THE CATABOLISM OF ARGININE AND ORNITHINE IN THE LIVER

DAN O'SULLIVAN

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THE CATABOLISM OF ARGININE AND ORNITHINE IN THE LIVER

by

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degree of Doctor of Philosophy.

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ABSTRACT

The liver is a key organ for the catabolism of amino acids in mammals. However, the processes for the catabolism of arginine and ornithine in liver have not been studied in great detail. Neither the extent to which these amino acids are catabolized, nor the location within the liver of this catabolism is known. With respect to the localization of these processes, certain metabolic pathways are restricted to specific regions within the liver. Thus, it is possible that the processes for catabolizing ornithine and arginine are not distributed homogeneously throughout the liver, but are contained within a specific region. Furthermore, the catabolism of amino acids such as glycine and glutamine in liver is known to be regulated by various dietary and hormonal stimuli; the rates of catabolism of arginine and ornithine may also respond to such stimuli.

It was discovered that the catabolism of both ornithine and arginine could be carried out, in their entirety, in the perivenous cells of the liver (those cells lining the central vein, where blood normally exits the liver). Also, the rates of catabolism of ornithine and arginine are subject to regulation by the amount of dietary protein. Rats fed a high protein diet over a period of days showed increased rates of catabolism of these amino acids, when compared with rats fed a diet with normal protein content. With respect to the effects of hormones, it was demonstrated that the catabolism of arginine, but not of ornithine, is subject to acute stimulation by glucagon, whereas insulin was without effect.

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CONTENTS

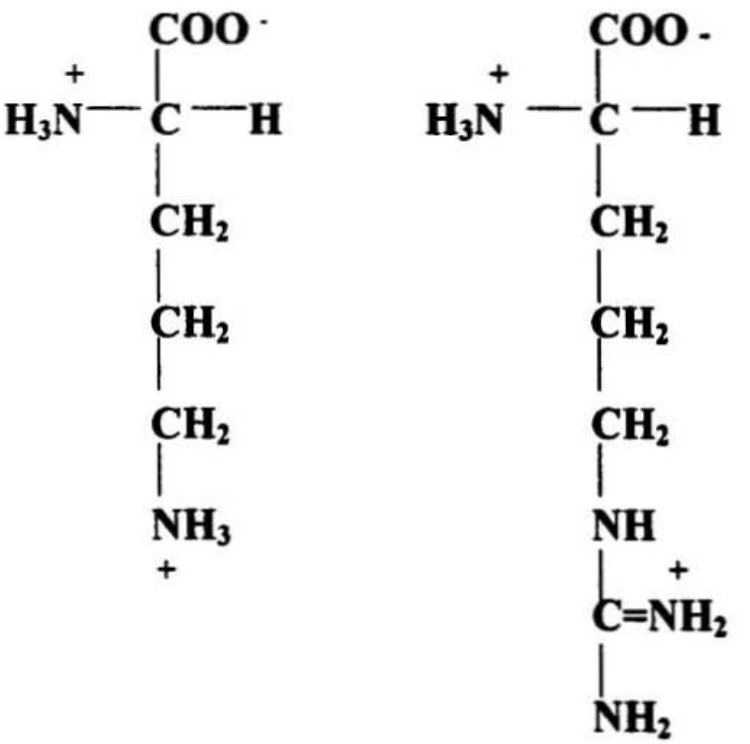
CHAPTER 1

INTRODUCTION

THE METABOLISM OF ARGININE AND ORNITHINE

THE DIETARY REQUIREMENT FOR ARGININE IN MAMMALS

The basic amino acid arginine (2-amino-5-guanidinovaleric acid), first isolated in the late nineteenth century, is shown in **Figure 1.1**. It is present in human and rat plasma at concentrations between 0.1mM and 0.2mM (Brusilow and Horwich, 1989; Rémésy et al, 1978). The dietary requirement for arginine differs among mammals and may vary during the development of individual members of a species. In adult humans, arginine was defined as a 'non-essential' amino acid (Rose et al, 1954). This is to say that endogenous *de novo* synthesis of arginine proceeds at a rate sufficient to meet the demand for arginine in humans. This study, in common with all the pioneering experiments of Rose, used the parameters of growth and nitrogen balance to assess the dietary requirement. In the same decade it was demonstrated that arginine was not required for growth or maintenance of nitrogen balance in human infants (Snyderman et al, 1959). In the immature rat arginine is required for optimum growth, but not for maintenance of nitrogen balance (Rose et al, 1948; Milner et al, 1974). In species such as cats (Morris, 1985) and ferrets (Deshmukh and Shope, 1983) there is an absolute requirement for this amino acid. The term 'conditionally essential' (Chipponi et al, 1982) is most often applied when discussing the requirement for arginine (for alternative nomenclature, see Laidlaw and Kopple, 1987; Young and El-Khoury, 1995). Arginine's requirement is conditional upon the species in question as well as the developmental stage under investigation. In this respect, it was acknowledged by Rose in his earlier studies that arginine may become an essential amino acid in humans under certain conditions (Rose et al, 1948).



L-ORNITHINE (pH 7.4)

L-ARGININE (pH 7.4)

Figure 1.1. Chemical structures of arginine and ornithine.

De novo synthesis of arginine is achieved principally by the concerted action of the intestine and kidney. Trivial amounts of the citrulline, produced from glutamine in the intestine (Windmueller and Spaeth, 1981), are catabolized in the liver leaving the majority to proceed to the kidney where it is converted, stoichiometrically, to arginine (Featherston et al, 1973; Dhanakoti et al, 1990). Arginine production from citrulline also occurs in endothelial cells (Sessa et al, 1990) and macrophages (Wu and Brosnan, 1992). This second and quantitatively minor route serves to recycle citrulline which is generated in the production of nitric oxide (NO) from arginine.

Considerable debate continues as to whether criteria based solely on the parameters of growth and nitrogen balance are adequate for the accurate assessment of the dietary requirement for arginine (Visek, 1986; Young and El-Khoury, 1995). Arising from this discussion is the proposal that orotic acid excretion should also be monitored, as this is a more sensitive indicator of arginine status (Milner et al, 1974). Briefly, as the urea cycle becomes depleted of arginine carbamoyl-phosphate accumulates within the mitochondria, the excess of which then leaks into the cytoplasm. This carbamoyl-phosphate is consumed in the production of orotic acid, an intermediate in pyrimidine biosynthesis. Orotic acid levels quickly exceed the renal threshold for this molecule and that amount excreted in the urine can be routinely quantified (Kesner, 1965). Orotic acid excretion is a more sensitive indicator, as the minimum arginine requirement to ensure basal orotic acid excretion is greater than that required for optimum growth (Milner et al, 1974). On a more general note, it is plausible that a wide range of specific metabolites

may be used in the assessment of the optimal requirements for the individual amino acids at some point in the future.

The currently recommended intake of protein (FAO/WHO/UNU, 1985) of 0.8g protein/kg body weight/day meets the requirement for growth and development in the human population at large and, as such, argues against the case for reanalysis of the dietary requirement of amino acids in humans. However, known pathophysiological situations exist in which arginine becomes an essential nutrient for humans. For example, patients with inborn errors of the urea cycle require dietary supplementation with arginine (Brusilow, 1984). Also, supplementation of diets with arginine has been shown to improve wound healing in rats (Seifter et al, 1978), and immune system function in humans (Barbul et al, 1981). Therapeutic administration of supra-dietary amounts of arginine is made feasible by the low toxicity associated with this amino acid. In human studies 30g/day of arginine-HCl have been administered orally over a period of 7 days (Barbul et al, 1981), or by a single i.v. injection over a 30 minute period (Goodner and Porte, 1972), the patients incurring only minor side effects. A key distinction should be made between the issue of requirement for the population at large, which appears to be adequate, from that of specialised needs which is at the heart of much of the current debate. Those processes which form the basis of a demand for arginine are now discussed.

ARGININE'S RÔLES IN MAMMALIAN METABOLISM

The metabolic processes in which arginine participates are outlined in **Figure 1.2.** The reaction catalyzed by arginase (EC 3.5.3.1) provides the principal link between arginine and ornithine. As a protein or common amino acid, arginine serves as a substrate for a specific tRNA-synthetase. In contrast, arginine can also function in the degradation of certain proteins; arginine is conjugated to the acidic N-terminal residues of proteins, in the form of an aminoacyl-tRNA, as a preparatory step in a ubiquitin-dependent protein degradation pathway (Ciechanover and Schwartz, 1989). Thus, competition for available arginyl-tRNA between these opposing processes may occur within the cell (Sivaram and Deutscher, 1990). Arginine is an intermediate of the urea cycle, a process for the detoxification of ammonia (Krebs and Henseleit, 1932) carried out in the livers of animals. *In vitro*, arginine is an activator of N-acetyl glutamate synthase (EC 6.2.3.11), the product, N-acetyl glutamate, can stimulate carbamoyl-phosphate synthesis (Meijer et al, 1990). In addition, increasing dietary protein which increases circulating arginine levels results in increased citrulline synthesis in isolated rat liver mitochondria. In these experiments, increasing dietary protein increased the mitochondrial content of N-acetyl glutamate (Morimoto et al, 1990).

Arginine can be converted to agmatine in a reaction catalyzed by arginine decarboxylase (EC 4.1.1.19). This reaction is quantitatively minor in terms of daily arginine consumption. The recent isolation of agmatine in bovine brain (Li et al, 1994) is the first demonstration of this substance in mammals. This molecule is capable of displacing the

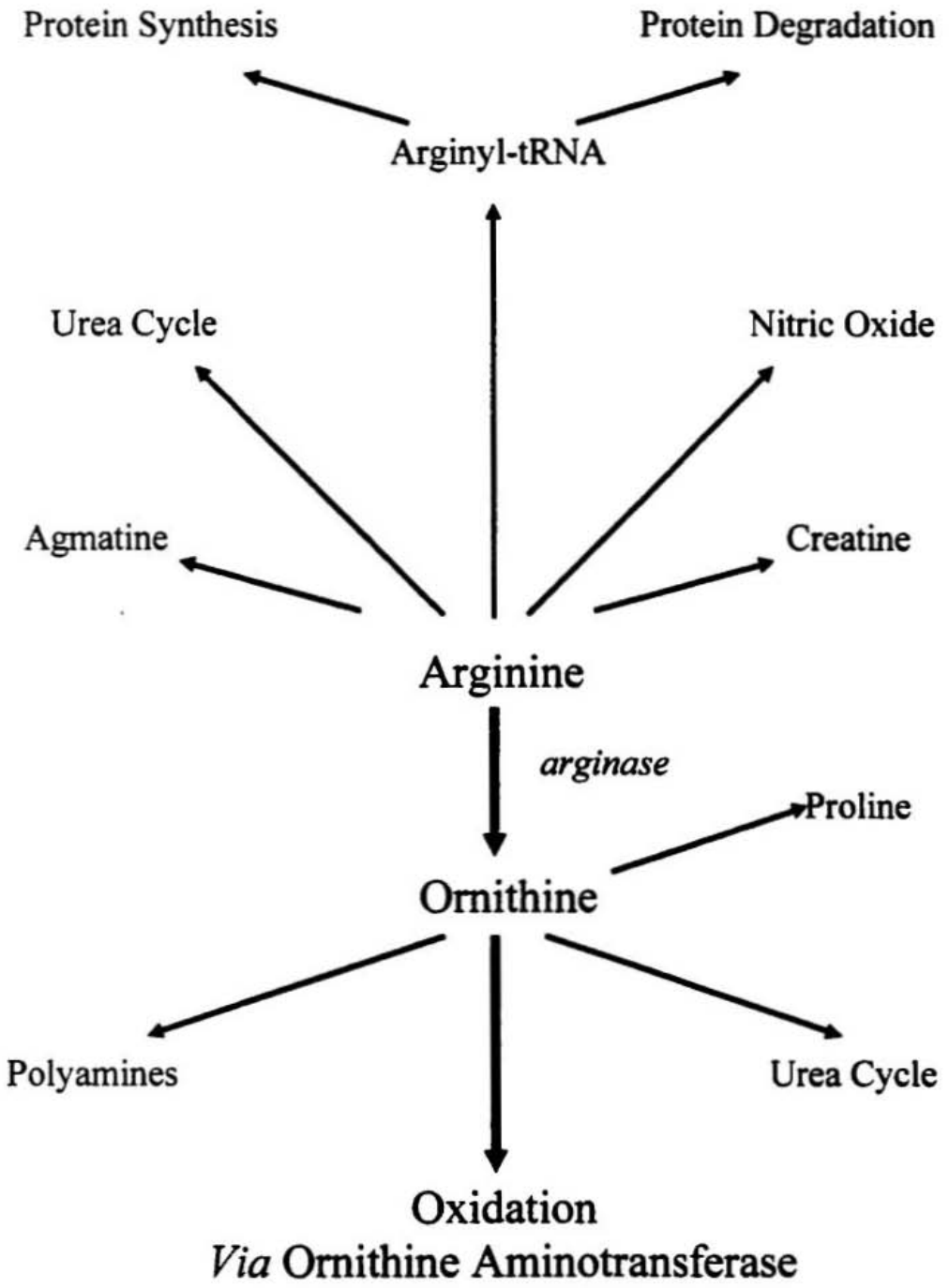


Figure 1.2. The metabolic roles of arginine and ornithine in mammals.

