GLUTAMINE SYNTHESIS AND SYNTHETASE IN GUINEA PIG KIDNEY

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by

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DEDICATION

This work is whole-heartedly dedicated to:

MY FATHER

for leading his children into intellectual pursuits

MY MOTHER

in recognition and appreciation for so much

MISS CHRISTINE WEE NA TAN

for making everything worthwhile

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ABSTRACT

It is now generally believed that kidneys of many species increase ammonia excretion (through the action of glutaminases) in the urine, in response to an acid challenge, and that the physiological significance of such enhanced ammoniagenesis is to primarily conserve extra-cellular fluid cations, especially Na^+. The source material for urine ammonia has been identified as glutamine. However, the physiological stimulus for renal ammonia excretion, the relative importance of enzymatic and physicochemical factors in the mechanism of ammonia excretion, and the rate-limiting metabolic step(s) which is (are) modified during acidosis, are still not clear.

In an attempt to unravel some of these mysteries, studies were conducted on renal synthesis of glutamine, and glutamine synthetase. Glutamine synthetase has the potential to regulate ammoniagenesis by removing ammonia. It is clear, therefore, that results of studies performed on a system which contains high activities of both the ammonia producing enzyme (glutaminase), and the ammonia consuming enzyme (glutamine synthetase), would be difficult to interprete in terms of net ammonia metabolism at any given time. A system with a relatively high activity of one or the other enzyme was necessary.

Earlier studies had implicated glutamine synthetase in the regulatory events of renal ammoniagenesis in the rat. The guinea pig was chosen as a suitable experimental animal model for the studies reported, because the guinea pig kidney has a relatively high activity of glutamine synthetase, and a low activity of glutaminase.

These studies showed that the guinea pig kidney cortex tubule

synthesizes glutamine rapidly at pH 7.4 (503±78μmoles/30 min/g dry wt), when incubated with glutamate and ammonium chloride. Furthermore, the high rates of glutamine synthesis were reduced by about 50% at pH 7.1, regardless of whether the lower pH was produced by decreasing the HCO_3^- concentration (metabolic acidosis) or increasing the P_{CO_2} (respiratory acidosis). The inhibition of glutamine synthesis was reversible.

Subcellular fractionation studies on guinea pig kidney cortex indicated an exclusive cytosolic localization of glutamine synthetase. Such localization was not altered when the pH of the homogenizing medium was changed, since glutamine synthetase almost consistently appeared in the soluble fraction of spun homogenates, using sucrose or KCl media.

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ADP	Adenosine 5'-Diphosphate
AMP	Adenosine 5'-Monophosphate
AOA	Aminooxyacetate
ATP	Adenosine 5'-Triphosphate
A-V	Arterio-venous difference
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
EGTA	Ethyleneglycol-bis-(-Amino-ethyl ether)N, Tetra Acetic Acid
GABA	γ-Amino Butyric Acid.
GDH	Glutamate Dehydrogenase
GHA	L-Glutamic acid γ-monohydroxamate
GKA	Glutamine-Keto Acid Transferase
GS	Glutamine Synthetase
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic Acid
MSO	L-Methionine DL-Sulphoximine
Min	Minute
NAD^+	Nicotinamide Adeine Dinucleotide
NADH	Nicotanamide Adenine Dinucleotide (reduced form)
PEPCK	Phosphoenolpyruvate Carboxykinase
PDG	Phosphate Dependent Glutaminase
Tris	Tris(hydroxymethyl)amino-methane

I. INTRODUCTION

1.1 Focus on the role of Glutamine in intermediary metabolism

1.1.1 Discovery and Functions of Glutamine

The existence of glutamine seems to have been first considered by Hlasiwetz and Habermann, who suggested in 1873 that the ammonia found in hydrolysates of protein arose by hydrolysis of protein-linked glutamine and asparagine (1); and by 1932, all of the other common amino acids of proteins except threonine had been isolated from protein hydrolysates (2). It is now known, that glutamine is widely distributed in virtually all species in protein-bound and free forms (3), and that it is the most abundant free amino acid constituent of mammalian blood plasma (4).

The central role of glutamine in nitrogen metabolism is illustrated in Fig. 1, which shows those pathways in which the amide group of glutamine is utilized as a preferred source of nitrogen for the biosynthesis of various amino acids, purine, pyrimidine nucleotides, glucosamine-6-P, and NAD^+. Thus it is a key intermediate in the ultimate synthesis of protein, nucleic acids and complex polysaccharides.

1.1.2 Glutamine as precursor of Urinary ammonia and Carrier of nitrogen between tissues

Nash and Benedict (5) in 1921 showed conclusively that ammonia excreted in the urine was formed in the kidneys. The source material was first believed to be blood urea, or plasma proteins, then later amino acids.

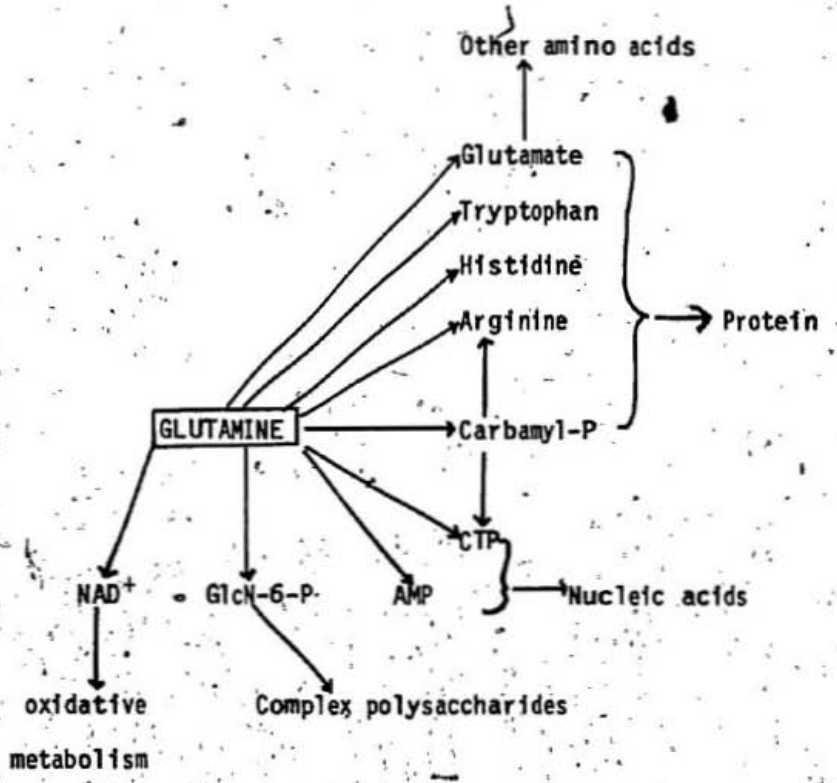


Fig. 1 Central role of Glutamine in intermediary metabolism

Van Slyke et al. demonstrated that, administration of glutamine to a dog in HCl acidosis markedly increased ammonia excretion, and that depressing the ammonia excretion by changing from hydrochloric acid acidosis to bicarbonate alkalosis was accompanied by a corresponding decrease in removal of glutamine from the renal blood (6). Table 1 indicates the relation between glutamine and ammonia concentrations in their experiments (6). These experiments provided the first direct clue, in vivo., of a specific physiological function of glutamine in animals, and established glutamine as an important link in the regulation of acid-base balance. Functionally, circulating glutamine has been viewed as an important vehicle (a) whereby nitrogen, during metabolic acidosis, is delivered to the kidneys for the production of urinary ammonia (6,7) and (b) where in ureotelic species, waste nitrogen derived from catabolic reactions in peripheral tissues is delivered directly to the liver for the biosynthesis of urea.

1.1.3 Interorgan metabolism of circulating glutamine: sources and utilization of glutamine

In addition to the kidneys, three major sites of glutamine metabolism (uptake or release) have been proposed: Liver (8,9), skeletal muscle (10), and Gut (8,11,12).

LIVER

The role of the liver in the supply and utilization of circulating glutamine is controversial. Addae and Lotspeich (8) reported that dog liver showed a net release of glutamine into the hepatic vein and that this release was significantly increased by metabolic acidosis. In other studies from Arterio-venous (A-V) difference measurements in

TABLE 1. Glutamine and NH_3 concentrations during acidosis and alkalosis.

Condition	Renal blood Plasma flow	Urine ammonia N	Glutamine amide N removed from blood	Preformed NH_3 N in blood	
				Arterial	Renal Venous
	cc.per min.	mg.per min.	mg.per min	mg.per 100cc	mg.per 100cc
Acidosis	245	0.562	0.33	0.02	0.10
	268	0.605	0.39	0.02	0.10
	262	0.615	0.41	0.04	0.10
Alkalosis	178	0.005	0.02	0.034	0.075
	200	0.004	0.02	0.039	0.075
	191	0.004	0.04	0.042	0.063

vivo., dog liver neither extracted nor released significant amounts of glutamine (13). Hills et al. (14) questioned the interpretation of the results of Addae and Lotspeich on technical grounds. Aikawa et al. (15) reported a net release of glutamine from rat liver, but the significance of their experiments was questioned by Schröck et al. (16). Studies on glutamine metabolism in the isolated perfused rat liver have also produced conflicting results. Lueck and Miller (17) found no net glutamine uptake or release from the normal isolated perfused rat liver when the liver was perfused with physiological concentrations of glutamine (approx. 0.5mM), and an increase in net glutamine synthesis when the pH of the perfusion medium was lowered. This was similar to the findings of Oliver et al. (18). However Kinlen and Bourke (19) could not detect net glutamine synthesis in perfused livers obtained from either control or acidotic rats. Lund reported a release of glutamine in her experiments on the isolated perfused rat liver only when glutamine was not added to the medium, and in fact, when 1mM glutamine was added at the beginning of the perfusion there was a net uptake rather than release for the first 30 min.

The isolated perfused guinea pig liver (20) utilized significant amounts of exogenous glutamine only when it was present in concentrations of 1 mM or higher. Among fasted animals only in the sheep has net hepatic glutamine uptake been demonstrated (21). Thus, the liver can either extract (normal postabsorptive liver (16)) or release glutamine and appears to play a regulatory role, restoring blood glutamine levels to normal when they are elevated or depressed, a view first expressed by Lund (9). Recent studies suggest that the concentrations of certain hormones and metabolites, in addition to

glutamine itself, may also influence hepatic glutamine utilization. Regulatory roles have been proposed for ammonia (22,23), bicarbonate (22), Leucine (24), and glucagon or cylic AMP (22). Häussinger et al. (25) and Oliver et al. (18), meanwhile believe that blood pH independent of the bicarbonate concentration is important.

MUSCLE

The concentration of circulating glutamine in normal animals is closely regulated within a narrow range. Since other organs extract glutamine from the blood continuously, there must be compensatory sources elsewhere in the body. An important source appears to be skeletal muscle, where glutamine and alanine are the predominant end products of ammonia fixation and amino acid catabolism, particularly leucine, isoleucine, and valine, for which muscle is a major catabolic site. A net release of glutamine by muscle has been observed in vivo from A-V difference measurements in the hindlimbs of dogs and monkeys (14), rats (15,18,26,27), sheep (21), and in the forearm of postabsorptive man (11). In addition glutamine is produced and released by the isolated perfused rat hindlimb (27) and by isolated strips of rat skeletal muscle in vitro (10). The effects of metabolic acidosis on glutamine metabolism in skeletal muscle are controversial. Oliver et al. (18) and Schröck et al. (26) report an increased glutamine release by rat skeletal muscle during acidosis, but no such increase was seen by Lund and Watford (28) or Lemieux et al. (29). Addae and Lotspeich (8) found no significant effect of metabolic acidosis on A-V glutamine differences in the hindlimb of the dog. However, in their experiments, there was also no demonstrable A-V glutamine difference in the hindlimbs of normal dogs, a finding inconsistent with that of more

recent investigations (14).

Ruderman et al. (30) reported that 78% of the rat hindlimb is skeletal muscle, and that this muscle accounts for 95% of the total oxygen consumed in the hindlimb. Thus, most of the metabolically active tissue in rat hindlimb is skeletal muscle. Therefore, it may be concluded that glutamine released from the hindlimb is synthesized in skeletal muscle. If this is true, then it is not unreasonable to assume that total body muscle contributes to the circulating glutamine pool in the rat, and that the rate of muscle glutamine production may be influenced by the dietary (28) and hormonal (31) status of the animal. The origin of the carbon skeleton for de novo synthesis of glutamine has not been determined (32,33).

INTESTINE

A-V difference measurements in fasted dogs (8,14,34), revealed a large net uptake of glutamine by the group of organs drained by the hepatic portal vein - the stomach, small and large intestine, cecum, spleen, and pancreas. Similar observations were soon reported in sheep (21), rat (15) and man (35,36). Subsequent studies (12,37,38) have made it apparent that the small intestine plays a dominant role in this phenomenon.

Mucosal epithelial cells of the intestine have access to glutamine from two sources: the arterial blood across the basolateral membrane of the cells and, after a meal, the intestinal lumen, across the brush border membrane. A-V difference measurements in vivo. across the tissues drained by the superior mesenteric vein, in most laboratory animals, indicated a net uptake of 20-30% of the total plasma glutamine by the small and large intestine; net uptake was less in rabbits and no

uptake was observed in guinea pigs and chickens (39). Schröck et al. (16) recently reported that the gut (nonhepatic splanchnic bed) does not appear to play a role in supplying additional glutamine during chronic acidosis or diabetic ketoacidosis. They reported some slight variation in glutamine uptake at this site, but the differences were not significant when compared to controls. They concluded then that, since glutamine is used as one of the main respiratory fuels in the gut (12, 40), it would be unreasonable to cut down glutamine uptake during acidosis to supply glutamine to the kidneys (16). However a most recent study by Brosnan et al. (41) indicates suppression of intestinal glutamine utilization in the diabetic ketoacidotic rat due probably to its replacement as a metabolic fuel by the ketone bodies, whose concentrations are greatly elevated (5.4 mM).

KIDNEY

While it is well known that during periods of metabolic acidosis the kidneys extract large amounts of glutamine from the circulation for the production of urinary ammonia (6,42), the magnitude of renal glutamine utilization under normal conditions remain uncertain. Squires et al. (43), Lund and Watford (28) and Schröck et al. (26), all found little or no glutamine uptake by normal rat kidneys. Recently, Hughey et al. (44) also measured A-V differences for glutamine across the kidneys of normal rats in whole blood and could not establish an A-V difference significantly different from zero. These findings essentially confirm the earlier work of Pitts (45) who found that glutamine hydrolysis was about exactly balanced by glutamine synthesis in the non-acidotic rat kidney.

Renal glutamine utilization appears to be concentration dependent, and even small increases in the plasma concentration may stimulate uptake considerably. No net renal glutamine uptake was observed in unanesthetized sheep with in-dwelling blood sampling cannulae (21). And in rats, removing the small intestine, liver, and spleen (46) or only the small intestine (12) from the circulation produced a much larger increase in the plasma glutamine concentration than did nephrectomy, evidence that the intestine is quantitatively a more important site for glutamine utilization.

OTHER TISSUES AND CULTURED CELLS

A-V difference measurements using the umbilical artery and vein in pregnant rats revealed a large utilization of plasma glutamine by the developing fetus (47). Extensive glutamine utilization by a tumor, also a rapidly growing tissue, was indicated from arterial-iliac vein differences in rats. Similar techniques, indicate that pancreas in dog utilizes glutamine, but unlike in the intestine, 40-50% of the glutamate produced from glutamine hydrolysis is released back into the blood (48). Glutamine constitutes the key metabolite for the elimination of ammonia in nervous tissue and plays an important role in the production of GABA (a neurotransmitter), in brain. A number of mammalian cell lines in culture actively metabolize glutamine to CO_2, including fibroblasts (49), bovine lens (50), 6C3HED tumor cells (51), oocytes (52), reticulocytes (53) and Hela cells (54).

1.1.4 Metabolic fate of Glutamine Carbon and Nitrogen

The quantitative contribution of glutamine oxidation in vivo. has been established only for the small intestine, in which the mucosa was

identified as the site of oxidation and arterial blood (as opposed to the lumen) as the main source of glutamine (37). In this tissue, arterial glutamine is a more important respiratory fuel than is glucose in both fed and starved rats (12,38,40,55). Major products (which is a reflection of the metabolism of the tissue, and not the intestinal microflora) other than CO_2 and NH_3 are citrate, lactate, alanine, citrulline and proline (37). The products reflect the extensive oxidation of glutamine and a small capacity by rat intestine for gluconeogenesis, contrary to that observed by Anderson and Rosendall in guinea pig intestine (56); they found that the guinea pig jejunal mucosa contained significant levels of the important gluconeogenic enzymes and that these enzymes show an adaptive increase after starvation (56).

Glutamine contributes both carbon and nitrogen for alanine formation and release by the intestine, so that glutamine is indirectly involved in hepatic gluconeogenesis insofar as it serves as a precursor of circulating alanine. Extraction of glutamine by the kidney is elevated in starvation and under acidotic conditions, and the carbon of glutamine may be oxidized or used for the synthesis of glucose (57); renal gluconeogenesis becomes of increasing significance with prolonged starvation (58). What contribution glutamine makes to the net release of alanine by the kidney (15) is presently not known, but both alanine and glutamine released by skeletal muscle will make significant contributions to body glucose formation, although it is only alanine that is involved directly in hepatic gluconeogenesis (see fig. 2).

