, across the antiluminal surface (101). Theoretically, there are several enzymes in the renal tubular cells capable of releasing ammonia from glutamine. However, only the enzymes of two pathways have been extensively considered as important in the production of ammonia.

The first pathway (glutaminase I pathway) involves the sequential action of phosphate dependent glutaminase and glutamate dehydrogenase:



Phosphate dependent glutaminase, so-called because its <u>in vitro</u> activity depends upon phosphate, removes the amide-N of glutamine as ammonia. Glutamate dehydrogenase then releases the remaining amino-N as ammonia by converting glutamate to α-ketoglutarate. The enzymes of the glutaminase I pathway are located inside the mitochondrial inner membrane (20, 56). Therefore, in order for glutamine to become available for ammonia synthesis, via this pathway, it must be transported across the inner mitochondrial membrane.

The second pathway (glutaminase II pathway) consists of two linked reactions, catalyzed by glutamine transaminase and ω-amidase. Glutamine transaminase catalyzes the transamination of glutamine with an α-keto receptor to yield α-ketoglutaramate. Although this reaction does not release ammonia, it forms an amino acid from an α-keto acid. The amino acid can then be transaminated, with α-ketoglutarate by ubiquitous transaminases, to form glutamate, the substrate for glutamate dehydrogenase:



ω-amidase releases the amide-N of glutamine as ammonia by converting α-ketoglutaramate to α-ketoglutarate.

Although both pathways are found in all mammalian kidneys examined (94), various in vitro estimates of their activities indicate the glutaminase I pathway to be predominant (7, 33, 93). A similar conclusion is drawn from studies using intact rat kidney cortex slices. Relman and Narins (93) have shown that an inhibitor of glutamine transaminase does not have any significant effect on the production of ammonia by these slices, whereas Goldstein (31) found that an inhibitor of glutaminase resulted in a marked reduction in ammonia production. Welbourne (112) has also promoted γ-glutamyl transferase as a possible source of renal ammonia but the physiological significance of this pathway is obscure.

2. Removal of Carbon Skeleton

At physiological pH glutamine is a neutral substance, whereas α-ketoglutaric acid is a strong dibasic acid. Consequently, the production of ammonia, via the glutaminase I pathway, results in the formation of

2 protons. In order for the ammonias to become available to buffer urinary acid, α-ketoglutarate must be concomitantly metabolized to a neutral substance in a manner that utilizes the protons. One means of accomplishing this is conversion of α-ketoglutarate to glucose. This requires that the carbon skeleton leave the mitochondrion and that NADH be generated in the cytosol for reversal of the triosephosphate dehydrogenase step during gluconeogenesis. Both of these requirements are met if the carbon skeleton leaves the mitochondrion as malate and is then oxidized to oxaloacetate via malate dehydrogenase. Another means of removing α-ketoglutarate is by complete oxidation to CO_2 and H_2O. In this case, the carbon skeleton of α-ketoglutarate must leave the Krebs cycle by a route which converts it to pyruvate so that it may re-enter the cycle as acetyl CoA. Pitts (82) and Goodman (39) have suggested that this might occur by conversion of oxaloacetate to pyruvate via the cytosolic enzymes phosphoenolpyruvate carboxykinase and pyruvate kinase. It appears, however, that oxaloacetate is not able to translocate from the mitochondrial matrix into the cytosol (44). Lardy and co-workers (67) have proposed that other metabolites serve as transport derivatives for oxaloacetate. Their evidence suggests that in a situation like this, where no cytosolic NADH is required, oxaloacetate is transaminated to aspartate which then passes into the cytosol and regenerates oxaloacetate by the converse reaction.

C. Regulation of Ammonia Production

1. Adaptation

In abnormal states (starvation, diabetes, ingestion of strong acids)

when more than normal amounts of H^+ ions must be eliminated, the kidney has the ability to increase the rate of excretion of H^+ ions up to about 10 times that of their normal rate. In part, this is due to the ability of the kidney to increase the amount of free acid in the urine up to the pH of maximal acidity, about 4.6. However, the acids requiring elimination in these abnormal states are usually relatively strong acids of low pK (for example, hydrochloric, β-hydroxybutyric, acetoacetic and sulfuric acids) and the device of acidifying the urine is not effective for eliminating these H^+ ions. What is important is the ability of the kidney to increase the rate of ammonia excreted. Apparently, the rate of ammonia excretion depends upon 2 factors (80): (1) urine pH and (2) cellular production. The adaptive increase in ammonia excretion is a slow process, taking place over several days. Urine pH in the chronically adapted stage is essentially the same as that in the acute stage, yet it is during this period of adaptation that the greatest increases in ammonia excretion are observed (80). Furthermore, Pitts has shown that the rate of ammonia excretion by the chronically adapted kidney is considerably greater at any given urinary pH value than that by the normal kidney (80). These observations are interpreted as meaning that the major determinant of ammonia excretion is cellular production.

2. Regulation

It is apparent that in order for the adaptive changes which occur in the production of ammonia to take place, the flux through the pathway and hence the activities of all the steps in the metabolism of glutamine must increase. In this sense, the activities of all the steps in the pathway

are "regulated". The crucial and yet unanswered questions are:

(1) which step in the sequence responds to the initial signal and thereby effects changes in the other steps.

(2) what is the original signal which triggers these changes.

It seems certain that availability of glutamine in the blood is not a rate-determining factor for the increase in ammonia production since the quantity of glutamine delivered to the kidneys in the arterial blood does not differ significantly in acidosis and alkalosis (2, 101, 106). Neither can increased transport of glutamine across the luminal surface be invoked, as essentially the same quantities are filtered and reabsorbed in acidosis and alkalosis, yet far greater quantities are utilized in acidosis (79). It therefore seems likely that the production of ammonia is regulated by an intracellular step in the metabolism of glutamine.

3. Hypotheses

At present there are three principal hypotheses regarding the identity of the regulatory step in the renal metabolism of glutamine:

(a) Transport hypothesis. One hypothesis, suggested by Pitts (83) proposes that the site of regulation of ammonia production is the transport of glutamine across the inner mitochondrial membrane. Although there is no irrefutable evidence for a specific carrier-mediated transport system for glutamine, the demonstration of other transport systems (62) involved in the regulation of mitochondrial metabolism lends credibility to this hypothesis. One of the major problems in studying this process, from the viewpoint of regulation, is that it is difficult, with the presently used techniques (62), to measure initial rates of entry of

glutamine into mitochondria. However, Crompton and Chappell (18) claim to have measured the initial rate of entry of glutamine into pig renal-cortex mitochondria. Their calculations reveal that the rate of glutamine entry into isolated mitochondria is considerably slower than the rate at which glutamine is deamidated by disrupted mitochondria. This is taken to suggest (33) that the transport of glutamine across the inner mitochondrial membrane is rate-limiting for the deamidation of glutamine.

The studies of Adam and Simpson (1) also support this hypothesis. In their experiments they were unable to demonstrate the accumulation of ^{14}C-glutamine in the mitochondrial matrix, when mitochondria, isolated from rat kidney cortex, were incubated with as much as 10 mM ^{14}C-glutamine and then rapidly centrifuged through silicone into perchloric acid. This suggests that the transport of glutamine across the inner mitochondrial membrane is slower than its subsequent deamidation inside the matrix. The same result was obtained using dog kidney cortex mitochondria (104). At variance with these results, Crompton and Chappell (18) found labelled glutamine inside the matrix space of pig kidney mitochondria when incubated with ^{14}C-glutamine in a different medium. However, Adam and Simpson, using a medium identical to that described by these authors, were still unable to demonstrate glutamine in the matrix space of either rat kidney mitochondria (1) or pig kidney mitochondria (104). In experiments using inhibitors of glutamine deamidation (low temperature, p-chloromercuribenzoate, mersalyl) glutamine was still not detectable inside the matrix space in either rat kidney mitochondria (1) or dog kidney mitochondria (104). It therefore appears that even at these reduced rates of deamidation, the transport step is still slower and, therefore, rate-

limiting. It is possible, however, that these inhibitors also affect glutamine transport, in which case, the inability to detect glutamine inside the matrix space under these circumstances cannot be taken as evidence for the idea that transport is rate-limiting. It is also possible that the transport process and deamidation are closely linked so that only glutamate is released into the matrix space. In such an instance, the regulation of the overall transport deamidation complex, rather than transport per se, would be important.

Other support for the transport hypothesis comes from what appears to be an increase in the uptake of ^{14}C-glutamine by kidney cortex mitochondria isolated from rat (1, 32) and dog (104) after these animals had received an acid load. These increases are based upon increases in the steady-state concentrations of matrix label after incubation 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. These may be hazardous assumptions. Accumulation of ^{14}C-glutamate could also be caused by either a decreased exit of glutamate from the mitochondria or a decreased affinity of the glutamate exit mechanism. Adam and Simpson (1) have already shown that the uptake of ^{14}C-glutamate is quite depressed in mitochondria isolated from rats given an acid load and hence the activity of the glutamate transport mechanism may be lowered. Goldstein (32) has also studied mitochondrial transport using 6-diazo-5-oxo-L-

norleucine (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. He found a 1.5 - 2.0 fold increase in the steady-state accumulation of this analogue 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 it must be emphasized that an increased steady-state accumulation of DON does not necessarily imply an increased rate of entry. Again alterations in the rate and affinity of DON effux may be responsible.

The increase in ^{14}C-glutamate accumulation by dog kidney cortex mitochondria incubated with ^{14}C-glutamine is consistent with the idea that a carrier system is involved in the regulation of ammonia production, since in this species there is no observable increase in the activity of PDG in vitro (104). Also, there was an increase in accumulation of label by rat renal cortex mitochondria incubated with ^{14}C-glutamine after only 3 hours of acid challenge, without a concomitant increase in extractable PDG activity (1). However, in contrast, Goldstein (32) 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 2 days of acid challenge, even though ammonia excretion in vivo was significantly increased.

(b) Phosphate dependent glutaminase hypothesis. A second hypothesis proposes that the deamidation of glutamine by phosphate dependent glutaminase is

the regulatory step in the renal production of ammonia. This hypothesis was originally postulated by Pitts, who suggested (80) that the excretion of ammonia was regulated by changes in the concentration or amount of glutaminase within the renal tubular cells. The plausability of this suggestion was strengthened by Davies and Yudkin (22). These workers demonstrated that kidney cortex slices taken from rats given an acid load produced considerably more ammonia from glutamine than slices taken from normal animals. Subsequently, it was shown that the activity of PDG increased in the kidney of the rat (6, 68, 87, 91, 92, 109) and the guinea pig (35, 36, 94) in response to an acid challenge. After the first 24 hours of acid challenge, enzyme activity and urinary ammonia excretion correlate well in both the rat (92) and the guinea pig (35). The lack of a measurable increase in PDG activity (6, 68, 92) during the earlier stage of acid challenge (i.e., up to 24 hours), despite increases in ammonia excretion (68, 92) suggests that changes in the amount of enzyme cannot be the sole means whereby the synthesis of ammonia is regulated. However, it may be, as demonstrated by Curthoys and Lowry (19), that since the kidney is such a heterogenous system, studies with the whole organ must tend to average out or partially mask the magnitude of adaptive responses in a small portion of the nephron. These workers showed, by microanalytical techniques, that the 2-4 fold increase observed in whole kidney homogenates is in reality caused by a 20-fold increase within the proximal convoluted tubules (19). Thus, it is certainly possible that changes in PDG activity do occur during the first 24 hours of acid challenge but are not detectable in whole kidney homo-

genates. In fact, alterations in the activity in the proximal convoluted tubules must occur before they are manifest in the whole homogenate.

More serious doubt is cast upon this hypothesis by other anomalous findings. The activity of PDG in canine kidney does not change during acid challenge despite large increases in ammonia production (87, 91). Although this finding, in itself, does not discount the validity of the hypothesis for other species, it does at least demonstrate that this hypothesis is not universally applicable. Evidence against this hypothesis for the rat comes from work by Goldstein (29) and Bignal et al. (11), who both found a small increase in PDG activity after 1 day of acid challenge. Goldstein's experiments showed that an antibiotic, actinomycin D, inhibited the adaptive rise in PDG activity, but failed to inhibit the increase in ammonia excretion. The experiments of Bignal et al. (11) also show (75), using actinomycin D, that an adaptive increase in ammonia excretion can occur in the rat without a change in enzyme activity. However, actinomycin D is very toxic and cannot be administered for periods longer than 24 hours. Therefore, these experiments using actinomycin D can only be taken as evidence against this hypothesis for the first day of acid challenge. It is still possible that ammonia production is regulated by changes in the amount of PDG after this period. A similar study (74) in the rat, using ethionine as an inhibitor of protein synthesis (amino acid anologue), failed to inhibit the adaptive rise in ammonia excretion. However, although ethionine has been shown to inhibit the adaptive increase in renal PDG activity in the guinea pig (35), it was found to be only partially effective in producing complete in-

hibition of PDG induction in the rat (37).

(c) Phosphoenolpyruvate carboxykinase (gluconeogenesis) hypothesis. A third hypothesis proposes that the regulatory step in the renal production of ammonia is that catalyzed by phosphoenolpyruvate carboxykinase (PEPCK). This enzyme is required for conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP) and is almost entirely cytoplasmic in the rat (27). According to this hypothesis, changes in the activity of PEPCK regulate the removal of the carbon skeleton of glutamine to glucose, thereby regulating the level of glutamate inside the mitochondrion, which in turn controls the activity of PDG by-product inhibition. This hypothesis is based upon the observations that glutamate is an effective inhibitor of rat renal PDG at concentrations normally found in the kidney (30, 37, 38) and that glutamate concentration in the kidney decreases (21, 30, 34, 37, 38) when the rat is given an acid load. The findings that an acid challenge also stimulated gluconeogenesis in rat kidney cortex slices (4, 17, 41, 42, 59) and that glucose production was a major fate of glutamine in the rat kidney (57, 59) lead to the proposal that changes in glutamate concentration was regulated by gluconeogenesis. This idea was further strengthened by the demonstration of a positive correlation between the adaptive increases in ammoniagenesis and gluconeogenesis in kidney cortex slices taken from rats given an acid load for at least 48 hours (4, 17, 42, 57, 59). Subsequently, it was shown that the conversion of OAA to PEP was enhanced (3) and that the adaptive increase in renal PEPCK activity directly correlated with urinary output of ammonia (6).

One difficulty in accepting this hypothesis is that the concentration of glutamate inside the mitochondrion is controlled by the activity of an enzyme (PEPCK) in the cytosol and that for changes to occur in glutamate levels, this enzyme must exert a "pull" across the inner mitochondrial membrane and through all the intervening reactions. This necessitates that all the intervening steps be poised quite close to equilibrium, which has never been demonstrated and appears most unlikely. Apart from this theoretical objection, other more demonstratable findings are inconsistent with this hypothesis. 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 and that increases in ammonia excretion during prolonged acid challenge occur without corresponding decreases in glutamate concentration (30, 38). 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 (77). However, in defense of this hypothesis, it may be that changes in the concentration of glutamate within the cortical mitochondrial compartment, in which PDG is located, are not reflected in total glutamate concentrations. On the other hand, Adam and Simpson (1) reported that the glutamate content of mitochondria isolated from rats given an acid load was higher, not lower, than that of mitochondria isolated from control rats. Another criticism of this hypothesis is the lack of a correlation between glucose and ammonia production in rat kidney cortex slices during the early stage of acid challenge (i.e., up to 6 hours) (4). This is somewhat disturbing, since it may indicate that the two processes are not directly linked and that increased

gluconeogenesis is a consequence rather than a cause of increased ammonia production.

Studies using Krebs cycle inhibitors also show a dissociation between ammoniagenesis and gluconeogenesis. Churchill and Malvin (17) inhibited gluconeogenesis from glutamine in rat kidney cortex slices with either malonate or phenylpyruvate without affecting ammoniagenesis (malonate) or stimulating it (phenylpyruvate). Preuss (88) found that the addition of malonate or arsenite to the incubation medium failed to block the increased ammonia production seen in rat renal cortical slices, when the medium pH was decreased. However, glucose production was almost completely abolished. Kamm and Asher (57) however, argue against the validity of these studies. They point out the possibility that products other than glucose may accumulate and these were not measured. Recently Bennett and Alleyne (9) used what is thought to be a more specific inhibitor, mercaptopicolinic acid, to inhibit PEPCK. Their results show that the decrease in glucose production coincides with a decrease in ammonia production, suggesting that the two processes may indeed by causally linked.

Iynedjian et al. (53) showed, using isotopic-immuno chemical procedures, that the adaptive increase in rat renal PEPCK activity is due to an increase in the synthesis of new enzyme. These workers also found that the increase in activity could be prevented by actinomycin D at doses much higher than those used by previous workers (27, 69), who failed to find inhibition. Since inhibition of PDG induction occured at doses which were found not to inhibit PEPCK induction (11, 29), it is not known at present whether the adaptive increase in PEPCK activity is necessary for the adaptive increase in ammonia excretion.

Glutamate has also been shown to be an effective inhibitor of dog renal PDG (8, 98) and its concentration in the dog kidney cortex also decreases when an acid load is administered to the animal (101, 107). The activity of PEPCK is known to increase under these circumstances (103). Thus, the PEPCK (gluconeogenesis) hypothesis has also been considered for the dog. However, the relationship between ammoniagenesis and gluconeogenesis is even more tenuous than in the rat. The studies of Malvin and Churchill (16) and Roxe et al. (97) did not detect a significant change in renal glucose output in vivo in dogs to which an acid load had been administered. However, this is not surprising since the blood flow of the kidney is so high that even if all the glutamine extracted was converted to glucose the resultant increase in renal venous glucose would be exceedingly small. Steiner et al. (107), using a more precise method for measuring blood glucose, found a very small but statistically significant increase in renal glucose output in vivo in dogs given an acid load. This increase in renal glucose output was in the range which would be expected if much or all of the glutamine giving rise to augmented ammonia production was ultimately converted to glucose. However, the increase in glucose production, although significant, was so small that this study cannot be taken as conclusive proof that renal gluconeogenesis is enhanced (39). In addition, glucose A-V differences are difficult to interpret because the medulla is a potentially important user of glucose (71). Therefore, an increase in glucose output by the kidney may also be interpreted as a decrease in glucose utilization by the medulla.

A correlation between the adaptive increases in glucose and ammonia production by dog kidney cortex slices has only been demonstrated (15)

at reduced medium pH (i.e., 7.0). Even then, cortex slices from dogs produce much less ammonia and glucose than slices from rats when comparable concentrations of glutamine are used as substrate (15). Most evidence (78, 82, 86) indicates that in the dog, unlike the rat, renal gluconeogenesis is a minor pathway for disposal of the glutamine carbon skeleton. However, PEPCK is probably also involved in the total oxidation of glutamine to CO_2. Therefore, this enzyme may also be of importance in ammonia production in the dog.

Yu et al. (116), in a recent study, observed that infusion of neutral sodium phosphate elicited a large increase in ammonia excretion by the acidotic rabbit. A similar effect could be observed in vitro (slices and isolated mitochondria) and in the case of mitochondria the effect was shown to involve the malate/phosphate antiporter. The exit of malate (the possible end product of mitochondrial metabolism) from the mitochondrion occurs via the malate/phosphate antiporter and is stimulated by elevated concentrations of extramitochondrial phosphate. This study directs attention to the role of mitochondrial transporters other than the glutamine transporter. Additionally, it shows that the three possibilities for regulation of ammonia production discussed above (i.e., PDG, glutamine transport and PEPCK-gluconeogenesis) are not the only ways in which control has been envisaged.

4. Metabobolic Signals

Although the nature of the metabolic signals involved in the regulation of ammonia production cannot be identified with certainty until the rate-determining step has been positively identified, a number

of possible candidates exist. One of the most obvious and primary effects of an acid challenge is a drop in blood pH. Such a drop in pH could be a signal for indirectly or directly activating ammonia production by the renal tubular cells. It is possible it may indirectly act by altering the plasma concentration of glucose precursors, such that glutamine is preferentially used. It is also possible that a drop in blood pH may effect the release of an extrarenal hormone. The involvement of a hormone was suggested by studies of Alleyne and Roobol (5). These workers demonstrated a dialyzable factor, present in the plasma of acutely acidotic rats, which increased the ammonia production of normal kidney cortex slices when added to the incubation medium. A change in systemic pH may also directly cause an alteration in the intracellular pH of the cells responsible for renal ammonia production. Evidence for a direct role of pH is conflicting. Ammonia production from endogenous substrates was shown to be increased when the pH of the medium bathing rat kidney cortex slices was decreased (48, 50, 95). Endogenous ammonia production by dog kidney cortex slices was shown either to be not affected (48) or to be increased (95) when the medium pH was lowered. When glutamine was added as substrate, lowering the medium pH either did not affect (52) or significantly decreased (40, 48, 93) ammonia production by rat kidney cortex slices, while ammonia production by dog kidney cortex slices was both increased (15, 78) and decreased (48). When glutamate was employed, ammonia production by rat kidney cortex slices was increased (88, 93) after the medium pH was lowered. Relman and Narins (73, 93) surmize, on the basis of the difference between ammoniagenesis from glutamine and glutamate by kidney cortex slices, that

lowering medium pH inhibits PDG, but enhances either the mitochondrial efflux of malate (63) or the activity of PEPCK. The relevance of this effect to the *in vivo* situation remains to be determined.

