REGULATION OF N-ACETYLGLUTAMATE LEVELS THROUGH GLUTAMINASE ACTIVITY- A POTENTIAL MEDIATOR OF UREA SYNTHESIS

STEPHEN WILLIAM DAMIAN BALL

Your file Votre référence
ISBN: 0-612-93010-6
Our file Notre référence
ISBN: 0-612-93010-6

Regulation of N-Acetylglutamate Levels Through Glutaminase Activity- A Potential Mediator of Urea Synthesis

By

Stephen William Damian Ball, B.Sc.

A thesis submitted to the
School of Graduate Studies
in partial fulfilment of the
requirements for the degree of
Master of Science

Department of Biochemistry
Memorial University of Newfoundland

2003

St. John's, Newfoundland

Table of Contents

ABSTRACT

N-Acetylglutamate synthetase catalyses the reaction of glutamate and acetyl-CoA to produce N-acetylglutamate (NAG), which is an essential activator of carbamoyl phosphate synthase I (CPS I). CPS I is the flux-controlling enzyme of the urea cycle. Hepatic glutaminase is believed to be associated with the urea cycle but the nature of this association is not fully understood. Our hypothesis is that hepatic glutaminase provides glutamate for N-acetylglutamate synthesis. Therefore, factors which increase glutaminase activity should activate CPS I by increasing NAG levels.

We investigated the effects of the known activators of glutaminase, glucagon and NH_4Cl, in perfused rat livers. Mitochondria were subsequently prepared from these livers and NAG levels and citrulline synthesis assessed. Both glucagon (10^{-7} M) and NH_4Cl (1 mM) increased mitochondrial NAG levels and the rate of citrulline synthesis. This was in agreement with previous results.

We proceeded to investigate whether a link between glutaminase activity and NAG levels existed.We employed 6-diazo-5-oxo-norleucine, DON, a compound we found to be a suitable inhibitor of glutaminase activity. Glucagon significantly increased NAG and citrulline synthesis in mitochondria prepared from hepatocyte incubations. However, pre-incubation of hepatocytes with DON significantly decreased the stimulatory effect of glucagon on NAG levels and subsequent citrulline synthesis. Hepatocyte incubations with 1 mM ammonia also yielded a significant increase in mitochondrial NAG levels. Once again, pre-incubation of hepatocytes with DON significantly inhibited the effect mediated by ammonia on mitochondrial NAG content. These results suggest a link between glutaminase activation and mitochondrial levels of

NAG.

The increase in mitochondrial NAG due to ammonia stimulation, however, did not correlate with an increase in the rate of citrulline synthesis. Therefore, there may exist some means by which mitochondria down regulate the activation of citrulline synthesis despite elevated levels of NAG. The mechanism by which the kinetic relationship between NAG levels and citrulline synthesis is disrupted remains unknown. Our results have led us to suggest that glutaminase may indirectly affect urea production through its effects on NAG levels.

Acknowledgements

There are numerous people who gave their help and support over the course of my thesis and I would like to thank them for all they have done for me. First of all, I would like to thank my supervisor, Dr. John.T Brosnan, for being supportive and open with me about my project and allowing me to experience a side of biochemistry that I may not have without him. I learned a lot not only about school but life as well. To Dr. Margaret E. Brosnan and Dr. Gene Herzberg, thank you for the open door policy and helping me through many of my problems which I could not have done without your guidance. The option to seek your help when in need was greatly appreciated. And teaching 4002 was a great experience...thank-you Dr. Brosnan.

To Bea Hall, there is nothing I can write that would capture my gratitude for what you have done for me. You're the best and will always be the best. I am glad I met you and hope all good things happen to you in the future.

To Shob and Donna, thanks for the friendship and the good times. You were always there when I needed you and it was my pleasure to spend the last two years in the lab with you. Thanks for keeping me grounded and smiling. Also, thanks to Barry for the laughs, you are definitely one of a kind.

To the Grad students and students that I crossed paths with during this time (in no particular order: Rene, Lori, Tim, Adam, Ally, Enoka, Mike, Cathy, Dave, Jim, Erin, Sarah, Jana...and everyone else), hope that all goes well in the future and I appreciated the good times we had which made my time in MUN a lot easier. All I can say is mixers...everything else should be understood. I view you all as good friends and wish you the best to you all.

Last but certainly not least, my family and friends, who never let me down when I needed them and always seemed to believe in me when sometimes I didn't. To my Mom and Dad, they made sure that nothing got to big for me and let me feel secure in my abilities. They believe in me so much its contagious and I am grateful for that. To Pat, Rob, Steph, my brothers Mark and Dave, and my sister Melissa, where do I begin.... I have never met a group of people like you before who always knew how to get my mind off all things that bothered me. You guys are golden and I am glad that we are still close friends and family although living so far apart. Thanks for just being there.

List of Figures

LIST OF ABBREVIATIONS

ASL	Argininosuccinate Lyase
ASS	Argininosuccinate Synthetase
cAMP	Cyclic Adenosine Monophosphate
CPS I	Carbamoyl-Phosphate Synthetase I
DON	6-diazo-nor-leucine
EDTA	Ethhylenediamine tetraacetic acid
EGTA	Ethyleneglycol- bis- (Beta-aminoethyl ether)- N, N, N ,N-tetraacetic acid
HEPES	N-2-ethanesulphonic acid
NAG	N-acetylglutamate
NAGS	N-acetylglutamate Synthetase
NO	Nitric Oxide
OTC	Ornthine transcarbamoylase

Chapter One

Introduction

Increased catabolism of proteins (endogenous or dietary) results in an increased production of amino acids that can be catabolized through a number of metabolic processes. These pathways lead to the production of ammonia, which at modest circulating concentrations is a potent neurotoxin (Meijer ***et al.*** 1990). In mammals, the major metabolic pathway for removal of ammonia is the urea cycle. This cycle, which is located in the periportal region of the liver acinus, assimilates two nitrogen atoms and one bicarbonate molecule into a non-toxic product, urea. Acute control of this pathway allows for ammonia levels to be tightly regulated to avoid toxicity and for safe excretion of nitrogen from the body.

Urea Cycle

The urea cycle is located in the periportal region of the hepatic acinus where it acts as a low affinity but high capacity system for the removal of ammonia (Haussinger ***et al.*** 1992) The cycle is made up of five major enzymes; carbamoyl-phosphate synthase I (CPS I), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (Watford, 1991). The enzymes of this cycle are not localized in the same compartment within the liver cell, as CPS I and OTC are co-localized in the mitochondria while the remaining enzymes are situated in the cytosol (Figure 1.1).

The first step in ureagenesis involves the CPS I reaction in which ammonia and bicarbonate are combined to form carbamoyl-phosphate. This is an energetically demanding step where two ATP molecules are required to facilitate the formation of the product. Carbamoyl-phosphate is released into the mitochondrial matrix where OTC can utilize it along with

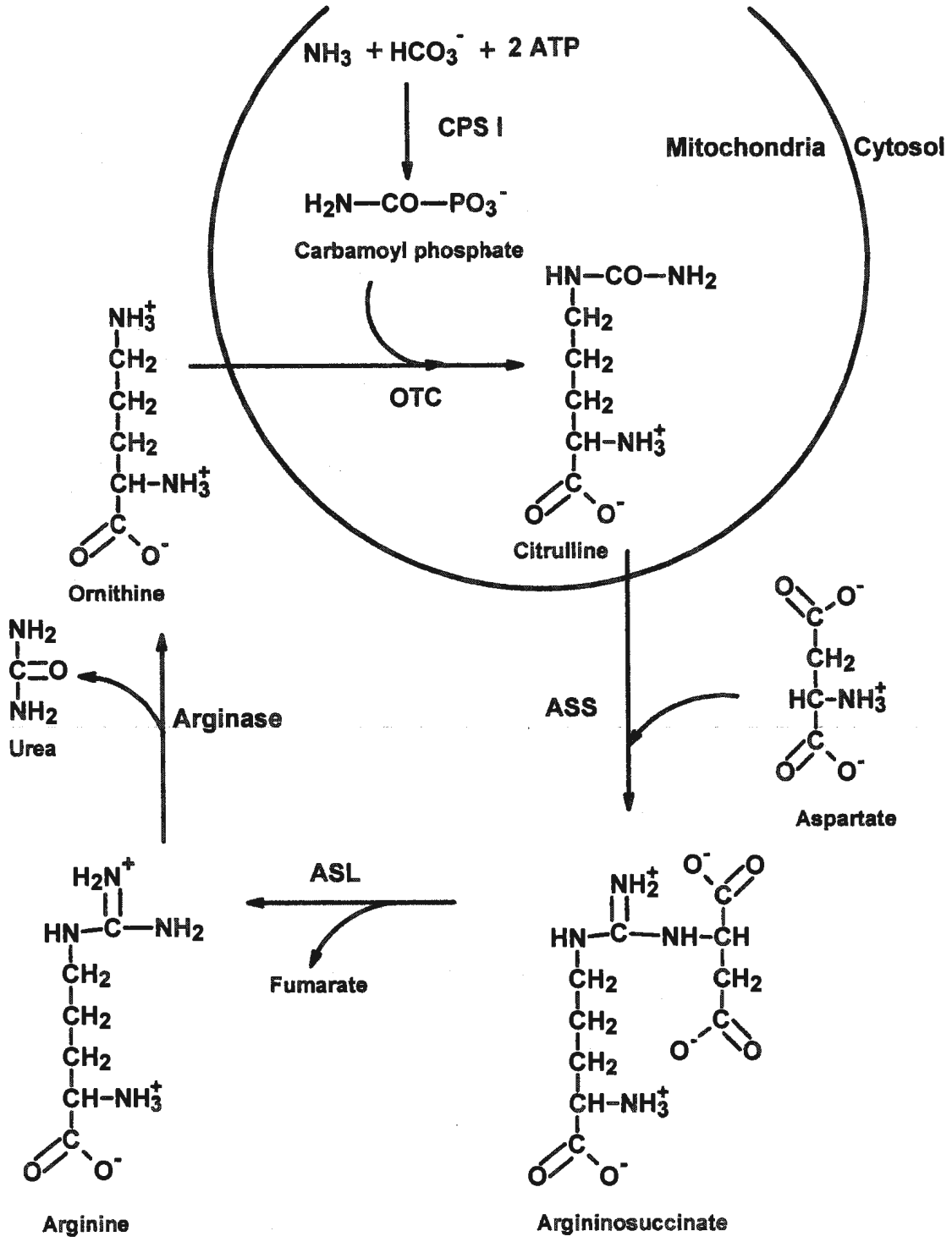


Figure 1.1: **Compartmentalization of Urea Cycle Enzymes.** Carbamoyl-phosphate synthase I (CPS I), Ornithine transcarbamoylase (OTC), Argininosuccinate Synthetase (ASS), Argininosuccinate Lyase (ASL) and Arginase.

ornithine, to produce citrulline. These enzymes, through the use of immuno-electronmicroscopy, have been found to be loosely associated with the inner membrane of the mitochondria (41 % of CPS I and 59 % of OTC are located within 15 nm of the cristae) (Powers-Lee and Corina, 1987; Yokota and Mori, 1986). It is believed that these enzymes are in close proximity with one another and that this localization allows for more efficient exchange of intermediates (Powers-Lee and Corina, 1987; Watford, 1991). OTC has also been reported to be closely associated with the mitochondrial ornithine-citrulline antiporter (Cohen ***et al.***, 1987). This antiporter moves ornithine from the cytosol into the mitochondria in exchange for a citrulline molecule. This leads to constant removal of OTC's product while providing the necessary substrate for further flux through this enzyme.

The first cytosolic reaction occurs when citrulline reacts with aspartate in the ASS-catalyzed reaction to form argininosuccinate. This reaction involves the incorporation of the second nitrogen through aspartate. Argininosuccinate can then be cleaved into arginine and fumarate by ASL. Fumarate is recycled to form aspartate in the cytosol while arginine is cleaved by arginase into ornithine and urea. Ornithine can be transported back into the mitochondria to become the substrate for OTC, thereby beginning another cycle. Urea, the other product of arginase, can now be released into the blood stream where 60-70% is filtered by the kidneys and released from the body in the urine. The remainder is degraded by microbial urease in the intestine (Metges, 2000). Jackson et al. (1993; 1995) stated that the colon can salvage urea nitrogen and that this nitrogen can be incorporated by intestinal microbes into amino acids that are then absorbed by the host. Although this certainly occurs, its quantitative importance in terms of amino acid nutrition has been debated.

Occurrence of Urea Cycle Enzymes Outside the Liver

In mammals, the liver is seen as the central site of ureagenesis as it contains all the enzymes involved in that cycle. These individual cycle enzymes, however, are not exclusively localized to the liver and have been found to exist in other tissues. For instance, the intestinal mucosa contains CPS I and OTC while ASS and ASL are found in the kidneys. The purpose of these partial pathways is the synthesis of arginine (in the kidney) from glutamine (converted to citrulline in intestinal mucosa) that can be used for such functions as protein synthesis and NO production (Dhanakoti ***et al.*** 1990). Furthermore, Wu (1995) identified that the enterocytes of post-weaning pigs had the capacity to synthesize urea from extracellular or intramitochondrially generated ammonia (Wu, 1995). This is the only other known existence of a complete urea cycle existing in mammalian cells other than liver cells.

Regulation of the Urea Cycle

Regulation of ureagenesis is essential in the maintenance of ammonia homeostasis within the body. Long-term regulation involves changes in the amount of enzyme protein while short-term regulation occurs when the activity of existing enzyme protein is altered.

Long-term changes in the enzymatic concentrations of the urea cycle occur over a relatively extended period of time due to the quite long half-lives of these enzymes (on the order of days) (Watford, 1991; Meijer ***et al.*** 1990). This regulation is an adaptive mechanism that responds to alterations in amino acid nitrogen flux due to changes in dietary protein intake or catabolism of endogenous protein (Morris, 2002). Hormones (glucagon, insulin, and glucocorticoids) play a major role in mediating changes in activity under these conditions,

largely through altering the rate of transcription of the urea cycle enzymes (Morris, 2002).

In addition, there is an essential role to be played by short-term regulation of the urea cycle. It has been shown that substrate availability is probably the most important influence on short-term changes in rates of ureagenesis (Beliveau ***et al.***, 1993). CPS-I, which catalyses the initial, committed step in ureagenesis, has an absolute requirement for N-acetylglutamate as an allosteric activator. This activator is synthesized within the mitochondrion by N-acetylglutamate synthetase, and its levels have been reported to correlate with the rate of ureagenesis (Beliveau ***et al.***, 1993; Stewart and Walser, 1980; Lund and Wiggins, 1987, Cohen ***et al.***, 1982). It is the short-term regulation of CPS I, NAG production and subsequent flux through the urea cycle which will be the focus of my thesis.

Carbamoyl-Phosphate Synthase I

As mentioned above, there exists one enzyme within the urea cycle, carbamoyl-phosphate synthase I, which requires an allosteric activator, N-acetylglutamate (NAG), to modulate its activity. This enzyme has also been identified as having a flux coefficient of ≈ 1 for this pathway, which means that all of the flux control of the urea cycle is vested in this enzyme (Meijer ***et al.*** 1990). This enzyme belongs to a class of CPSases which differ in their substrates and activators. Other CPSases (CPS II and CPS III) use glutamine as the ammonia donor for carbamoyl-phosphate synthesis (McCudden and Powers-Lee, 1996). The inherent glutaminase-like activities found in these enzymes have been lost in CPS I. However, a residual glutaminase domain remains in the protein structure but a mutation has occurred which has replaced an essential cysteine with a serine rendering the site inactive (Rubio ***et al.***, 1983). It is the inability

to use glutamine as the nitrogen donor which distinguishes CPS I from the other CPSases.

Purified CPS I has been shown to exist in a state of rapid, reversible monomer-dimer equilibrium (Lusty, 1978; Lusty 1981). The allosteric activator NAG displaces this equilibrium toward the catalytically active monomer form (160 kDa) (Lusty, 1978; Lusty 1981). Also, CPS I is the most abundant protein (15-26 % of total matrix protein; concentration of 1-1.5 mM) in liver mitochondria and is thought to be loosely associated with the inner membrane together with another urea cycle enzyme, OTC (Lusty, 1981; Powers-Lee and Corina, 1987). This association with the inner membrane has been proposed as an effective means of channelling of intermediates between these two enzymes (Powers-Lee and Corina, 1987; Cohen ***et al.*** 1987; Watford 1991).

The reaction catalyzed by CPS I occurs as a step-wise process. There exist two distinct ATP binding sites on this enzyme referred to as domains B and C (Simmer ***et al.***, 1990; Hong ***et al.***, 1994; Lusty, 1983; Nyunoya ***et al.***, 1985). The binding of ATP to these sites occurs in discrete steps in the reaction pathway. ATP_B binds to domain B and activates the bicarbonate to accept the ammonia molecule (McCudden and Powers-Lee, 1996). Then, ATP_C reacts with domain C and donates a phosphate group to the carbamide to form carbamoyl-phosphate (McCudden and Powers-Lee, 1996). The substrates which donate the carbon and nitrogen are specific as well. NH_3 rather than NH_4^+ is the true substrate for this reaction, with a Km for NH_3 of 38 μM.(Cohen ***et al.***, 1985). Also, HCO_3^- not CO_2 is the carbon donor, with bicarbonate (Km = 4-5 mM) being preferentially supplied by the enzymatic reaction of carbonic anhydrase. This enzyme scavenges CO_2 released in the mitochondria via the citric acid cycle and converts it to bicarbonate (Dodgson ***et al.***, 1983).

Regulation of Carbamoyl-phosphate Synthase

The regulation of CPS I is mediated by its absolute requirement for N-acetylglutamate (NAG). This activator is produced within liver mitochondria by the enzyme N-acetylglutamate synthetase (NAGS) which catalyses the following reaction (Shigesada ***et al.***, 1978):

$$\text{Acetyl CoA} + \text{Glutamate} \Longrightarrow \text{N-acetylglutamate} + \text{CoASH}$$

Mitochondrial production of NAG is the only source of this regulator for CPS I, as cytosolic NAG is impermeable to the outer mitochondrial membrane (Meijer and Van Woerkom, 1978). The catabolism of NAG depends on transport out of the mitochondria and deacylation in the cytosol (Meijer and Van Woerkom, 1978).

NAGS exists as a trimer with a molecular weight of 160 kDa. The Km for glutamate is 3 mM and the Km for acetyl-CoA is 0.7 mM (Souba and Tatibana, 1983). Arginine has been reported to be a potent activator of NAGS (Ka = 5-10 mM) (Shigesada ***et al.***, 1978). Long-term exposure to saturating arginine concentrations increased the activity of NAGS by increasing the Vmax without altering the Km for substrates (Morimoto ***et al.***, 1990; Shigesada ***et al.***, 1978; Kawamoto ***et al.***, 1985). Short-term changes in arginine concentration did not exhibit similar results. Stewart and Walser (1980) injected amino acids mixtures (20 amino acids) into rats to observe short-term changes in ureagenesis. They reported that the increase in NAG was not attributable to increased activation of NAGS by arginine. As arginine levels increased within the liver, the activity of NAGS remained unchanged (Stewart and Walser, 1980). These results suggested that moderate loads of amino acids activated ureagenesis via a rapid increase in NAG

levels, which were secondary to the increase in hepatic glutamate content, and independent of arginine stimulation of NAGS (Stewart and Walser, 1980). Since acute changes in mitochondrial NAG in response to an amino acid load are not a result of the activation of NAGS, there must be some other mechanism to account for this phenomenon. One suggestion is that the sudden increase in the liver concentration of glutamate, as seen by Stewart and Walser (1980), may play an important role in NAG synthesis.