anti-hypertensive drug clonidine by binding to both α2-adrenergic receptors as well as both classes of imidazoline receptors (I_1 and I_2). Agmatine stimulates the production of epinephrine and norepinephrine in adrenal chromaffin cells *via* an interaction with the I_2 class of imidazoline receptors (Li et al, 1994). A further study reports the presence of agmatine (10^{-12}M-10^{-9}M) in rat brain, kidney and liver as well as plasma (Raasch et al, 1995). Agmatine production may occur within the tissues as arginine decarboxylase mRNA has been found in rat kidney, brain, gut, adrenal gland and liver (Morrissey et al, 1995). However, the presence of a particular mRNA species is not definitive evidence for the existence of the corresponding protein. Particular attention has focused upon the rôle of this molecule in the regulation of kidney function; agmatine increases the single nephron glomelular filtration rate and absolute proximal reabsorption *via* independent mechanisms (Lortie et al, 1996). In bacteria, agmatine serves as precursor to the polyamines, however, recent evidence suggests that this molecule reduces the production of the polyamines in mammals (Satriano et al, 1998).

Arginine is consumed in the production of nitric oxide (NO). Currently, this is one of the most actively researched areas in the field of amino acid metabolism. Originally known as the "endothelium derived relaxing factor" (Furchgott and Zawadski, 1980) this gas has been shown to be an important regulator of blood pressure (Palmer et al, 1987), neurotransmission (Bredt and Snyder, 1989) and is also responsible for the cytotoxic activity of macrophages (Hibbs et al, 1988). Nitric oxide synthase, which requires the cofactors tetrahydrobiopterin, FMN, NADPH and FAD (Stuehr, 1997), catalyzes the

reaction in which arginine and molecular oxygen are converted to citrulline and NO. NO production appears to be a feature of most mammalian cell types (Nathan and Xie, 1994). The three isoforms of nitric oxide synthase which have been characterized to date are endothelial (type 1) and neuronal (type 3), the calcium-calmodulin dependent constitutively expressed isoforms, and the inducible (type 2) which is calcium independent (Stuehr, 1997). NO production may not always be beneficial. For example, NO plays a protective rôle in a model of acute kidney dysfunction (Waddington et al, 1996) as well as natural killer cell activity (Hibbs et al, 1988) whereas excessive NO production may be a key factor in the development of a number of neurodegenerative diseases (Hantraye et al, 1996).

NO production, in the normal rat liver, is below the level of detection in both a recirculating perfusion (Pastor et al, 1995) and a single-pass perfusion model (Wettstein et al, 1994). (The lower limit of detection of the standard assays for NO is between 10^{-9} M and 10^{-7} M). Thus, hepatic NO production has little impact on the daily consumption of arginine in the normal liver. However, hepatic NO production does increase during a septic insult, principally due to the action of the inducible NO-synthase isoform. NO production is also stimulated by cytokines, and by treatment of rats with killed Corynebacterium-parvum (Pastor et al, 1995) or bacterial endotoxin (Wettstein et al, 1994; Pastor et al, 1995). Under these conditions, NO production assumes a more significant rôle in terms of daily arginine consumption.

Arginine is a secretagogue, stimulating the secretion of insulin (Mulloy et al, 1982), glucagon (Assan et al, 1977) and growth hormone (Alba-Roth et al, 1988). Endogenous steroid secretion also increases with increased arginine supply (Barbul et al, 1983). Arginine serves as the sole source of amidino groups for the synthesis of creatine, a molecule important in muscle and energy metabolism. A fraction of this creatine spontaneously degrades to creatinine (in clinical biochemistry creatinine is the most widely used indicator of kidney function). Visek contends that the currently accepted daily requirement for arginine is barely sufficient to replace daily obligatory losses of this molecule (Visek, 1986). However, for every molecule of creatine formed from arginine, there is a concomitant production of an ornithine molecule in this process which may then be recycled to arginine. Dietary arginine is the chief source of the amino acid ornithine and thus arginine must be viewed as a primary precursor to the processes of ornithine metabolism. These processes are now discussed.

THE RÔLE OF ORNITHINE IN MAMMALIAN METABOLISM:

The non-protein amino acid ornithine (2,5-diamino-pentanoic acid, **Figure 1.1**), derived principally from dietary arginine, is present in human and rat plasma at concentrations of approximately 0.1mM (Valle and Simmell, 1995; Rémésy et al, 1978). Ornithine is an intermediate of the urea cycle (Krebs and Henseleit, 1932). Reactions which consume ornithine are catalyzed by ornithine aminotransferase (EC 2.6.1.13; OAT) and ornithine decarboxylase (EC 4.1.1.17), the former being the major catabolic enzyme of ornithine and the latter an enzyme required for polyamine production, a quantitatively minor route

for disposal of the daily ornithine load. Polyamines function in cell growth and differentiation, but much remains unknown concerning the physiological rôles of these polycationic molecules (Tabor and Tabor, 1984). Ornithine may be converted to proline or glutamate, these three amino acids are all linked *via* the intermediate pyrroline-5-carboxylate (Jones, 1985; Herzfeld et al, 1977). Based on the distribution of the enzymes P5C-reductase and ornithine aminotransferase the capacity to produce proline from ornithine is common in rat tissues, particularly in rat fetal tissues (Herzfeld et al, 1977) and in the lactating rat mammary gland (Mezl and Knox, 1977; Jones, 1985). In addition, production of proline from P5C, catalyzed by P5C-reductase in cells such as human erythrocytes may function to regulate the ratio of oxidized to reduced nucleotide pyridines i.e. NADP/NADPH. This is based on the enzymes preferential use of NADPH within these cells and the observation that NADP, but not proline, can inhibit this enzyme (Merrill et al, 1989). As emphasized in **Figure 1.2** arginine may be oxidized through ornithine aminotransferase, after its conversion to ornithine. This process is the primary concern of this thesis.

INBORN ERRORS OF METABOLISM OF ARGININE AND ORNITHINE IN HUMANS

Frequently, clues concerning the physiological rôle of a particular process arise from the situations in which the process malfunctions. Studies investigating the genetic disorders of arginine and ornithine metabolism have provided answers to two questions that are of particular relevance to this thesis. These deal with the presence of isoforms of arginase

within mammalian tissues, and the importance of the OAT reaction to the disposal of the daily loads of arginine.

Argininemia, a deficiency of the liver-type arginase (AI type), is the least common of the inborn errors of urea cycle metabolism (Prasad et al, 1997; Brusilow and Horwich, 1989), with 27 cases reported in the literature to date. It is an autosomal recessive disease produced by a variety of mutations in the AI arginase gene (Uchino et al, 1995; Uchino et al, 1992); this heterogeneity at the gene level results in clinical symptoms of varying severity among sufferers. In addition to greatly elevated plasma arginine concentrations (400-1500μM), clinical symptoms include progressive mental retardation, spasticity, decreased motor function and episodic hyperammonemia. Near-normal urea production persists due to the functioning of the kidney-type arginase (AII type), an important factor in the relatively mild clinical course of the disease. The limited human biopsy samples available to date show elevated AII arginase activity; the range of this increase is from 2-30 fold, relative to normal individuals (Spector et al, 1980; Grody et al, 1993). Raised circulating levels of arginine may be responsible for the induction of AII; *in vitro* studies with a human embryonic kidney cell line show that elevated arginine concentrations in the medium cause an induction of the AII enzyme (Grody et al, 1989). The presence of a substantial kidney arginase activity in these patients, concomitant with the complete absence of the AI isozyme, was, at the time, a substantive piece of evidence in favour of the presence within humans of at least two independently regulated arginase genes. The human genes for the AI (Ohtake et al, 1988) and AII (Vockley et al, 1996) arginase genes

have since been cloned.

With respect to ornithine metabolism, gyrate atrophy of the choroid and retina is an autosomal recessive disease, characterized by a deficiency of OAT, in which plasma ornithine levels reach 400-1400μM (Valle and Simell, 1995) and 0.5 to 10 mmoles of ornithine may be excreted daily. There have been 150 cases documented, with a particularly high prevalence among Finnish people. Clinical symptoms include myopia and night blindness leading to tunnel vision in the second decade of life. Cataracts may also occur with retinal damage usually resulting in complete blindness by the fourth decade. Tubular aggregates are also apparent in type-II muscle fibers. Creatine administration is effective in dealing with the tubular aggregates in type-II fibres. The most efficacious of the therapeutic measures employed to date has been the provision of an arginine-restricted diet since OAT is a major consumer of the daily arginine, and thus, ornithine load. In cases in which residual OAT activity exists supra-physiological doses of pyridoxine and pyridoxal phosphate (the cofactor for this enzyme) have shown promise in reducing circulating levels of ornithine. The non-uniformity of response to the various treatments is a reflection of the heterogeneity of this disease at the level of the OAT gene (Park et al, 1992). The other inborn error of metabolism involving ornithine metabolism which has so far been described in only 40 patients (Valle and Simell, 1995) is thought to occur as a result of a defective mitochondrial transporter for ornithine. This autosomal recessive disease is known as hyperornithinemia-hyperammonemia-homocitrullinuria.

THE RATIONALE FOR THIS THESIS

Adult mammals in nitrogen balance must oxidize an amount of protein equal to that which is absorbed from the diet. A balance is achieved between the supply and the consumption of protein. Arginine balance could, in principle, be achieved by altering the metabolic processes which produce or consume this amino acid. In this regard, the existing studies show that the catabolic, rather than the synthetic, processes fluctuate under conditions of varying arginine intake in humans (Castillo et al, 1994a; Castillo et al, 1994b), rats (Dhanakoti et al, 1990) and pigs (Prior and Gross, 1995). The processes involved in the catabolism of arginine are the subject of this thesis.

Quantitatively, the most important pathway for the catabolism of arginine and ornithine in rats and humans occurs through OAT. In patients with the genetic disease gyrate atrophy OAT deficiency leads to immense increases in blood and tissue levels of ornithine as well as a substantial ornithinuria (Valle and Simell, 1995). As discussed earlier, one of the most effective treatments for this disease is restriction of dietary arginine. Inhibition of OAT in mice leads to substantial increases in the levels of ornithine in all tissues studied (Alonso and Rubio, 1989; Seiler et al, 1989). In addition, in adult mice in which the OAT gene has been deleted similar increases in ornithine are seen (Wang et al, 1995). The other processes in which these amino acids are consumed, as discussed earlier, are quantitatively either minor or result in the production of a substrate which may be recycled back to arginine. The catabolism of arginine and ornithine through OAT is the focus of this thesis.

Liver contains all the enzymes necessary for the complete catabolism of arginine and ornithine to CO_2 through OAT and is the key organ for post-prandial amino acid clearance (Jungas et al, 1992). This work was undertaken with the goal of understanding more fully the factors (nutritional and hormonal) which regulate these processes in the rat liver, and to determine their localization within rat liver. The oxidation of arginine and ornithine through OAT is now discussed as well as the localization of metabolic processes within the mammalian liver and possible sites of regulation.