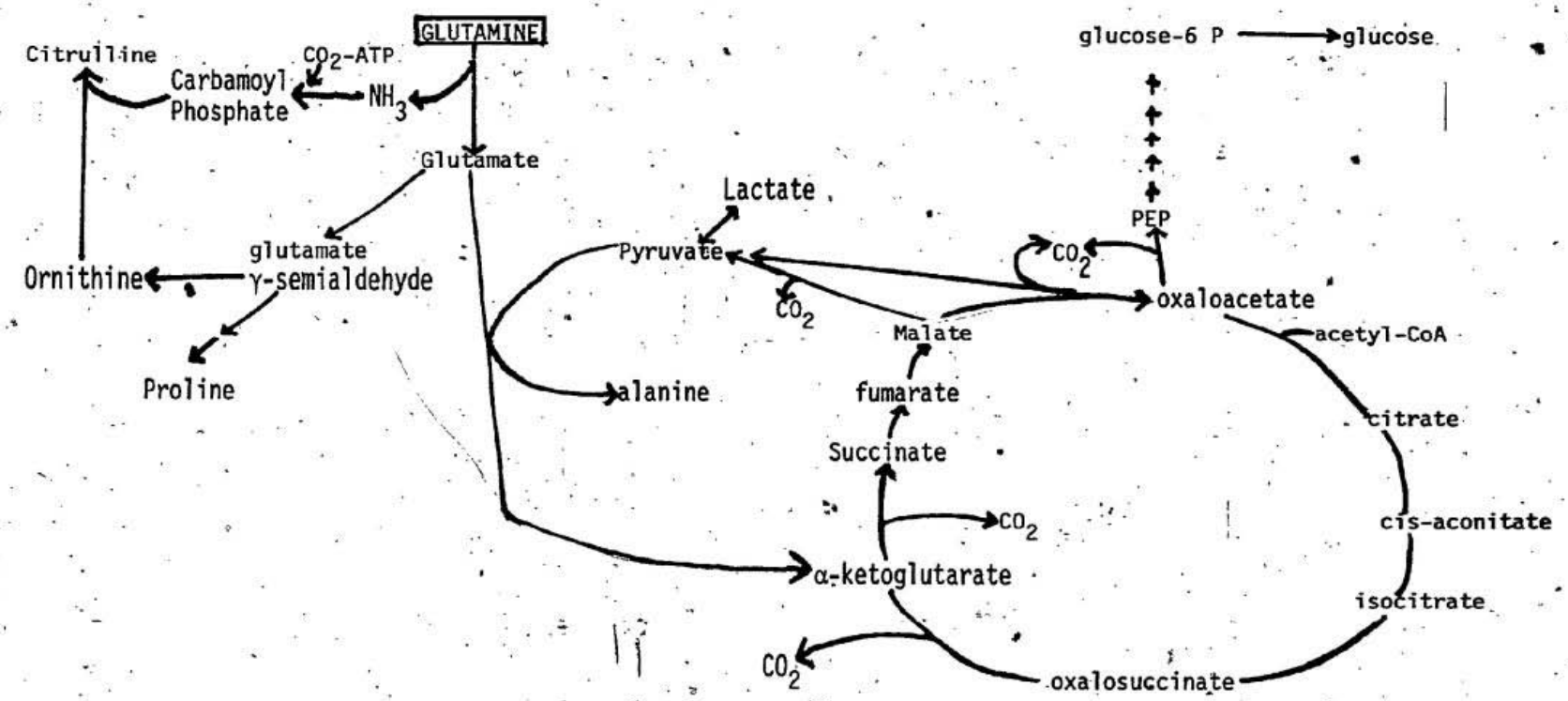


Fig. 2 Suggested Pathway for glutamine metabolism by rat intestinal mucosa (Ref. 39)

1.1.5 Renal glutamine, glutamate, and NH_3 metabolism in different species

Since the initial work of Krebs (59), and that of Richterich-van Baerle (60), Klahr et al. (61) and Preuss (62), it is now known that, in contrast with kidney cortex slices of dog, cat, pig, rat, sheep, and pigeon, slices of guinea pig and rabbit, do not release ammonia when incubated with glutamate. Ammonia excretion of guinea pig and rabbit was also low, and in this herbivores, unlike dogs, rats, and humans, was no different during acute acidosis than during acute alkalosis (63). The latter all carnivores, increase ammonia excretion markedly in acidosis (42,64). A correlation between renal glutamine synthesizing ability of guinea pig, rabbit, rat, dog, cat, and pig (average urine pH range, 8.1-5.8), and urinary pH have been noted by Janicki et al. (65). Rabbits and guinea pigs with alkaline urines and rats with neutral urines have high abilities to synthesize glutamine, whereas cats, dogs and hogs with acidic urines had no detectable glutamine synthesizing activity in their kidneys. In contrast, there was a direct relation between renal glutaminase activity and the urinary acidity in these animals.

Since it has a high capacity for glutamine synthesis, and a low glutaminase activity (66), it might be expected that in vivo., the guinea pig kidney would not take up, but rather release glutamine into the circulating blood. That this is indeed the case has been shown by Baveral et al. (67). Until lately, the pathway of renal metabolism of glutamate in these species was uncertain. Baeveral et al (67) have made an attempt to clarify this subject.

In fact, as far back as 1950, Smith (68) had recognized the great

variation in renal physiological function between herbivores, carnivores and omnivores, and between water-conserving desert animals and temperate species; it is therefore clear then, that, glutamine metabolism in relation to ammoniagenesis and its metabolic fate differs between species, so that results from rat kidney may not be necessarily applicable to other species.

1.2 Important enzymes of Glutamine metabolism

The synthesis of glutamine provides many types of cells with a mechanism for removal of NH_3 and for its storage. Although not essential in the diet of mammals, plants, and most micro organisms, glutamine plays a central role in amino acid and nitrogen metabolism. It is evident therefore that this amino acid amide must be synthesized by a wide variety of living cells, and hence the enzymes that catalyse the synthesis and breakdown of this ubiquitous amino acid must be of major importance in cellular metabolism and function.

1.2.1 Glutamine synthetase (EC 6.3.1.2)

Glutamine synthetase (GS), which catalyzes the formation of L-glutamine from L-glutamate, NH_3, Mg^{2+}, and ATP (2,69,70) appears to have a very wide distribution in nature. Its activity has been found in micro organisms and plants (71), and in mammals, it is found in many tissues, especially brain, liver, skeletal muscle and in the kidneys of some species (59,60,72,73). The enzyme probably plays a dual role in the economy of the animals, namely the synthesis of glutamine and the detoxication of NH_3. Although these two functions are inseparable, it is possible, nonetheless that one

function or the other predominates in an animal or a particular organ of the animal. A survey of the distribution of GS in the organs of different species of animals might help to elucidate the relative importance of its two functions, and also shed some light on the relation of its distribution to the phylogenetic classification.

GS IN BRAIN

Brain is more susceptible to NH_3 poisoning than any other tissue; high concentrations of blood NH_3 lead to coma and convulsions in man (74), and inhibition of brain GS causes seizures (75). Thus, it is essential for the brain to have some mechanism by which NH_3 can be effectively detoxicated. Perhaps the three most effective systems engaged in the removal of ammonia are glutamine synthetase, carbamyl phosphate synthetase and glutamate dehydrogenase. Carbamyl phosphate synthetase has not been described, thus far in brain, and it is not surprising therefore that brain possessed GS activity in all vertebrate species studied (76), and that in the lower animals (Ureotelic reptiles amphibians and fishes), it became the only tissue to possess the enzyme activity.

GS IN LIVER

Second only to brain in GS activity is liver. The enzyme was found in liver of all species above the reptilian level on the phylogenetic scale (76). A difference has been observed between the livers of a chelonian reptile (turtle) and that of an ophidian reptile (snake). The ureotelic turtle did not possess GS in liver, nor in any other tissue, but brain; while the uricotelic snake, possessed the enzyme not only in the liver but also in other tissues. GS activity in the snake liver was the highest of all livers. By and large the enzyme

activity in the liver of uricotelic animals (snake and birds) was considerably higher than that of ureotelic mammals. This difference, can be explained on the basis of ureotelism and uricotelism in these animals. In the uricoteles, uric acid forms the bulk of nitrogenous end products in the excreta, and 2 moles of glutamine are consumed for each mole of the uric acid excreted. Uricoteles obviously would need more glutamine than ureoteles, which require no direct participation of glutamine in the synthesis of urea for excretion.

GS IN MUSCLE

Glutamine has been known to be released from human forearm (11) and rat skeletal muscle (15,18,30) in amounts higher than could be accounted for by muscle proteolysis alone; and while the existence of GS has been confirmed in skeletal muscle (30,31,77), the observation by Blackshear et al. (78), that methionine sulphoximine (MSO), a specific inhibitor of GS (79), markedly inhibited glutamine accumulation after functional hepatectomy in the rat, indicate that the GS pathway is the source of most of the glutamine released by the extrasplanchnic tissues, and that glutamine may be synthesized de novo in skeletal muscle, and other extrasplanchnic tissues.

GS IN KIDNEY

GS was not found in the kidney of any animals below the level of mammals, and even in some mammals this tissue contained no detectable enzyme activity (76). The absence of demonstrable GS in dog (76,80) and human (81) kidneys may make it seem unlikely that the enzyme serves a crucial function. The difference between animals with and without renal GS may be related to a difference in urinary acidity. In man and dog the urine is habitually acid and therefore more urinary ammonia is

required than in rats, rabbits, guinea pigs and hamsters, with usually neutral or alkaline urines. The latter group may use GS to retrieve NH_4^+ not needed in the urine.

LOCALIZATION, PROPERTIES, AND REGULATION OF GS

GS has been shown, through immunochemical studies to be located in astrocytes of rat brain (82), and to be largely membrane bound, through cell fractionation studies (83). In the rat liver, GS is mainly associated with microsomes during cellular fractionation in isotonic sucrose (84,85) and in the chicken liver it is localized in the mitochondrial fractions (85).

Detailed subcellular studies on GS in guinea pig kidney is lacking. But studies in the rat and rabbit show exclusive localization of GS in the proximal straight tubule of rat nephron (86), while activity is high in both proximal convoluted and straight tubules of rabbit nephron (86), with some activity in the glomeruli and distal straight tubules.

The wide variety of the metabolic functions of glutamine suggests that GS must be subject to regulatory mechanisms, which can control the supply of glutamine available for different purposes. It would also be expected, since glutamine performs different functions in different cells, that the nature of the regulatory mechanisms would be different and therefore that the glutamine synthetases of different cells would differ in structure and function. It is evident that the function of brain GS would be related intimately to the metabolism of the putative neurotransmitters, GABA and glutamic acids. On the other hand, liver GS probably functions in the general amino acid and nitrogen metabolism of the mammalian organism. In the kidney, GS would also be expected to

partake in one of its most active biochemical functions - NH_3 production.

A comparison of certain regulatory and structural features of bacterial and mammalian glutamine synthetases shows that the GS of E. coli is inhibited by tryptophan, histidine, AMP, and glucosamine-6-P (87), while the GS of rat liver and ovine brain are not. Furthermore, the GS of E. coli can exist in adenylylated forms, while no such forms of mammalian glutamine synthetases have been found. KG and Citrate are inhibitory of GS activity when divalent cations like Mg^{2+} and Mn^{2+} are limiting in concentration in the assay media. Excess cations reverse the effect of αKG and citrate, and other compounds chelating divalent cations mimic the effect of both (88).

One might also expect to find differences between the glutamine synthetases of various mammalian tissues; however, since all of the glutamine synthetases catalyze the same chemical reaction, one would expect that a number of the properties of the glutamine synthetases obtained from various cells would be similar, especially those properties which are functions of the active centers of the enzymes. It is notable that all of the mammalian glutamine synthetases thus far examined have eight subunits (4), with subunit molecular weights in the range 44,000-50,000. It is also of interest that glutamine synthetases obtained from widely different sources are irreversibly inhibited by methionine sulphoximine and carbamyl phosphate (79,89), indicating that the mechanisms of the reactions catalyzed are probably the same or similar.

MECHANISM OF THE GLUTAMINE SYNTHETASE REACTION

The mechanism of the GS reaction is that proposed originally by Krishnaswamy et al. (90) and supported by Meister (89) and Tate and Meister (91).

In this proposal, GS binds negatively charged ATP first, by virtue of a positively charged microenviroment on its surface, and reacts with glutamate to give a complex which promptly rearranges to one that contains enzyme, ADP, and γ-glutamyl phosphate (activated glutamate). The γ-glutamyl phosphate complex may react with nucleophilic agents (90) to give a corresponding γ-glutamyl product; glutamine, if the nucleophile is ammonia, and γ-glutamyl hydroxamate if the nucleophile is hydroxylamine (Fig. 3a).

Methionine sulphoximine can exist in conformations which is very similar to that proposed for L-glutamate (Fig. 3b). A clue as to the mode of inhibition of GS by MSO emerged when preincubation of GS with MSO, ATP, and Mg^{2+}, produced marked inhibition (89), which could not be reversed by addition of glutamate. The irreversible inhibition of GS by MSO is now known to be associated with the phosphorylation of this amino acid analogue, and with its tight binding to the enzyme. The initial attachment of MSO to the active site of the enzyme in essentially the same manner as does glutamate is possible because of the conformational similarity between glutamate and MSO (Fig. 3b).

1.2.2 Glutaminase isoenzymes (EC 3.5.1.2)

In his paper, entitled, "The synthesis of glutamine from glutamate and ammonia, and the enzymic hydrolysis of glutamine in animal tissues" published in 1935 (59), H.A. Krebs perceptively recognized: "Liver of mammals (pig, guinea pig, rat) contain a

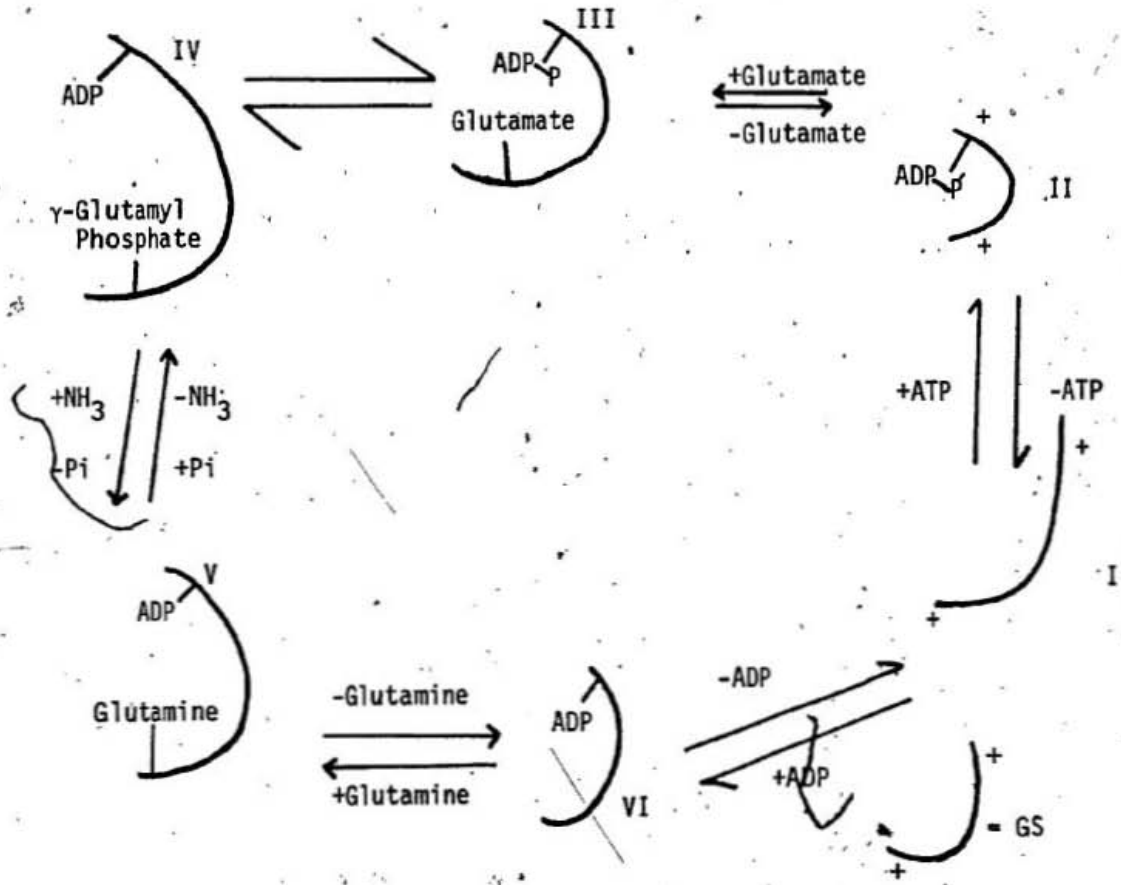
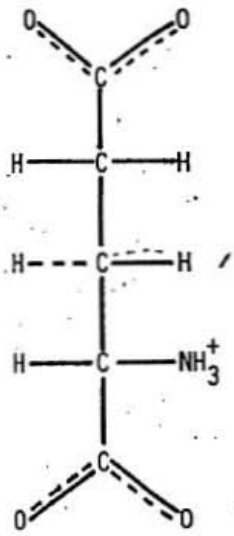
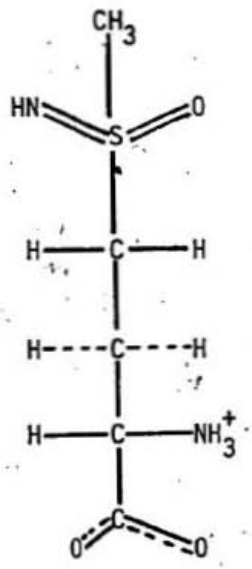


Fig. 3a Scheme for the enzymatic synthesis of glutamine



L-glutamate



L-Methionine Sulphoximine

Fig. 3b Relationship between L-glutamate and L-methionine sulphoximine

glutamine-splitting enzyme which is not inhibited by glutamate, and shows a pH optimum different from the optimum of glutaminase from brain, kidney and retina.

The mitochondrially located (92) "phosphate-dependent" isoenzymes are considered to be the true glutaminases, and are designated glutaminase I. Liver, and possibly lung, possess the "liver-type" isoenzyme, all other tissues, including brain and lung, have the kidney-type" (93). The two are distinguished on the basis of P_i requirement (the liver type requires a low concentration for activation, the kidney type, a high concentration), pH optima, affinity for glutamine, reactions with activators and inhibitors (93) and inhibition by glutamate (59). The tissue distribution of glutaminase activity reported by different workers is not identical, but highest activity is present in kidney, brain, and possibly small intestine, with much lower activity in liver and other tissues (93, 94). The two enzymes of the glutaminase II pathway, glutamine ketoacid transferase (GKA) and ω-amidase, are located primarily in the cytosol (92).

The glutaminase I (PDG) pathway commences with the entry of glutamine into the mitochondrial matrix. There glutamine is deamidated by PDG, yielding ammonia and glutamate, which can subsequently be oxidatively deaminated by glutamate dehydrogenase (GDH) to give rise to ammonia and αKG (see Fig. 3c).