Mitochondrial NAG levels have been shown to be positively correlated with the rate of mitochondrial citrulline synthesis by a number of researchers (Beliveau ***et al.***, 1993; Stewart and Walser, 1980; Lund and Wiggins, 1987, Cohen ***et al.***, 1982). This correlation has been shown to occur in response to acute changes in ammonia levels such as after a single protein meal or shortly after an ip injection of an amino acid mixture (Beliveau ***et al.***, 1993; Stewart and Walser, 1980). In addition, the half-life of NAG is approximately 20 minutes as it is transported out of the mitochondria for deacylation (Meijer and Van Woerkom, 1978; Morita ***et al.***, 1982). Thus, changes in NAG content can be achieved quickly compared with the time required to increase the protein concentration of urea cycle enzymes. It is believed that short-term control of ureagenesis can be mediated through regulation of NAG levels in the mitochondria.

One molecule of NAG is bound per molecule of CPS I; this results in complete activation of the enzyme. This activator binds to CPS I via the C-terminal end of the protein which changes the conformation of the enzyme (Rodriguez-Aparicio ***et al.***, 1989). Previously, it had been suggested that NAG bound in the N-terminal end in proximity to the residual non-functional glutaminase subunit but this theory was discarded after radio-labeled experiments (cleaved portions of CPS I were incubated with U - ^{14}C- NAG) proved that the C-terminal end was key in

the binding of this activator (McCudden and Powers-Lee, 1996). The importance, if any, of this residual non-functional glutaminase domain is still unknown. The binding of NAG to CPS I facilitates the binding of ATP_B to domain B by inducing a conformational change in CPS I which exposes essential S-H groups at Domain B; it does not influence the binding of ATP_C (Potter and Powers-Lee, 1993; Rubio ***et al.***, 1983).

The kinetic relationship between this enzyme and activator has been described as positive V- type allosteric kinetics (Lusty, 1981). This type of regulation, as described by Monod and Wymann (1965), involves an allosteric ligand which exerts a direct effect on the catalytic activity rather than on the affinity of the enzyme for its substrates and implies that cooperative substrate interaction should not be observed (Monod and Wymann, 1965).

Throughout the literature, there have been disagreements on the exact relationship between NAG and flux through CPS I. Stewart and Walser (1980) reported a hyperbolic relationship between rat liver mitochondrial NAG levels and the rate of mitochondrial citrulline synthesis. Their experiment measured acute changes in NAG levels and citrulline synthesis in rats exposed to an ip load of different mixtures of 20 amino acids. They reported a Ka of 0.8 mM for NAG which corresponds to half the concentration of CPS I in the mitochondria (Stewart and Walser, 1980). This hyperbolic relationship was also reported by Lund and Wiggins (1987). They analysed CPS I activity in freeze -thawed mitochondria as a function of NAG concentration. The Ka reported varied depending on the isolation and assay techniques employed (Lund and Wiggins, 1987). In contrast, Morimoto ***et al.*** (1990) reported a linear relationship between NAG and CPS I up to 1.5 mM mitochondrial concentration of NAG. Their study investigated three test groups of rats which were fed different protein diets for 24 hours *ad*

libitum (Morimoto ***et al.***,1990). These results were also supported by Rabier ***et al.*** (1982) who found a similar linear relationship when rats were exposed to glucagon for extended periods. Although discrepancies exist, the hyperbolic relationship was reported to occur in experiments where short-term changes in either ureagenesis or NAG levels were measured.

As previously mentioned, short-term changes in ureagenesis correspond to increased NAG levels but not an increase in NAG synthase activity (Stewart and Walser, 1980). Therefore, it is plausible that the limiting factor is substrate availability. As mentioned previously, Stewart and Walser (1980) showed a direct correlation between the glutamate concentration and the amount of NAG within liver mitochondria. We intend to investigate whether glutamate produced through the hydrolysis of glutamine by liver-type glutaminase, another mitochondrial enzyme, has any influence on the production of NAG within the mitochondria.

Glutamine Metabolism

Glutamine is a nonessential amino acid which plays an important role in the interorgan transport of nitrogen and carbon in mammals. It is the most abundant amino acid in the blood, and accounts for about 1/3 the circulating amino acid nitrogen (Brosnan ***et al.***, 1983; McGivan and Lynch, 1988). Glutamine serves as a key substrate for renal ammoniagenesis, an important precursor for pyrimidine, purine, NAD+, and glucosamine synthesis, and a substrate for gluconeogenesis and ureagenesis in the liver (Souba, 1991). Synthesis of this amino acid scavenges free ammonia which serves to protect the brain against toxic side effects.

The intracellular metabolism of glutamine is regulated by two key enzymes. The synthesis of glutamine is carried out by the enzyme glutamine synthetase which utilizes a

glutamate and an ammonia molecule to form glutamine. Skeletal muscle and lung which synthesize and release net amounts of glutamine have substantial amount of this enzyme (Souba, 1991). On the other hand, the catabolism of glutamine is catalyzed by the enzyme glutaminase which hydrolyses glutamine to glutamate and ammonia. Replicating cells such as enterocytes, lymphocytes, reticulocytes, oocytes, and cancer cells contain greater activities of glutaminase as they tend to be large consumers of this amino acid (Souba, 1991). The liver has the capacity to consume or release net amounts of glutamine.

Heterogeneous Distribution of Glutamine Metabolism in Liver

In the liver acinus (functional unit of liver), the periportal and perivenous regions differ in the expression of enzymes and, consequently, in metabolic functions. Haussinger (1998) (Figure 1.2) has described three separate zones of ammonia and glutamine metabolism. The first zone (Zone 1) is found in the periportal region (sinusoidal inflow) and it contains the enzymes that are involved in ureagenesis and glutamine catabolism. Within this zone, circulating ammonia and ammonia released from the catabolism of glutamine can be detoxified through the urea cycle. The second zone described by Haussinger (1998) was Zone 2, which lies in the middle of the liver acinus, between the periportal (sinusoidal inflow) and perivenous (sinusoidal outflow). This zone also contains the enzymes of the urea cycle and can further remove toxic ammonia from circulation. The last zone described was Zone 3, which is located in the perivenous end of the liver acinus which contains the enzyme glutamine synthetase (Haussinger, 1998). The location of this enzyme allows for circulating levels of ammonia to be scavenged by the perivenous cells and assimilate it along with glutamate into a less toxic carrier, glutamine.

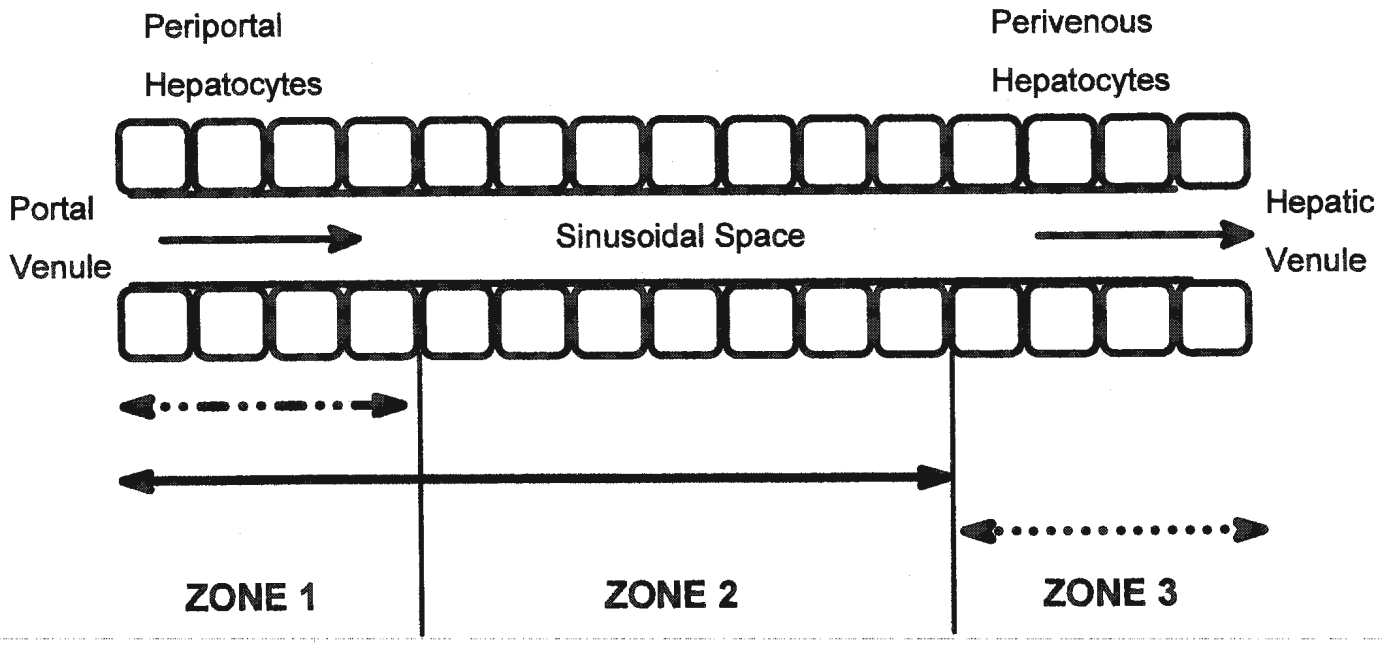


Figure 1.2: **Organization of Hepatic Glutamine and Ammonia Metabolism.** The metabolic zones and enzyme localization within the liver, as depicted above, were described by Haussinger (1998). The legend illustrates those enzymes that play an essential role in hepatic glutamine and ammonia metabolism.

Figure 1.2 depicts this zonation of ammonia and glutamine metabolism.

These enzyme distributions serve a physiological function in ammonia metabolism. The localization of glutaminase and the urea cycle together at the inflow allows for effective metabolism of amino acid nitrogen. As mentioned previously, glutamine is the most abundant amino acid in the blood, and accounts for about 1/3 the circulating amino acid nitrogen (Brosnan ***et al.***, 1983; McGivan and Lynch, 1988). The breakdown of glutamine mediated by glutaminase will provide ammonia for immediate detoxification via the urea cycle. The presence of glutamine synthetase in the perivenous region allows for excess toxic ammonia to be scavenged from the blood and a non-toxic carrier glutamine is produced. This glutamine, in turn, can be carried to other parts of the body or circulated back to the periportal region. The hepatic catabolism of glutamine and its link to ureagenesis is the central focus of my thesis.

Glutamine Transport

The transport of glutamine into the liver occurs through a liver specific sodium-dependent transporter referred to as System *N* (Kilberg ***et al.*** 1980). This transporter has specificity for two other amino acids, asparagine and histidine (Tamarappoo ***et al.*** 1994). System *N* is composed of a100-kDa plasma membrane protein that mediates the transport of these amino acids (Tamarappoo ***et al.*** 1994). Glutamine can also be transported via System *A*, but this only constitutes 5-10 % of total hepatic glutamine transport (Low ***et al.***, 1990). While the influx of glutamine into the liver is performed by System *N*, the efflux is regulated by another transporter which is Na+-independent and termed "System *n*" (Pacitti ***et al.*** 1993). It is believed that System *n* is involved in the facilitated diffusion of glutamine from hepatocytes in the perivenous region

of the liver.

The transport of glutamine via System *N* can be induced by prolonged exposure to insulin and glucagon (Gebhardt and Kleeman, 1987) but short-term hepatocyte incubations with these hormones do not induce changes in System *N* activity (Kilberg ***et al.***, 1980). Diabetes (streptozotocin-induced) caused an increase in System *N* activity in isolated hepatocytes (Barber ***et al.***, 1982) but not in isolated sinusoidal membrane vesicles (Low ***et al.*** 1992). The opposite effect was seen with corticosteroid treatment of rats which induced glutamine transport in sinusoidal membrane vesicles (Low ***et al.***, 1992). Regulation is also mediated by the extracellular pH, as a decreased pH resulted in lowered glutamine uptake (Lenzen ***et al.***, 1987).

Work on glutamine transport revealed that steady state glutamine concentration gradients are built up within the liver (Haussinger ***et al.***, 1985; Lenzen ***et al.***, 1987.; Remesy ***et al.***, 1988). The physiological concentration of glutamine in the portal vein is 0.6 mM while within the cytosol of the liver it is 4-6 mM and 20 mM in the mitochondrial matrix (Haussinger ***et al.***, 1985a). Haussinger ***et al.*** (1985b) found that histidine at near physiological concentrations inhibits influx (periportal) and efflux (periportal) of glutamine in the liver. This group continued to study the influence of transport inhibition by histidine on the metabolism of glutamine in isolated hepatocytes. It was determined that System *N* had a flux control coefficient of 0.31 for glutamine catabolism. This suggests that glutamine transport is involved in the regulation of hepatic glutamine metabolism.

Glutaminase

History

Glutaminase was first identified by Hans Krebs in 1935, when his analyses of

mammalian extracts from a number of tissues demonstrated the capacity to hydrolyse glutamine into glutamate and ammonia (Krebs, 1935). Further work allowed Krebs to describe two glutaminases which are distinguishable by their optimal pH and inhibition by glutamate. The two isozymes were originally referred to as brain-type (now referred to as kidney-type) and liver-type glutaminase. It is now evident that differences exist not only in kinetic properties but also protein structure as the enzymes are the products of two different but related genes (Smith and Watford, 1988).

Studies on the localization of glutaminase within various tissues have shown that it is a mitochondrial enzyme. Guha's work on liver mitochondria identified glutaminase activity within this organelle (Guha, 1962). Other studies have found similar results for glutaminase in the kidney, brain, and intestine (Kvamme ***et al.***, 1991; Curthroys and Weiss, 1974). The location within the mitochondria was further studied by Kalra and Brosnan (1973). Their use of detergents and sonication methods to disrupt liver mitochondria showed that glutaminase was released to the same extent as mitochondrial matrix markers. They suggested that glutaminase must be located in the mitochondrial matrix (Kalra and Brosnan, 1973). McGivan ***et al.*** (1980) furthered the study on localization by the use of freeze thawing to disrupt liver mitochondria. Their work suggested that liver glutaminase was associated with the mitochondrial membrane fraction. Curthoys and Weiss (1974) furthered the investigation on the submitochondrial localization of glutaminase through their use of digitonin and fractionation methods. Their studies led to the discovery that glutaminase was loosely associated with the inner surface of the inner membrane of mitochondria.

General Properties

Glutaminase (L-glutamine amidohydrolase) (EC 3.5.1.2.) is referred to by a number names such as glutaminase I, phosphate activated glutaminase, and /or phosphate-dependent glutaminase. This enzyme catalyses:

$$\text{L-glutamine} + H_2O \Longrightarrow \text{L-glutamate}^- + NH_4^+$$

There are two isoenzymes of glutaminase which perform this irreversible reaction. This distinction was first made by Krebs in 1935 (Krebs, 1935).

Kidney-Type

The kidney-type isozyme of glutaminase is widely distributed among glutamine utilizing tissues such as the kidney, brain, intestine, heart, skeletal muscle and lung. In addition, this enzyme is found in the liver during fetal development but is not present in the adult liver (Curthoys and Weiss, 1974). Reports of the Km for glutamine vary slightly depending on the tissue location of the enzyme. The kidney enzyme has a Km of 4 to 5 mM, within the brain the Km is 2 to 8 mM and in the intestine the Km is about 2.2 mM (Klingman and Handler, 1958;, Svenneby ***et al.***, 1973; Pinkus and Windmueller, 1977). This isozyme of glutaminase is activated by inorganic phosphate (Ka = 20-30 mM) (Errera and Greenstein, 1949; Curthoys and Watford, 1995) and inhibited by its end-product, glutamate. Kinetic studies performed on glutamate's inhibition showed that it exhibits non-competitive inhibition with respect to glutamine and exhibits competitive inhibition in relation to inorganic phosphate (Sayre and Roberts, 1958). These results show that glutamate inhibits the function of this enzyme by occupying the binding

site of phosphate rather than the binding site of glutamine.

The activity of kidney-type glutaminase can be influenced by a number of other metabolites and compounds. Studies involving a number of sulfhydryl blocking agents such as mercuric chloride, mersalyl, and 3,3'- dithiobis[6-nitro-]benzoic acid have shown their capacity to inhibit this enzyme (Kvamme ***et al.***, 1970). Also, the leucine analog 6-diazo-5-oxo-L-norleucine has been shown to be an effective inhibitor of this enzyme (Conti and Minelli, 1994; Shapiro ***et al.***, 1979). Activation of this isozyme has been noted to occur in the presence of a number of metabolites. ATP, ADP, ITP, CTP, and some carboxylic acids such as malate, succinate, and α-ketoglutarate have exhibited the ability to activate this enzyme (Kovacevic and McGivan, 1983). It should be noted that activation by these metabolites occurs at concentrations which are not of physiological relevance. The activity of renal glutaminase has also been shown to increase in response to metabolic acidosis (Curthoys ***et al.***, 1984) , streptozotocin-induced diabetes (Lemieux ***et al.***, 1984), and long term feeding of high protein diet (Brosnan ***et al.***, 1978).

Liver- Type

The liver-type glutaminase, unlike the kidney-type, is only found in the postnatal liver and accounts for nearly all of the glutamine catabolized within the liver (Low ***et al.***, 1993). As mentioned previously, it is loosely associated with the inner membrane of the mitochondria. The kinetic properties of liver type glutaminase are different from kidney-type. For instance, liver-type glutaminase has a relatively high Km (17 mM) for glutamine, a lower Ka for phosphate and a pH optimum from 7.8 to 8.2 (Smith and Watford, 1988). Also, liver-type glutaminase is not inhibited by its end-product glutamate (Krebs, 1935). However, -SH blocking agents which

inhibit kidney-type glutaminase also inhibit the activity of the liver-type glutaminase (Joseph ***et al.***, 1981). Its activity has also been reported to be inhibited by a number of amino acid analogs such as N-acetylglutamine and 6-diazo-5-oxo-L-norleucine (Conti and Minelli, 1994). Purification has shown that this enzyme has an apparent subunit molecular mass of 58 kDa (Smith and Watford, 1988).

Short Term Regulation of Liver-Type Glutaminase

Liver-type glutaminase has another regulatory property which distinguishes it from other glutaminases as it is activated by its end-product, ammonia. This phenomenon was first identified by Charles (1958) in intact mitochondria, in isolated hepatocytes by Joseph and McGivan (1978) and in perfusion studies of rat livers (Haussinger ***et al.***, 1983). These studies showed that ammonia influenced the activity of the enzyme but never identified whether the ammonia was working directly on the enzyme or by some other indirect mechanism. Work performed on disrupted mitochondria where the enzyme is exposed directly to the substrate demonstrated that ammonia evokes its effect directly on the enzyme (McGivan and Bradford, 1983). In perfused rat liver, the activation of glutamine hydrolysis is half-maximal at 0.2 - 0.3 mM NH_4^+ (Haussinger and Sies, 1979). This activation of glutaminase by NH_4Cl is of physiological relevance as the portal vein ammonia concentration has been measured to be approximately 0.6 mM (Haussinger and Sies, 1979).