THE OXIDATIVE CATABOLISM OF ARGININE AND ORNITHINE IN MAMMALIAN LIVER

Transport of the cationic amino acids into the liver

In mammalian liver the y^+ transporter is responsible for the transport of the cationic amino acids (White, 1985). This Na^+-independent, electrogenic, amino acid transporter, first described by Christensen in the 1960's (Christensen, 1964), is a low affinity transporter for arginine, ornithine, histidine and lysine. This transporter was shown to be specific for the L-amino acids, subject to trans-stimulation, and, in the presence of Na^+, may also carry neutral amino acids (Christensen, 1984). The y^+ transporter has since been cloned (Kim et al, 1991), a serendipitous discovery arising from the study of murine retrovirus receptors. One of these viruses subverts this transporter to gain access to the cell interior. This transporter/viral receptor was named the murine cationic amino acid transporter-1 (MCAT1). This gene codes for a protein with 622 amino acid residues containing 14 putative hydrophobic membrane-spanning regions, a motif prevalent

among nutrient transporters. Subsequent studies revealed the presence of a related gene, with high sequence homology to MCAT1, that is differentially transcribed to produce the MCAT2A and MCAT2B transporters (Closs et al, 1993). MCAT1 is expressed in all cell types studied to date with the exception of adult hepatocytes (Maillard et al, 1995). Only the low affinity MCAT2A transporter is expressed in normal adult hepatocytes (Kakuda et al, 1993). This may explain the low uptake of the cationic amino acids in the perfused rat liver (Pardridge and Jefferson, 1975). This transporter has an apparent K_m between 2-5mM and, thus, is not saturated at the physiological concentrations of the cationic amino acids (cumulative concentration for arginine, ornithine and lysine in humans is 0.25mM). Net movement of arginine via the Na^+-independent y^+ transporter is made possible by the decreasing concentration gradient of this amino acid which exists between extracellular and intracellular compartments of the liver, due to the high arginase activity within hepatocytes. Also, since the cell interior is electrochemically negative (membrane potential for cells are typically -70mV) relative to the cells exterior, arginine flow would be attracted inwards to achieve electrical neutrality. Unlike the MCAT1 and MCAT2B transporters, MCAT2A is a low affinity transporter that is not subject to regulation by trans-stimulation. Most of the studies concerned with the transport of the cationic amino acids measure the cumulative action of all MCAT gene products expressed in a particular tissue (designated y^+ transporter activity). In the cases where a particular MCAT gene product has been studied I will refer to the specific transporter in question (MCAT1, MCAT2A or MCAT2B), otherwise the transporter activity will be referred to as y^+.

Key enzymes involved in the catabolism of arginine ornithine

Arginase (L-arginine ureohydrolase EC 3.5.3.1), **Figure 1.3.**, best known for its participation in the urea cycle, carries out the irreversible reaction converting arginine to ornithine. Arginase activity is present in kidney, mammary gland, intestine, brain, submaxillary gland, as well as a variety of other tissues (Reddi et al, 1975; Aminlari and Vaseghi, 1992). The extra-hepatic arginases must function in processes other than the urea cycle as the liver is the only tissue in which this process is fully functional (Meijer et al, 1990). Arginase requires Mn^{2+} for activity and the crystal structure for trimeric rat liver arginase has been refined to a 2.1 A° resolution (Kanyo et al, 1996). Early comparisons made between rat liver arginase and partially purified preparations from rat mammary gland (Glass and Knox, 1973) and rat kidney (Kaysen and Strecker, 1973; Reddi et al, 1975) demonstrated differences in kinetic parameters, charge properties, inhibition profiles, cofactor requirements, immunological characteristics, electrophoretic mobility, subcellular location and solubility characteristics. Marked differences in the properties of hepatic *versus* non-hepatic arginases were also demonstrated in humans (Spector et al, 1982). These studies lended credance to an earlier speculation (Cabello et al, 1965) that mammalian tissues contained different forms of this enzyme.

The number of isozymes of arginase present in human and rat tissues has since been reported to be between two and five (Spector et al, 1994; Zamecka and Porembska, 1988). It is clear that at least two isozymes of arginase exist, and that the activity of these accounts for the vast majority of the arginase activity within mammalian tissues (Kaysen

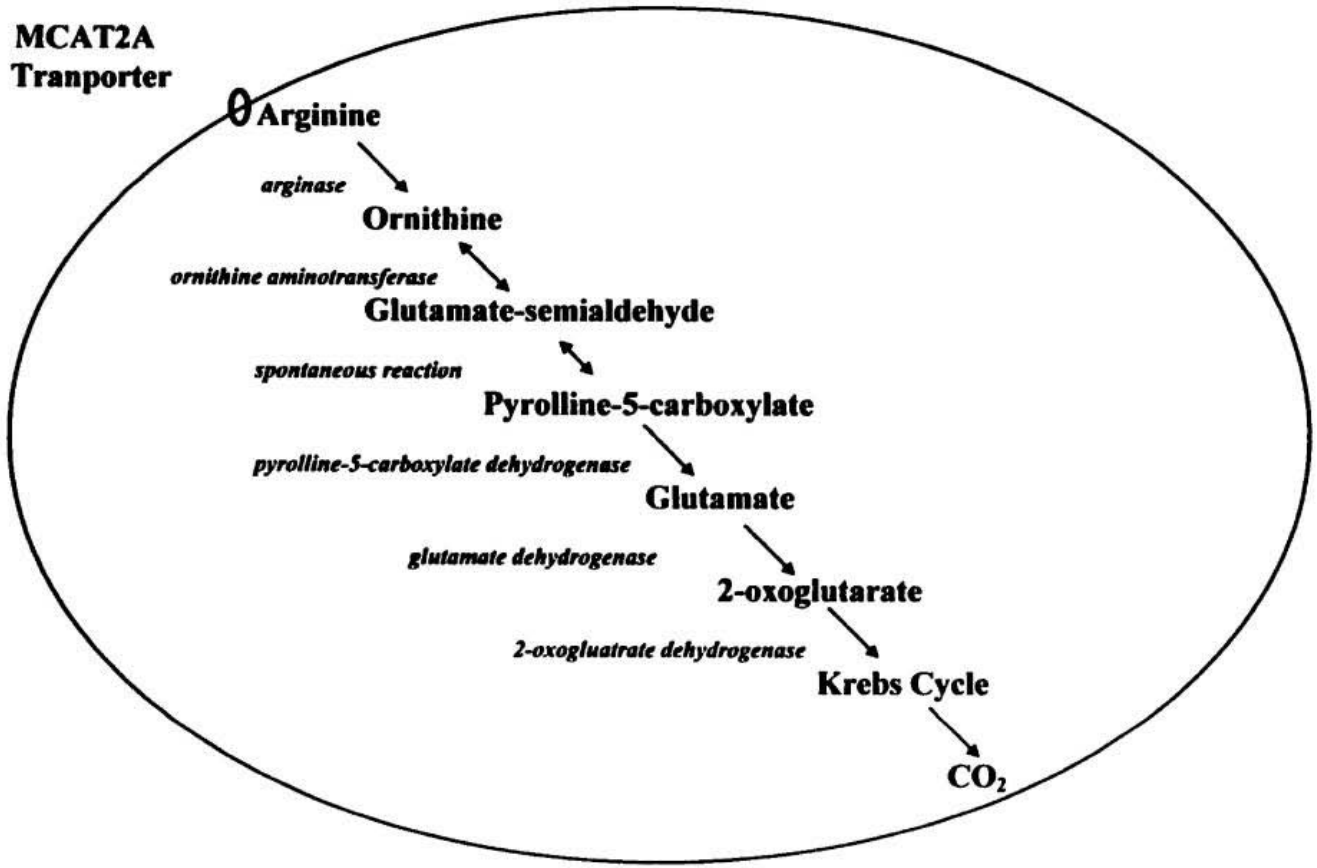


Figure 1.3. The metabolic pathway for the catabolism of arginine and ornithine through ornithine aminotransferase.

and Strecker, 1973; Reddi et al, 1975). However, current evidence does not preclude the possibility of the presence of still minor forms. Quantitatively, the two principal isozymes are AI ('liver type') and AII ('kidney type'). AI, expressed in liver and in erythrocytes, is cytoplasmic while AII, present in many other tissues, is mitochondrial (Spector et al, 1994). Cederbaum's group, using immunoassays, produced evidence for the presence of AI arginase in the kidney (Spector et al, 1994) but in recent studies, using *in situ* hybridization, the same group showed only the presence of the AII isozyme (Vockley et al, 1996); this result is in agreement with data produced by other groups (Kaysen and Strecker, 1973; Glass and Knox, 1973). Based on the distinct characteristics and differential regulation of these isozymes, it was predicted that the two arginases were the products of separate genes.

The cloning of distinct genes for the AI and AII arginases confirmed this view. The AI gene was cloned from rats (Kawamoto et al, 1986) and humans (Haraguchi et al, 1987) subsequent to its assignment to chromosome band 6q23 in humans (Sparkes et al, 1986). The deduced amino acid sequence for the human and rat gene products both contain 322 amino acids (M.W. 35,000 daltons) and share high sequence homology. Previous protocols for purification of human liver and erythrocyte AI arginase yielded a homotrimer (Ikemoto et al, 1989). Interestingly, expression of the human AI gene in E-coli yielded a monomer; apart from this difference in quaternary structure it possessed many of the characteristic properties of the AI arginase (Ikemoto et al, 1990). However, when concentrated in an alkaline environment, this monomer associated to form dimeric

and trimeric protein species. Standard purification procedures for liver arginase based on that developed by Schimke (Schimke, 1964) involve concentration in an alkaline environment. A newly developed purification scheme for the isolation of human hepatic arginase also yielded a monomer (Kuhn et al, 1995). Thus, it is possible that the quaternary conformation of arginase is an experimental artefact and that *in vivo* this protein exists as a monomer. The gene for AII has been cloned recently (Vockley et al, 1996); this gene codes for a protein of 355 amino acids and contains a mitochondrial signal sequence. In humans, the gene has been assigned to chromosome position 14q24.1-24.3 (Gotoh et al, 1997).

Several functions have been proposed for the AII arginase, including proline and glutamate production, arginine and ornithine catabolism as well as a rôle in the regulation of NO production. In the mammary gland of a lactating rat, arginase AII functions in the production of proline and glutamate for milk production (Yip and Knox, 1972; Mezl and Knox, 1977). With increased milk production there is a co-ordinate increase in the activity of arginase, OAT and P5C-reductase (these enzymes, operating in concert, are capable of catalyzing the conversion of arginine to proline). These studies demonstrated that in tissue minces and homogenates of mammary gland there was a significant labelling of both proline and glutamate from [U-^{14}C] arginine, whereas incubation with [U-^{14}C] proline showed that the production of arginine from proline was negligible. Neither was there a significant proline oxidase or P5C dehydrogenase activity within this tissue, enzymes responsible for the degradation of proline (Adams and Frank, 1980).

Several studies indicate that the AII arginase is more sensitive to inhibition by proline than the AI isozyme (Kaysen and Strecker, 1973; Carjaval and Cederbaum, 1986), a property which may enable feedback inhibition of proline synthesis at the first step of this process.

The pyridoxal-phosphate dependent enzyme ornithine aminotransferase (L-ornithine:2-oxoglutarate aminotransferase, EC 2.6.1.13) catalyzes the conversion of ornithine and 2-oxoglutarate to pyrroline-5-caboxylate and glutamate. This reaction was first described in mammals in studies using rat liver preparations (Meister, 1953). OAT is a mitochondrial matrix protein in mammalian tissues (Peraino and Pitot, 1962; Strecker, 1965). The K_{eq} for this reaction in rat liver, using a partially purified preparation was calculated to be 71; thus the forward reaction is somewhat favoured (Strecker, 1965). However, earlier attempts to reverse this reaction using partially purified preparations showed negligible reverse reaction (Meister, 1953). The production of citrulline from glutamine within enterocytes means that the reverse reaction proceeds *in vivo* in rats (Windmueller and Spaeth, 1981). Glutamate (formed from glutamine in the reaction catalyzed by glutaminase) and pyrroline-5-carboxylate are converted to 2-oxoglutarate and ornithine; the ornithine is then converted to citrulline in a reaction involving ornithine transcarbamoylase. In addition, a study using gabaculine (3-amino, 2,3,-dihydrobenzoic acid) to inhibit OAT demonstrated that the reaction catalyzed by OAT is involved in both the synthesis of ornithine as well as its degradation. Infusion of ^{14}C-glutamate to mice resulted in the recovery of significant amounts of the radioactive label in the amino acids

arginine and ornithine; gabaculine administration reduced significantly the incorporation of [^{14}C] into arginine and ornithine in mouse tissues. Infusion of [^{14}C]-ornithine resulted in the production of $^{14}CO_2$; gabaculine administration reduced significantly the production of $^{14}CO_2$ in these mice (Alonso and Rubio, 1989). It is likely that OAT functions in different directions in the different tissues. The pI was determined to be 5.38 indicating that this protein is acidic at the physiological pH (Peraino et al, 1969). The gene for rat OAT has been sequenced (Shull et al, 1992) as has the human (Dougherty et al, 1992). In humans the OAT gene has been mapped to 10q26 (but several pseudogenes are also present in the genome (Geraghty et al, 1993)). Crystal structures for native human OAT (Shen et al, 1998) and OAT complexed with the inhibitors gabaculine and canaline (Shah et al, 1997) have been determined recently.

