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STIMULATION OF THE UREA CYCLE IN ENDOTOXEMIC RATS

by

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Abstract

Muscle wasting is common in infection. There is increased muscle proteolysis without concomitant increased muscle protein synthesis so negative nitrogen balance results. Much work has focused on this aspect of catabolic states. However, increased release of amino acids into circulation leads to elevated amino nitrogen levels. The liver is the site for detoxification of amino nitrogen wastes, particularly ammonia. The urea cycle, located only within the periportal hepatic acinus, converts neurotoxic ammonia to urea which may be excreted in the urine. The response of the urea cycle subsequent to endotoxin administration was of specific interest to this work.

In this study, a single intraperitoneal injection of *E. coli* lipopolysaccharide (LPS, 0127 : B8) at a sublethal dose (3 mg/kg) resulted in a nearly three-fold increase in urea excretion from fasted rats between 12-24 hours after injection, compared to saline-injected controls. Substrates for urea synthesis were also measured. Ammonia levels were not changed in liver, muscle, blood or plasma 18 hours after endotoxin injection. There were significant changes in the total concentrations of amino acids in both arterial plasma and in the liver. Levels of alanine and glutamine, the primary glucogenic amino acids, were also affected subsequent to LPS injection. Alanine was significantly increased in all sample groups (skeletal muscle, liver, blood and plasma from hepatic portal vein and abdominal aorta)in response to LPS. Glutamine concentrations were not increased in the liver nor arterial blood but were significantly elevated in hepatic portal venous plasma

and blood and in arterial plasma. There was, however, a significant decrease in muscle glutamine which is in agreement with findings in the literature. There appears to be an increase in circulating ureagenic substrates as well as an increase in the hepatic content of amino acids between 12-24 hours after LPS-injection in rats.

The urea cycle exhibits compartmentation of metabolism. The first two enzymes (carbamyl phosphate synthetase I, CPS I; ornithine transcarbamylase, OTC) are located within the mitochondrion while the remaining enzymes (argininosuccinate synthetase, AS; argininosuccinate lyase, AL; arginase) are found in the cytosol. Assays of the enzymes of the urea cycle were carried out 24 hours after injection with LPS or saline. There was no increase in total activity, *in vitro*, of any of the enzymes post-LPS treatment. A previous study in our lab has found that liver protein increases by 18% in response to LPS but this increase is not sufficient to account for the three-fold increase of urea excretion from rats.

Regulation of flux through the urea cycle enzymes has been previously reported to be limited by the activity of CPS I. In isolated intact rat liver mitochondria, the rate of citrulline synthesis was doubled in rats 24 hours after injection of LPS compared to the saline controls. The mitochondrial portion of the urea cycle, therefore, was stimulated in response to LPS. *N*-acetylglutamate (NAG), an allosteric activator of CPS I, was hypothesized to play a role in the increased citrulline-synthesizing capacity of mitochondria isolated from endotoxemic rats. NAG levels were measured in quick frozen mitochondrial samples from endotoxemic and saline control rats in which citrulline

synthesis had also been measured in a separate aliquot. GC-MS analysis by Dr. Kratz (Baltimore, MD) determined that mitochondria from endotoxemic animals contained significantly more NAG (50%) compared to saline controls. Analysis of the relationship between citrulline synthesis from these mitochondria and the NAG content showed a significant positive correlation between NAG and the rate of citrulline synthesis. Finally, to assess the effect of NAG upon citrulline synthesis, we carried out experiments utilizing uncoupled mitochondria in the presence of varying concentrations of exogenous NAG. At 24 hours post-injection, mitochondria from LPS-injected animals synthesized citrulline at double the rate of mitochondria from the saline control animals (as seen in intact, coupled mitochondrial studies) but as the concentration of exogenous NAG reached 2.0-10.0 mM the difference in citrulline synthesizing capacity between the two groups was abolished. We propose, therefore, that by 24 hours after injection with bacterial lipopolysaccharide the increased urea synthesis in rats involves both increased ureagenic substrate load (amino acids) and CPS I activation by increased mitochondrial NAG levels.

This work is dedicated to my guardian angel.

Craig Purchase

June 28th, 1965 - September 29, 1990

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To properly acknowledge contributions made to any work one must reflect upon what has been achieved or gained. I see this work as a journey, partly as a new scientist and, more importantly as a person. So from this vantage point I must thank those persons who have helped me and taught me along this road.

I would first like to thank the people I worked with daily. Specifically, Gehan Mabrouk, Steven Ewart, Bea Hall and, particularly, Dan O'Sullivan who became a friend along the journey. I learned a great deal from these people. I would also like to acknowledge the support I received from Barry Walters and Desmond Pink from our neighboring lab.

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During my Masters Programme I was also afforded the opportunity to teach, first as a student lab demonstrator, and then in the position of Lab Instructor. The latter position presented several defining moments for me. Working closely with Dr. Margaret Brosnan I learned much about how professional integrity and basic human compassion can be mixed and applied to students who really need it-not just those who show obvious promise. I admire this quality and I will remember it no matter where this road takes me. Of course, Dr. Brosnan has been integral in the completion of my research, and I thank her for this as well as helping me in many, many other situations.

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As for my supervisor, Seán Brosnan, what can I say? It is difficult to put into words what I have learned from him. As a scientist I have learned to demand excellence from myself as he does. This extends from research through teaching and I, truly, respect him as a scientist and as a teacher. I will strive to become a teacher of his caliber throughout my career. Under Dr. Brosnan I received so many opportunities to teach and to travel and I am grateful. But, I have also learned a great deal about what I can expect of myself and

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Abbreviations

AEC: amino-ethyl-cysteine
AL: argininosuccinate lyase
AS: argininosuccinate synthetase
cAMP: cyclic adenosine monophosphate
CPS I: carbamyl phosphate synthetase I; ammonia-specific
DAG: diacylglycerol
EDL: extensor digitorum longus; muscle
EDTA: ethylenediamine tetraacetic acid
EGTA: ethyleneglycol - bis - (β-aminoethyl ether) - *N, N, N',N'*-tetraacetic acid
Hepes: *N*-2-ethanesulphonic acid
HepG2: hepatoma G2 cell culture
IF: interferon
IL: interleukin (1, 2, 4, 6)
IP_3: inositol triphosphate
LBP: LPS binding protein
LDL: Low density lipoprotein
LPS: lipopolysaccharide; endotoxin
mRNA: messenger ribonucleic acid
NAG: N-acetylglutamate
NO: nitric oxide
NOS: nitric oxide synthase (i-inducible; c-constitutive)
OTC: ornithine transcarbamylase
PEP: phosphoenolpyruvate
PIP_2: phosphatidylinositol bisphosphate
PKA: protein kinase A
PKC: protein kinase C
PMA: phorbol myristate acetate
TNF: tumour necrosis factor

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Chapter 1
General Introduction

His flesh is consumed away , that it cannot be seen; and his bones that were not seen stick out.

(Job 33:21-King James version of the Holy Bible.)

General Introduction

It has long been known that in conditions such as sepsis (Watters *et al*, 1986; Beisel, 1975), burn injury (Nygren *et al*, 1995), post-surgical states (Heindorff *et al*, 1991, 1992, 1994), and during cancer cachexia (Balkwill *et al*, 1987) many homeostatic mechanisms are disrupted. Most notable is the outpouring of lean body mass, in the form of amino acids, from muscle, their metabolism in liver and the resulting negative nitrogen balance. This is in direct contrast with the events surrounding starvation in which the body takes great care to spare its peripheral protein depots choosing, rather, to deplete the liver of its excess protein first and then to mobilize fat from adipose tissue.

In 1876 Robert Koch conclusively determined that infections were the direct result of invading microbes. Upon invasion by a pathogen, the host's immune system initiates a host response which may be mild, comprising a mild fever and chills, or severe, leading to irreversible shock and death (Siegal *et al*, 1995; Watters *et al*, 1986). Within the host, microbes may colonize a specific tissue, such as the lung in pneumonia. However, in dire situations the organism may enter the bloodstream, a condition known as sepsis (Downs *et al*, 1995).

Of greatest clinical importance are gram-negative bacteria. They possess potent cell wall lipopolysaccharides (LPS) or endotoxins (Old, 1987) which evoke the host immune response. Originally, LPS was believed to exert its toxicity directly (Reitschel & Brade, 1992). It is now known, however, that LPS enlists help within the host via endogenous cytokines (Old, 1987). Cytokines, or some of the "polypeptide hormones

which transmit signals between cells" such as tumour necrosis factor (TNF), (Fong *et al*, 1989) and interleukins 1 (IL-1) (Fong *et al*, 1989) and 6 (IL-6) (DeBandt *et al*, 1994a) have been identified as important mediators of the pathogenesis of endotoxemia. Recently another cachectic factor has been isolated during the progression of cancer (Tisdale *et al*, 1996) but this appears to be specific for cancer cachexia.

Endotoxin is the most potent stimulus for TNF production (Old, 1988). Studies which utilized administration of human recombinant TNF (hr-TNF) reproduced the physiological symptoms of endotoxemia (Beutler & Cerami, 1988). IL-1, though structurally different from TNF, elicits host responses similar to those elicited by LPS and TNF. Clearly, the immune system is equipped with mediators with overlapping function. This, likely, provides a failsafe as well as a potent net of defence when all mediators act in concert to defeat an infection.

Concomitant with the immune response and alteration in cytokine levels, there are also disruptions in the hormonal milieu. During endotoxemia there are elevated levels of insulin (Yelich & Filkins, 1980), glucagon (Zenser *et al*, 1974), glucocorticoids (Melby & Spink, 1958), and catecholamines (Groves *et al*, 1973). These disruptions in hormone levels may lead to disruptions in fuel metabolism and may play significant roles in the redistribution of body proteins during infection as the body works to regain metabolic homeostasis.

A major shift in protein metabolism begins at the onset of the host response to infection. Sparing of peripheral protein is replaced by the mobilization of these amino

acids for the requirements of the acute phase response to infection (Powanda, 1977).

The host acute phase response includes: (Wannemacher, 1977)

1. fever;
2. rapid multiplication of white blood cells;
3. increased hepatic gluconeogenesis (particularly from amino acids);
4. decreased hepatic synthesis of housekeeping proteins (eg., albumin) and increased hepatic synthesis of immunoproteins (eg., complement); and
5. increased free amino acid liberation from skeletal muscle (Wannemacher, 1977): coupled with increased transport into the liver (Pacitti *et al*, 1992; Inoue *et al*, 1995).

Metabolism in these animals shifts from economy to survival mode.

Bacterial Endotoxins

History

In 1892, Richard Pfeiffer discovered that *Vibrio cholerae* produce a toxin which is neither heat-labile, nor excreted by the intact bacteria (Pfeiffer, 1892). Pfeiffer concluded that the toxin must be inside the microbe and coined the term endotoxin from the Greek word, 'endo' for within.

Classification of bacteria began with the application of the Gram stain, a blue dye. The bacteria which retained the dye subsequent to treatment were termed gram positive and those which did not retain the blue dye, gram-negative (Con, 1969). Only gram-negative bacteria possess endotoxins (Boivin & Mesrobeanu, 1935; Westphal *et al*, 1952).

Analytical studies of gram-negative bacteria during the 1930s and 1940s determined that the heat-stable toxin was comprised of polysaccharide, lipid and protein (Boivin & Mesrobeanu, 1935). The term lipopolysaccharide resulted from a similar analysis of *S. marcescens* (Shear & Turner, 1943). Interest in endotoxin was fuelled by a turn of the century physician. William Coley discovered that malignant tumours often regressed if the patient was treated with a mixture of killed bacteria (Coley-Nauts, 1980).

Lipopolysaccharide

Pure LPS can be obtained by extraction from cell walls. Phenol-water and phenol-chloroform-petroleum ether solvent extractions are commonly used. The lipid component of LPS, lipid A, is embedded on the outer membrane of bacterial cells. Lipid A constitutes much of the external surface of the membrane. The polysaccharide protrudes from the surface like a branch and it is composed of two distinct parts: **(Figure 1.1)**

1. the core oligosaccharide which attaches to lipid A
2. the O-specific chain: Comprised of many repeating oligosaccharide units; it serves as the surface antigen and is unique to a given LPS and the strain of bacteria. There are as many distinct LPSs as there are gram-negative strains. The core region has little structural variability compared to the O-specific chain. *Eschericia coli* (*E.coli*) and *Salmonella typhimurium*, together, present more than one hundred serotypes, yet only 6 and 1 core types, respectively (Brade *et al*, 1988).

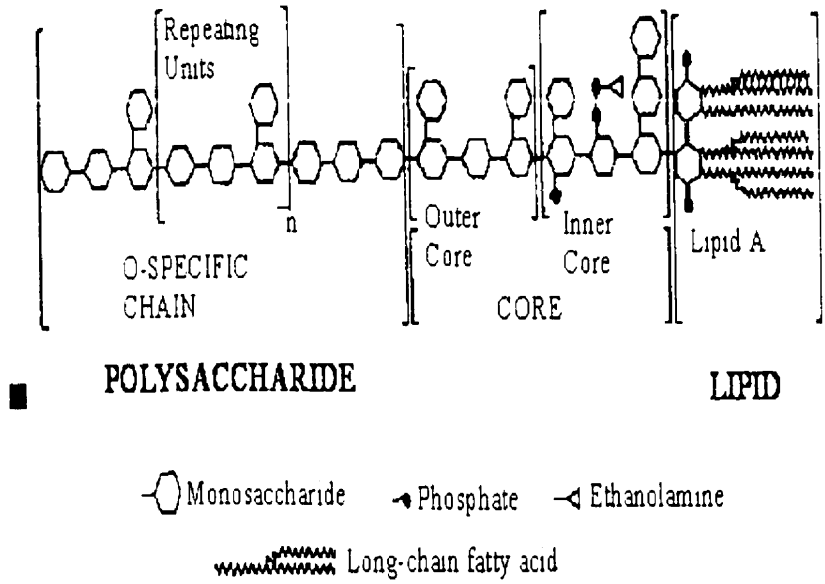


Figure 1.1 Schematic Structure of Bacterial Lipopolysaccharide. (Modified from Brade *et al*, 1988)

The region of lipid A proximal to the inner oligosaccharide core has been reported to signal production of TNF and IL-1 (Levin *et al*, 1988). Lipid A possesses the minimal structure requirement for full expression of endotoxicity (Brade *et al*, 1988). The structure of lipid A consists of a hydrophilic region containing bisphosphorylated D-glucosamine disaccharide and a hydrophobic region containing fatty acids (Westphal *et al*, 1952).

Biological Activity of Endotoxins

The effects of endotoxins, within the host, begin once the LPS is actually released from the cell walls of gram-negative bacteria (Pfeiffer, 1892). Liberation of LPS may be accomplished in a number of ways:

1. natural death of the microbe;
2. bacteriolysis by antibiotics, complement or phagocytosis (Levin *et al*, 1988);
3. and multiplication of the colony.

Biological effects of LPS are elicited through immunocyte activation (Paul, 1993). These host cells, such as macrophages, then secrete mediators: TNFα, IL-1 and IL-6 activating a myriad of physiological responses collectively known as an immune response. The effects of endotoxin include: non-specific activation of the immune system, activation of the complement cascade, and induction of the characteristic shock syndrome (Morrison & Ryan, 1987).

The severity of the effects of endotoxemia is roughly proportional to the level of

systemic LPS. Mild, localized infections give rise to low to moderate levels of LPS release and a moderate, controlled response with beneficial effects for the immune system in future infections. In severe infections, however, invading bacteria may gain access to the bloodstream.

In this temperate environment bacteria thrive and multiply quickly and circulating LPS is markedly elevated. As a direct consequence there is exaggerated release of TNFα (Old 1988), IL-1 (Libby *et al*, 1986), IL-6 (Paul, 1993) and nitric oxide (Clancy & Abramson, 1995). Multiple organ system failure and irreversible shock often result (Downs *et al*, 1995).

Endotoxin Interacts With Host Cell Receptors

LPS is not believed to interact, directly, with circulating immunocytes. In many species, including humans, there is a soluble serum protein which binds to LPS. It is known as LPS binding protein (LBP). Levels of LBP prior to immune challenge are <0.5 μg/ml but they increase approximately 100-fold within 24 hours of induction of the acute phase response (Schumann *et al*, 1990). The structure of LBP has been deduced via cDNA sequencing. Human and rabbit LBP are 60 kD, hepatically synthesized glycoproteins (Schumann *et al*, 1990). LBP binds endotoxin via lipid A so that LBP serves mainly as a carrier protein for LPS in which the variable O-chains remain exposed (Cohen *et al*, 1995). The LPS-LBP complex interacts with membrane receptors (CD 14)

on monocytes/macrophages (Wright *et al*, 1990) and neutrophils (Cohen *et al*, 1995). Tissues which lack a membranous CD14 receptor, such as endothelial and epithelial cells, utilize a serum CD14 to engage local immune responses. These cells do not utilize LBP (Cohen *et al*, 1995).

CD14 maps to chromosome 5, near several myeloid-specific growth factors (Goyert *et al*, 1988) and the protein is a 5 kD glycoprotein. CD14 receptors are mobile within the lipid phase of the membrane (Wright *et al*, 1990). They have, however, no transmembrane domain but are linked to the bilayer via a phosphatidylinositol linkage (Haziot *et al*, 1988).

Interaction of the LPS-LBP complex with CD14 'activates' the macrophage and stimulates rapid and abundant transcription of TNF-α and IL-6 genes (Cohen *et al*, 1995). Monoclonal antibody abolition of CD14 prevents the induction of TNF-α (Wright *et al*, 1990). *In vivo* studies using transgenic mice have demonstrated that if CD14 is overexpressed the animal is highly susceptible to the lethal effects of LPS (Ferrero *et al*, 1993). Conversely, genetically altered mice who lack CD14 expression are 10-100-fold less sensitive to LPS than normal mice (Cohen *et al*, 1995).

There are membrane proteins other than CD14 which bind LPS but their functions are, as yet, unknown (Cohen *et al*, 1995). Acetylated LDL receptors, which are important in cholesterol metabolism, can bind lipid A in liver cells but the purpose is strictly to speed clearance. There is no immune activation (Hampton *et al*, 1991). B cells (lymphoma culture), which are devoid of CD14 also respond to LPS (Sibley *et al*, 1988).

Signal Transduction: From Endotoxin to Action

The LPS-LBP-CD14 complex initiates the first signal from bacterial endotoxin to the macrophage. The results include: the respiratory burst, increased levels of cytokines and differentiation of the inactive macrophage; yet only a little is known about the post-membrane events which effect them. Evidence has, however, accumulated which implicates protein kinase C (PKC) as integral in subcellular signalling during interaction with LPS. Activation of macrophage PKC with phorbol myristate acetate (PMA) and studies involving elevation of intracellular calcium via the ionophore A23187, resulted in marked increases of inflammatory species as well as a respiratory burst (Hamilton & Adams, 1987).

Activation of PKC occurs via agonist binding to receptors, coupled to a G-protein (G_q). Membrane phosphatidylinositol-bisphosphate (PIP_2) is converted into inositol trisphosphate (IP_3) and diacylglycerol (DAG), each with specific second messenger roles. IP_3 effects an efflux of calcium from intracellular stores while DAG interacts with PKC and helps translocate it to the plasma membrane where it is active. LPS or lipid A, in concentrations as low as 10 ng/ml, can initiate hydrolysis of PIP_2 to IP_3 (Hamilton & Adams, 1987). Recent studies have verified that PKC activity during immune challenge is blocked if PKC is prevented from translocating to the plasma membrane (Seguin *et al.* 1995).

Stimulation of cyclic AMP (cAMP) production within macrophages has an inhibitory effect upon the action of LPS. Agents which cause the G-protein, $G\alpha_s$, to be

locked in an activated form, such as cholera toxin, suppress LPS-induced production of TNFα mRNA in a time- and dose-dependent manner (Tannenbaum & Hamilton, 1989). Similar effects have been attributed to dibutyryl cAMP, a synthetic, cell permeable analogue of cAMP. Stimulation of protein kinase A (PKA) is associated with decreased TNFα mRNA (Seguin *et al,* 1995).