It has been proposed by Welbourne (1987) that the ammonia activation of glutaminase is an interorgan "feed-forward" system. Ammonia produced from intestinal catabolism of glutamine would ensure that the liver would be able to deal with increased glutamine absorption

from the diet by activating glutaminase (Welbourne, 1987). This system would allow the liver to maintain homeostasis during fluctuations in dietary uptake. Experimentally, "feed-forward" activation of glutamine by ammonia was first identified by Haussinger ***et al.*** (1975). His study involved the perfusion of 5 mM glutamine through rat livers and he discovered that glutamine catabolism occurred after a lag period of 30 minutes. This was interpreted by Haussinger ***et al.*** (1975) as the time required for self activation through end-product accumulation. A brief perfusion of ammonia prior to the perfusion with glutamine showed no lag period in glutamine catabolism. This quick response to ammonia illustrates the importance of this end-product in the regulation of liver-type glutaminase activity.

Glutamine catabolism in the liver is also influenced by a number of hormones and other metabolites. Hormones such as glucagon (Brosnan ***et al.*** 1995), adrenaline, angiotensin II, catecholamines and vasopressin (Corvera and Garcia-Sainz, 1983) have been reported to quickly activate glutaminase. Mitochondria isolated from rats which were exposed to these hormones have altered metabolism compared to mitochondria isolated from control (saline-injected) rats. Increases in mitochondrial adenine nucleotides, membrane potential, matrix volume, pH gradient, citrulline synthesis, and alteration in mitochondrial respiratory rates have been reported after glucagon injection (Halestrap ***et al.***, 1986). Halestrap has argued that activation by these hormones occurs via mitochondrial swelling (Halestrap 1989), as similar results were illustrated by incubating mitochondria in hypotonic media. However, the precise link between the signaling pathway of these hormones, the altered metabolic conditions and the activation of glutaminase has yet to be elucidated.

Brosnan ***et al.*** (1995) studied the glucagon signaling pathway in glutaminase activation.

Their study showed that glutaminase was activated by a cell permeable protein kinase A activator (Sp-cAMPS) and by a cell permeable protein phosphatase 1 and 2A inhibitor okadaic acid. However, the activation of glutaminase by glucagon was not inhibited by a protein kinase A inhibitor. This study, alone, illustrates the complexity and redundant elements that may be involved in the signaling pathway of hormones in the activation of glutaminase.

Objectives of this Study

The intent of this study is to investigate whether liver-type glutaminase plays a indirect role in ureagenesis through its effect on NAG production. As mentioned previously, NAG is an essential activator of CPS I which is the rate limiting step of the urea cycle. Hepatic glutaminase is believed to be associated with the urea cycle but the nature of this association is not fully understood. My hypothesis is that hepatic glutaminase provides glutamate for N-acetylglutamate synthesis (Figure 1.3). Therefore, factors which influence glutaminase activity should activate CPS I by increasing NAG levels. We intend to investigate known activators of liver-type glutaminase (1 mM NH_4Cl and 10^{-7} M glucagon) in perfused rat livers and isolated hepatocytes to examine their influence on mitochondrial NAG levels. From the perfused livers and isolated hepatocytes, we shall prepare mitochondria and measure both the rate of citrulline synthesis and concentration of NAG within the mitochondria. These measures will allow us to assess the influence of liver-type glutaminase activators on mitochondrial NAG levels.

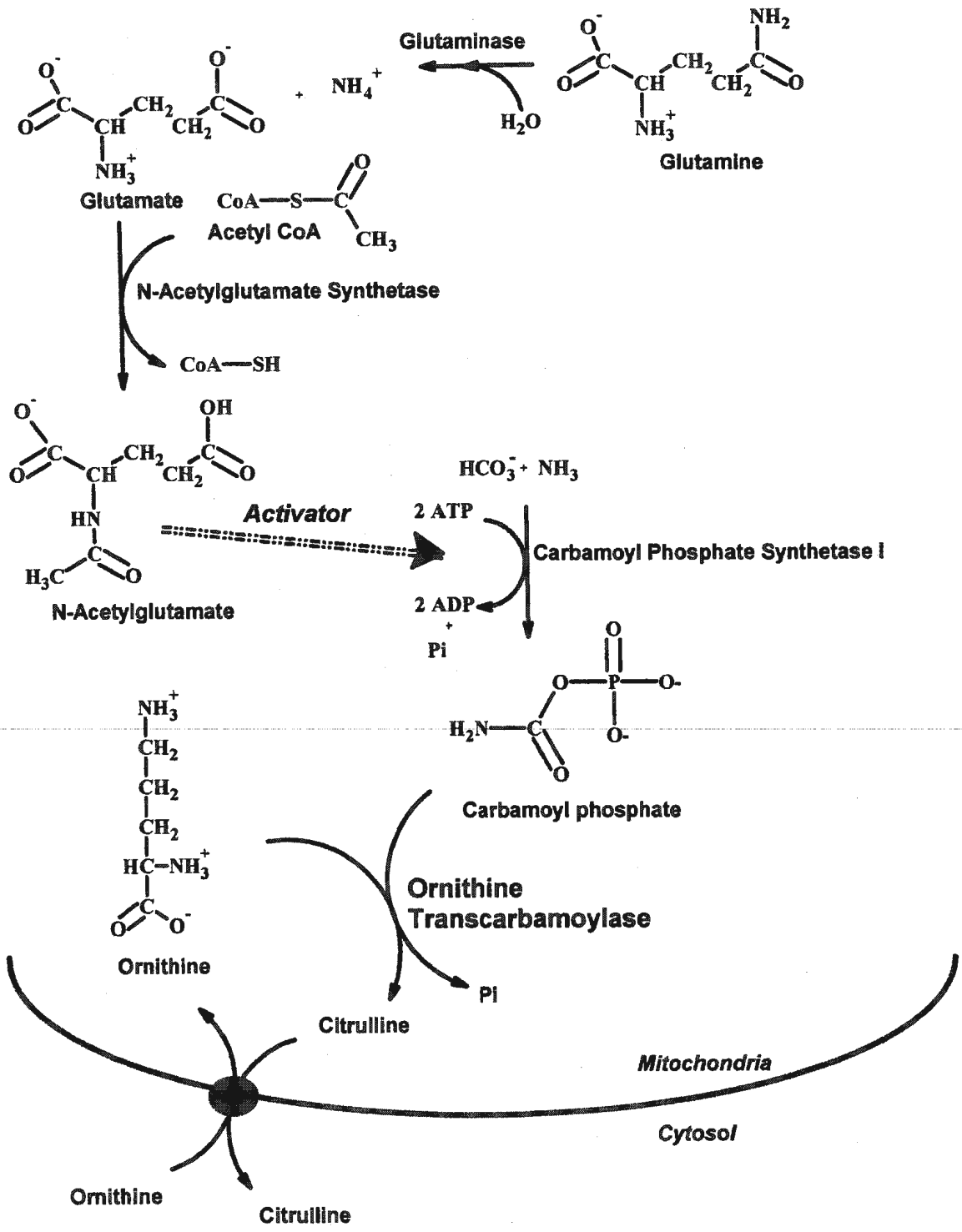


Figure 1.3: **Proposed Involvement of Liver-type Glutaminase in the Regulation of the Urea Cycle.**

Chapter Two

Materials and Methods

MATERIALS

Chemicals

Collagenase A, glutamate dehydrogenase in glycerol and bovine serum albumin (prepared from fraction V, essentially fatty acid free) were obtained from Boehringer-Mannheim (Laval, Quebec). [1-^{14}C] Glutamate was from Dupont New England Nuclear (Mississauga, Ontario). Toluene and Omnifluor were purchased from Fisher Scientific (Nepean, Ontario). QAE Sephadex A-25 was from Pharmacia LKB (Baie D'Urfe, Quebec). Glucagon was obtained from Sigma (St. Louis, USA). All other chemicals were obtained from Sigma and were of the highest grade available.

Preparation of [1-^{14}C] glutamine

To prepare [1-^{14}C] glutamine from [1-^{14}C] glutamate, a 15 000 x g supernatant from a rat liver homogenate of one part tissue plus three parts 0.9 % NaCl was used as a crude preparation of glutamine synthetase. The incubation was for 3 hours at 37° C. The incubation mixture, in a final volume of 0.5 ml, consisted of: Tris/HCl buffer, pH 7.4 (78 mM); 15.6 mM NH_4Cl; 15.6 mM $MgCl_2$; 7.8 mM ATP; 5.6 mM [1-^{14}C] glutamate; 7.8 mM phosphocreatine; 10 units /ml creatine kinase and 0.1 ml of liver sample in 0.5 ml incubation mixture (0.2 ml /ml) (Baverel and

Lund, 1979). At the end of the incubation, the mixture was deproteinized with 0.05 ml of 10 % perchloric acid (w/v). The pH was adjusted to 7.0 using 50 % K_2CO_3 (w/v). [1-^{14}C] Glutamine was separated from any unreacted [1-^{14}C] glutamate by column chromatography using Sephadex QAE. This is an anion exchanger that will bind glutamate but not glutamine. This gel has been equilibrated with 40mM Tris-HCl, pH 5.7 (Haussinger ***et al.***, 1983). The radioactivity of our sample was assessed before adding to the column. This was performed by placing 10 µl of our sample in Scintiverse and measuring the radioactivity using a LKB Wallac liquid scintillation counter. The sample was placed onto the column and 2 ml fractions were collected. The fractions were analysed for radioactivity, and those with positive results (tubes 13-20) were pooled and freeze dried. The samples were resuspended in H_20 (approx. 25 ml) and were, once again, freeze- dried and stored in the freezer.

Animals

Male Sprague-Dawley rats weighing 250-350g were obtained from Memorial University breeding colony. Animals had access to water and Purina chow (Ralston Purina of Canada, Don Mills, Ontario) *ad libitum*. All procedures were approved by the President's Committee on Animal Bioethics and Care of Memorial University and were in accordance with the guidelines of the Canadian Council on Animal Care.

METHODS

Rat Liver Perfusion

Rats were anaesthetised by i.p. administration of 0.3 ml of sodium pentobarbitol (0.1 ml/ 100g body weight). The femoral vein was exposed and 0.1 ml of heparin (1000 Units /ml) was injected. Rat livers were then perfused *in situ* in non-recirculating mode as described by Sies (1978) with 2.1 mM lactate and 0.3 mM pyruvate present in the perfusate (Krebs-Henseleit medium) as respiratory substrates. The flow rate in all cases was approximately 40 ml/minute; all media were gassed with O_2: CO_2 (95:5) for 20 minutes prior to use and gassing continued throughout the perfusion. NH_4Cl (1 mM), 10^{-7} M glucagon, and/or 1 mM glutamine were added to the perfusate as stimulants of glutaminase activity. The perfusion was allowed to run for 75 minutes; then the liver was removed and mitochondria prepared as described below.

Preparation of Intact Mitochondria

Each rat was killed by cervical dislocation and the liver rapidly removed and immersed in an ice-cold isolation medium that was modified from Hampson ***et al.*** (1983). This medium consists of mannitol (225 mM); sucrose (75 mM); ethyleneglycolbis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) (1 mM); and *N*-2-ethanesulphonic acid (Hepes) (5 mM); pH 7.4. The method, essentially that of Jois ***et al.*** (1989), required that the liver be quickly and finely minced with scissors and then homogenized in 19 volumes of cold medium in a hand-held teflon, Potter-Elvejhem homogenizer, clearance of ~0.3 mm. The homogenate was then centrifuged in a

Beckman J2-MC centrifuge (JA- 20 rotor) at 600 g for 10 minutes at 4° C to pellet any erythrocytes, connective tissue, etc. The subsequent spins were carried out at 8200g. The second centrifugation provided a crude mitochondrial pellet that was re-suspended in isolation medium with one or two passes of a Dounce homogenizer. The mitochondria were purified by centrifuging this suspension at 8200 g for 10 minutes, re-suspending the pellet, and then repeating this procedure. A standard biuret procedure was used for determining the protein concentration of the mitochondrial suspension (Gornall ***et al.***, 1949). Bovine serum albumin (BSA) was used as standard.

Respiratory Control Ratio and Succinate Oxidation

Mitochondrial oxygen uptake was measured using a Clark-type electrode (Estabrook, 1967) in a medium which contained: 140 mM KCl; 4 mM KH_2PO_4; 1 mM EDTA; 2.5 mM $MgCl_2$; 5 mM Hepes; 1 mg/ml BSA; pH 7.4 at 30°C. The medium was equilibrated with air. Mitochondria (1-2 mg mitochondrial protein) were incubated in this medium with 5 mM succinate and state 3 respiration was begun by addition of ADP (0.26 mM; final concentration). The mitochondria were accepted if they exhibited respiratory control ratios (State 3/ State 4 respiration) of greater than 3.

Flux Through Glutaminase in Intact Mitochondria

Isolated mitochondria were incubated in 25 ml Erlenmeyer flasks for 10 minutes at 30°C. All incubations were in triplicate and each flask contained 2 mg mitochondrial protein and a 2.0 ml final volume. The incubation medium consists of 100 mM KCl, 20 mM L-glutamine, 5 mM

succinate, 10 mM KH_2PO_4, 1 mM EGTA, 20 mM Tris, 10 mM $KHCO_3$, pH 7.4 (Lacey ***et al.***, 1987). Rotenone was also present in the incubation medium to prevent further glutamate oxidation. After 10 minutes, the reaction was stopped with 0.3 ml of perchloric acid (7 % w/v). For zero time samples, 0.3 ml of perchloric acid was added before the mitochondria. The protein was removed by centrifugation in an Eppendorf microcentrifuge for 2 minutes at 12,000 x g. The supernatant was taken and adjusted to pH 8.8 with 3 M K_3PO_4 and was used for the glutamate assay (Bernt and Bergmeyer, 1974). Glutamate formation was calculated as the difference between the total flask content of glutamate at zero time and after the 10 minute period of incubation. The amount of glutamate formed was therefore a measure of flux through glutaminase.

Glutaminase Assay in Freeze-thawed Mitochondria

Glutaminase is located within the mitochondrion and, to assay its activity, disruption of the mitochondrial inner membrane is required. This is achieved by three cycles of freezing the mitochondria in liquid nitrogen followed by thawing in warm water (less than 37 °C). The freeze-thawed mitochondria were then incubated for 10 minutes at 37 °C in an incubation medium which consisted of 300 mM mannitol, 20 mM L-glutamine, 0.7 mM NH_4Cl, 20 mM Hepes, and 20 mM KH_2PO_4, pH 7.4 (Szweda and Atkinson, 1990). The final volume was 1.0 ml with a final concentration of 1 mg /ml freeze-thawed mitochondria. The incubation was terminated by the addition of 0.3 ml of 7 % $HClO_4$ (w/v). The supernatant was adjusted to pH 8.8 with K_3PO_4 and glutamate was determined by using an enzymatic assay, employing a glutamate dehydrogenase assay (Bernt and Bergmeyer, 1974). This assay measures glutamate levels

through the conversion of glutamate and NAD+ to ketoglutarate and NADH. The increase in the absorbency at 340 nm (measured using UV-vis) resulting from the reduction of NAD+ to NADH, gives an accurate assessment of the amount of glutamate in our samples.

Preparation of Hepatocytes

Hepatocytes were prepared essentially as described by Krebs ***et al.*** (1974) except that hyaluronidase was not present in the perfusate. Rats were anaesthetised by i.p. administration of 0.3 ml of sodium pentobarbitol (0.1 ml/ 100g body weight). The femoral vein was exposed and 0.1 ml of heparin (1000 Units /ml) was injected. The isolation of hepatocytes involves a two step perfusion of the liver. First, the liver was perfused with 500 ml of calcium-free Krebs-Henseleit bicarbonate medium containing 2 mM EGTA, 20 mM glucose, 2.1 lactate, and 0.3 mM pyruvate. The flow rate in all cases was approximately 40 ml/minute; all media were gassed with O_2: CO_2 (95:5) for 20 minutes prior to use and gassing continued throughout the isolation procedure. The liver was then perfused with 500 ml of Krebs-Henseleit medium that contained 2.5 mM calcium, 20 mM glucose, 2.1 lactate, and 0.3 mM pyruvate. Calcium-containing Krebs-Henseleit medium with 2.5 % BSA and 30 mg collagenase/100 ml was then recirculated for 15 to 20 minutes in a total volume of 200 ml. After the recirculation, the liver began to leak medium and was observed to break apart. At this point the liver was removed and minced in a petri dish. This suspension was then shaken in a Dubnoff metabolic shaker at 37°C under O_2: CO_2 (95:5) for 10 minutes. The cells were spun at 30 g and washed two more times with Krebs-Henseleit medium. The final wash was in Krebs-Henseleit medium containing 2.5 % BSA and cells were also resuspended in this medium. Cell viability was determined by 0.1 % trypan blue exclusion and was greater than

95 % in all instances. Cells were quantified by determination of dry weight. A 3 ml aliquot of the cell suspension was placed in a pre-weighed metal weighing pan and a 3 ml aliquot of the 2.5 % BSA resuspension medium was placed in a separate pre-weighed weighing pan. Both were weighed and dried in an oven at 50°C for 24 hours. The difference in weight of the cell suspension and the medium was used to determine the dry weight of the cells present in our suspension.

Measurement of Flux through Glutaminase in Isolated Hepatocytes

The procedure for determining flux through glutaminase involves the trapping and counting of $^{14}CO_2$ after incubation of cells with [1-^{14}C] glutamine (1 mM) (Vincent ***et al.***, 1989). This procedure has been shown previously by Brosnan ***et al.*** (1995) to be a valid measurement of glutaminase flux. Incubations were performed in triplicate in 25 ml Erlenmeyer flasks containing between 4-6 mg dry weight of hepatocytes at a final volume of 1 ml. Cells were preincubated in Krebs-Henseleit medium for 20 minutes at 37°C before addition of [1-^{14}C] glutamine. Where indicated, 6-diazo-nor-leucine (DON) or glutamate-γ-hydrazide were added during this preincubation. Also, in some incubations glucagon or ammonium chloride were added at the same time as [1-^{14}C] glutamine. Each flask was gassed with O_2: CO_2 (95:5) for 20 seconds after the addition of the cells and also after addition of [1-^{14}C] glutamine. The incubation flasks were equipped with rubber septa in which empty plastic centre wells were suspended. NCS tissue solublizer was introduced into the centre wells through the septa just before termination of the incubation with 0.15 ml of 30 % (w/v) perchloric acid. $^{14}CO_2$ was collected for one hour and the centre wells were then transferred to scintillation vials containing 15 ml of scintillation fluid

(Omnifluor)

The cellular production of $^{14}CO_2$ was quantitated by a LKB Wallac liquid scintillation counter. Blanks included both zero time and zero protein incubations.