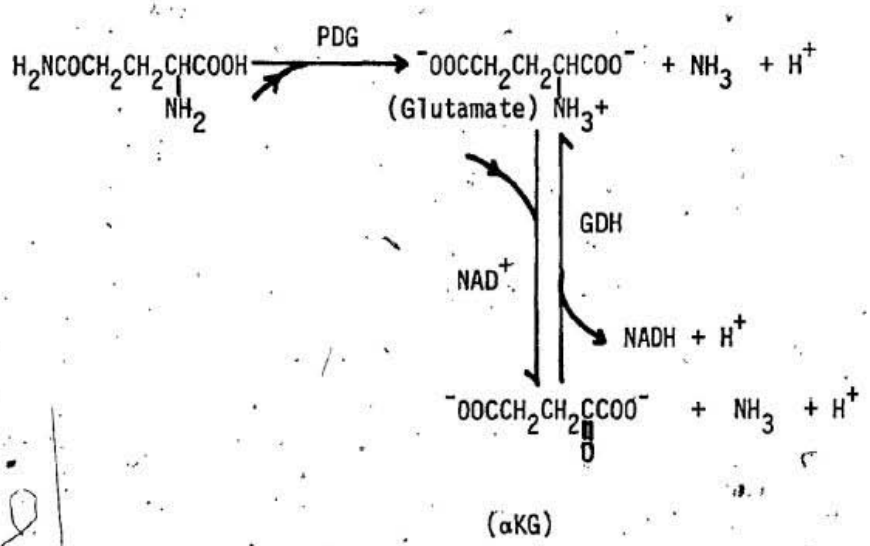


Fig. 3c The glutaminase I pathway

The glutaminase II pathway involves the initial transfer of the amino group of glutamine to a ketoacid, in a reaction catalyzed by GKA, to give the respective amino acid and α-ketoglutaramate, whose deamidation is catalyzed by ω-amidase, yielding ammonia and αKG.

In the kidney, it seems significant that the segment of the rat nephron which is the exclusive locus of the enzyme which makes glutamine has the lowest activity of the enzyme which degrades it, i.e., PDG (86).

1.3. Renal metabolism of Glutamine and ammonia

1.3.1 Relation of ammonia excretion to Urine pH

In 1856, Claude Bernard (95) observed that the urinary pH of carnivorous animals is markedly lower than that of herbivorous animals. Some years later (96) Walter described the excretion of ammonia in various animals that had been rendered acidotic and stressed the protective and base-saving features of this mechanism. Since then, numerous studies on the patterns of ammonia excretion in man, dog, and rat, especially during acidosis have been published (29,45,65,67,81, 97). On the other hand, very few studies on these patterns in the guinea pig and rabbit have been made; the current theories of ammonia excretion are, therefore, largely based on studies in species that normally excrete acid urine.

In spite of much work in this field, three major questions await conclusive answers. What is the physiological stimulus for ammonia excretion? What is the relative importance of enzymatic and physico-chemical factors in the mechanism of ammonia excretion? What is the rate-limiting metabolic step(s) modified during acidosis?

1.3.2 Regulation of ammonia production: Focus on current views

The metabolic pathways involved in enhanced ammonia formation during metabolic acidosis utilize glutamine as the primary ammoniagenic precursor in man, dog, and rat (6,29,81,97), and result in virtually complete production of ammonia from both nitrogens of glutamine. PDG is considered the key enzyme in ammoniagenesis (97-101) in most species studied. Inherent in this role of glutaminase, are the activities of other enzymes considered important in the regulation of ammonia production; the enzymes are; glutamate dehydrogenase, α KG dehydrogenase, and PEPCK. The permeability of mitochondria to glutamine, the rate of gluconeogenesis and the presence of a plasma factor (97-101), are also considered important components in the regulation of renal ammonia production.

1.3.3 Evidence for a role of GS in the regulation of renal ammoniagenesis.

Glutamine synthesis, which catalyzes the reaction;

Glutamate + ammonia + ATP $\longrightarrow$ Glutamine + ADP + Pi has the potential for regulating ammoniagenesis by removing ammonia. A necessary and sufficient condition for GS to be a significant regulator of ammoniagenesis is that, it must be relatively active in kidney to ensure enough recycling between the ammonia producing and consuming pathways, and must be regulated, directly or indirectly by the acid-base status of the animal.

Although measurements of renal GS activity indicate that it is either unchanged or minimally diminished in chronic metabolic acidosis (45), quantitation of this reaction in vivo with [^{14}C] glutamate injection, by Damian and Pitts (45), suggests diminished flux through

this ammonia-consuming pathway in metabolic acidosis, even though this in vivo approach may be criticized on the grounds that the measured intrarenal specific activities of glutamate and glutamine represent the average of many individual pools. Additionally, a decrease in glutamine synthesis by the isolated perfused kidney, and tissue slices from acidotic rats have been reported by Hems (57). One problem with Hem's data is that the glutamine that accumulated in his perfusion is the resultant of glutamine production and utilization, and it is not possible to be certain at any given time, which of the two processes is predominant.

It seems obvious, against this background that inhibition of the GS pathway may play a role in the response to acidosis in the rat. However the finding that GS is absent from the kidneys of both dog and man (76,80,81), and that enhanced ammonia production occurs in rat in the presence of the GS inhibitor, MSO, indicate that modulation of GS activity cannot be the sole or primary factor in the response to acidosis (97).

1.3.4 Model for study of renal ammoniagenesis and its regulation: Use of gunea pig kidney tubules.

The high activities of both GS and glutaminase in rat kidney have made interpretation of results on in vivo and in vitro studies difficult, mainly because one cannot delineate which of the two reactions was predominant at any given time. The guinea pig and rabbit, therefore offer a more appropriate experimental animal models for studies on renal GS, since they contain high activities of GS and relatively low activities of glutaminase (see table 2).

TABLE 2. Renal GS and glutaminase activities in representative mammals (Taken from Ref. 65.)

Mammal	Renal GS Activity	Renal Glutaminase Activity
	Units*	Units*
Rabbit	44.6±3.1	185±27
Guinea Pig	39.6±2.4	210±6
Rat	37.5±2.5	1,455±99
Cat	BLD+	1,557±27
Dog	BLD+	1,392±5
Pig	BLD+	1,337±125

Values are means ± SE of 4 animals. *Units = μmoles product/g tissue wet wt x hr + Below limits of determination.

Kidney cortex metabolism has been extensively studied in vitro with slices (102-105) which exhibited some advantages compared with the isolated perfused organ or cell-free preparations. The method presented here offers an *in vitro* system of kidney cortex cells which combines the high metabolic rates of cortex slices with the technical advantages of broken cell preparations. The method is based on the principle first employed by Rodbell (106), who separated fat cells by enzymatic treatment with collagenase. This method was first used for the preparation of kidney tubules by Burg and Orloff (107). The results of *in vitro* studies, using isolated guinea pig cortical tubules are shown in the early parts of this work.

Since detailed subcellular studies on GS in guinea pig kidney is lacking, a number of cell fractionation studies was carried out, with

the aim of providing further insight into the exact subcellular localization of GS in guinea pig kidney, and also to supplement information gathered from renal tubule studies, in an attempt to offer explanations or answers to the mechanism of renal ammoniagenesis during acute acidosis.

2. MATERIALS AND METHODS

2.1 Materials

ANIMALS

Male guinea pigs (700-900g), of the Dunkin-Hartley albino strain, were obtained from Canadian Farms and Laboratories Ltd., St. Constant, La Prairie, Quebec. The animals were fed a standard guinea pig chow, obtained from Supersweet Farms, St. John's, Newfoundland, and had free access to food and water.

CHEMICALS

L-glutamic acid, α-ketoglutaric acid, L-glutamic acid γ-monohydroxamate, (GHA) hydroxylamine, L-lactic acid, and creatine phosphate were purchased from Sigma Chemical Company, St. Louis, Missouri.

NAD^+, NADH, NADP, NADPH, L-methionine DL-Sulphoximine were also obtained from Sigma Chemical company; ADP and ATP were purchased from Boehringer Mannheim GmbH. All other biochemicals were of the highest purity available, and were obtained from J.T. Baker Chemical Co., Phillipsburg, N.J., British Drug House (BDH) Chemicals Ltd., Poole, England, or from Fisher Scientific Company, Fair Lawn, N.J.

ENZYMES

The following enzymes were obtained from Sigma Chemical Company; Glucose 6-phosphate dehydrogenase; L-glutamate dehydrogenase (Type II, in 50% glycerol containing sodium phosphate buffer, pH 7.3), lactate dehydrogenase, pyruvate kinase (Type II), creatine phosphokinase, and glutaminase (Grade II). (Hexokinase and Collagenase, grade II were obtained from Boehringer Mannheim GmbH)

OTHER CHEMICALS

"Sommotol" sodium pentobarbital (65 mg/ml), was obtained from

M.T.C. pharmaceuticals. Water was treated by the Culligan Reverse osmosis system, RDSI.

2.2 Methods

Guinea pigs were anesthesized by intraperitoneal injection of sodium pentobarbital (6.5 mg/100g body weight), and the kidneys were quickly removed; kidney cortex slices, or homogenates were prepared.

2.2.1 Preparation of isolated kidney cortex tubules and incubations.

PREPARATION OF ISOLATED TUBULES

The tubule isolation technique was adopted from that of Guder et al. (108), with the modification of Baverel et al. (67). After the guinea pigs were anesthesized, the two kidneys were excised through a midline abdominal incision and placed in ice-cold Krebs-Henseleit phosphate medium. The cooled kidney was hemisected after the capsule and fatty tissues had been removed; medullary tissue, as well as the tissue forming the corticomedullary junction, was scooped out and discarded. Thin slices (2mm thick) prepared from 4 to 8 g cortex tissue were placed in a 250 ml Erlenmeyer flask containing 10 ml of Krebs-Henseleit medium (pH 7.4) (109), and 25 mg collagenase (grade II). The flask was incubated for 50 min at 37°C with 95% oxygen and 5% carbon dioxide as a continuous gassing phase, in a water-bath which oscillated at 100 cycles/min. The homogenate was filtered through a plastic tea strainer (mesh size 0.5 x 0.5 mm^2), and the tubules were collected by centrifugation at x 50g for 40 sec., at 4°C. The packed tubules were then washed twice by resuspension in Krebs-Henseleit medium and centrifuged at x 50g for 40 sec. The final pellet was resuspended in Krebs-Henseleit medium, which yielded a

concentration of 2-10 mg of tubules (dry weight) per milliliter.

Where the effect of Ca^{++} was of interest, the washing and final resuspension of the tubular fragments was done by using a Ca^{++}-free Krebs-Henseleit medium. Alkaline pH conditions (pH 7.7) was obtained by using 40.4 ml of $NaHCO_3$ (0.154M) and 80.6 ml of NaCl (0.154 M) in the Krebs medium, and the final resuspension was in this medium.

The entire procedure took 90-100 min from the time the kidney was removed until the tubules were placed in the incubation flasks. Fig. 4 shows a light micrograph of a typical tubule preparation.

INCUBATION PROCEDURES

Incubations were carried out at 37°C for 30 min. in a shaking water bath, in 25 ml stoppered Erlenmeyer flasks in an atmosphere of O_2:CO_2 (19:1). Each flask contained 0.5 ml of the tubule suspension plus 2 ml of Krebs-Henseleit medium supplemented or not with substrates or inhibitor, plus lactate (5mM) as respiratory substrate. L-methionine-DL Sulphoximine (MSO, 2mM) and/or HCl (12 mM) were added to obtain inhibition and/or pH 7.1 effect. In all experiments, incubations for each experimental condition was carried out in duplicate or triplicate. Incubations were terminated after 30 min. by adding perchloric acid, $HClO_4$ (3% v/v, final concn.) to each flask. In all experiments, zero-time flasks with substrates, were prepared by adding $HClO_4$ to the flasks before the tubules. Media were centrifuged for 10 min. at X 4000 g to remove the denatured protein, and the supernatant was neutralized with 40% K_2CO_3. Metabolite assays were conducted on the neutralized supernatant.

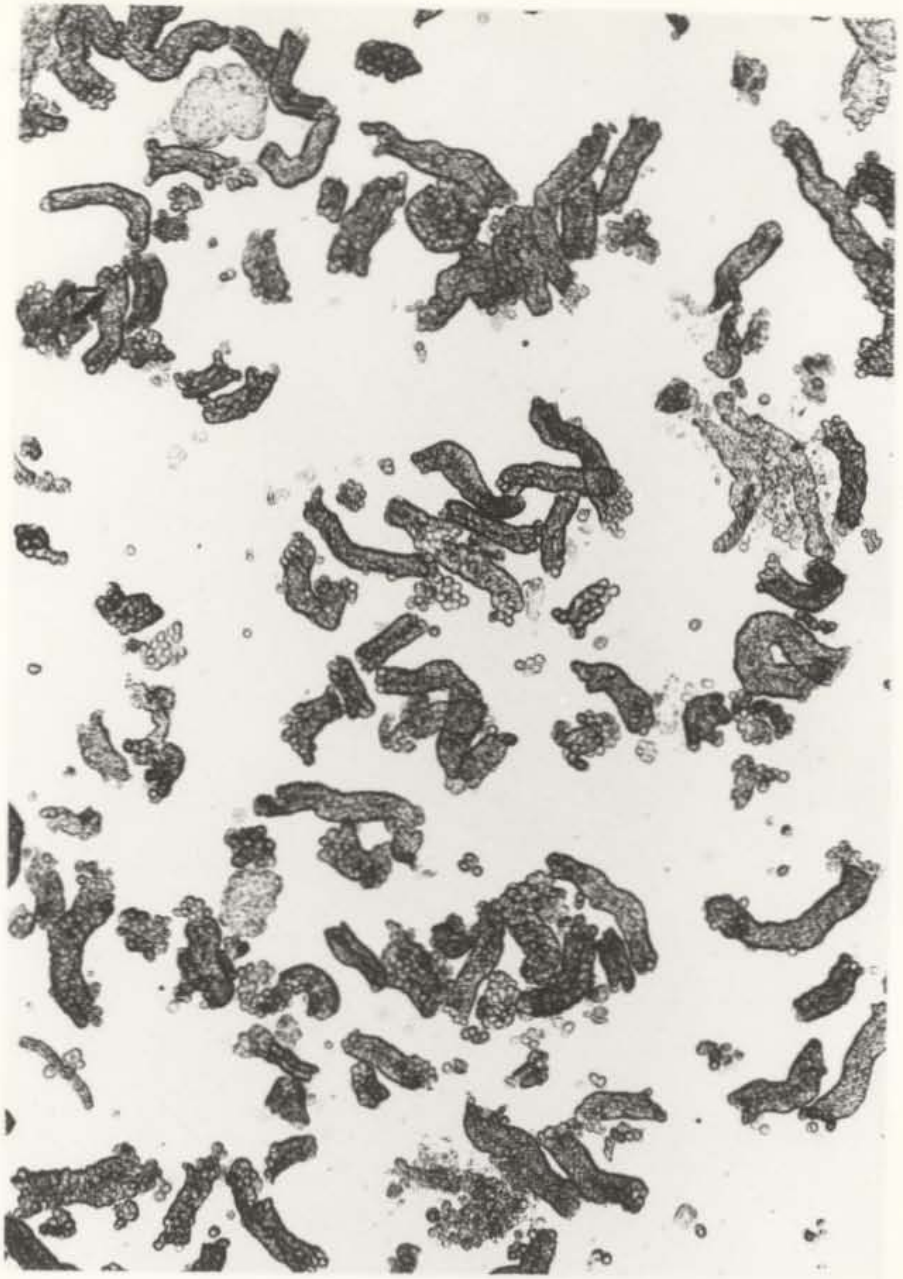


Fig. 4. Light micrograph of a typical tubule preparation (x 64).

2.2.2 Analytical methods

Glucose, lactate, NH_3, glutamate, and glutamine were spectrophotometrically assayed by enzymatic methods. Glucose was measured by the hexokinase technique (110). Lactate by the method of Gutmann and Wahlefeld (111), glutamate and glutamine by the method of Lund (112), and NH_3 by the method of Kun and Kearney (113). ATP was measured by a bioluminescent technique (114).

In each experiment, the dry weight of the amount of tubules added to the flasks was determined. For this, 1 ml of the tubule suspension was placed in a preweighed glass beaker and dried in an oven at 70°C. After cooling, the beaker was reweighed, and the weight of tubules was corrected for the electrolytes contained in 1 ml of Krebs-Henseleit medium.

Preliminary experiments as depicted in Fig. 5 and Fig. 6 show respectively that, 0.5 ml of tubule suspension (corresponding to a dry weight of about 5 mg), incubated for 30 min., offer suitable linear ranges for glutamine synthesis with respect to tissue concentration, and incubation time.