Relatively less is known concerning the characteristics of pyrolline-5-carboxylate dehydrogenase (P5CDH). The cDNA sequence for human P5CDH is known (Hu et al, 1996). This enzyme is a mitochondrial matrix protein and has been purified from rat liver mitochondria (Small and Jones, 1990).

HEPATOCYTE HETEROGENEITY: "METABOLIC ZONATION" ACROSS THE LIVER

This section deals with the localization of the processes for the catabolism of ornithine and arginine within the liver. The most widely accepted model describing the functional unit of mammalian liver is the 'hepatic acinus' model first described by Rappaport

(Rappaport, 1954; Rappaport, 1973). In this model blood is supplied to the liver *via* a dual afferent system, comprised of the portal vein and the hepatic artery, the blood then flows through the sinusoidal system of channels and leaves the liver through the terminal hepatic vein. The acinus describes a micro-circulatory unit in which the hepatocytes are classified based on their position relative to the blood supply. Those hepatocytes proximal to the affluent portal vein are termed 'periportal' while those surrounding the effluent hepatic vein are called 'perivenous.' The term 'metabolic zonation' originated with Jungermann (Katz and Jungermann, 1976). Seminal studies in the field of hepatic carbohydrate matabolism produced evidence for a non-uniform distribution of the enzymes for gluconeogenesis and glycolysis across the hepatic acinus. The processes of gluconeogenesis and glycogenolysis are known to be primarily periportal in location while glycolytic activity is more abundant in the perivenous region. Subsequent studies demonstrated that in regard to hepatic metabolism, zonation is the rule rather than the exception.

Experiments using a variety of techniques demonstrated the differential distributions of enzymes, amino acid transporters, hormone receptors etc. across the hepatic acinus (for reviews see Jungermann and Keitzmann, 1996; Gebhardt, 1992; Häussinger et al, 1992; Jungermann and Thurman, 1992; Katz, 1992 ; Häussinger, 1990; Jungermann and Katz, 1989). There is no strict anatomical demarcation of periportal from perivenous cells, rather the dimensions of these zones are specific to the individual metabolic process under consideration. Distribution patterns across the acinus follow two basic designs for

compartmentation. These are 'strict compartmentation' and 'gradient compartmentation.' In strict compartmentation the individual process or enzyme is present in certain cells across the acinus while is absent in others, and there is a clearly defined boundary separating these two cell types. In the adult rat, glutamine synthetase and carbamoyl phosphate synthetase 1 follow a strict compartmentation pattern, the former's distribution being restricted to a small population of hepatocytes surrounding the central vein (Gebhardt and Mecke, 1983) while the latter is present only in the periportal cells (Gaasbeek-Jansen et al, 1984). A recent mRNA *in situ* hybridization study simultaneously investigated the localization of all five urea cycle enzymes and showed that the transcripts predominated in the periportal region (Dingemanse et al, 1996); thus, the urea cycle is a periportal process. Gradient compartmentation is characterized by a gradual increase or decrease in the abundance of an enzyme across the acinus. It is likely that a combination of factors operates to maintain the pattern of gene expression, such as hormone and oxygen gradients, cell to cell interactions etc. At the gene level, glutamine synthetase may be restricted to the perivenous region through a transcriptional regulator interaction with an upstream enhancer element (Lie-Venema, 1995); in transgenic rats in which a DNA construct containing this element and a reporter gene (chloramphenicol acetyltransferase) were integrated into the host DNA the expression of the reporter gene was limited to cells of the perivenous region. The co-ordinate regulation of the urea cycle enzymes may be achieved by the presence of such elements in the genome (Morris, 1992).

The distribution pattern of a particular enzyme or metabolic process in the liver may alter during normal development or with varying physiological status (a process known as 'dynamic zonation'). The pattern and extent of expression of the various urea cycle enzymes changes across the hepatic acinus during development, particularly during the perinatal period (Dingemanse et al, 1996). In rats subjected to cold exposure (4°C for 15 hours) the periportal/perivenous ratio of long chain fatty acid oxidation changed from 1.4 to 0.5 in enriched populations of perivenous and periportal hepatocytes (Guzman et al, 1995). The localization of other enzymes appears to be refractory to such changes in the studies carried out to date i.e. these exhibit a static type of zonation e.g. glutamine synthetase (Matsuzawa et al, 1994).

With respect to amino acid metabolism Häussinger brilliantly elucidated the pattern of distribution of the processes for ammonia detoxification across the hepatic acinus (Häussinger, 1983). In this study, in which rat livers were perfused in a nonrecirculating manner, he demonstrated a reciprocal distribution of the activities of the urea cycle and glutamine synthetase to the periportal and perivenous regions, respectively. The key to the success of these experiments lay in the ability to perfuse rat liver in both the normal physiological direction (antegrade) and in the direction opposite to this (retrograde), see **Figure 1.4**. The sinusoidal system for carrying blood through the liver is valveless. The urea synthesizing periportal region is much larger than the glutamine producing perivenous region. Häussinger demonstrated that the principal nitrogenous product formed in antegrade perfusions was urea while in the retrograde perfusions glutamine

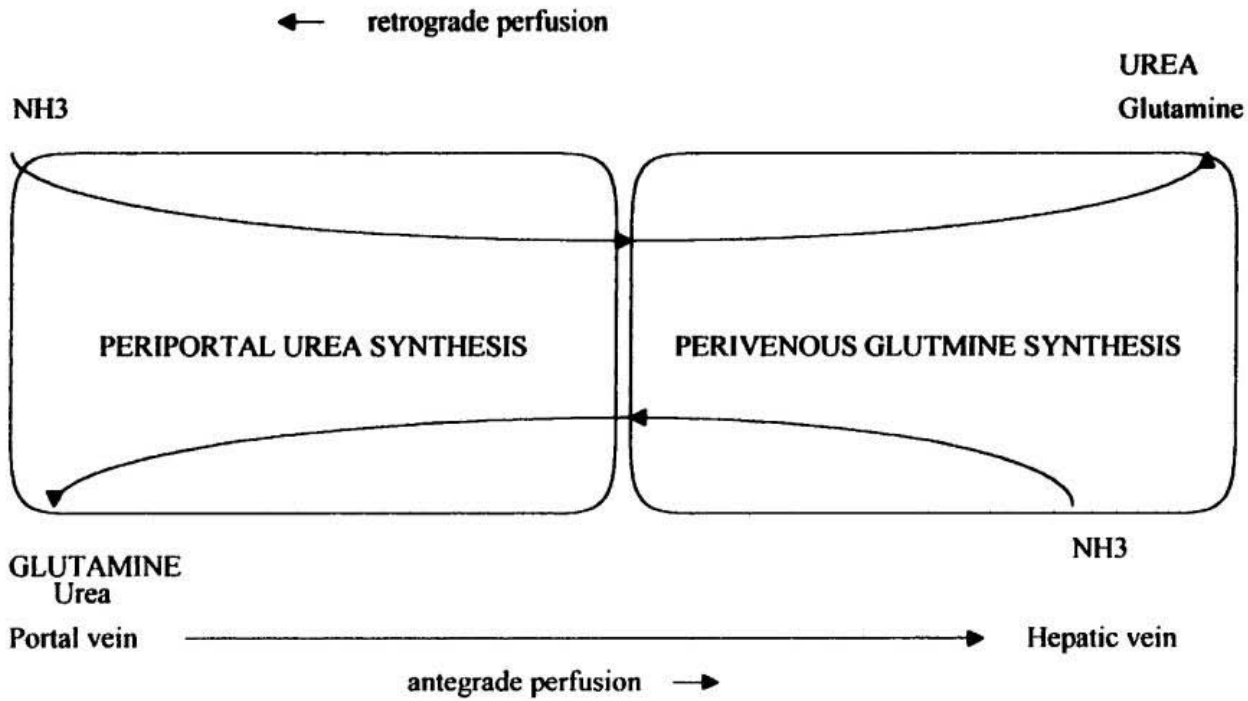


Figure 1.4. The zonation of urea synthesis and glutamine production in the liver.

production predominated. In rat livers in which glutamine synthesis was inhibited, using methionine sulfoximine, urea production was identical in antegrade and retrograde perfusions. (In all experiments, concentrations of ammonia (0.2mM) were used which were not saturating for either urea production or glutamine synthesis). This proved that the processes were physically separated and that urea production occurs in the periportal region while glutamine synthesis is a function of the perivenous region.

Particularly relevant to this thesis is that OAT was found to be strictly compartmented to a cell population surrounding the hepatic vein by mRNA *in situ* hybridization (Kuo et al, 1991). An earlier study had shown OAT to be preferentially expressed in a particular subpopulation of hepatic mitochondria, characterized by a smaller diameter and thought to be localized to a small region surrounding the central vein (Swick et al, 1970). Immunohistochemical studies also localized OAT to this subpopulation of hepatocytes (Matsuzawa et al, 1994). The separation of OAT from the urea cycle enzymes (Dingemanse et al, 1996) precludes the possibility of OAT depleting the urea cycle of ornithine, thus giving primacy to the vital function of the urea cycle. The distribution of OAT within the liver, therefore, defines ornithine catabolism as a perivenous process. Whether or not arginine catabolism through OAT is a perivenous process depends on the presence or absence of an arginase in this region. P5C dehydrogense distribution within the liver has not yet been studied.

The localization of arginase within the liver: Is there a perivenous arginase?

It has been held that the arginase in liver is cytosolic and restricted in location to the periportal cells. The sole function of hepatic arginase, it is widely believed, is as a catalyst in the urea cycle. Raijman, however, has reported that there is a small amount of arginase, with properties resembling those of the AII isozyme, which is mitochondrially associated (Cheung and Rajman, 1981). Recent studies show that the mRNA for the AII isozyme is present in human and mouse liver (Gotoh et al, 1997; Morris et al, 1997). One proposal is that this mitochondrially-associated arginase, due to the magnitude of its activity and its location, could provide a mechanism by which the arginine that is produced in the cytosolic reactions of the urea cycle could "channel" (for a review on metabolic channelling see Srere, 1987) ornithine back into the mitochondria to participate in the urea cycle reactions of this compartment (Cheung et al, 1989; Watford, 1991). Experimental evidence suggests that there is channelling of both the cytosolic and the mitochondrial reactions of the urea cycle, as well as the transport of ornithine back into the mitochondria (Cheung et al, 1989; Cohen et al, 1987). Whether or not this mitochondrially-associated arginase functions in this manner has not been proven. Raijman's group report that the cytosolic enzymes of the urea cycle are concentrated around the mitochondrion rather than being homogenously distributed throughout the cytosol (Cohen and Kuda, 1996; Cohen, 1996). Alternatively, this mitochondrially associated arginase may be involved in some other process within the liver. In this thesis we investigate whether an arginase is present in the perivenous region which may be involved in the catabolism of arginine.

NUTRITIONAL AND HORMONAL REGULATION OF THE CATABOLISM OF ARGININE AND ORNITHINE IN THE PERFUSED RAT LIVER: POSSIBLES SITES FOR REGULATION.

Regulation by hormonal and dietary factors is a common feature of amino acid metabolism. Alterations may occur at the level of substrate availability, allosteric or covalent modification of enzymes, or by enzyme induction or degradation. As one of the foci of this thesis is the regulation of the processes for the catabolism of arginine and ornithine the following section deals with possible sites for the regulation of these processes.