Isolation of Mitochondria from Hepatocytes

Hepatocytes were isolated from livers of fed rats as indicated previously. Two separate preparations of hepatocytes were pooled. This was necessary in order to provide a sufficient amount of mitochondria. The pooled cells were resuspended in a final volume of 90 ml with Krebs-Henseleit medium containing 2.5 % BSA. The cells were then preincubated with or without 5 mM 6-diazo-nor-leucine (dissolved in DMSO) at 37°C under O_2: CO_2 (95:5) in a shaking bath for 20 minutes. Glutamine (1 mM) was then added to each of the flasks along with 10^{-7} M Glucagon (dissolved in 10 mM HCl) and/or NH_4Cl (1mM) and allowed to incubate for another 30 minutes. All concentrations listed are the final concentrations in incubation flasks. Along with incubations previously described, control incubations containing only glutamine (1mM) and with or without 5 mM DON were performed with each experiment. All incubations were in a final volume of 15 ml in a 50 ml Erlenmeyer flask which was gassed with O_2: CO_2 (95:5). The incubation was terminated by spinning the hepatocytes at 30 g for 2 minutes to remove the Krebs-Henseleit medium. A modification of a procedure used by Corvera and Garcia-Sainz (1983) was used to obtain the mitochondria. This method uses digitonin to disrupt the plasma membrane of hepatocytes and permits the release of intact mitochondria. The cells were resuspended in isolation medium containing 0.255 M mannitol, 0.075 M sucrose, 5 mM

Hepes, 1 mM EGTA, 1 % BSA, and 0.4 mg digitonin/ ml (dissolved in DMSO). The cell suspension was kept on ice for 30 minutes with occasional inversion to resuspend the cells. This incubation in digitonin provides sufficient time for the chemical to adequately disrupt the plasma membrane. The cells were then subjected to a high-speed spin (8200 X g) for five minutes. The cell pellet was homogenized in digitonin-free isolation medium using 8-10 soft strokes of the Potter-Elvejhem hand-held homogenizer. The suspension was then subjected to the same procedure as used for the isolation of mitochondria from intact liver. Again, respiratory control ratios were checked using a Clark oxygen electrode with 5 mM succinate as a substrate and acceptable preparations had a ratio greater than 3.

Citrulline Synthesis in Intact Mitochondria

Flux through the mitochondrial portion of the urea cycle was measured by incubation of mitochondria in a 25 ml Erlenmeyer Flask placed in a Dubnoff metabolic shaker at 30°C with a medium which comprised (final concentration): 80 mM KCl; 20 mM Tris (hydroxymethyl) methylamine (TRIS); 5 mM K_2PO_4; 5 mM succinate HCl; 20 mM $KHCO_3$; 5 mM NH_4Cl; 20 mM L-ornithine; 1 mM EGTA; pH 7.4 in a final volume of 1.0 ml (Lacey ***et al.***, 1987). The pH was adjusted using 0.1M NaOH. The final concentration of mitochondria was 3 mg protein /ml. The reaction was started by the addition of mitochondria, incubated for 6 minutes and then stopped by the addition of 0.3 ml of 30 % (w/v) perchloric acid. All incubations were done in triplicate.

Citrulline produced during the incubation was assayed using the colorimetric method of Herzfeld and Raper (1976). The deproteinized supernatant of the incubation is first transferred to 5 ml test tubes. Then 0.1 ml of the sample is added to a test tube containing 0.15 ml of H_2O. To

each of the tubes 3.0 ml of the chromogenic reagent was added and the tubes were mixed by inversion. The chromogenic reagent consists of 5.0 mg of thiosemicarbazide dissolved in 50 ml of diacetyl monoxime to which 100 ml of ferric acid solution was added. The tubes were incubated in a water bath at 100°C for five minutes. They were removed, cooled, and absorbance was measured at 530 nm.

Mitochondrial NAG Measurements

Measurement of N-acetylglutamate levels in isolated mitochondria was performed as described by Brosnan ***et al.*** (1996). Aliquots of mitochondria were deproteinized using 0.3 ml of perchloric acid (7 % w/v). The protein was removed by centrifugation in an Eppendorf microcentrifuge for 2 minutes at 12,000 x g. The supernatant was taken and neutralized with KOH. Analyses of samples were performed by Dr. Itzhak Nissim at the University of Pennsylvania in Philadelphia, Pennsylvania, USA. N-acetylglutamate was determined following separation from other amino acids present in the sample. This was done by applying a 500-µl aliquot of mitochondrial extract to an AG-1 column (Cl^- X-8; 100-200 mesh; 0.5 × 2.5 cm). The column was washed with 3 ml of deionized water. NAG was eluted off the column with 3 ml of 1 N HCl. NAG concentrations were determined through the use of a modification of the conventional isotope dilution technique. GC-MS measurements were performed on a Hewlett-Packard 5970 MSD coupled with a 5890 HP-GC. NAG enrichment in our sample was measured against a *t*-BDMS-derivative of ^{13}C-NAG.

Determination of Ammonia (Phenol-Hypochlorite Method)

Ammonia was determined by the phenol-hypochlorite method (Chaney and Marbach, 1962). In a 10 ml test tube, 0.5 ml of the supernatant from the hepatocyte incubation was added along with 1 ml phenol colour reagent and 1 ml alkali-hypochlorite reagent. The phenol colour reagent was prepared by adding 50 g of phenol and 0.25 g of sodium nitroprusside to one litre of H_2O and stored in an amber bottle. The alkali-hypochlorite reagent was prepared by the addition of NaOH (25 g) and sodium hypochlorite (1g) to 1 litre of H_2O. Samples were incubated in a shaker bath at 37°C for 20 minutes. After incubation, the samples were allowed to cool and their absorbance was measured at 630 nm. A standard curve for ammonia was also constructed and used to calculate ammonia concentration.

Statistical Analysis

All values are expressed as Mean ± SEM as indicated. Statistical analysis was executed by Graphpad Instat 3™ (Graphpad Software, San Diego, California, USA) and standard curves and graphs were produced using Graphpad (also of the Graphpad Software package). Both Student's t-test and the paired t-test were carried out using Instat 3™, as indicated. Multiple comparisons were tested using ANOVA followed by Neuman-Keul's post test and 3-factor analysis of variance was employed on the perfusion studies. Statistically significant differences were deemed to be $P < 0.05$.

Chapter Three

Results and Discussion

RESULTS

Rationale

N-acetylglutamate is an essential activator of the enzyme carbamoyl-phosphate synthase I (CPS I) which is the rate limiting enzyme of ureagenesis (Hensgens ***et al.***, 1980). Therefore, the rate of citrulline synthesis is markedly influenced by the mitochondrial concentration of NAG. This activator is produced by the mitochondrial enzyme N-acetylglutamate synthetase which catalyses the reaction of glutamate and acetyl-CoA to produce NAG and CoASH.

Liver-type glutaminase, another mitochondrial enzyme, catalyses the hydrolysis of glutamine to glutamate and ammonia. The work presented here is focused on the investigation of glutamate, the product of glutaminase, and its potential role in regulating the urea cycle. We suggest that the glutamate released from glutaminase will become the substrate for N-acetylglutamate synthetase. This will increase the mitochondrial concentration of CPS I's activator, NAG, and subsequent flux through the urea cycle. This activation of glutaminase should result in an increased rate of ureagenesis.

Effect of Glutaminase Activation on N-Acetylglutamate Concentration

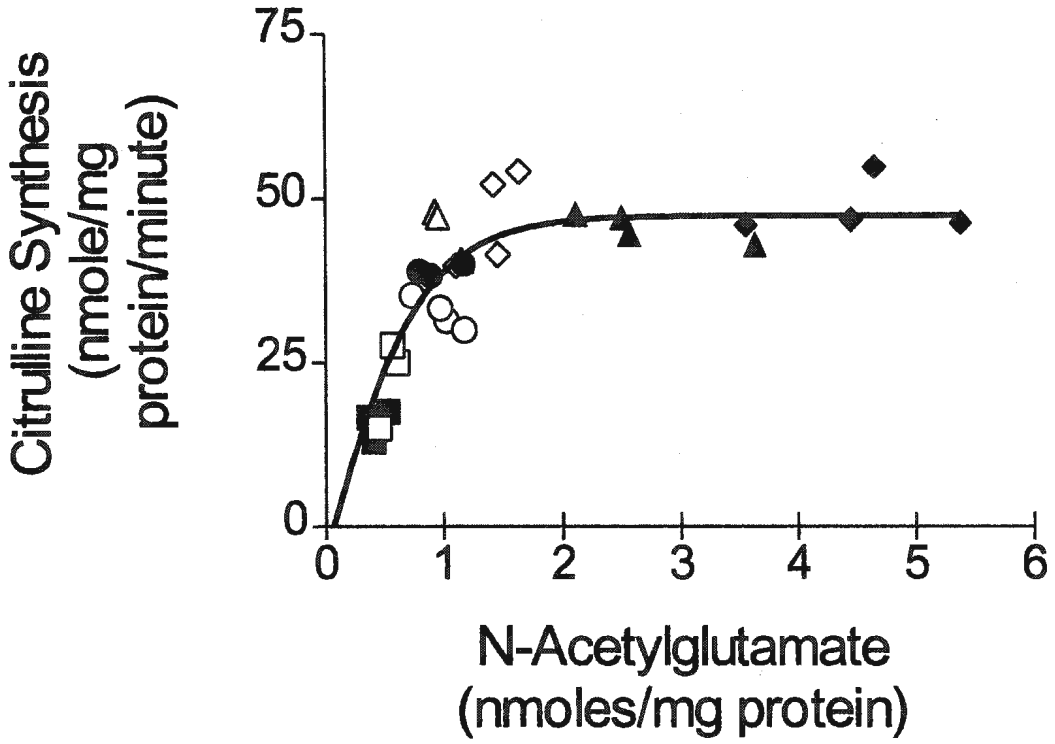
Our initial experiments involved rat liver perfusions where known activators of glutaminase (glucagon and NH_4Cl) and its substrate glutamine were introduced to examine their influence on mitochondrial NAG content and citrulline synthesis, which is a measure of flux through the first two enzymes of the urea cycle (CPS I and OTC).

When glucagon (10^{-7} M) was present in the perfusion, we found that the concentration of NAG in mitochondria increased significantly by 49 % (Figure 3.1). Correspondingly, the rate of citrulline synthesis within these same mitochondria also increased. These results of glucagon's influence on NAG production are consistent with previous work on this hormone (Rabier ***et al.***, 1982; Cathelineau ***et al.***, 1982).

When NH_4Cl (1 mM) was perfused through the liver, there was a significant effect on the mitochondrial NAG content (100% increase; Figure 3.1) and a corresponding increase in the rate of citrulline synthesis. The effect of NH_4Cl suggests that this urea cycle substrate influences its own removal, in part, by increasing production of NAG. A similar effect of ammonium chloride on NAG levels had been reported by Zollner (1981) but that study used concentrations of the activator, ammonia, which were 10 times higher than those used here. Our study, at near physiological concentrations, suggests that the influence of NH_4Cl on the urea cycle activator NAG is of physiological importance.

Additionally, when both activators of glutaminase, NH_4Cl (1 mM) and glucagon (10^{-7} M), were perfused together there was an increase in the amount of NAG that was greater than with either activator alone (Three Factor Analysis of Variance). Since both are known activators of glutaminase, our initial results indicate that there may be a possible role to be played by this enzyme in the regulation of the urea cycle. The data also showed that the third factor we were analysing, glutamine (1 mM), did not alter the NAG content of the liver mitochondria when perfused either alone or in the presence of activators (Three Factor Analysis of Variance). We assume that, even in the absence of glutamine in the perfusate, there was sufficient intracellular glutamine presumably arising from proteolysis.

Figure 3.1: **Comparison of Citrulline Synthesis and N-Acetylglutamate Levels in Mitochondria Prepared from Livers that were Perfused with Activators and Substrates of Liver-type Glutaminase.** Measurement of the rate of citrulline synthesis and N-acetylglutamate levels were performed as described in Material and methods section: □ - Medium alone; ■- 1 mM Glutamine; ○ - 10^{-7}M Glucagon; ● - 10^{-7}M Glucagon and 1 mM Glutamine; △ - 1 mM NH_4Cl; ▲ - 1 mM NH_4Cl and 1 mM Glutamine; ◇- 10^{-7}M Glucagon and 1 mM NH_4Cl; ◆ - 10^{-7}M Glucagon and 1 mM NH_4Cl and 1 mM Glutamine. All concentrations reported are the final concentrations in the perfusion medium.


Citrulline Synthesis
(nmole/mg
protein/minute)
75
50
25
0
0
1
2
3
4
5
6
N-Acetylglutamate
(nmoles/mg protein)

This initial study was consistent with previous work that showed a positive correlation between NAG content and the rate of citrulline synthesis in isolated mitochondria. The data fit a rectangular hyperbola that coincides with observations by Stewart and Walser (1980) and Lund and Wiggins (1987). The K_m for this relationship was 0.9 nmole NAG /mg or 0.9 mM (using conventional values of 1 µl matrix volume for 1 mg mitochondrial protein) and the V_{max} was found to be 48 nmole/mg protein /minute. This K_m value reported here is consistent with the value reported by Stewart and Walser (1980) of 0.8 mM within intact mitochondria while our V_{max} is within the reported range for intact mitochondria (Meijer ***et al.*** 1990). A linear relationship between NAG and citrulline synthesis was reported by Morimoto ***et al.*** (1990) and Rabier ***et al.*** (1982). However, this was based on mitochondrial NAG levels up to 1.6 nmole/mg. They had no values that extended beyond this point. Thus, their data were fitted to a straight line. In our experiment, however, we were able to identify a rectangular hyperbolic relationship as levels of mitochondrial NAG exceeded 1.6 nmole/ml.

Glutaminase Inhibition

To determine whether NH_4Cl and glucagon's effect on NAG content was the result of activation of glutaminase or some other unrelated mechanism, we decided to investigate potential inhibitors of glutaminase. First we needed to validate a suitable inhibitor of glutaminase. We examined two compounds, glutamate-γ-hydrazide and 6-diazo-nor-leucine (DON), which had been previously reported to be effective inhibitors of glutaminase (Low ***et al.***, 1993; Conti and Minelli, 1994; Shapiro ***et al.***, 1979, Matsuno and Hirai, 1989). It should be noted that DON had

been previously reported to effectively inhibit kidney-type glutaminase and glutaminase in hepatoma cells (Conti and Minelli, 1994; Matsuno and Hirai, 1989). Therefore, our investigation of DON's effect directly on liver-type glutaminase is the first to our knowledge.

We found that glutamate-γ-hydrazide did not inhibit glutaminase activity from freeze-thawed mitochondria (Figure 3.2), or from intact mitochondria (Figure 3.3), or in isolated hepatocytes (Figure 3.4). These results contrast those reported by Low ***et al.*** (1993), who identified this compound as having a greater than 80 % inhibitory effect on glutaminase activity. However, we are confident of our results, which were reproducible with batches of glutamate-γ-hydrazide obtained from two different sources. On the other hand, my work with DON showed promising results as the compound significantly inhibited glutaminase activity in both freeze-thawed (Figure 3.5) and intact mitochondria preparations (Figure 3.6).

We extended our investigation of DON to hepatocytes. As seen in Figure 3.7, flux through glutaminase was significantly inhibited by DON in the presence and absence of known activators of this enzyme, NH_4Cl and glucagon. Although DON does not completely inhibit glutaminase activity (≈ 40%), We felt that its effect would be sufficient for our investigation of whether a link exists between the activation of liver-type glutaminase and increased NAG levels. We changed our experimental protocol from liver perfusions to hepatocyte incubations because of the prohibitive cost involved in perfusing our inhibitor at the concentrations required to significantly inhibit glutaminase. The amount of inhibitor required for each experiment using hepatocyte incubations is only a fraction of that required for perfusion studies. Furthermore, isolated hepatocytes have provided comparable results to those in the perfused liver in investigations of glucagon and NH_4Cl and their effects on NAG levels in liver mitochondria

Figure 3.2: **The Effect of Glutamate -γ-Hydrazide on Glutaminase Activity in Freeze-thawed Liver Mitochondria.** Activity was measured in rat liver mitochondria that had been exposed to three cycles of freezing (-70°C) and thawing. This was performed to break the mitochondrial membrane. Mitochondrial extracts were then incubated with or without 2.5 mM Glutamate -γ-Hydrazide (G-γ-H) for 10 minutes. Results are means ± SEM of 3 separate experiments. Results were compared using Student *t*-test ($P < 0.05$). No significant difference was found.

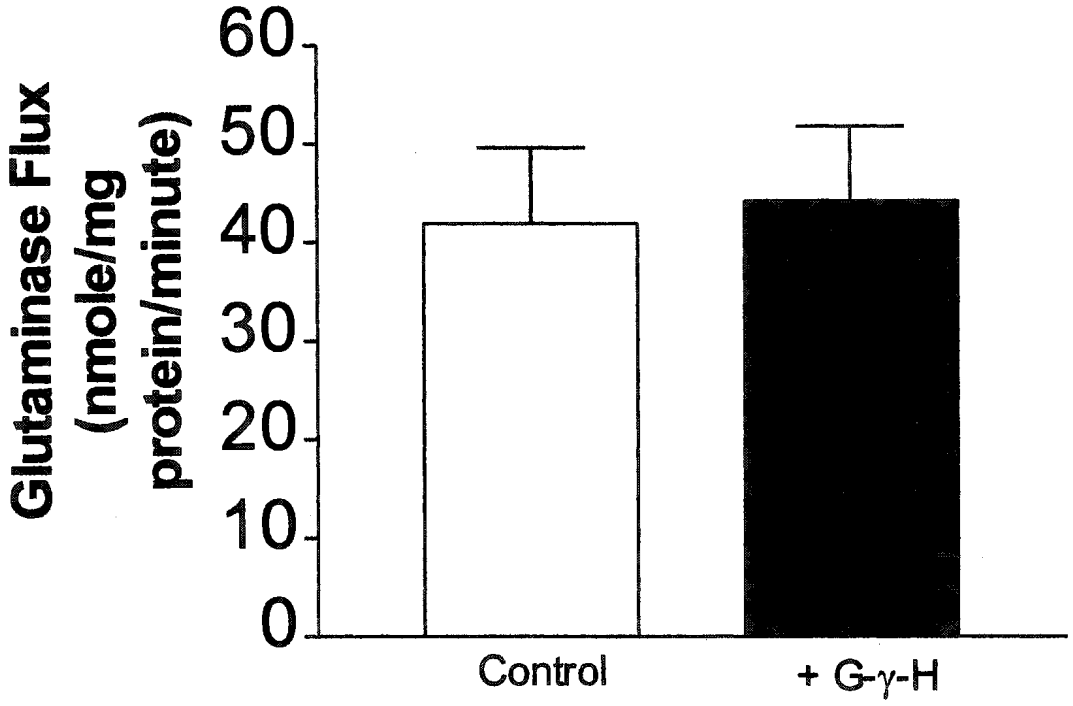

Glutaminase Flux
(nmole/mg
protein/minute)
60
50
40
30
20
10
0
Control
+ G-γ-H

Figure 3.3: **The Effect of Glutamate -γ-Hydrazide on Flux Through Glutaminase in Isolated Rat Liver Mitochondria.** Flux was measured in freshly isolated, intact rat liver mitochondria. Mitochondria were incubated in a final concentration of 2.5 mM Glutamate -γ-Hydrazide. Results are means ±SEM of 3 separate experiments. Results were compared using the Student *t*-test ($P < 0.05$). No significant difference was found.