The effect of pH on the subsequent metabolism of glutamine is even more confusing. Glucose production by rat kidney cortex slices, using glutamine as substrate, is either not affected (5) or increased (52, 58) by acidifying the medium. Glucose production from glutamate was shown to be increased (58, 88). Decreasing medium pH increased glucose production from glutamine (15, 78) by dog kidney cortex slices. CO_2 production from both glutamine and glutamate by rat renal cortex tubules was shown not be to affected (64, 65) by lowering medium pH, while CO_2 production by isolated rat kidney cortex mitochondria was inhibited (64, 65). On the other hand, CO_2 production from glutamine by dog kidney cortex slices and mitochondria were both shown to be increased on acidifying the medium (105). The difference in the effect of lowered medium pH on CO_2 production, between the dog and the rat, is explained by Kurokawa and Rasmussen (65) as being due to differences in the Ca^{++} ion concentrations used. These same authors conclude from studying the metabolism of a number of different substrates by rat kidney cortex mitochondria and tubules (65, 105), that changes in H^+ ion concentrations influence primarily intramitochondrial processes.

The effect of pH on renal glutamine metabolism remains unclear, especially since most of the studies are performed using a bicarbonate buffer system, designated to resemble physiological conditions. A bicarbonate buffer consists of 3 variables: pH, HCO_3^- and pCO_2. It is not possible to maintain two of these variables constant while studying the

effect of the third. CO_2 production from glutamine by rat kidney cortex mitochondria (65) and dog kidney cortex mitochondria (105) was also increased when $[HCO_3^-]$ and pCO_2 were lowered and pH kept constant. Cade et al. (14) found a good correlation between plasma pCO_2 and urine ammonia excretion in the acidotic dog. Kamm et al. (60) have shown that a positive correlation exists between total plasma CO_2 content (plus bicarbonate) and the activity of renal PEPCK in the rat. However, the increase in CO_2 production, observed by lowering $[HCO_3^-]$ and pCO_2, at constant pH, is not as great as that which occurs when pH is lowered (65, 105). Also, the relevance of bicarbonate to ammonia excretion is unclear, since adaptive increases in ammonia excretion are observed at low plasma bicarbonate (90) as well as at high plasma bicarbonate (51, 99) and at normal plasma bicarbonate (92).

The changes which occur in blood pH, $[HCO_3^-]$ and pCO_2 in relation to ammonia excretion during continuous acid challenge have not, as yet, been well investigated. Denning et al. (24) found that when an acid load was administered to humans for 14 days, the pH of the blood, after initially dropping, gradually returned to normal. Hilton administered an acid load for 30 days (47). He observed that acidosis developed and thereafter subsided, so that, after 4 weeks the plasma pH and $[HCO_3^-]$ had returned to normal, despite continued administration of acid. Wood (115) has described the effects of giving a daily acid load to a human subject for 44 days. He states that at no time during the experiment did plasma pH or $[HCO_3^-]$ return to normal. However, it is quite apparent from his results that after reaching minimum values, after 3-4 days, these parameters then gradually returned towards normal. Plasma pCO_2 also re-

turned towards normal after a drop during the first 3 days. Ammonia excretion, on the other hand, remained elevated after reaching a maximum at about 5 or 6 days. After the acid load was discontinued, plasma pH and $[HCO_3^-]$ rose above normal and then gradually fell, returning to normal after about 10 days. Plasma pCO_2 had returned to normal values after 3 or 4 days. Clearly, these results do not reveal any simple correlation between any of these possible regulatory parameters (i.e., pH, $[HCO_3^-]$, pCO_2) and ammonia excretion.

D. Purpose and Approach of this Study

Despite the widespread interest in the control of renal ammoniagenesis over the past 25 years, abundant enthusiasm has not yet been matched by corresponding progress in understanding the specific control mechanisms involved. Pitts recently remarked (83) that "no presently proposed theory of control of ammonia production is intellectually satisfying".

As can be gathered from the foregoing introduction, all of the hypotheses are based mainly on correlative studies of events which take place during adaptation to an acid challenge. A correlation between two events does not necessarily mean that they are causally related. On the other hand, a dissociation or lack of correlation can be taken as evidence that two events are not causally related. However, most of the dissociations taken as evidence against the presently discussed hypotheses, occur in unphysiological situations or where the methods of detecting changes are subject to question.

Dies and Lotspeich (25) observed that, when rats were returned to

tap water after 5 days of acid challenge, the ammonia excretion abruptly fell to normal values within 24 hours. It therefore seemed possible that this phase of adaptation might be rewarding in either establishing dissociations or confirming correlations. It was anticipated that the return of ammonia excretion to normal would be accompanied by a return to normal values of regulatory parameters, but not necessarily by a return to normal values of non-regulatory parameters. This search for such relationships was carried out at different levels of organization; i.e., from the level of enzyme activities through isolated organelle function and slice metabolism to the whole animal. Although studying the recovery phase was the original impetus for this work, changes which occur during acid challenge were also investigated in an attempt to fill in some of the gaps presently found in the literature.

MATERIALS AND METHODS

Materials

A. Animals

Male Sprague-Dawley rats, weighing 250-500 g, were used in all experiments. These animals were obtained from Canadian 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 22.0% protein, a minimum 4.0% fat and a maximum 5% fibre. The remainder of the rat chow was mainly carbohydrate plus mineral and vitamin supplements.

B. Cofactors and Nucleotides

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

C. Enzymes

The following enzymes were purchased from Sigma Chemical Company: lactic dehydrogenase (type II), glutamic dehydrogenase (type II), pyruvate kinase (type II), hexokinase (type C-300) and PGO enzymes.

D. 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.

E. Other Chemicals

"Somnotol", sodium pentobarbital (65 mg/ml), was obtained from M.T.C.

Pharmaceuticals. "Siliclad" (silicone) was obtained from Clay Adams, Parsippancy, New Jersey. Cesium chloride (biological grade) was purchased from Schwarz-Mann, Orangeburg, New York. Cation exchange resin (AG 50W-X8, 50-100 mesh) was purchased from Bio Rad Laboratories, Richmond, California. Phenol was obtained from BDH Chemicals, Toronto, Ontario. Commercial bleach (Javex), a source of sodium hypochlorite, was obtained from Bristol-Meyers Canada Ltd., Toronto, Ontario. Water was treated by the Culligan Reverse Osmosis System, RDS 1. All other chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri; BDH Laboratory Chemicals Ltd., Poole, England; Fisher Scientific Company, Fawn Lawn, New Jersey or J.T. Baker Chemical Company, Phillipsburg, New Jersey and were of analytical grade purity.

Methods

A. Treatment of Animals

On arrival, the animals were placed in standard rat cages (not more than 3 rats to a cage) containing hardwood bedding chips. The animals were allowed free access to food and water for a minimum of 2 days. This period allowed them to adjust to their new environment. The animal room was continually lighted and maintained at constant temperature.

At the end of this period the animals were apportioned to either a control group or an experimental group. The control group was allowed free access to tap water and, depending on the experiment, either fed *ad libitum* until used or deprived of food for a period of up to 2 days. The treatment of the experimental group depended on the nature of the

experiment. When adaptation to acid challenge was being examined, the animals were given free access to 1.5% (w/v) NH_4Cl, in place of tap water, and fed ad libitum for periods of up to 7 days. When recovery from acid challenge was being examined, rats were given free access to 1.5% (w/v) NH_4Cl for either 3 days or 7 days. Animals which had been drinking 1.5% (w/v) NH_4Cl for 3 days were returned to tap water for another 3 days. Animals which had been drinking 1.5% (w/v) NH_4Cl for 7 days were either returned to tap water for up to 2 days, or, in some experiments, returned to tap water and deprived of food for up to 2 days.

B. Use of Metabolic Cages

The use of metabolic cages entails certain errors. For the measurement of urinary ammonia excretion the principal potential errors are probably:

(1) spillage of 1.5% (w/v) NH_4Cl solution into the urine.

(2) spillage of food and feces into the urine.

(3) evaporation of urine on the collecting funnel.

(4) evaporation of collected urine.

(5) bacterial degradation of urea or other nitrogenous substances in the urine to yield ammonia.

Measures were taken to minimize some of these errors. Also in some cases, the extent of the errors were estimated.

(1) Spillage

The rats used in these experiments drank between 15 and 34 ml* of fluid (1.5 % (w/v) NH_4Cl) each day. The rat metabolic cages (obtained from Acme Metal Products, Chicago) deliver the drinking fluid to the rat

* The volume of fluid drunk refers to that consumed by individual rats of different body weights. There was much less variability in fluid consumption when expressed as per 100 g body weight.

via a spout that reaches into the cage through an opening (3/4 inch in diameter) in the back wall. Hence, any spillage of drinking fluid can drop into the cage and is automatically collected along with the urine. Therefore, the drinking fluid container was moved so that the end of the spout was located on the outside of the cage, about 1 inch away from the back wall. The opening was enlarged to 1 1/4 inch in diameter, so that the rat could reach out and drink, but could not escape. Spillage was collected in a small cup placed beneath the spout.

The rats used in these experiments ate between 13 and 22 g of food each day. There was considerable spillage into the urine when cubed rat food was used since the rats withdrew the cubes from the feeding dish into the cage proper and pieces broken off readily fell into the urine. This problem was minimized by grinding the food in a mill. However, inevitably, some food (about 1 g/day) found its way into the urine. That this could not be a significant artifactual source of ammonia (ammonium) in the urine was shown by experiments in which 1 g of food was soaked in 15 ml of water for 24 hours. The ammonia, subsequently found in the water (by the method of Kirsten *et al.* (61)) was trival in comparison with normal daily excretion (i.e., less than 1%).

A similar problem involves feces. No device for separating feces from urine was present in the metabolic cages used. Therefore, metal grids, cut to size, were placed in the collecting funnels. However, some feces still dropped into the urine. In addition, it is also possible that the urine comes in contact with the feces on the metal grid. To determine whether feces could be a significant source of ammonia (ammonium), ground feces was soaked in 15 mls of water in amounts com-

parable to that found in the collected urine. Again, the ammonia found in the water after 24 hours does not represent a significant artifact (i.e., less than 1%).

(2) Evaporation

The rats used in these experiments excreted between 10 and 20 ml of urine each day. The collecting funnels were treated every two or three days with "Siliclad" (silicone) to minimize evaporation of urine. "Siliclad" makes the funnels more water repellent and improves drainage. The extent of evaporation, which did occur, was estimated by experiments in which the collecting funnels were washed with water after 24 hr of urine collection. Creatinine was measured in the washes and collected urines by a colorimetric method based on the Jaffe reaction (26). By comparing the amount of creatinine in the washes with the total amount of creatinine excreted, the average evaporation was calculated to be 6.8% of the total urine excreted.

Urine was collected in 30 ml glass tubes. Evaporation of the collected urine was reduced by adding mineral oil to the collecting tubes. Mineral oil forms a layer on top of the urine so that the urine is not exposed to the air.

(3) Bacterial Contamination

No protection against bacterial contamination, other than mineral oil, was used. Therefore, it was important to know if ammonia is formed, under these conditions, by bacterial degradation of urea or other nitrogenous substances. For this purpose, experiments were carried out in which one portion of each urine, collected under ordinary conditions,

was frozen while the other portion was allowed to stand for 24 hr, under mineral oil, in the animal room. The lack of a significant difference between the differently treated portions is taken as evidence that bacterial degradation is not a significant source of urinary ammonia.

C. Studies in vivo

1. Urine

(a) Ammonia excretion. Rats were individually placed in carefully washed metabolic cages 24 hr prior to the period of urine collection. This period was allowed for the rat to become accustomed to the cage. The urine was collected under mineral oil for 24 hr. The ammonia in the urine was measured, after appropriate dilution, by the method of Kirsten et al. (61).

2. Plasma

(a) Glutamine arterio-renal venous differences. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (6.5 mg/100 g body weight). The renal vein and abdominal aorta were exposed by midline incision. Blood samples (approximately 1 ml) were withdrawn into heparinized plastic syringes, first from the renal vein and then from the abdominal aorta. The blood samples were centrifuged and a portion of each plasma was added to a half volume of 12% (w/v) $HClO_4$, mixed vigorously with a vortex mixer and allowed to stand on ice for 15 min. After removing the precipitated proteins by centrifugation, an aliquot of each supernatant was added to an equal volume of 0.3 N lithium citrate buffer (pH 2.2) and the pH adjusted to 2.2 with 2 N LiOH.

Samples of 0.5 ml from the buffered solutions were then used to measure glutamine on a Beckman Model 121 Automatic Amino Acid Analyzer (106). The concentrations of glutamine from paired samples were used to calculate arterio-renal venous differences.

3. Blood

(a) pH, pCO_2 and $[HCO_3^-]$. Rats were coaxed into a restraining cage and, after tail prick with a sharp scalpel, blood samples were drawn into 100 µl heparinized capillary tubes. Care was taken to minimize stress from handling. The capillary tubes were immediately plugged, mixed with a magnetic bar and magnet and placed on ice. The pH and pCO_2 were measured as rapidly as possibly and always within one hour with the model 213 pH Blood Gas Analyzer (Instrumentation Laboratories). The $[HCO_3^-]$ was calculated from the pH and pCO_2 measurements by the formula:

$$[HCO_3^-] = (.03\ pCO_2) \times \text{antilog}\ (pH - 6.1)$$

D. Studies in vitro

1. Entire Kidney Homogenates

(a) Preparation of homogenates. The entire right kidney of each animal was weighed, chopped into small pieces and suspended in 50 volumes of ice-cold 0.25 M sucrose containing 3 mM Tris (HCl) buffer (pH 7.4), 1 mM EGTA and 0.15 M NaF. The suspensions were then homogenized in a smooth glass Potter-Elvehjem homogenizer with a close-fitting motor driven Teflon pestle (clearance .15 mm).

(b) Phosphoenolpyruvate carboxykinase assay. One part of each homogenate was centrifuged at 30,000 g and 0° C for 30 min. Aliquots of

0.1 ml of the supernatants were then used to assay the activity of phosphoenol-pyruvate carboxykinase (PEPCK) in the direction of decarboxylation by a modification of the method of Seubert and Huth (100). In the method used NaF was added to the homogenizing medium instead of to the incubation medium. Fluoride ion is included to inhibit phosphatases and enolases which can remove nucleoside triphosphate and phosphoenolpyruvate (PEP), respectively. The effectiveness of NaF was demonstrated by recovery experiments in which PEP was added to incubations using homogenates with and without NaF. In incubations using homogenates without NaF, the recovery of PEP was 94%, while incubations using homogenates containing NaF, the recovery of PEP was 100%. Figs. 2(a) and 2(b) show the formation of PEP by 2% homogenates prepared from the kidneys of normal rats and rats receiving 1.5% (w/v) NH_4Cl for 7 days as functions of incubation time (0.1 ml aliquot) and amount of enzyme (15 min incubation), respectively. The incubation time and amount of homogenate used for the measurement of PEPCK activity are within the linear range of this assay.

(c) Phosphate dependent glutaminase assay. Aliquots of 0.1 ml of the other part of each kidney homogenate were used, without centrifugation to assay phosphate dependent glutaminase (PDG) by the method of Curthoys and Lowry (19). It was previously shown that sonication of this preparation was not necessary to obtain maximum activity. The inclusion of 0.15 M NaF in the homogenates is shown in Table 1 not to affect this assay when using kidney homogenates prepared from either normal or acidotic rats.

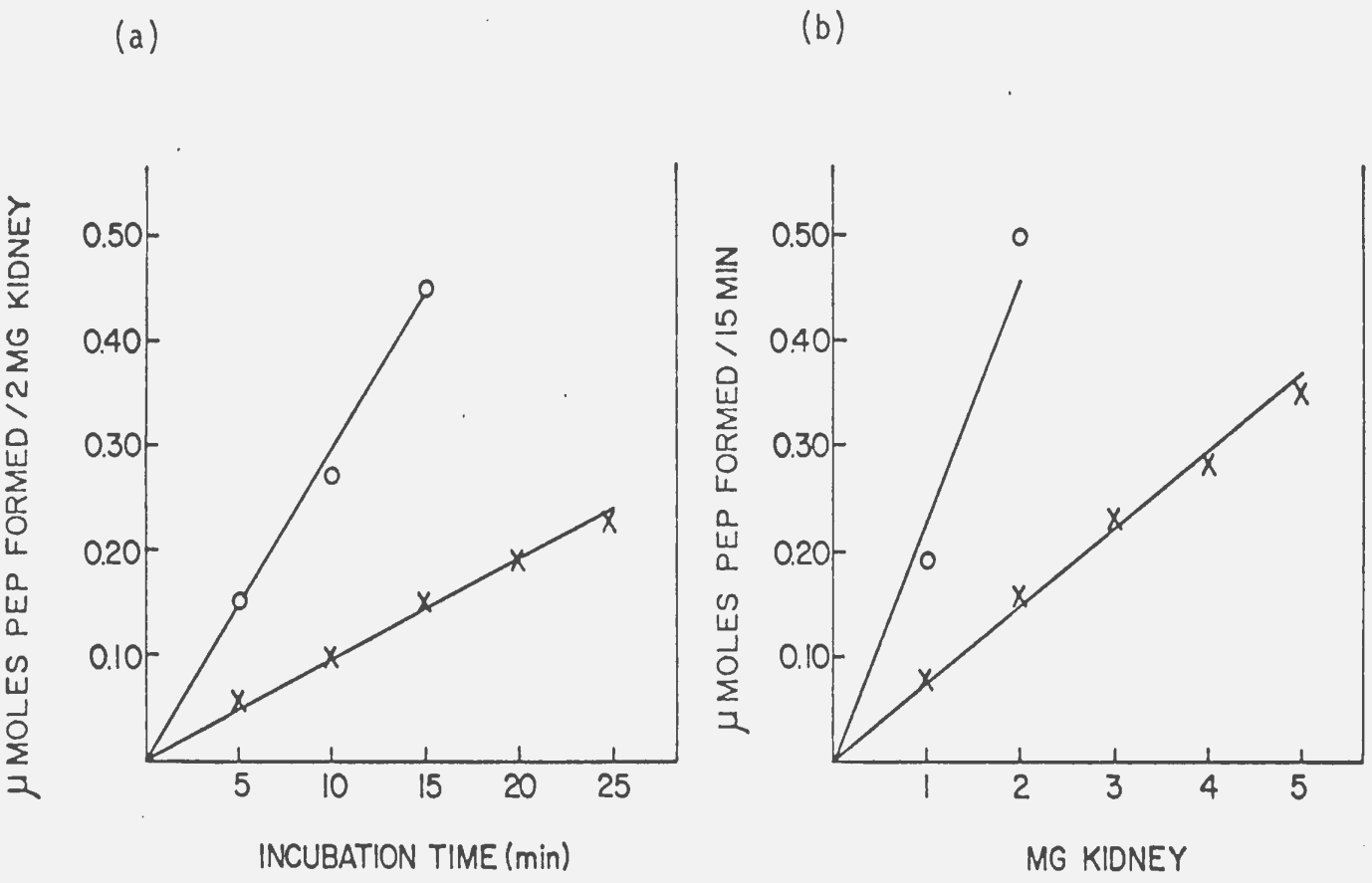


Figure 2 Formation of Phosphoenolpyruvate by Homogenates as a Function of Incubation Time and Amount of Kidney

X——X Normal Rat

O——O Acidotic Rat

Table 1 Effect of NaF on Phosphate Dependent Glutaminase Assay

0.15M NaF in Homogenizing Medium	GLUTAMATE FORMED (μmoles)	
	Normal	Acidotic
-	0.73	2.16
+	0.69	2.16

This table shows the amount of glutamate formed by 2% (w/v) entire rat kidney homogenates (with and without NaF) from a normal rat and a rat administered 1.5% (w/v) NH_4Cl for 7 days.

2. Kidney Cortex Mitochondria

(a) Preparation of mitochondria. The kidneys of each rat were demedullated, chopped into small pieces and suspended in 0.25 M sucrose containing 5 mM Hepes buffer (pH 7.4) and 1 mM EGTA. Mitochondria were prepared by homogenization and centrifugation according to the method described by Kalra and Brosnan (56). Mitochondrial protein was measured by the biruet method (43) after solubilization of lipid with deoxycholate (55). Bovine serum albumin (fatty acid free) was used as a standard. The mitochondrial suspension was then adjusted to a protein concentration of between 10 and 15 mg/ml. All operations were carried out at 0-4° C.

(b) Incubation procedure and ammonia production. The following is a modification of the incubation procedure used by Hird and Marginson (49). Aliquots of 0.1 ml of the mitochondrial suspensions (1.0 - 1.5 mg of mitochondrial protein) were incubated with 20 mM Hepes buffer (pH 7.4), 2 mM glutamine, 10 mM sucrose (from mitochondrial suspension), 0.14 M KCl, 15 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mg/ml BSA (fatty acid free), 2 mM EDTA, 23 mM glucose, 2 mM ADP and dialyzed hexokinase (at least 8 units) all in a final volume of 2.4 ml. The incubations were carried out at 30° C in unstoppered 25 ml Erlenmeyer flasks, shaking at a rate of approximately 100 oscillations per minute. After 15 min, the incubations were stopped by the addition of 0.2 ml of 70% (w/v) $HClO_4$ and neutralized to pH 7.0 with 5 M KOH. The contents of the incubation flasks were centrifuged to remove precipitated $KClO_4$ and the supernatants used to measure ammonia (ammonium) as described below.