Regulation of transport

Mammalian amino acid transporters are subject to regulation by a range of different of factors (for reviews see Macleod, 1996; McGivan, 1996; Maillard et al, 1995; Kilberg et al, 1993; White, 1985). Alterations in substrate supply may regulate transporter activity. The portal vein concentrations for many amino acids are below the K_ms for their respective transporters (Meijer et al, 1990). Therefore, fluctuations in the levels of these amino acids in this vessel will alter the rates of their transport into the cell. The concentration of arginine in the portal vein of normal rats and humans is 0.1-0.2mM (Brusilow and Horwich, 1989; Rémésy et al, 1978) and the K_m for the MCAT2A transporter (the only MCAT gene expressed in normal hepatocytes) is between 2 and 5mM (Closs et al, 1993). Thus, any alteration in the concentration of arginine in this vessel, within the physiological range, will result in changes in the rate of its transport by the MCAT2A transporter.

In rats that were fed either a high carbohydrate containing diet (79% w/w starch; 13% w/w casein) or a high protein containing diet (42% w/w starch; 50% w/w casein) for a period of 10 days the rat portal vein concentration of arginine increased from 0.14mM to 0.27mM in the rats fed the high protein diet (Rémésy et al, 1978). The data from this experiment would lead one to predict increased transport of arginine by the MCAT2A transporter in the rats fed the high protein diet. In patients with hyperargininemia and gyrate atrophy (discussed earlier) one would also expect altered transport of arginine and ornithine into the liver, and the effects of this increased supply of substrate on processes such as NO production and polyamine production would make an interesting study.

In the streptozotocin-induced diabetic rat, y^+ transporter activity in primary rat hepatocyte cultures is increased five fold when compared to cells isolated from non-diabetic controls ; ip administration of glucagon (2mg/100g prior to killing) also increased y^+ transporter activity by five fold in these cells when compared to saline treated rats (Handlogten and Kilberg, 1984). A single high protein meal, and feeding a high protein diet over a period of days, raises the circulating levels of glucagon (Robinson et al, 1981). Thus, it is possible that feeding rats a high protein diet could increase y^+ transporter in the short-term by increasing substrate supply (direct mechanism) and in the long-term by inducing the y^+ transporter (indirectly by increasing the concentration of circulating glucagon).

Increasing the arginine content alone in the diet may increase y^+ transporter activity ; in

rats and humans fed supradietary amounts of arginine and glutamine, the activities of the transporters system N and y^+ increases in hepatic plasma membrane vesicles (Espat et al, 1996). There is a discriminate induction of the transporters for the cationic amino acids; in rat brain astroglial cultures, induction of y^+ transporter activity by endotoxin and interferon-γ is effected *via* an increase in the high affinity CAT2B transporter without altering the expression of either CAT1 or CAT2A (Stevens et al, 1996). In untreated hepatocytes, where the low affinity MCAT2A transporter is expressed, any stimulus which could induce either the MCAT1 of MCAT2B would, by virtue of the higher affinities of these transporters, alter dramatically the transport characteristics of these cells at the physiological concentrations of the cationic amino acids. It is plausible that alterations in the delivery of arginine and ornithine into liver, by the y^+ transporter, could affect the rate of catabolism of these amino acids. The recent discovery of the MCAT genes, and the current interest in the regulation of NO biosynthesis by the y^+ transporter provide the impetus for rapid advances in this area.

Regulation at the level of individual enzymes

Arginase catalyses the first non-equilibrium reaction in the pathway outlined in **Figure 1.2.** and, thus, regulation at this step would be an efficient means of regulating arginine catabolism. Previous studies investigating the regulation of rat liver arginase would have, necessarily, been primarily concerned with the regulation of the AI isozyme; any AII activity present in the liver would be quantitatively minor when compared to the AI isozyme (as discussed earlier). Rat liver arginase is subject to regulation by the level of

dietary protein (Schimke, 1962; Morris et al 1987), treatment with glucagon and by ip injection of dibutyryl cAMP (Morris, 1992). Possible contributions to these effects by a rat liver AII arginase are obscured by the relatively larger AI activity.

With respect to the regulation of the AII arginase, it is increased in kidney in patients with hyperargininemia (Grody et al, 1989), compared with normal patients, and results from cell culture studies suggest that this increase may be caused by increased circulating levels of arginine. A high protein diet increases circulating levels of arginine (Rémésy et al, 1978) and this may induce any AII which may be present in the liver. In a murine macrophage-like cell line (Gotoh et al, 1997) and in bone marrow-derived macrophages (Corraliza et al, 1997) arginase (AII) is induced by agents which increase the concentration if intracellular cAMP, including dibutyryl-cAMP. The caveat to this type of extrapolation is the possibility of differential tissue regulation of enzymes. For example, OAT is regulated differently in liver than it is in kidney, and this difference is not due to the presence of isozymes (Dougherty et al, 1992). Much remains unknown concerning the regulation of the arginases in the liver and kidney, however, the advent of advanced molecular biological techniques, and the recent cloning of the AI and AII genes should allow rapid progress to made in this field.

OAT is induced in rat liver upon feeding rats a high protein diet (Volpe et al, 1969), or by treatment with glucagon (Lyons and Pitot, 1976). The OAT induction may be effected through glucagon; a high protein diet increases the circulating levels of glucagon (Peret et

al, 1981). In female rats, kidney OAT is induced by estrogen (Herzfeld and Knox, 1968). P5CDH is regulated by increasing the amount of protein in the diet (Matsuzawa et al, 1994).

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Animals

Male Sprague Dawley rats were used. They were purchased from Charles River Ltd. (Montreal, P.Q.). The rats, weighing between 100-150g, were housed in the Biotechnology Animal Care facility at Memorial University in cages containing no more than 3 animals, and were maintained under a 12:12 hour light:dark cycle. The normal light period extended between 8a.m. and 8p.m. Rats were provided free access to standard Purina® chow diet and tap water. The majority of food intake occurred during the dark cycle. All the studies described used rats weighing between approximately 200 and 350g.

Purified diets and feeding regimen

Diets were prepared based on AIN76 recommendations with modifications (Bieri et al, 1977). The diets used were either normal protein (containing 15% casein) or high protein (containing 60% casein), and were isocaloric. Diet constituents and amounts are detailed in **Table 2.1.** The reduction in protein derived calories in the normal protein diet, relative to the high protein diet, was compensated for by a commensurate increase in cornstarch and sucrose-derived calories. Purified diets were provided to the rats in detachable metal containers placed inside the cages. Free access to these diets was provided for a period lasting between 3 and 7 days and normal weight gain was a prerequisite for participation within a study. **Table 2.2.** shows data typical for the

Table 2.1. Composition of the 15% and 60% casein diets.

	15% casein diet (g/kg)	60% casein diet (g/kg)
Sucrose	533	192
Casein	148.5	598.5
Cornstarch	170	61
Alphacel	50	50
Vitamin Mix (AIN 76) [1]	10	10
Mineral Mix (AIN 76) [1]	35	35
Corn oil	50	50
Choline Bitartrate	2	2
L-methionine	1.5	1.5

[1] Details of the composition of the vitamin and mineral mix are provided in Bieri et al (1977).

Table 2.2. Body weights of rats fed the 15% and 60% casein diets. Results are expressed as the means ± S.D. for groups of 3 rats. Day 1 denotes the initial weight.

Diet	Day 1	Day2	Day3	Day 4	Day 5	Net Gain (g)
15% Casein	194.3 ± 1.1	199.3 ± 1.1	205.3 ± 1.5	215.3 ± 1.5	223.3 ± 1.5	29.0
60% Casein	200.3 ± 3.8	196.0 ± 3.5	209.3 ± 2.1	218.0 ± 2.0	223.3 ± 1.5	23.0

changes in weight of rats fed either a normal protein (15% casein) or high protein (60% casein) diet.

Chemicals

Diet components were purchased from ICN (Cleveland, Ohio) with the exception of cornstarch and L-methionine which were purchased from Sigma Chemical Co., Ltd. (St. Louis, Mo.). Mazola corn oil was purchased from Best Foods Canada (Etobicoke, Ont.). [U-^{14}C]-arginine, [U-^{14}C]-ornithine and [1-^{14}C]-ornithine and Omnifluor® were obtained from Dupont-New England Nuclear (Mississauga, Ont.). [U-^{14}C]-ornithine and [1-^{14}C]-ornithine were also purchased from Amersham Canada Ltd. Insulin, glucagon, orthoaminobenzaldehyde, gabaculine and bovine serum albumin (prepared from fraction V; essentially fatty acid-free) were purchased from Sigma Chemical Co., Ltd. (St. Louis, Mo.). Silicone oil was from Dow Corning (William F. Nye, Inc. New Bedford, Ma. USA). Heparin (sodium injection USP) was purchased from Allen and Hanburys (Glaxo Canada, Ltd., Montreal, P.Q.). All other reagents were of analytical grade.

METHODS

Perfusion apparatus

The perfusion system used was a nonrecirculating type, essentially as described by Sies (Sies, 1978). The apparatus used is outlined in the schematic in **Figure 2.1**. As shown, the medium (gassed and maintained at 37°C) is pumped from the reservoir to the

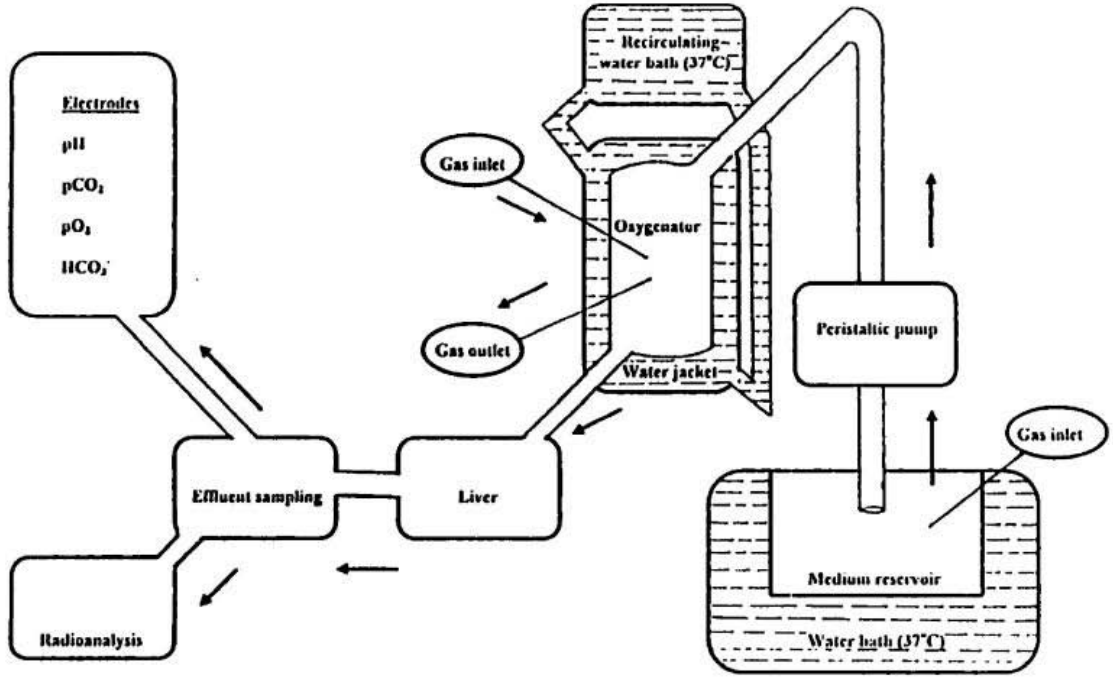


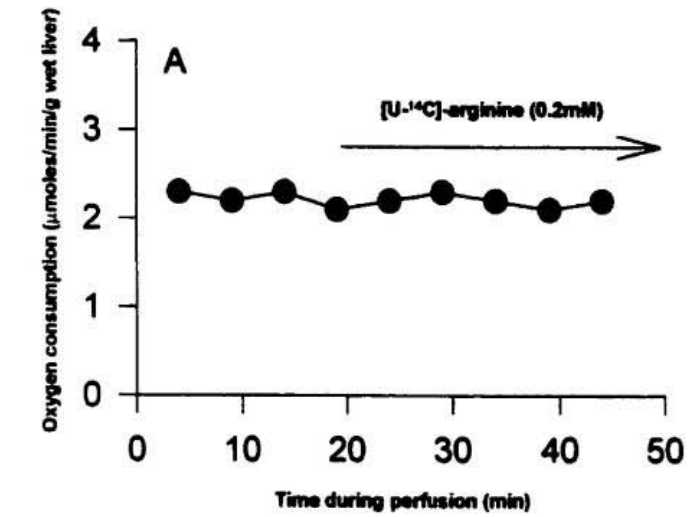
Figure 2.1. Schematic for the apparatus used for liver perfusions.