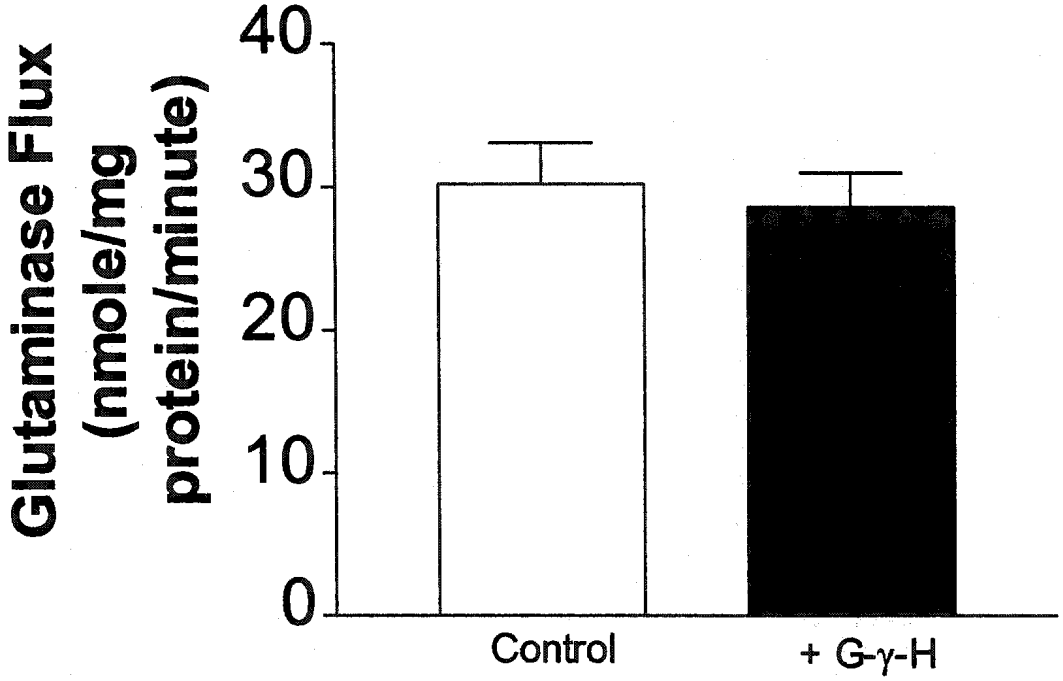
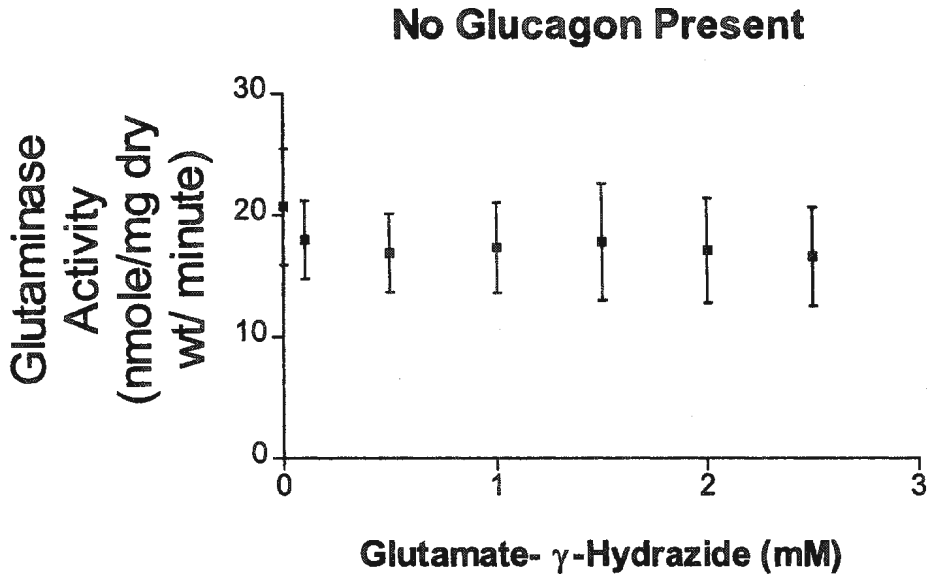

Glutaminase Flux
(nmole/mg
protein/minute)
40
30
20
10
0
Control
+ G-γ-H

Figure 3.4: **The Effect of Glutamate-γ-Hydrazide on Flux Through Glutaminase in Isolated Rat Liver Hepatocytes.** Results shown are the effect of this compound on glutaminase in the presence and absence of glucagon. 1 mM NH_4Cl was also present in both sets of experiments to increase the activation of glutaminase. All concentrations reported are the final concentrations within the hepatocyte incubation. Results are Means ± SEM of three separate experiments. Results were analysed using ANOVA followed by Neuman-Keul's post test. No significant difference was observed.


No Glucagon Present
Glutaminase Activity (nmole/mg dry wt/ minute)
30
20
10
0
0
1
2
3
Glutamate- γ-Hydrazide (mM)

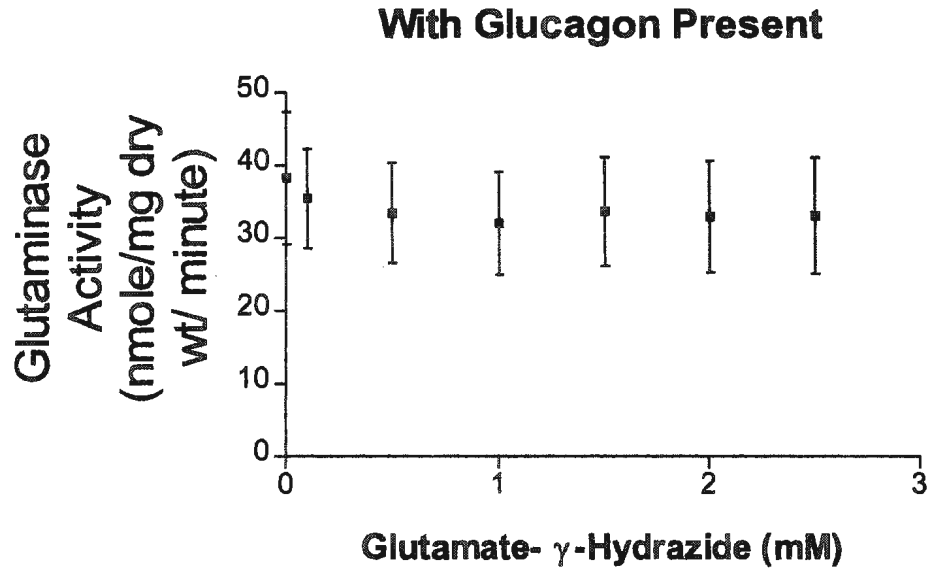

With Glucagon Present
Glutaminase Activity (nmole/mg dry wt/ minute)
50
40
30
20
10
0
0
1
2
3
Glutamate- γ-Hydrazide (mM)

Figure 3.5: **The Effect of 6-Diazo-nor-leucine on Glutaminase Activity in Freeze-thawed Liver Mitochondria.** Activity was measured in rat liver mitochondria that had been exposed to three cycles of freezing (-70°C) and thawing. This was performed to break the mitochondrial membrane. Mitochondrial extracts were then incubated with or without 5 mM DON (final concentration) for 10 minutes. Results are means ± SEM of 3 separate experiments. Results were compared using Student *t*-test ($P < 0.05$): * denotes significant differences between groups with or without 5 mM DON present.

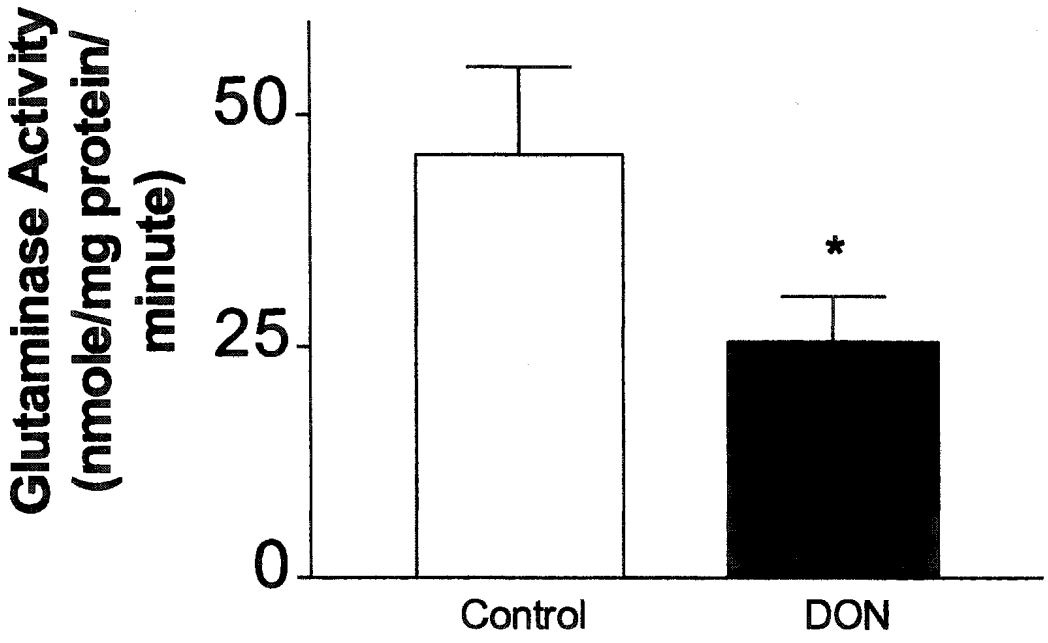

Glutaminase Activity
(nmole/mg protein/
minute)
50
25
0
*
Control
DON

Figure 3.6: **The Effect of 6-Diazo-nor-leucine on Flux Through Glutaminase in Isolated Rat Liver Mitochondria.** Flux was measured in freshly isolated, intact rat liver mitochondria. Mitochondria were incubated with a final concentration of 5 mM DON. Results are means ± SEM of 3 separate experiments. Results were compared using Student *t*-test ($P < 0.05$): * denotes significant differences between groups with or without 5.0 mM DON present.

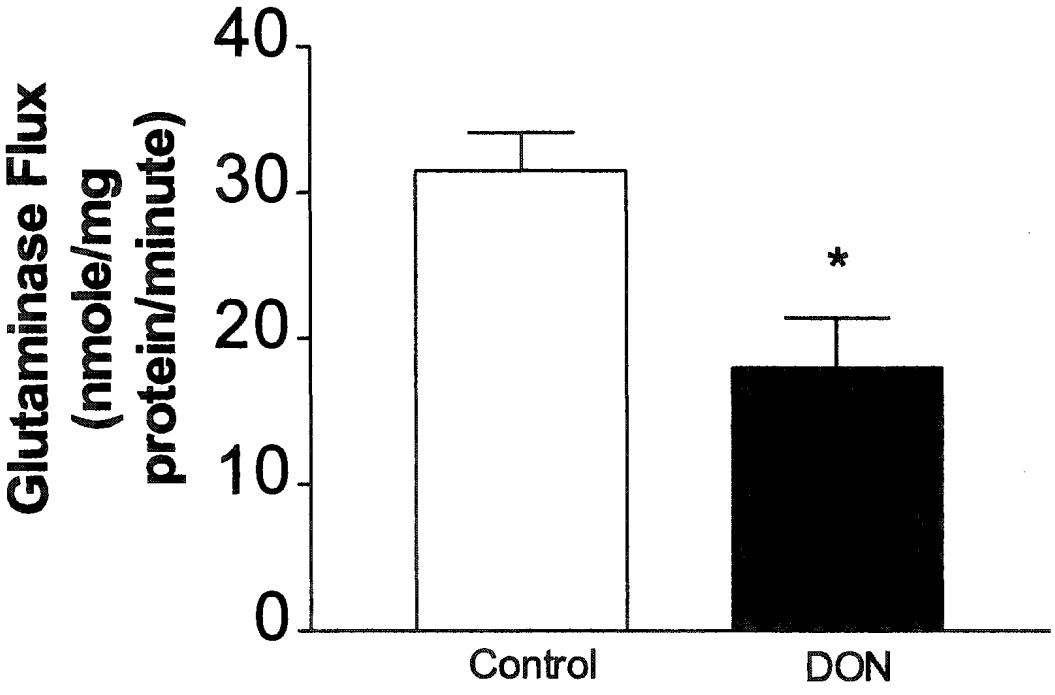
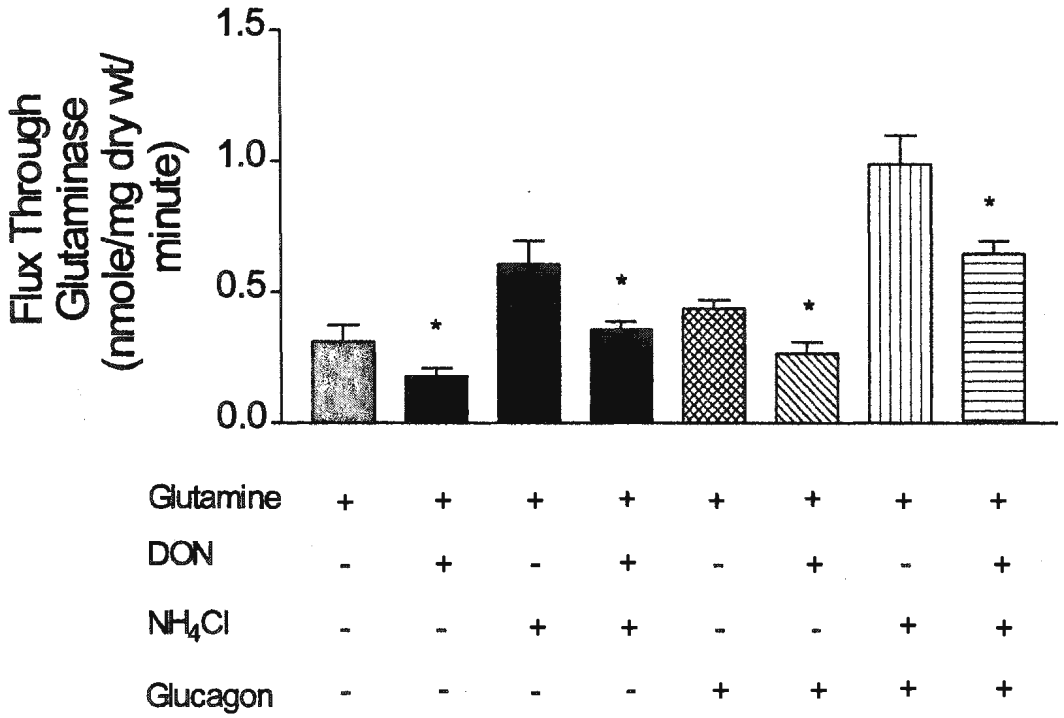

Glutaminase Flux
(nmole/mg
protein/minute)
40
30
20
10
0
*
Control
DON

Figure 3.7: **The Effect of 6-Diazo-nor-leucine on Flux Through Glutaminase in Isolated Rat Liver Hepatocytes.** All concentrations reported are the final concentrations within the hepatocyte incubation. Results are Means ± SEM of three separate experiments. Results were analysed using Student paired t-test ($p < 0.05$): * denotes significant differences between groups without DON present.


Flux Through Glutaminase (nmole/mg dry wt/ minute)
1.5
1.0
0.5
0.0
*
*
*
*
Glutamine + + + + + + + +
DON - + - + - + - +
NH4Cl - - + + - - + +
Glucagon - - - - + + + +

(Hensgens *et al.*, 1980; Zollner, 1981). Therefore, the use of hepatocyte incubations provided us with a cost effective method to investigate the inhibitory effects of DON on these known activators.

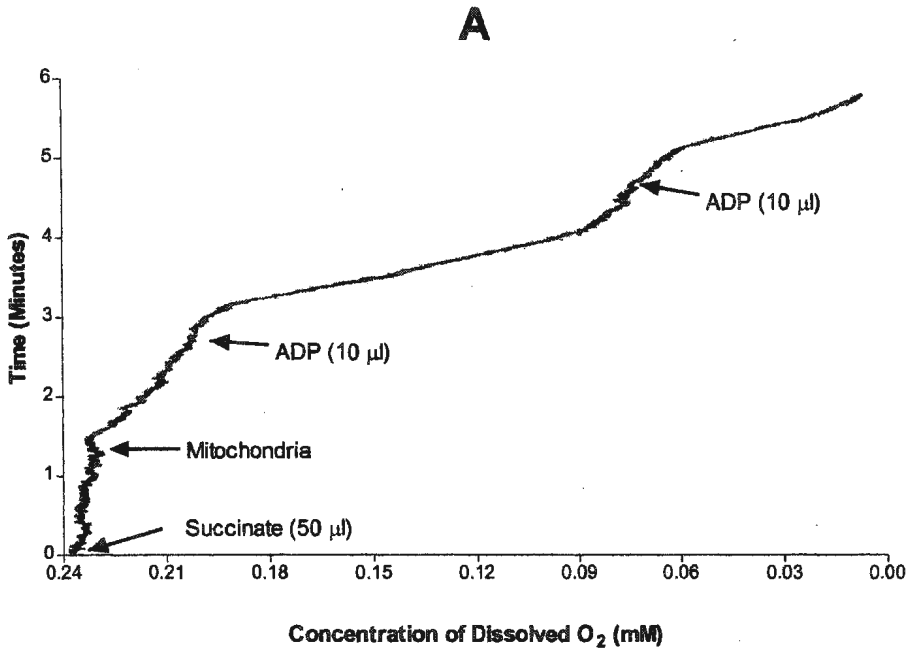
Does DON Affect Mitochondrial Integrity?

Hepatocyte incubations were performed as described in Materials and Methods section. After incubation, mitochondria were prepared and analysed for NAG content and the rate of citrulline synthesis. It was essential that we determine whether DON treatment of hepatocytes affected mitochondrial integrity. We did this by examining mitochondrial respiration. There was no effect of DON on mitochondrial respiration in the presence or absence of ADP (Figure 3.8 A vs. 3.8 B). In all cases, respiratory control ratios were greater than 3. Therefore, DON did not compromise the assay for citrulline synthesis, which is dependent upon mitochondrial membrane integrity.

Inhibition of Glucagon Stimulated NAG Production by DON

Mitochondria isolated from hepatocytes which had been incubated with glucagon showed significantly higher levels of NAG as expected from our earlier work (0.71 ± 0.04 vs. 0.40 ± 0.07 nmole/mg mitochondrial protein). In addition, glucagon-treated cells that had been pre-incubated with DON were shown to have significantly lower levels of NAG than glucagon treated cells incubated without DON (Figure 3.9; $p < 0.05$). The lowered NAG content due to DON's

Figure 3.8: **Effect of 6-Diazo-nor-leucine on Mitochondrial Respiration.** Mitochondria were prepared from isolated hepatocytes as described in Chapter 2. 5 mM succinate was used as substrate for oxidative phosphorylation and state 3 respiration was begun by addition of ADP (0.26 mM; final concentration).The mitochondrial concentration was 0.2 mg protein /ml. The concentration of oxygen in solution in equilibrium with air at 30°C was taken as 0.24 mM. **A**- Control Mitochondria; **B** - Mitochondria from DON treated hepatocytes. The figure shows an individual experiment but three separate experiments were carried out. Respiratory control ratios (~ 4) were not significantly different from one another ($n = 3$), Student's paired t-test ($p < 0.05$).