Glucose and hexokinase were added to regenerate ADP from ATP produced during the incubation. The yeast hexokinase (purchased as a crystalline suspension in 3.2 M ammonium sulfate) was prepared by dialyzing with constant stirring at 0^{o} C against 1 liter of 10 mM potassium phosphate buffer (pH 7.0). The phosphate buffer was changed eight times. The protein concentration of hexokinase after dialysis was determined by measuring its absorption at 260 nm and 280 nm.

The determination of ammonia (ammonium) is complicated by the presence of glutamine. Glutamine breaks down non-enzymatically to yield ammonia. Therefore ammonium (ammonia) was measured by the following method. This method is based upon that used by Sherrard and Simpson (102) for the separation of ammonium from blood and urine. Ammonium present in 0.5 ml aliquots of the supernatants (from incubations) was absorbed to batches (approximately 0.3 g) of cation exchange resin. The resin used was Bio-Rad AG 50W-X8, 50-100 mesh, sulfonic acid type, Na form. After washing several times with deionized ammonia free water, ammonium was eluted with 1 ml of 2 M CsCl and measured colorimetrically by the indophenol reaction (26). Standard curves were constructed by taking known quantities of ammonium chloride through the entire procedure described above. Figs. 3(a) and 3(b) show the production of ammonia by mitochondria isolated from normal rats and rats receiving 1.5% (w/v) NH_4Cl for 7 days as a function of incubation time (0.10 ml aliquot) and mitochondrial protein (15 min incubation), respectively. The production of ammonia is linear under the conditions employed up to at least 1.5 mg of mitochondrial protein and 15 min incubation time.

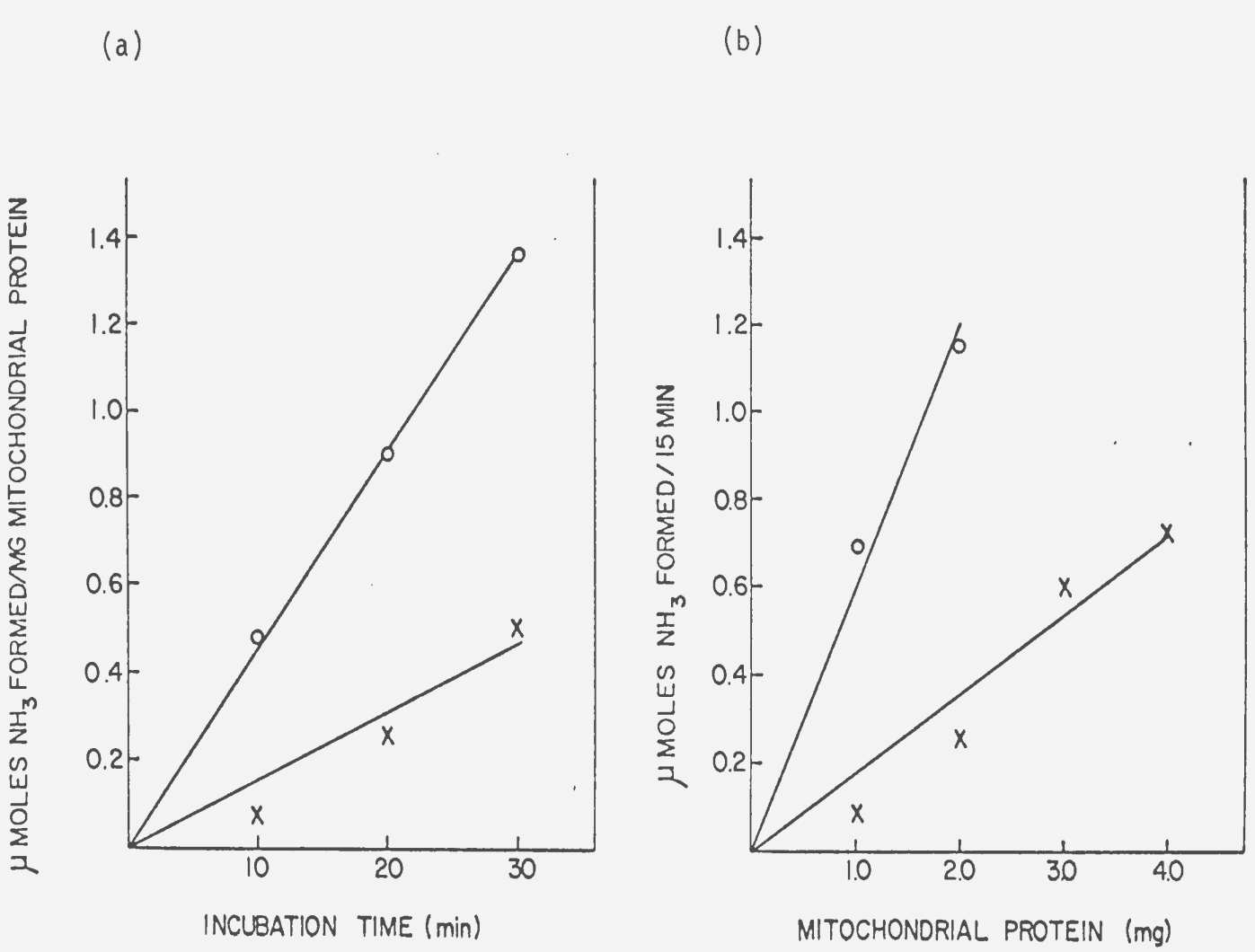


Figure 3 Formation of Ammonia by Isolated Mitochondria as a Function of Incubation Time and Amount of Mitochondrial Protein

X——X Normal Rat

O——O Acidotic Rat

(c) Incubation procedure and oxygen consumption. The utilization of oxygen by mitochondria was measured with a Clark-type oxygen electrode by incubating, at 30° C, aliquots of 0.1 ml of the mitochondrial suspensions in a medium containing 5 mM Tris (HCl) buffer (pH 7.2), 0.14 M KCl, 15 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EDTA, 2 mM ADP, 1 mg/ml BSA (fatty acid free) and either 2 mM glutamine, 2 mM glutamate, 2 mM α-ketoglutarate or 2 mM succinate.

3. Kidney Cortex Slices

(a) Preparation of slices and incubation procedure. Kidney cortex slices of approximately 10-30 mg were cut free-hand by the method of Deutch as described by DeLuca (23). Each slice was individually preincubated at room temperature in a saline medium of Krebs Ringer for 10-15 min. The Krebs Ringer solution was made up such that the concentration of Ca^{++} was equivalent to the concentration of ionized Ca^{++} in serum. This preincubation procedure was carried out to remove preformed glucose and glucose produced from endogenous substrates. The slices were then blotted free of excess saline, weighed on a torsion balance and placed in 25 ml Erlenmeyer flasks containing 5 ml of gassed (95% O_2 - 5% CO_2) Krebs-Henseleit medium and either no substrate (slice blank) or with one of the following substrates: 2 mM glutamine, 10 mM oxaloacetate, 10 mM malate, 10 mM lactate or 10 mM pyruvate. The Krebs-Henseleit medium also contained Ca^{++} concentration equal to the concentration of ionized Ca^{++} in serum. The glutamine concentration used was 2 mM because the results of experiments by Welbourne (110) suggest that higher substrate concentration leads to glutamine utilization other than by the glutaminase I pathway. A flask containing 2 mM glutamine in 5 ml of gassed Krebs-Henseleit and no

slice was used as a glutamine blank. The flasks were then flushed with 95% O_2 - 5% CO_2, stoppered, and incubated at 37° C in a Dubnoff metabolic shaker at approximately 100 oscillations per minute. After 90 min the incubations were stopped by the addition of 0.45 ml of 70% (w/v) $HClO_4$. The contents of each flask were then homogenized in a smooth glass Potter-Elvehjem homogenizer with a close-fitting motor-driven Teflon pestle (clearance .15 mm) and neutralized to pH 7.0 with 3 M K_3PO_4. The resulting precipitate was removed by centrifugation.

(b) Ammonia production, glutamate formation, glutamine utilization and glucose production. Ammonium (ammonia) was removed from 0.1 ml aliquots of the supernatants with cation exchange resin, and then measured by the indophenol reaction as previously described. Glutamate was measured in 0.5 ml aliquots according to the enzymatic method of Bernt and Bergmeyer (10). Glutamine was measured in 1.0 ml aliquots of the supernatants by hydrolyzing it to pyrollidone carboxylate with heating (100° C) for 5 min in 2N H_2SO_4, cooling on ice, and then converting it to glutamate by heating (100° C) for 45 min in a final concentration of 1N KOH. After cooling, the alkaline solutions were then adjusted to pH 9.0, centrifuged and 0.1 ml aliquots of the supernatants used to measure glutamate by the method described above. The amount of glutamine was calculated by subtracting the amount of glutamate present before hydrolysis from the amount of glutamate present after hydrolysis. Glucose was measured in 2.0 ml aliquots of the supernatants (obtained by centrifuging the incubation mediums after homogenization and neutralization) by the specific glucose oxidase method described in Sigma Technical

Bulletin, No. 510.

E. Statistical Treatment

Results are expressed as means $\pm$ standard deviations. The significance of differences was calculated by the Student "t" test. "P" values of 0.05 or less were taken to indicate a significant difference between means.

RESULTS AND DISCUSSION

A. Changes in Renal Ammonia Production

So as to investigate the mechanisms involved in the regulation of ammonia production, it was decided to compare the time course of metabolic changes, which are thought to be of regulatory importance in the rat, with changes in the production of ammonia *in vivo*. It was therefore necessary to establish the pattern of ammonia production during the experiments designed for this study. The protocol involved giving an acid load by substituting 1.5% (w/v) NH_4Cl for drinking water. NH_4Cl, which is commonly employed for this purpose, is metabolized in the liver to hydrochloric acid and urea:

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

Since HCl cannot be metabolized further, it must be eliminated from the body via the kidneys. It was estimated that each rat drank in one day about 7 ml of this solution per 100 g body weight*. This means that about 2 mmoles of acid per 100 g body weight was taken in each day and required excretion.

1. Urinary Ammonia Excretion

In general about 75% of the ammonia produced by the kidney is excreted in the urine. The other 25% diffuses into the renal venous blood (84). Table 2 shows that the excretion of ammonia in the urine is elevated in rats given 1.5% (w/v) NH_4Cl. This increase is statistically significant at day 1 ($P < 0.005$ compared to day 0) and continued to climb ($P < 0.005$ day 2 compared to day 1) until day 3 ($P <$

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* Although the quantity of fluid drunk was quite constant during the late onset and plateau phases, somewhat less was consumed during the first day. However, the slow adaptation in ammonia excretion cannot be accounted for by assuming a slow increase in the amount of acid consumed. The sharp fall in blood pH on day 7 (Table 4) attests to the fact that more acid was consumed than could be excreted.

Table 2 Uninary Ammonia Excretion by Rats after Different Treatments

Day of Treatment	TREATMENT		UNINARY AMMONIA EXCRETION
	Days on NH_4Cl 1.5%(w/v)	Days on Water	mmoles/24 h/100g body wt ±SD (no of animals)
0	-	∞	0.30±0.18(12)
1	1	-	0.70±0.23(6)
2	2	-	1.37±0.29(7)
3	3	-	1.91±0.51(7)
4	4	-	2.03±0.51(7)
5	5	-	1.81±0.78(4)
6	6	-	1.84±0.64(5)
7	7	-	2.22±0.45(10)
8	7	1	0.74±0.32(6)
9	7	2	0.35±0.27(6)

0.05 compared to day 2). No further change in ammonia excretion is observed until the NH_4Cl was replaced by water. The rates of ammonia excretion for days 5 and 6, however are not significantly different from that for day 2. This is probably because of the small number of animals used and the large variability between animals. After day 7, when the rats were returned to tap water, ammonia excretion abruptly fell ($P < 0.001$ day 8 compared to day 7) but was still significantly higher than normal ($P < 0.005$). However, since the value reported for day 8 is a measure of the ammonia excreted over the previous 24 hours, it is probable that the rate of ammonia excretion had returned to normal values by the end of day 8. The value reported for day 9 is not significantly different from that for either day 8 or day 0.

The time course of adaptation to administration of NH_4Cl appears to vary in different laboratories. In addition, the maximal response of ammonia excretion is not consistent with the dose administered. Welbourne (111) found that rats receiving, ad libitum, approximately 1 mmole/100 g body weight daily, required about 4 days to reach maximum rates of ammonia excretion (approximately 4-5 fold above normal). Rector et al. (92) found that rats intubated with approximately 1.3 mmoles NH_4Cl/100 g body weight each day also required about 4 days to reach maximal ammonia excretion, but that the maximal response was only about 2 fold above normal. Dies and Lotspeich (25) intubated rats with a much larger dose, 3 mmoles NH_4Cl/100 g body weight/day, yet their results show that the increase in ammonia excretion was complete by the end of 2 days and increased only 2-3 fold. It is possible that differences in diet contribute to the observed differences in ammonia ex-

cretion. Dies and Lotspeich (25) compared the response of ammonia excretion to the dose of NH_4Cl administered. Their results show that this relationship is not linear as might be expected, but rather, curvilinear with steeper increases in ammonia excretion occuring at higher doses of NH_4Cl used. Consequently, it may not be justified to directly compare the response obtained here with those obtained in other laboratories.

2. Glutamine Arterio-Renal Venous Differences

It was important to know if the abrupt drop in ammonia excretion on day 8 is due to a similarly abrupt fall in ammonia production, or due, at least in part, to a diversion of produced ammonia into the renal-venous blood. If the latter explanation was true, then it would be expected that the animals would become quite sick during recovery. Since this did not appear to be the case it was thought likely that the production of ammonia did parallel the excretion of ammonia in the urine. To ensure that this conclusion was correct, the utilization of glutamine by the kidney was measured. This was determined by measuring glutamine arterio-renal venous differences.

Table 3 shows that glutamine A-V differences increase in rats given 1.5% (w/v) NH_4Cl. This increase is statistically significant at day 3 ($P < 0.05$ compared to day 0) and remains elevated for the remainder of the 1.5% (w/v) NH_4Cl regimen. On return to tap water, the glutamine A-V differences fell abruptly ($P < 0.025$ day 8 compared to day 7) to very low levels. The negative A-V seen on day 9, although not significantly different from zero, may indicate that more glutamine leaves the kidney than enters it. This suggests that an increase in the synthesis of

Table 3 Glutamine Arterio Renal Venous Differences in Rats after Different Treatments

Day of Treatment	TREATMENT: Days on NH_4Cl 1.5%(w/v)	TREATMENT: Days on Water	Glutamine Arterio Renal Venous Differences: µmoles/ml plasma ±SD (no of animals)	P Value (Arterio Venous Difference Different from Zero)
0	-	∞	0.067±0.012(4)	<0.005
1	1	-	0.104±0.113(3)	NS
3	3	-	0.146±0.046(3)	<0.050
5	5	-	0.147±0.038(3)	<0.025
7	7	-	0.160±0.057(3)	<0.050
8	7	1	0.016±0.042(5)	NS
9	7	2	-0.025±0.034(3)	NS

NS indicates not significant ($P>0.05$)

glutamine from glutamate, via glutamine synthetase, may be involved in reducing net ammonia production.

Figure 4 compares ammonia excretion with glutamine A-V differences of rats after the treatment described in Tables 2 and 3. The 6-7 fold increase in ammonia excretion is accompanied by a 2-3 fold increase in the utilization of glutamine. The abrupt fall in glutamine A-V differences coincides with a similarly abrupt drop in ammonia excretion. It is concluded from this correlation that the abrupt drop in ammonia excretion is due to an abrupt drop in net ammonia production.

The response of the kidney in the production of ammonia can now be divided into 3 phases (Figure 4). The onset phase lasts for approximately 3 days during which there is a slow progressive increase in ammonia production. The plateau phase begins with the attainment of plateau levels of ammonia excretion on day 3 and lasts until the end of day 7. The rate of ammonia excretion returns to normal during the recovery phase which begins at the end of day 7 and is complete by the end of day 8.

B. Changes in Acid-Base Parameters in the Blood

Blood $[H^+]$, $[HCO_3^-]$ and pCO_2 comprise a group of interrelated parameters that define the acid-base status of the animal. It is not unreasonable to assume that changes in these parameters could be transmitted to the intracellular environment of the cells responsible for producing urine ammonia and thus may potentially be of regulatory importance. To help assess their role in this regard, it was necessary to know how these parameters changed in relation to ammonia production. For this purpose,

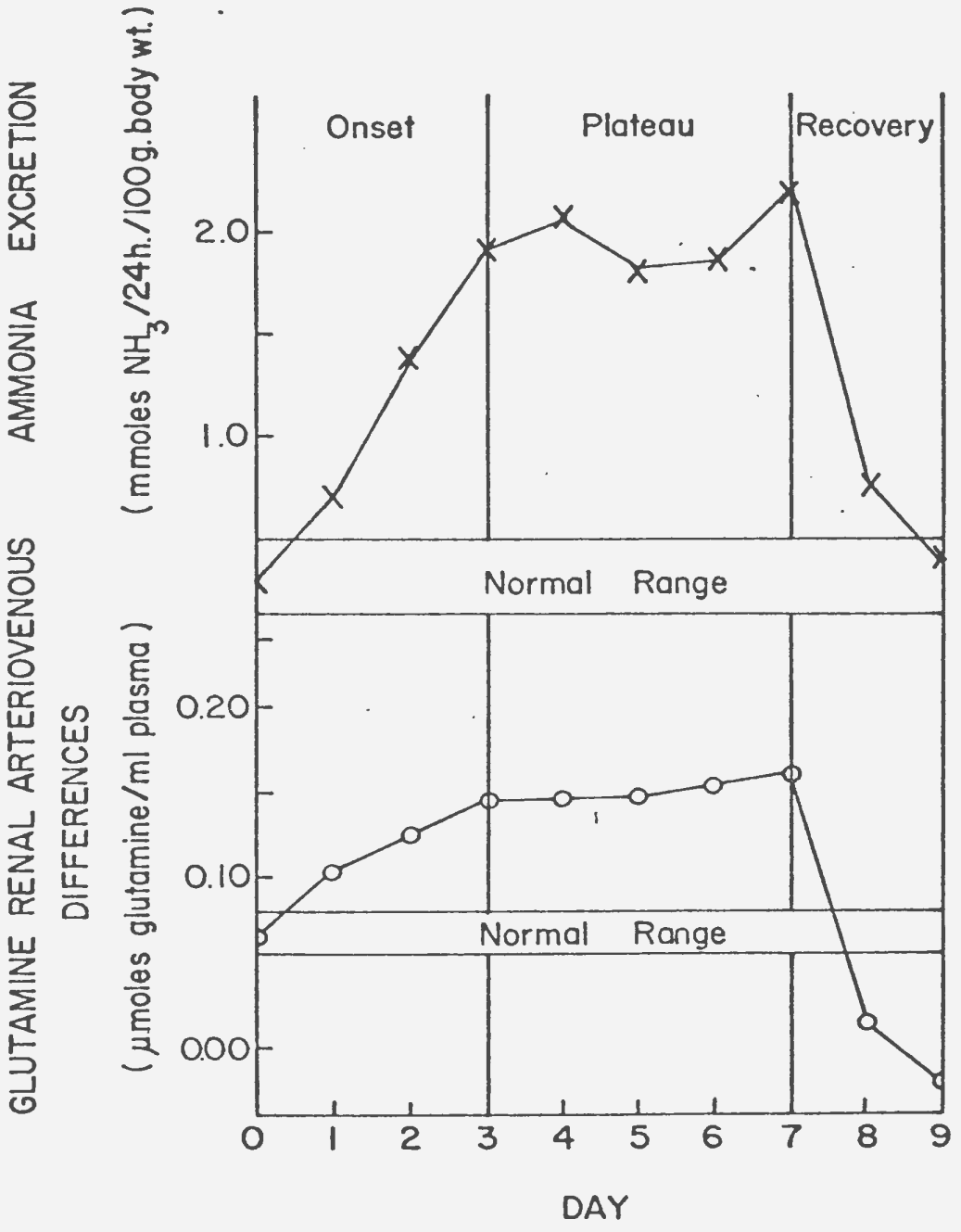

Figure 4 Comparison of Glutamine Renal Arteriovenous Differences with Uninary Ammonia Excretion during Onset, Plateau and Recovery

experiments were performed in which these parameters were measured during the different phases of adaptation and recovery. This required taking samples of blood from the animals. It was undesirable to use an anesthetic because even light anesthesia affects respiration and hence alters the pCO_2. Instead, blood was sampled, without anesthesia, from the tail. To reduce variability the same animals were used throughout the experiment. This required that blood be sampled from each rat every day for 9 consecutive days. Such treatment could possibly affect respiration and cause metabolic changes due to stress. To control for this, the acid-base parameters were also determined in a control group which was allowed tap water to drink. The animals in the control group were handled and the blood sampled in the same manner as the animals of the experimental group.