2.2.3 Calculations and Statistical Analysis

Net substrate utilization and product formation were calculated as the difference between the total flask contents (tissue + medium) at the start (zero-time flasks) and after the period of incubation. The metabolic rates are expressed in micromoles of substance taken up or produced per gram dry weight of tubules per incubation time. They are reported as mean ± S.E. The results were analysed by use of

(i) student's t-test for paired data

or (ii) ANOVA-one and two-way

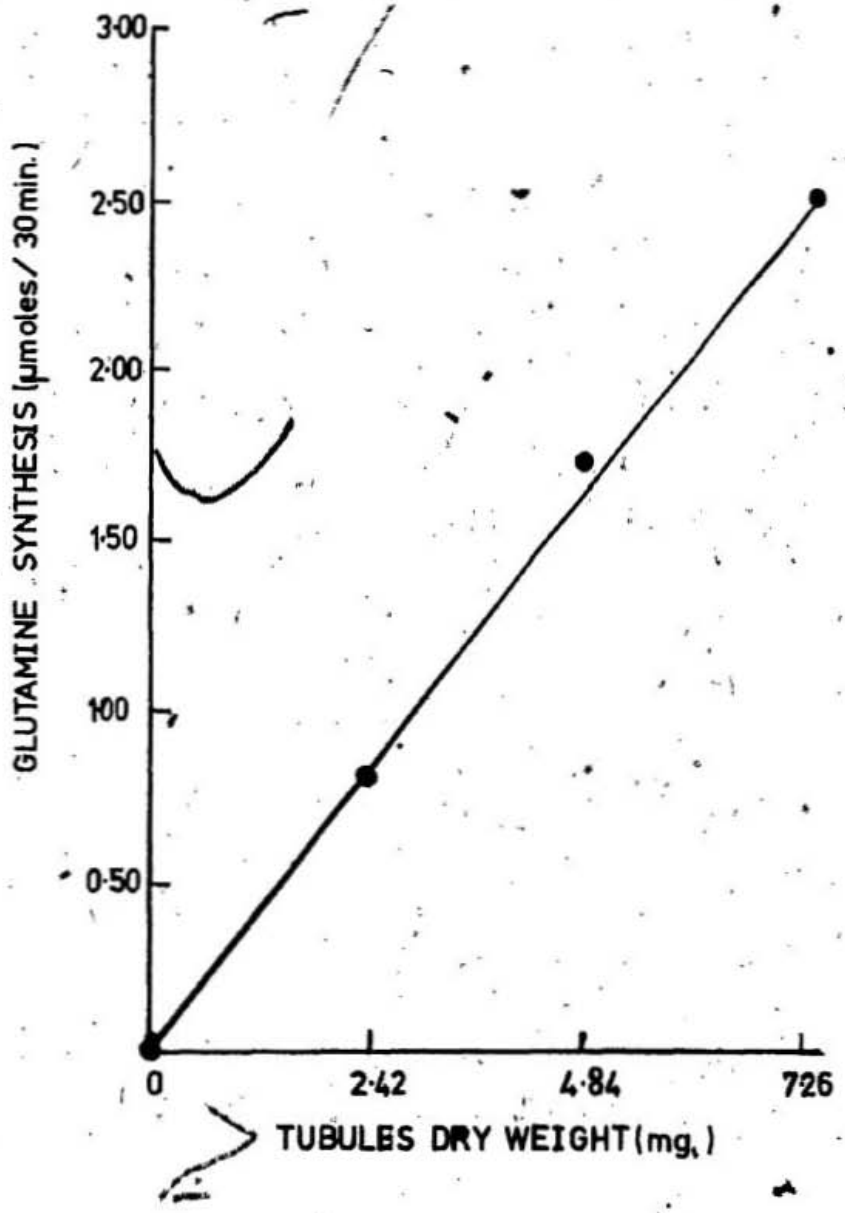


Fig. 5 Glutamine synthesis versus tubules dry wt.
Data for a typical experiment

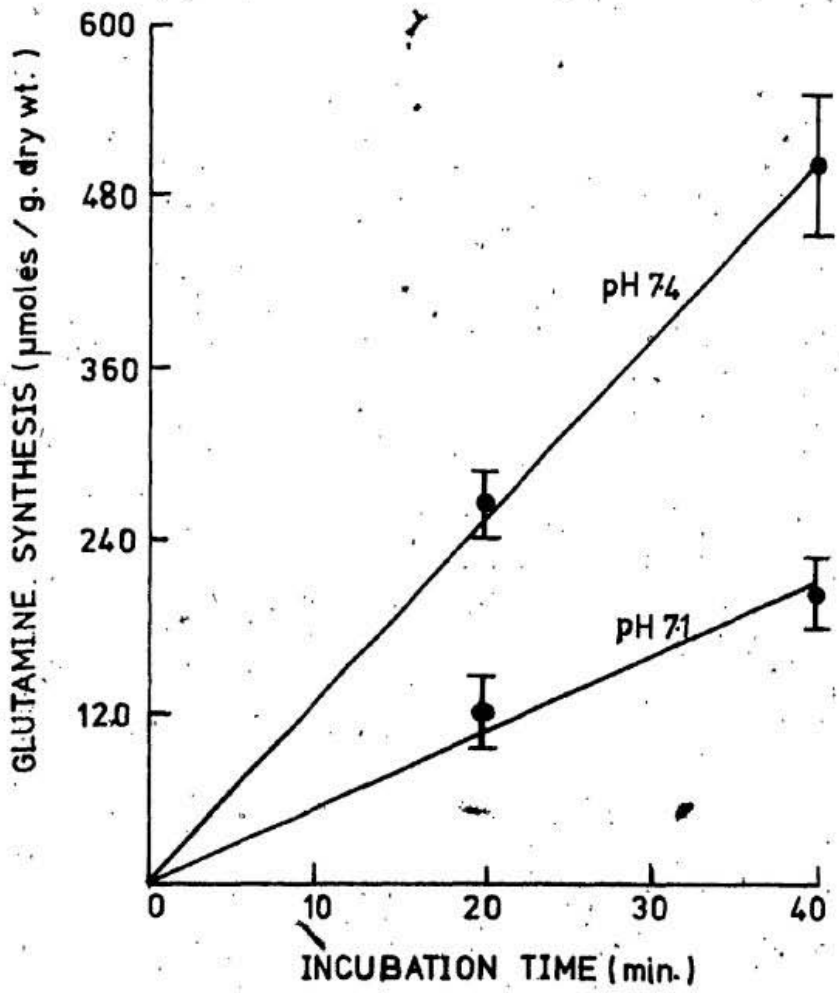


Fig. 6 Glutamine synthesis versus incubation time

Data represents mean±S.E for 3 experiments

and (iii) Duncan's or Newman-Keuls multiple range test

2.2.4 Homogenization and Subcellular fractionation of kidney cortex

HOMOGENIZATION PROCEDURE

The demedullated kidney (described earlier), was weighed and chilled in ice-cold 0.33 M sucrose, containing 2 mM HEPES, pH 7.4, and cut coarsely with a pair of scissors. A smooth glass Potter-Elvehjem homogenizer, with a loose-fitting teflon pestle (clearance 0.30 mm) served to prepare the cortex homogenate containing 100 mg of tissue per ml of medium. Homogenization was achieved by hand, with 5 or 6 strokes of the pestle.

The medium employed for homogenization depended on the object of the study. The following media had been employed:

(i) 0.33M sucrose, 2mM HEPES, and/or 5mM $MgCl_2$, pH 7.4

(ii) 0.33M sucrose, 2mM HEPES, 5mM $MgCl_2$, and/or 5mM EDTA, or 5mM EGTA, pH 7.4

(iii) 0.33M sucrose, 2mM HEPES, 5mM EGTA and Ca^{++} (2-4mM), pH 7.4

Homogenization was also done in a 0.154 M KCl solution, pH 7.4

After filtration through three layers of cheese-cloth, the homogenate was fractionated by differential centrifugation. During the entire process, the temperature was maintained near 5°C.

SUBCELLULAR FRACTIONATION PROCEDURE

The method of de Duve et al. (115) was used with modifications to obtain the subcellular fractions. The nuclear fraction (N) was collected on centrifuging the homogenate at 450xg for 2 min. in an International B-20 refrigerated centrifuge. It was washed once by resuspending in about one half its original volume of homogenizing

medium and centrifuged again. The original supernatant fluid and the supernatant fluid from the washing were combined and centrifuged at 13,000 x g for 10 min. to obtain the mitochondrial and lysosomal fraction (M+L); this was also washed once with the homogenizing medium and centrifuged again. The original supernatant fluid and the supernatant fluid from washing the mitochondria were combined and centrifuged at 105,000 x g for 60 min. in a Beckman L-50 ultracentrifuge to obtain the microsomal fraction (P). The supernatant fluid from the last operation formed the soluble or S fraction. The pellets of the nuclear, mitochondrial and lysosomal fractions, and microsomal fraction were finally resuspended in suitable volumes of homogenizing medium for analysis.

In other experiments, the homogenate was spun once at 105,000 x g on a Beckman L-50 ultracentrifuge to obtain particulate and soluble fractions; the particulate fraction in this case was also resuspended in an appropriate volume of homogenizing medium.

2.2.5 Enzyme assays

ASSAY OF GLUTAMINE SYNTHETASE

Suitable assay conditions were determined as a function of incubation time, and amount of tissue homogenate and/or fractions, in preliminary experiments (see Fig. 7 and Fig. 8).

GS activity was routinely assayed by the method of Herzfeld (116), in a medium having the following composition per millilitre: 32 µmoles Tris buffer (pH 7.4), 65 µmoles L-glutamate, 65 µmoles hydroxylamine. HCl, 16 µmoles $MgCl_2$, 6.4 µmoles ATP, 150 µl enzyme source, 10 µmoles creatine phosphate and 15 EC units of creatine phosphokinase, all in a final volume of 1.05 ml. In some experiments, the enzyme

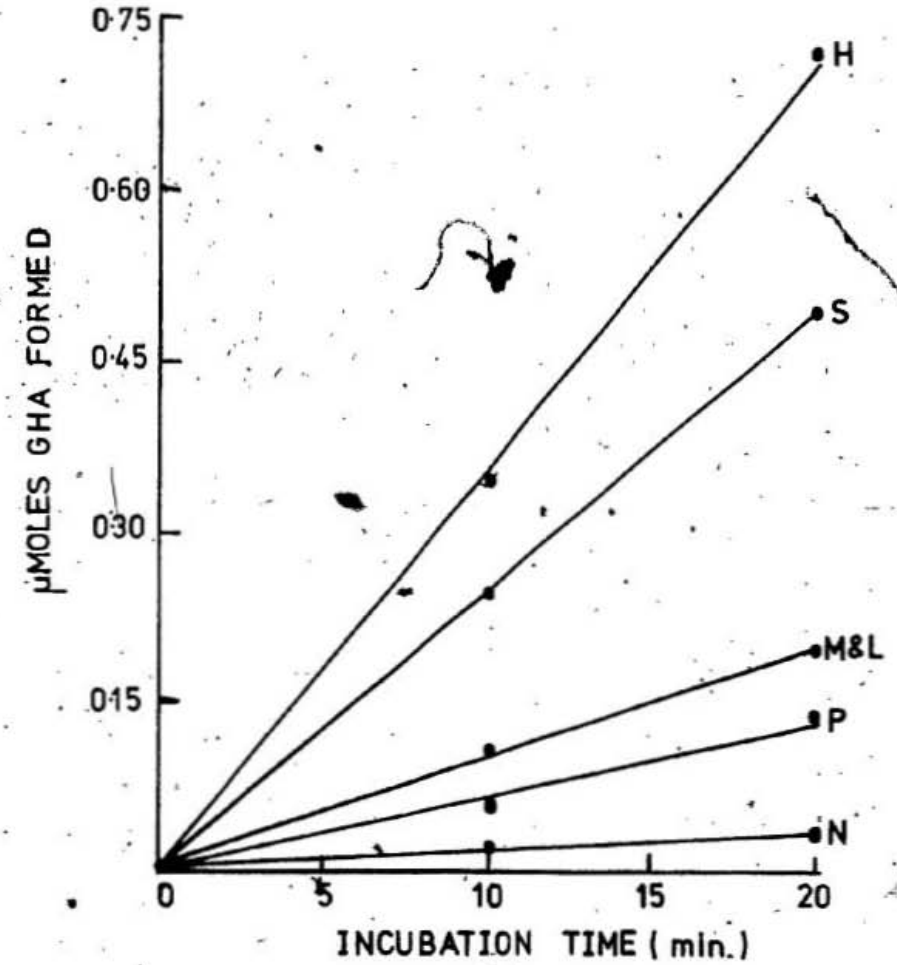


Fig.7 Progress curves for GS reaction, using 100 µl of sample

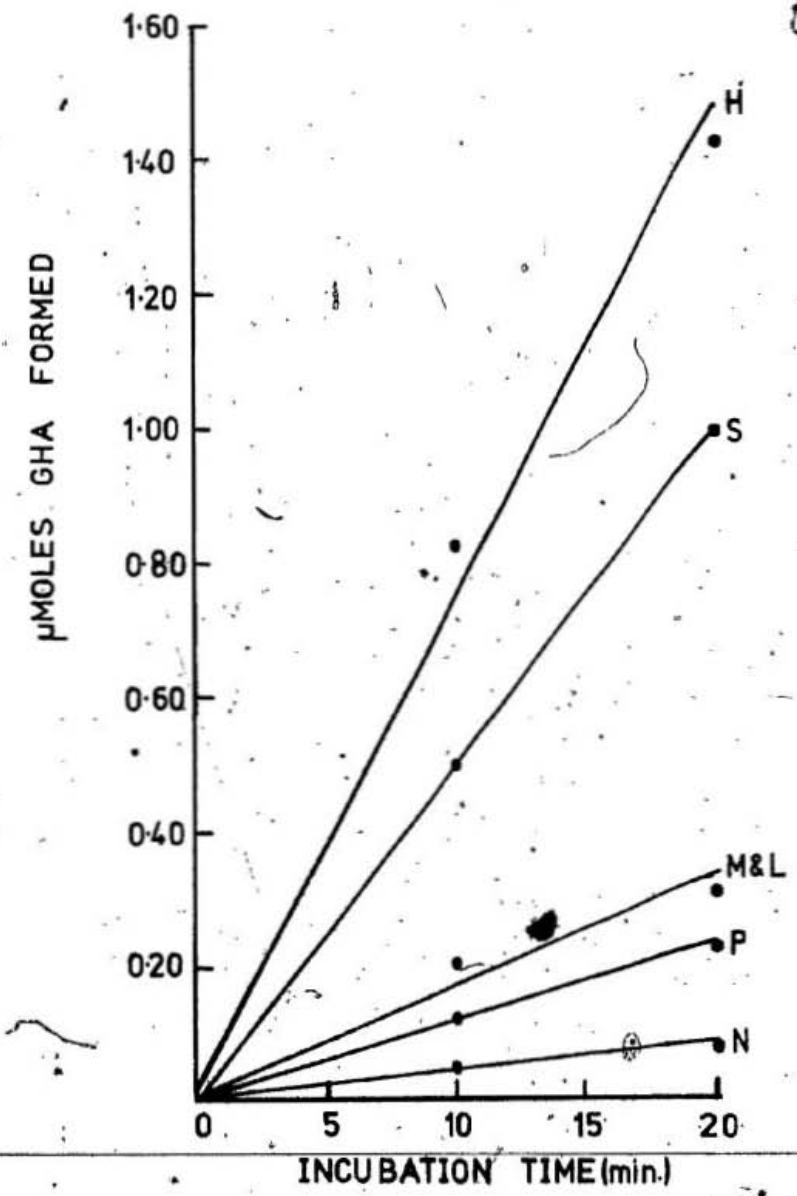


Fig. 8 Progress curves for GS reaction, using 200µl of sample

source was preincubated with MSO (5mM), for 5 min, before the addition of L-glutamate.

The method is based on the colorimetric determination of the product of the reaction between L-glutamate and hydroxylamine, γ-glutamylhydroxamate, when the latter reacts with ferric chloride (117). High ADP/ATP ratios inhibit GS (71) and it is customary to include an ATP-generating system in the reaction mixture; this was satisfied by use of creatine phosphate and creatine phosphokinase. All acidic or basic compounds were individually neutralized to pH 7.4. The reaction was started by addition of the enzyme source and the mixture was then incubated at 37°C for 15 min. It was stopped by the addition of 2 ml of ferric nitrate reagent (1:1 ratio of 40% TCA and 0.6 M ferric nitrate). After mixing, the precipitated proteins were removed by centrifugation and the absorbance of the ferric-hydroxamate colour was measured at 500nm and the readings converted to μmoles of γ-glutamylhydroxamate by comparison with a standard curve prepared from pure GHA. An absorbance spectrum was also determined for the ferric hydroxamate colour produced by both the enzyme source and pure GHA, within the wavelength range 400-750nm. Pure GHA showed only one absorption maximum at 500nm, while all the enzyme sources showed two absorption maxima, at 500nm and 750nm. The absorption at 500nm is attributed to GHA, while that at 750nm might be due to unidentified chromophores.

All values reported have been corrected by the subtraction of suitable blank values obtained from control tubes in which the stopping reagent was added before enzyme source.

ASSAY OF LACTATE DEHYDROGENASE

The enzyme activity was assayed spectrophotometrically at room

temperature by following the oxidation of NADH at 340 nm as described by Morrison et al. (118). The assay mixture in a final volume of 2.1 ml contained: 5-50 µl of enzyme source, 100mM Tris-HCl buffer (pH 7.4), 0.30 mM NADH, and 20 mM nicotinamide. The reaction was started by the addition of sodium pyruvate (pH 7.4) to give a final concentration of 2 mM. The enzymatic rate was calculated by using an extinction coefficient for NADH at 340 nm of 6.22×10^3 litre . mol^{-1} cm^{-1} (119).

The reaction was shown to be linear with respect to tissue concentrations, for all fractions and homogenate within the run period.

ASSAY OF GLUTAMATE DEHYDROGENASE

The enzyme activity was assayed spectrophotometrically at room temperatue by following the oxidation of NADH at 340nm, as described by Brdiczka et al. (120). The assay mixture in a final volume of 3 ml contained: 5-50 µl of enzyme source, 5mM EDTA, 0.22mM NADH, 3mM αKG, 2mM ADP, 5µM rotenone, and 50 mM triethanolamine buffer (pH 7.6). The reaction was started by the addition of $(NH_4)_2 SO_4$ to give a final concentration of 200 mM. The enzymatic rates were calculated by using an extinction coefficient for NADH at 340nM of 6.22×10^3 litre . mol^{-1} cm^{-1} (119).

The reaction was shown to be linear with respect to tissue concentrations, for all fractions and homogenate within the run period.

ASSAY OF NADPH-CYTOCHROME C REDUCTASE

The enzyme activity was measured spectrophotometrically at room temperature by following the reduction of cytochrome C at 550nm as described by Sottocasa et al. (121). The reaction mixture in a final volume of 3 ml contained: 10-50 µl of enzyme source, 0.1 mM oxidized

cytochrome c, 0.3 mM KCN, 50 mM potassium phosphate buffer (pH 7.5), and 5 μM rotenone. The reaction was initiated by the addition of NADPH to a final concentration of 0.1 mM. A reduced-oxidized extinction coefficient for cytochrome c of 18.5×10^3 litre. mol^{-1} cm^{-1} (122) was used in the calculation of reaction rates.

The reaction was shown to be linear with respect to tissue concentration, for all fractions and homogenate within the run period.

DNA EXTRACTION AND ESTIMATION

DNA was extracted by a modification of the Schneider method (123). 0.5 ml of homogenate or supernatant, and 0.2 ml of mitochondrial or nuclear fractions was mixed with ice-cold 0.5 M perchloric acid (PCA) to a final volume of 1.0 ml. The suspension was gently mixed and allowed to stand on ice for 10 min. The mixture was centrifuged at top speed, on a clinical centrifuge, at 4°C for 15 min. The supernatant was discarded and the pellet was broken up. 2 ml of 0.5 M PCA was added to the pellet and heated at 70°C for 25 min. The cloudy suspension was centrifuged again on the clinical centrifuge, and the supernatant decanted carefully for DNA estimation.