A
Time (Minutes)
6
5
4
3
2
1
0
ADP (10 µl)
ADP (10 µl)
Mitochondria
Succinate (50 µl)
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0.00
Concentration of Dissolved O_2 (mM)

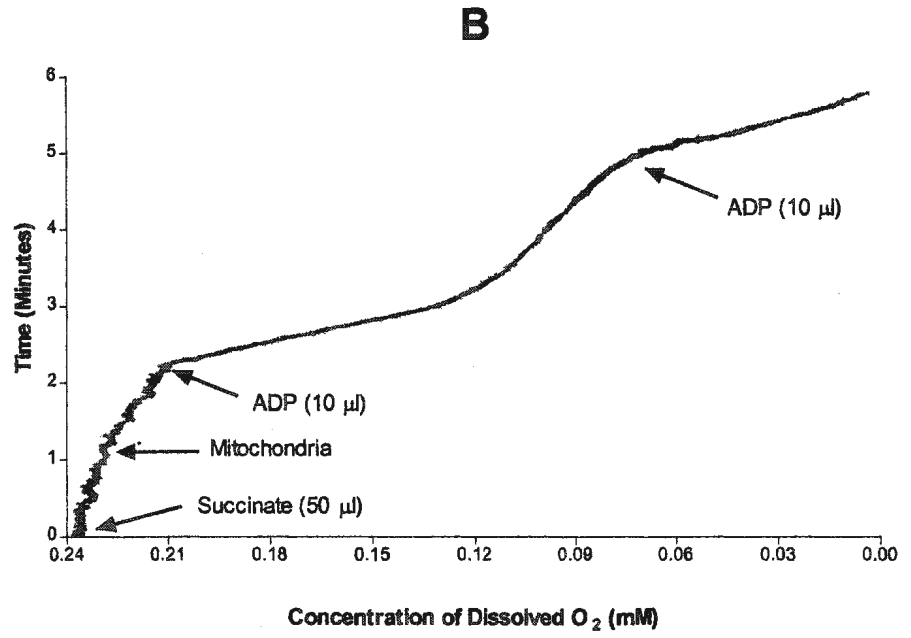
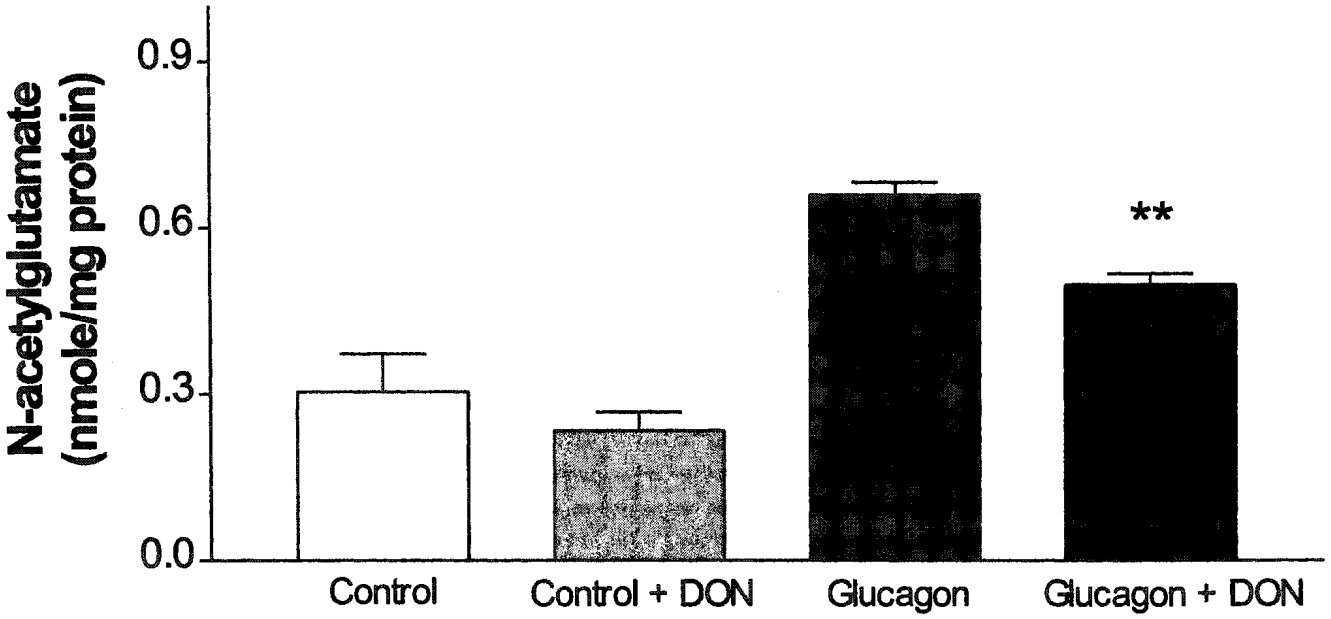

B
Time (Minutes)
6
5
4
3
2
1
0
ADP (10 µl)
ADP (10 µl)
Mitochondria
Succinate (50 µl)
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0.00
Concentration of Dissolved O_2 (mM)

Figure 3.9: **N-Acetylglutamate Levels in Mitochondria Isolated from Hepatocytes Incubated with/without Glucagon or 6-Diazo-nor-leucine.** All incubations contained 1 mM Glutamine. Incubation concentrations of Glucagon and DON were 10^{-7}M and 5 mM respectively. Cells were pre-incubated with or without DON for 20 minutes, then incubated 30 minutes with or without Glucagon. Results are means ± S.E.M. of 5 separate experiments. Results were compared using a Student t-test: ** denotes a significant difference ($p < 0.01$) compared to the corresponding incubations without DON.


N-acetylglutamate
(nmole/mg protein)
0.9
0.6
0.3
0.0
**
Control
Control + DON
Glucagon
Glucagon + DON

inhibition of glucagon's effect was also reflected in the rate of citrulline synthesis in those same mitochondria. As seen in Figure 3.10, the rate of citrulline synthesis was higher in the glucagon treated cells than control (34.6 ± 5.2 nmole/mg/minute compared to 16.6 ± 1.3 nmole/mg/minute) but was significantly reduced by our inhibitor (34.6 ± 5.2 nmole/mg/minute compared to 24.7 ± 3.1 nmole/mg/minute). In our control incubations, DON did not decrease the NAG content and rate of citrulline synthesis.

When the rate of citrulline synthesis in individual mitochondrial preparations is related to NAG content (Figure 3.11), the inhibitory effect of DON on glucagon's stimulation becomes quite evident. Preparations that were incubated with DON had significantly lower levels of NAG and thus citrulline synthesis. This effect of DON is highly suggestive of a link between activation of glutaminase activity by glucagon and subsequent production of NAG.

Inhibition of NH_4Cl-Stimulated NAG Production by DON

The next step in our investigation was to determine whether the stimulatory effect of NH_4Cl on mitochondrial NAG levels is influenced by inhibition of glutaminase. Similar methods were employed. Hepatocytes were first pre-incubated for 20 minutes, with or without DON. Then those cells were incubated with 1 mM glutamine, with or without 1 mM NH_4Cl for 30 minutes. NH_4Cl was found to significantly increase NAG levels in the mitochondria (Figure 3.12; $p < 0.001$). NAG levels were 180 % greater in mitochondria isolated from cells incubated with NH_4Cl than in control incubations (1.12 ± 0.106 vs. 0.400 ± 0.067 nmole/mg mitochondrial protein). In fact, NH_4Cl's effect on mitochondrial NAG was greater than the effect mediated by glucagon (57

Figure 3.10: **The Rate of Citrulline Synthesis in Mitochondria Isolated from Rat Liver Hepatocytes Incubated with/without Glucagon and/or 6-Diazo-nor-leucine.** All incubations contained 1 mM Glutamine. Incubation concentrations of Glucagon and DON were 10^{-7}M and 5 mM respectively. Cells were pre-incubated with or without DON for 20 minutes, then incubated 30 minutes with or without Glucagon. Results are the means ± S.E.M from 5 separate experiments. Results were compared using Student t-test ($p < 0.05$): * denotes a significant difference compared to the corresponding incubations without DON.

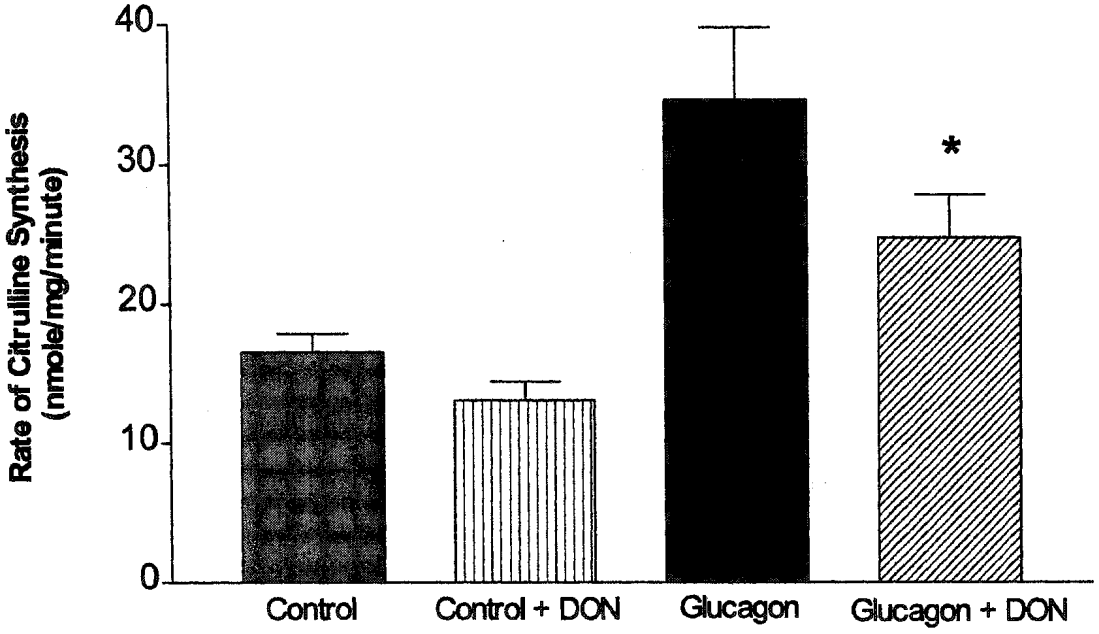

Rate of Citrulline Synthesis
(nmole/mg/minute)
40
30
20
10
0
*
Control
Control + DON
Glucagon
Glucagon + DON

Figure 3.11: **Relationship between of Citrulline Synthesis and N-Acetylglutamate Levels In Mitochondria Isolated from Incubated Hepatocytes.** Preparation of mitochondria from hepatocytes, measurement of the rate of citrulline synthesis and N-acetylglutamate content as described in Material and Methods section: ○ - 1 mM glutamine; ● - 1mM glutamine and 5 mM DON; □ - Glucagon (10^{-7} M) and 1 mM glutamine; ■ - 1mM glutamine and Glucagon (10^{-7} M) and 5 mM DON. Incubation concentrations of Glucagon and DON were 10^{-7} M and 5 mM respectively. $R = 0.729$. (n = 5).

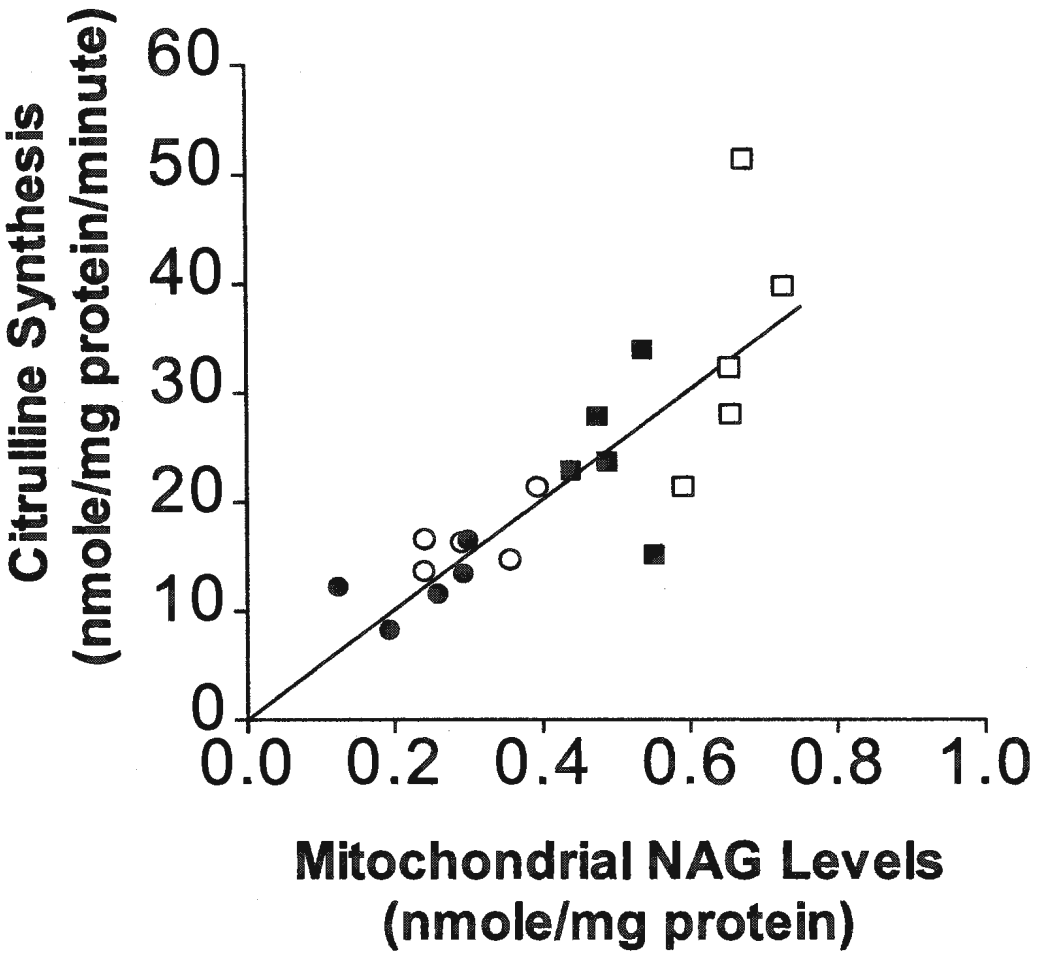
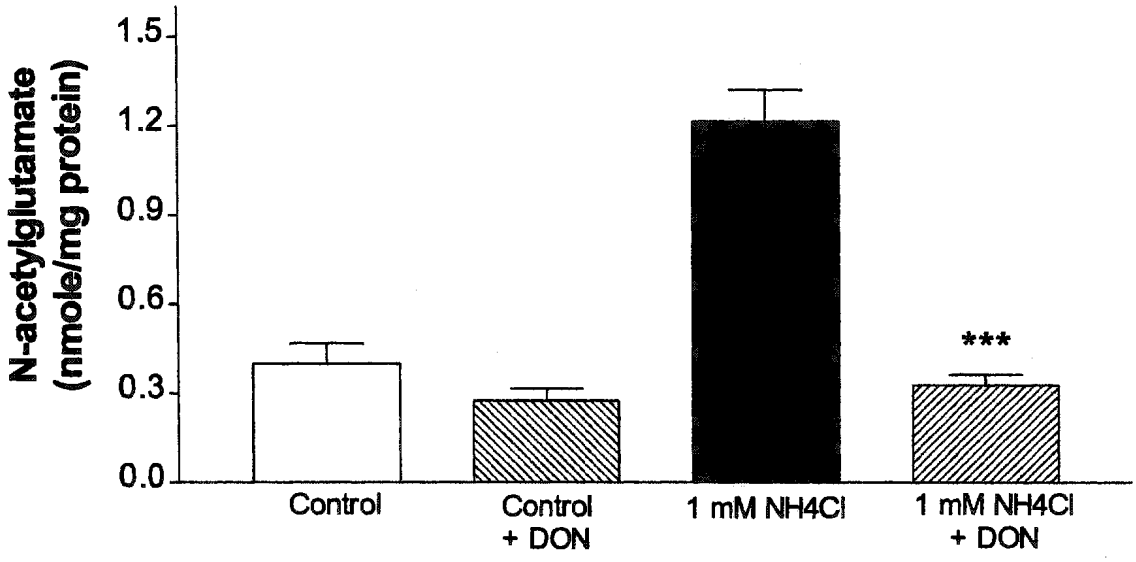

Citrulline Synthesis
(nmole/mg protein/minute)
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Mitochondrial NAG Levels
(nmole/mg protein)

Figure 3.12: N-Acetylglutamate Levels in Mitochondria Isolated from Hepatocytes Incubated with/without NH_4Cl or 6-Diazo-nor-leucine. All hepatocyte incubations contained 1 mM Glutamine. Final Concentrations of DON and NH_4Cl in the incubations were 5 mM and 1 mM respectively. Cells were pre-incubated with or without DON for 20 minutes, then incubated 30 minutes with or without NH_4Cl. Results are means ± S.E.M. of 5 separate experiments. Results were compared using a Student t-test: *** denotes a significant difference ($p < 0.001$) compared to the corresponding incubations without DON.


N-acetylglutamate
(nmole/mg protein)
1.5
1.2
0.9
0.6
0.3
0.0
***
Control
Control
+ DON
1 mM NH4Cl
1 mM NH4Cl
+ DON

% increase). These results suggest that ammonium chloride is not only a substrate for ureagenesis but also seems to be a potent stimulant for the production of the CPS I's essential activator, NAG. As NAG levels increase due to stimulation by ammonia, CPS I activity should subsequently increase resulting in ammonia removal from the mitochondrial matrix. Mitochondria that had been prepared from cells pre-incubated with DON did not show increased NAG levels when incubated with NH_4Cl (0.328 ± 0.034 nmole/mg mitochondrial protein in DON treated vs. 1.12 ± 0.106034 nmole/mg mitochondrial protein without DON) (Figure 3.12).

Measurements of citrulline synthesis in those same mitochondria, however, led to unexpected results. Our perfusion study showed a rectangular hyperbolic relationship between mitochondrial NAG levels and the rate of citrulline synthesis. This relationship was not observed in mitochondria isolated from cells that had been incubated with NH_4Cl. The mitochondrial NAG content was greatly increased compared to controls but there was no increase in the rate of citrulline synthesis (Figure 3.13 and Figure 3.14).

We further investigated these anomalous results (compared to previous results from perfused livers) by measuring the change in ammonia concentration in the hepatocyte incubation medium over the 30-minute incubation period. As depicted in Figure 3.15, the levels of ammonia became depleted quite rapidly in the incubation medium. This rapid decrease in ammonia from the medium over the first five minutes coincided with a significant increase in the rate of citrulline synthesis (Figure 3.16). After the initial increase in the rate of citrulline synthesis, there was an eventual return to control rates over the course of the incubation (Figure 3.16). The rates of citrulline synthesis in the control experiments also decreased with time. After 30 minutes of incubation with ammonia, rate of citrulline synthesis returned to control levels while NAG levels

Figure 3.13: The Rate of Citrulline Synthesis in Mitochondria Isolated from Rat Liver Hepatocytes Incubated with/without NH_4Cl or 6-Diazo-nor-Leucine. All hepatocyte incubations contained 1 mM Glutamine. Final Concentrations of DON and NH_4Cl in the incubations were 5 mM and 1 mM respectively. Cells were pre-incubated with or without DON for 20 minutes, then incubated 30 minutes with or without NH_4Cl.Results are the means ± S.E.M from 5 separate experiments. Results were compared using Student t-test: *denotes a significant difference ($p < 0.05$) compared to the corresponding incubations without DON.