1. Blood Hydrogen Ion Activity

Table 4 shows the blood hydrogen ion activity (a_H) of the control group and the experimental group. The a_H values were calculated* from measured pH values before determining the mean $\pm$ standard deviation (SD). In the control group, the a_H did not change significantly throughout the duration of the experiment. In the experimental group, the a_H rose sharply during the first day of treatment and remained elevated at this level over the next 2-3 days. Thereafter, the a_H gradually returned

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* 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 (13).

Table 4 Blood Hydrogen Ion Activity in Control and Experimental Rats

Day of Treatment	CONTROL GROUP		EXPERIMENTAL GROUP		
	Blood a_H nEq/l±SD (no of animals)	P Different from Day 0	Blood a_H nEq/l±SD (no of animals)	P Different from Day 0	P Experimental Group Different from Control Group
0	38.7±2.3(4)		39.6±1.8(8)		NS
1	37.8±3.7(4)	NS	54.5±5.9(8)	<0.001	<0.001
2	40.3±3.5(4)	NS	57.1±8.6(8)	<0.001	<0.01
3	37.5±3.1(4)	NS	52.9±4.9(8)	<0.001	<0.001
4	38.2±1.4(4)	NS	48.0±4.7(8)	<0.001	<0.005
5	36.4±4.7(3)	NS	45.8±3.7(8)	<0.005	<0.025
6	38.0±3.3(4)	NS	44.5±5.8(4)	NS	NS
7	37.7±0.1(4)	NS	46.1±3.7(8)	<0.001	<0.005
8	36.8±1.6(4)	NS	35.6±2.2(8)	<0.005	NS
9	38.3±2.3(4)	NS	37.1±2.0(8)	<0.05	NS

The control group drank tap water for the duration of the experiment. The experimental group drank 1.5% (w/v) NH_4Cl for the first 7 days and then was returned to tap water for the remainder of the experiment. NS indicates not significant (P>0.05)

towards normal. Day 6 is not significantly different from either day 0 of the experimental group or day 6 of the control group. However, the mean for day 6 of the experimental group was calculated from only 4 determinations. Consequently, variability prevents this mean from being significantly different from normal. It is expected, in light of the fact that the a_H for day 7 is still significantly higher than normal, that day 6 is also above normal and would prove significant after more values are obtained. When the experimental group was returned to tap water after drinking 1.5% (w/v) NH_4Cl for 7 days, there was an abrupt fall in blood a_H from slightly above normal on day 7 to slightly below normal on day 8. Although the blood a_H on days 8 and 9 are both lower than normal, they are not significantly different from the blood a_H of days 8 and 9 of the control group.

Figure 5 compares blood a_H of the control and experimental groups with ammonia excretion during the onset, plateau and recovery phases. It is evident from this comparison that ammonia excretion is not a simple function of blood a_H. Although it is possible that the elevated blood a_H during the onset phase is an initial stimulus for the increase in ammonia production, it is clear that there is no delay in acidification of the blood, similar to the time lag for the adaptive increase in ammonia excretion. Also, since a_H falls during the plateau phase while the rate of ammonia excretion remains elevated, then it would appear that a_H is not responsible for the sustained adaptive response. Obviously, the fall in blood a_H towards normal during the plateau phase occurs because the animal has developed its ability to excrete acid, as a result of its in-

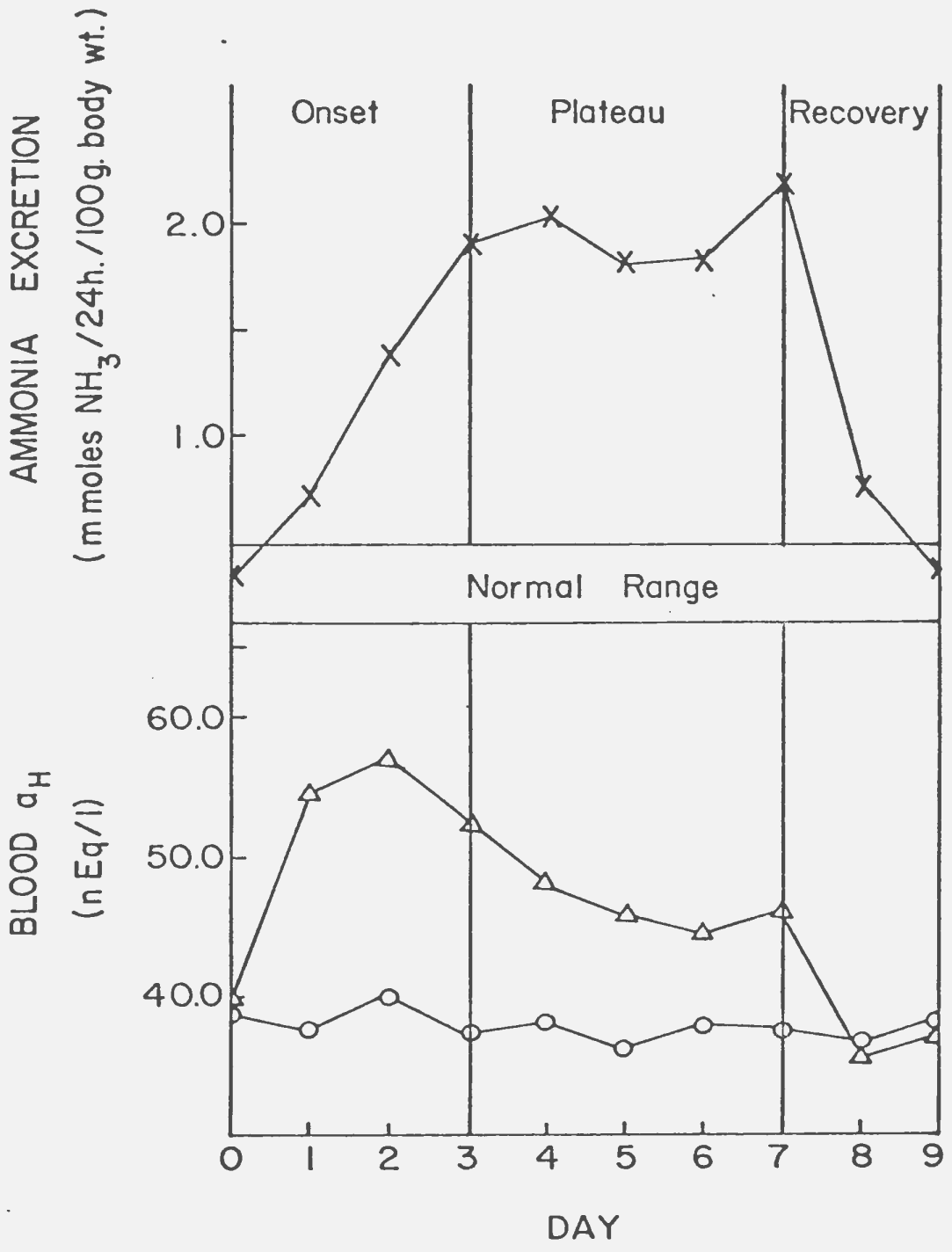

Figure 5 Comparison of Blood a_H with Urinary Ammonia Excretion during Onset, Plateau and Recovery

O——O Control Group

Δ——Δ Experimental Group

creased renal capacity to synthesize ammonia. However, it appears that a_H does not completely return to normal during the plateau phase. Therefore, although a_H is probably not directly responsible for sustaining the maximal adaptive response it may be indirectly important for continued ammonia excretion. Thus, the further fall in a_H observed during the recovery phase may play a role in turning off ammonia production.

Masoro and Siegel (70) define acidosis "as an abnormal condition or process which would produce a fall in the pH or 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 an acidosis with a normal blood pH or H^+ concentration". Thus, even though the blood a_H has returned to near normal within 5 or 6 days, these animals should still be regarded as acidotic.

2. Blood Bicarbonate Ion Concentration

Table 5 shows the blood bicarbonate ion concentration $[HCO_3^-]$ in control and experimental rats. In the control group the $[HCO_3^-]$ increased significantly on days 7 and 8. The reason for this is not known. However, it is unlikely that these increases can be attributed to errors in their measurement since the blood-gas machine was calibrated before each determination. It is possible that the method of sampling the blood (i.e., by tail prick) contributes to the variation in pCO_2.

In the experimental group the blood $[HCO_3^-]$ drops abruptly during the first day of treatment and remains at this level for another 2-3 days. Thereafter, the $[HCO_3^-]$ gradually returns to normal by day 5 and remains

Table 5 Blood Bicarbonate Ion Concentration in Control and Experimental Rats

Day of Treatment	CONTROL GROUP		EXPERIMENTAL GROUP		
	Blood $[HCO_3^-]$ mEq/l±SD (no of animals)	P Different from Day 0	Blood $[HCO_3^-]$ mEq/l±SD (no of animals)	P Different from Day 0	P Experimental Group Different from Control Group
0	24.2±2.6(4)		24.8±2.2(8)		NS
1	26.3±2.2(4)	NS	19.4±4.0(8)	<0.01	<0.025
2	24.3±3.5(4)	NS	19.4±3.9(8)	<0.01	NS
3	25.9±2.7(4)	NS	19.4±2.4(8)	<0.001	<0.005
4	25.5±2.1(4)	NS	21.2±2.7(8)	<0.025	<0.05
5	28.1±3.2(3)	NS	23.2±2.4(8)	NS	<0.05
6	27.1±2.4(4)	NS	25.6±4.3(4)	NS	NS
7	29.7±1.4(4)	<0.025	23.8±3.7(8)	NS	<0.025
8	29.2±2.0(4)	<0.05	35.2±1.9(8)	<0.001	<0.001
9	28.0±2.1(4)	NS	28.2±3.1(8)	<0.05	NS

The control group drank tap water for the duration of the experiment. The experimental group drank 1.5% (w/v) NH_4Cl for the first 7 days and then was returned to tap water for the remainder of the experiment. NS indicates not significant (P>0.05)

within the normal range for the duration of the 1.5% (w/v) NH_4Cl regimen. After day 7, when the rats were returned to tap water, there is a highly significant increase in blood $[HCO_3^-]$. The reason for this overcompensation is not clear.

Figure 6 compares the blood $[HCO_3^-]$ of the control and experimental groups with ammonia excretion during the onset, plateau and recovery phases. The blood $[HCO_3^-]$ of the experimental group falls abruptly to a minimum during day 1 and remains at this level for the duration of the onset phase. Since during the onset phase ammonia excretion only slowly increases while no comparable delay in blood $[HCO_3^-]$ changes occurs, then it is clear that a rapid direct control of renal ammoniagenesis by blood bicarbonate cannot be invoked. It is also obvious that low blood $[HCO_3^-]$ is not responsible for the sustained adaptive response of ammonia production since, during the plateau phase, the $[HCO_3^-]$ returns to normal while the rate of ammonia excretion remains elevated. However, it is possible that the drop in blood $[HCO_3^-]$ during the onset phase is an initial stimulus for the increase in ammonia production. It is also possible that the abrupt rise in $[HCO_3^-]$ in the recovery phase is involved with turning off the production of ammonia.

3. Blood Carbon Dioxide Tension

Table 6 shows the blood carbon dioxide tension (pCO_2) of the control and experimental groups. In the control group, there is a significant increase in pCO_2 on day 7. The reason for this is not known, but it may be purely fortuitous. In the experimental group there are significant increases in pCO_2 on days 6 and 8. However, only the pCO_2 for day 8

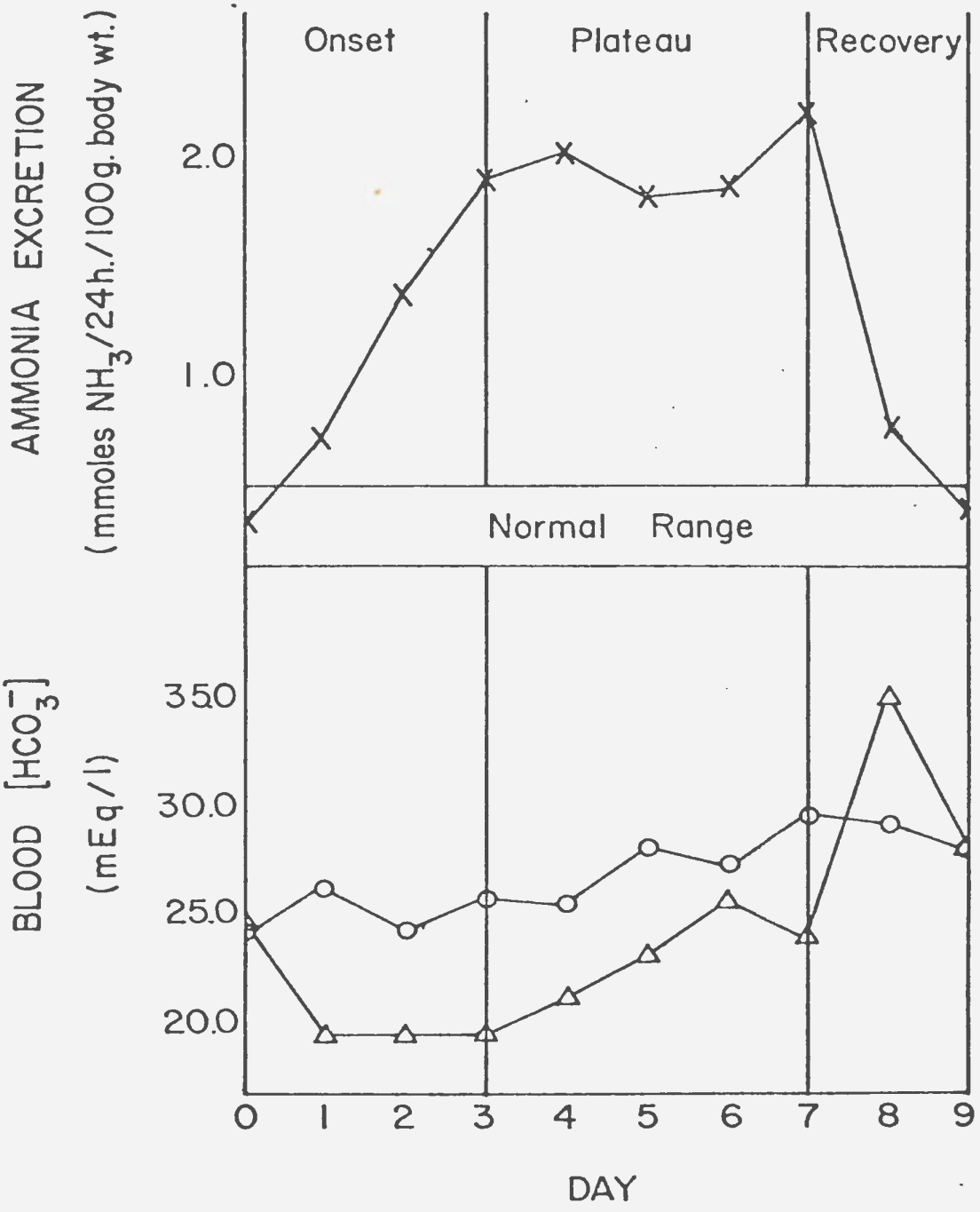

Figure 6 Comparison of Blood $[HCO_3^-]$ with Urinary Ammonia Excretion During Onset, Plateau and Recovery

O—O Control Group

Δ—Δ Experimental Group

Table 6 Blood pCO_2 in Control and Experimental Rats

Day of Treatment	CONTROL GROUP		EXPERIMENTAL GROUP		
	Blood pCO_2 mmHg ±SD (no of animals)	P Different from Day 0	Blood pCO_2 mmHg ±SD (no of animals)	P Different from Day 0	P Experimental Group Different from Control Group
0	39.2±4.0(4)		41.2±3.6(8)		NS
1	41.7±5.5(4)	NS	43.8±6.1(8)	NS	NS
2	40.6±3.5(4)	NS	45.5±6.3(8)	NS	NS
3	40.6±3.9(4)	NS	42.9±4.8(8)	NS	NS
4	40.9±3.6(4)	NS	42.6±4.7(8)	NS	NS
5	42.9±7.8(3)	NS	44.4±2.5(8)	NS	NS
6	43.3±5.1(4)	NS	47.2±3.0(4)	<0.05	NS
7	46.9±3.2(4)	<0.05	45.6±4.1(8)	NS	NS
8	45.1±4.5(4)	NS	51.6±3.0(8)	<0.001	<0.025
9	45.0±3.0(4)	NS	43.8±4.4(8)	NS	NS

The control group drank tap water for the duration of the experiment. The experimental group drank 1.5% (w/v) NH_4Cl for the first 7 days and then was returned to tap water for the remainder of the experiment. NS indicates not significant ($P>0.05$)

is significantly different from the pCO_2 for the corresponding day of the control group.

Figure 7 compares the blood pCO_2 of the control and experimental groups with ammonia excretion during the onset, plateau and recovery phases. No respiratory adaptation (i.e., decreased pCO_2) during onset was observed although it is possible that one occured during the first day since pCO_2 was only measured every 24 hours. During recovery pCO_2 increases above normal. This may have been a respiratory response involved in stabilizing the blood $[H^+]$ during this phase. Since this increase in pCO_2 coincides with the return of ammonia excretion to normal, it may also be possible that pCO_2 is involved with turning off ammonia production.

C. Response of Enzymes Involved in the Renal Metabolism of Glutamine

Phosphate dependent glutaminase (PDG) and phosphoenolpyruvate carboxykinase (PEPCK) have both been implicated as rate-determining steps in the renal metabolism of glutamine. PDG activity, measured *in vitro*, has been shown to correlate with ammonia excretion *in vivo* during the onset and plateau phases of adaptation (92). Therefore, it was of interest to see what would happen to the activity of this enzyme during the recovery phase. The activity of PEPCK has also been shown to increase during the onset phase (6). However, the activity of this enzyme has not been studied in relation to ammonia production beyond 48 hours of acid challenge. This is surprising since adaptation of ammonia production can take longer than 2 days. It was, therefore, of interest to investigate the activity of this enzyme during adaptation and recovery.

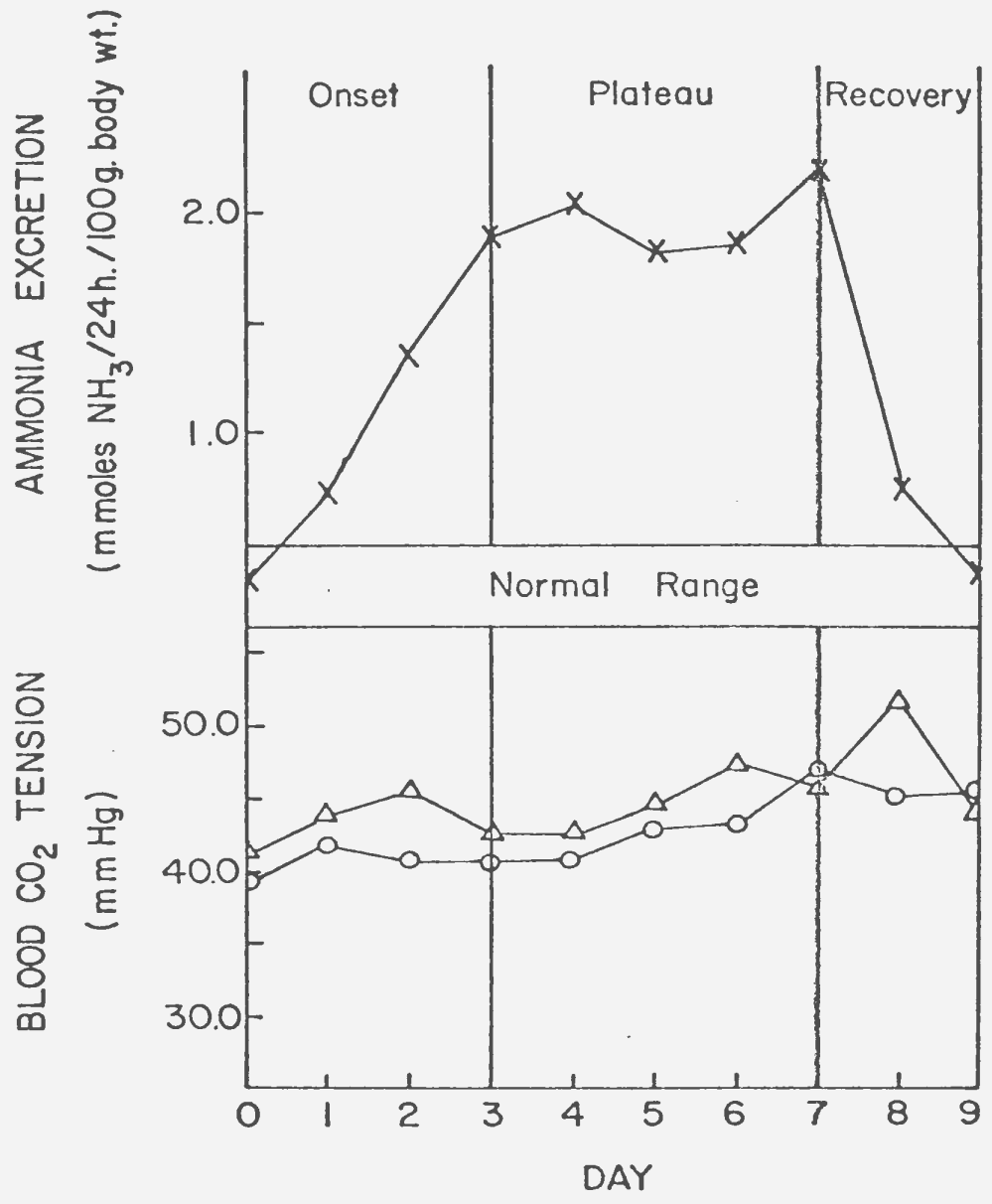

Figure 7 Comparison of Blood CO_2 Tension with Urinary Ammonia Excretion during Onset, Plateau and Recovery

O—O Control Group

Δ—Δ Experimental Group

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.