DNA was measured with diphenylamine reagent as described by Burton (124). Calf thymus DNA (10-40 μg) was used as standard.

PROTEIN ESTIMATION

Protein was determined by the biuret method (125), following solubilization with deoxycholate. Bovine serum ablumin (BSA) was used as standard.

2.2.6 Calculations and analysis of data

One unit of enzyme activity has been defined as that amount of enzyme that will cause the removal/formation of 1 μmol of substrate/

product in 1 min at either 37°C or room temperature. Tissue activity is expressed as units/ml of tissue homogenate or fraction, and the specific activity in units/mg protein. Statistical analysis, where necessary, were performed as described in section 2.2.3.

3. RESULTS AND DISCUSSION

3.1 Kidney Cortex Tubules Studies

3.1.1. Acute acidosis and glutamine synthesis

Isolated rat kidney tubules have been used extensively for the study of ammoniagenesis from glutamine, and a stimulation by acidic pH has been demonstrated (29, 126). Lanctin and Brosnan (unpublished results) found that in the presence or absence of lactate, ammoniagenesis from glutamine was significantly stimulated at pH 7.1 compared with pH 7.4, and that MSO stimulated ammoniagenesis to a much greater extent than the low pH. They also noted that, in the presence of MSO, acidification of the medium did not produce any additional stimulation of ammoniagenesis, suggesting therefore, that an appreciable rate of NH_3 removal for glutamine synthesis occurs in the rat renal tubules, and that the mechanism whereby a decreased pH stimulates ammoniagenesis is by inhibiting this glutamine synthesizing activity.

A suitable experimental model for testing this hypothesis, could be found in a study on the effect of reduction of pH on glutamine synthesis, using a system which has high GS activity and low glutaminase activity. The results of such experiments, carried out using guinea pig kidney cortex tubules, are shown in Table 3. The experiments show a high rate of glutamine synthesis (compared with that of Baverel et al. (67), using 5mM NH_4Cl and 5mM glutamate, under similar conditions), which correlates well with glutamate and NH_3 removal, at pH 7.4. This synthesis was significantly decreased by about 50% at pH 7.1, regardless of whether the lower pH was produced by decreasing the bicarbonate concentration (metabolic acidosis, depicted

TABLE 3. Effect of acute metabolic and respiratory acidosis, and Methionine Sulphoximine (MSO) on glutamine synthesis

Metabolite removal (-) or production (+). Results expressed as μmoles/30 min/g. dry weight cortex tubules (mean ± SE); n = number of separate tubule preparations.

		Metabolites		
pH	n	Glutamate	NH_3	Glutamine
7.4	7	-438±80	-400±62	+503±78
7.1	7	-214±62*	-228±36*	+246±45*
7.4+MSO	4	-97±28*	+6.5±17.1*	+39±47*
7.1+MSO	4	-67±10**	+27±18**	-20±31**
7.1(+)CO_2	3	-102±113*	-169±41*	+226±61*

* $P \leq 0.05$ compared with pH 7.4 incubations
** $P \leq 0.05$ compared with pH 7.1 incubations

as pH 7.1 in Table 3), or by increasing the P_{CO_2} (respiratory acidosis, shown as pH 7.1 (+)CO_2 in Table 3). The removal of glutamate and NH_3 was also comparably inhibited. The presence of MSO almost completely abolished glutamine synthesis, and comparably inhibited the removal of both glutamate and NH_3.

Thus, in vitro, guinea pig kidney tubules, incubated with glutamate and NH_3, synthesize glutamine. That this is not due to protein degradation, is shown by the effect of MSO in the incubations and by the stoichiometry of glutamate and ammonia removal with glutamine production. Furthermore, decreasing the incubation pH of the tubules leads to an inhibition of glutamine synthesis. The observation that guinea pig kidney synthesizes glutamine rapidly, is in agreement with that made first by Krebs in 1935 (59), and confirmed by Baverel et al. (67).

If increased hydrogen ion concentration, $[H^+]$, inhibits glutamine synthesis by tubules, what is the effect of changing bicarbonate ion concentrations $[HCO_3^-]$, on renal glutamine synthesis?

3.1.2 Acute metabolic alkalosis and glutamine synthesis

Table 4 shows that, while the effect of MSO is also evident at high $[HCO_3^-]$ (i.e. pH 7.7), glutamine synthesis at pH 7.7 was not significantly different from that at pH 7.4, although slightly greater (646 µmoles/30 min/g dry wt, compared to 582 µmoles/30 min/g dry wt.). Fig. 9 shows the result of an experiment where incubation medium pH was varied from 6.85 to 7.60 and the actual pH of each flask measured. The

TABLE 4. Effect of acute metabolic alkalosis and MSO on glutamine synthesis

Metabolite removal (-) or production (+). Results expressed as μmoles/30 min/g. dry weight cortex tubules (mean ± SE); n = number of separate tubule preparations.

Buffer System	n	Metabolites		
		Glutamate	NH_3	Glutamine
25mM HCO_3^-; 5% CO_2 in O_2 (pH=7.4)	3	-506±81	-534±164	+582±117
50mM HCO_3^-; 5% CO_2 in O_2 (pH=7.7)	3	-567±103	-530±138	+646±134
25mM HCO_3^-; 5% CO_2 in O_2 (pH=7.4) + MSO	3	-68±32*	-62±39*	+89±41*
50mM HCO_3^-; 5% CO_2 in O_2 (pH=7.7) + MSO	3	-51±52**	-79±87**	-33±144**

* $P \leq 0.05$ compared with pH 7.4 incubations
** $P \leq 0.05$ compared with pH 7.7 incubations

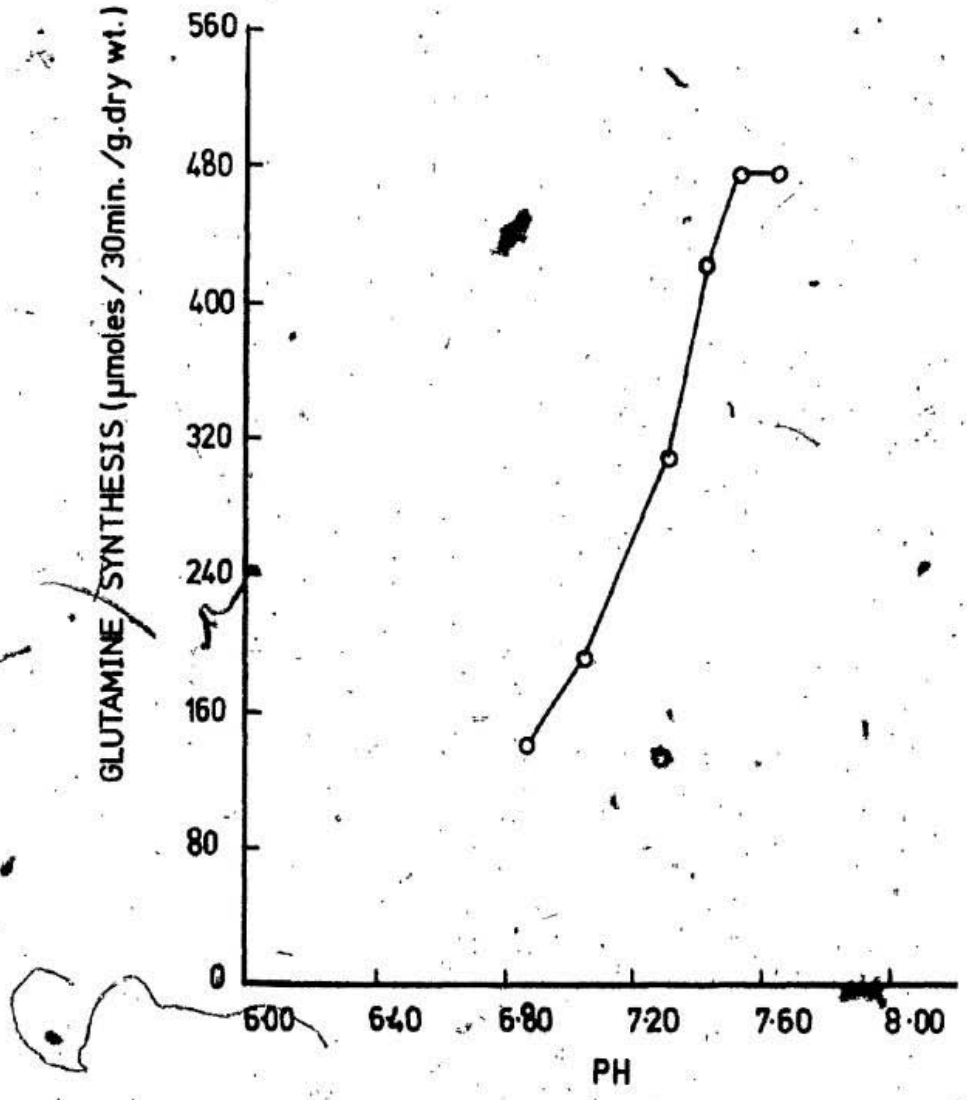


Fig.9 Effect of pH on glutamine synthesis
Data for a typical experiment

data confirms that in Tables 3 and 4, in that a sharp fall in glutamine synthesis occurs as acidic pH values are approached from pH 7.4 and a modest increase towards alkaline pH values, which levels off at about pH 7.5.

The data so far thus indicate a direct involvement of [H^+] per se (as opposed to [HCO_3-], in the inhibition of glutamine synthesis by renal tubules. The experiment reported in Fig. 10 was designed to test the reversibility of the inhibition. In this experiment tubules were incubated at pH 7.4 and pH 7.1. In some flasks sufficient $NaHCO_3$ was injected at 15 min to raise the pH from 7.1 to 7.4. Thus, whatever mechanism is involved in the inhibition of glutamine synthesis by acid, cannot be said to involve a permanent, irreparable damage to the glutamine synthesizing machinery of the tubules. Rather it represents a rapidly responsive reversible regulatory system.

3.1.3 Metabolic viability of tubular cells: Gluconeogenesis as an index

Kidney cortex is known to have a large capacity to synthesize glucose from non-carbohydrate precursors (80, 102), and in fact, apart from the liver, it is the only organ which has a high gluconeogenic capacity (103). With the development and use of isolated kidney tubules in metabolic studies, gluconeogenic rates, higher than rates in slices and perfused kidney, have been consistently reported (102-108).

As a test of the metabolic viability of my tubule preparations, the rates of gluconeogenesis from various substrates were measured in guinea pig kidney tubules, to ascertain whether glucose synthesizing

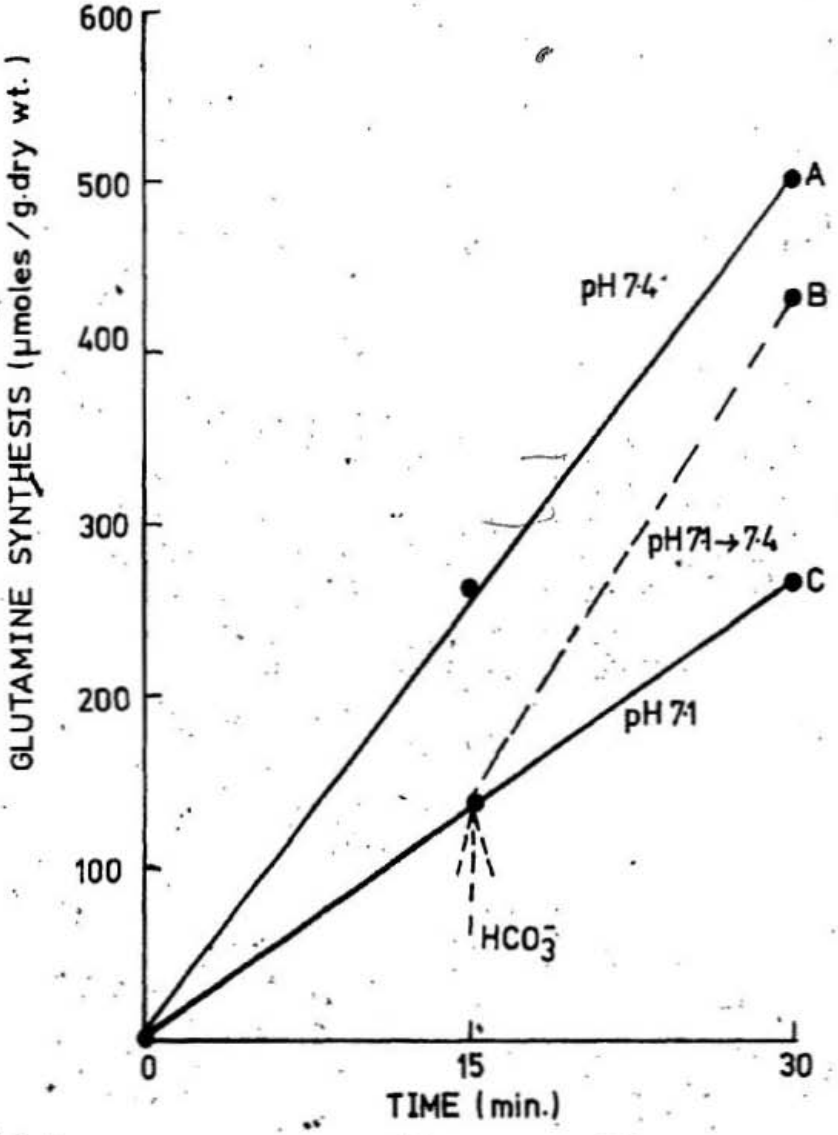


Fig.10 Glutamine synthesis inhibition by HCl, and reversal of inhibition by HCO_3^-

Gradient : A = 16·7 B = 18·6 C = 7·9

Data for a typical experiment

ability was comparable to that reported by other workers and also to see whether the rate of glucose production would be different at pH values of 7.1, 7.4 and 7.7 (pH values employed in the incubation systems). If there were a significant decrease in gluconeogenesis at pH 7.1 it could be argued that this could result from an interference with ATP production at the low pH since gluconeogenesis is an ATP-requiring process. This would then suggest that the inhibition of glutamine synthesis seen at pH 7.1 was due to interference with ATP production. This possibility was tested in two sorts of experiments. In one, we examined the effect of acidic pH on gluconeogenesis, itself an ATP-requiring process, and in the second, we measured ATP levels.

Table 5 shows that the rate of gluconeogenesis from malate and pyruvate was not significantly different at the three pH values. These values compare favourably with those reported by other workers, under similar conditions (102, 103, 108, 131, 132), and therefore, one can conclude that there was no inhibitory effect of acidic pH on the endergonic process of gluconeogenesis.

3.1.4 Metabolic viability of tubular cells: steady state levels of ATP as an index

A number of methods describing the measurement of subpicomole quantities of ATP by firefly luciferase have been published (114). The measurement of ATP is finding wide application as a criterion of cell viability. The sensitivity and convenience of this method has been utilised to assess the viability of a number of cells (114). ADP can be readily measured, using the same technique, by using pyruvate kinase to convert the ADP to ATP.

TABLE 5. Rate of gluconeogenesis from malate and pyuvate as substrates

Results expressed as μmoles/30 min/g. dry wt. tubules (mean ± S.E); n=4

	pH		
Substrates	7.1	7.4	7.7
Malate	79±7	90±26	89±23
Pyruvate	41±9	47±11	51±12

$P \geq 0.05$ compared with pH 7.4 incubations for both substrates

As a further test of the metabolic viability of the tubule preparation therefore, the steady state concentrations of ATP in tubules incubated under various conditions, for 30 min, and at zero time, were determined. Table 6 shows that the steady state level of ATP had risen, after a period of 30 min. incubation, from an initial value of 5.57±1.67 μmoles/g dry wt., to 12.56±2.74 μmoles/g. dry wt. at pH 7.4, and to 13.14±3.86 μmoles/g dry wt. at pH 7.1. There was no statistical significance between the ATP levels under these two conditions, thus emphasizing the fact that, the tubule preparations were metabolically active, and that the ATP generating systems were unaffected by acid. The increase in the sum of (ATP+ADP) in these experiments does not necessarily mean that a net synthesis of adenine nucleotides has occured. It is well appreciated that during anoxia, ATP decreases due to continued ATP utilization in the absence of adequate ATP synthesis and that AMP, rather than ADP, accumulates due to the action of adenylate kinase. During the preparation of tubules some anoxia inevitably occurs and this accounts for the relatively low ATP concentration at 0'. However upon incubation in well-oxygenated media ATP synthesis occurs and ATP levels are restored (127).

3.1.5 Mechanism of acid inhibition of glutamine synthesis: role of the NH_4^+-NH_3, system and the concentration of glutamate

The data now available, for the mechanism of the GS reaction, support the view that the enzyme binds the substrates in ordered sequence, i.e. ATP Mg^{2+}, L-glutamate, and ammonia (89, 90, 91); with the rigid requirement for the presence of the nucleotide, before any activation of glutamate. According to this mechanism, activation

TABLE 6. ATP and ADP concentrations in tubules

Results expressed as μmoles/g. dry wt. (mean ± S.E); n=3

Incubation time (min)	Medium pH	[ATP]	[ADP]
0	7.4	5.57±1.67	6.25±0.94
30	7.4	12.56±2.74	6.09±2.35
30	7.1	13.14±3.86*	5.42±1.29

*P>>0.05 compared with pH 7.4 incubations.

of glutamate to form γ-glutamyl phosphate constitutes the first step, and the second step involves reaction of the activated intermediate with ammonia (or hydroxylamine). The first step is less optically specific than the second, which becomes rate-limiting with D-glutamate and ammonia. Thus, whereas the rate of synthesis of D- and L-γ-glutamyl hydroxyamate were found to be similar, the synthesis of D-glutamine occurred at a significantly lower rate than that of L-glutamine (128). Furthermore, in the reaction with L-glutamate, ammonia and hydroxylamine react equally well, the rate of nucleophile addition not being limiting. On the other hand, hydroxylamine shows a much greater reactivity as compared to ammonia with D-glutamate and other glutamate analogues. This enhanced reactivity of hydroxylamine has been attributed to it being a better nucleophile than ammonia (89).