Inhibition of G-protein G_i, with pertussis toxin inhibited the response of B cells and macrophages to LPS (Jakway & DeFranco, 1986). Clearly, multiple second messenger systems are affected by LPS. Concurrent activation of PKC and inactivation of PKA lead to increased transcription of TNFα, IL-1 and IL-6 and an immediate, cytotoxic respiratory burst. It is noteworthy that the effects of LPS in macrophages may be amplified if they are first 'primed' by interacting with low levels of interferon gamma (IFNγ) (Hamilton & Adams, 1987). Now macrophages begin to produce three groups of powerful mediators:

1. proteins: TNF, IL-1, IL-6 and IL-8. Produced de novo, not from a 'store';
2. reactive Oxygen Species: Superoxide, hydrogen peroxide and nitric oxide; and
3. lipids: Prostaglandin E_2, thromboxane A_2, and platelet activating factor.

Low levels of these endogenous mediators are beneficial to the host. As levels elevate so, too, do the potential dangers.

Cachexia

The term cachexia arose from the Greek, *kakos* (bad) plus *exein* (state or habit of the body). It is a wasting syndrome characterized by rapid and persistent weight loss even when adequate nutrition is available (Beutler, 1988). The condition is present in sepsis (Old, 1987), malignant states (Balkwill *et al.* 1987; Tisdale & Smith, 1993), after surgery (Heindorff *et al.* 1991), and in the progression of AIDS (Grunfeld & Feingold, 1992). Weight loss from *Trypanosoma bruceii* infected rabbits may reach 50% of initial body weight (Rouzer & Cerami, 1980). Fat stores are depleted during cachexia (Winter *et al.* 1995; Beutler *et al.* 1985b) but the primary source of weight loss is the peripheral protein, i.e. skeletal muscle, which accounts for approximately 45-50% of total body protein (Tessitore *et al.* 1993).

During cachexia the body adapts to systemic dyshomeostasis. Oxygen consumption increases as does hepatic gluconeogenesis. In adipose tissue, genes for enzymes such as lipoprotein lipase, glycerolphosphate dehydrogenase, fatty acyl binding protein, acetyl CoA carboxylase and fatty acid synthase are suppressed by TNF-α (Winter *et al.* 1995) thus, the deposition of fat in adipose tissue is prevented. There are elevated serum triglycerides while hepatic β-oxidation and ketogenesis are suppressed. And, as tissues such as the erythrocytes and the brain are obligatory glucose users, gluconeogenesis from amino acids is markedly elevated. The brain, which can adapt to ketone bodies as fuel, is not presented with the option due to low blood ketone concentrations. Another drain upon body protein depots is the increased demand for acute

phase protein synthesis within the liver at the onset of the acute phase response.

Cachexia is a complicated metabolic sequence of events orchestrated by a common factor which was, historically, termed cachectin (Old,1987). Cloning technology has shown that cachectin and TNF-α, are the same molecule (Beutler *et al*, 1985a; Beutler *et al*, 1985b; Aggarwal *et al*, 1985; Pennica *et al*, 1984). TNF-α, was originally known as a cytotoxic agent capable of causing necrosis and regression of mouse sarcoma tumours (Green *et al*, 1976).

The Structural Characteristics of TNF

TNF can be further classified into TNFα and TNFβ. The latter is also known as lymphotoxin as it is secreted from lymphocytes rather than macrophages. The two TNFs share 50% sequence homology and 35% identity. TNFα and β can interact with the same receptors in some tissues (Aggarwal *et al*, 1985).

The genes coding for human TNFα are located on the short arm of chromosome 6 and are around 3 kilobases in length. Murine TNFα is located on chromosome 17 (Beutler, 1988). The protein, itself, is a single polypeptide of 157 amino acids, with a homotrimeric tertiary structure comprised of 17 kilodalton subunits (Beutler, 1988).

Biological Action of TNFα

TNFα levels within the body are markedly elevated during chronic bacterial infection (Waage *et al*, 1987), parasitic infection (Scuderi *et al*, 1986) malignant diseases

(Balkwill *et al*, 1987), and AIDS (Patry *et al*, 1995). The levels of TNFα , produced in rabbits in response to an LPS injection, peak within 2 hours. TNFα is cleared from the blood rapidly, as well, with a half-life of 6-7 minutes (Beutler *et al*, 1985b). The elevated cytokine has been implicated in many aspects of the host response to bacterial endotoxin. The degree of detriment to the host directly correlates with the level of TNFα produced (Waage *et al*, 1987).

There is a specific, high affinity receptor for TNFα in many tissues of the body. It has been shown, using intravenously-injected, radioiodinated recombinant TNFα, that within 8 minutes 31%, 30%, 8.8%, 7.8%, 1.8% of injected label is bound to receptors on rabbit liver, skin, gastrointestinal tract, kidney and lung cells, respectively. The bound TNFα is then rapidly degraded (Beutler *et al*, 1985b). There are two receptor types which are known to bind TNF: 55 kD (TR55) and 75 kD (TR75) (Wiegmann *et al*, 1992).

The signal transduction pathways of TNFα have yet to be fully elucidated. Given the pleiotrophic nature of TNFα action it is likely that multiple signal transduction pathways are involved. It has been suggested that several of the biological activities of TNFα occur via cyclooxygenase pathways (Evans *et al*, 1989). Recently, evidence has arisen for a novel pathway, known as the sphingomyelin pathway, which may mediate some of the effects of TNFα and IL-1 (Liu *et al*, 1994).

Sphingomyelin is found in the outer leaflet of most mammalian cells (Kolesnicke & Golde, 1994). Binding of TNFα or IL-1 to surface receptors results in hydrolysis of membrane sphingomyelin to ceramide which acts as second messenger. Ceramide then

stimulates a serine/threonine kinase, CAP kinase, seven-fold (Liu *et al*, 1994). The sphingomyelin pathway has been implicated in translocation of transcription factors to the nucleus (NFkB), HIV-1 replication, IL-2 transcription, and apoptotic cell damage and, thus, in the signalling of TNF and IL-1 (Kolesnick & Golde, 1994). The postmembrane events can be replicated using synthetic ceramide analogues (Liu *et al*, 1994). It is noteworthy that diacylglycerol also activates CAP kinase but ceramide does not activate PKC (Kolesnick & Golde, 1994). CAP kinase has recently been shown to interact with *Raf 1*. *Raf 1* interacts with GTP-*ras* thereby linking the cell surface with important signalling cascades (Yao *et al*, 1995).

Metabolic Adaptation During Sepsis: The Pivotal Role of Skeletal Muscle.

Fuel Dyshomeostasis

Host metabolism during the anorectic state which accompanies sepsis differs both qualitatively and quantitatively from prolonged fasting (Powanda, 1977). The overall variety of nutritional responses is broad and may include most major metabolic pathways of most cells (Beisel, 1977). During starvation, as nitrogen intake decreases, bodily protein is spared, in part, by the substitution of fat for carbohydrate as the principal fuel. This spares muscle protein and urinary nitrogen excretion decreases (Young *et al*, 1973). In severe infection, however, excretion of nitrogenous compounds increases (Beisel 1975, 1977; Wannemacher, 1977; Hasselgren *et al*, 1988). It has been calculated from literature sources that starvation-adapted individuals lose approximately 4 g nitrogen per day while

a septic patient loses nearly 15 g of nitrogen per day (Wannemacher, 1977). As there are no protein 'stores' within the body and skeletal muscle comprises ~ 45% (De Bandt *et al*, 1994a; Tessitore *et al*, 1993) of bodily protein, its wasting has a profound effect upon body mass.

The metabolic responses to bacterial infection begin promptly following the initiation of the host response and evolve as a series of interrelated events (Beisel, 1975; Powanda, 1977):

1. protein catabolism proceeds at a level which exceeds that which can be accounted for by anorexia;
2. ketogenesis is depressed and levels of hepatic and plasma ketones decrease;
3. septic patients have inappropriately elevated plasma insulin as there is concomitant hypoglycaemia;
4. gluconeogenesis is stimulated early in the host response (Halestrap, 1989; Felig, 1973);
5. there are profound differences in protein metabolism in muscle such as increased proteolysis (Beisel, 1975; Hasselgren *et al*, 1988) with decreased (Vary & Kimball, 1992) or unchanged levels of synthesis. Therefore, muscle protein is depleted;
6. in the liver there is a markedly increased influx of amino acids (Inoue *et al*, 1995) as well as increased synthesis of acute phase proteins, proliferation of phagocytic cells, and elevated production of peptide cytokine mediators.

Ultimately, every aspect of the host defence process is dependent upon the ability of host cells to synthesize proteins. In this light, skeletal muscle protein depletion can be viewed as a sacrifice necessary to meet the precursor needs of the emergent anabolic processes of the host defence. If proteolysis were blocked via clinical intervention it would be

deleterious to the patient's recovery (Wannemacher, 1977; Fischer & Hasselgren, 1991).

Certain metabolic processes may follow a biphasic pattern of sequential change. In the early stages of infection there is hyperglycemia in the presence of peripheral insulin resistance as well as impaired sensitivity to glucose in the pancreatic β cells (Rocha *et al.* 1973) which inevitably gives way to hypoglycemia as the condition worsens and body glycogen depletes (Beisel, 1975). Glucose metabolism also plays an important role in the metabolic sequelae of sepsis and will be discussed further in the next section.

It has also been shown that injection of bacterial endotoxin into rats results in an increased hepatocyte cell volume (Qian & Brosnan, 1996). Cell volume increases are reported to stimulate gluconeogenesis, glycogenolysis and urea synthesis within the liver (Halestrap, 1989; Häussinger & Lang, 1991).

Gluconeogenesis and the Endocrine Hormonal Milieu

During sepsis the energy requirements of the host dramatically increase. The hormonal alterations in early infection have been interpreted as an adaptation to the demands of the immune challenge. In severely injured patients cortisol, catecholamines and glucagon are elevated (Hasselgren *et al.* 1988). Increased glucocorticoids are also present during gram negative infections (Melby & Spink, 1958). During early sepsis plasma levels of glucose, insulin, glucagon (Rocha *et al.* 1973; Rayfield *et al.* 1977), and catecholamines (Groves *et al.* 1973) are notably increased. The elevated stress hormones, in the presence of peripheral insulin resistance, serve to increase hepatic glucose output

through stimulation of glycogenolysis (Beisel, 1975) and depressed glycogen synthesis. Gluconeogenesis is also increased (Felig, 1973).

The increased glycogenolysis, in the absence of food intake, leads to depletion of glycogen stores. Obligatory glucose users such as erythrocytes and the brain require a continuous supply of glucose for survival. This need for glucose, particularly in the brain, is enhanced by the lack of ketone production and serious demands are placed upon peripheral sources for gluconeogenic amino acids. Glucose is also required as the only source of ATP in those tissues which become anoxic as a result of infection-induced damage. As sepsis progresses, gluconeogenic capacity decreases and evidence suggests that phosphoenolpyruvate carboxykinase (PEPCK) is inhibited within 18 hours after endotoxin injection with little change in the amount of enzyme present (Horton *et al*, 1994).

Protein Metabolism in Skeletal Muscle During the Host Response

Synthesis Versus Proteolysis

Skeletal muscle plays a pivotal role in the metabolic sequelae of a variety of catabolic states. In fact, the utilization of amino acids encompasses virtually every aspect of the host's defence against invading microbes. The total protein content of skeletal muscle depends upon the balance between protein synthesis and degradation. Generally, emphasis is placed upon the difference of the two processes, rather than the individual synthetic or proteolytic rates. Clearly, peripheral protein is lost in these states but there is

some controversy as to how the final negative balance is reached. Increased rates of proteolysis of muscle protein have been widely reported during sepsis (Beisel, 1975; Beisel, 1977; Wannemacher, 1977; Hasselgren *et al*, 1988), in response to IL -1 *in vitro* (Baracos *et al*, 1983; Cooney *et al*, 1994), after endotoxin administration to rats (Beutler *et al*, 1985a; Fong *et al*, 1990), after TNFα treatment (Flores *et al*, 1990; Fong *et al*, 1989), after elective surgery (Heindorff *et al*, 1991), and during AIDS cachexia (Patry *et al*, 1995). There are also reports in which muscle protein synthetic rates remain unchanged (Hasselgren *et al*, 1986c) or increase (Clowes, 1988) during sepsis. However, even when protein synthesis was reported to increase there was an even greater increase in proteolysis so that net protein loss resulted (Clowes, 1988).

Sepsis affects individual muscle fibre types differently (Hasselgren *et al*, 1986c). Depressed protein synthesis is more pronounced in fast twitch rat muscle fibres. The decrease in protein synthesis appears to result from inhibition of chain elongation during translation (Vary & Kimball, 1992).

There is also evidence of insulin resistance in septic rat muscle. Increased proteolytic breakdown of extensor digitorum longus (EDL) muscle from septic rats is maintained even in the presence of elevated insulin levels (Hasselgren *et al*, 1987). Amino acid uptake is also impaired in septic rat soleus muscle by 30-50% within 5 hours following a *Salmonella enteriditis* insult (Wannemacher *et al*, 1974).

A Mechanism for Increased Skeletal Muscle Proteolysis During Catabolic States

Evidence, which has been accumulating recently, is shedding light upon the post-membrane events which lead to wasting of, particularly, myofibrillar proteins. Ubiquitin, a peptide involved in targeting cellular proteins for degradation via an ATP-dependent, nonlysosomal proteosome in the cytoplasm (Hilt & Wolf, 1996) has been studied. Research has shown that rat skeletal muscle possessed significantly elevated ubiquitin mRNA if treated with TNFα (Garcia-Martinez *et al*, 1994) and during cancer cachexia (Llovera *et al*, 1995) but not in response to IL-1 (Garcia-Martinez *et al*, 1995). It has also been shown that glucocorticoids cause increases in rat ubiquitin mRNA as well as in both free and conjugated ubiquitin (Hasselgren *et al*, 1996). There was excessive ubiquitinization of myofibrillar proteins while free ubiquitin was in excess in the sarcoplasm (Hasselgren *et al*, 1996). Treatment of the rats with RU38486, a potent glucocorticoid antagonist, attenuated the response only partially, however, suggesting a more complex regulation of ubiquitin induction than can be explained by glucocorticoids alone.

Regulation of Skeletal Muscle Wasting

Increased muscle protein breakdown, particularly of myofibrillar proteins, and increased hepatic protein synthesis have long been hallmarks of sepsis and severe injury. Survival in these states is correlated with increased visceral amino acid uptake and increased hepatic protein synthesis (Clowes, 1988). Cytokines and glucocorticoids are,

likely, key mediators (Fischer & Hasselgren, 1991) but catecholamines and glucagon may have roles in the sequelae of sepsis as well (Bessey *et al*, 1984).

In muscle, there is conflicting evidence for the role of glucocorticoids in severe infection. In *in vitro* studies, in which the hindquarters of normal rats are perfused with glucocorticoids at concentrations comparable to those seen during sepsis, there is a 30-50% decrease in myofibrillar protein synthesis but an increase in proteolysis (Kayali *et al*, 1987). However, the increased myofibrillar protein breakdown in septic rats was not normalized when treated with the glucocorticoid antagonist RU38486 (Hall-Ångeras *et al*, 1990).

Reports of the effects of glucocorticoids upon the liver have been more consistent in the literature. Plasma glucocorticoid concentrations increase during gram negative infections (Melby & Spink, 1958). *In vitro*, dexamethasone potentiates acute phase protein synthesis if isolated rat hepatocytes are co-incubated in the presence of LPS (Koj *et al*, 1984). Dexamethasone alone, however, does not stimulate the increased protein synthesis (Baumann *et al*, 1987). There is also increased sodium-dependent transport of amino acids into hepatic plasma membrane vesicle preparations (HPMVs) from endotoxin-treated rats which is attenuated by 20-60% if the rats are pre-treated with RU38486 (Inoue *et al*, 1995).

There is interplay between hormones and cytokines. Cytokines can increase production and secretion of glucocorticoids while glucocorticoids can potentiate the effects of cytokines. It has been suggested that there may be an integrated response

between skeletal muscle and liver during sepsis. In fact, some of the effects generally attributed to cytokines may well reflect glucocorticoid activity (Fischer & Hasselgren, 1991). Septic rats exhibit increased total muscle protein loss and myofibrillar protein loss but this is significantly blunted in adrenalectomized rats (Fischer & Hasselgren, 1991). In isolated hepatocytes, or in an hepatoma cell line, a combination of IL-6, IL-1, IFβ2 and TNFα are required to induce the entire acute phase response (Baumann *et al*, 1987). Within the liver, Kuppfer cells are likely the most important source of acute phase-regulating cytokines in sepsis and endotoxemia (Fischer & Hasselgren, 1991).

The role of cytokines in muscle protein turnover is less clear. It has been proposed that muscle proteolysis, during sepsis, is brought about by a circulating factor which is a cleavage product of IL-1 (Clowes, 1988). It was found that a partially purified supernatant from stimulated monocytes added *in vitro* to rat muscles increased proteolysis (Baracos *et al*, 1983; Moldawer *et al*, 1987). However, when IL-1 activity derived from activated monocyte supernatant was blocked using IL-1 antibodies, protein breakdown was unchanged (Moldawer *et al*, 1987).

The availability and use of recombinant cytokines have also yielded conflicting results. Recombinant TNFα (rTNFα) infused into cancer patients resulted in increased amino acid efflux from the forearm (Starnes *et al*, 1988). However, neither rTNFα nor rIL-1 had any effect upon muscle protein breakdown or synthesis in mice (Moldawer *et al*, 1987). Similarly in rats, administration of rTNFα caused fever but did not affect protein turnover (Kettlehut *et al*, 1988). The reliability of recombinant cytokines in

mediating *in vivo* responses has been debated. Recombinant technology does not account for post-translational modifications which may be necessary for bioactivity. In addition, when administered on their own, neither glucocorticoids nor other possibly important mediators may be present at appropriate concentrations (Hall-Angerås *et al*, 1990). rTNFα has, however, been implicated in inducing a concomitant increase in adrenal weight and plasma corticotrophin in rats while decreasing carcass nitrogen and causing negative nitrogen balance (Mealy *et al*, 1990). *In vitro*, muscle amino acid uptake is reduced by incubation of muscle with the catabolic hormones but not when the hormones are infused *in vivo* thereby suggesting that cytokines play the more important role *in vivo*.

Individual Amino Acids Play Key Roles

During infection in man the pattern of individual plasma free amino acid concentrations differs markedly from that during simple starvation. Phenylalanine and tryptophan are released into the free amino acid pool of muscle but are non-metabolizable in that tissue so plasma concentrations rise (Wannemacher, 1977). Studies show that there is marked incorporation of these amino acids into hepatic proteins synthesized *de novo* but plasma levels remain high as synthetic rates proceed more slowly than the release of phenylalanine and tryptophan from skeletal muscle (Wannemacher *et al*, 1974).

Branched chain amino acids serve a dual role within the muscle. The intramuscular levels of valine, isoleucine and leucine increase as proteolysis proceeds but their plasma concentrations are depressed during sepsis (Wannemacher, 1977) Branched

chain amino acids may be utilized both as an energy source for the muscle (Wannemacher, 1977; Beisel, 1977) and may provide amino nitrogen for transamination of pyruvate to alanine (Beisel, 1977).

Alanine, the principle gluconeogenic substrate (Felig *et al*, 1973), is released in large quantities, by muscle, during the host response (Beisel, 1975; Beisel, 1977; Wannemacher, 1977) while alanine re-uptake by the muscle is impaired (Hasselgren *et al*, 1986b). It also serves as a nitrogen carrier from the periphery to the liver. The free alanine is, subsequently, taken up by the liver at an accelerated rate (Inoue *et al*, 1995; Wannemacher *et al*, 1974). There is an elevation of muscle to liver alanine exchange via the glucose-alanine cycle (Felig, 1973).

Glutamine also serves a variety of vital functions. In addition to its role as an inter-tissue carbon and nitrogen carrier, it serves as a fuel for cells both of the intestine (Souba, 1991) and of the immune system (Newsholme *et al*, 1985). It has also been surmised to provide much of the nitrogen during ureagenesis (Nissim *et al*, 1992) while there is some evidence which runs conversely to this notion (Moorman, *et al*, 1994). Rennie and associates have hypothesized that there is a net efflux of glutamine from muscle which limits protein synthetic rates during catabolic states. They found a positive correlation between muscle glutamine content and the rate of protein synthesis (MacLennan *et al*, 1987).