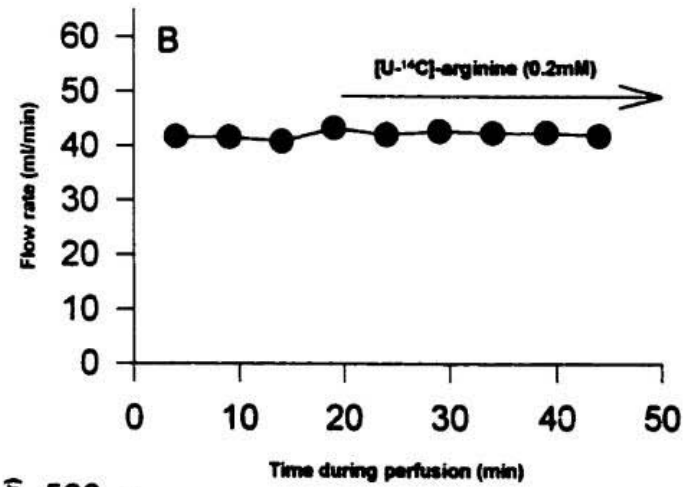
oxygenator where equilibration with the gas mixture of O_2/CO_2 (19/1) occurs. The medium is then passed through the liver and the effluent sampled. The viability of the liver was assessed by effluent analysis of O_2 consumption using a Ciba Corning 238 pH/blood gas analyzer. Any errors in medium preparation/gassing can also be detected within a relatively short time by monitoring the pH. During all perfusions, effluent samples were taken at 5-minute intervals to assess pO_2, pCO_2, pH and HCO_3^-. Perfusions were discontinued upon detection of any abnormal reading in these parameters. Following perfusion the entire liver was removed and dried to constant weight in an oven set at 50°C. Typical data are shown for a rat fed a standard chow diet for the parameters of oxygen consumption, flow rate and urea production in **Figure 2.2**.

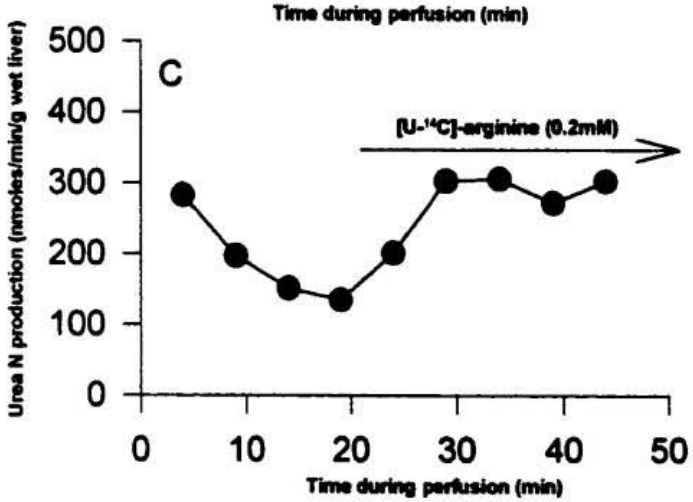
Surgical procedure

Rats were anaesthetized with Somnotol® (Na-pentobarbital, 65mg/kg). An incision was made to expose the femoral vein and 500IU of heparin was injected into this blood vessel. An incision was then made to expose the abdomen. The fur was cut away with a midline incision from below the abdomen to above the diaphragm. A saline swab was used to remove any remaining fur from the flesh. An incision was made immediately above the bladder towards the diaphragm. The surgical scissors were used to position the liver away from the path of the incision, and the cut completed to the base of the diaphragm. With the aid of hemostats, incisions perpendicular to the midline incision were made on both sides; this exposed the peritoneal cavity. The stomach and intestine

Figure 2.2. Typical data from an antegrade perfusion carried out in a rat fed a normal protein diet (15% casein) for 3 days. Oxygen consumption (A), flow rate (B) and urea N production (C) were measured as described in **Materials and Methods**. [U-^{14}C]-arginine (0.2mM) was provided for the duration of the perfusion indicated by the arrows.


A
[U-14C]-arginine (0.2mM)
Oxygen consumption (μmoles/min/g wet liver)
0
1
2
3
4
0
10
20
30
40
50
Time during perfusion (min)


B
[U-14C]-arginine (0.2mM)
Flow rate (ml/min)
0
10
20
30
40
50
60
0
10
20
30
40
50
Time during perfusion (min)


C
[U-14C]-arginine (0.2mM)
Urea N production (nmoles/min/g wet liver)
0
100
200
300
400
500
0
10
20
30
40
50
Time during perfusion (min)

were moved to the left allowing access to the portal vein. Ligatures #1 and #2 (see **Figure 2.3** for numbering and arrangement of ligatures) were placed around the portal vein close to its point of entry to the liver and ligature #3 was attached further behind on the portal vein. Ligature #4 was placed around the inferior *vena cava* between the right kidney and the liver. The portal vein ligature #3 liver was tied and the ends pulled gently towards the tail to produce a tension on the portal vein. The portal vein was then quickly cannulated and connected to the perfusate inflow. The perfusate flow rate at this point was maintained at approximately 10ml/min. Ligatures #1 and #2 on the portal vein were tightened and knotted and the abdominal aorta was cut to allow flow through of the perfusate. The ends of all ligatures were cut and all hemostats were removed. The thorax was cut through, to expose the heart, and ligatures #5 and #6 were attached. The cannula was then run through the right atrium and the ligatures tightened and knotted. Finally, ligature #4 was tied. During a wash period of approximately 5 minutes the liver was gently agitated to help removal of blood and the flow rate is adjusted to 40-45 ml/min which ensured adequate delivery of dissolved oxygen to all parts of the liver. The flow rate was maintained between 40-45ml/min throughout the perfusion.

Medium preparation for perfusions

Krebs-Henseleit medium (pH 7.4), maintained at 37°C and gassed with O_2/CO_2 (19/1), served as the basic medium for all perfusion procedures. This medium contained 118mM NaCl, 4.8mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25.0mM $NaHCO_3$ and

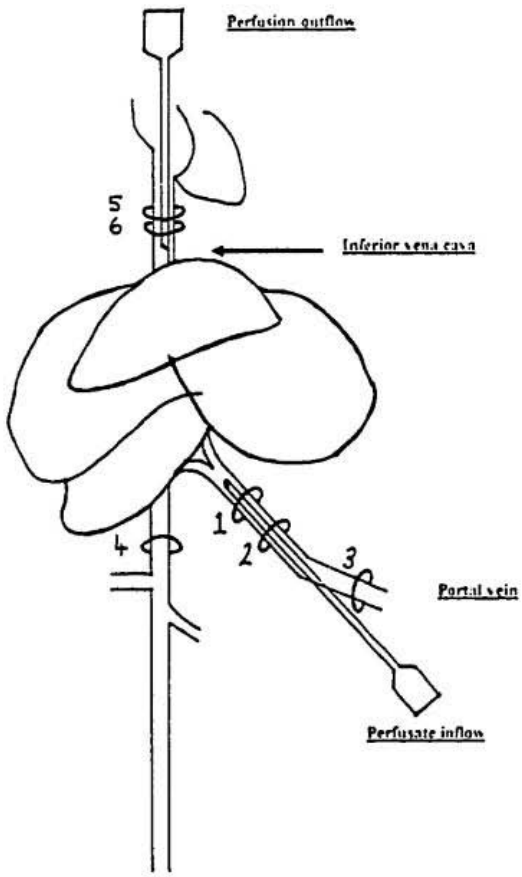


Figure 2.3. Outline of surgical procedure for rat liver perfusions.

2.5mM $CaCl_2$. To this 2.1mM lactic acid and 0.3mM pyruvate was added to provide oxidizable substrates and to ensure correct balance of the NAD/NADH ratio; this did not result in any change in the pH of the medium. The amino acids [U-^{14}C]-L-arginine and [U-^{14}C]- or [1-^{14}C]-L- ornithine were added at various concentrations. In cases where amino acids were added in amounts resulting in a final concentration of >1mM the medium osmolarity was adjusted by varying the NaCl concentration. Similarly, in studies which investigated the effects of altered osmolarity the various media differed in the final concentration of NaCl. All media was filtered through a preparative filter (1μm) before use. In experiments in which the pancreatic hormones glucagon and insulin were infused an infusion pump was used to introduce these into the stream of the influent perfusion medium. The concentration of each hormone in the infused stock solution was chosen to provide a final concentration of these hormones to the liver of approximately 10^{-7} M and was based on a calculation of the rate of delivery of the infusion pump and the approximate perfusate flow rate (43ml/min). Thus, fluctuations in the perfusate flow rate will alter somewhat the nominal final concentration of these hormones which is delivered to the liver. However, the fluctuation in perfusate flow rates in the perfusions did not exceed 10% and in the majority of cases was lower than 5%.

Antegrade/retrograde perfusions

Antegrade/retrograde perfusions were carried out as described by Häussinger

(Häussinger, 1983). *In vivo*, blood flows into the liver though the portal vein (and to a lesser extent hepatic artery) and leaves the liver through the hepatic vein. Perfusions in which various media are supplied through the portal vein and the effluents are collected leaving the hepatic vein are known as antegrade perfusions. Perfusions carried out in the opposite direction are called retrograde perfusions. During the course of a single perfusion procedure it is possible to switch from an antegrade to a retrograde perfusion and *vice versa*. **Figure 2.4** outlines the apparatus used for alternating the flow between antegrade and retrograde perfusions. A loop is constructed using silicon tubing and a series of clamps, as represented in **Figure 2.4**. **Panel A** shows the configuration of this loop when one is perfusing in the antegrade direction. When the direction needs to be reversed to the retrograde (**Panel B**) the open clamps are closed and those that were closed opened. The opening and closing of clamps must be performed simultaneously to ensure uninterrupted supply of the perfusate to the liver; this required two people.