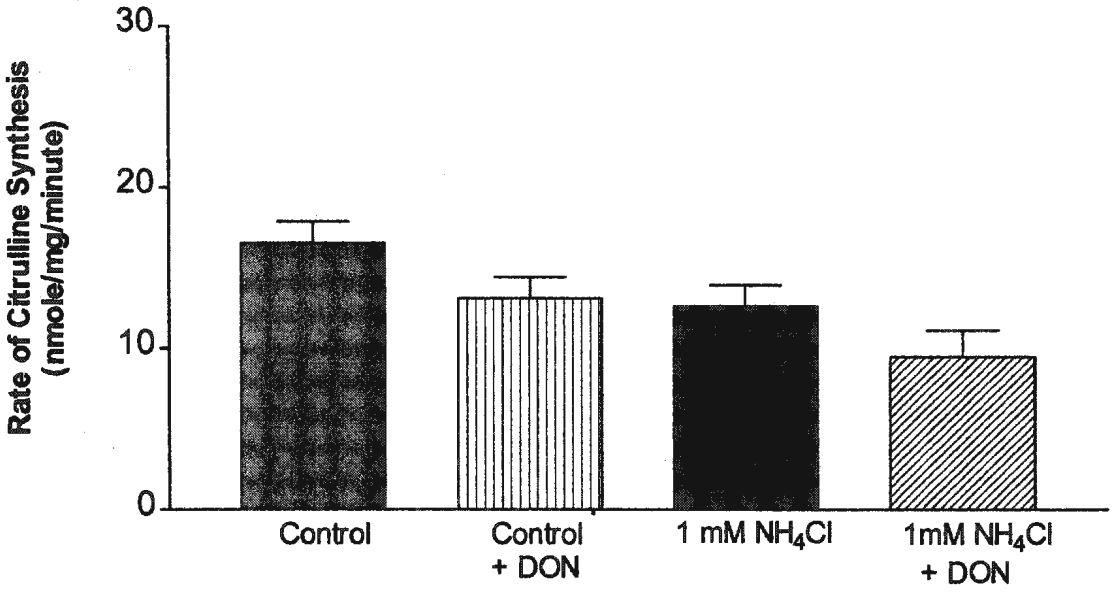

Rate of Citrulline Synthesis
(nmole/mg/minute)
30
20
10
0
Control
Control
+ DON
1 mM NH4Cl
1mM NH4Cl
+ DON

Figure 3.14: **Relationship between Citrulline Synthesis and N-Acetylglutamate Levels in Mitochondria Isolated from Incubated Hepatocytes.** Preparation of mitochondria from hepatocytes, measurement of the rate of citrulline synthesis and N-acetylglutamate content as described in Materials and Methods section: ○ - 1 mM glutamine; ● - 1mM glutamine and 5 mM DON; □ - 1 mM NH_4Cl and 1 mM glutamine (10^{-7} M); ■ - 1mM glutamine and 1 mM NH_4Cl and 5 mM DON. Each data point represents an individual mitochondrial preparation.

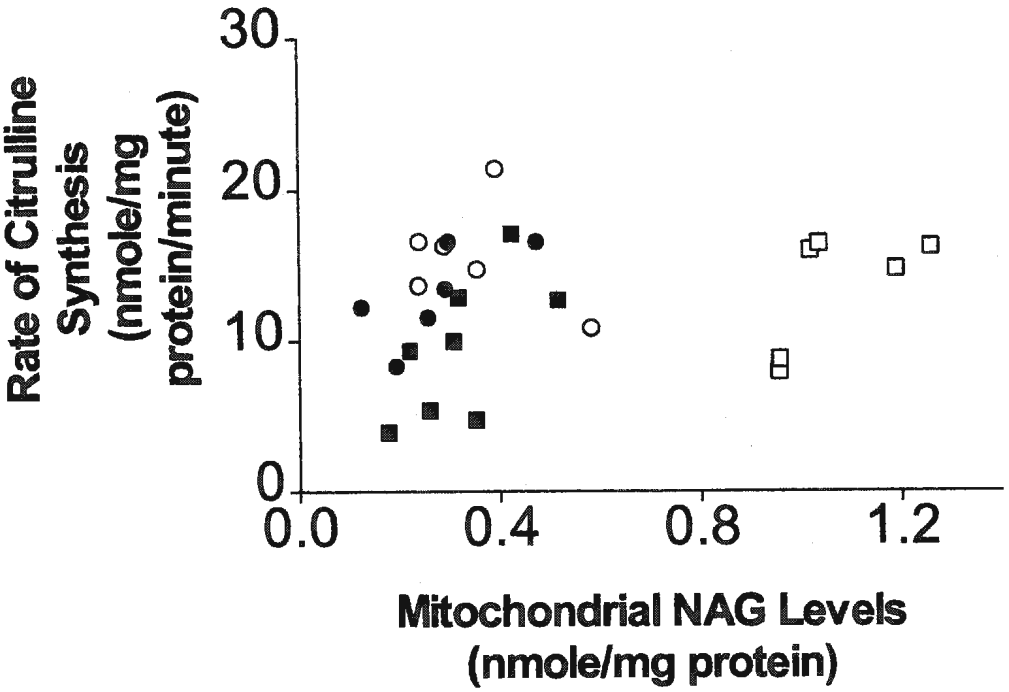

Rate of Citrulline Synthesis (nmole/mg protein/minute)
30
20
10
0
0.0
0.4
0.8
1.2
Mitochondrial NAG Levels (nmole/mg protein)

Figure 3.15: Removal of Ammonia during Hepatocyte Incubation. Ammonia determination was performed on supernatant as described in Materials and Methods: □ - 1 mM glutamine; ■ - 1mM glutamine and NH_4Cl (1 mM). Results are the means ± S.E.M from 3 separate experiments.

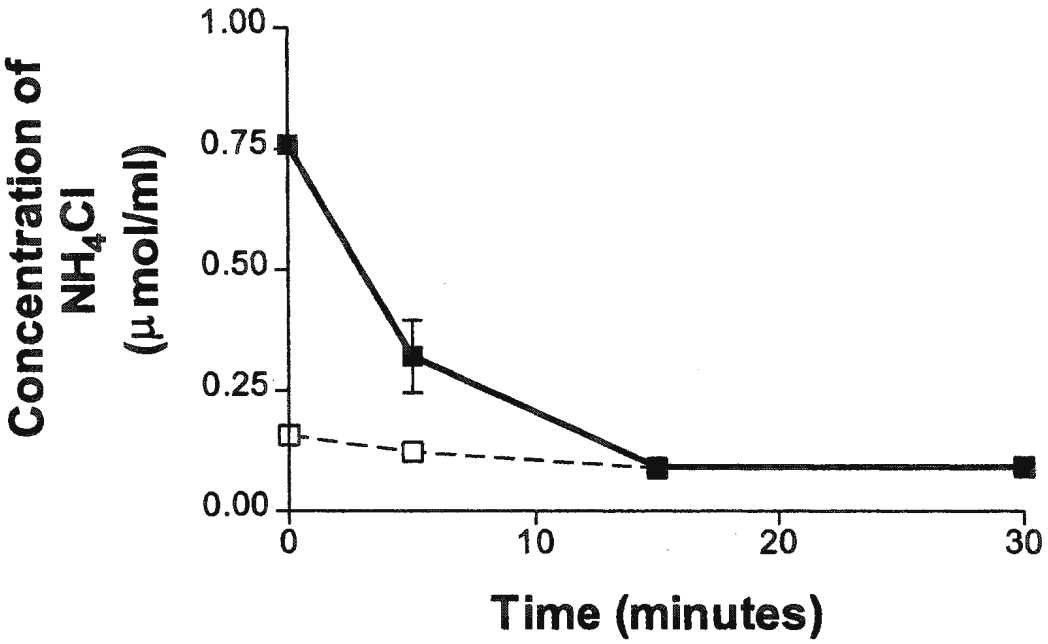
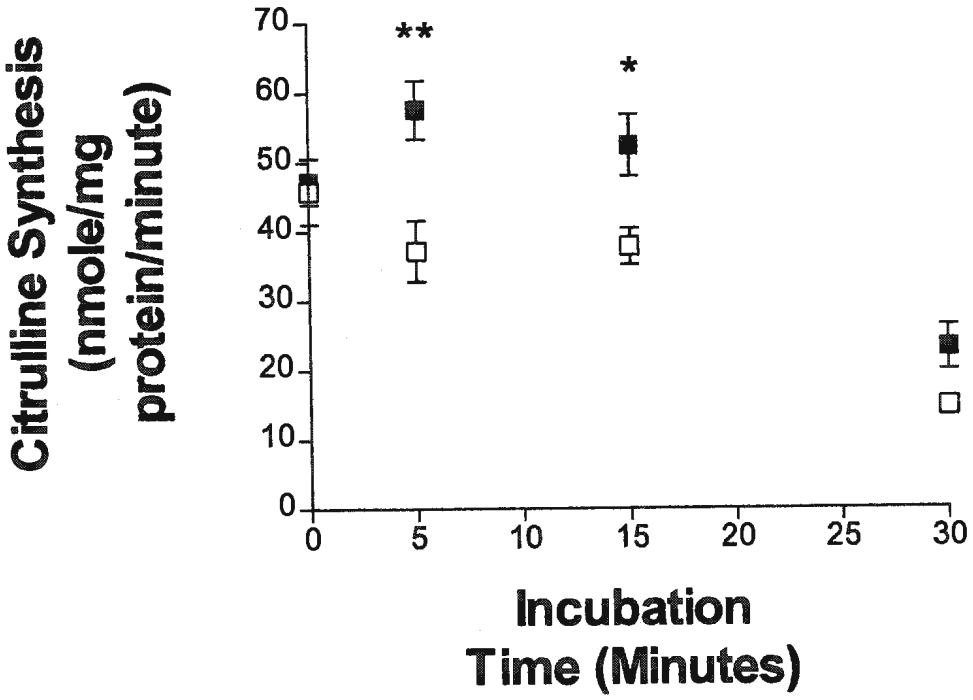

Concentration of NH_4Cl (μ mol/ml)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
Time (minutes)

Figure 3.16: Citrulline Synthesis in Mitochondria Isolated from Hepatocytes Incubated for Different Time Intervals. Incubations were stopped at each specified time interval and preparation of mitochondria from hepatocytes, measurement of the rate of citrulline synthesis were performed as described in Materials and Methods section. Initial incubation concentrations: □ - 1 mM glutamine; ■ - 1mM glutamine and NH_4Cl (1 mM). Results are the means ± S.E.M from 3 separate experiments. Results were compared using Student t-test: *denotes a significant difference compared to the corresponding incubations without NH_4Cl.


Citrulline Synthesis (nmole/mg protein/minute)
70
60
50
40
30
20
10
0
**
*
0
5
10
15
20
25
30
Incubation Time (Minutes)

in the mitochondria were still elevated (Figure 3.10).

These results could be explained if NAG is sequestered within the mitochondria. It may be that NH_4Cl initially activates the production of NAG, which coincides with an increase in citrulline synthesis, but as NH_4Cl becomes depleted in the medium there is no need to further activate the urea cycle. Therefore NAG may become sequestered by some unknown mechanism. Conceivably, it may be transported out of the matrix to the inter-membrane space. This decreased rate of citrulline synthesis cannot be attributed to catabolism of NAG as levels in the mitochondria are still elevated after 30 minutes (Figure 3.12). Hence, there may exist some unknown mitochondrial down-regulation mechanism which can disrupt the relationship between CPS I and NAG.

DISCUSSION

Glucagon's action to increase NAG levels has been well documented but the mechanism of this induction has yet to be elucidated (Hensgens ***et al.***, 1980, Rabier ***et al.***, 1982,Cathelineau ***et al.***, 1980, Cathelineau ***et al.***, 1982). Our results confirmed glucagon's effect on NAG levels both in the liver perfusion and in hepatocyte incubations (Hensgens ***et al.***, 1980, Rabier ***et al.***, 1982). Furthermore, the positive correlation between NAG content and citrulline synthesis seen with our study is in accordance with other work and illustrates the dependence of CPS I on NAG as an obligatory activator. Since it has been shown previously that glucagon does not increase the activity of NAG synthetase (short term), this hormone may exert control by influencing the intramitochondrial concentrations of substrates for this enzyme, in particular glutamate (Rabier ***et al.***, 1982; Cathelineau ***et al.***, 1982).

One of the key contributors to intramitochondrial glutamate within the liver is glutaminase. This enzyme is activated by glucagon and ammonia. We investigated ammonia activation of glutaminase in relation to NAG content. Our results showed that ammonia, when perfused through the liver, increased the levels of NAG and subsequent citrulline synthesis in mitochondria isolated from rat liver. The effect mediated by ammonia suggests that this urea cycle substrate influences its removal, in part, by increasing the production of NAG. These results agree with work performed by Zollner (1981), who investigated the effects of 10 mM NH_4Cl on NAG levels. Although the

concentration employed was much greater than in our study (and not physiological), there was a significant increase in NAG levels due to ammonia. The use of near physiological concentrations (relative to portal vein) of ammonia (1 mM) in our study illustrates that this activation is of physiological importance. Zollner (1981) also showed that ammonia stimulated a corresponding increase in mitochondrial glutamate levels, which he suggested acted as the mediator of ammonia's effect (Zollner, 1981). He also suggested that the increase in glutamate levels might be attributed to glutamate synthesis from ammonia and α-ketoglutarate by the enzyme glutamate dehydrogenase (Zollner, 1981). Since this enzymatic reaction is reversible, he believed that ammonia might mediate its effect by stimulating the reverse reaction. On the other hand, we suggest that since ammonia is known to be a potent activator of glutaminase activity, increased glutamine hydrolysis may account for elevated mitochondrial glutamate levels and subsequent NAG levels.

We examined the relationship between glutaminase and NAG levels through the use of the leucine analog 6-diazo-nor-leucine which we found to be a suitable inhibitor of glutaminase activity both in mitochondrial (Figure 3.5 and 3.6) and hepatocyte (Figure 3.7) preparations. Hepatocyte incubations with this compound showed that glutaminase inhibition significantly lowered the effects of glucagon and ammonia on NAG levels. This inhibition suggests that NAG levels are mediated by increased provision of glutamate as a result of an increase in glutaminase activity. Within glucagon-treated cells, the increased levels of NAG led to an increased rate in citrulline synthesis (Figure 3.11). The lowered NAG levels resulting from pre-incubation of glucagon-treated cells with DON had a predictable effect on the rate of citrulline synthesis within those same

mitochondria. This, however, was not seen in incubations where ammonia was present. The increase in mitochondrial NAG due to ammonia stimulation did not correlate with an increase in the rate of citrulline synthesis. As shown in Figure 3.15, ammonia (1 mM) becomes exhausted in the incubation medium quite rapidly during the initial five minute of incubation. This rapid uptake of ammonia was accompanied by a significant increase in the rate of citrulline synthesis (Figure 3.16). With ammonia exhausted in the medium, however, the rate of citrulline synthesis decreased until rates similar to control were reached (at 30 minutes). These results explain our earlier findings with citrulline synthesis where the 30-minute hepatocyte incubation with ammonia had similar rates as control although NAG levels were much higher (Figure 3.14). This decreased rate of citrulline synthesis cannot be attributed to the catabolism of NAG as levels in the mitochondria are still elevated after 30 minutes. Therefore, there may exist some means by which mitochondria down-regulate the activation of citrulline synthesis despite elevated levels of NAG and optimal assay conditions for citrulline synthesis. The mechanism by which the kinetic relationship between NAG levels and citrulline synthesis is disrupted remains unknown.

In our mitochondrial experiments on citrulline synthesis, we assumed that CPS-I is the rate-limiting enzyme and that the concentration of NAG, as CPS-I activator, is the primary determinant of flux through this enzyme. The literature supports the idea that ornithine transcarbamoylase is unlikely to offer any rate limitation (Meijer, 1990). The role of the ornithine/citrulline antiporter, however, should be considered with respect to the conclusions of this thesis. At present, there exists no evidence that this transporter is a target for acute regulation of the urea cycle, however, Morris and Kepka-Lenhart (2002)

found that this transporter may be regulated chronically. They found that an increased induction of mRNA levels of this transporter occurred when cultured hepatocytes were incubated with a cell-permeable cAMP analogue and/or dexamethasone (combination of both of these agents was synergistic). This induction occurred over several hours (8-12 hours) which is much longer than any incubation performed in my study with glucagon (30 minutes). These findings by Morris and Kepka-Lenhart (2002) provided no evidence that suggested the ornithine/citrulline transporter was a target for acute regulation by glucagon (cAMP). Nevertheless, it is not inconceivable that acute regulation of this transporter could occur and that it could be important for some of the observations in this thesis. In particular, the data in Figure 3.14 suggests that the concentration of NAG cannot be the sole regulator of citrulline synthesis. It is conceivable that an acute effect on the transporter could explain these results, however, there is exists no evidence to support acute regulation of this transporter.

The relationship between liver-type glutaminase and NAG was investigated by Staddon ***et al.*** (1984). Their study involved measuring the change in concentration of whole liver metabolites (glutamate, glutamine, and NAG) as a result of glucagon stimulation. The idea was that a decrease in glutamine levels with a subsequent increase in NAG would suggest the involvement of glutaminase in NAG production. Their results suggested that no such relationship exists as NAG levels increased with no evident decrease in glutamine levels during the first 10 minutes of exposure to glucagon (Staddon ***et al.***, 1984). It is possible that the measurement of whole liver rather than mitochondrial metabolite concentration may not provide an accurate assessment of this relationship. Also, Staddon ***et al.*** (1984) proposed that a lag phase occurred in glucagon's stimulation

of glutaminase that corresponded to a decrease in glutamine seen after the initial ten minutes. They suggested that glucagon must act through another mechanism to increase NAG content because of this lag in glutamine hydrolysis. As seen in our studies, DON's inhibition of glutaminase significantly decreased the effect mediated by glucagon. Our results provided evidence for the existence of a link between NAG content and the stimulation of liver-type glutaminase induced by glucagon and ammonia.

The existence of this relationship within mitochondria increases our understanding of the function of liver-type glutaminase. Glucagon and ammonia increase the amount of ammonia and glutamate in the mitochondrial matrix through stimulation of glutaminase. Ammonia, in turn, activates further hydrolysis of glutamine by a feedback activation mechanism. This type of activation maybe seen as unstable and unsustainable but in the context of our findings this regulatory mechanism makes logical sense. As ammonia levels increase in the liver (e.g. from the intestine), it activates glutaminase to produce glutamate. This glutamate can then be preferentially used by NAG synthetase, increasing the intramitochondrial concentration of NAG. This, in turn, would activate carbamoyl-phosphate synthetase I which will mediate the removal of ammonia. Therefore, activation of glutaminase will indirectly mediate the removal of its product ammonia by its direct provision of glutamate for NAG synthesis.

Chapter Four

References

Barber, E.F., Handlogten, M. E., Vida, T. A., & Kilberg, M. S. (1982) Neutral amino acid transport in hepatocytes isolated from streptozotocin-induced diabetic rats. J. Biol. Chem., 257,14960-7.

Baverel, G. & Lund, P. (1979). A role for bicarbonate in the regulation of mammalian glutamine metabolism. Biochem. J., 184, 599-606.

Beliveau, C. G., Cheung, C. W., Cohen, N. S., Brusilow, S., & Raijman, L. (1993). Regulation of urea and citrulline synthesis under physiological conditions. Biochem. J., 292 , 241-247.

Bernt, E. & Bergmeyer, H. U. (1974). L-Glutamate: UV-assay with glutamate dehydrogenase and NAD. In H.U.Bergmeyer (Ed.), Methods of Enzymatic Analysis (Vol. 4 ed., pp. 1704-1708). New York: Academic Press Inc.

Brosnan, J. T., McPhee, P., Hall, B., & Parry, D. M. (1978). Renal glutamine metabolism in rats fed high-protein diets. Am. J. Physiol, 235, E261-E265.

Brosnan, J. T., Man, K. C., Hall, D. E., Colbourne, S. A., & Brosnan, M. E. (1983). Interorgan metabolism of amino acids in streptozotocin-diabetic ketoacidotic rat. Am. J. Physiol, 244, E151-E158.

Brosnan, J. T., Ewart, H. S., & Squires, S. A. (1995). Hormonal control of hepatic glutaminase. Adv. Enzyme Regul., 35, 131-146.