The activities of PDG and PEPCK were measured using entire kidney preparations. Entire kidney preparations were employed since it would be difficult to quantitatively excise the cortex from all kidneys and also such measurements allow calculation of total kidney activities. The main disadvantage is that the magnitude of any change is reduced. However, this masking is only small since, in the rat, the bulk of the kidney is cortex tissue.

The activity of each enzyme is expressed in 2 ways: first, as mmoles/24 hr/g kidney and second, as mmoles/24 hr/100 g body weight. The activities were 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 activities should also be expressed per 100 g body weight. This mode of expression takes into account the total activity of the enzyme and the work load required of it. It also has the advantage of making comparisons with ammonia excretion more meaningful.

1. Phosphate Dependent Glutaminase

PDG activity increased in rats drinking 1.5% (w/v) NH_4Cl. Table 7

Table 7 The Activities of Phosphate Dependent Glutaminase and Phosphoenolpyruvate Carboxykinase (expressed in mmoles/24h/g kidney) in the Kidneys of Rats after Different Treatments

Day of Treatment	TREATMENT		PHOSPHATE DEPENDENT GLUTAMINASE ACTIVITY	PHOSPHOENOL-PYRUVATE CARBOXYKINASE ACTIVITY
	Days on 1.5%(w/v) NH_4Cl	Days on Water	mmoles/24h/g Kidney ±SD (no of animals)	
0	-	∞	28.3± 3.7(14)	9.2±1.6(15)
1	1	-	34.2± 4.3 (5)	19.8±4.9 (7)
2	2	-	46.8± 8.8 (6)	25.1±6.6(10)
3	3	-	64.7±10.7 (6)	28.4±8.3(10)
4	4	-	64.2±12.9 (6)	26.7±7.2 (7)
5	5	-	70.3±16.4 (6)	28.7±7.7 (7)
6	6	-	67.4±24.9 (5)	24.6±5.7 (6)
7	7	-	78.5±17.7(10)	26.7±6.3(10)
8	7	1	85.9±32.9 (5)	12.7±5.3 (5)
9	7	2	77.0±40.8 (5)	7.5±1.3 (4)

shows the activity of PDG in mmoles/24 hr/g kidney. When expressed in these units the increase is statistically significant at day 1 ($P < 0.025$ compared to day 0). The activity continued to climb ($P < 0.05$ day 2 compared to day 1) to reach a plateau level at about day 3 ($P < 0.0025$ compared to day 2). After day 7, when the rats were returned to tap water, the activity of PDG remained elevated ($P < 0.05$ day 8 compared to day 2). However, the standard deviation of the mean on day 9 increased such that the activity was significantly different only from that for day 0 ($P < 0.001$).

Table 8 shows the activity of PDG expressed in mmoles/24 hr/100 g body weight. A similar pattern is observed. However, the increase is not statistically significant until day 2 ($P < 0.001$ compared to day 0). The plateau level was achieved at day 3 ($P < 0.050$ compared to day 2). The activity of PDG remained at plateau levels ($P < 0.05$ day 8 compared to day 2) after the rats were returned to tap water, but again, the standard deviations of the means increased such that the means were significantly different only from that for day 0 ($P < 0.005$ for both days 8 and 9 compared to day 0).

Figure 8 compares PDG activity with urine ammonia excretion during the onset, plateau and recovery phases. There is a good correlation during the onset and plateau phases. Significant increases in PDG activity occur with significant increases in ammonia excretion. Both reach a plateau level by the end of day 3. On the other hand, a marked dissociation is observed during the recovery phase. Whereas PDG activity remains at high plateau levels, ammonia excretion abruptly falls to normal during this period. It is obvious from this dissociation that,

Table 8 The activities of Phosphate Dependent Glutaminase and Phosphoenolpyruvate Carboxykinase (expressed in mmoles/24h/100g body wt) in Rats after Different Treatments

Day of Treatment	TREATMENT Days on 1.5%(w/v) NH_4Cl	TREATMENT Days on Water	PHOSPHATE DEPENDENT GLUTAMINASE	PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY
			mmoles/24h/100g body wt ±SD (no of animals)	
0	-	∞	21.6± 4.1(14)	7.0±1.5(15)
1	1	-	24.9± 6.4 (5)	15.5±4.6 (7)
2	2	-	34.3± 8.6 (6)	17.7±5.6(10)
3	3	-	48.0± 9.4 (6)	21.7±6.6(10)
4	4	-	50.1±10.1 (6)	20.1±5.6 (7)
5	5	-	56.2±15.1 (6)	22.3±7.1 (7)
6	6	-	55.4±23.3 (5)	19.8±5.7 (6)
7	7	-	60.3±16.2(10)	21.2±6.3(10)
8	7	1	62.7±25.2 (5)	9.2±4.0 (5)
9	7	2	63.5±42.0 (5)	5.6±0.8 (4)

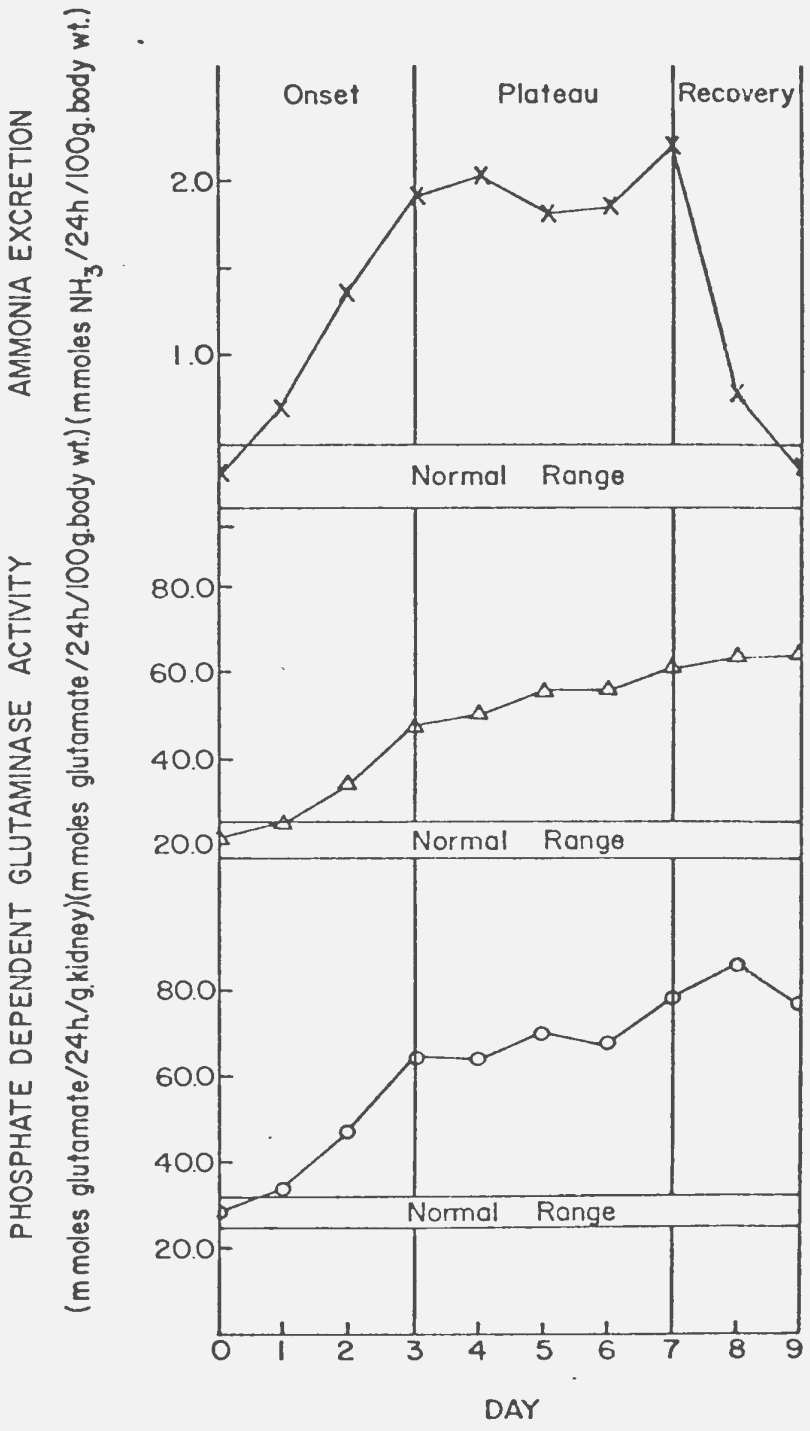

Figure 8 Comparison of Phosphate Dependent Glutaminase Activity with Urinary Ammonia Excretion during Onset, Plateau and Recovery

although the measured activity of this enzyme is such that it is capable of metabolizing glutamine at the high rates seen during the plateau phase, this activity is not expressed *in vivo* during recovery. Clearly then ammoniagenesis from glutamine is not regulated solely by the total amount of PDG present.

2. Phosphoenolpyruvate Carboxykinase

The activity of PEPCK also increased in the kidneys of rats given 1.5% (w/v) NH_4Cl. The activities of both PDG and PEPCK increased approximately 2-3 fold. Table 7 shows the activity of PEPCK in mmoles/24 hr/g kidney. As seen in this Table, the increase is statistically significant at day 1 ($P < 0.001$ compared to day 0). However, further increases over day 1 are significant only on day 3 ($P < 0.05$), day 5 ($P < 0.05$) and day 7 ($P < 0.05$) and no significant increase over the activity of day 2 is observed. Unlike PDG, PEPCK activity fell immediately ($P < 0.001$ day 8 compared to day 7) when the rats were returned to tap water. Although the decrease from day 8 to day 9 is not significant, the activity at the end of day 9 is back to that of day 0. In Table 8, PEPCK activity is expressed in mmoles/24 hr/100 g body weight. When expressed in these units the increase in PEPCK activity is significant at day 1 ($P < 0.001$ compared to day 0), however, no further increase over the activity of day 1 is observed, possibly due to the scatter of the data. PEPCK activity returns to normal on day 8 ($P < 0.001$ day 8 compared to day 7). Although a further decrease occurs on day 9 ($P < 0.005$ compared to day 8) the activity of PEPCK at the end of day 9 is not significantly different from that of day 0.

Figure 9 compares PEPCK activity with urine ammonia excretion during the onset, plateau and recovery phases. PEPCK attains maximum activity within the first 2 days of the 1.5% (w/v) NH_4Cl regimen whereas ammonia excretion does not reach its plateau until the end of day 3. Thus, an increase in ammonia excretion occurs on the third day of treatment without a detectable increase in PEPCK activity. This dissociation suggests that the maximal activity of PEPCK cannot be the sole rate-limiting step for the production of ammonia during this phase. On the other hand, the abrupt fall in ammonia excretion during the recovery phase coincides with a similarly abrupt drop in PEPCK activity. This correlation is consistent with an important role for this enzyme in regulating ammonia production during recovery.

D. Effect of Fasting on Urine Ammonia Excretion, Phosphate Dependent Glutaminase and Phosphoenolpyruvate Carboxykinase During the Recovery Phase

The marked correlation between PEPCK activity and urine ammonia excretion is consistent with this enzyme playing a role in the regulation of ammonia production during the recovery phase. It is also well known that starvation is accompanied by an increase in renal PEPCK activity (45, 54). It was therefore of interest to examine the effect of fasting during the recovery phase on ammonia excretion, PDG activity and PEPCK activity.

1. Starved Control

To serve as a control, rats were deprived of food for 24 and 48

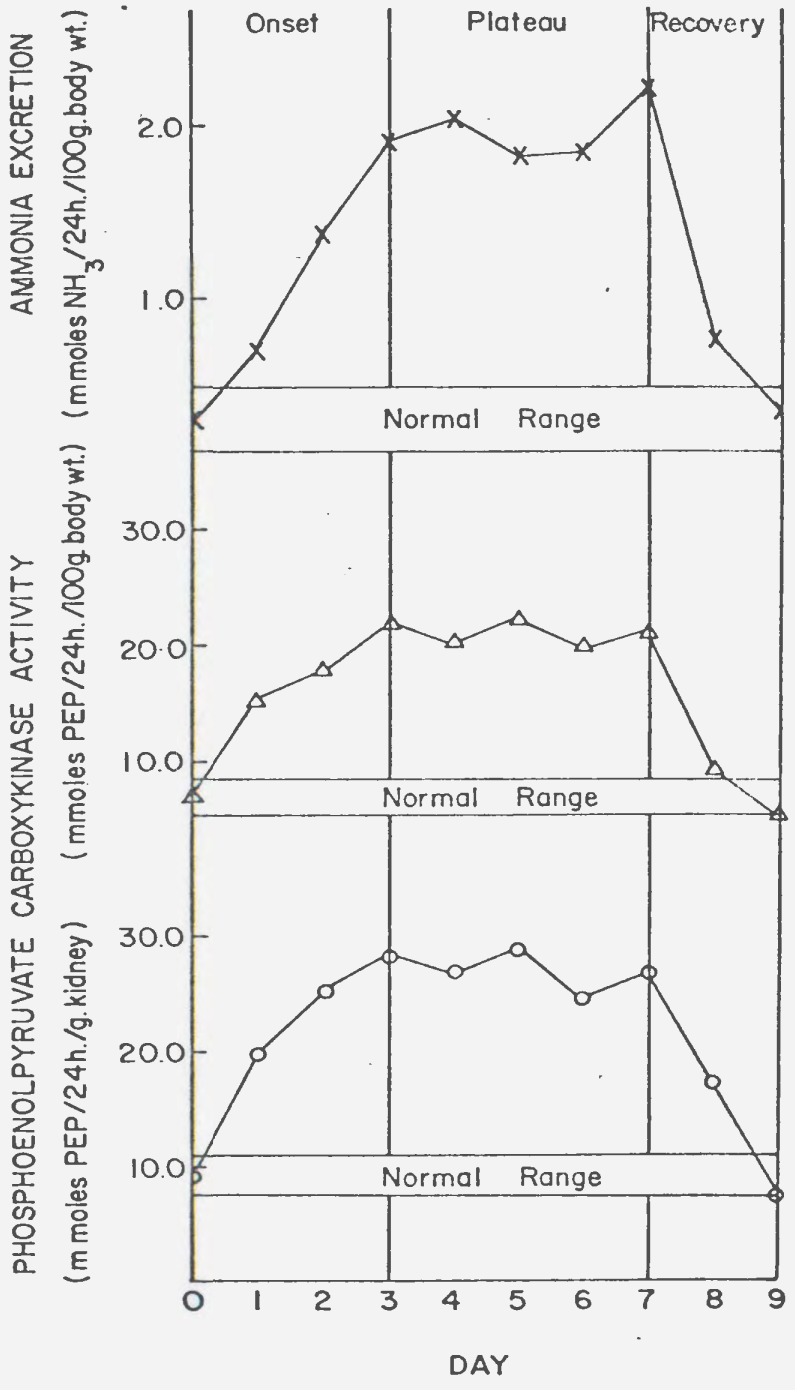

Figure 9 Comparison of Phosphoenolpyruvate Carboxykinase Activity with Urinary Ammonia Excretion during Onset, Plateau and Recovery

hours. Table 9 shows the urinary ammonia excretion, PDG activity and PEPCK activity after this treatment. No change in urinary ammonia excretion is observed. There is a decrease in PDG activity after 24 hours of fasting. The P value for day 1 compared to day 0 is < 0.05 when the activity is expressed per g of kidney and is < 0.025 when expressed per 100 g body weight. However, after 48 hours of fasting, the activity of PDG is not significantly different from either day 0 or day 1. Therefore, it is quite probable that the small drop in PDG activity after 24 hours is due to the large variability between animals and the small number of rats used in this study, rather than to the effect of fasting. PEPCK activity increases after 24 hours of fasting. The P value for day 1 compared to day 0 is < 0.005 when PEPCK activity is expressed per g of kidney and is < 0.025 when expressed per 100 g body weight. No further increase in activity above that attained by the end of day 1 is observed.

2. Starved Recovery

Table 10 shows ammonia excretion, PDG activity and PEPCK activity in rats which, after drinking 1.5% (w/v) NH_4Cl for 7 days, were returned to tap water and deprived of food for 24 and 48 hours. The urinary ammonia excretion fell abruptly after the 1.5% (w/v) NH_4Cl was removed ($P < 0.001$ day 8 compared to day 7) but was still significantly higher than normal ($P < 0.005$) on day 8. Although there was no further significant drop, the ammonia excretion on day 9 was back to normal.

The activity of PDG remained elevated at high plateau levels. The P values for days 8 and 9 compared to day 0 in both modes of expression are < 0.001. PEPCK activity dropped after the rats were returned to tap

Table 9 Urinary Ammonia Excretion and the Renal Activities of Phosphate Dependent Glutaminase and Phosphoenolpyruvate Carboxykinase in Rats during Starved Control

Day of Treatment	TREATMENT	URINARY AMMONIA EXCRETION	PHOSPHATE DEPENDENT GLUTAMINASE ACTIVITY		PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY	
		mmoles/24h/ 100g body wt	mmoles/24h/ g kidney	mmoles/24h/ 100g body wt	mmoles/24h/ g kidney	mmoles/24h/ 100g body wt
		±SD (no of animals)				
0	Normal	0.30±0.18(12)	28.3±3.7(14)	21.6±4.1(14)	9.2±1.6(15)	7.0±1.5(15)
1	Starved(24h)	0.36±0.24(10)	23.2±1.9 (3)	15.0±1.2 (3)	14.8±4.4 (3)	9.6±2.9 (3)
2	Starved(48h)	0.32±0.16(11)	23.4±2.9 (3)	16.2±1.3 (3)	14.4±3.0 (3)	9.9±1.8 (3)

Table 10 Urinary Ammonia Excretion and the Renal Activities of Phosphate Dependent Glutaminase and Phosphoenolpyruvate Carboxykinase in Rats during Starved Recovery

Day of Treatment	TREATMENT: Days on 1.5%(w/v) NH_4Cl	TREATMENT: Days on Water	URINARY AMMONIA EXCRETION: mmoles/24h/100g body wt	PHOSPHATE DEPENDENT GLUTAMINASE ACTIVITY: mmoles/24h/g kidney	PHOSPHATE DEPENDENT GLUTAMINASE ACTIVITY: mmoles/24h/100g body wt	PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY: mmoles/24h/g kidney	PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY: mmoles/24h/100g body wt
			±SD (no of animals)				
7	7	-	2.22±0.45(10)	78.5±17.7(10)	60.3±16.2(10)	26.7±6.3(10)	21.2±6.3(10)
8	7	1(starved)	0.80±0.31 (4)	86.0±18.3 (4)	80.4±23.8 (4)	16.9±5.3 (5)	15.7±6.7 (5)
9	7	2(starved)	0.52±0.23 (4)	81.1± 9.9 (3)	67.0±13.5 (3)	14.5±2.0 (5)	11.6±1.0 (5)

water, but remained elevated above normal. This fall in activity is significant after 1 day on tap water ($P < 0.025$ compared to day 7) when the activity is expressed per g of kidney. No further significant drop is observed when expressed in these units. The P values for days 8 and 9 compared to day 0 are both < 0.001. When PEPCK activity is expressed in mmoles/24 hr/100 g body weight, the drop in activity is not significant until day 9 ($P < 0.010$ day 9 compared to day 7). The P value for day 9 compared to day 0 is < 0.001.

Figure 10 compares the effect of fasting on urine ammonia excretion with the effect of fasting on PDG activity during the recovery phase. Ammonia excretion during starved recovery returns to normal with the same time course as ammonia excretion during fed recovery. Similarly, PDG has the same maximal activity during starved recovery as it does during fed recovery. Therefore, the same relationship between ammonia excretion and PDG activity exists in the starved recovery as it does in the fed recovery.