It is obvious therefore, that any attempt at explaining the observed effect of acid on glutamine synthesis, must take cognisance of the mechanism of the reaction.

One may express the concentration of free base ammonia in either of two ways; in terms of micromoles, calculated from pH and total ammonia concentration (free base plus ammonium ion), by the Henderson-Hasselbalch equation, or as P_{NH_3} in mm Hg (129). Using the former method and assuming a pK for the reaction.

$NH_4^+ \rightleftharpoons H^+ + NH_3$ of 9.4 (130), a plot of varying ammonia concentration versus rate of glutamine synthesis is shown in Fig. 11. At no concentration of free base is the inhibitory effect of acid obscured, and therefore, the concept of a limiting nucleophile (or free base) concentration in going from an incubation pH of 7.4 to pH 7.1, cannot be invoked as a mechanism to account for the observed

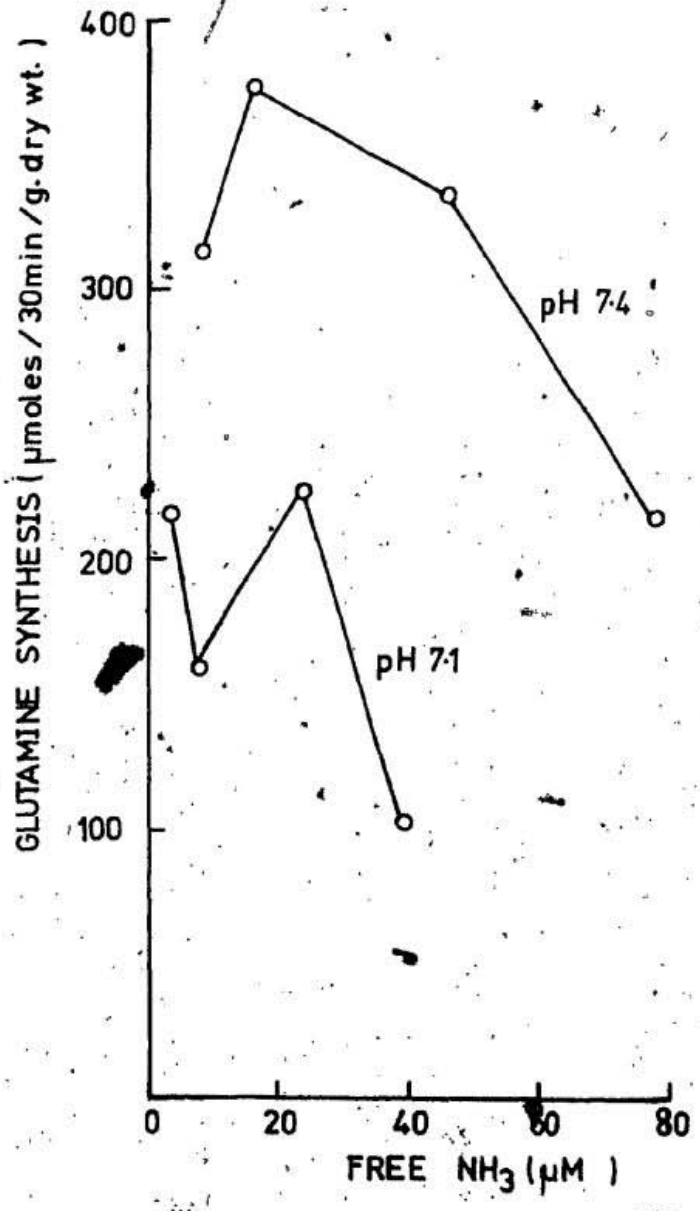


Fig.11 : Effect of NH_3 concentration on glutamine synthesis

Data for a typical experiment

effect of acid. I next examined glutamine synthesis as a function of glutamate concentration. Fig. 12 shows that, acidification of the incubation medium, inhibits glutamine synthesis at all glutamate concentrations tested. However the degree of inhibitioin was less at the higher glutamate concentrations. Thus at 0.5 mM the degree of inhibition was 66% whereas at 10mM it was only 32%. It was not possible to carry out experiments at higher glutamate concentrations but it is possible that the effect of acidic pH is to decrease the affinity of glutamine synthetase for glutamate. However, this should be regarded as a tentative suggestion rather than a firm conclusion. It should be borne in mind that the glutamate concentrations plotted in Fig. 12 are in the incubation medium and these are not necessarily the same as those in the renal cells.

3.1.6 Ca^{++}, norepinephrine, and glutamine synthesis

The question as to whether or not glutamine synthesis is stimulated by norepinephrine (a catecholamine), arose. Since norepinephrine works via Ca^{++} fluxes, a series of experiments were started to find out what effect ommission of Ca^{++} from the Krebs-Henseleit medium would have on the rate of glutamine synthesis. Table 7 shows an interesting observation. The 50% inhibition in glutamine synthesis observed at pH 7.1 in previous experiments may only occur in the presence of Ca^{++}. In the absence of Ca^{++}, glutamine synthesis could not be demonstrated at either pH. Measurement of ATP levels (Table 8), shows that the steady state level of ATP after 30 min incubation was not significantly different from that at zero time. Thus the increase in ATP that occurs during incubation (Table 6) did

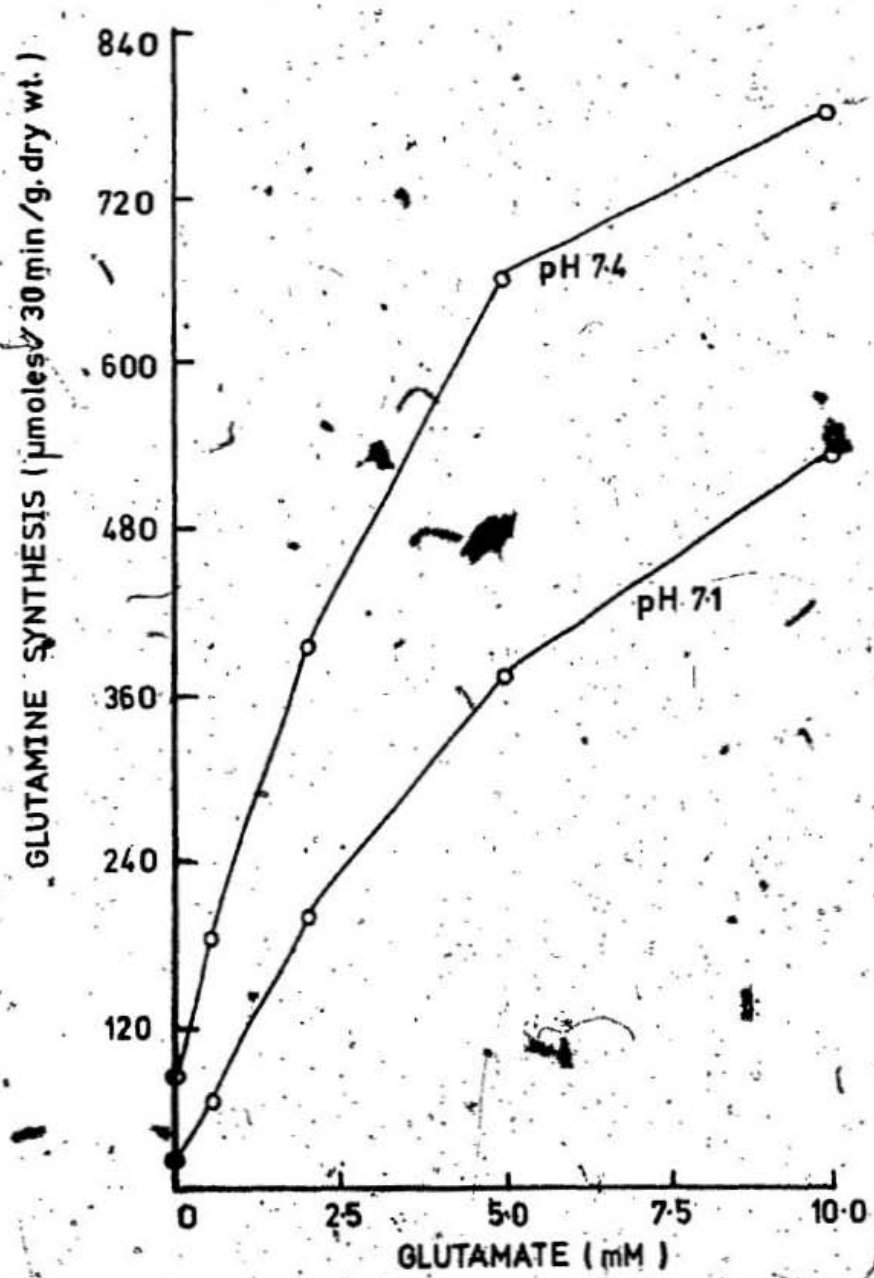


Fig.12 Effect of Glutamate concentration on glutamine synthesis
Data for a typical experiment

TABLE 7. Glutamine synthesis in the presence or absence of calcium

Metabolite removal (-) or production (+). Results expressed in μmoles/30 min/g dry wt. cortex tubules (mean ± S.E.); n=3

		Metabolites		
	Medium pH	Glutamate	NH_3	Glutamine
(a)	7.4+Ca	-242±30	-300±29	+305±56
(b)	7.4-Ca	-128±30	-81±88	+11±117
(c)	7.1+Ca	-134±17	-148±42	+148±47
(d)	7.1-Ca	-62±27	-55±77	+71±77

$P \leq 0.05$ for comparisons between (a) and (b); (c) and (d) (all metabolites).
$P \leq 0.05$ for comparisons between (a) and (c); (b) and (d) (all metabolites).

TABLE 8. ATP and ADP concentrations in tubules incubated without calcium ions

Results expressed as μmoles/g. dry wt. tubules (mean ± S.E); n=3

Incubation time (min)	Medium pH.	[ATP]	[ADP]
0	7.4	5.57±1.67	6.25±0.94
30	7.4	5.71±2.53	5.59±1.70
30	7.1	7.54±4.61	6.72±2.84

not occur. Therefore, the inhibition of glutamine synthesis in this case, is not a specific effect. How lack of Ca^{++} affects ATP levels is not understood.

The tissue response to catecholamines seems to depend on the presence of adrenergic receptor sites, classified as alpha and beta, on the basis of their response to sympathomimetic amines and adrenergic blocking agents (133). The α-adrenoceptors are classified into α_1 and α_2 subtypes (134). Gluconeogenesis in rat renal cortex slices or tubules is stimulated by exogenous 3'5'-cyclic AMP (135) and catecholamine hormones (136), which is believed to be exerted through α-adrenoceptors of the α_1 subtype (134).

Results of experiments to determine how the interplay of Ca^{++} and norepinephrine might influence glutamine synthesis is summarized in Table 9. Norepinephrine at 1 µm final concentration did not affect glutamine synthesis significantly (compared to controls without hormone). ATP concentrations at the end of the incubations were not different from that of the controls. Except for a recent report on the effect of norepinephrine on renal gluconeogenesis in guinea pig tubules (132), the hormonal regulation of metabolism in the guinea pig has not been studied.

3.2 Subcellular studies on GS

3.2.1 A prelude to subcellular studies on GS

As a preliminary to understanding the regulation of glutamine synthetase in guinea pig tubules, it was necessary to establish the subcellular localization of this enzyme.

TABLE 9. Effect of norepinephrine on Glutamine Synthesis and ATP concentrations

Metabolite removal (-).or production (+). Results in μmoles/30 min/g dry wt (mean + S.E); n=3. The adenine nucleotide concentrations are in μmoles/g dry wt of tubules

	Metabolites				
Condition	Glutamate	NH^3	Glutamine	ATP	ADP
7.4+Ca+H	-344±78	-263±63	+283±32	11.66±4.68	4.53±1.02
7.4+Ca-H	-283±30	-258±60	+222±94	10.05±3.85	4.89±1.86

H = norepinephrine (1 μM final conc.)

P>>0.05 for all metabolites and ATP

GS has been found to be mainly associated with microsomes during cellular fractionation (84, 85), and to be attached to the surface of these particles, in rat tissues. However no information is available for guinea pig tissues.

The a priori assumption that the intracellular distribution of an enzyme is an essentially invariant characteristic of that enzyme and which forms the basis for the assignment of various enzyme activities to certain subcellular locations, during centrifugal fractionation, has been questioned. There is the belief now, that certain enzymes may not have a fixed intracellular distribution, but rather, may be rapidly and reversibly interconverted between soluble and membrane-bound forms with the distribution between these forms being a function of the concentration of certain metabolites, typically, substrates, products, allosteric effectors, etc. (137). This postulated movement of the enzyme molecules onto and off of membranes had been noted earlier (138), and might well be important in the regulation of metabolism (138).

Against this background therefore, a number of subcellular fractionation studies were carried out on guinea pig renal cortex.

3.2.2 De Duve fractionations in sucrose medium

Table 10 shows the intracellular distribution of GS and marker enzymes in the guinea pig kidney cortex, when HEPES (2mM, pH 7.4) and $MgCl_2$ (5mM) were present in the homogenizing medium. Represented as a de Duve plot in Fig. 13, for a typical experiment, the marker enzymes are well separated and enriched in their respective fractions. GS was predominantly found in the S fraction and was enriched here.

TABLE 10. Intracellular distribution of GS and marker enzymes of guinea pig kidney cortex

Enzymes were assayed as described under "materials and methods". The homogenizing medium contained 0.33mM sucrose, 2mM HEPES (pH 7.4), 2mM $MgCl_2$. The absolute value in the orignal homogenate are given in nmoles of substrate metabolized per mg kidney cortex protein per min. Results are expessed as mean + S.E. H = total homogenate, N = nuclear fraction; M L = mitochondrial plus lysosomal fraction; P = microsomal fraction; S = final supernatant (n=4).

	Original homogenate	% of Recovery					
		H	N	M L	P	S	Total % Recovery
Protein	-	100	12.2±0.5	35.6±2.8	5.6±0.9	46.5±5.2	76.5±2.1
Glutamine Synthetase	39.5±1.6	100	4.4±1.2	7.2±1.4	26.6±2.8	61.9±3.3	82.7±5.4
Lactate Dehydrogenase	646.5±63.2	100	4.5±0.7	2.2±0.7	6.8±1.6	86.4±0.9	86.4±2.2
Glutamate Dehyro-genase	125.5±11.1	100	18.9±2.7	65.0±5.3	15.9±5.6	0.2±0.0	84.5±4.4
NADPH-Cytochrome C reductase	6.0±0.7	100	11.0±0.9	26.3±4.5	58.3±3.6	4.3±1.5	71.3±8.8
DNA	12.0±0.7	100	90.4±2.8	8.0±2.1	1.2±0.4	0.4±0.2	78.7±6.2

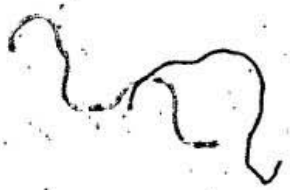
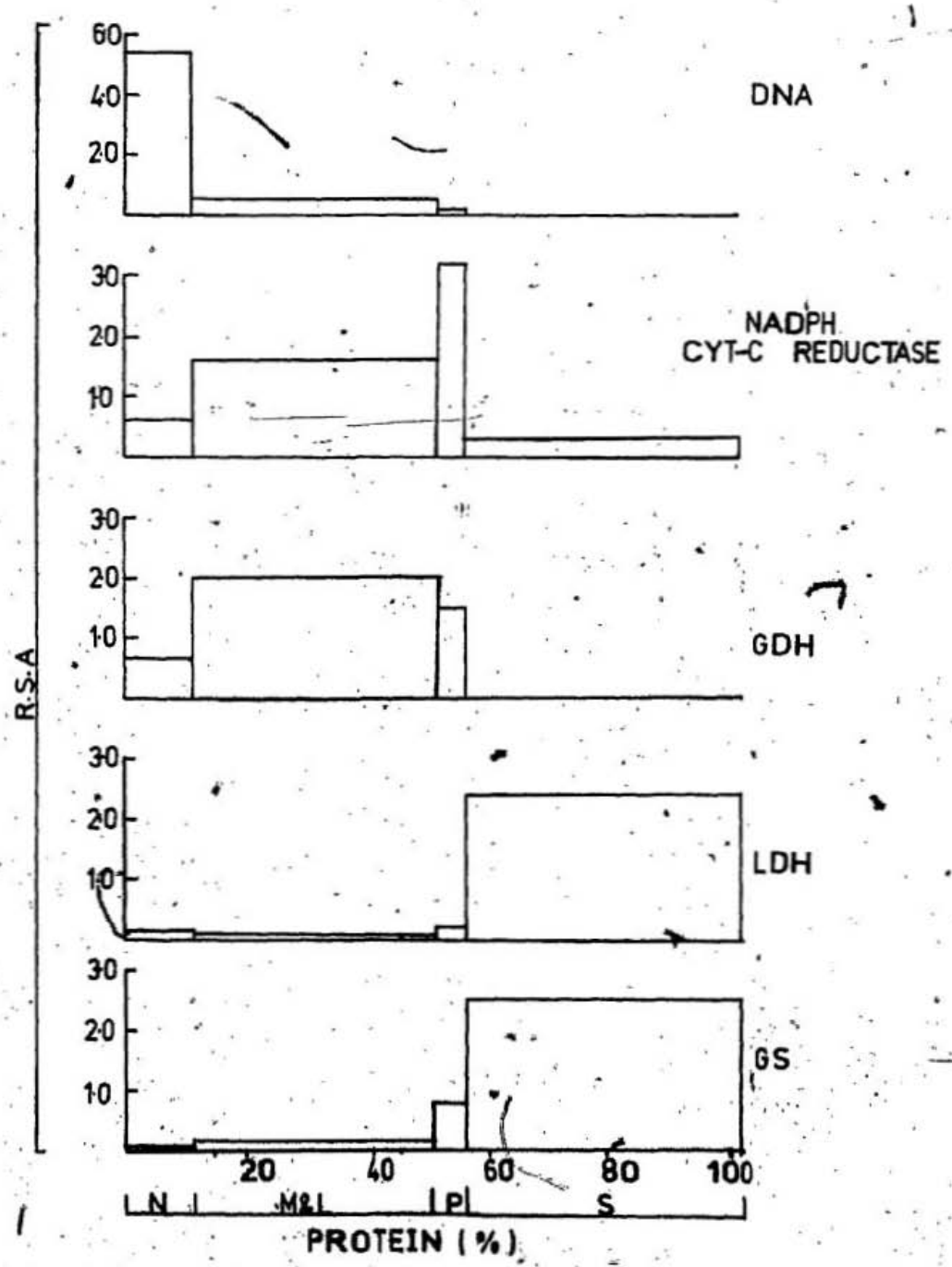


Fig. 13. Subcellular distribution of GS and marker enzymes represented as a DeDuve plot.