Arginine is another key amino acid during infection. It is an intermediate in the urea cycle (Krebs & Henseleit, 1932) and an allosteric activator for *N*-acetylglutamate

synthetase, whose product serves as an allosteric activator for the first enzyme of the urea cycle, CPS I (Tatibana & Shigesada, 1976). Arginine is also substrate for NOSs (Clancy & Abramson, 1995). Nitric oxide (NO) is a labile free radical which provides non-specific immunity against invading microorganisms (Clancy & Abramson, 1995). NO is also known as endothelium relaxing factor, (Palmer *et al*, 1987) which helps regulate blood pressure, and has been implicated in irreversible shock during sepsis (Downs *et al*, 1995; Clancy & Abramson, 1995).

There are two NO synthases. Constitutive NO synthase (cNOS) is calcium-dependent and produces low levels of NO (Clancy & Abramson, 1995). Inducible NOS (iNOS), which is expressed in response to LPS, IL-1, and TNFα produces high, sustained levels of NO (Clancy & Abramson, 1995). iNOS is expressed in many tissues including macrophages (Clancy & Abramson, 1995) as well as hepatocytes (Curran *et al*, 1989) and Kupffer cells (Marletta *et al*, 1988). In perfused livers of rats which have been injected with endotoxin there is a time- and dose-dependent increase of NO synthesis (Wettstein *et al*, 1994).

The Urea Cycle

Introduction

All of the evidence surrounding dyshomeostasis and accelerated protein catabolism inevitably leads to a discussion on the disposal of protein catabolic waste products. Urea was first isolated from urine in 1773 by Rouille and gained fame as the

first organic compound to have been synthesized from inorganic precursors. In ureotelic species it is synthesized from ammonia and bicarbonate in the liver. The pathway of urea synthesis, originally termed the ornithine cycle, was elucidated in 1932 by Sir Hans Krebs (Krebs & Henseleit, 1932). It is localized to the periportal region of the hepatic acinus (Häussinger *et al*, 1992).

In mammals, the primary role of urea synthesis has been discussed and two schools of thought have emerged. The first: urea serves as an irreversible ammonia removal pathway (Krebs & Henseleit, 1932; Meijer *et al*, 1990). The second: urea is a way of removing excess bicarbonate produced during amino acid catabolism and maintaining acid-base homeostasis (Bean & Atkinson, 1984).

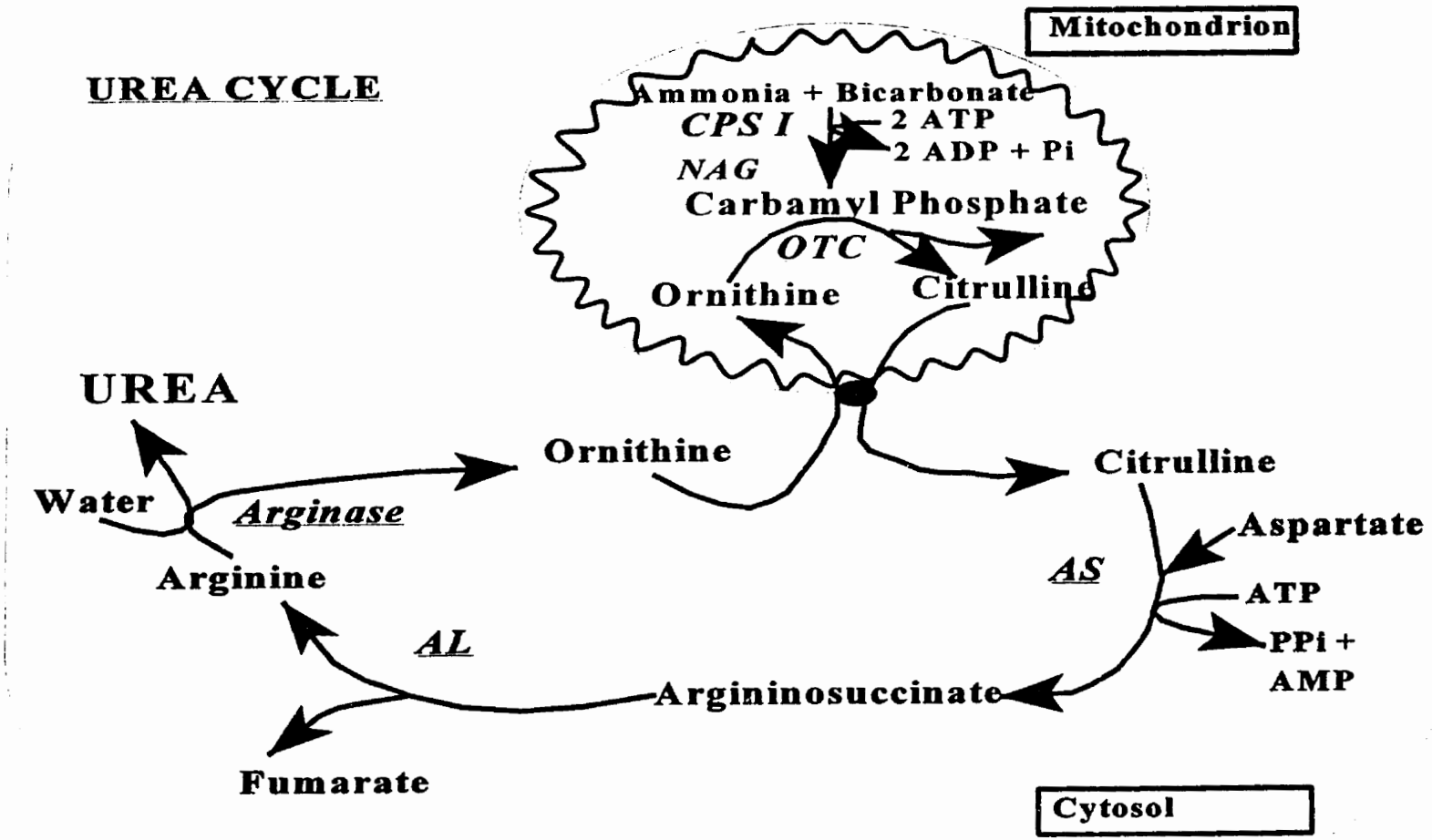
Protein catabolism results in increased levels of free amino acids which are, then, subject to a variety of fates. They may be: (1) oxidized; (2) used as gluconeogenic substrates; and (3) recycled into protein synthesis. During catabolic states there is increased oxidation and gluconeogenesis from amino acids as well as acute phase protein synthesis. As gluconeogenesis proceeds excess ammonia is cleared by the urea cycle. Similarly, as oxidation proceeds, excessive amounts of ammonium and bicarbonate ions as well as carbon dioxide are evolved (Meijer, 1995). These must be removed from circulation. Normal fasting arterial ammonia is < 50 μM (Cooper & Plum, 1987). Ammonia is a potent neurotoxin at greater than 200 μM (Cooper & Plum, 1987) thus irreversible removal of ammonia by the urea cycle is crucial.

Zonation of Ammonia Metabolism in the Liver

The urea cycle is located only within liver periportal hepatocytes where it acts as a low affinity but high capacity system of ammonia removal (Häussinger *et al*, 1992). Within these cells the urea cycle enzymes are compartmentalized. The first two enzymes of the cycle proper, CPS I (EC 6.3.4.16) and OTC (EC 2.1.3.3) are located in mitochondria (Nuzum & Snodgrass, 1976) in proximity to the inner mitochondrial membrane (Cheung *et al*, 1989). Also located within the mitochondrial matrix is *N*-acetylglutamate synthetase (NAG synthetase; EC 2.3.1.1) which synthesizes NAG, the obligatory allosteric activator of CPS I (McCudden & Powers-Lee, 1996). The remaining enzymes of the cycle are located within the cytosol. These include AS (EC 6.3.4.5), AL (EC 4.3.2.1) and arginase (EC 3.5.3.1) (Nuzum & Snodgrass, 1976). The cycle is presented in **Figure 1.2**.

The metabolism of glutamine is also involved in ammonia detoxification. It, too, exhibits zonation of metabolism. There is some evidence that catabolism of glutamine, via glutaminase, may present CPS I with the bulk of amino acid-derived ammonia in an in vitro liver perfusion model (Nissim *et al*, 1992). Glutaminase is hypothesized to be in close proximity to CPS I within mitochondria (Häussinger *et al*, 1992). Synthesis of glutamine occurs in the perivenous hepatocytes via glutamine synthetase (Häussinger *et al*, 1992). Glutamine synthetase is a low capacity, high affinity ammonia scavenging enzyme. The enzyme is localized to the perivenous liver and it acts as a fail safe by

Figure 1.2: The Urea Cycle (hepatocyte)



removing ammonia, which has not been converted to urea by the urea cycle (Häussinger *et al*, 1992).

Occurrence of Urea Cycle Enzymes Outside the Liver

Traditionally, the urea cycle is believed to exist exclusively within the liver, however recent evidence has arisen which supports an entire urea cycle within the developing intestinal enterocytes of pigs (Wu, 1995). CPS I and OTC are co-expressed in intestinal mucosa cells (Ryall *et al*, 1986) while AS and AL are expressed in kidney proximal tubules (Dhanakoti *et al*, 1992) and in testis (Yu *et al*, 1995).

The Enzymes of the Urea Cycle

CPS I accounts for approximately 22-26% of soluble mitochondrial protein in liver (Raijman & Jones, 1976) and nearly 5% of soluble liver protein (Raijman & Jones, 1976). The enzyme is a homodimer of 222,000 ± 10,000 Da total weight (Raijman & Jones, 1976). The human CPS I gene has, recently, been re-mapped to chromosome 2q35 (Summar *et al*, 1995).

OTC is the second enzyme of the urea cycle. Like CPS I, it is a major component of the mitochondrial matrix protein comprising 3-4% *in vivo* (Raijman & Jones, 1976). OTC is a trimeric protein whose molecular weight is 36,000 Da. The OTC gene maps to Xp21.1 region of the human genome (Obbliger-Leibundgut *et al*, 1996).

AS is the third enzyme of the pathway. Its gene maps to chromosome 9q34 (Surh

et al, 1991; Dennis *et al*. 1989) and the bovine protein is a tetramer of 180,000 Da (Ratner, 1982).

The fourth step of the urea cycle is catalyzed by AL whose gene is found on chromosome 7cen-q11.2. It is also a tetrameric protein whose molecular weight is ~200,000 Da (Powers-Lee & Meister, 1988).

The final reaction in the urea cycle is the production of urea and regeneration of ornithine. The enzyme arginase is found in abundance in the liver. Its activity is reported in the literature as 35000 ± 1500 μmol/hour/g (Schimke, 1962). The human arginase gene is located on chromosome 6q23 (Sparkes *et al.* 1986). Regulation occurs at each of these five enzymes but the molecular mechanisms are not the same for each (Morris *et al*. 1987).

Channelling and the Urea Cycle

The enzymes of the urea cycle are hypothesized to form a 'metabolon' (Watford, 1991) in which the substrate of each enzyme is formed from the previous enzymic reaction and undergoes limited mixing with the general pool. Studies using radiolabelled ornithine and isolated rat liver mitochondria showed that exogenous ornithine is used, preferentially, to that generated within the mitochondrial matrix (Cohen *et al*, 1987). There is reported channelling of citrulline from the active site of OTC within the mitochondrion to the active site of AS in the cytosol (Cheung *et al*, 1989). Furthermore, these authors provide evidence for channelling between AS and AL as well as very tight

channelling between AL and arginase.

The Urea Cycle and Endotoxemia

As mentioned earlier, during endotoxemia protein catabolism significantly increases. Skeletal muscle amino acids are released at an accelerated rate (Hasselgren *et al*, 1988) to meet the increasing energy demands of the body as well as to furnish substrates for protein synthesis for the immune response. But how is the liver affected during endotoxemia?

Is there increased flux through the enzymes of the urea cycle? Are the enzyme activities increased? Is the cycle in some way activated during endotoxemia? Or, is there an effect which results from a combination of two or more of these factors?

Objectives of this Work:

1. To determine whether the urea cycle is affected by endotoxin treatment. Specifically, does endotoxin administration to rats result in a net change in urinary urea excretion?
2. If urea excretion is changed, to determine the nature of the effect, i.e., is there an increased flux through the enzymes of the urea cycle? Are the activities of the enzymes increased? Is there an elevation of ureagenic substrate concentrations within the liver? Or is there an activation of one or more of the enzymes of the pathway?

Chapter 2

Materials and Methods

Treatment of Animals

Male Sprague-Dawley rats weighing 250-350g were obtained from Charles River Co. (Montréal, P.Q.). Animals were maintained in a constant environment at 24 Celsius with a 12 hour light/dark cycle (light: 8:00 am-8:00 pm) and they had access to Purina chow (Ralston Purina of Canada, Don Mills, Ontario) and tap water *ad libitum*. The rats were housed, two or three per cage, in Nalgene Cages unless specified otherwise. Animals were housed in this manner for at least 5 days before experimental use so as to become acclimatised. All procedures were approved by the President's Committee on Animal Bioethics & Care of Memorial University and were in accordance with the guidelines of the Canadian Council on Animal Care.

Chemicals

Enzymes, *E. coli* endotoxin serotype 0127:B8, and unlabelled amino acids were purchased from Sigma Chemical Co., (St. Louis, Mo). [^{14}C]-Ureidocitrulline was obtained from DuPont-New England Nuclear (Mississauga, Ont.) Glutamate dehydrogenase in glycerol and bovine serum albumin (prepared from fraction V, essentially fatty acid-free.) were purchased from Boehringer-Mannheim Canada (Laval, P.Q.). Toluene and Omnifluor were purchased from Fisher Scientific (Nepean, Ont.). Other chemicals were of analytical reagent grade.

The Experimental Model

Animals utilized in endotoxin studies were subjected to a uniform protocol. Rats, weighing 200-350g, were weighed and fasted for 8-12 hours, but retained free access to tap water. They then received a single intraperitoneal injection of 0.3 mg/100g body weight bacterial lipopolysaccharide (*Eschericia coli* serotype 0127:B8) in 0.9% pyrogen-free sterile saline (1.0 mg/ml). They continued to have free access to water but were given no food. Paired controls were injected with vehicle alone. Subsequent to injection all animals were housed individually.

The protocol for this experimental model was based upon prior studies in our lab. Endotoxemic animals are anorexic and there is constriction of the pyloric sphincter (Evans *et al*, 1989). Therefore, the LPS-treated animals are in a fasted state and, for a suitable control, fasted animals must also be used. The dose of endotoxin chosen was that determined to be the lowest dose which would induce the symptoms of endotoxemia in rats (Qian & Brosnan, 1996).

Animals were excluded from the experimental group if they did not display the external physical manifestations of endotoxemia. These include: piloerection, lethargy, lack of preening, exudate around the eyes and nostrils, and diarrhea (Qian, 1993). Less than 10 animals were rejected during the course of these studies because they did not display the symptoms associated with endotoxemia. It is noteworthy that livers excised from treated rats were increased in mass compared to paired controls and they had a characteristic maroon colour (Qian, 1993).

In vivo Study: Measurement of Urinary Urea

Rats were housed individually in Nalgene metabolic cages for a total of 4 days. Rats were acclimatized to the metabolic cages for 36 hours with free access to ground chow and tap water. After 36 hours the food was removed. After 12 hours of fasting the rats were injected with endotoxin or vehicle and returned to the cages for urine collection. After a further 48 hours all animals were sacrificed.

Urine was collected at 12 hour intervals throughout the study including the initial acclimatization period, taking care to rinse all plastic surfaces for complete urine sample collection and measurement. The volume was measured and then aliquots were frozen for urea determination. Urine samples were uniformly diluted but not chemically treated in any way prior to freezing. At the end of the study urea was measured in all samples by the method of Geyer and Dabich (1989).

Preparation of Intact Mitochondria

Each rat was killed by cervical dislocation and the liver rapidly removed and immersed in an ice-cold isolation medium which was modified from Hampson *et al* (1983). This medium consisted of mannitol, 225 *mM*; sucrose, 75 *mM*; ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 1 *mM*; *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 2250 rpm (600 *g*) for 10 minutes at 4°C to pellet any erythrocytes, connective tissue, etc. The subsequent spins were carried out at 8250 rpm (8200*g*). The second centrifugation provided a crude mitochondrial pellet which 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. The final mitochondrial suspension had a concentration of 25-70 mg/ml of mitochondrial protein as determined by the biuret reaction (Gornall *et al*, 1949) using bovine serum albumin (BSA) as standard. The yield of mitochondria was similar for endotoxin-treated and saline-treated rat livers. The mitochondria were then evaluated for respiratory control.

Respiratory Control Ratio and Succinate Oxidation

Mitochondrial oxygen uptake was measured using a Clark-type electrode (Estabrook, 1967) in a medium which contained: KCl, 140 *mM*; KH_2PO_4, 4 *mM*; EDTA, 1 *mM*; $MgCl_2$, 2.5 *mM*; Hepes, 5 *mM*; BSA, 1 mg/ml; 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 begun by addition of ADP (0.26 *mM*, final concentration). The mitochondria were acceptable if they exhibited respiratory control ratios (state 3/ state 4 respiration) of greater than 4.

Disruption of Mitochondria

The first two enzymes of the urea cycle are located within the mitochondrion and, thus, assays of CPS I and OTC required disruption of the mitochondrial inner membrane. They were disrupted by three cycles of freezing in liquid nitrogen followed by thawing in warm water (less than 37 °C). As a control, to compare maximal membrane breakage via this method, the mitochondrial marker enzyme glutamate dehydrogenase (GDH; Bernt & Bergmeyer, 1974) was measured in samples of mitochondria which were disrupted by 0.5 % Triton-X 100. The activity of GDH at maximal disruption by Triton-X 100 was a little more than the activity displayed following 3x freeze-thaw using liquid nitrogen. By measuring the disappearance of NADH at 340 nm under varying disruptive conditions it was found that after 2x freeze-thaw there was no additional enzyme activity. This control was reproducible. 3x freeze-thaw was, subsequently, used to ensure that the mitochondria were adequately broken.

Table 2.1: Disruption of Mitochondrial Membranes Via Freezing / Thawing Using 0.5 Triton-X 100 as Standard.

Experimental Conditions	Disappearance Of NADH (µmol/min/mg mitochondrial protein)
0.5% Triton-X 100	0.178
1x Freeze-thaw	0.111
2x Freeze-thaw	0.133
3x Freeze-thaw	0.133
4x Freeze-thaw	0.133

Blood, Liver, Skeletal Muscle and Plasma Sampling

Rats were anaesthetized by an intraperitoneal injection of nembutal (sodium pentobarbitol) at a dose of 6.5 mg/100 g body weight. For blood and plasma determinations about 1 ml of blood was drawn into an heparinized syringe from the hepatic portal vein and from the abdominal aorta. Liver and muscle samples were obtained from animals which were not used for blood sampling. The procedure of removing tissue samples was stepwise. The rats were surgically prepared, under anaesthesia, such that the abdominal cavity was open and the liver exposed. Next, the skin from one thigh was removed and a portion of the muscle cut out and freeze-clamped using pre-cooled aluminum tongs. Immediately, a lobe of liver was removed and freeze-clamped. Each sample was wrapped in labelled aluminum foil and stored, in liquid nitrogen, until analysis.

Amino Acid Determination

Liver and muscle amino acids were extracted as described by Brosnan *et al* (1983). The frozen tissue samples were pulverized in pre-cooled ceramic mortars. 0.5 g of tissue powder was added to a pre-cooled, tared centrifuge tube and was homogenized with 2.5 ml of 0.5 *M* perchloric acid using a motor-driven teflon pestle that just fitted the centrifuge tube. The homogenate was then centrifuged at 18000*g* for 15 minutes at 4 C. The supernatant was neutralized, dropwise, with 10 *M* KOH and 3 *M* K_3PO_4 then placed on ice for 30 minutes. The precipitated potassium perchlorate was then removed by

centrifugation. The neutralized supernatant was diluted with lithium citrate sample dilution buffer (0.2 N Li^+ with 1% thiodiglycol and 1% phenol, pH 2.2 ± 0.2; Pierce Chemical, Rockford, IN) and the pH adjusted to that of the buffer with 3.0 *M* LiOH. 100 µl of amino-ethyl-cysteine (AEC) was added as an internal standard and amino acids were measured on a Beckman model 121-M amino acid analyzer as described by Lee (1974) by Doug Hall and Sonia Banfield of Memorial University's Amino Acid Analysis Facility.