Figure 11 compares the effect of fasting on ammonia excretion with the effect of fasting on PEPCK activity. PEPCK activity, instead of returning to normal as it does in the fed recovery, drops from high plateau levels to starved control levels, which are significantly higher than normal. The P value for day 9 of the starved recovery compared to day 9 of the fed recovery is < 0.005 when expressed per g kidney and < 0.001 when expressed per 100 g body weight. In the starved control study, elevated PEPCK activity was not accompanied by an increase in ammonia excretion. In this starved recovery study, despite the elevated PDG activity, increased PEPCK activity is still not accompanied by increased

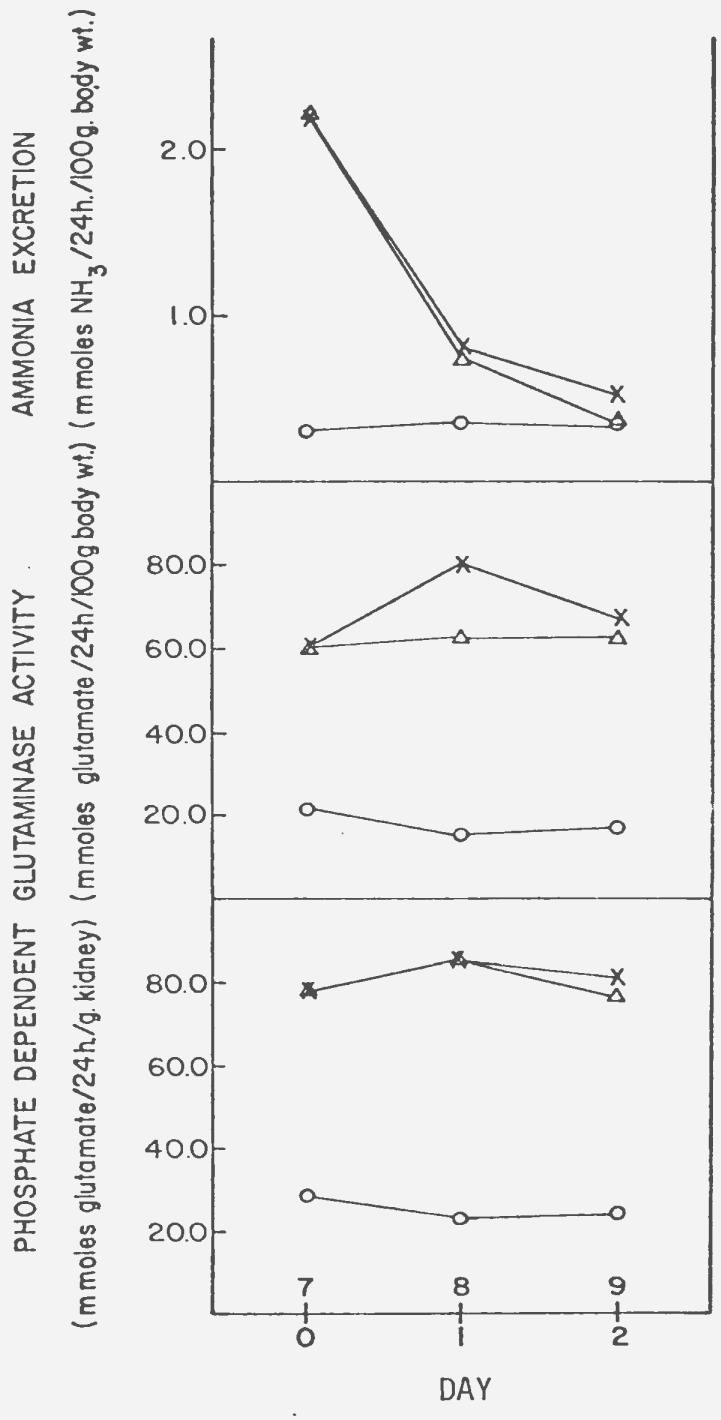

Figure 10 Comparison of Phosphate Dependent Glutaminase Activity with Urinary Ammonia Excretion during Starved Control, Starved Recovery and Fed Recovery

O——O Starved Control

X——X Starved Recovery

Δ——Δ Fed Recovery

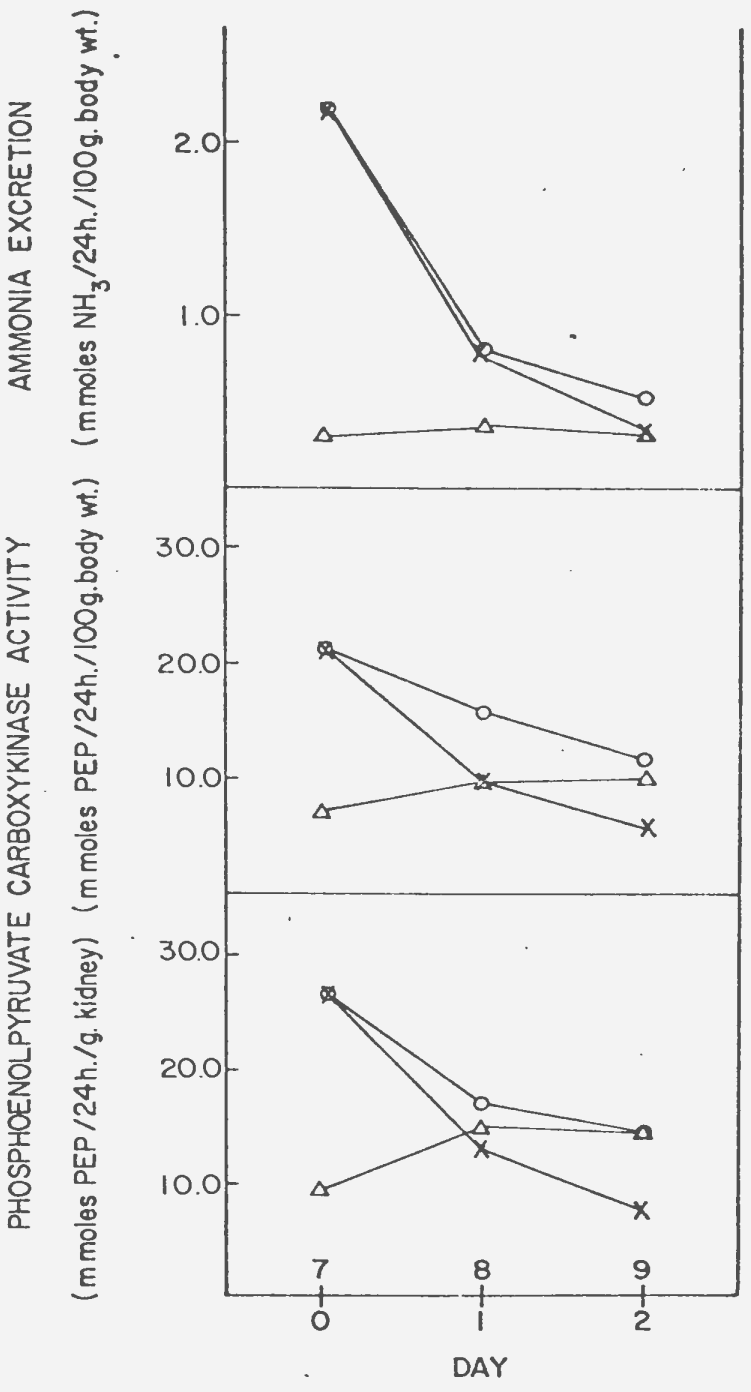

Figure 11 Comparison of Phosphoenolpyruvate Carboxykinase Activity with Urinary Ammonia Excretion during Starved Control, Starved Recovery and Fed Recovery

Δ——Δ Starved Control

O——O Starved Recovery

X——X Fed Recovery

urine ammonia. The lack of a correlation between PEPCK activity and urine ammonia excretion is inconsistent with this enzyme being a sole rate-determining step in ammonia production. The possibility still exists that the production of ammonia is increased but that the increased amount of ammonia formed is not trapped in the urine. However, this is considered unlikely. It seems pointless for the rat to have evolved a control mechanism which would allow large amounts of highly toxic ammonia to be released into the systemic circulation.

E. Recovery from the NH_4Cl Regimen before the Acid-Base Status of the Animal had an Opportunity to Improve

The abrupt drop in urine ammonia excretion, found in the present work, agrees with the results of Dies and Lotspeich (25). In both studies, rats had been kept on NH_4Cl long enough so that the plateau phase of ammonia excretion had been established for at least 2 days. Blood $[HCO_3^-]$ and pH measurements indicate that it is during this phase that the acid-base status of the animal improves. Therefore, it seemed possible that by the time the NH_4Cl was removed, the acid-base status of the animal had returned, almost to normal, such that continued excretion of ammonia during the recovery phase was not required.

In contrast, in experiments with humans (80, 114) high levels of ammonia were excreted in the urine for several days after the NH_4Cl regimen was discontinued. However, it appears that in these experiments the urine ammonia excretion was continually increasing over the duration of NH_4Cl injestion (4 or 5 days). It was, therefore, suspected that the ammonia excretion had not yet achieved a plateau phase or possibly had

just reached the plateau phase. Thus, since the onset phase is a situation where there is an excess of acid injestion over acid excretion, there would be an excess of acid in the body fluids and tissues. Consequently, it was thought that, when the NH_4Cl regimen was terminated, continued ammonia excretion was required to restore acid-base balance (i.e., to excrete excess acid and restore body sodium). The results of an experiment by Wood (115) support this conclusion. 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 for only 4 or 5 days (80, 114). Therefore, it was of interest to follow ammonia excretion in the rat during a recovery from a regimen of 1.5% (w/v) NH_4Cl before the acid-base status of the animal had an opportunity to recover during the plateau phase.

1. Early Recovery

It was shown in previous experiments that the plateau phase is not achieved until about the end of the third day of the 1.5% (w/v) NH_4Cl regimen. Therefore, in order to study recovery before the plateau phase had been well established, rats were returned to tap water after only 3 days on 1.5% (w/v) NH_4Cl. Table 11 shows the time course for the return of ammonia excretion to normal. The fall in ammonia excretion is not significant until day 5 ($P < 0.005$ compared to day 3). Although the difference between day 4 and day 5 is not significant, the P value is less than 0.10. Since the standard deviation for day 5 is relatively large, it is likely that this difference would prove significant if more rats

Table 11 Uninary Ammonia Excretion in Rats during Early Recovery

Day of Treatment	TREATMENT		UNINARY AMMONIA EXCRETION
	Days on 1.5%(w/v) NH_4Cl	Days on Water	mmoles/24h/100g body wt ±SD (no of animals)
3	3	-	1.91±0.51(7)
4	3	1	1.80±0.75(4)
5	3	2	0.77±0.22(4)
6	3	3	0.53±0.27(4)

are used. The ammonia excretion for day 5 is still significantly higher than normal ($P < 0.005$). However, the rate of ammonia excretion is probably back to normal by the end of day 5 since the value for this day is, of course, the cumulative excretion during the previous 24 hours. Ammonia excretion for day 6 is no longer significantly different from normal although the drop from day 5 to day 6 is not statistically significant.

Figure 12 compares the return of ammonia excretion to normal during recovery in rats after drinking 1.5% (w/v) NH_4Cl for 3 days with that during recovery in rats after drinking 1.5% (w/v) NH_4Cl for 7 days. The earlier recovery takes a day longer than does the later recovery. This suggests that the persistence of elevated levels of ammonia excretion in the recovery phase is necessary to restore the acid-base balance of the animal.

F. Response of Kidney Cortex Mitochondria

In the rat kidney PDG is located inside the mitochondrion (20, 56) whereas PEPCK is situated primarily in the cytosol (27). Therefore, isolated mitochondria afford a means of studying the metabolism of glutamine via PDG, *in situ*, unaffected by the influence of PEPCK. This level of organization also provides a system with some physiological integrity, so that the metabolism of glutamine can be studied as it may be affected by mitochondrial activators and inhibitors, transport processes and competing pathways.

The metabolism of glutamine by isolated mitochondria was studied in 2 ways: first, by measuring the production of ammonia from glutamine

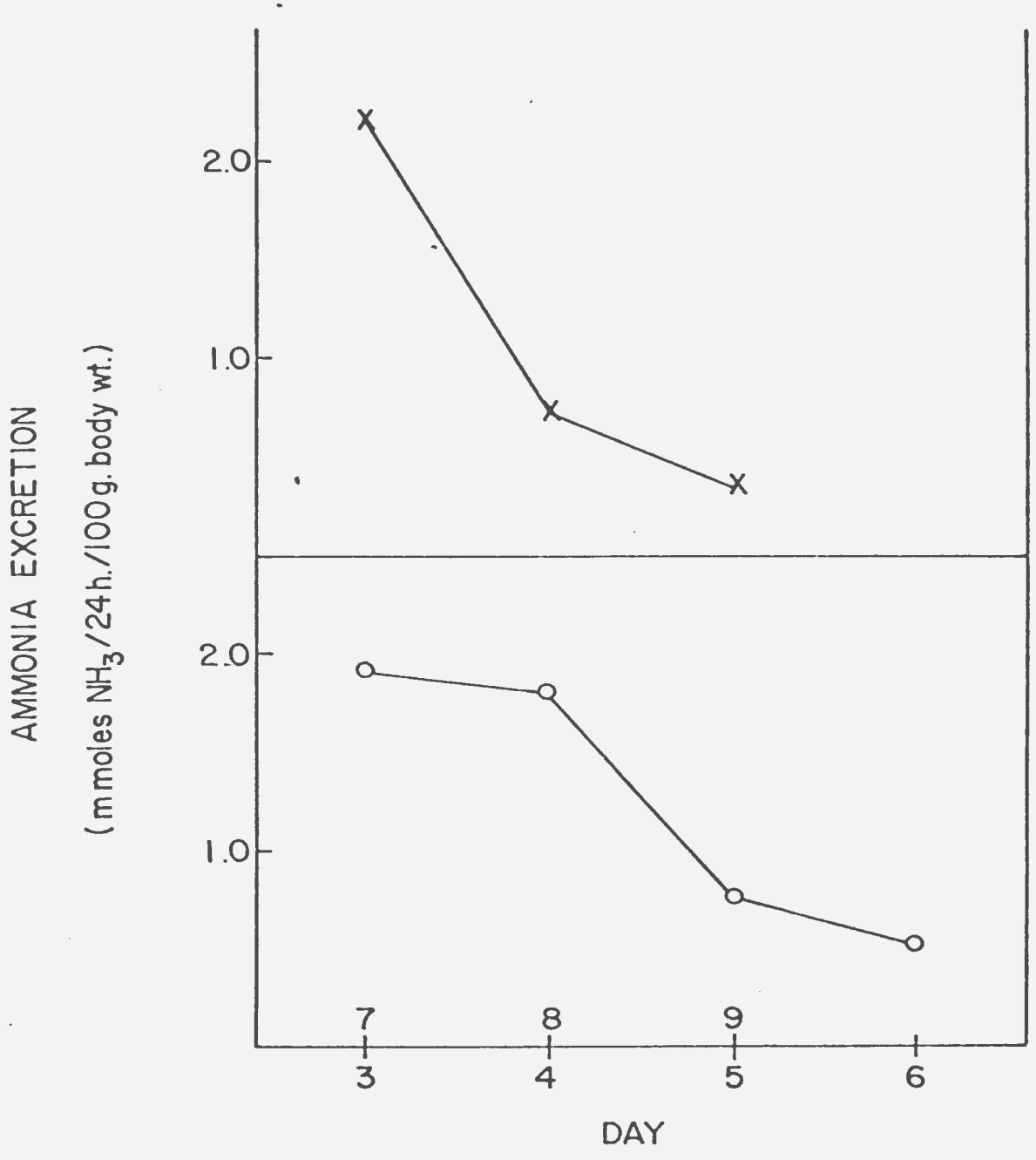

Figure 12 Comparison of Urinary Ammonia Excretion during Recovery after 3 Days on 1.5% NH_4Cl with that during Recovery after 7 Days on 1.5% NH_4Cl

and second, by measuring oxygen consumption during the metabolism of glutamine and related substrates (glutamate, α-ketoglutarate and succinate). The object of measuring oxygen consumption was to allow comparison between glutamine metabolism and the metabolism of the other mitochondrial substrates. In both studies the incubation temperature was 30° C. This temperature was used, although unphysiological, because higher temperatures have been shown to uncouple isolated mitochondria (12).

1. Ammonia Production

Table 12 shows that the capacity of mitochondria to produce ammonia from glutamine is elevated in mitochondria isolated from rats given 1.5% (w/v) NH_4Cl. This increase is statistically significant after 1 day of treatment ($P < 0.001$ compared to day 0). Plateau levels were achieved by day 3 ($P < 0.001$ day 3 compared to day 1). After day 7, when the rats were returned to tap water the mitochondrial ammonia production remained elevated above normal ($P < 0.005$ for days 8 and 9 compared to day 0), but was no longer significantly higher than that for day 1. Although days 8 and 9 are not statistically significantly lower than day 7, the P values of less than 0.10 tend to suggest a possible drop in mitochondrial ammonia production.

Figure 13 compares the rate of ammonia production by isolated mitochondria from glutamine with the rate of ammonia excretion *in vivo* during onset, plateau and recovery. There is a good correlation between ammonia production and ammonia excretion during the onset and plateau phases. Both increase significantly on the first day and continue to in-

Table 12 Ammonia Production from Glutamine (2mM) by Kidney Cortex Mitochondria from Rats after Different Treatments

Day of Treatment	TREATMENT		AMMONIA PRODUCTION
	Days on NH_4Cl 1.5%(w/v)	Days on Water	mmoles/24 h/100 mg mitochondrial protein ±SD (no of animals)
0	-	∞	2.7±0.5(5)
1	1	-	5.2±0.6(6)
3	3	-	8.8±1.5(3)
5	5	-	8.3±0.3(3)
7	7	-	10.2±2.7(5)
8	7	1	6.9±2.1(5)
9	7	2	6.6±1.8(5)

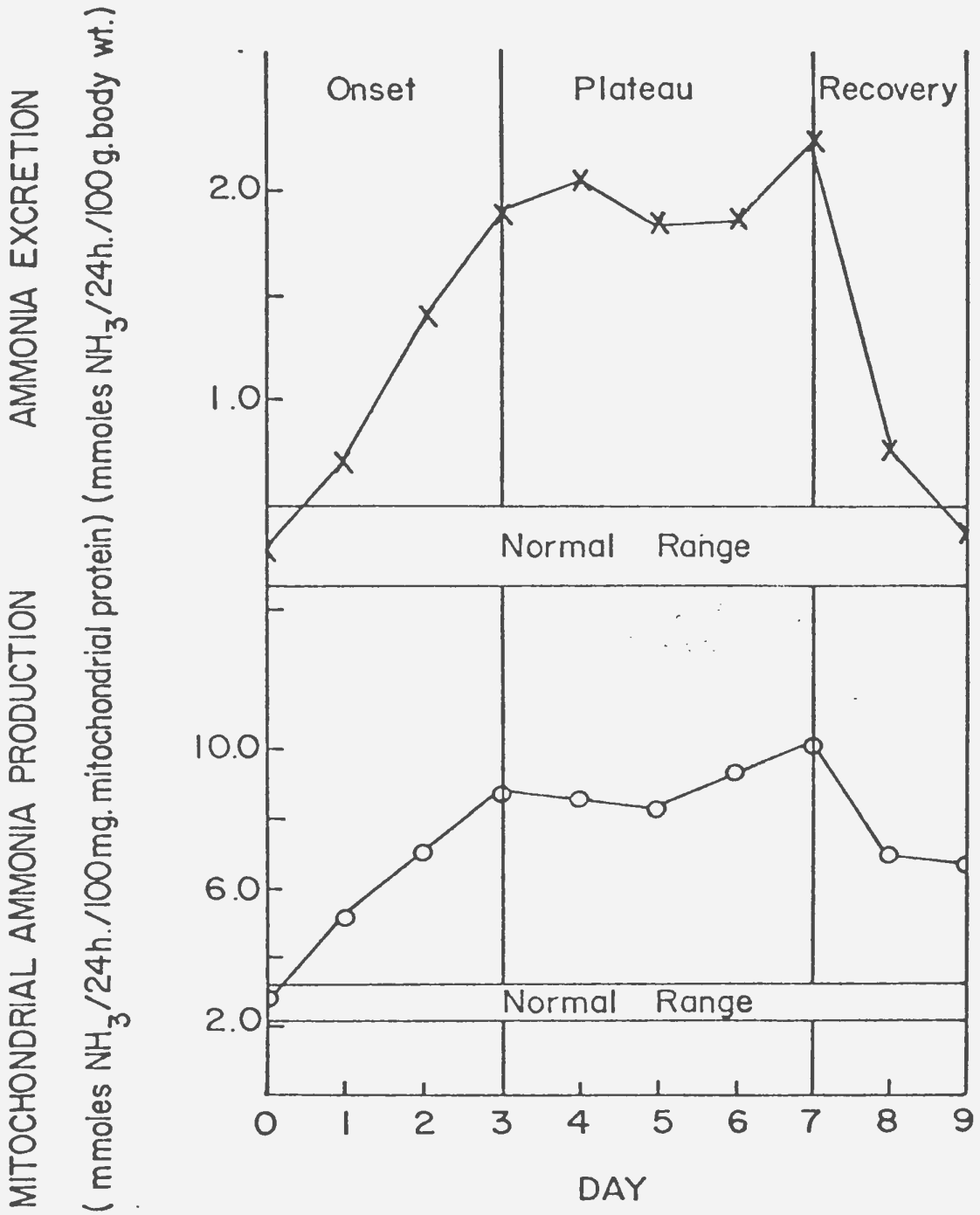

Figure 13 Comparison of Mitochondrial Ammonia Production with Urinary Ammonia Excretion during Onset, Plateau and Recovery

crease until day 3. However, whereas the increase in ammonia excretion is 6-7 fold, the increase in ammonia production is only 2-3 fold. This could be taken to indicate that an extramitochondrial event may be required for full expression of the mitochondrial capacity or it could possibly mean that an increase in extramitochondrial ammonia production may also be an important part of the adaptive response.