R.S.A
60
40
20
DNA
30
20
10
NADPH
CYT-C REDUCTASE
30
20
10
GDH
30
20
10
LDH
30
20
10
GS
20
40
60
80
100
N
M&L
P
S
PROTEIN (%)

62% of GS, and 86% of LDH were found in this fraction. However 26% of GS was found in the P fraction, as opposed to only 7% of LDH. Thus the conclusion from this experiment is that, GS in guinea pig kidney cortex is predominantly a cytosolic enzyme, but a small proportion may be associated with the microsomes. This is essentially the reverse situation compared with that found in rat kidneys, where the enzyme is primarily microsomal but may be released from these organelles by certain treatments (92). Accordingly, I carried out a number of experiments in which the homogenization medium was altered so as to see whether the relative distribution of GS between cytoplasm and particulate fractions could be altered.

3.2.3 Fractionation in different media

In these series of experiments, homogenates were centrifuged at 105,000 x g for 1 hour so that particulate and soluble fractions were obtained. The activity of GS and of the marker enzymes, LDH (cytosol) and NADPH-cyt c reductase (microsomes) were assayed. The various homogenization media were:

(i) 0,33 M sucrose, 2mM HEPES (pH 7.4), and 0-10 mM $MgCl_2$.

(ii) 0.33 M sucrose, 2mM HEPES (pH 7.4), and 2mM EDTA

(iii) 0.33 M sucrose, 2mM HEPES (pH 7.4), 5mM EGTA and 0-4mM $CaCl_2$ (in these experiments, the free Ca^{++} concentration varied from 0-210nM).

(iv) 0.15 M KCl, 2mM HEPES (pH 7.4) and 0-5mM EDTA.

(v) 0.15 M KCl, 2mM HEPES (pH 7.4), 5mM EGTA and 0-4mM $CaCl_2$

(vi) 0.33 M sucrose, 5mM EGTA, 2mM HEPES (pH 7.1-7.7)

In all of these media 70-80% of GS was found in the soluble fraction. In two experiments, the presence of EGTA seemed to have caused the enzyme to appear in the particulate fraction, but this was not a reproducible phenomena and many subsequent experiments failed to duplicate the observation. The conclusion is therefore being made, that glutamine synthetase is predominantly a cytoplasmic enzyme in the guinea pig kidney.

4. GENERAL DISCUSSION

4.1 Glutamine metabolism in guinea pig

The presence in many animal tissues of enzymes that catalyze the synthesis of glutamine from glutamate and also the hydrolysis of glutamine to glutamate and ammonia was first reported by Krebs in 1935 (59), and as stated in section 1.2.2, it has recently become clear that the kinetic properties of the glutaminases in liver kidney and intestinal mucosa possess characteristic differences which relate to the physiological function of glutamine in these different tissues.

Even though A-V difference measurements *in vivo* across the tissues drained by the superior mesenteric vein in the guinea pig indicated no net uptake of arterial glutamine (37, 38, 39), the guinea pig jejunal mucosa has been shown to contain significant levels of the important gluconeogenic enzymes, which show an adaptive increase after starvation (56). Because glutamate did not accumulate in the serosal fluid when guinea pig intestinal segments were luminally perfused *in vitro* with a glutamine-containing medium (139), it was erroneously concluded that glutamine was not deamidated during its translocation from the intestinal lumen into the blood. Not appreciated was the large capacity of the guinea pig intestine to metabolize further the glutamate produced. The general opinion, however is that, in the guinea pig (and the chicken) unlike in all other animals studied, there is a net output, rather than uptake of circulating glutamine by small intestine (39).

Relatively low activity of PDG in the small intestine of guinea pig, compared to that in the small intestine of all other animals surveyed, has been reported (39). Plasma glutamine levels were also

observed to be low (about 0.25 mM), in the guinea pig. These findings cast doubt on the quantitative importance of glutamine as energy source for the small intestine in the guinea pig. Riklis and Quastel (140) have however implicated glutamine in intestinal transport. They reported that addition of 10mM glutamine to a solution perfusing the lumen of guinea pig intestinal segments *in vitro* increased the mucosal-to-serosal glucose translocation rate by 53%.

The liver (and spleen) of the guinea pig did not synthesize glutamine (59) *in vitro*, and therefore glutamine metabolism in this tissue might be centered on its uptake and breakdown. Guinea pig liver glutaminase is not inhibited by glutamate.

Experimental data has been presented (20) showing that glutamine degradation in the perfused guinea pig liver, as judged on the basis of ammonia, urea, and glucose formation may be subject to several regulatory mechanisms which are likely to act *in vivo* too. The isolated perfused guinea pig liver utilized statistically significant amounts of exogenous glutamine only when it was present in concentrations of 1mM or higher; half maximal rate of glucose and urea production was attained at 0.5mM glutamine, which corresponds to twice its physiological blood concentration (39). Armed with the knowledge that liver cells accumulate glutamine (22), and assigning a value of 3-5 for the intracellular to extracellular glutamine concentration ratio (22, 28), Deaciuc *et al.* predicted an intracellular glutamine concentration of about 12-20mM in their experiments. This range of concentration is obviously unphysiological (the *in vivo* situation should correspond to between 0.75 and 1.15mM), and they concluded therefore that at its blood concentrations of 0.25mM, glutamine is not likely to be

effectively degraded by the liver. The stimulation of ammonia production, and the concomitant inhibition of ureogenesis and gluconeogenesis when aminooxyacetate (AOA) (a known inhibitor of transaminases) was used in the perfusion medium makes it unlikely that a transaminase reaction is involved as the first step of glutamine degradation, when this glutamine is presented to the liver at concentrations higher than 0.25mM.

The essential role of GDH in providing ammonia for urea synthesis has been pointed out recently (141). Consequently, changes of the mitochondrial redox state of the NAD^+-NADH couple could affect the rate of ammonia urea and glucose production from glutamine. For example, a shift to a more oxidized state in the mitochondria could increase the flux through GDH in the direction of deamination and ultimately, the rate of formation of final metabolites. By changing the redox state of the NAD^+-NADH couple in the mitochondria in their study (20), Deaciuc et al. were able to effect changes in the rate of formation of ammonia, urea, and glucose, in the perfused guinea pig liver. Such alterations in metabolite formation were in agreement with the proposed role of GDH (141) in providing ammonia for urea synthesis, when it acts in the direction of deamination. The conclusion was also made that degradation of glutamine is eventually under the control exerted by the respiratory chain. An interesting observation which arose from the work of Deaciuc et al. is the failure of NH_4Cl to activate glutaminase in guinea pig liver mitochondria (20), as it does in the rat (22). Inorganic phosphate however stimulated the rate of glutamate formation from glutamine.

Low glutaminase activity has been detected in guinea pig brain,

lung, and kidney (59), while brain, retina and kidney have high activity of GS (59, 65).

4.2 Glutamine metabolism in guinea pig kidney

Glutaminase I (142) and the glutamine α-keto acid transamination-deamination systems (143) of guinea pig kidney have been extensively studied as counterparts to in vivo studies of ammonia excretion in the same species (63). Glutaminase I was characterized as a hydrolase with an optimal pH of 7.4, which was activated by phosphate. Glutaminase II was characterized as having an optimum pH of 8.8, and is dependent on the presence of α-keto acids; it is similar to the glutamine transaminase-deamidase of the liver studied extensively by Meister (2, 3). GS in guinea pig kidney has been shown to form glutamine from glutamate and ammonia, has an optimal pH of 7.4 in homogenates and is dependent on ATP and Mg^{++} (59, 60, 63, 66, 142, 143). It is now fully established that the glutaminase activity of guinea pig kidney is low and the GS activity is high (65, 67).

4.2.1 Glutamine synthesis in guinea pig kidney

Since the initial work of Krebs (59), and that of Richterich-van Barle (60), Klahr et al. (61), and Preuss (62), it is now known that, in contrast with kidney cortex slices of other animals, slices of guinea pig (and rabbit), do not release ammonia when incubated with glutamate. Ammonia excretion of guinea pig was also found to be low, and was not different during acute acidosis than during acute metabolic alkalosis (63). A correlation has been made between the high glutamine synthesizing ability of guinea pig kidney and its high urine pH (65).

In an attempt to elucidate the pathway of glutamine synthesis in guinea pig kidney, Baverel et al. studied the fate of glutamate carbon

and nitrogen (67) and alanine (144) in isolated guinea pig kidney cortex tubules. When 1mM glutamate was the sole substrate, glutamate removal was linear with time over a 60 min. incubation period (67). Linearity was achieved in my experiments (see Fig. 6, page 34) over a 40 min. incubation period, in the presence of 2mM glutamate, 1mM NH_4Cl, and 5mM lactate as oxidizable substrate. Furthermore, the high endogenous synthesis of glutamine (i.e. in the absence of substrate) reported by Baverel et al., was not observed by me. Rather, extremely low rates of endogenous glutamine synthesis was achieved in all my experiments (results not shown). Net conversion of glutamate carbon and nitrogen into glutamine is however obvious in both experiments. The fact that glutamine synthesis under similar conditions was higher in my experiments could be due to the fact that glutamate functioned both as an oxidizable substrate for the generation of ATP, as well as carbon and nitrogen donor for glutamine synthesis, in the experiments of Baverel et al. They reported also, that, when 5mM MSO was added to the incubation system, glucose production was inhibited. I did not assess alterations in Krebs cycle intermediates, or alanine and aspartate concentrations in my experiments, but Baverel et al. (67) argued that since alanine, aspartate, and lactate concentrations increased, the inhibition of glucose synthesis might be due to preferential utilization of the glutamate carbon via oxaloacetate for the synthesis of aspartate and pyruvate, from which lactate and/or alanine could be formed. However, I'm of the opinion, that since competition exists between glucose synthesis from noncarbohydrate precursors and glutamine synthesis, inhibition of one pathway should favour the operation of the other. In other words, in the presence of

MSO, the carbon skeleton of glutamate should be channeled into synthesis of glucose. Glucose synthesis increased in the presence of MSO in my experiments, but I cannot compare my results with those of Baverel et al., since the glucose formed could have come from the exogenous lactate.

The most important finding in the work of Baverel et al., however, is the importance of GDH in glutamate metabolism in guinea pig kidney. In the absence of exogenous NH_4Cl, the supply of ammonia via GDH is the rate-limiting step in glutamine formation from glutamate, and addition of NH_4Cl increases both glutamate and ammonia removal and glutamine synthesis (compare with my results (Tables 3 and 4, pages 44 & 46) and observe the excellent stoichiometry between substrate removal and product formation). The physiological relevance of Baverel's work is that, in vivo, the guinea pig kidney would most likely release, rather than take up, glutamine into circulating blood. That, this is infact the case has been shown by A-V difference measurements (67). The guinea pig kidney also releases ammonia and alanine into venous blood (67).

Baverel et al. showed that the guinea pig renal cortex contains significant activity of alanine aminotransferase (67), and that alanine synthesis by isolated guinea pig tubules metabolizing glutamate, was inhibited by the presence of AOA. The conclusion was therefore drawn by Forissier et al. (144), that alanine synthesis occured via alanine aminotransferase. They also inferred that, since alanine aminotransferase catalyzes a reaction which is close to equilibrium, alanine could also be a substrate of the guinea pig renal cortex, Forissier et al. (144) therefore investigated the metabolism of alanine

in guinea pig kidney cortex tubules, and stressed the critical role of CO_2 fixation by pyruvate carboxylase, in the presence of sufficient HCO_3^-. At 1mM and 5mM concentrations of alanine, glutamine was found to be the main carbon and nitrogenous product of the metabolism of alanine. Furthermore, there was no accumulation of Krebs cycle intermediates, glycogen, glucose, pyruvate, lactate or aspartate. This results are diametrically opposed to those reported by Krebs in 1935 (59). Krebs reported that, glutamine was formed in renal cortex slices of the guinea pig, only when the slices were incubated with glutamate, proline, and hydroxyproline, but not alanine. In parenthesis, it should be noted that Krebs used DL-alanine as substrate, and incubated in phosphate-saline medium with an atmosphere of pure oxygen. Such conditions have been criticized (144).

Fig. 14 is a scheme of the pathway proposed by Forissier and Baverel to account for the complete transformation of alanine carbon and nitrogen into glutamine. Besides the critical role of pyruvate carboxylase, which was ignored by Krebs, the role of GDH in furnishing a second molecule of αKG, is also essential. Other enzymes involved are; alanine aminotransferase, GS, pyruvate dehydrogenase, citrate synthase, as well as the operation of the enzymes of the Krebs cycle responsible for the conversion of citrate into KG.

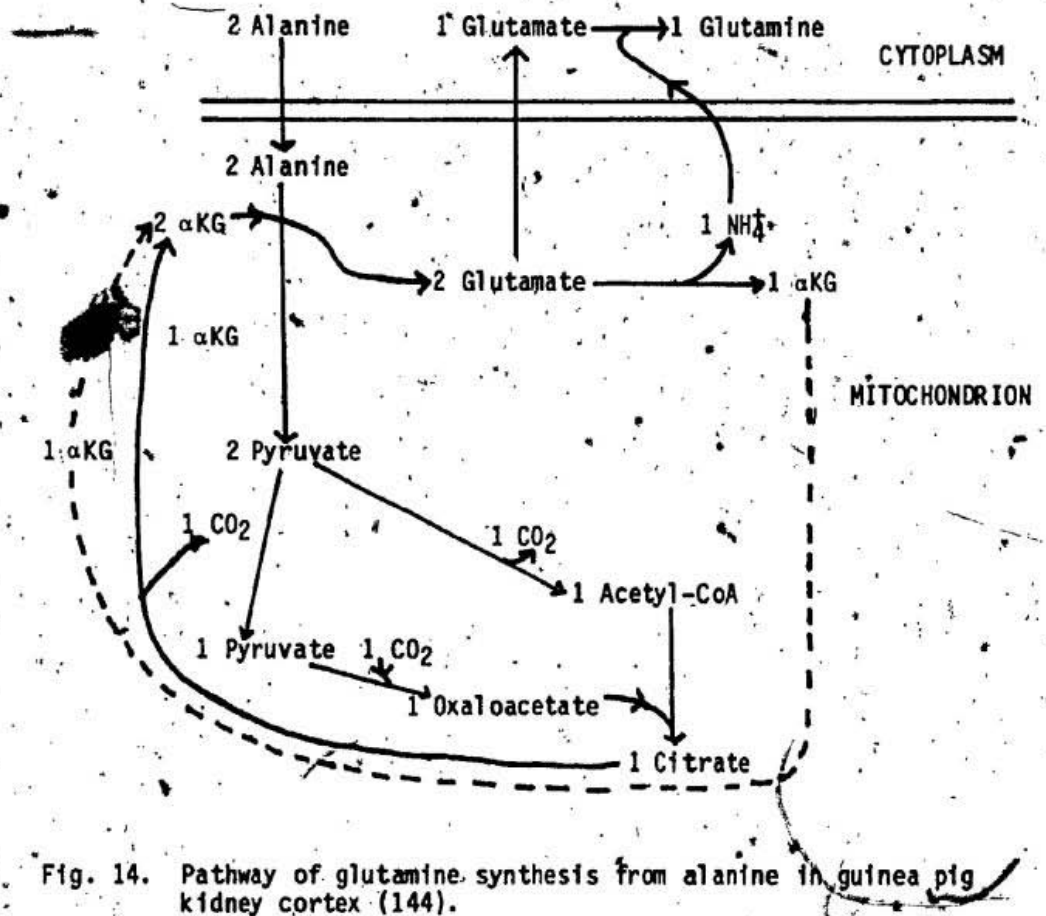


Fig. 14. Pathway of glutamine synthesis from alanine in guinea pig kidney cortex (144).

Three experiments I performed (results not shown) are in agreement with these findings. High rates of glutamine synthesis from 5mM alanine were observed. The inhibitory effect of acid on glutamine synthesis observed earlier, using glutamate as substrate was evident. This gives full confirmation to the proposed direct role of increased tissue [H^+] per se on the enzyme moiety (GS), as opposed to an inhibitory effect of acid on the transport of glutamate into the cytoplasm, across the cellular membrane.

4.3 The role of ammonia production and excretion in regulation of acid-base balance in carnivores and omnivores

Stabilization of the extra-cellular fluid (E.C.F.) within narrow limits of pH 7.35 and 7.45, by regulating the relative amount of fixed cation in E.C.F. that is in combination with HCO_3^-, is the chief role of the kidney in acid-base balance. The kidney must work constantly in order to maintain the normal concentration of bicarbonate-bound cation (25-28m Eq1L). However, the electrolyte content of the average diet, containing excess of inorganic anions over inorganic cations, is far different than that of the E.C.F. Moreover, the oxidation of sulphur- and phosphorus- containing foods leads to the production of the fixed anions $SO_4^=$ and $PO_4^=$ which displace HCO_3^- in the E.C.F. The kidney meets this challenge by conserving fixed cation (mainly Na^+) and HCO_3^-, through the complete reabsorption of all the filtered $NaHCO_3$, and by excretion of fixed anions in combination with NH_4^+ rather than Na^+. The kidney excretes an acid urine, so that the freely diffusible base, NH_3 is trapped in the lumen as a relatively

nondiffusible ammonium ion, NH_4^+. The current theories on the mechanism of elimination of 30-50 mEq of metabolic acid produced per day, as ammonium salts, and the capacity to increase renal ammonia excretion 5- to 10-fold when acid production is abnormally increased, have been developed with omnivores and carnivores, which excrete acid urine in mind. What happens in herbivores, which excrete alkaline urine?