We validated our antegrade/retrograde perfusion protocol by repeating the experiments of Häussinger on ammonia metabolism (Häussinger, 1983). In these experiments Häussinger provided a low concentration of ammonia to the perfused rat liver and altered the direction of perfusion antegrade to retrograde. When he did this the system that encountered the ammonia first (i.e. the urea cycle in the antegrade direction and glutamine synthetase in the retrograde direction) provided the primary nitrogenous product (i.e. urea in the antegrade and glutamine in the retrograde). The experiments

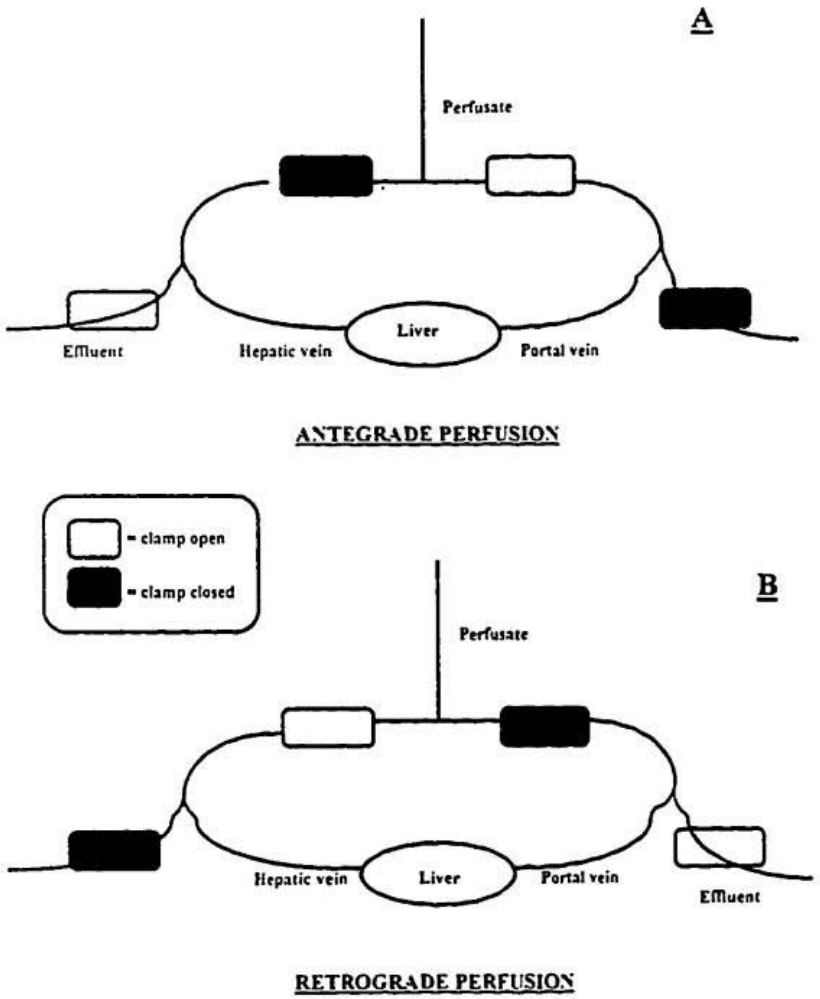


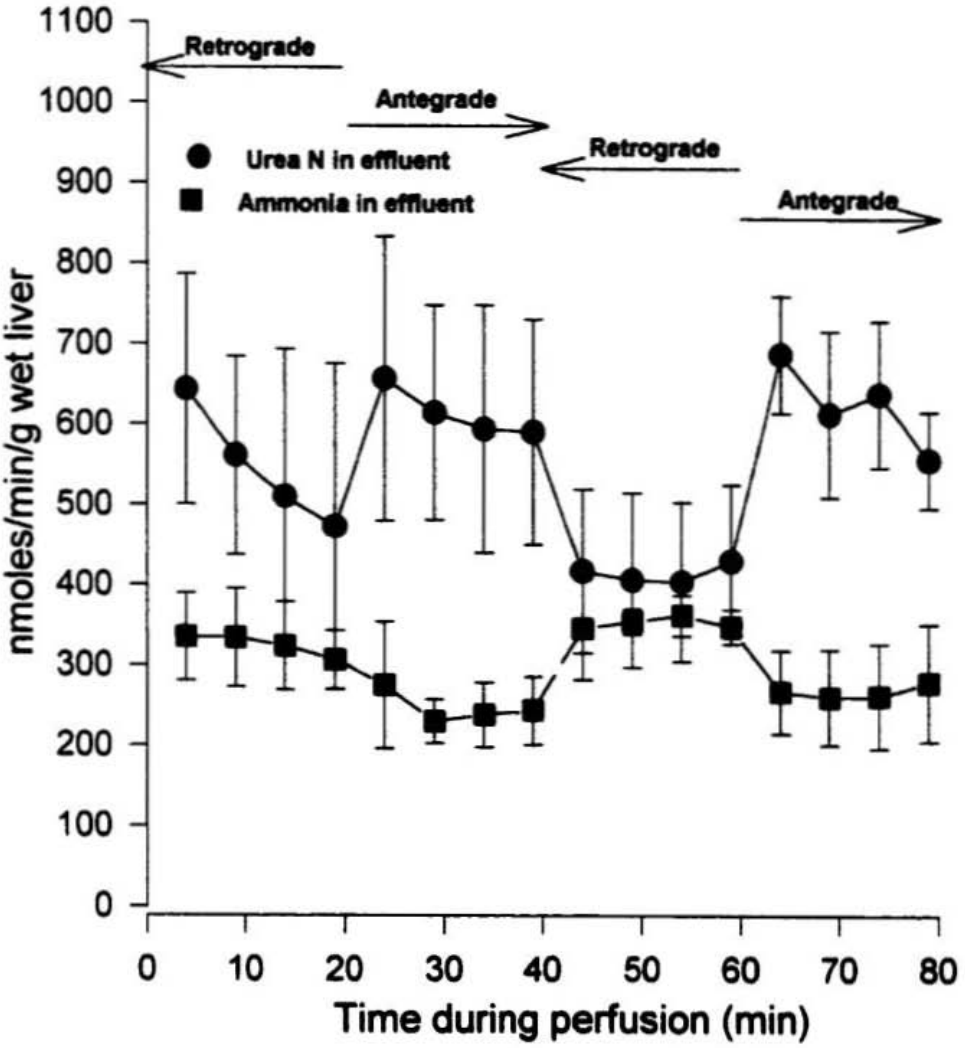
Figure 2.4. Configuration of clamps for antegrade and retrograde perfusions.

shown in **Figure 2.5** show the pattern of urea-N and ammonia production from ammonia as the direction of the perfusion is changed. The increased rate of urea-N at the beginning of the perfusion is thought occur as a result of the emptying of the urea pool present in livers rather than a change in the rate of urea cycle activity (this pattern is typical, as shown in **Figure 2.2**). The rate of urea-N production was about 600 nmoles/min/g wet liver in the antegrade direction and this fell to about 400 nmoles/min/g wet liver in the retograde direction. Thus, we were able to reproduce Häussinger's results and have confidence that our antegrade/retrograde perfusions were reliable. The pattern of ammonia concentrations in the effluent were also consistent with Häussinger's results.

Measurement of $^{14}CO_2$ in solutions

Perfusate samples were taken under mineral oil to prevent loss of CO_2. 5ml of each perfusate sample were injected into a stoppered 25ml Erlenmeyer flask, containing 0.4ml of 0.1M HCl. The flasks were fitted with center wells containing filter paper and 0.4ml of NCS tissue solubilizer. The evolved CO_2 was trapped in the center wells during incubation in a shaking water bath at 37°C for 1 hour. The center wells were transferred to scintillation vials containing 10ml of scintillation fluid (Omnifluor®), and counted in a scintillation counter (LKB-rack beta) for ^{14}C. Medium blanks were also prepared so as to account for any preformed $^{14}CO_2$ which might be present in the radioactive compounds. Cpm were corrected to dpm in the scintillation counter using an external standard. In

Figure 2.5. Antegrade/retrograde perfusions and the pattern of urea-N and ammonia in the effluent. Rat livers were perfused with Krebs-Henseleit medium (pH 7.4) containing lactic acid (2.1mM), pyruvic acid (0.3mM), NH_4Cl (0.2mM) and ornithine (2mM). Rats were fed a standard Purina® chow diet. Results are shown as mean ± S.D. for four independent experiments.


1100
1000
900
800
700
600
500
400
300
200
100
0
nmoles/min/g wet liver
Retrograde
Antegrade
Retrograde
Antegrade
Urea N in effluent
Ammonia in effluent
0
10
20
30
40
50
60
70
80
Time during perfusion (min)

antegrade perfusions carried out with 0.2mM [U-^{14}C]-arginine, no alteration in the rate of $^{14}CO_2$ production occurred upon the addition of 0.2mM α-ketoglutarate; this demonstrated that α-ketoglutarate was not limiting for the catabolism of arginine in our experiments; α-ketoglutarate is required in the transamination reaction of OAT).

Determination of urea nitrogen in the perfusate

Urea was determined using the thiosemicarbazide-diacetlymonoxime method developed by Gayer and Dabich (Gayer and Dabich, 1971). Briefly, an aliquot (0.1-0.5ml) of perfusate brought to a total volume of 0.5ml, if necessary with distilled water, was added 1.0ml of the color reagent (61.7mM Butane-2,3-monoxime and 3.6mM thiosemicarbazide) followed by 1.5ml of the acid reagent (3.6M H_2SO_4, 0.12mM $FeCl_3$ and 38.6mM H_3PO_4). The solutions were mixed thoroughly and incubated at 100°C for 10 minutes. The tubes were cooled to room temperature and the absorbance was measured at 520nm. Standard urea-N containing solutions (0, 0.0833 and 0.166 mM) were prepared during each assay and were used to calculate the concentration in the effluent. The aliquot of perfusate which was assayed, depended on the experimental conditions in question, and was chosen to fall within the range of the standards. The influent perfusate (to which no urea was added) served as the medium blank for these assays.

Determination of ammonia uptake across the perfused rat liver

Perfusate samples were taken from the influent and effluent perfusion media. An aliquot of the perfusate (0.1ml) was brought to a final volume of 0.5ml with water in a glass test tube. This was mixed with 1ml of a phenol containing reagent (0.53M phenol and 0.84mM sodium nitroprusside) and 1ml of an alkali-hypochlorite reagent (0.625M sodium hydroxide and 28.2mM sodium hypochlorite) and incubated at 37°C for 20 minutes. The solutions were allowed to cool to room temperature and the absorbance was read at 630nm. Standard solutions containing known amounts of ammonia (0, 0.1, 0.25 and 0.5mg ammonia per tube) were prepared during each assay and used to calculate the ammonia concentration in the perfusate sample. The aliquot of perfusate which was assayed, which depended on the experimental conditions in question, was chosen to fall within the range of the standards. To calculate the ammonia uptake across the liver the effluent perfusate concentration of ammonia was subtracted from the influent (as a known amount of ammonia was added, this served as a further check on the accuracy of this assay) and the difference multipled by the flow rate and then divided by the wet liver weight.

Assay for ornithine aminotransferase activity

100µl of sonicated liver homogenate (10% w/v in buffer containing 0.25M sucrose and 50mM Tris, pH 7.6) was added to the ortho-aminobenzaldehyde containing reagent mixture (0.125M KH_2PO_4, 0.02M α-ketoglutarate, 0.25mM pyridoxal phosphate, 0.1M

ornithine and 0.01M ortho-aminobenzaldehyde, pH 7.6). The solutions were mixed thoroughly and incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 2.0ml of a 7.5% trichloroacetic acid solution. The particulate matter was sedimented in a clinical centrifuge and the absorbance of the supernatant was measured at 440nm. A molar extinction coefficient of 2.71 (Herzfeld and Knox, 1968) was used to calculate the production of pyrroline-5-carboxylate. **Figure 2.6** contains data from preliminary studies which were carried out to determine whether the assay was linear with respect to time and protein content.

Administration of gabaculine (2,3,-dihydrobenzoic acid)

Gabaculine (OAT inhibitor), in 0.9% saline, was administered (20mg/ml) intraperitoneally at a dose of 50mg/kg body weight, 2 hours prior to using the rats. Control rats were administered the saline vehicle by i.p. injection.

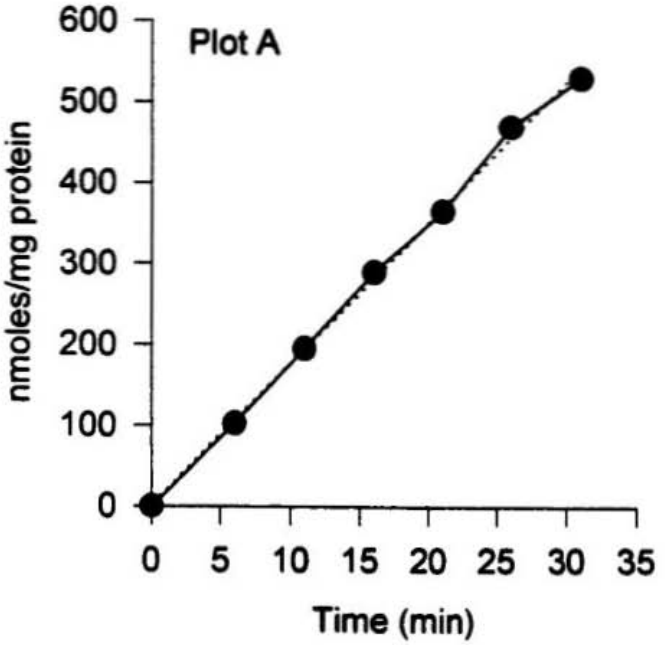
Assay for perfusate amino acids

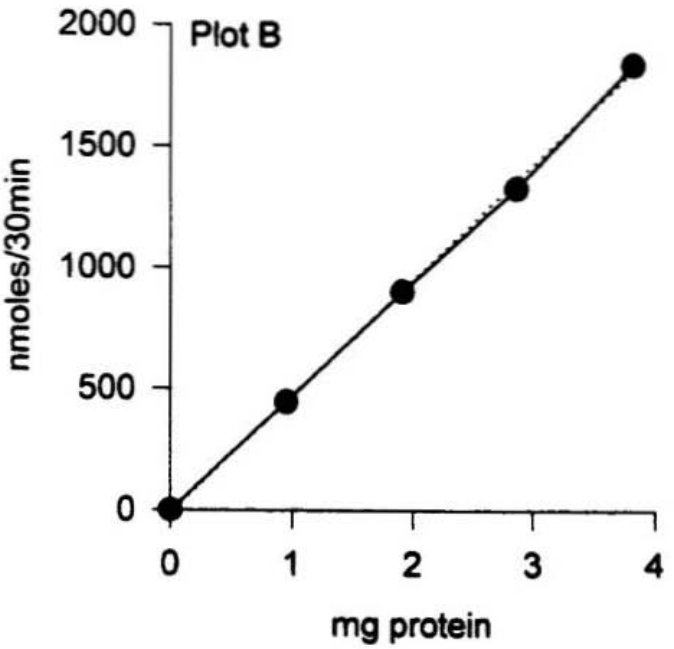
Effluent amino acids were measured on a Beckman model 121-M amino acid analyzer as described by Lee (1974) following adjustment of pH to 2.2 using lithium citrate (0.2N).