In the recovery phase a lack of correlation exists. Mitochondrial ammonia production remains elevated at above normal levels, whereas ammonia excretion drops abruptly to normal during this phase. This dissociation suggests that the regulation of ammonia production is not brought about solely by alterations in the mitochondrial capacity to metabolize glutamine.

2. Oxygen Consumption

Kidney cortex mitochondria isolated from rats drinking 1.5% (w/v) NH_4Cl have a greater capacity to metabolize glutamine. This was shown in studies measuring mitochondrial ammonia production. This is also evident from studies in which oxygen consumption was measured. Table 13 shows that oxygen consumption, using glutamine as substrate, is increased in mitochondria isolated from rats drinking 1.5% (w/v) NH_4Cl. This increase is statistically significant at day 1 ($P < 0.025$ compared to day 0). However, no further significant increase over day 1 is observed. This lack of a further significant increase may be due to the fact that the process being measured (i.e., oxygen consumption) is relatively far removed from the actual hydrolysis of glutamine, so that significant changes in the metabolism of glutamine may not involve

significant changes in oxygen consumption. In any case, it is evident that an increase in glutamine metabolism is reflected by an increase in oxygen consumption. It is also evident that the capacity of isolated mitochondria to metabolize glutamine remains elevated during the recovery phase. The P values comparing ammonia production for days 8 and 9 with that of day 0 are both < 0.001.

Table 13 also shows oxygen consumption by isolated mitochondria using glutamate, α-ketoglutarate and succinate as substrates. No adaptive change in the metabolism of these substrates is observed. Glutamine and glutamate are metabolized by mitochondria from control rats at approximately the same rate. However, glutamine is metabolized at a much faster rate than glutamate by mitochondria isolated from rats drinking 1.5% (w/v) NH_4Cl. Since removal of intramitochondrial glutamate is required for the oxidation of glutamine, it is probable that the transport of glutamate into mitochondria is the rate-limiting step for its metabolism. This being the case, then the lack of a detectable change in the metabolism of glutamate by isolated mitochondria cannot be taken to mean that the metabolism of intramitochonrial glutamate is not accelerated during adaptation.

Figure 14 compares the rate of oxygen consumption by isolated mitochondria, using glutamine as substrate, with *in vivo* utilization of glutamine by the kidney. The major point of interest in this comparison is that glutamine metabolism by isolated mitochondria, as reflected by oxygen consumption, remains elevated during the recovery phase whereas *in vivo* glutamine utilization falls abruptly. This implies that although mitochondria isolated from rats during the recovery phase retain the

Table 13 Oxygen Consumption During the Metabolism of Glutamine and Related Substrates by Kidney Cortex Mitochondria from Rats after Different Treatments

Day of Treatment	TREATMENT		OXYGEN CONSUMPTION			
	Days on 1.5%(w/v) NH_4Cl	Days on Water	Glutamine (2mM)	Glutamate (2mM)	αKetoglutarate (2mM)	Succinate (2mM)
			ng atoms O/min/mg mitochondrial Protein ±SD (no of animals)			
0	-	∞	18.2± 9.6 (8)	12.5± 7.9 (8)	61.9±18.3 (8)	61.7±16.8 (8)
1	1	-	34.6±13.4(12)	12.9± 6.3(12)	71.1±14.0(12)	46.3±10.4(12)
3	3	-	45.0±11.1 (9)	14.8±10.5 (9)	78.2± 8.6 (9)	54.4±15.9 (9)
5	5	-	34.1± 7.3 (9)	10.6± 5.8 (9)	57.2±28.8 (9)	51.7±13.4 (9)
7	7	-	38.5±14.1 (8)	10.1± 6.3 (8)	63.0±25.6 (8)	46.9± 8.3 (8)
8	7	1	43.8± 8.4 (7)	12.6± 5.1 (8)	68.1±11.2 (8)	59.6±17.8 (8)
9	7	2	42.4± 5.6 (8)	12.9± 5.5 (8)	73.7±28.8 (8)	62.8±10.0 (8)

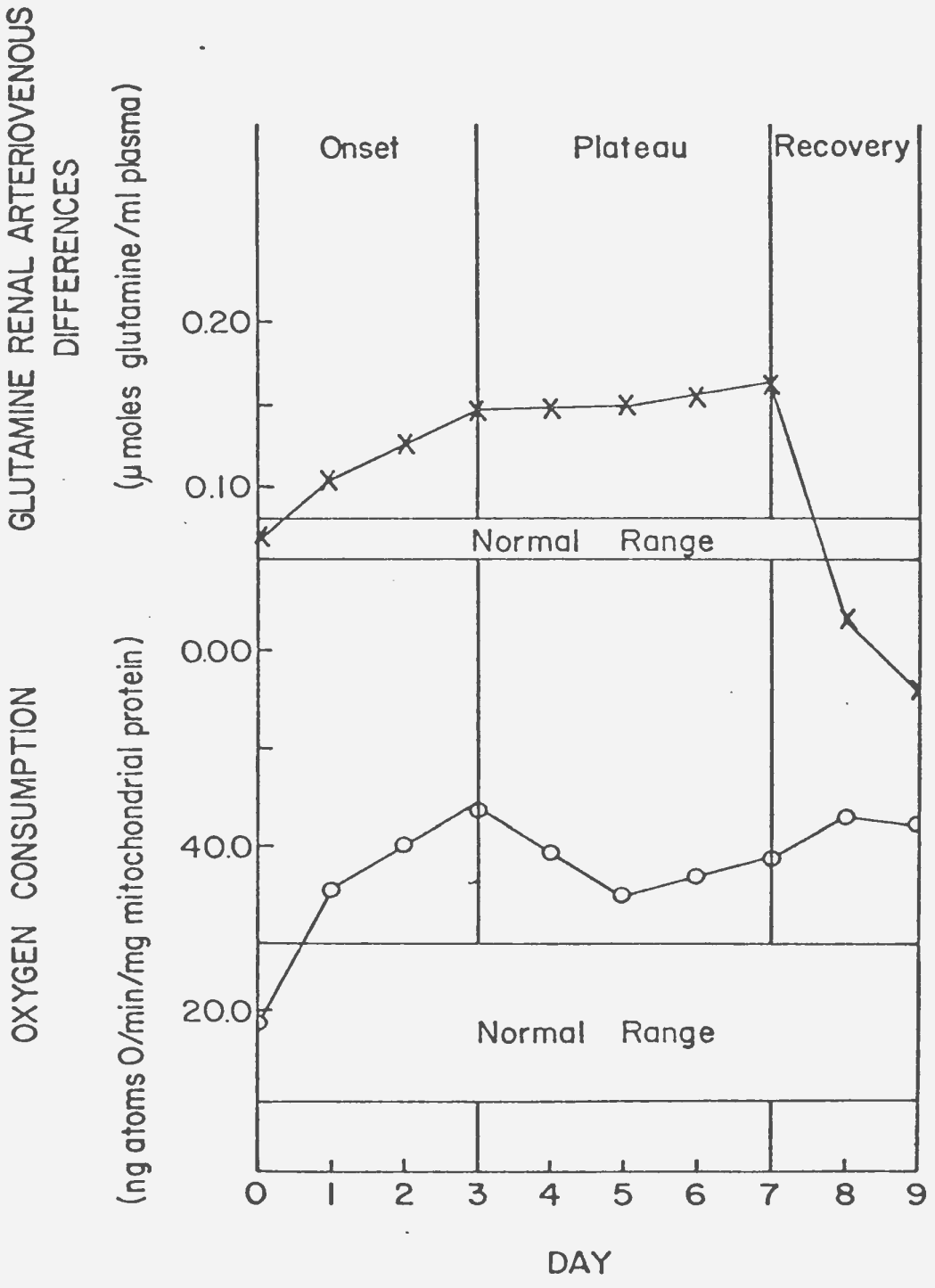

Figure 14 Comparison of Mitochondrial Oxygen Consumption with Glutamine Renal Arteriovenous differences during Onset, Plateau and Recovery

capacity to metabolize glutamine at the high rates seen during the plateau phase, this capacity is not expressed *in vivo* and must be controlled by some other means.

G. Response of Kidney Cortex Slices

The next level at which the metabolism of glutamine was studied is the intact kidney cortex slice. The tissue slice has the advantage in that it is thought to represent organized surviving tissue, the metabolism of which reflects that of the original tissue. Kidney slices, however, still suffer from the one major drawback from which nearly all biochemical work on the kidney suffers; that is, the kidney is a highly heterogenous organ and slices of the kidney inevitably comprise many different cell types. From the results of the previous experiments, studying changes which occur during the recovery phase proved to be an interesting approach. The correlations and dissociations between isolated steps in the metabolism of glutamine and *in vivo* ammonia production shed some new light on the problem of how renal ammoniagenesis is regulated. It was, therefore, felt appropriate to study how glutamine metabolism responded during the recovery phase at the level of the intact cell. At this cellular level the *in vitro* metabolism of glutamine would be expected to best approximate the metabolism of glutamine *in vivo*.

1. Glutamine Metabolism

The metabolism of glutamine by kidney cortex slices was followed by measuring the utilization of glutamine and the production of its major products: ammonia, glutamate and glucose. Table 14 shows that the

Table 14 Metabolism of Glutamine (2mM) by Kidney Cortex Slices from Rats after Different Treatments

Day of Treatment	TREATMENT: Days on 1.5%(w/v) NH_4Cl	TREATMENT: Days on Water	GLUTAMINE UTILIZATION	AMMONIA PRODUCTION	GLUTAMATE FORMATION	GLUCOSE PRODUCTION
			µmoles/90 min/g wet wt ± SD (no of animals)			
0	-	∞	78.1±13.4(5)	106.0±14.8(5)	13.6±6.1(5)	4.6±1.6(5)
7	7	-	248.8±42.8(3)	196.3±41.4(4)	28.2±4.3(3)	39.7±4.6(4)
8	7	1	112.7±68.3(5)	112.9±19.4(5)	13.8±3.1(5)	19.6±1.0(3)
9	7	2	85.4±16.3(2)	94.9±22.6(4)	14.0±2.6(4)	8.9±1.9(4)

metabolism of glutamine is faster in kidney slices taken from rats given 1.5% (w/v) NH_4Cl than in slices taken from the kidneys of control rats. This elevated rate of metabolism abruptly fell after the rats were returned to tap water.

(a) Glutamine utilization. The utilization of glutamine was increased approximately 3 fold ($P < 0.001$ day 7 compared to day 0) in kidney slices taken from rats drinking 1.5% (w/v) NH_4Cl for 7 days. When the rats were returned to tap water, glutamine utilization fell abruptly back to normal ($P < 0.050$ for days 8 and 9 compared to day 7). Figure 15 compares the *in vivo* utilization of glutamine by the kidney with the *in vitro* utilization by kidney cortex slices during the recovery phase. Both drop 2-3 fold, returning the utilization of glutamine back to normal by the end of day 8.

(b) Ammonia production. The production of ammonia from glutamine by kidney cortex slices, taken from rats drinking 1.5% (w/v) NH_4Cl for 7 days increased approximately 2 fold ($P < 0.010$ day 7 compared to day 0). The increase in *in vivo* ammonia excretion is in the order of 6-7 fold. This discrepency suggests a lack of physiological integrity in the slices and clearly needs explaining. Very recently Welbourne *et al.* (113) observed that the 2 fold elevation in ammonia production by kidney cortex slices taken from rats administered NH_4Cl was greatly increased (compared with control slices) when the incubation time was lowered from 45 minutes to 15 minutes. He also observed, on the basis of dry to wet weight ratios, that swelling occurs in slices incubated longer than 15 minutes. On the basis of these observations, Welbourne suggests that after 15 minutes in-

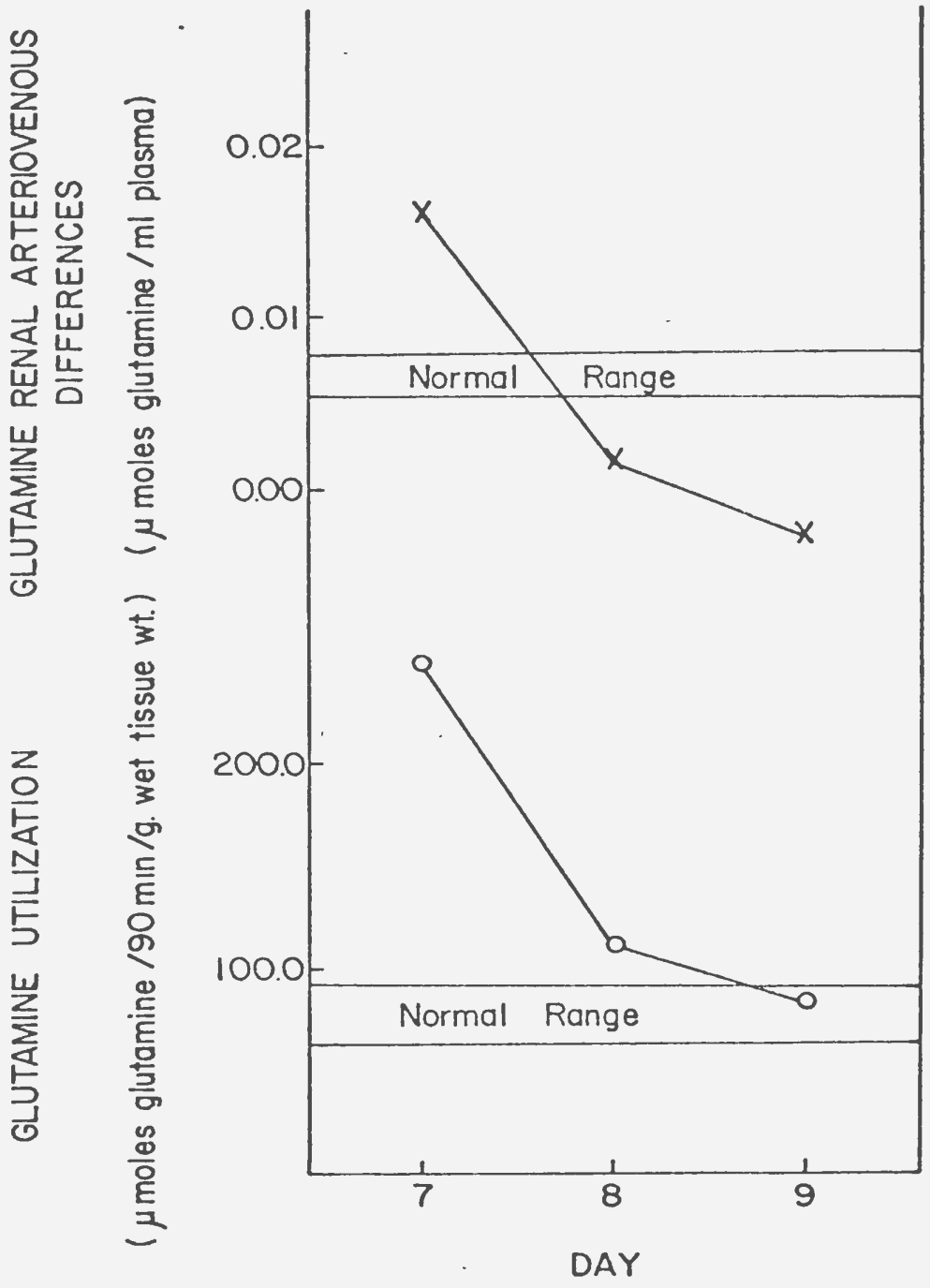

Figure 15 Comparison of Glutamine Utilization by Kidney Cortex Slices with Glutamine Renal Arteriovenous Differences during Recovery

cubation the swelling of the tissue slices activates the mitochondrial glutaminase I pathway, such that the difference between control slices and slices taken from acidotic rats is much less. Such a non-physiological activation *in vitro* would, therefore, mask the magnitude of the *in vivo* activation. Accordingly, the tissue slice appears not to reflect the true physiological situation. The perfused kidney is probably a better system since the *in vivo* increase in ammonia excretion can be exactly duplicated (110).

Figure 16 compares *in vivo* ammonia excretion with *in vitro* ammonia production by kidney cortex slices during the recovery phase. Although the production of ammonia by the tissue slice does not behave physiologically in quantitative terms, it does qualitatively coincide with ammonia excretion *in vivo*. Both are back to normal by the end of day 8. This would tend to discount the importance of the "plasma factor" of Alleyne and Roobol (5) in regulating renal ammonia production, especially if the action of the putative factor is of short duration.

(c) *Glutamate formation*. Glutamate formation was also examined (Table 14). Increased glutamate formation was evident in the acidotic animals ($P < 0.025$ day 7 compared to day 0) and this increase had disappeared on day 8. The increased glutamate accumulation in acidosis would tend to indicate that the removal of glutamate may not keep pace with its formation. However, since glutamate is released by neither the normal kidney or the acidotic kidney *in vivo* (106), this glutamate accumulation may again indicate the unphysiological handling of glutamine by slices.

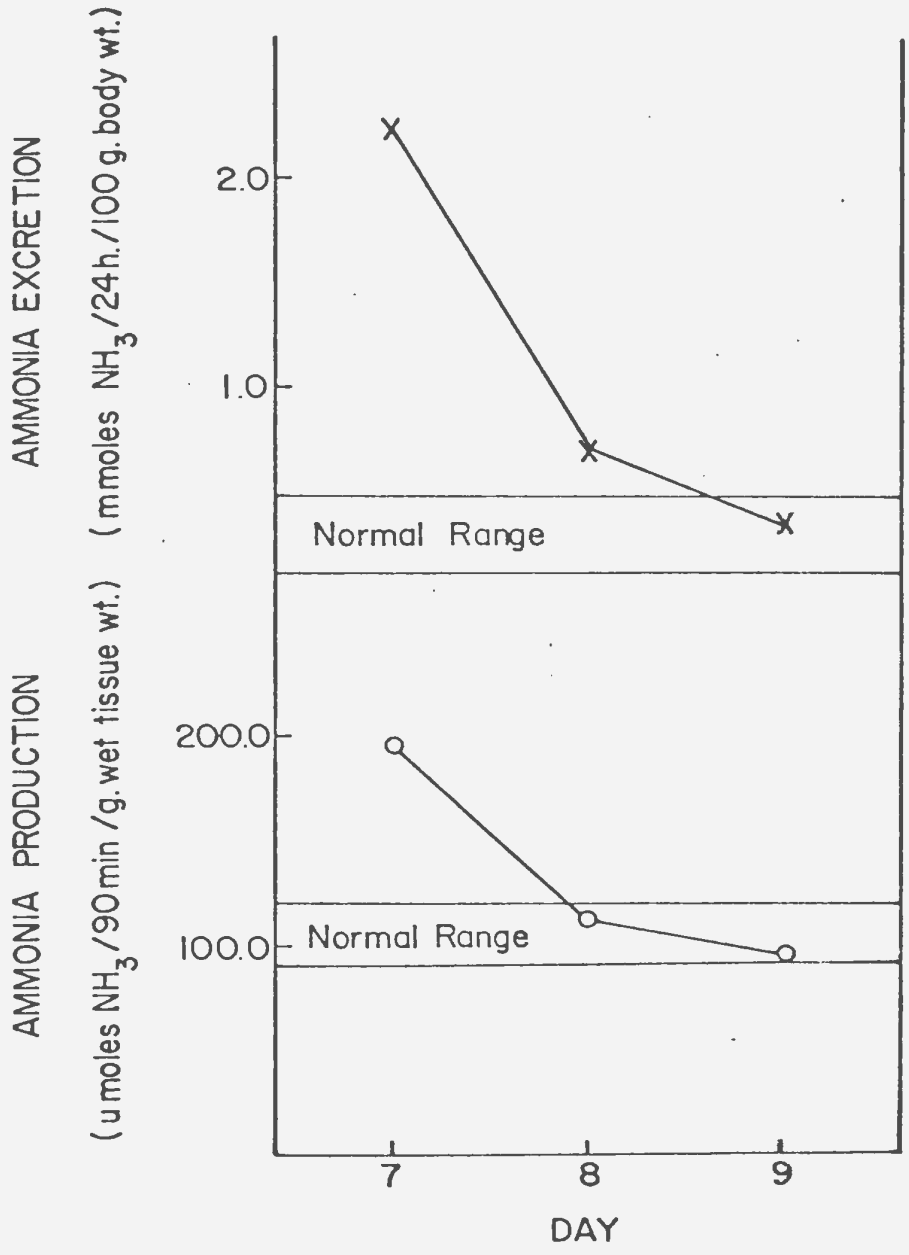

Figure 16 Comparison of Ammonia Production by Kidney Cortex Slices with Urinary Ammonia Excretion during the Recovery Phase

(d) Glucose production. Gluconeogenesis increases substantially (Table 14) in kidney slices taken from rats drinking 1.5% (w/v) NH_4Cl ($P < 0.001$ day 7 compared to day 0). When the rats were returned to tap water glucose production fell significantly on day 8 ($P < .005$ compared to day 7) and continued to fall on day 9 ($P < .001$ compared to day 8). It is probable that glucose production is back to normal by the end of day 9. Figure 17 compares glucose production by kidney cortex slices with the activity of PEPCK in vitro during the recovery phase. The fall in glucose production coincides with the fall in PEPCK activity. This is as expected since PEPCK is thought to be the rate-limiting enzyme for renal gluconeogenesis. This correlation is also consistent with the PEPCK-gluconeogenesis hypothesis for the regulation of renal ammonia production.