4.4 Acid-base balance in guinea pig: a herbivore

The ability of herbivores to protect themselves effectively against acidosis was in doubt until 1898, when the important work of Winterberg appeared (145). That showed conclusively that rabbits fed on oats could protect themselves against ingested mineral acids by coupling these with ammonia, and excreting the ammonium salts in an acid urine. Accompanying this utilization of ammonia for purposes of neutralization, there was a reduction in the output of urea. This work, and the earlier report by Bernard (95), that fasted rabbits excreted an acid urine, which reverted to alkaline when they were given grass to eat, have established the principle that there is no difference in the ability of carnivores or herbivores to maintain tissue neutrality through the production and/or utilization of excess ammonia under conditions of acidosis, and also that, whether an animal excretes acid or base in its urine depends not on the species of animal, but on the diet consumed by the animal.

In such herbivorous animals, the fraction of total nitrogen excreted as ammonia is low (146), for in these forms dietary intake of available inorganic cations or organic anions equals or exceeds meta-

bolic production of acids. Many plant materials, such as fruits and leaves, on which they feed contain salts of organic acids, oxidation of which ultimately leads to HCO_3^-. Achievement of acid-base balance, therefore demands the excretion of alkali, not acid and the higher pH of herbivore urine is probably a means of minimizing loss of NH_4^+, and allowing as much HCO_3^- as can be tolerated in the urine to be excreted by this route.

4.5 Acidosis and inhibition of glutamine synthesis in guinea pig kidney: a possible explanation

The finding by me, that <u>in vitro</u>, the systhesis of glutamine from glutamate and NH_4Cl by guinea pig kidney tubules is inhibited when the pH of the incubation medium is lowered, is to the best of my knowledge novel. The inevitable question then is, "what might this discovery mean for the guinea pig?"

In the course of evolution, the guinea pig, excreting alkaline urine in its capacity as a herbivore, had had to develop alternate routes via the blood for ammonia, since the diffusion gradient for ammonia is less from cell to urine than from cell to blood (considering pH and flow differences). This necessitated the evolution of a means by which metabolic energy could be used to drive a process that is in effect the conservation of an acidic species (NH_4^+), and the simultanous conversion of the same toxic acidic species into a less toxic form, namely glutamine. The GS pathway was developed. But since metabolic acidosis, disguised as starvation, was (and it probably still is) the commonest perturbation of acid-base balance, in the evolution of man and animals, it is not surprising that the guinea pig

has developed a way of coping with it. During starvation, the guinea pig can be transformed, so to speak, into a carnivore, living on its own tissue proteins! The results of such endogenous protein metabolism would be the production and excretion of acid urine, as a way of eliminating excess H^+. Under these conditions, the complete reabsorption of all filtered $NaHCO_3$, and the excretion of ammonia in urine becomes necessary. With a very low activity of an ammonia producing enzyme (i.e. glutaminase), the only option left for the guinea pig kidney would be to turn off, or inactivate the otherwise efficient ammonia consuming pathway, in order to make ammonia available for excretion. This has made the glutamine synthesizing system of guinea pig kidney vulnerable to acid.

My data indicate that the highly sensitive nature of GS to acid is evident even under acutely acidotic conditions, as compared to a chronic situation like starvation. The response to acid is rapid (occuring within 30 min!) and reversible. Such a rapid response to acid is necessary if GS is to be considered truely regultory in ammoniagenesis during acute acidosis. After all acute metabolic acidosis is a much more common and serious problem, even in herbivores. The high rates of glutamine synthesis in guinea pig kidney indicate that GS probably operates maximally and efficiently at removing ammonia. It will be a shame if such a process cannot be made to occur again after acute administration of acid. No wonder the inhibition by acid is clearly reversible.

4.6 Alkalosis and glutamine synthesis in guinea pig kidney

Alkalosis, in contrast, has little effect on the GS reaction.

Glutamine synthesis during metabolic alkalosis was not significantly different from normal conditions, although slightly greater. Tentatively, one can make the proposition that HCO_3^- *per se* has no direct effect on GS. The earlier reports (65, 143) that the output of ammonia in the urines of guinea pig increases not only during acidosis, but also during alkalosis, is not in complete harmony with my findings.

4.7 Acid inhibition of GS in guinea pig kidney: extrapolation of results to other species

A summary of the current views on the distribution and activities of GS and glutaminase is as follows: Janicki and Goldstein (65) report significant GS activity in the kidneys of rabbit, guinea pig, and rat, but not those of cat, dog, and pig. Glutaminase activity, present in all animals studied, was greatest in those excreting the more acid urine (cat, dog, and pig), least in those excreting an alkaline urine (rabbit and guinea pig), and intermediate in the rat. GS is not present in the kidneys of man and dog (57, 76, 80, 81).

Glutamine is the major source of urinary ammonia (6, 7). Lyon and Pitts (147) readily incorporated label from KG in the renal glutamate pool of all species studied (dog, cat, rat, and guinea pig), indicative of either reversal of the GDH step or transamination with alanine or aspartate. Only in the rat and guinea pig was renal glutamine labeled. Accordingly, urinary ammonia of the dog and cat cannot be derived from the amide nitrogen of intrarenally synthesized glutamine. In other words, obligatory coupling of deamidation and glutamine synthesis to ammonia excretion cannot be the case in the pig, cat, or dog, which has

no synthetase activity whatsoever. Even in the rat, obligatory synthesis of glutamine from ammonia and glutamate cannot be a prerequisite for excretion of ammonia, since if it were synthetase activity would be greatest in acidosis, which it is not (45). In Lyon and Pitts (147) study, labeling was significantly greater in tissue from rats in metabolic alkalosis than in tissue from those in metabolic acidosis.

My fndings are in consonance with the view expressed by Damian and Pitts (45). They argue, that even though the glutamine synthetase reaction in thermodynamically reversible and the glutaminase I reaction is not, the two reactions can be seen as parts of one reaction, which can in a technical sense be reversed; with a net shift to the left or right being determined by the acid-base status of the animal. Such a view implies that acidosis would simultanously exert reciprocal effects on the glutaminase I reaction and the GS reaction, in relation to normal acid-base balance. On the other hand, alkalosis reduces the rate of the glutaminase I reaction, without having any significant effect on the GS reaction.

Both Kamin and Handler (148) and Orloff and Berliner (149) have expressed the view, that the minute by minute production of ammonia within tubular cells might be best regulated if a system which removes ammonia also existed. In the guinea pig and rat, the synthesis of glutamine can therefore be seen as a mechanism, which in essence is a negation of the effect of the ammonia producing enzyme. The capacity to consume ammonia then, affords GS the potential for regulating ammoniagenesis, in the rat, guinea pig, and probably in the rabbit and sheep also, but not in dog, cat or pig.

The views expressed in the preceeding paragraphs demand a discussion on the renal localizations of the two major reactions. GS is found exclusively in the proximal straight tubule of rat, and in the proximal convoluted and straight tubules of the rabbit kidney (86), and is a cytosolic enzyme. Glutaminase I is a mitochondrial enzyme (92), that is widely distributed along the nephron (150). However the bulk of ammoniagenesis probably occurs in the proximal tubule (150). Since the adaptive increase in renal glutaminase that occurs in metabolic acidosis is confined to the proximal convoluted tubule (92-94), this is probably the major site of ammoniagenesis and gluconeogenesis, and hence the locus of PDG. The segregation (intercellular and intracellular) of synthetic and degradative capacities is a useful arrangement which makes the operation of a classical substrate cycle unlikely, in the rat.

4.8 Proposed mechanism for acid inhibition of GS in guinea pig kidney

Since the mechanism for the GS reaction proposed by Meister (89, 90, 91) has not been seriously questioned by others, I wish to discuss my findings on the basis of the scheme on the following page.

It is clear, from Fig. 15, that increased tissue H^+ can contribute to inhibition of glutamine synthesis by:

(i) reducing the proportion of available nucleophile (NH_3), and/or

(ii) reducing the affinity of GS for glutamate and/or ATP.

This later effect can be achieved by inducing a conformational change in the enzyme molecule.

My experiments have shown (Fig. 11) that the free base concen-

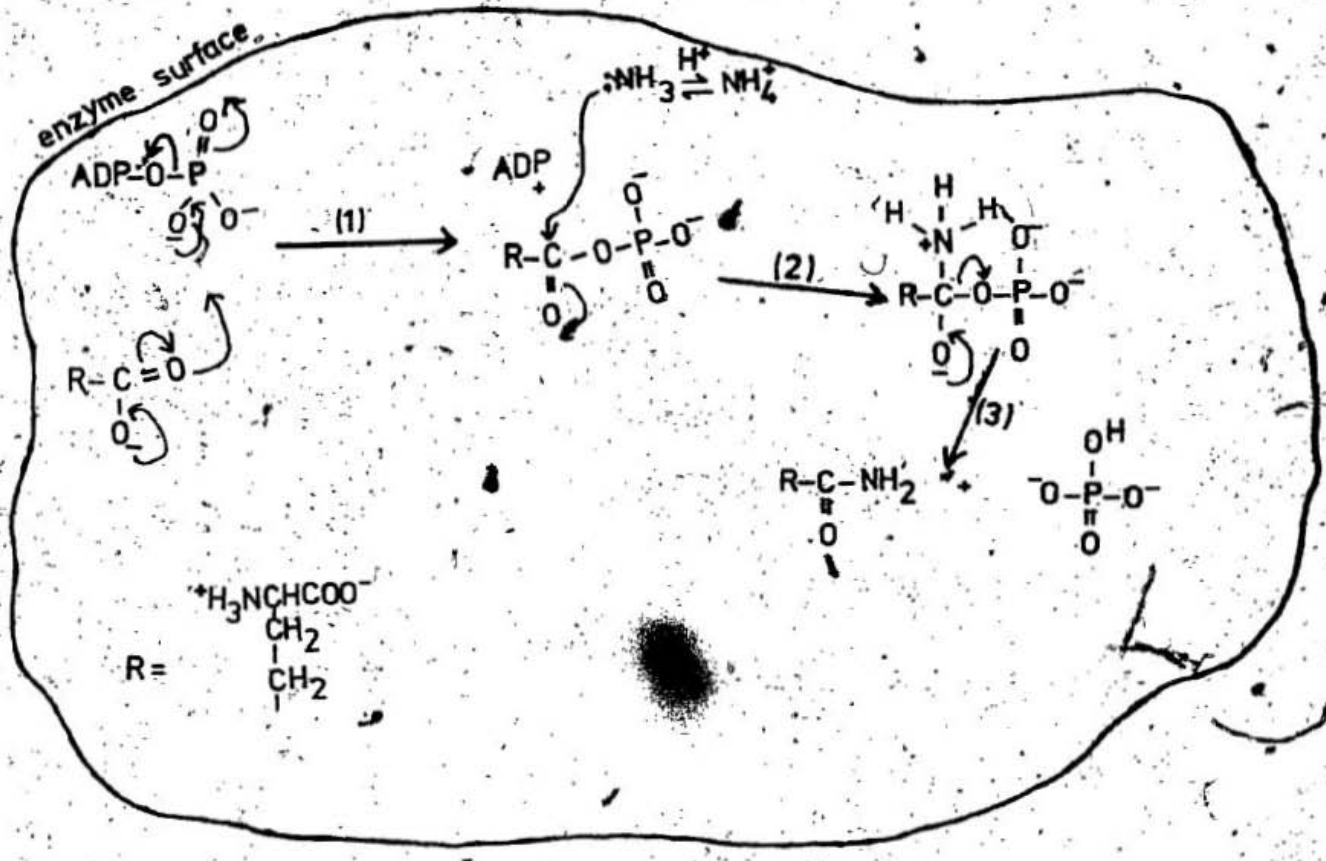


Fig. 15 Chemistry of the GS reaction

tration is not limiting under the conditions employed. Furthermore, the inhibitory effect of acid occurs irrespective of the concentration of glutamate (Fig. 12).

The hypothesis for the enzymatic mechanism I'm proposing is simple; increased tissue concentrations of H^+ affect GS directly, probably by a change in conformation of the enzyme molecule, and/or by alterations in the electrostatic nature of the microenvironment at the active site. The consequence of such changes would be a lowering of the affinity of GS for glutamate and/or ATP.

4.9 Models of acidosis for studies in guinea pig

Induction of acidosis by use of NH_4Cl or mineral acid is obviously artifical. A much more natural situation would be to make an animal acidotic by excess endogenous production of acid. This is especially necessary in guinea pigs which have been reported to appear lethargic and eat and drink little while on acid load (62). One such model is the acidosis of severe exercise induced by swimming (151). Renal cortical slices from rats wich had been forced to swim at 23°C; for 30 min. produced significant amounts of glucose and ammonia, when incubated with glutamine as substrate (151). These metabolic changes must have been caused by acidosis (lactic), because pretreatment of the rats with HCO_3^- before swimming prevented the changes. It goes without saying, that starvation should also be employed as a more physiological acidotic condition.

Two works, "Anesthesia in guinea pigs" (152), and "Acid-base status in unanesthetized, unrestrained guinea pigs", (153), should provide useful reference materials for future investigations involving

the guinea pig.

4.10 The choice of animal species for studies of metabolic regulation

The rat has been the choice of most biochemists in the majority of studies on the regulation of metabolism in mammals, and therefore the foundation for the development of much of biochemical knowledge has been built from studies with this animal. The gigantic amount of information available in the literature on the metabolism of the rat, in itself perpertuates the use of this animal in metabolic studies. However, evidence is accumulating that certain specific patterns of metabolic control are not the same among species. This can have far reaching effects if we try to apply the information gathered from research on one species to another, perhaps unrelated animal.

4.10.1 Rate of gluconeogenesis and PEPCK activity as factors involved in the regulation of renal ammoniagenesis

Virtually all of the research on renal or hepatic gluconeogenesis have been carried out using the rat, and a set of general concepts for the regulation of the process has emerged. But various aspects of PEPCK involvement in kidney metabolism, notably its unique intracellular distribution among species, its activity in relation to acid-base balance, and its activity in relation to hormones and other physiological effectors, has made a reassessement of the universality of such concepts necessary.

In the liver, PEPCK is almost totally mitochondrial in birds and rabbits (95%), whereas in the rat, mouse and hamster, it is cytosolic (90%) (132). The vast majority of species studied to date, however exhibit a ratio of cytosolic to mitochondrial enzymes of about 1:1. In

guinea pig kidney, PEPCK was found to be about 60% cytoplasmic (131), and in human liver, it is about 50-60% cytoplasmic (131), and in human liver, it is about 50-60% mitochondrial.

While the metabolic consequences of the pattern of PEPCK distribution is immediately obvious, vis a vis, substrate for PEPCK, carrier systems for Krebs cycle anions, nature of substrate (i.e. reduced or oxidized), etc, the interaction between gluconeogenesis and glutamine synthesis, made necessary by the existence of a cycle from which both processes can draw cycle intermediates, cannot be overlooked in the attempt to derive mechanisms of renal ammonia production. The inevitable differences in mitochondrial and cytosolic control mechanisms between different animals must be appreciated.

In diabetes, the NAD/NADH ratio of 725 in rat liver decreases to 300. The NAD/NADH ratio for guinea pig liver is 290, and the metabolic consequences of this difference between rats and guinea pigs have not been fully appreciated. However, while no one experimental animal model can be considered ideally suited for studies on metabolic regulation, we must be aware of the implications of our choice, and the extent to which we can extrapolate. The rat for example will not be a good model for study of human metabolic disorders, directly or indirectly concerned with glucose synthesis. On the other hand, an integrated model approach, which considers the primary differences between the rat and the guinea pig, should be more useful in extrapolation to human metabolism.

4.11 Future experiments

The hypothesis that increased tissue H^+ causes inhibition of

glutamine synthesis must be tested by kinectic experiments. Further experiments would probe the enzymatic basis for altered kinectics. For example, conformational changes could be detected by circular dichroism spectroscopy. These experiments would, of course, require purified GS from guinea pig kidneys.

Glutamate can be utilized via the GS or GDH pathway; but the operation of the GDH pathway was not of importance in my experiments, since the tubules were incubated with NH_4Cl. However, the proposed limiting role of the GDH pathway during glutamine synthesis in the absence of NH_4Cl, and the reported inhibition of GDH by increasing concentrations of HCO_3^- in the rat kidney (24), makes a study of the effect of varying HCO_3^-, independent of any pH change, also necessary, in the guinea pig kidney.

For this study, the tubules should be incubated at pH 7.4 and the HCO_3^- and P_{CO_2} varied accordingly. MSO should be employed to ensure that glutamine formed is not due to protein degradation. Aminooxyacetate can be added in some experiments to ensure full utilization of the glutamate carbon through the GS pathway. Metabolite determinations should be made on glutamate, glutamine, aspartate, alanine, ammonia, and glucose.

It would be interesting also to perform <u>in vivo</u> studies on the guinea pig kidney and liver. Metabolite levels in freeze-clamped livers and kidneys should be measured in normal and NH_4Cl acidotic animals. The effect of starvation on the levels of such metabolites, the activity of GS, and the urinary output of urea and ammonia under such conditions should also be examined.

In addition, tubules from acidotic or starved animals should be

incubated *in vitro* with glutamate, plus or minus NH_4Cl, and the rate of glutamine synthesis determined, and compared with results from normal animals.

The use of NH_4Cl for the induction of acidosis in animals depends on its conversion into urea and acid (HCl) in the liver. This ability of the liver to "generate" acid, means that hepatic nitrogen metabolism and renal ammonia excretion are interlinked (154). Liver perfusion studies, aimed at elucidating the exact role of the liver in pH homeostasis would also be interesting; even more so, in the light of the reinterpretation of conventional views on urea and ammonia excretion, being asked for (18), by Oliver *et al*.

4.12 Summary and conclusion

The rate of formation of glutamine in the guinea pig kidney is decreased during acute metabolic or respiratory acidosis, as has been shown in cortex tubules, *in vitro*. This effect is not due to lack of availablity of ATP, since the tissue ATP concentration in tubules incubated at pH 7.1 is not significantly different from that at pH 7.4. There is no change in the subcellular distribution of GS during acidosis. The inhibitory effect of H^+ on glutamine synthesis suggests that increased tissue H^+ could contribute to the inhibition by acidosis of glutamine synthesis. Thus GS is being implicated in the regulatory events connected with the acute production of ammonia in the kidney. Studies which could define the role of the liver in pH homeostasis has been suggested. It is possible that regulation of the various pathways of glutamine metabolism is more significant in terms of ammoniagenesis than is the regulation of GS

itself.

Much remains to be learned, but there is already much evidence that control of ammonia production in acidosis is multifactorial, involving a variety of mechanisms at multiple sites. That should not be surprising, since nature rarely entrusts the regulation of important processes to a single mechanism.

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