Blood samples were deproteinized by addition to cooled, pre-weighed centrifuge tubes containing 0.75 ml of 0.5 *M* perchloric acid and then vortexed vigorously. Centrifugation at 16000 rpm (31000*g*) pelleted the precipitated proteins. 0.4 ml of the supernatant was mixed with 0.1 ml SSA LiOH (10% sulfosalicylic acid in 3 N LiOH), 0.05 ml 1.25 *mM* AEC and 0.25 ml lithium citrate sample dilution buffer. pH was adjusted with LiOH to 2.2 ± 0.2 for amino acid determination. Approximately 1 ml of blood was centrifuged for 2 minutes in an Eppendorf benchtop microcentrifuge to pellet the cells. 0.4 ml of plasma was then prepared for amino acid analysis in the same manner as deproteinized blood. The straw colour of the plasma indicated very little haemolysis.

Ammonia Determination

Ammonia was measured in hepatic portal and abdominal aortic blood as well as in liver samples 18 hours after injection with LPS or vehicle. Supernatants of deproteinized blood and tissue samples were neutralized to pH 7.0 using 3 *M* K_3PO_4 and 10 *M* KOH.

Samples were assayed for ammonia by the method of Buttery & Rowsell (1971) in a Beckman DU65 spectrophotometer, using Kinetic Pac™ software which tabulated the results and traced the course of the reaction. This method coupled ammonia removal by GDH to NADH consumption at 340 nm (Bernt & Bergmeyer, 1974). Scrupulously clean, i.e. ammonia-free, glass spectrophotometric cuvettes were used in these measurements. Glutamate dehydrogenase, suspended in glycerol (120 U/mg in 50% glycerol v/v; Boehringer Mannheim), was added to the cuvette to begin the reaction.

Enzyme Assays

A summary of time and protein requirements for the assays employed in this work is appended (**Table A-1**).

Citrulline Synthesis in Intact Mitochondria

Flux through the mitochondrial portion of the urea cycle was measured by incubation of mitochondria in a shaking water bath at 30°C with a medium which comprised (final concentration): potassium chloride, 80 *mM*; tris (hydroxymethyl) methylamine (TRIS), 20 *mM*; dibasic potassium phosphate, 5 *mM*; succinate HCl, 5 *mM*; potassium bicarbonate, 20 *mM*; ammonium chloride, 5 *mM*; *L*-ornithine, 20 *mM*; ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 *mM*; pH 7.4 in a final volume of 1.0 ml (Lacey *et al.* 1987). The reaction was started by adding the mitochondria and stopped with 0.3 ml of 30% perchloric acid.

Assessments of linearity for protein concentration as well as incubation time were carried out. Representative experiments can be seen in **Figure 2.1**. Subsequent incubations were within these linear ranges and used 3.0 mg of mitochondrial protein per flask, at 30 C for 6 minutes. Incubations were terminated with perchloric acid and citrulline assayed in the deproteinized incubation mixtures using the colorimetric method of Herzfeld and Raper (1976).

Carbamyl Phosphate Synthetase I

CPS I was assayed as described by Nuzum and Snodgrass (1976). The principle of this assay is that all carbamyl phosphate is converted to citrulline (via the added ornithine and OTC) which is measured. Pyruvate kinase is included so as to regenerate ATP from ADP using PEP as phosphoryl donor. Preparation of the incubation cocktail involved 20 minutes of gassing with 95% 5% O_2 / CO_2 of a 'working pool.' This pool consisted of (final concentrations): ammonium bicarbonate, 50.4 *mM*; *N*-acetylglutamate (NAG), 5.04 *mM*; L-ornithine, 5.04 *mM*; ATP, 12.1 *mM*; magnesium sulphate, 24.2 *mM*; phospho(enol)pyruvate (PEP), 40.3 *mM*; pH 7.0. Once gassed, and immediately prior to assay, supplementary enzymes OTC, (168 units from *Streptococcus faecalis*:) and pyruvate kinase (Type II from rabbit muscle, 2233 units; final values) were added to form the 'assay pool.'

Freeze-thawed mitochondria was added to start the reaction. The total volume was 0.3 ml and the reaction was terminated by precipitation using 0.3 ml of 30% perchloric

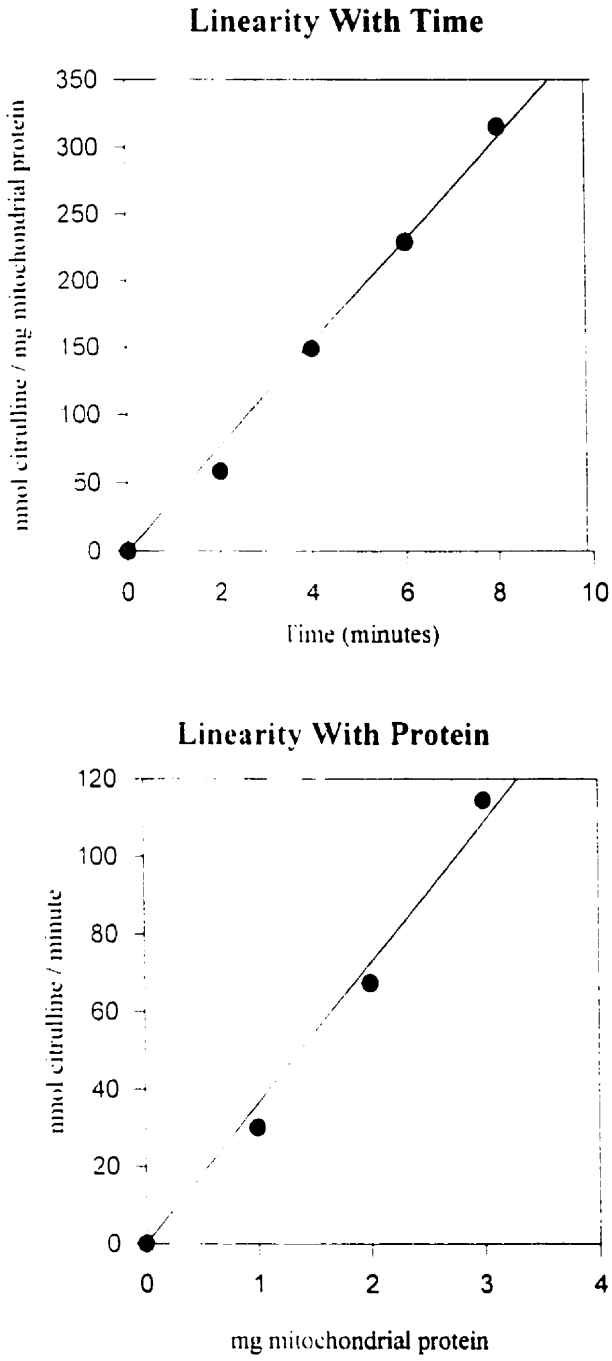


Figure 2.1: Linearity With Protein Concentration and Time for Citrulline Synthesis in Intact Mitochondria. The time linearity curve for mitochondrial citrulline synthesis utilized 3.0 mg of mitochondrial protein. The protein linearity evaluation was carried out for 6 minutes.

acid. Citrulline was measured in the deproteinized supernatant (Herzfeld & Raper, 1976).

The linear ranges for both protein and time were determined and representative experiments are included in **Figure 2.2**. From these experiments we chose 0.3 mg of protein per incubation flask and an incubation time of 6 minutes for all experiments.

Ornithine Transcarbamylase

OTC catalyses the transfer of the carbamyl group from carbamyl phosphate to ornithine. OTC was also measured in freeze/thawed broken mitochondrial preparations. The mitochondria were incubated at 37°C in: ornithine, 2.5 *mM* and 180 *mM* triethanolamine, pH 7.6 (final concentrations). The reaction was started by the addition of the substrate carbamyl phosphate, 5.0 *mM* (final concentration). The volume of incubation was 0.3 ml and the reaction was stopped upon addition of 0.3 ml perchloric acid. As in the CPS I assay, citrulline was quantitated in the deproteinized supernatant (Herzfeld & Raper, 1976). The results of a representative experiment to assess both time and protein linearity are presented in **Figure 2.3**. Six minutes was chosen as assay time and 20µg as the protein amount. To avoid inaccuracy associated with transferring minute volumes, the mitochondrial extract was diluted with homogenization medium to a concentration of 0.2 mg/ml and 0.100 ml was added to each flask

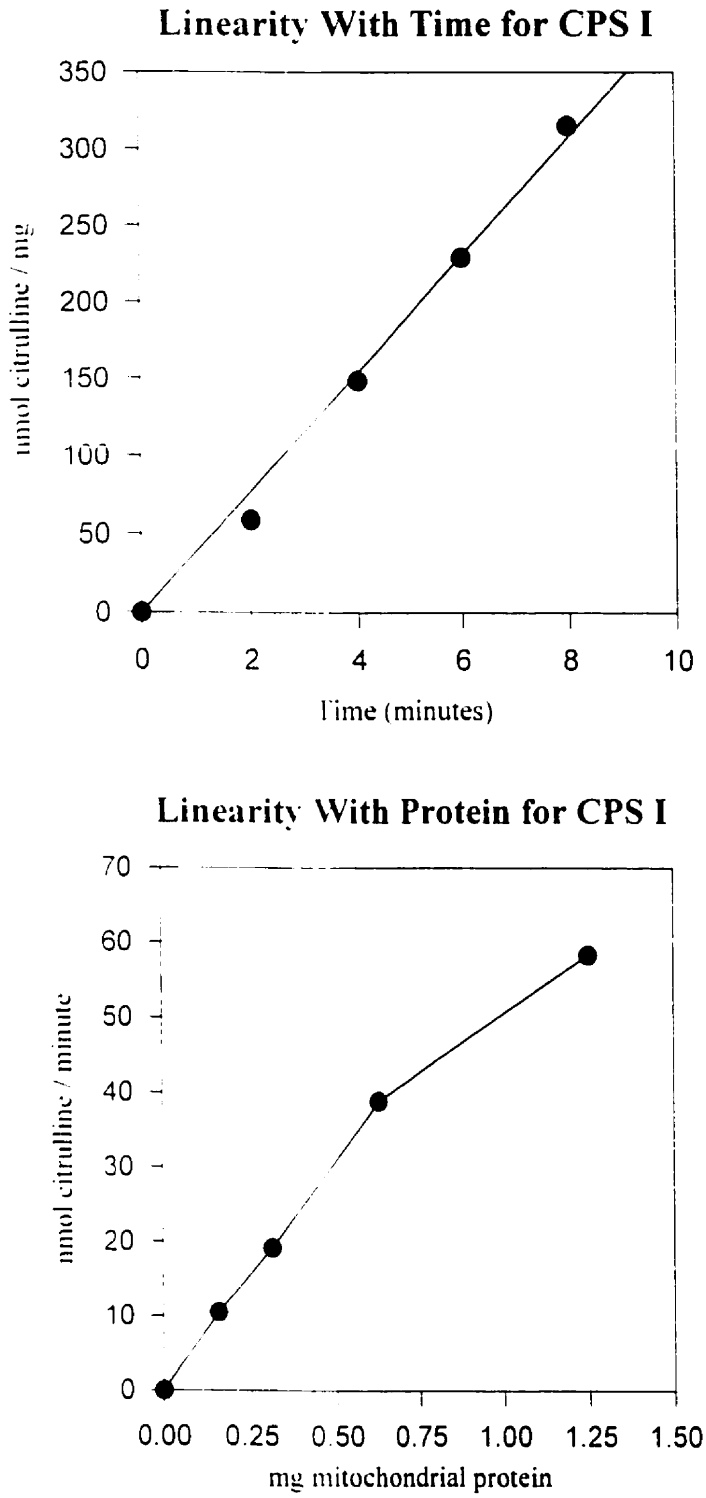


Figure 2.2: **Time and Protein Linearity for Carbamyl Phosphate Synthetase I.** The time linearity curve for CPS I used 0.30 mg of mitochondrial protein. The protein linearity assessments were carried out for 6 minutes.

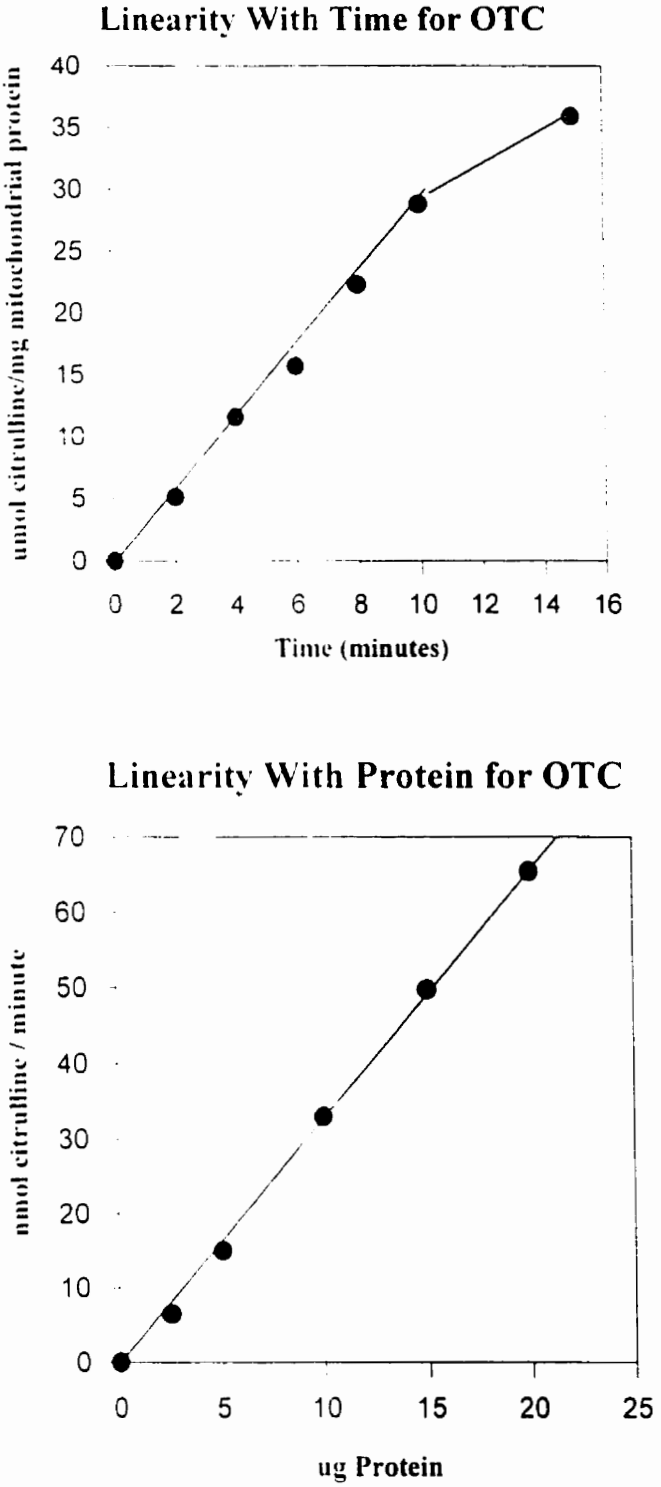


Figure 2.3: **Time and Protein Linearity for Ornithine Transcarbamylase.** Protein linearity was carried out for 6 minutes. Time linearity assessments utilized 0.02 mg of mitochondrial protein.

Argininosuccinate Synthetase

The third enzyme of the urea cycle, which catalyzes the condensation of citrulline and aspartate, was measured in liver homogenates using a slightly modified version of Nuzum and Snodgrass' method (1976). The modification of the method required using labelled [^{14}C]-ureidocitrulline [$H_2NC^*ONH(CH_2)_3CH(NH_2)COOH$]. The [^{14}C]-argininosuccinate was converted to labelled arginine, urea and CO_2 by successive addition of AL, arginase and urease. Supplementary enzymes also had to be added to the incubation for ATP regeneration.

The incubation medium consisted of (final concentrations): dibasic potassium phosphate, 50 *mM* pH 7.5; aspartate, 5 *mM*; ATP, 2 *mM*; magnesium sulphate, 2 *mM*; PEP, 2 *mM*; [^{14}C]-ureidocitrulline, 5 *mM*, 3.7 *kBq*/mmole; pyruvate kinase (Type II, rabbit muscle), 3 units; AL (Type IV, bovine liver) 0.8 units; arginase (bovine liver), 5 units; urease (Jack bean), 14 units. The incubation was carried out at 37° C. As in all previous enzyme assays, time and protein linearity assessments were determined. Representative experiments can be seen in **Figure 2.4**. The chosen protein amount in the AS assay was 3 mg and the time of incubation in subsequent experiments was 10 minutes. The incubation total volume was 1.0 ml.

The incubation flasks were fitted with rubber caps which positioned centre wells

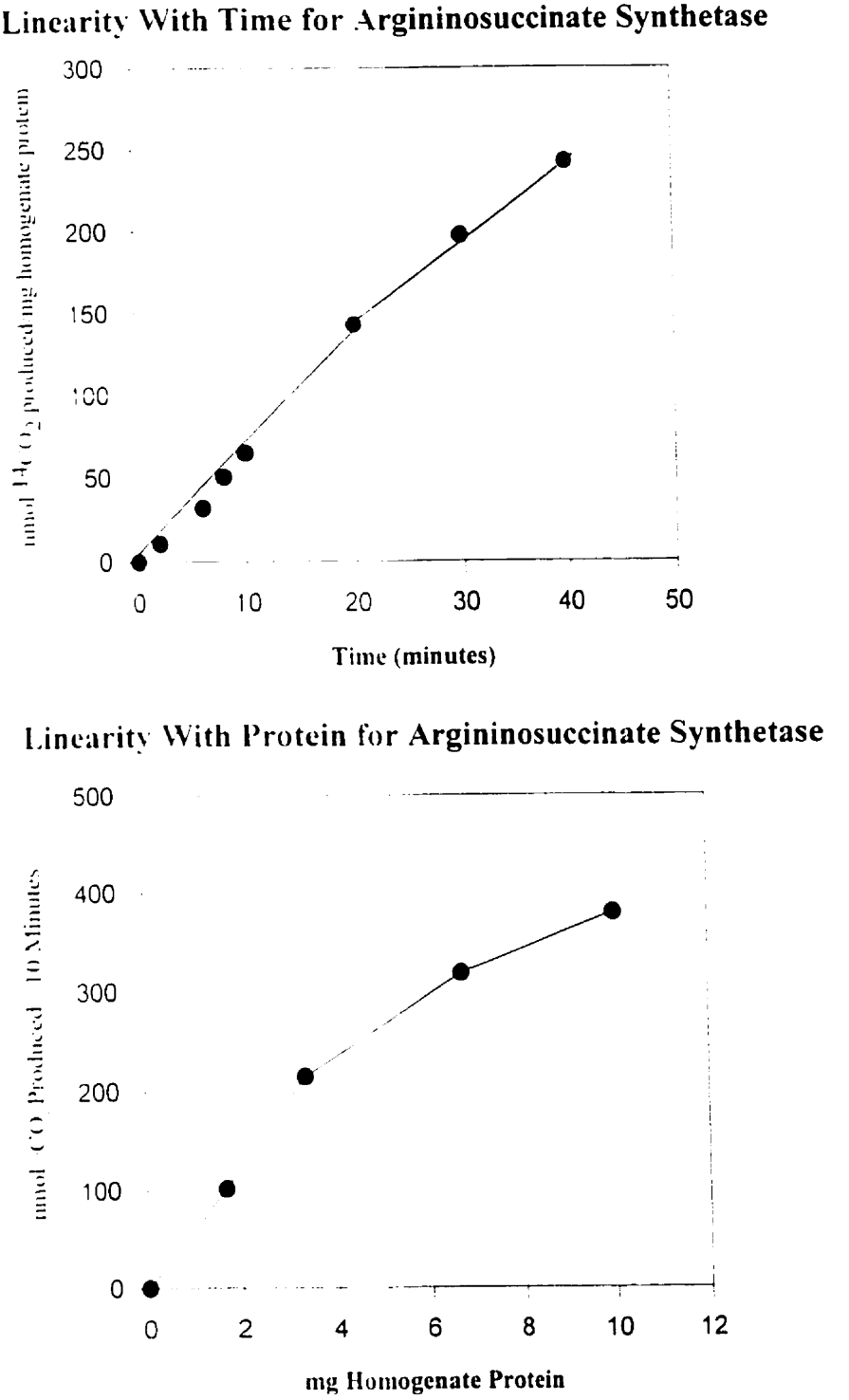


Figure 2.4: **Time and Protein Linearity for Argininosuccinate Synthetase.** Protein linearity was determined in 10 minute incubations. Linearity with time utilized 1.0 mg of liver homogenate protein.

above the incubation mixture. Each well contained 0.4 ml NCS, a tissue solubilizer comprised of a mixture of quaternary ammonium hydroxides and toluene. NCS acted as a sink for $^{14}CO_2$ which was liberated from solution upon addition of perchloric acid at the end of incubation. The 0.2 ml of acid was added to the incubation solutions through the rubber cap via a 1.0 ml syringe and thus loss to atmosphere was prevented. 0.4 ml NCS is in excess of the volume which is required to trap the liberated labelled CO_2 and the incubated flasks remain in the shaking water bath for 30 minutes post-acid injection to ensure trapping of all labelled gas. The centre wells were then immersed in 10 ml toluene-omnifluor scintillant and left overnight to allow dissipation of any chemiluminescence. The following morning samples were counted in an LKB liquid scintillation counter model 1214. Counts per minute (cpm) were converted to disintegrations per minute (dpm) via an internal standard.