2. Metabolism of Related Substrates

(a) Glucose production. Kidney cortex slices were also used to measure glucose production from other substrates (oxaloacetate, malate, lactate and pyruvate). This study was possible because tissue slices from the kidney are permeable to these substrates. Each one of these substrates is thought to share part of the pathway for the metabolism of glutamine. Lactate and malate are on the same oxidation level as glucose. Consequently, the reducing equivalents required for reversal of the triosephosphate dehydrogenase step can be supplied via cytoplasmic reactions. In the case of malate, the enzyme required to generate the reducing equivalents is malate dehydrogenase. This enzyme converts malate to oxaloacetate. Conversion of oxaloacetate to glucose is

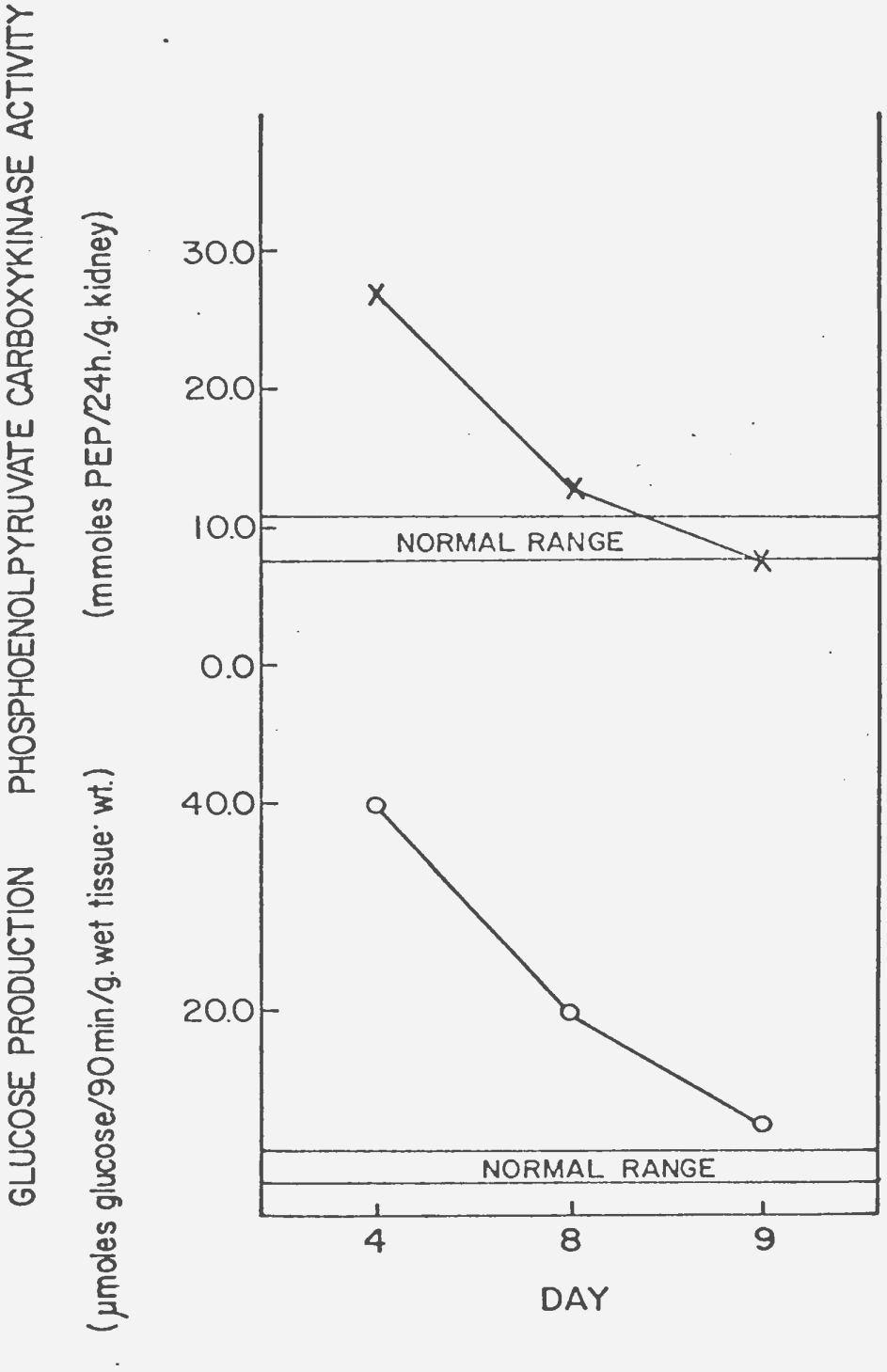

Figure 17 Comparison of Glucose Production by Kidney Cortex Slices with Phosphoenolpyruvate Carboxykinase Activity during Recovery

probably part of the pathway for gluconeogenesis from glutamine. In the case of lactate, the enzyme required to generate the reducing equivalents is lactate dehydrogenase. Lactate dehydrogenase converts lactate to pyruvate. However, conversion of pyruvate to glucose requires participation of mitochondrial enzymes. This means that pyruvate is probably converted inside the mitochondrion to oxaloacetate (pyruvate carboxylase) and then to aspartate (glutamate oxaloacetate transaminase) which leaves the mitochondrion to regenerate cytoplasmic oxaloacetate (96). This oxaloacetate is converted to phosphoenolpyruvate and then to glucose. The conversion of intramitochondrial oxaloacetate to cytoplasmic phosphoenolpyruvate is probably part of the pathway for conversion of glutamine to CO_2 and H_2O.

On the other hand, the substrates pyruvate and oxaloacetate are more highly oxidized than glucose and consequently require, in their conversion to glucose, mitochondrially produced reducing equivalents to reverse the triosephosphate dehydrogenase step. In the case of pyruvate, this is probably accomplished by intramitochondrial conversion to oxaloacetate (pyruvate carboxylase) and then reduction via malate dehydrogenase to malate (96). The malate so formed can then pass into the cytosol where it is converted to oxaloacetate by cytoplasmic malate dehydrogenase, thus generating the cytoplasmic reducing equivalents at the expense of intramitochondrial reducing equivalents. In the case of oxaloacetate as substrate, it may first be converted to pyruvate (phosphoenolpyruvate carboxykinase and pyruvate kinase) which then follows the same route to glucose as the substrate pyruvate. However, oxaloacetate may also enter the mitochondrion itself, where it is reduced to malate

(malate dehydrogenase) which then leaves the mitochondrion and is converted back to oxaloacetate, thus generating cytoplasmic reducing equivalents. With both substrates (pyruvate and oxaloacetate), conversion of intramitochondrial malate to glucose occurs, which is probably part of the pathway for gluconeogenesis from glutamine.

Table 15 shows the production of glucose by kidney cortex slices from each of these substrates. There are significant increases in glucose production from oxaloacetate ($P < 0.05$), lactate ($P < 0.005$ and pyruvate ($P < 0.025$) by slices taken from the kidneys of rats drinking 1.5% (w/v) NH_4Cl for 7 days compared to control slices. Such increases are expected since these substrates are converted to glucose via PEPCK. Glucose production from malate was also elevated but this elevation is not statistically significant.

There are significant decreases in glucose production from malate ($P < 0.025$), lactate ($P < 0.050$) and pyruvate ($P < .025$) by kidney slices taken from rats on day 8 compared with slices taken from rats on day 7. Glucose production from malate and pyruvate are back to normal by the end of day 8. Gluconeogenesis from lactate is back to normal by the end of day 9 ($P < 0.025$ day 9 compared to day 8). Although glucose production from oxaloacetate does not significantly decrease on day 8, it is back to normal by the end of day 9 ($P < 0.05$ day 9 compared to day 7).

Figure 18 compares glucose production from oxaloacetate, lactate, pyruvate and malate with glucose production from glutamine during the recovery phase. The return to control values of gluconeogenesis from all five substrates followed a similar time course. This suggests that the

Table 15 Glucose Production from Various Substrates by Kidney Cortex Slices from Rats after Different Treatments

Day of Treatment	TREATMENT		GLUCOSE PRODUCTION			
	Days on 1.5%(w/v) NH_4Cl	Days on Water	Oxaloacetate (10mM)	Malate (10mM)	Lacate (10mM)	Pyruvate (10mM)
			µmoles/90 min/g wet tissue wt ±SD (no of animals)			
0	-	∞	14.3± 5.3(4)	42.2± 5.1(4)	12.2±5.4(5)	44.8± 8.3(5)
7	7	-	36.6±11.5(3)	51.8±11.0(4)	37.9±8.4(4)	120.5±27.9(4)
8	7	1	23.9± 8.5(3)	35.4± 6.1(4)	24.9±3.8(5)	63.8±19.0(5)
9	7	2	11.4± 7.8(4)	28.1± 9.5(3)	16.2±3.2(4)	49.0±15.2(4)

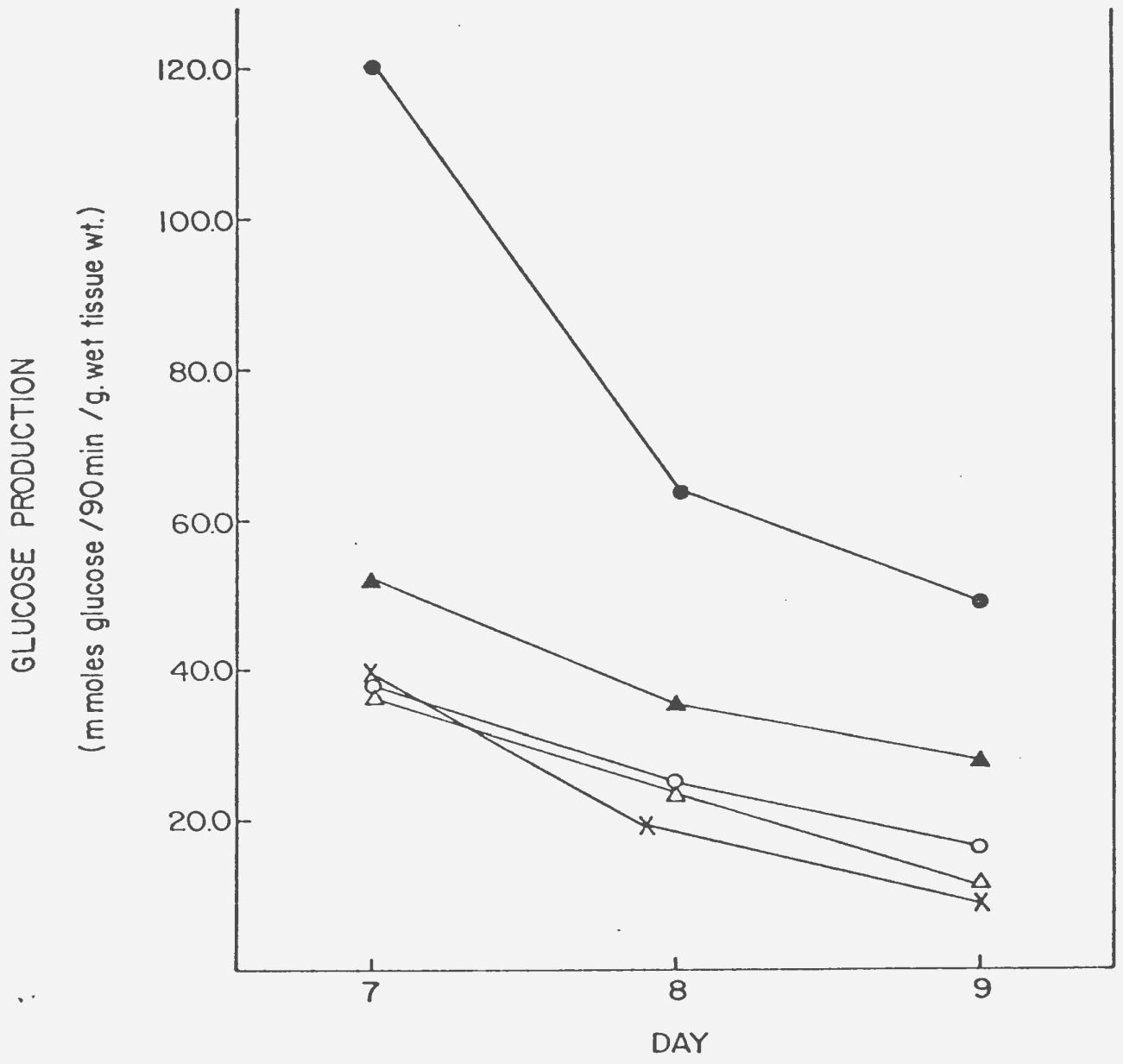

Figure 18 Comparison of Glucose Production from Glutamine and Related Substrates by Kidney Cortex Slices during the Recovery Phase

X——X Glutamine

Δ——Δ Oxaloacetate

O——O Lactate

▲——▲ Malate

●——● Pyruvate

factor responsible for the return to normal is common to the gluconeogenic pathway of all five substrates. In view of the similar time course in the return of PEPCK to control levels (Fig. 17), it seems probable that decreases in gluconeogenesis is brought about by the decrease in PEPCK.

GENERAL DISCUSSION

A. Complexity of Ammonia Regulation

For an animal to survive an acid challenge, the kidney must be capable of dealing with the acid load on both an immediate, acute basis and on a long term, chronic basis. The kidney deals with the need to excrete increased amounts of strong acid on an acute basis by increasing the rate of ammonia production within the pre-existing capabilities of the metabolic steps involved. This might be accomplished by increasing the substrate level of glutamine in the renal tubular cells or possibly by altering the concentration of activitors or inhibitors. As well, conversion of an inactive to an active protein could serve to regulate the production of ammonia on an acute basis. Such changes occur rapidly and do not require changes in amount of specific proteins. Effectively, they involve an increase in the activity of the step which is rate-determining under steady-state conditions. The kidney deals with the need to excrete increased amounts of strong acid on a chronic basis by increasing the capacities of the slower steps in the metabolic sequence for ammonia production. Increasing the capacities presumably involves increasing the total protein responsible for carrying out these steps. These chronic changes require more time. The rate-determining step during chronic adaptation need not necessarily be the same as the rate-determining step during acute adaptation.

An important part of these renal mechanisms must be an ability to cope with the termination of the strong acid load. Normal blood levels are already close to half the values at which ammonia toxicity begins (46).

Since ammonia which is not trapped in the urine will diffuse into the blood, then in order to survive termination of a strong acid challenge, a control mechanism must exist, whereby the production of ammonia is closely coupled to its excretion in the urine.

Although the control of ammonia production appears to be rather complex, the proposed hypotheses are, in principal, relatively simple. It should be recognized that although postulating simple mechanisms of control is initially appropriate 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. None of the proposed hypotheses alone account for the changes observed in ammonia excretion during both the onset and recovery phases. This does not necessarily mean that the hypotheses are invalid. It may also mean that more than one regulatory step exists. Therefore, on the basis that the previously discussed hypotheses describe the three most likely rate determining steps, it should be possible to construct a model for the regulation of ammonia production during onset and recovery, using where justified the features of more than one hypothesis. Such an approach is probably more appropriate than trying to fit the results of the present work within the narrow confines of one particular hypothesis. However, it is recognized that other regulatory steps may exist whose importance is not currently appreciated.

B. Model for Regulation of Ammonia Production

The adaptive changes which occur in the kidney in response to an acid challenge can be divided into two types: (1) mitochondrial (i.e., increases in glutamine transport and in amounts of PDG) and (2) extramitochondrial (i.e., increases in amounts of PEPCK). Apparently, in the rat, both responses are required for the kidney to reach maximal rates of ammonia excretion. In the present study, it was shown that PEPCK reached fully adapted levels before the onset phase was complete. This suggests that PEPCK alone is not responsible for the slow adaptive increase in ammonia excretion and therefore may not be regulatory for the production of ammonia during this phase. On the other hand, the correlations between ammonia excretion and both maximal PDG activity and mitochondrial ammonia production *in vitro* are consistent with the mitochondrial response being responsible for the slow adaptation in ammonia excretion. However, at present, there is insufficient evidence to discern which mitochondrial step (i.e., glutamine transport, or conversion of glutamine to glutamate via PDG) if either, is rate-limiting. The hypothesis suggesting that PDG amount is regulatory is weakened by the studies by Goldstein (29). The results of his studies show that an adaptive increase in ammonia excretion, during the first 24 hours of acid challenge, can occur without an increase in PDG activity. However, it is certainly not justified to reject this hypothesis for the entire onset phase solely on the basis of a dissociation which occurs in only one part of this phase. Nor is it justified to accept the transport hypothesis as correct solely on the basis that no evidence has yet been produced against it. The transport hypothesis has not yet been subject to serious critical

study, probably because of the technical difficulties involved.

During recovery, apparently only the extramitochondrial response is required to return ammonia production to normal. The dissociations between ammonia excretion and both maximal PDG activity and mitochondrial ammonia production *in vitro* suggest that the mitochondrial capacity is not rate-limiting for ammonia production during this phase. On the other hand, the apparent correlation between ammonia excretion and maximal PEPCK activity is consistent with this enzyme being involved in the regulation of ammoniagenesis during recovery. One problem with this interpretation is that during starved recovery the maximal activity of PEPCK remains elevated, yet ammonia excretion for the same day has returned to normal. If ammonia excretion on this day actually reflects ammonia production (and it may not since extra ammonia may diffuse into the blood instead) then this dissociation may suggest that events other than those presently discussed, either mitochondrial or extramitochondrial are important. However, for the purpose of this model it appears that ammonia production is regulated by a mitochondrial factor (possibly glutamine transport or PDG amount) during the onset phase and by a cytoplasmic factor (possibly PEPCK amount) during the recovery phase.

C. Possible Future Studies

1. Early Recovery

The results of the present work indicate that there is a difference in the time course for the return of ammonia excretion to normal in rats drinking 1.5% (w/v) NH_4Cl for 3 days compared to rats drinking 1.5% (w/v) NH_4Cl for 7 days. It would be of interest to see if PEPCK activity still

correlates with ammonia excretion during this "early" recovery.

2. Recovery

Considering the results of the present study, a close look at the proposed regulatory events during recovery in rats after drinking 1.5% (w/v) NH_4Cl for 7 days might be revealing. The present work indicates that both ammonia excretion and PEPCK activity return to normal with the same time course. However, neither of these events were measured at times less than 24 hr of recovery. Therefore, it would be important to know if PEPCK activity still correlates with ammonia excretion at recovery times less than 24 hr. It might also be worthwhile to see if administration of $NaHCO_3$, at the start of recovery, would reduce the time required for these events to return to normal, without dissociating them.

If, during recovery, PEPCK amount is regulatory for ammonia production (by the proposed mechanism) then glutamate levels must increase substantially so as to inhibit the elevated levels of PDG and these elevated levels must persist until PDG has returned to normal. Measurement of cortical glutamate levels during recovery is therefore important. However, although it is not expected that cortical glutamate levels necessarily reflect mitochondrial glutamate levels, it is expected, because of the large increase in mitochondrial glutamate required to inhibit PDG, that the change in cortical [glutamate] would be even greater than is observed during onset. Another important aspect of the possible role of PEPCK in the regulation of ammonia production during recovery is the mechanism whereby PEPCK activity returns to normal. This would initially involve measuring the rates of synthesis and degradation of PEPCK during recovery.

The maximal activity of PDG does not fall significantly, upon recovery, for the length of time that it was followed (i.e., 2 days). It would be interesting to know how long it takes for PDG activity *in vitro* to return to normal. It would also be of interest to measure the rates of synthesis and degradation of PDG during onset, plateau and recovery. PDG activity and mitochondrial ammonia production need not necessarily return to normal with the same time course. If mitochondrial ammonia production returns to normal faster than the activity of PDG, then this would be consistent with the concept that adaptation of the transport process does occur.

The negative glutamine A-V differences observed during recovery suggest that glutamine synthesis is involved with reducing net ammonia production. It would therefore be of interest to look for changes in glutamine synthetase activity (GS) and to study the effect of methionine sulfoximine (an inhibitor of GS (66)) on the activity of glutamine synthetase (*in vivo*) during the recovery phase.

3. Rechallenge

Dies and Lotspeich (25) found that there was no lag in the excretion of acid when rats were rechallenged with NH_4Cl immediately after recovery from a previous challenge. Presumably, this is because the capacity of the mitochondria to produce ammonia from glutamine is still elevated and that PEPCK can adapt rapidly. If this does prove to be the case, then it might be taken to suggest that adaptation of PEPCK during onset is not rate-limiting because it is capable or adapting rapidly, but that PEPCK is regulatory, after the mitochondrial capacity has adapted, during recovery and rechallenge. At the end of recovery, PDG is elevated while

PEPCK has returned to normal. If the elevated level of PDG is the result of a long half life, then it should be possible to use the high doses of actinomycin D, required to inhibit PEPCK adaptation (53), during rechallenge. This would help discern if an adaptive increase in PEPCK (or some other protein) is required for an adaptive increase in ammonia production during this phase.

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