Brosnan, J. T., Brosnan, M. E., Charron, R., & Nissim, I. (1996). A mass isotopomer study of urea and glutamine synthesis from 15N- labeled ammonia in the perfused rat liver. J. Biol. Chem., 271, 16199-16207.

Cathelineau, L., Rabier, D., Petit, F., & Kamoun, P. (1980). [Role of acetylglutamate in the stimulation of citrullinogenesis by glucagon]. C.R.Seances Acad.Sci.D., 291, 625-627.

Cathelineau, L., Rabier, D., & Coude, F. X. (1982). Acute glucagon treatment in rats fed various protein diets effect on n- acetyl glutamate concentration. Adv. Exp. Med. Biol., 153, 217-228.

Chaney, A. L. & Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. Clin. Chem., 8, 130-132.

Charles, R. (1958). Mitochondriale Citrulline Synthese: een Ammoniak Fixerende en ATP Vebruikend Proces. Ph.D.Thesis University of Amsterdam, Amsterdam.

Cohen, N. S., Cheung, C. W., Kyan, F. S., Jones, E. E., & Raijman, L. (1982). Mitochondrial carbamyl phosphate and citrulline synthesis at high matrix acetylglutamate. J. Biol. Chem., 257, 6898-6907.

Cohen, N. S., Kyan, F. S., Kyan, S. S., Cheung, C. W., & Raijman, L. (1985). The apparent Km of ammonia for carbamoyl phosphate synthetase (ammonia) in situ. Biochem. J., 229, 205-211.

Cohen, N. S., Cheung, C. W., & Raijman, L. (1987). Channeling of extramitochondrial ornithine to matrix ornithine transcarbamylase. J. Biol. Chem., 262, 203-208.

Conti, F. & Minelli, A. (1994). Glutamate immunoreactivity in rat cerebral cortex is reversibly abolished by 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of phosphate-activated glutaminase. J. Histochem. Cytochem., 42, 717-726.

Corvera, S. & Garcia-Sainz, J. A. (1983). Hormonal stimulation of mitochondrial glutaminase. Effects of vasopressin, angiotensin II, adrenaline and glucagon. Biochem. J., 210, 957-960.

Curthoys, N. P. & Weiss, R. F. (1974). Regulation of renal ammoniagenesis. Subcellular localization of rat kidney glutaminase isoenzymes. J. Biol. Chem., 249, 3261-3266.

Curthoys, N. P. & Watford, M. (1995). Regulation of glutaminase activity and glutamine metabolism. Annu. Rev. Nutr., 15, 133-159.

Dhanakoti, S. N., Brosnan, J. T., Herzberg, G. R., & Brosnan, M. E. (1990). Renal arginine synthesis: studies in vitro and in vivo. Am. J. Physiol, 259, E437-E442.

Dodgson, S. J., Forster, R. E., Schwed, D. A., & Storey, B. T. (1983). Contribution of matrix carbonic anhydrase to citrulline synthesis in isolated guinea pig liver mitochondria. J. Biol. Chem., 258, 7696-7701.

Errera, M. & Greenstein, J. P. (1949). Phosphate-activated glutaminase in kidney and other tissues. J. Biol. Chem., 178, 495-509.

Estabrook & R.A. (1967). Mitochondrial respiratory control and polarographic ADP:O ratios. Methods Enzymol., 10, 41.

Gebhardt, R. & Kleemann, E. (1987). Hormonal regulation of amino acid transport system N in primary cultures of rat hepatocytes. Eur. J. Biochem., 166, 339-344.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., 177, 751-766.

Guha, S. R. (1962). Glutaminases of guinea pig liver mitochondria. Enzymologia, D. Haag, 24, 310-326.

Halestrap, A. P. (1989). The regulation of the matrix volume of mammalian mitochondria in vivo and in vitro and its role in the control of mitochondrial metabolism. Biochim. Biophys. Acta, 973, 355-382.

Halestrap, A. P., Quinlan, P. T., Whipps, D. E., & Armston, A. E. (1986). Regulation of the mitochondrial matrix volume in vivo and in vitro. The role of calcium. Biochem. J., 236, 779-787.

Hampson, R. K., Barron, L. L., & Olson, M. S. (1983). Regulation of the glycine cleavage system in isolated rat liver mitochondria. J. Biol. Chem., 258, 2993-2999.

Haussinger, D. (1998). Hepatic glutamine transport and metabolism. Adv.Enzymol.Relat Areas Mol.Biol., 72, 43-86.

Haussinger, D. & Sies, H. (1979). Hepatic glutamine metabolism under the influence of the portal ammonia concentration in the perfused rat liver. Eur. J. Biochem., 101, 179-184.

Haussinger, D., Weiss, L., & Sies, H. (1975). Activation of pyruvate dehydrogenase during metabolism of ammonium ions in hemoglobin-free perfused rat liver. Eur. J. Biochem., 52, 421-431.

Haussinger, D., Gerok, W., & Sies, H. (1983). Regulation of flux through glutaminase and glutamine synthetase in isolated perfused rat liver. Biochim. Biophys. Acta, 755, 272-278.

Haussinger, D., Soboll, S., Meijer, A. J., Gerok, W., Tager, J. M., & Sies, H. (1985). Role of plasma membrane transport in hepatic glutamine metabolism. Eur. J. Biochem., 152, 597-603.

Haussinger, D., Lamers, W. H., & Moorman, A. F. (1992). Hepatocyte heterogeneity in the metabolism of amino acids and ammonia. Enzyme, 46, 72-93.

Hensgens, H. E., Verhoeven, A. J., & Meijer, A. J. (1980). The relationship between intramitochondrial N-acetylglutamate and activity of carbamoyl-phosphate synthetase (ammonia). The effect of glucagon. Eur. J. Biochem., 107, 197-205.

Herzfeld, A. & Raper, S. M. (1976). Enzymes of ornithine metabolism in adult and developing rat intestine. Biochim. Biophys. Acta, 428, 600-610.

Hong, J., Salo, W. L., Lusty, C. J., & Anderson, P. M. (1994). Carbamyl phosphate synthetase III, an evolutionary intermediate in the transition between glutamine-dependent and ammonia-dependent carbamyl phosphate synthetases. J. Mol. Biol., 243, 131-140.

Jackson, A. A. (1995). Salvage of urea-nitrogen and protein requirements. Proc. Nutr. Soc., 54, 535-547.

Jackson, A. A., Danielsen, M. S., & Boyes, S. (1993). A noninvasive method for measuring urea kinetics with a single dose of [$^{15}N^{15}N$] urea in free-living humans. J. Nutr., 123, 2129-2136.

Jois, M., Hall, B., Fewer, K., & Brosnan, J. T. (1989). Regulation of hepatic glycine catabolism by glucagon. J. Biol. Chem., 264, 3347-3351.

Joseph, S. K. & McGivan, J. D. (1978). The effect of ammonium chloride and glucagon on the metabolism of glutamine in isolated liver cells from starved rats. Biochim. Biophys. Acta, 543, 16-28.

Joseph, S. K., McGivan, J. D., & Meijer, A. J. (1981). The stimulation of glutamine hydrolysis in isolated rat liver mitochondria by Mg2+ depletion and hypo-osmotic incubation conditions. Biochem. J., 194, 35-41.

Kalra, J. & Brosnan, J. T. (1973). Localization of glutaminase in rat liver. FEBS Lett., 37, 325-328.

Kawamoto, S., Sonoda, T., Ohtake, A., & Tatibana, M. (1985). Stimulatory effect of arginine on acetylglutamate synthesis in isolated mitochondria of mouse and rat liver. Biochem. J., 232, 329-334.

Kilberg, M. S., Handlogten, M. E., & Christensen, H. N. (1980). Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. J. Biol. Chem., 255, 4011-4019.

Klingman, J. D. & Handler, P. (1958). Partial purfication and properties of renal glutaminase. J. Biol. Chem., 232, 369-380.

Kovacevic, Z. & McGivan, J. D. (1983). Mitochondrial metabolism of glutamine and glutamate and its physiological significance. Physiol Rev., 63, 547-605.

Krebs, H. A. (1935). Metabolism of amino acids IV. The synthesis of glutamine from glutamic acid and ammonia and the enzymic hydrolysis of glutamine in animal tissues. Biochem. J., 29, 1951-1969.

Krebs, H. A., Cornell, N. W., Lund, P., & Hems, R. (1974). Isolated liver cells as experimental material. In Regulation of Hepatic Metabolism (pp. 726-750). Munksgaard, Copenhagen.

Kvamme, E., Tveit, B., & Svenneby, G. (1970). Glutaminase from pig renal cortex. I. Purification and general properties. J. Biol. Chem., 245, 1871-1877.

Kvamme, E., Torgner, I. A., & Roberg, B. (1991). Evidence indicating that pig renal phosphate-activated glutaminase has a functionally predominant external localization in the inner mitochondrial membrane. J. Biol. Chem., 266, 13185-13192.

Lacey, J. H., Bradford, N. M., Joseph, S. K., & McGivan, J. D. (1987). Increased activity of phosphate-dependent glutaminase in liver mitochondria as a result of glucagon treatment of rats. Biochem. J., 194, 29-33.

Lemieux, G., Aranda, M. R., Fournel, P., & Lemieux, C. (1984). Renal enzymes during experimental diabetes mellitus in the rat. Role of insulin, carbohydrate metabolism, and ketoacidosis. Can. J. Physiol Pharmacol., 62, 70-75.

Lenzen, C., Soboll, S., Sies, H., & Haussinger, D. (1987). pH control of hepatic glutamine degradation. Role of transport. Eur. J. Biochem., 166, 483-488.

Low, S. Y., Salter, M., Knowles, R. G., Rennie, M. J., & Pogson, C. I. (1990). Effect of L-glutamate-gamma-hydrazide on the transport and metabolism of L-glutamine in rat liver cells and isolated mitochondria. Biochem. Soc. Trans., 18, 1239-1240.

Low, S.Y, Taylor, P. M., Hundal, H. S., Pogson, C. I, Rennie, M. J. (1992). Transport of L-glutamine and L-glutamate across sinusoidal membranes of rat liver. Effects of starvation, diabetes and corticosteroid treatment. Biochem. J., 284, 333-40

Low, S. Y., Salter, M., Knowles, R. G., Pogson, C. I., & Rennie, M. J. (1993). A quantitative analysis of the control of glutamine catabolism in rat liver cells. Use of selective inhibitors. Biochem. J., 295, 617-624.

Lund, P. & Wiggins, D. (1987). Inhibition of carbamoyl-phosphate synthase (ammonia) by Tris and Hepes. Effect on Ka for N-acetylglutamate. Biochem. J., 243, 273-276.

Lusty, C. J. (1978). Carbamoyl-phosphate synthetase I of rat-liver mitochondria. Eur. J. Biochem., 85, 373-383.

Lusty, C. J. (1981). Catalytically active monomer and dimer forms of rat liver carbamoyl-phosphate synthetase. Biochemistry, 20, 3665-3675.

Lusty, C. J. (1983). The molecular structure and function of carbamoyl phosphate synthetase I. Trans. N.Y. Acad. Sci., 41, 103-115.

Matsuno, T. & Hirai, H. (1989). Glutamine synthetase and glutaminase activities in various hepatoma cells. Biochem. Int., 19, 219-225.

McCudden, C. R. & Powers-Lee, S. G. (1996). Required allosteric effector site for N-acetylglutamate on carbamoyl- phosphate synthetase I. J. Biol. Chem., 271, 18285-18294.

McGivan, J. D. & Bradford, N. M. (1983). Characteristics of the activation of glutaminase by ammonia in sonicated rat liver mitochondria. Biochim. Biophys. Acta, 759, 296-302.

McGivan, J. D. & Lynch, A. (1988). Characterisation and partial purification of the Na+-dependent neutral amino acid transporting system from bovine kidney brush border membranes. Contrib. Nephrol., 63, 37-42.

McGivan, J. D., Lacey, J. H., & Joseph, S. K. (1980). Localization and some properties of phosphate-dependent glutaminase in disrupted liver mitochondria. Biochem. J., 192, 537-542.

Meijer, A. J. & van Woerkom, G. M. (1978). Control of the rate of citrulline synthesis by short-term changes in N- acetylglutamate levels in isolated rat-liver mitochondria. FEBS Lett., 86, 117-121.

Meijer, A. J., Lamers, W. H., & Chamuleau, R. A. (1990). Nitrogen metabolism and ornithine cycle function. Physiol Rev., 70, 701-748.

Metges, C. C. (2000). Contribution of microbial amino acids to amino acid homeostasis of the host. J. Nutr., 130, 1857S-1864S.

Monod, J. & Wyman, F. C. J. P. (1965). Kinetic Relationships between enzyme and activators. J. Mol. Biol., 12, 88-118.

Morimoto, B. H., Brady, J. F., & Atkinson, D. E. (1990). Effect of level of dietary protein on arginine-stimulated citrulline synthesis. Correlation with mitochondrial N-acetylglutamate concentrations. Biochem. J., 272, 671-675.

Morita, T., Mori, M., & Tatibana, M. (1982). Regulation of N-acetyl-L-glutamate degradation in mammalian liver. J. Biochem.(Tokyo), 91, 563-569.

Morris, S. M., Jr. (2002). Regulation of enzymes of the urea cycle and arginine metabolism. Annu. Rev. Nutr., 22, 87-105.

Morris, S. M., Jr. & Kepka-Lenhart D. (2002) Hormonal induction of hepatic mitochondrial ornithine/citrulline transporter mRNA. Biochem Biophys Res Commun., 294, 749-52.

Nyunoya, H., Broglie, K. E., Widgren, E. E., & Lusty, C. J. (1985). Characterization and derivation of the gene coding for mitochondrial carbamyl phosphate synthetase I of rat. J. Biol. Chem., 260, 9346-9356.

Pacitti, A. J., Inoue, Y., & Souba, W. W. (1993). Characterization of Na(+)-independent glutamine transport in rat liver. Am. J. Physiol, 265, G90-G98.

Pinkus, L. M. & Windmueller, H. G. (1977). Phosphate-dependent glutaminase of small intestine: localization and role in intestinal glutamine metabolism. Arch. Biochem. Biophys., 182, 506-517.

Potter, M. D. & Powers-Lee, S. G. (1993). Direct photoaffinity labeling of rat liver carbamoyl phosphate synthetase I with ATP. Arch. Biochem. Biophys., 306, 377-82

Powers-Lee, S. G. & Corina, K. (1987). Photoaffinity labeling of rat liver carbamoyl phosphate synthetase I by 8-azido-ATP. J. Biol. Chem., 262, 9052-9056.

Rabier, D., Briand, P., Petit, F., Parvy, P., Kamoun, P., & Cathelineau, L. (1982). Acute effects of glucagon on citrulline biosynthesis. Biochem. J., 206, 627-631.

Remesy, C., Morand, C., Demigne, C., & Fafournoux, P. (1988). Control of hepatic utilization of glutamine by transport processes or cellular metabolism in rats fed a high protein diet. J. Nutr., 118, 569-578.

Rodriguez-Aparicio, L. B., Guadalajara, A. M., & Rubio, V. (1989). Physical location of the site for N-acetyl-L-glutamate, the allosteric activator of carbamoyl phosphate synthetase, in the 20-kilodalton COOH- terminal domain. Biochemistry, 28, 3070-3074.

Rubio, V., Britton, H. G., & Grisolia, S. (1983). Mitochondrial carbamoyl phosphate synthetase activity in the absence of N-acetyl-L-glutamate. Mechanism of activation by this cofactor. Eur. J. Biochem., 134, 337-343.

Sayre, F. W. & Roberts, E. (1958). Preparation and some properties of a phosphate-activated glutaminase from kidney. J. Biol. Chem., 233, 1128-1134.

Shapiro, R. A., Clark, V. M., & Curthoys, N. P. (1979). Inactivation of rat renal phosphate-dependent glutaminase with 6-diazo- 5-oxo-L-norleucine. Evidence for interaction at the glutamine binding site. J. Biol. Chem., 254, 2835-2838.

Shigesada, K., Aoyagi, K., & Tatibana, M. (1978). Role of acetylglutamate in ureotelism. Variations in acetylglutamate level and its possible significance in control of urea synthesis in mammalian liver. Eur. J. Biochem., 85, 385-391.

Sies, H. (1978). The use of perfusion of liver and other organs for the study of microsomal electron-transport and cytochrome P-450 systems. Methods Enzymol., 52, 48-59.

Simmer, J. P., Kelly, R. E., Rinker, A. G., Jr., Scully, J. L., & Evans, D. R. (1990). Mammalian carbamyl phosphate synthetase (CPS). DNA sequence and evolution of the CPS domain of the Syrian hamster multifunctional protein CAD. J. Biol. Chem., 265, 10395-10402.

Smith, E. M. & Watford, M. (1988). Rat hepatic glutaminase: purification and immunochemical characterization. Arch. Biochem. Biophys., 260, 740-751.

Sonoda, T. & Tatibana. M.(1983). Purification of N-acetyl-L-glutamate synthetase from rat liver mitochondria and substrate and activator specificity of the enzyme. J. Biol. Chem., 258, 9839-44

Souba, W. W. (1991). Glutamine: a key substrate for the splanchnic bed. Annu. Rev. Nutr., 11, 285-308.

Staddon, J. M., Bradford, N. M., & McGivan, J. D. (1984). Effects of glucagon in vivo on the N-acetylglutamate, glutamate and glutamine contents of rat liver. Biochem. J., 217, 855-857.

Stewart, P. M. & Walser, M. (1980). Short term regulation of ureagenesis. J. Biol. Chem., 255, 5270-5280.

Svenneby, G., Torgner, I. A., & Kvamme, E. (1973). Purification of phosphate-dependent pig brain glutaminase. J. Neurochem., 20, 1217-1224.

Szweda, L. I, & Atkinson, D. E. (1990). Response of rat liver glutaminase to pH, ammonium, and citrate. Possible regulatory role of glutaminase in ureagenesis. J. Biol. Chem., 265, 20869-73

Tamarappoo, B. K., Singh, H. P., & Kilberg, M. S. (1994). Protein modification of glutamine transporters in SV40-transformed hepatocytes and immunodetection of proteins associated with hepatic system N transport activity. J. Nutr., 124, 1493S-1498S.

Vincent, N., Martin, G., & Baverel, G. (1989). Simultaneous synthesis and degradation of glutamine in isolated rat liver cells. Effect of vasopressin. Biochim. Biophys. Acta, 1014, 184-188.

Watford, M. (1991). The urea cycle: a two-comparment system. In K.F.Tipton (Ed.), Essays in Biochemistry (pp. 49-58). London, UK: Portland Press.

Watford, M., Smith, E. M., & Erbelding, E. J. (1984). The regulation of phosphate-activated glutaminase activity and glutamine metabolism in the streptozotocin-diabetic rat. Biochem. J., 224, 207-214.

Welbourne, T. C. (1987). Interorgan glutamine flow in metabolic acidosis. Am. J. Physiol, 253, F1069-F1076.

Wu, G. (1995). Urea synthesis in enterocytes of developing pigs. Biochem. J., 312 (Pt 3), 717-723.

Yokota, S. & Mori, M. (1986). Immunoelectron microscopical localization of ornithine transcarbamylase in hepatic parenchymal cells of the rat. Histochem. J., 18, 451-457.

Zollner, H. (1981). Regulation of urea synthesis. The effect of ammonia on the N-acetylglutamate content of isolated rat liver cells. Biochim. Biophys. Acta, 676, 170-176.