Argininosuccinate Lyase

Argininosuccinate lyase cleaves argininosuccinate into arginine and fumarate. Its activity was also measured in liver homogenate using the method of Nuzum and Snodgrass (1976) with minor modifications. The incubation cocktail required, in final concentrations: argininosuccinate, 31 *mM*; dibasic sodium phosphate buffer, 89 *mM*, pH 7.0; EDTA, 44 *mM*; at 37° C and pH 7.0 and arginase (Jack bean), 100 units. The reaction was started by adding the liver homogenate. The total volume was 0.3 ml and the reaction was terminated by acid precipitation using 0.1 ml 30% perchloric acid. The

product, urea, was measured in the deproteinized supernatant after centrifuging samples in a benchtop centrifuge (Geyer & Dabich, 1989).

The curves which represent the experiments carried out to ascertain time and protein linearity ranges are found in **Figure 2.5**. There is excellent linearity with time. However, linearity with protein only occurred above 1.2 mg homogenate protein. This was highly reproducible in all conditions employed although we do not understand why. For routine assays we used 15 minutes and 2.0 mg of homogenate protein.

Arginase

The final enzyme of the urea cycle is found in abundance in the rat hepatocyte, its maximal activity *in vitro* reported as 47000 µmol/min/mg at 37° (Powers-Lee & Meister, 1988). For this reason liver homogenates, had to be diluted 1:42 with 0.5% bovine serum albumin prior to assay in accordance with the method of Nuzum and Snodgrass (1976).

The assay itself requires two stages: a pre-incubation, 'activation' of the arginase and an incubation which determines the rate of arginase activity. The pre-incubation involves adding 0.5 ml diluted homogenate to 1.0 ml 30 *mM* manganese chloride (12 *mM* final) and 1.0 ml 300 *mM* glycine (120 *mM* final), vortexing and incubating at 55°C for 20 minutes. Upon completion of activation 0.1 ml of 'activated' homogenate is incubated immediately, at 37°C with 375 *mM* arginine (125 *mM* final), pH 9.8. It is appreciated that

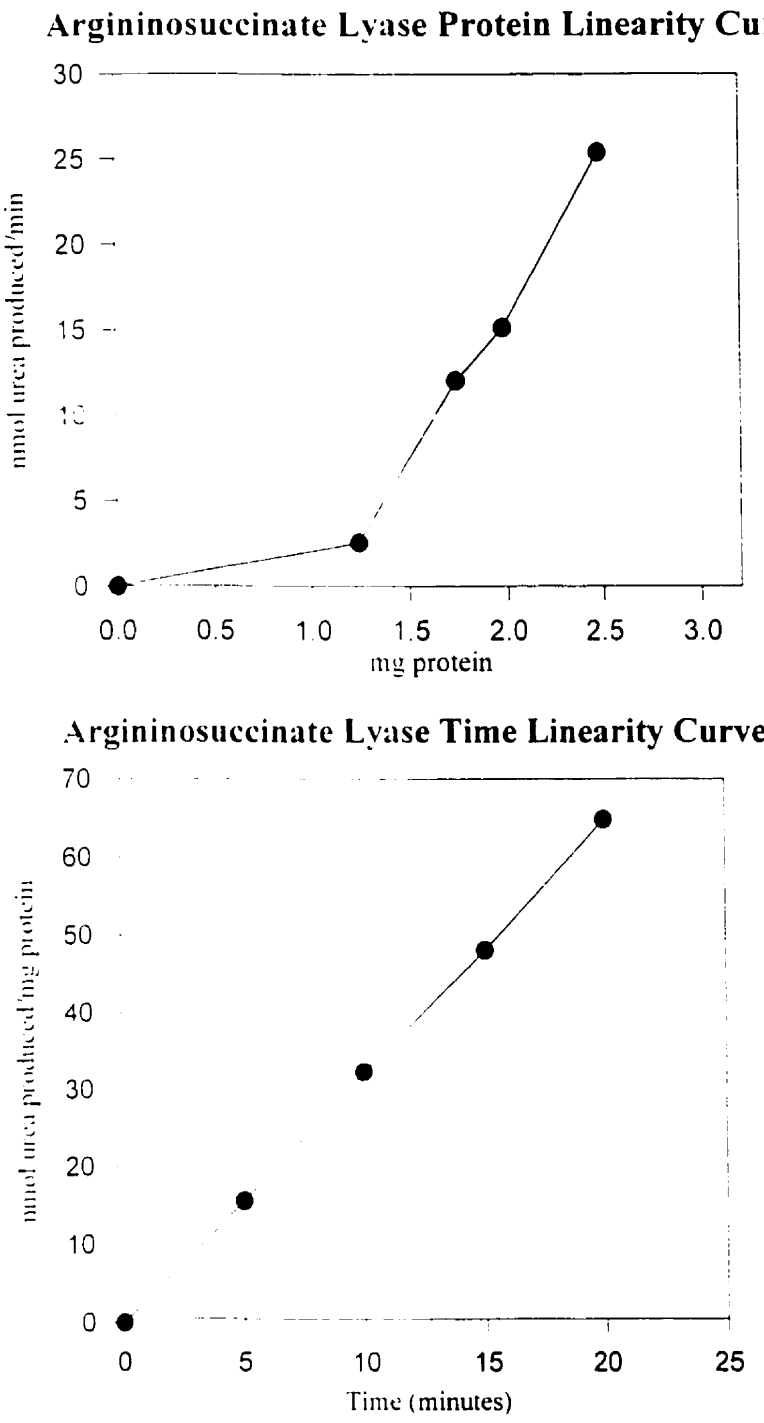


Figure 2.5: **Time and Protein Linearity for Argininosuccinate Lyase.** Protein linearity was determined in 15 minute incubations. Linearity with time incubations utilized 2.0 mg of liver homogenate protein.

these are very unphysiological conditions but this is a standard arginase assay. Reaction is terminated with perchloric acid and urea is measured in the deproteinized supernatant (Geyer & Dabich, 1989).

Linearity with respect to homogenate protein concentration and time was determined for arginase. Representative curves are shown in **Figure 2.6**. The time of incubation was chosen to be 10 minutes. The accepted protein amount per flask was 0.005-0.020 mg homogenate. The protein determination was linear through 0.012 mg in this curve but another protein linearity determination for arginase carried out in our lab has shown this assay to remain linear through 0.03 mg of homogenate protein (unpublished results).

N-Acetylglutamate Studies

Exogenous *N*-Acetylglutamate Incubations

These studies were conceived to assess the possible role played by the activator of CPS I, *N*-Acetylglutamate (NAG), within our experimental situation.

The inner mitochondrial membrane of coupled mitochondria is impermeable to NAG but this molecule can, nevertheless, be introduced into mitochondria by a method devised by Cheung *et al.* (1989). Using the mitochondrial uncoupler, 2,4-dinitrophenol (2,4-DNP), and the ATP synthase blocker, oligomycin, the isolated liver mitochondria were incubated with varying concentrations of NAG as well as exogenous ATP. The

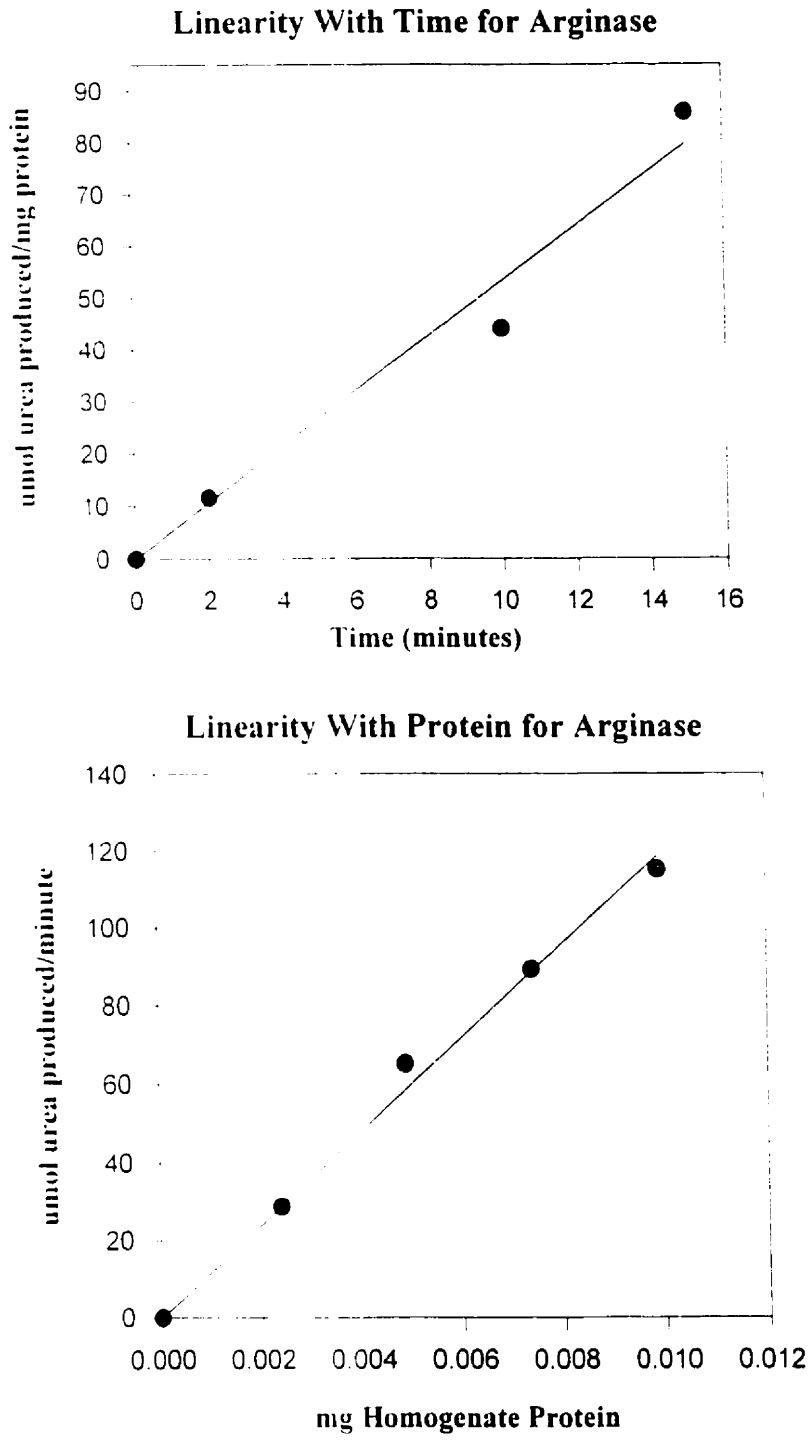


Figure 2.6: **Time and Protein Linearity for Arginase.** Protein linearity determinations were carried out in 10 minute incubations. The time linearity incubations used 0.015 mg of liver homogenate protein.

authors (Cheung *et al.* 1989) measured the intramitochondrial concentrations of NAG achieved by this type of incubation. Additions of 0, 0.5, and 5.0 *mM* NAG to the media resulted in internal NAG concentrations of: 0, 0.3, and 4.3 *mM*, respectively. The mitochondria were pre-incubated for 10 minutes at 30° in the presence of (final concentrations): 2,4-dinitrophenol, 0.3 *mM*; oligomycin, 10 μg/ml; Tris-HCl, pH 7.0, 50 *mM*; magnesium chloride, 5 *mM*; ATP, 5 *mM*; N-acetylglutamate, varying concentrations; the added mannitol concentration was adjusted to achieve isoosmolarity in the incubation.

Immediately following the pre-incubation with NAG, citrulline synthesis by the mitochondria was determined. The reaction was started by adding a cocktail containing (final concentrations): potassium chloride, 15 *mM*; ornithine, 10 *mM*; potassium bicarbonate, 15 *mM*; ammonium chloride, 10 *mM*; dibasic potassium phosphate, 5 *mM*; EDTA, 2 *mM*; TRIS-HCl, 50 *mM*; mannitol, 69 *mM* and bovine serum albumin, 0.1% w/vol. Volume of incubation was 1.0 ml. The reaction was terminated with perchloric acid and citrulline was measured in the deproteinized supernatant (Herzfeld & Raper, 1976). Assessments for linearity with respect to time and protein were carried out and the final protein concentration chosen was 3.0 mg mitochondrial protein/ml and time of incubation was 2 minutes.

Mitochondrial Matrix *N*-Acetylglutamate Levels

The levels of mitochondrial matrix NAG were measured at the Kennedy-Krieger Institute of Baltimore by Dr. Lisa Kratz. Liver mitochondria were isolated and quick frozen in liquid nitrogen and stored at -80°C (low temperature freezer) until shipping. Samples were sent to Baltimore in dry ice. Dr. Kratz analyzed the mitochondrial samples for NAG by ion ratio gas chromatography mass spectrometry by solvent extraction and trimethylsilyl derivitization, utilizing *N*-[$^{2}H_3$]acetyl-L-3-[^{13}C]aspartate as an internal standard.

Treatment of Data

Experimental data are reported as the mean of at least four experiments ± standard deviation. Statistical analysis utilized the Students' unpaired t-test in comparisons between two test groups (saline versus LPS animals) and the Students paired t-test was used when comparing results within the same group of animals (A-V differences). A statistically significant difference was deemed to be $p<0.05$. Quantitation of urea and citrulline from standard curves was calculated by GraphPad (GraphPad Software, San Diego, California, USA) and paired t-tests were analyzed by InStat (also of GraphPad Software package).

Chapter 3: Stimulation of the Urea Cycle After Bacterial Endotoxin Administration

Introduction

During times of metabolic stress, such as sepsis, negative nitrogen balance occurs. Administration of bacterial endotoxin elicits many of the effects seen in these circumstances. Using a model that was previously refined (Qian, 1993) we injected fasted rats with a single injection of LPS (0.3 mg/100 g body weight) or with sterile, pyrogen-free saline. There were two questions: (1) Is there an increased flux through the enzymes of the urea cycle in rats which have been injected with endotoxin? And if so (2) what is the nature of this effect?

Protocols

All animals used in these experiments were subjected to the same housing conditions. They experienced a 12 hour light/12 hour dark cycle (8:00-20:00 light and 20:00-8:00 dark) with free access to standard lab rodent chow and tap water for approximately 1 week prior to experimentation. The animals in the LPS studies were pair matched with a saline control of similar age and mass. Animals were deprived of food for a minimum of 12 hours prior to injection (between 17:00-20:00 food was removed). *E.coli* endotoxin was injected by a single i.p. dose of 0.3 mg/100 g body weight, in sterile saline, at 8:30 am. Fasted controls were injected with the vehicle alone. Animals were sacrificed 24 hours after treatment for the *in vitro* studies. Plasma and tissue samples for substrate measurement were taken 18 hours after endotoxin.

For the *in vivo* experiments animals were housed in individual metabolic cages for

96 hours. Urine was collected at 12 hour intervals. Food was removed at 36 hours, and the animals were injected with LPS or saline at 48 hours. The rats were sacrificed at 96 hours.

Results

Urinary Urea Excretion 24 Hours After LPS-Treatment

Fasted endotoxemic rats excreted nearly three times as much urea as did the paired fasted controls (1.13 ± 0.34 in the controls versus 2.93 ± 0.79 mmol urea/100g body weight in the LPS-treated rats) during the 12-24 hour interval after LPS injection (**Figure 3.1**, 60-72 hours). Urea output remained significantly elevated until approximately 36 hours after treatment. Urea excretion can be increased for a variety of reasons: (1) increased dietary protein intake (Folin, 1905); (2) increased mitochondrial content of specific amino acids, such as arginine or glutamate, which results in elevated synthesis of *N*-Acetylglutamate (NAG), the positive allosteric effector of CPS I (Shigesada & Tatibana, 1971); and (3) catabolic illnesses during which the liver may have to deal with increased amounts of amino acids which are released from skeletal muscle (Beisel, 1975).

Within the context of this study there are several possibilities that may account for the observed increase in urea cycle flux: (1) increases in the activities of the five urea cycle enzymes; (2) increases in substrate presentation to the liver for urea synthesis; (3) increased uptake of ureagenic substrates by the liver; (4) activation of flux-generating

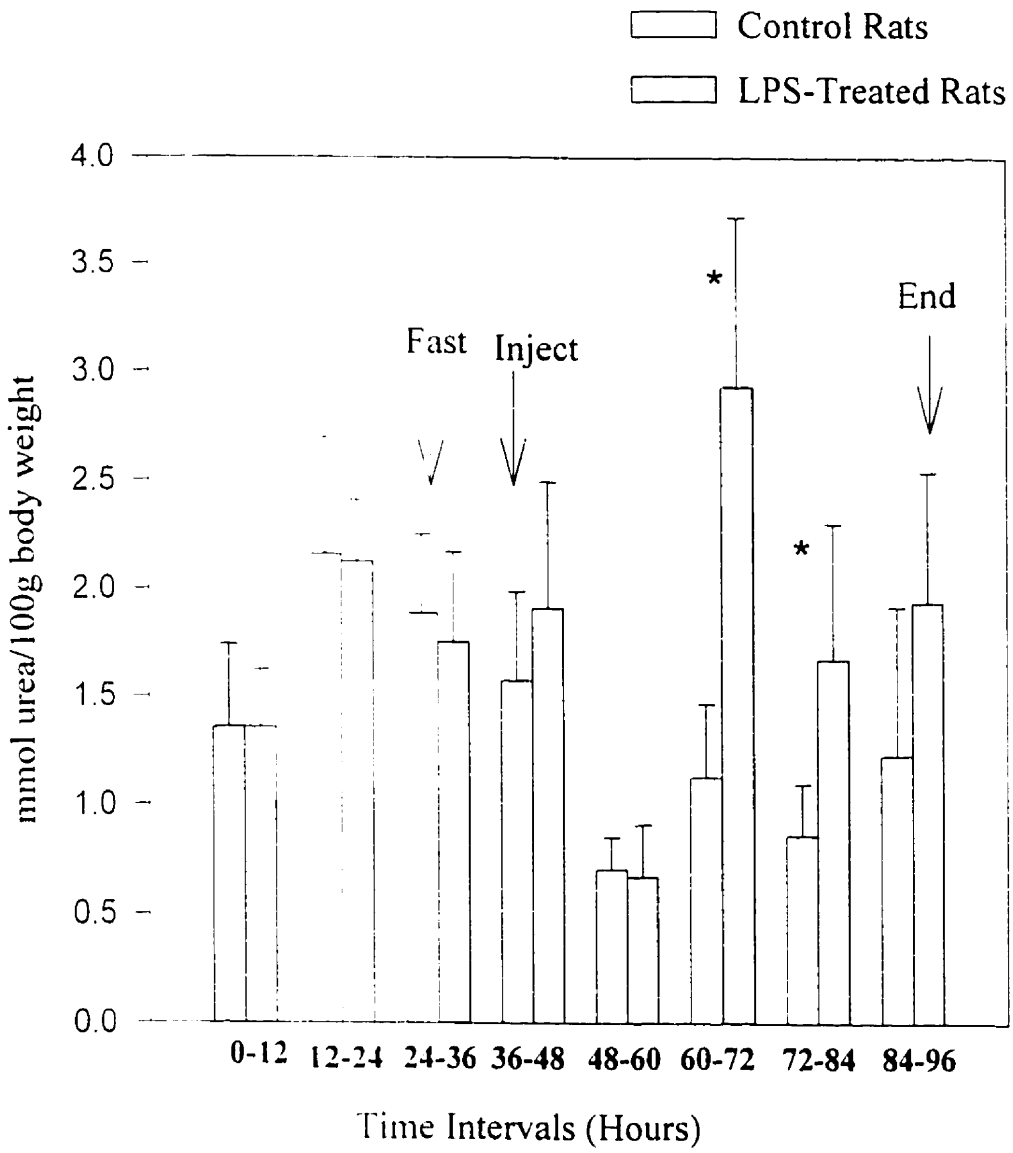


Figure 3.1: **Time Course of Urinary Urea Excretion in Saline Injected Control Rats and in LPS Injected Rats.** The results are shown as means ± standard deviation, n=6. The asterisk denotes statistical significance. *$p<0.05$; two-tailed unpaired t-test.

enzyme(s) of the urea cycle. As these possibilities are not mutually exclusive it may be that there is contribution from any or all of them. Having found a 3-fold increase in urea excretion in LPS-treated rats we focused on the next question: What is the basis for this increase subsequent to LPS treatment?

Is There an Increase in Ureagenic Substrate Concentrations and Presentation to the Liver After Endotoxin Administration?

Ammonia

CPS I, the first enzyme of the urea cycle, is ammonia-specific. Ammonia levels were measured in arterial blood, hepatic portal venous blood (both of which supply the liver) as well as in the liver and skeletal muscle. Because the effects of endotoxin were determined to be maximum at 12-24 hours after the insult we chose to measure all ureagenic substrates at 18 hours. **Table 3.1** presents the ammonia values which were obtained from blood, as well as liver and muscle. These values are in close agreement with those obtained by previous experimenters (Brosnan, 1968). Ammonia levels in blood, liver and skeletal muscle were not different in the LPS-treated rats compared with the controls.

Table 3.1: Ammonia Levels in Whole Blood, Quadriceps Muscle and Liver Samples 18 Hours After Administration of Bacterial Endotoxin or Sterile Saline. Blood was sampled from the hepatic portal vein or the abdominal aorta. Results are presented as means ± standard deviations of 3 or 4 individual measurements; two-tailed p values by unpaired t-test; *p<0.05. There were no significant differences.

Sample	Saline Control	LPS-Treated
Portal Venous Blood (μmol/ml)	0.17 ± 0.070	0.18 ± 0.031
Abdominal Aortic Blood (μmol/ml)	0.054 ± 0.011	0.055 ± 0.018
Quadriceps Muscle (μmol/g)	0.25 ± 0.16	0.17 ± 0.01
Liver (μmol/g)	0.69 ± 0.08	0.66 ± 0.24

Free Amino Acid Concentrations

Free amino acid concentrations were quantified in samples which were taken from: (1) hepatic portal venous whole blood and plasma; (2) abdominal aortic whole blood and plasma; (3) freeze-clamped liver; and (4) freeze-clamped quadriceps muscle. As this amounts to a large body of data the key data will be summarized and appended as tables of individual free amino acid concentrations.

Table 3.2 presents a summary of the total free amino acid concentrations in blood, plasma, skeletal muscle and liver. Total circulating free amino acids were not changed in the blood and portal venous plasma of LPS-treated rats compared to controls. Neither were there any differences in the skeletal muscle of the treated rats. However, the total free amino acid concentrations measured in arterial plasma and liver were significantly increased in LPS-treated animals.

There was a large net release of amino acids across the gut in the blood of both control (-648.9 nmol/ml) and LPS-treated (-687.4 nmol/ml) rats (**Table A-2**) presumably because of the increased protein catabolism in their fasted state.

Blood and Plasma Amino Acids

It is generally believed that plasma, rather than erythrocytes, is the vehicle for amino acid exchange between tissues (Munro, 1970). However, there are studies which report that red blood cells can play plasma-independent and, often, opposing roles in amino acid exchange in the liver and gut (Elwyn *et al*, 1968). There is an increase in the

Table 3.2: Total Free Amino Acid Concentrations in Blood, Plasma, Skeletal Muscle and Liver 18 Hours After Endotoxin Administration. Blood was collected from the hepatic portal vein and from the abdominal aorta. Skeletal muscle and liver samples were quickly excised and freeze-clamped. Values are presented as means ± standard deviation of 7 or 8 animals per group. An asterisk denotes a significant difference as determined by unpaired t-test. (*$p<0.05$)

Sample	Saline Control	Endotoxin-Treated
Blood (nmol/ml)		
Hepatic Portal Venous	4735 ± 468	5027 ± 1214
Abdominal Aortic	4177 ± 396	4312 ± 1419
Plasma (nmol/ml)		
Hepatic Portal Venous	3829 ± 382	4116 ± 527
Abdominal Aortic	3546 ± 393	4223 ± 525*
Skeletal Muscle (nmol/g)	21519 ± 468	20130 ± 2306
Liver (nmol/g)	13672 ± 2059	19650 ± 4394*

total concentration of amino acids in arterial plasma of LPS-treated rats (**Table 3.2**). There are also significant increases in the circulating levels of key amino acids alanine (**Table 3.3**) and glutamine (**Table 3.4**) the main end products of muscle protein degradation (Felig, 1975). The importance of these changes will be discussed in further sections.

Liver Amino Acids

The liver is the site of ureagenesis and the main organ of gluconeogenesis thus it carries out significant amino acid catabolism. Total free amino acid concentration is significantly increased in endotoxemic rat livers (13672 ± 2059 nmol/g in controls, 19650 ± 4394 nmol/g in LPS group; *$p<0.05$; **Table 3.2**). **Table A-3** gives data on the individual liver amino acids. Glutamine is not increased in liver whereas alanine is (**Tables 3.3 and 3.4**).

Key Amino Acids During Endotoxemia

Alanine

Alanine is the principal gluconeogenic amino acid and participant in the glucose-alanine shuttle (Felig, 1973). In muscle, alanine may be: (1) released via proteolysis or (2) produced via transamination reactions. Muscle alanine levels in LPS-treated rats were significantly increased (1017 ± 202 nmol/g in controls versus 1475 ± 333 nmol/g in

Table 3.3: Summary of Free Alanine Data From Amino Acid Analyses 18 Hours Post LPS or Saline Injection. Values are presented as means ± standard deviation, n=7 or 8. Statistical significance : A-V differences across the gut were determined to be significantly different from zero using paired t-test. All other groups were subjected to unpaired t-test. (*p<0.05, **p<0.01)

Sample	Saline Controls	LPS-Treated
Skeletal Muscle (nmol/g)	1017 ± 202	1475 ± 333**
Liver (nmol/g)	320 ± 56	777 ± 447*
Hepatic Portal Venous Blood (nmol/ml)	399 ± 74	614 ± 338*
Abdominal Aortic Blood (nmol/ml)	293 ± 52	506 ± 251*
A-V Blood (nmol/ml)	-102 ± 121	-107 ± 458
Hepatic Portal Plasma (nmol/ml)	432 ± 56	552 ± 113*
Abdominal Aortic Plasma (nmol/ml)	340 ± 42	496 ± 104**
A-V Plasma (nmol/ml)	-93 ± 79	-56 ± 42*

Table 3.4: Summary of Free Glutamine Concentrations 18 Hours After Injection With Saline or Endotoxin. Values are presented as means ± standard deviations, n=7 or 8. Statistical evaluation of A-V values (across the gut) was by paired t-test while all other sample groups were compared by unpaired t-test. (*p<0.05; **p<0.01; ***p<0.001)

Sample	Saline Controls	LPS-Treated
Skeletal Muscle (nmol/g)	2478 ± 500	1819 ± 397*
Liver (nmol/g)	3972 ± 658	4316 ± 461
Hepatic Portal Venous Blood (nmol/ml)	511 ± 68	651 ± 108**
Abdominal Aortic Blood (nmol/ml)	518 ± 44	614 ± 120
A-V Blood (nmol/ml)	5.9 ± 72	-37 ± 72
Hepatic Portal Venous Plasma (nmol/ml)	539 ± 47	675 ± 92**
Abdominal Aortic Plasma (nmol/ml)	574 ± 82	799 ± 82***
A-V Plasma (nmol/ml)	34.5 ± 114	124 ± 71*

LPS-treated rats; **p<0.001). In the circulation, i.e., in blood and plasma, there were also marked, significant increases of alanine in LPS-treated rats (**Table 3.3**). In the livers of LPS-treated rats there was twice as much alanine as in the control rats livers (**Table 3.3**). All of this is relevant to the effect(s) of endotoxin treatment upon urea synthesis because, as the animal attempts to cope with disrupted fuel homeostasis the liver may be presented with more alanine and, in turn, more ammonia. As a substrate for CPS I ammonia could stimulate ureagenesis.

Glutamine

Glutamine is also a primary gluconeogenic amino acid and its catabolism may provide ammonia directly to CPS I (Häussinger *et al*, 1992; Meijer, 1985). Therefore, it is also particularly relevant to these studies. Glutamine concentrations were affected post-LPS as shown in **Table 3.4**. Muscle glutamine concentration significantly decreased in LPS-treated rat muscle (2478 ± 500 nmol/g in control versus 1819 ± 397 nmol/g in LPS group; *p<0.05). In hepatic portal venous blood, the amino acid concentration was elevated (511 ± 68 nmol/ml control, 651 ±108 nmol/ml LPS group; **p<0.01) but not in the arterial supply. Glutamine concentrations were markedly elevated both in hepatic portal venous (539 ± 47 nmol/ml control, 675 ± 92 nmol/ml LPS group; **p<0.01) and abdominal aortic plasma (574 ± 82 nmol/ml control, 799 ±82 nmol/ml LPS group; ***p>0.001). The concentration of glutamine was not changed in rat liver 18 hours post-treatment, however.

In rats, the liver receives a greater supply of amino acids within 24 hours of injection with endotoxin, particularly the gluconeogenic amino acids, glutamine and alanine and these may have an effect upon the rate of urea synthesis during endotoxemia.

Are The Urea Cycle Enzymes Increased in Total Activity?

Urea Cycle Enzyme Activity *In Vitro* 24 Hours After LPS Injection

Table 3.5 shows the activities of the urea cycle enzymes in livers from rats injected with either LPS or vehicle (sterile saline). There was no change in the activity of any of these enzymes suggesting that changes in total enzyme amount cannot explain the increased urea synthesis seen after LPS administration.

Flux Through the Mitochondrial Urea Cycle Enzymes

There was no measurable difference in the activities of the individual enzymes of the urea cycle (**Table 3.5**) yet there was a nearly 3-fold increase in urea synthesis *in vivo* (**Figure 3.1**) subsequent to endotoxin injection. The enzyme activities are expressed per milligram of protein. Qian (1993) has shown, in this laboratory, that livers from LPS-treated rats have 18% more protein than do livers from controls. Therefore, it is possible that the total potential activity of these enzymes in the LPS-treated rats are increased by about 18%. This would contribute to the increased urea production *in vivo* in the LPS-treated animals but can, in no way, account for a 3-fold increase. Direct assay of the flux

Table 3.5: Summary of the Values Obtained Through Direct Measurement of the Five Urea Cycle Enzymes 24 Hours After LPS Administration. Values are presented as means ± standard deviations, n≥4. There were no significant differences subsequent to endotoxin administration as determined by unpaired t-test.

Activities of the Urea Cycle Enzymes 24 Hours After Endotoxin Administration		
Enzyme	**Control**	**Endotoxin**
Carbamyl Phosphate Synthetase (CPS I) nmol/min/mg†	47.1 ± 26.0	47.3 ± 21.3
Ornithine (OTC) Transcarbamylase µmol/min/mg†	2.6 ± 1.2	2.7 ± 1.2
Argininosuccinate Synthetase (AS) nmol/min/mg‡	2.4 ± 0.36	2.4 ± 0.32
Argininosuccinate Lyase (AL) nmol/min/mg‡	34.2 ± 9.7	35.8 ± 12.7
Arginase µmol/min/mg‡	22.6 ± 2.2	21.6 ± 3.9

† Denotes mg of mitochondrial protein; ‡ denotes mg of liver homogenate protein..

through CPS I and OTC in intact LPS-treated rat liver mitochondria resulted in flux rates double those of the control (**Figure 3.2**) even though the total activities of CPS I and OTC assayed *in vitro* were unchanged under these conditions. This increased flux in intact mitochondria suggests that there may have been activation of the intramitochondrial portion of the urea cycle after LPS injection.

We next carried out a series of experiments to examine further this doubling of citrulline synthesis in intact mitochondria (**Figure 3.2**; 18.23 ± 8.59 controls; 35.47 ± 12.60 nmol/min/mg LPS-treated, *$p<0.05$). CPS I, though not shown to increase in total activity as a result of LPS injection, is subject to allosteric modulation by *N*-acetylglutamate (NAG).Therefore, we examined the effects of LPS on mitochondrial NAG levels as well as the relationship between NAG and flux through the urea cycle.

N-Acetylglutamate: Increased Activation of CPS I After LPS?

Mitochondrial NAG Concentrations

Mitochondrial NAG was measured by GC-MS to assess directly whether there might be an increase in this key regulatory molecule in endotoxemic rats. **Figure 3.3** shows a significant elevation of NAG in the mitochondria of LPS-treated rats 24 hours post-injection (0.32 ± 0.080 controls; 0.46 ± 0.15 LPS-treated, nmol/mg; *$p<0.05$). One value was excluded from the LPS group. The mitochondria from this rat had the lowest NAG content of all the animals (0.18 nmol/mg) and also had a very low rate of citrulline synthesis (6.06 nmol/min/mg) in the intact mitochondria. This may have resulted from a

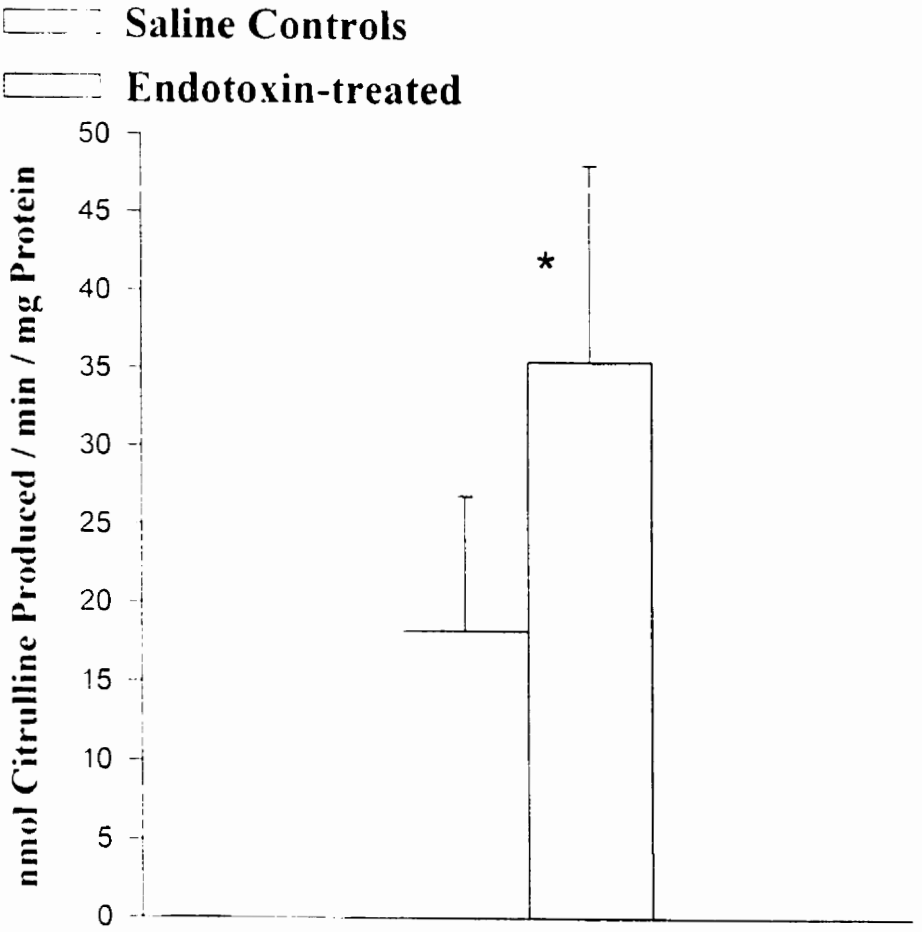


Figure 3.2: **Flux Through the Mitochondrial Enzymes of the Urea Cycle 24 Hours Post-LPS Injection.** Flux was measured in freshly isolated, intact rat liver mitochondria 24 hours after injection with saline or LPS. Values are presented as means ± standard deviations, n=9. Significance is denoted by an asterisk; *$p<0.05$ as determined by unpaired t-test.

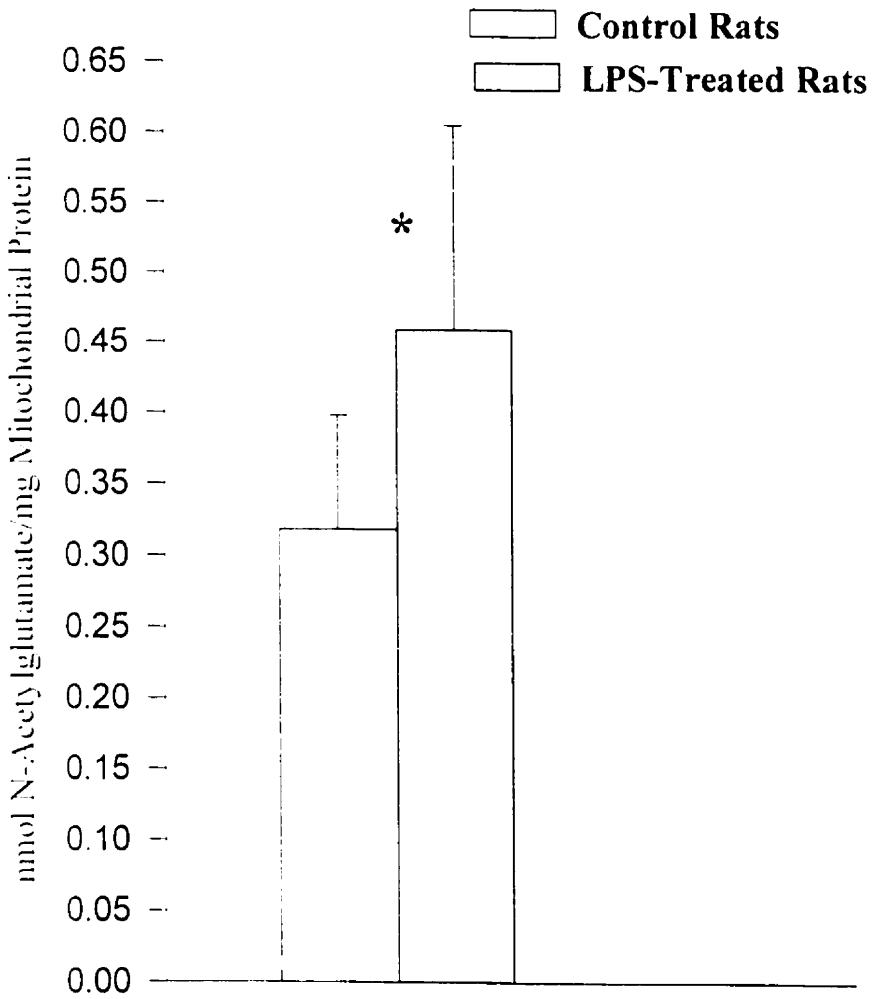


Figure 3.3: Rat Liver Mitochondrial NAG Content.
Mitochondria were freshly isolated and an aliquot was quick-frozen in liquid nitrogen. GC-MS analysis was carried out by Lisa Kratz, Ph.D, Baltimore, Md. Values are presented as means ± standard deviation; n=7 or 8, *$p<0.05$.

faulty injection of LPS.

Exogenous *N*-Acetylglutamate Studies

Figure 3.4 presents the citrulline synthesis rates by mitochondria in the presence of 0-10 *mM* exogenous NAG. These mitochondria were uncoupled to facilitate NAG entry. At zero exogenous NAG the liver mitochondria from endotoxin-treated rats synthesized citrulline at double the rate of the saline control animals (12.28 ± 6.46 in controls versus 28.84 ± 9.82 in LPS-treated rats; nmol/min/mg; *$p<0.05$) which recalls our data from **Figure 3.2** in which the rate of citrulline synthesis is doubled in LPS-treated rat liver mitochondria. The rate increased in both groups but the increase was greater in the mitochondria from the saline-injected rats such that there were no longer significant differences between the groups (35.62 ± 11.43 in controls versus 42.59 ± 11.23 in LPS-treated rats; nmol/min/mg) at 10 *mM* NAG. There is also a significant positive relationship between citrulline synthesis rates and intramitochondrial NAG levels (**Figure 3.5**; $r^2=0.610$, *$p<0.05$). Thus, increased intramitochondrial NAG may be responsible for the activation of flux through the urea cycle through its effect on CPS I.

Discussion

Much work has focused upon the processes which lead to muscle loss during endotoxemia. The focus of this work was to evaluate hepatic response to elevated amino nitrogen influx due to protein catabolism in muscle during endotoxemia. Amino acids

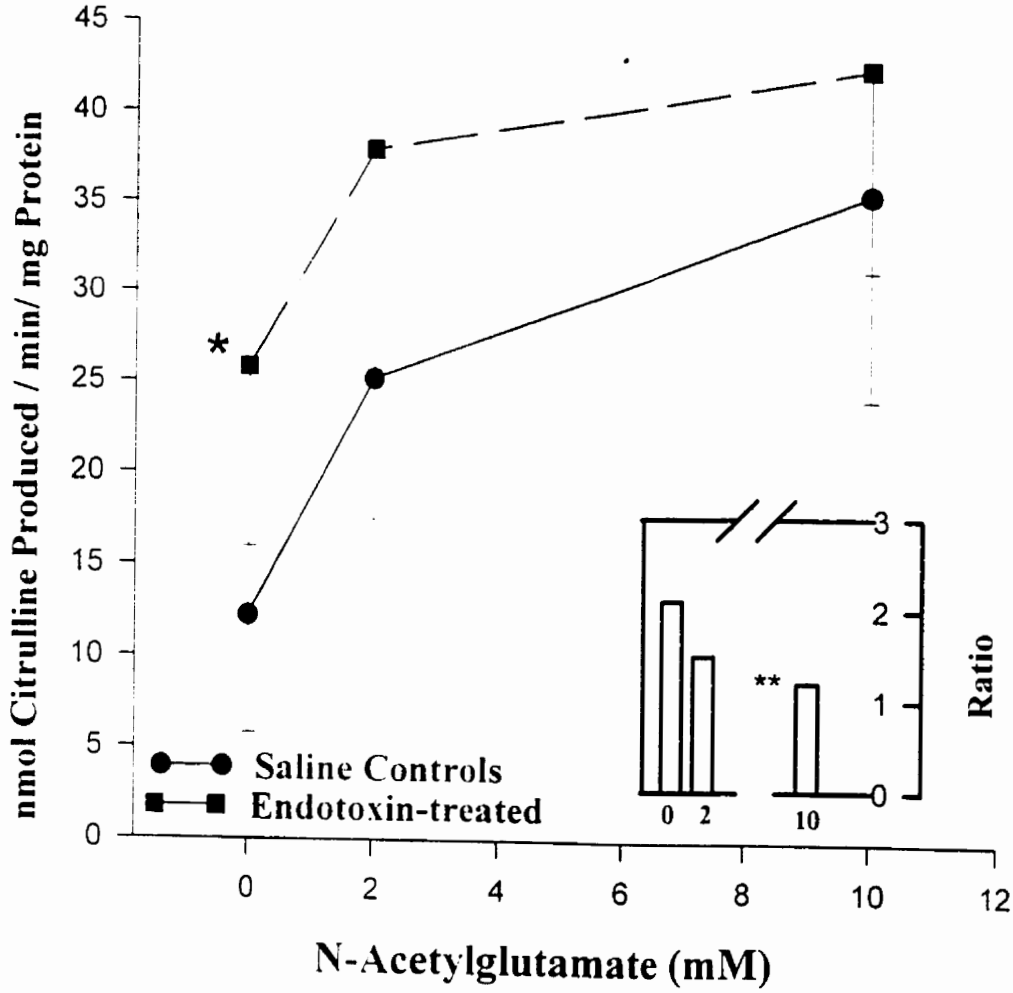


Figure 3.4: **Citrulline Synthesis in Uncoupled Rat Liver Mitochondria in the Presence of Exogenous N-acetylglutamate 24 Hours After Treatment With Bacterial Endotoxin (LPS) or Sterile Saline.** Mitochondria were freshly isolated 24 hours after treatment and uncoupled with 2,4-dinitrophenol and incubated with exogenous NAG. Values are presented as means ± standard deviation, n=6. *p<0.05
***Inset**: Ratio of endotoxin-treated: saline control values. **Ratio is significantly different from one as determined by paired t-test; n=6.*

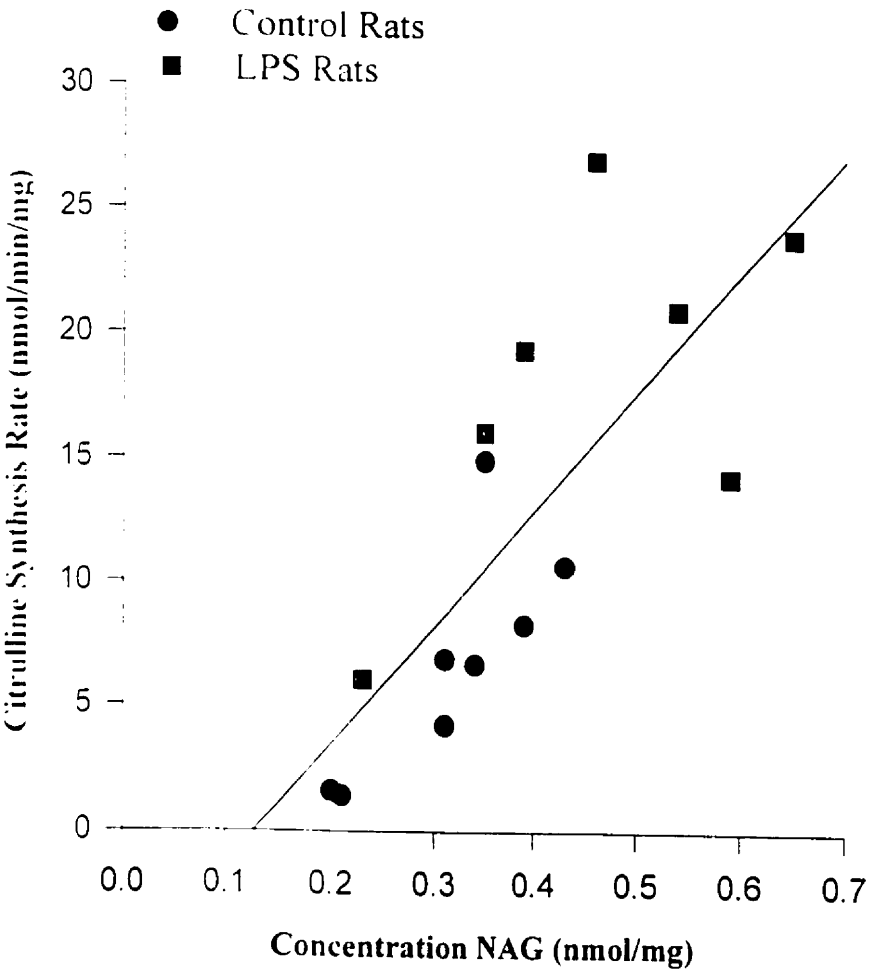


Figure 3.5: **The Relationship Between Citrulline Synthesis and Mitochondrial NAG Content.** The NAG content was measured by GC-MS by Lisa Kratz, Ph.D and the correlation between NAG concentration and citrulline synthetic rate of individual samples was plotted. n=7 or 8; r^2=0.610, *p<0.05.

may be: (1) oxidized; (2) used as gluconeogenic precursors; and (3) used for synthesis of proteins as well as a variety of other functions. Within the liver, during endotoxemic stress, amino acids may meet any or all of these fates. Protein synthesis generally involves acute phase protein synthesis (Wannemacher, 1977). Oxidation and gluconeogenesis liberate amino nitrogen. This is handled by the urea cycle (Krebs & Henseleit, 1932).

Urinary Urea Excretion

Injection of rats with LPS resulted in a 3-fold increase in urea excretion (**Figure 3.1**) 24-36 hours later. Total urea production over the first 24 hours in the metabolic cages was identical in both groups (control: 3.52 ± 0.922; LPS-treated: 3.49 ± 0.551 mmol/100 g body weight/24 hours). During 60-84 hours, or 24-48 hours after injection with LPS or saline there was marked increases in urinary urea excretion (control: 1.99 ± 0.924; LPS-treated: 4.60 ±1.42 mmol urea/100 g body weight/24 hours). This *in vivo* experiment answered our first question: Is there an increased flux through the enzymes of the urea cycle in rats which have been injected with endotoxin? Yes, clearly, ureagenesis is stimulated in endotoxemic rats, but the nature of the effect was unknown.

Is There an Increase in Ureagenic Substrates Post-LPS?

Ammonia

The true substrate for CPS I is ammonia (Cohen *et al*, 1985). We measured the level of ammonia in the liver as well as in the blood vessels which supply the liver. There

were no changes subsequent to the injection of LPS (**Table 3.1**). Ammonia was not increased in the livers of endotoxemic rats therefore it does not appear to be exerting a push upon the urea cycle.

Amino Acids

Amino acids are important precursors for urea synthesis. In fact, an increase in ureagenesis will only occur if there is an increase in the amino acid concentration in the liver (Meijer *et al*, 1985) even if the enzymes of the cycle are activated or increased.

Total Free Amino Acids

Endotoxin treatment did not significantly alter the total free amino acid concentrations in: arterial blood, portal venous blood, portal plasma or skeletal muscle (**Table 3.2**). There was a significant increase in the total concentration of amino acids in the livers from endotoxemic rats (~30%) and in abdominal aortic plasma from these rats (~16%). There were also important differences in individual amino acid concentrations in each of these sources, specifically alanine and glutamine. The specific changes in each of these tissues will be discussed in subsequent sections.

Skeletal Muscle Amino Acids

The skeletal muscle did not show a significant change in its total free amino acid pool after injection of endotoxin (**Table 3.2**). There is evidence for increased muscle

proteolysis during febrile states (Baracos *et al,* 1983) and influx of amino acids is impaired in muscles of endotoxemic and septic rats (Hasselgren *et al*, 1986a). There are significant alterations in the intramuscular levels of alanine and glutamine.

Alanine is significantly elevated (~30%) in the quadriceps of LPS-treated rats (**Table 3.3**). Regulation of the alanine movement in muscle is, in part, via transporters. During severe infection (sepsis and endotoxemia) alanine influx via system A is impaired by 90% in rat soleus muscle within 16 hours of immune challenge (Hasselgren *et al,* 1986a). System A transport of alanine into muscle, which is stimulated by insulin in normal rats (Karlstad & Sayeed, 1985) exhibits insulin resistance in endotoxemic rats (Hasselgren *et al,* 1986c). Cortisol and epinephrine, are elevated in rats in the catabolic states noted and may further depress transport via system A (Watters *et al,* 1986). Thus increased alanine release would require increased alanine concentrations in muscle cells, which is observed.

Glutamine concentration, also an important glucogenic and ureagenic amino acid, was significantly decreased (~36%; **Table 3.4**) in LPS-treated rat muscle. Glutamine is a fuel for intestinal cells (Souba, 1991), whose absorption of glutamine is impaired in septic rats (Salloum *et al*, 1991), and for immunocytes (Newsholme *et al,* 1985). During endotoxemia, the demand for fuels for immunocytes increases. Glucose, which may also be used by these cells, becomes limiting so there is extra demand for glutamine. Dietary supplementation of glutamine improves survival rate in septic mice (Adjei *et al*, 1994). As there are no protein stores *per se* the bulk of the necessary glutamine must come from

muscle in endotoxemic rats. This is also true under such catabolic states as sepsis (Roth *et al*, 1982) and after surgery (Vinnars *et al*, 1975) during which muscle glutamine depletes and muscle mass is decreased.

A positive relationship has been found between intramuscular glutamine concentration and the rate of muscle protein synthesis (Jepson *et al*, 1988; MacLennan *et al*, 1987). It is hypothesized that depletion of intramuscular glutamine results in decreased protein synthetic rates and net loss of mass. In rats, extensor digitorum longus (EDL) muscles exhibit greatest glutamine loss during endotoxemia (James *et al*, 1993; Hasselgren *et al*, 1986c). Our observation of decreased muscle glutamine in a situation where there is known to be net muscle proteolysis (Jepson *et al*, 1988) is consistent with these literature reports. The basis of this is not known. However decreased intramuscular glutamine may result from: (1) decreased muscle glutamine synthetase activity which is unlikely under these circumstances (Wannemacher, 1977); (2) decreased muscle proteolysis, hence less glutamine in the free amino acid pool. This does not occur since proteolysis is elevated in muscle in response to endotoxin (Jepson *et al*, 1986); (3) increased glutaminase activity; in rat muscle glutaminase activity is significantly lower than that of glutamine synthetase (Muhlbacher *et al*, 1984); (4) increased glutamine efflux due to alterations in the transporter (Babij *et al*, 1986a; Jepson *et al*, 1986; Rennie *et al*, 1986). The fact that muscle glutamine concentration is decreased, whereas that of alanine is increased, points to some specificity to the loss of glutamine - it is not due to a generalized effect on amino acid transport. Transport of glutamine across sarcolemmal

membranes is via system N^m (Ahmed *et al*, 1993) which has characteristics distinct from the liver glutamine transporter N (Kilberg *et al*, 1980). Human N^m is similar to rat N^m (Hundal *et al*, 1986).

The actual mechanism of glutamine uptake is one of co-transport of glutamine and sodium (Ahmed *et al*, 1993) in a 1:1 ratio (Hundal *et al*, 1986). ^{22}Na uptake via N^m by human muscle vesicles is increased in the presence of excess glutamine. Glutamine transport may depend upon the electrochemical gradients of both Na and glutamine (Hundal *et al*, 1986; Hundal *et al*, 1987). Further, uptake of glutamine by human sarcolemmmal vesicles is decreased under a variety of circumstances (such as when osmolarity of surrounding medium increases or if pH of the medium decreases) (Ahmed *et al*, 1993). However, influx of glutamine is accelerated if there is a negative potential on the inside of cells (Ahmed *et al*, 1993) or if there is an inwardly directed Na^+ gradient. During severe illnesses there are significant increases in intramuscular Na^+, Ca^{++}, and decreased K^+ (Askanaski *et al*, 1980). It is postulated that this influx of Na^+, by means yet unknown, may 'short-circuit' Na^+/glutamine co-transport. N^m also exhibits glucocorticoid sensitivity in perfused rat hindlimb (Babij *et al*, 1986b; Kayali *et al*, 1987) as glutamine influx decreases significantly. LPS and live bacteria injections in rats also result in decreased glutamine transport (Lynn *et al*, 1986). This discussion focuses on decreased glutamine transport into muscle cells which may result in net glutamine loss if outward glutamine transport is not affected. It must also be recognized that there may be an acceleration of outward glutamine transport, a process which is little understood.

Liver Amino Acids

The liver is a key site for amino acid metabolism. It is the site of ureagenesis (Krebs & Henseleit, 1932) and of the bulk of gluconeogenesis. Both of these pathways are vital during catabolic states such as endotoxemia. The liver also synthesizes acute phase proteins at an accelerated rate in immune-challenged animals (Beisel, 1977; Wannemacher *et al,* 1974). Amino acid concentrations within the liver are of specific importance to the effect(s) of bacterial endotoxin injection on urea synthesis in rats.

The total concentration of amino acids was significantly increased (**Table 3.2**) in liver while there were no differences in the total concentration of amino acids measured in the blood vessels that supply the liver. Transport of amino acids into the liver increases in endotoxemic rats (Inoue *et al,* 1993) as does blood flow to the liver (Augsten *et al,* 1991). Endotoxin administration results in an increase in rat hepatocyte volume (Qian & Brosnan, 1996) which, in turn, may stimulate amino acid uptake, protein synthesis, and ureagenesis from amino acids (Häussinger & Lang, 1991; Halestrap, 1989).
There were five amino acids which were significantly increased (**Table A-3**). Three of these, glutamate, alanine, citrulline, have specific roles in ureagenesis. Glutamate and ammonia are hydrolysis products of glutamine. Glutamate may also stimulate production of NAG and this will be discussed further in a later section. Citrulline is an intermediate in the urea cycle.

LPS-treated rat livers contained approximately twice as much alanine as did the control rat livers (**Table 3.3**). Studies have shown that nearly 50% of the total amino acid

uptake by liver is alanine (Felig, 1973). As the key glucogenic substrate during endotoxemia (Wannemacher, 1977), there is increased alanine demand to ameliorate the hypoglycaemia (Rayfield *et al,* 1973) and to supply obligate glucose-requiring tissues. In post-absorptive rats alanine catabolism favours glucose production over oxidation to CO_2 by 87% (Felig, 1973).

In post-absorptive man glutamine is taken up by liver in amounts comparable to alanine (Marliss *et al,* 1971). Glutamine was not found to be significantly increased in rat liver after administration of LPS (**Table 3.4**). However, Pacitti *et al,* (1992) determined that glutamine delivery to the liver doubled in endotoxemic rats without resulting in increased liver glutamine concentrations. In periportal rat hepatocytes starvation and LPS treatment stimulate glutamine uptake independently (1.6 and 2.6 fold, respectively) and synergistically (6.6-fold) (Fischer *et al,* 1997). It is also known that hepatic glutaminase is activated in response to endotoxin injection in rats (Ewart *et al,* 1995) and glutamate levels are increased (Pacitti *et al,* 1992; **Table A-3**). Cell swelling may stimulate hepatic glutaminase activity as well as ureagenesis (Halestrap, 1989; Häussinger *et al,* 1992). In effect, there is likely an elevated glutamine uptake but concurrent acceleration of glutamine catabolism.

Regulation of liver amino acid uptake following endotoxin treatment involves transport and may involve hormones and/or cytokines. LPS treatment of rats results in a time- and dose-dependent increase in transport of amino acids in hepatocyte plasma membrane vesicles (Inoue *et al,* 1995). The effects include: system A (5-fold), N (2.5-

fold), ASC (2.6-fold), y^+ and b^+ (2-fold). TNFα injection in rats resulted in increases in systems A, N and ASC (Pacitti *et al*, 1993). If rats were pre-treated with anti-TNFα antibodies prior to endotoxin administration (Inoue *et al*, 1994), there was a marked decrease in amino acid transport via decreased Vmax. Systems L, asc and n were unchanged by LPS injection.

Regulation of the effects of LPS on amino acid transport is likely to involve glucocorticoids, as well. Pre-treatment of rats with RU38486, a potent glucocorticoid antagonist, prior to endotoxin insult attenuated transport activity 20-60% (Inoue *et al*, 1995), also via decreased Vmax.

The Enzymes of the Urea Cycle

Flux through the entire cycle is increased after endotoxin administration, as urinary urea excretion triples in the LPS-treated rats (**Figure 3.1**). This was not found to result from increased total activity of any of the urea cycle enzymes, including the mitochondrial enzymes CPS I and OTC (**Table 3.5**). However, citrulline synthesis was doubled in mitochondria isolated from LPS injected animals (**Figure 3.2**). It was possible that elevated citrulline synthesis resulted from activation of CPS I after administration of LPS.

CPS I is an ATP-requiring enzyme (2 ATP-Mg^{2+}) and is subject to allosteric activation. *N*-acetylglutamate (NAG) is formed in the mitochondrial matrix from acetyl CoA and glutamate by *N*-acetylglutamate synthetase (EC 2.3.1.1). This enzyme is also

allosterically activated by arginine (Tatibana & Shigesada, 1976). In isolated rat liver mitochondria citrulline synthesis may be regulated by NAG (Meijer & VanWoerkom, 1978) while citrulline synthesis has been postulated as the key regulatory step in ureagenesis in isolated hepatocytes (McGivan *et al*, 1976). Specifically, CPS I has been hypothesized to be flux-generating for the urea cycle (Wanders *et al*, 1984; Meijer *et al*, 1985b) as it is insensitive to feedback by carbamyl phosphate (Meijer *et al*, 1990).

There has been much work on the actual binding of NAG to CPS I (McCudden & Powers-Lee, 1996; Rodriguez-Aparicio *et al*, 1989; Rubio *et al*, 1983) and the nature of its allosteric effect. When NAG binds near the carboxyl terminus of CPS I (McCudden & Powers-Lee, 1996) a binding site for the MgATP used to 'activate' bicarbonate is exposed. It is possible, therefore, that increased mitochondrial NAG concentration subsequent to LPS treatment may stimulate urea synthesis in the presence of elevated substrates, such as amino acids (**Tables 3.2** and **A-3**). Our work has shown a significant increase in mitochondrial NAG subsequent to LPS-treatment (**Figure 3.4**; control: 0.32 ± 0.080 nmol/mg versus LPS-treated: 0.46 ± 0.15 nmol/mg). We hypothesize that an accumulation of NAG may stimulate CPS I and, hence, urea synthesis. Other studies using intact, isolated rat liver mitochondria appear to support these findings. A positive correlation was determined between mitochondrial NAG and citrulline synthesis in control mitochondria (Beliveau-Carey *et al*, 1993) and a similar relationship was shown in this study for mitochondria from control and LPs-treated rats (**Figure 3.5**). Also, in mitochondria, citrulline synthesis was linearly dependent on NAG content up to about 1.5

mM (intramitochondrial) under various hormonal and nutritional conditions (Rabier *et al*, 1985; Hensgens *et al*, 1980). In our GC-MS-determined concentrations, the concentration of NAG was always well below 1.5 mM. (Values obtained from Dr. Kratz were presented as nmol/mg as well as nmol/ml for each mitochondrial sample).

Increases in mitochondrial NAG levels may be affected, in part, by: (1) mitochondrial glutamate levels (liver glutamate was found to increase by 46% in endotoxin-treated rats; **Table A-3**); and (2) possible inhibition of NAG efflux into cytosol (Meijer *et al*, 1982). We did not measure acetyl CoA but its level is unlikely to increase under these circumstances (Winter *et al*, 1995). Also, total liver arginine (the allosteric activator of NAG synthetase) was not found to be increased in this study. We could not measure mitochondrial arginine.

Glucagon, which is elevated in endotoxemic and septic rats (Watters *et al*, 1986), has been shown to increase liver NAG content by 30% *in vivo* in rats (Meijer & Van Woerkom, 1978). Also, there was increased citrulline synthesis in mitochondria isolated from the livers of glucagon-injected rats (Meijer & Van Woerkom, 1978). In addition they found a positive correlation between the rate of ureagenesis in hepatocytes and the NAG content of mitochondria isolated from these hepatocytes. Interestingly, NAG has also been found to activate glutaminase (Meijer & Verhoeven, 1986). We propose that the elevated ureagenesis in whole animals as well as the increased citrulline synthesis in isolated mitochondria results from at least two main sources of regulation: 1) increased mitochondrial NAG, thus increased available CPS I catalytic sites; and 2) increased

availability of ureagenic substrates.

Summary and Conclusions

Summary and Conclusions

1. A single intraperitoneal injection of *E.coli* LPS (0127: B8) at a sublethal dose (3mg/kg) significantly stimulates flux through the urea cycle *in vivo*. Urinary urea excretion, effectively, triples (1.13 ± 0.34 controls versus 2.93 ± 0.79 LPS ; mmol urea/100g body weight). This tripling is observed from 12-24 hours post treatment and the elevation remains for 36 hours post-injection.

2. There are elevated circulating concentrations of ureagenic substrates, particularly alanine and glutamine. Liver free amino acid totals are also significantly increased after endotoxin adminstration (13672 ± 2057 controls versus 19650 ± 4394 LPS; nmol/mg). An increased delivery of ureagenic amino acids such as alanine and glutamine will contribute to the stimulation of flux through the urea cycle in the LPS-treated rats. Ammonia levels remain unchanged subsequent to endotoxin treatment.

3. The urea cycle enzymes, themselves, are not changed in total activity subsequent to treatment.

4. Citrulline synthesis rates of intact isolated mitochondria from LPS-treated rat livers (35.47 ± 12.60 nmol/min/mg; $p<0.05$) are twice those of saline-treated control rat liver mitochondria (18.23 ± 8.59 nmol/min/mg). This significant stimulation of flux through the mitochondrial enzymes is not due to increased CPS I nor OTC total activity as these

remain unaltered post-LPS.

5. GC-MS measurement of mitochondrial matrix NAG determined that mitochondria isolated from the livers of LPS-treated rats have significantly increased NAG concentrations relative to the mitochondria isolated from saline-treated control rats (0.318 ± 0.080 controls versus 0.459 ± 0.146 LPS-treated; nmol/mg, $p<0.05$). NAG accumulates in the mitochondria of LPS-treated rats within 24 hours of injection and will, in conjunction with the elevated ureagenic substrate concentration in the liver, contribute to the increased flux through the urea cycle.

6. Exogenous NAG activates citrulline synthesis in uncoupled rat liver mitochondria. At NAG concentrations of 2.0 *mM*-10.0 *mM* the difference between the two groups' citrulline synthesis rates disappears. At 10 mM external NAG the rates of citrulline synthesis were 35.6 ● 11.4 nmol/min/mg and 42.6 ± 11.2 nmol/min/mg, respectively, in the control and LPs-injected rats.

7. Citrulline synthesis rates of mitochondria are positively correlated with the NAG content of each mitochondrial preparation ($r^2=0.610$, $p<0.05$). NAG, which is increased in mitochondria from LPS-treated rats, stimulates flux through the mitochondrial enzymes of the urea cycle *in vitro* and contributes to the elevated flux through the cycle proper *in vivo*.

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Appendix-Amino Acid Analyses and Enzymic Assay Conditions

Table A-1: Summary of Enzymic Assays Utilized in this Study
Established Protein and Time Intervals for Marker Enzymes and for the Enzymes of the Urea Cycle.

Enzyme	Protein (mg)	Time (minutes)
Glutamate Dehydrogenase	0.003	~12
Flux Through Mitochondrial Enzymes	3.0	6
CPS I	0.3	6
OTC	0.02	6
Argininosuccinate Synthetase	3.0	10
Argininosuccinate Lyase	2.0	15
Arginase	1.0-2.5	10
N-Acetylglutamate preincubation	3.0	10
Citrulline Synthesis (exogenous NAG)	3.0	2

Table A-2: Arterio-Venous Free Amino Acid Concentration Differences Across the Intestine in Whole Blood of Saline- Versus LPS-Treated Rats 18 hours Post-injection. Blood samples were taken from the abdominal aorta or the hepatic portal vein. Values a presented as means ± standard deviation. Statistical significance was determined by paired t-test to be different from zero, *p<0.05, and is denoted by an asterisk. (n=8.)

Amino Acid	A-V Control (nmol/ml)	A-V LPS-Treated (nmol/ml)
Taurine	-127.7 ± (103.7)*	-86.8 ± (297.2)
Aspartate	-133.2 ± (321.7)	-67.9 ± (299.6)
Threonine	-25.2 ± (53.2)	-26.6 ± (143.8)
Serine	-27.9 ± (28.3)	-19.2 ± (84.2)
Asparagine	1.4 ± (22.3)	19.2 ± (84.2)
Glutamate	-16.4 ± (42.3)	-23.7 ± (69.3)
Glutamine	5.9 ± (72.4)	-37.7 ± (72.4)
Proline	5.6 ± (50.8)	-25.6 ± (150.4)
Glycine	-65.8 ± (77.2)	-58.9 ± (198)
Alanine	-102.2 ± (121.8)	-107.6 ± (458)
Citrulline	-5.8 ± (29)	-30.2 ± (60.2)
Valine	-8.7 ± (44.9)	-41.5 ± (121.4)
Cystine	1.0 ± (15)	3.87 ±(10.7)
Methionine	-8.13 ± (11.2)	-9.9 ± (28.7)
Isoleucine	-6.7 ± (18.5)	-10.8 ± (22.4)
Leucine	-7.2 ± (33.7)	-13.8 ± (57.5)
Tyrosine	-13.9 ± (22.7)	-7.4 ± (48)
Phenylalanine	-7.4 ± (14.3)	-11.0 ± (46.6)
Tryptophan	1.56 ± (12.7)	-4.12 ± (15.1)
Ornithine	-9.1 ± (19.2)	-15.7 ± (59.4)
Lysine	-75.0 ± (88.2)	-71.6 ± (360.8)
Histidine	-5.24 ± (11.1)	-7.90 ± (49.0)
Arginine	-18.8 ± (49.5)	-32.6 ± (33.1)*

Table A-3: Free Amino Acid Concentrations Determined in Rat Liver 18 Hours After Saline- or LPS-Treatment. Liver samples were freezed clamped and stored in liquid nitrogen prior to analysis. Values are presented as means ± standard deviations, n=8. Significance is determined by unpaired t-test and denoted with an asterisk. (*$p<0.05$)

Amino Acid	Control (nmol/g)	LPS-Treated (nmol/g)
Taurine	2227 ± (1255)	4175 ± (1894)*
Aspartate	NA	NA
Threonine	385 ± (139)	944 ± (731)
Serine	601 ± (173)	1194 ± (826)
Asparagine	38 ± (25)	51 ± (31)
Glutamate	1051 ± (195)	1831 ± (501)*
Glutamine	4316 ± (461)	3972 ± (658)
Proline	99 ± (31)	134 ± (56)
Glycine	1139 ± (244)	1519 ± (721)
Alanine	320 ± (56)	771 ± (447)*
Citrulline	77 ± (19)	138 ± (61)*
Valine	138 ± (46)	142 ± (49)
Cystine	52 ± (16)	52 ± (18)
Methionine	48 ± (19)	59 ± (19)
Isoleucine	99 ± (41)	93 ± (31)
Leucine	124 ± (50)	126 ± (34)
Tyrosine	72 ± (31)	72 ± (35)
Phenylalanine	49 ± (35)	62 ± (28)
Tryptophan	12 ± (18)	13 ± (19)
Ornithine	192 ± (58)	306 ± (164)
Lysine	431 ± (81)	617 ± (225)*
Histidine	389 ± (55)	414 ± (68)
Arginine	32 ± (40)	30 ± (38)

Table A-4: Free Amino Acid Concentrations in Skeletal Muscle of Saline-Treated Versus LPS-Treated Rats 18 Hours Post-injection. Skeletal muscle was dissected from rat quadriceps. Values are expressed as means ± standard deviations; 7 or 8 animals per group. An asterisk denotes statistical significance as determined by unpaired t-test.(two-tailed ; *$p<0.05$).

Amino Acid	Control (nmol/g)	LPS-Treated (nmol/g)
Taurine	8724 ± 1311	9615 ± 2284
Aspartate	389 ± 55	518 ± 63*
Threonine	436 ± 71	490 ±144
Serine	586 ±90	498 ± 64*
Asparagine	112 ± 22	127 ± 25
Glutamate	1249 ± 204	1261 ± 198
Glutamine	2478 ± 500	1819 ± 397*
Proline	151 ± 66	210 ± 31
Glycine	3941 ± 906	2478 ± 429*
Alanine	1017 ± 202	1475 ± 333*
Citrulline	147 ± 38	85 ± 22*
Valine	169 ± 34	207 ± 40
Cystine	38 ± 6.3	38 ± 6.2
Methionine	51 ± 10	89 ± 21*
Isoleucine	94 ± 16	109 ± 18
Leucine	124 ± 20	164 ± 33*
Tyrosine	119 ± 26	121 ± 18
Phenylalanine	61 ± 9.4	93 ± 23*
Tryptophan	8.8 ± 7.1	16 ± 8.6
Ornithine	77 ± 10	72 ± 24
Lysine	882 ± 33	561 ± 219*
Histidine	153 ± 31	146 ± 22
Arginine	294 ± 98	177 ± 38*

Table A-5: Free Amino Acid Concentrations Determined in Hepatic Portal Venous and Abdominal Aortic Blood in Saline-Treated Versus LPS-Treated Rats. Values are presented as means ± standard deviations. Statistical significance is denoted by an asterisk; n=8. Two-tailed p values by unpaired t-test;*p≤0.05.

Amino Acid	Abdominal Aortic (nmol/ml)		Hepatic Portal Venous (nmol/ml)	
	Control	LPS	Control	LPS
Taurine	393 ± (102)	319 ± (152)	472 ± (99)	423 ± (155)
Aspartate	242 ± (116)	22 ± (166)	355 ± (232)	292 ± (189)
Threonine	241 ± (19)	244 ± (85)	263 ± (48)	271 ± (45)
Serine	234 ± (18)	198 ± (47)	250 ± (24)	217 ± (45)
Asparagine	41 ± (7.0)	45 ± (23)	40 ± (16)	55 ± (18)
Glutamate	206 ± (48)	162 ± (35)	220 ± (31)	185 ± (50)
Glutamine	518 ± (44)	613 ± (120)	511 ± (68)	651 ± (108)*
Proline	131 ± (53)	128 ± (92)	128 ± (16)	154 ± (720)
Glycine	366 ± (35)	300 ± (85)	426 ± (75)	358 ± (77)
Alanine	293 ± (52)	506 ± (251)*	399 ± (74)	614 ± (238)*
Citrulline	63 ± (7.0)	72 ± (42)	70 ± (25)	102 ± (28)*
Valine	167 ± (65)	166 ± (71)	186 ± (48)	207 ± (54)
Cystine	35 ± (12)	38 ± (14)	30 ± (8.6)	34 ± (5.3)
Methionine	37 ± (4.8)	53 ± (17)*	45 ± (8.4)	63 ± (15)
Isoleucine	78 ± (15)	74 ± (17)	86 ± (9.2)	85 ± (7.3)
Leucine	114 ± (24)	134 ± (40)	120 ± (15)	148 ± (21)*
Tyrosine	77 ± (7.8)	83 ± (32)	88 ± (23)	90 ± (21)
Phenylalanine	48 ± (13)	82 ± (29)*	56 ± (9.4)	93 ± (19)*
Tryptophan	29 ± (11)	24 ± (11)	27 ± (4.0)	28 ± (5.0)
Ornithine	88 ± (7.1)	102 ± (38)	97 ± (15)	118 ± (35)
Lysine	523 ± (64)	475 ± (210)	593 ± (62)	547 ± (197)
Histidine	45 ± (13)	67 ± (30)	52 ± (8.0)	75 ± (20)*
Arginine	207 ± (16)	183 ± (39)	207 ± (16)	183 ± (39)

Table A-6: Free Amino Acid Concentrations Determined in Abdominal Aortic and Hepatic Portal Venous Plasma of Saline- Versus LPS-Treated Rats 18 Hours Post-Injection. Values are presented as means ± standard deviations, n=8. An asterisk denotes statistical significance, two-tailed *p<0.05, as determined by unpaired t-test.

Amino Acid	Abdominal Aortic (nmol/ml)		Portal Venous (nmol/ml)	
	Control	LPS-Treated	Control	LPS
Taurine	160 ± (40)	220 ± (64)	191 ± (42)	261 ± (66)
Aspartate	13 ± (3.0)	16 ± (3.0)	19 ± (4.0)	22 ± (3.0)
Threonine	234 ± (26)	267 ± (44)	248 ± (31)	224 ± (45)
Serine	199 ± (16)	186 ± (37)	199 ± (25)	166 ± (35)*
Asparagine	45 ± (5.0)	60 ± (11)*	48 ± (5.0)	57 ± (8.0)*
Glutamate	90 ± (28)	67 ± (9.0)*	117 ± (44)	72 ± (12)*
Glutamine	574 ± (82)	799 ± (82)*	539 ± (47)	675 ± (92)*
Proline	121 ± (13)	159 ± (29)*	138 ± (15)	168 ± (26)*
Glycine	272 ± (36)	277 ± (48)	324 ± (36)	294 ± (50)
Alanine	340 ± (42)	496 ± (104)*	432 ± (56)	552 ± (113)*
Citrulline	61 ± (14)	75 ± (12)	76 ± (16)	91 ± (13)
Valine	188 ± (29)	210 ± (29)	191 ± (22)	196 ± (29)
Cystine	30 ± (20)	35 ± (13)	28 ± (18)	38 ± (8.0)
Methionine	48 ± (6.0)	78 ± (14)*	52 ± (4.0)	76 ± (14)*
Isoleucine	86 ± (7.0)	95 ± (14.5)	92 ± (7.5)	91 ± (16)
Leucine	132 ± (15)	158 ± (23)*	139 ± (13)	150 ± (25)
Tyrosine	81 ± (10)	85 ± (12)	85 ± (12)	82 ± (12)
Phenylalanine	59 ± (10)	97 ± (14)*	63 ± (8.6)	93 ± (14)*
Tryptophan	70 ± (12)	77 ± (14)	72 ± (6.4)	72 ± (16)
Ornithine	42 ± (11)	58 ± (15)*	45 ± (9.0)	57 ± (14)*
Lysine	445 ± (90)	409 ± (82)	475 ± (44)	394 ± (78)*
Histidine	60 ± (10)	73 ± (11)*	62 ± (9.0)	70 ± (10)
Arginine	125 ± (19)	146 ± (36)	126 ± (14)	132 ± (31)

Table A-7: Arterio-Venous Free Amino Acid Concentration Differences in Plasma From Saline- Versus LPS-Treated Rats 18 Hours Post-Injection. Plasma samples were obtained from blood taken from the abdominal aorta and the hepatic portal vein. Values are represented as means ± standard deviations, n=8. An asterisk denotes a difference which is significantly different from zero as determined by paired t-test. (*$p<0.05$)

Amino Acid	A-V Control (nmol/ml)	A-V LPS-Treated (nmol/ml)
Taurine	-29.9 ± (42.0)	-41.1 ± (20.4)*
Aspartate	-6.64 ± (5.59)*	-6.22 ± (2.54)*
Threonine	-45.0 ± (33.1)*	12.2 ± (14.3)*
Serine	-0.75 ± (24.6)	20.5 ± (10.6)
Asparagine	-2.46 ± (7.21)	2.83 ± (4.71)
Glutamate	-27.3 ± (66.3)	-5.06 ± (8.35)
Glutamine	34.5 ± (114.1)	124.1 ± (70.8)*
Proline	-16.3 ± (20.6)	-9.58 ± (11.1)*
Glycine	-51.8 ± (42.2)*	-17.4 ± (13.3)*
Alanine	-92.8 ± (79.4)*	-56.4 ± (42.4)*
Citrulline	-15.1 ± (22.1)	-16.0 ± (9.56)*
Valine	-3.18 ± (37.9)	14.6 ± (10.4)
Cystine	2.85 ± (34.1)	0.12 ± (17.4)
Methionine	-4.19 ± (7.72)	2.08 ± (5.19)
Isoleucine	-5.25 ± (10.0)	4.61 ± (5.68)
Leucine	10.3 ± (21.6)	8.71 ± (7.9)*
Tyrosine	-3.91 ± (12.9)	3.39 ± (4.14)
Phenylalanine	-3.44 ± (17.2)	4.11 ± (5.08)
Tryptophan	-1.13 ± (17.2)	5.67 ± (4.63)*
Ornithine	-2.88 ± (18.6)	0.73 ± (4.86)
Lysine	-30.2 ± (69.2)	15.1 ± (21.0)
Histidine	-2.07 ± (19.1)	2.98 ± (3.65)
Arginine	-0.29 ± (24.0)	14.0 ± (9.37)*

"Whatsoever thy hand findeth to do, do it with thy might; for there is no work, nor device, nor knowledge, nor wisdom, in the grave, whither thou goest."

Solomon