Preparation of rat liver mitochondria

Rats were killed by cervical dislocation. The liver was removed and cut into approximately 1mm-2mm sections using a pair of surgical scissors and homogenized in a

Figure 2.6. Ornithine aminotransferase activities in rat liver homogenates as a function of time (0-31 minutes with 4mg protein, Plot A) and protein concentration (0-3.8 mg liver protein, 30 minute incubation, PlotB). Experimental data from both plots correlated well ($r^2 > 0.99$) with the lines of best-fit from regression analysis.


Plot A
600
500
400
300
200
100
0
nmoles/mg protein
0
5
10
15
20
25
30
35
Time (min)


Plot B
2000
1500
1000
500
0
nmoles/30min
0
1
2
3
4
mg protein

hand-held Teflon homogenizer. The homogenization buffer used (Hampson et al, 1983) contained 225mM mannitol, 75mM sucrose, 1mM ethyleneglycolbis'-aminoethylether)-N,N,N,N-tetraaceticacid (EGTA),5mM-2-T-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), at pH 7.4. The homogenate (total volume 100ml) was centrifuged at 600Xg for 10 minutes. The decanted supernatant was centrifuged at 8,200Xg for 10 minutes resulting in the pelleting of the mitochondrial fraction. The mitochondrial fraction was resuspended in the homogenization medium (total volume ~ 20ml) and centrifuged for 10 minutes at 8,200Xg. This last step was repeated. The pellet was then resuspended in the medium to give a final mitochondrial protein concentration between 60 and 80 mg/ml. Protein concentration was determined by the Biuret method (Gornall et al, 1949) using BSA as standard protein and deoxycholate to solubilize lipids.

Respiratory control ratio and proline oxidation

Oxygen consumption was measured using a Clark-electrode. The respiratory control ratio (RCR), using the substrate 2-oxoglutarate, served as an indicator of the quality of the mitochondrial preparations. Those preparations with RCR values less than 4.0 were discarded. This was rarely necessary. The composition of the respiration medium was as follows: 1mg/ml BSA, 140mM KCl,1mM ethylenediamine-tetraaceticacid, 4mM KH_2PO_4, 5mM $MgCl_2$, 5mM HEPES, pH 7.4. This medium was equilibrated with respect to atmospheric oxygen concentration at a temperature of 30°C. The volume of the electrode chambers used were between 1.7ml and 1.8ml. The mitochondrial protein

amount in the chamber was between 1mg and 1.5mg and the adenosine-5'-diphosphate (ADP) concentration was 0.5mM. Where proline oxidation was studied, its concentration was varied.

Studies involving the effects of feeding a single meal

Rats were provided free access to the normal protein diet for 3 days during which time they were maintained under a reversed 12:12 hour light cycle. The light period extended between 8p.m. and 8a.m. The rats were subdivided into 3 groups; the control group, the normal protein meal group and the high protein meal group. On the fourth day at 12 midnight food was removed but the animals continued to be provided with water. At 8 a.m. the following morning, the normal protein meal group were provided with the normal protein diet, the high protein group with the high protein diet and the control group continued to receive no food; the rats, being in the post-absorptive state, ate promptly. At 10 a.m. rats from the 3 groups were killed.

Intraperitoneal administration of glucagon

An intraperitoneal injection of glucagon, dissolved in 0.9% saline and 0.05% BSA, was administered to rats at a dose of 0.1mg/100g body weight. Control rats were administered the saline BSA vehicle. Rats were killed 25-30 minutes after the injection.

Treatment of data

Experimental results were reported as means ± standard deviations. Comparisons between data groups were made using the Student t-test. In all cases a probability, $p < 0.05$ was regarded as indicating a statistical significance. The data reported in **Figures 3.1, 3.2, 4.1** and **4.2** were plotted using the computer program GraphPAD (GraphPAD Software, San Diego, California, USA) where the curve of best fit (rectangular hyperbola) is arrived at by an iterative process using an algorithm that minimizes the sum of the squares of differences between the dependent variable in the equations and the observations. Regression analysis was carried out using the Sigmaplot® for Windows version 1.0 program (Jandel Corporation).

CHAPTER 3

CHARACTERIZATION OF THE CATABOLISM OF ORNITHINE AND ARGININE IN THE PERFUSED RAT LIVER

SYNOPSIS

The production of $^{14}CO_2$ from [^{14}C]-arginine] and [^{14}C]-ornithine was measured to determine the rates of catabolism of these amino acids in the isolated nonrecirculating perfused rat liver. At physiological rat portal vein concentrations of these amino acids, the catabolic processes are not saturated. Treatment with gabaculine (an inhibitor of OAT) demonstrated that the majority of ornithine's, and arginine's, hepatic catabolism is carried out through OAT. A substantial catabolism of the carbon backbone of ornithine occurs; results suggest complete oxidation of this amino acid. Antegrade/retrograde perfusions show that the rate of catabolism of arginine is independent of perfusion direction. This final observation means that there must be a perivenous arginase.

INTRODUCTION

A necessary step towards a comprehensive understanding of whole body arginine and ornithine catabolism is the determination of the rôles of the individual organs in these processes. A study of particular relevance to this thesis, carried out in mice (Alonso and Rubio, 1989), showed that treatment with gabaculine (50mg/kg) decreased the production of $^{14}CO_2$ from [1-^{14}C]-ornithine by more than 80%, indicating that OAT is a key enzyme in whole body ornithine catabolism in this mammal. The liver is a key organ in clearance of amino acids post-prandially (Jungas et al, 1992), and is known to contain all the enzymes necessary to complete the catabolism of arginine and ornithine through OAT (for pathway see **Figure 1.3**.). This chapter presents data relating to the catabolism

of arginine and ornithine, through OAT, in the isolated nonrecirculating perfused rat liver.

The presence of a mitochondrially-associated rat liver arginase protein that differs in its properties from the cytosolic arginase (Cheung and Raijman, 1981) suggested that liver may contain various forms of arginase, or isozymes. Arginase II mRNA has been detected in human (Gotoh et al, 1997; Morris et al, 1997) and mouse liver (Morris et al, 1997). Cederbaum's group failed to detect human arginase II mRNA in the liver (Vockley et al, 1996); however, they also failed to demonstrate the presence of this transcript in tissues e.g. spleen and thymus that have been shown to contain the transcript (Morris et al, 1997). The distribution of these and, perhaps, other arginase isozymes within rat liver is not clear. As discussed in **Chapter 1**, the distribution of certain enzymes and entire metabolic processes may be restricted to certain areas within the liver. The urea cycle and the enzyme glutamine synthetase (EC 6.3.1.2) are spatially separated within the liver (Häussinger, 1983). The process for the catabolism of arginine may also be contained within a specific sub-population of cells. Swick determined by density gradient centrifugation that only a specific subset of liver mitochondria contained OAT (Swick, 1970) and proposed that these were contained within cells of the perivenous region. Darnell's group (Kuo et al, 1991) using the technique of *in situ* hybridization subsequently confirmed this. Thus, the catabolism of ornithine is a perivenous process. Whether or not arginine catabolism can be carried out in this region

depends on the presence, or absence of a perivenous arginase. A key question posed in this chapter is: Is the catabolism of arginine, through OAT, a perivenous process?

OBJECTIVES

(1) To determine some kinetic characteristics of the catabolism of arginine and ornithine in the isolated perfused rat liver.

(2) To determine the importance of OAT to the catabolism of arginine and ornithine in the isolated perfused rat liver.

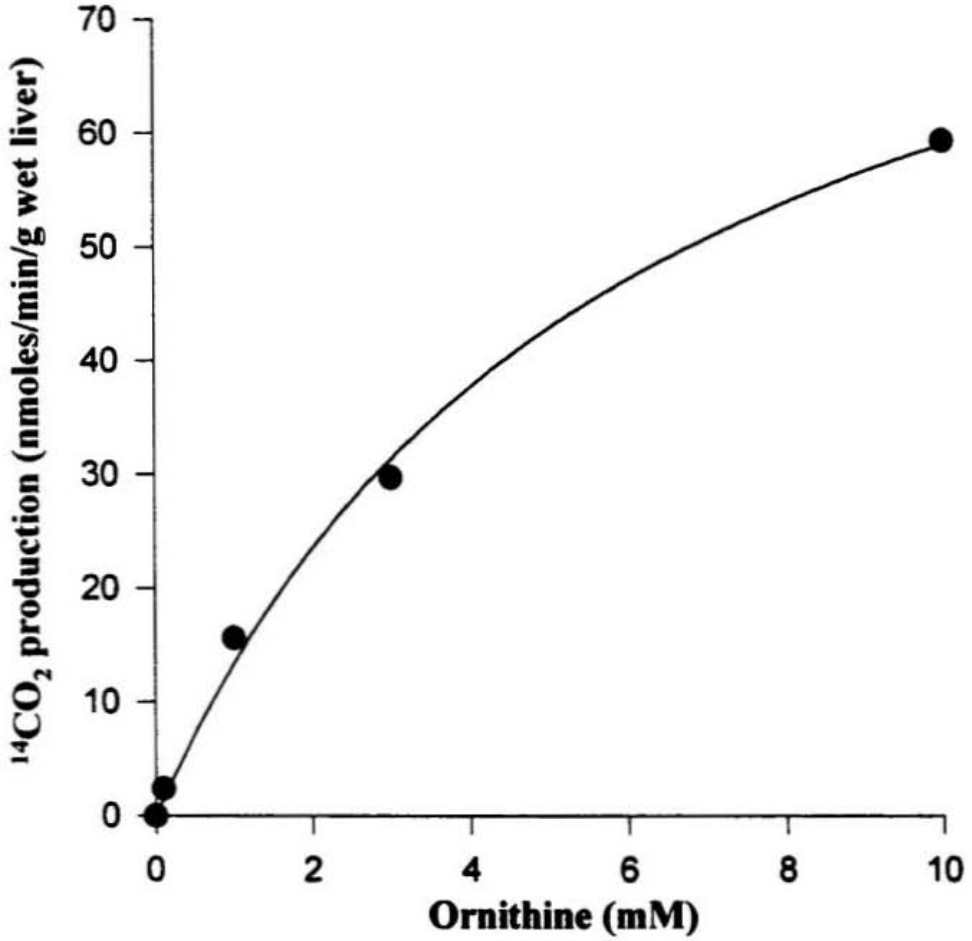
(3) To determine the extent of the catabolism of the carbon backbone of ornithine in the isolated perfused rat liver.

(4) To determine the rates of catabolism of arginine in antegrade and retrograde perfusions.

Kinetic characteristics of the catabolism of ornithine and arginine

Experiments were carried out with varying concentrations of labeled arginine and ornithine to determine the kinetic characteristics of the catabolism of these amino acids. In the case of ornithine (**Figure 3.1**) the data fit well to a rectangular hyperbola (r^2=0.994). A V_{max} of 94 nmoles/min/g wet liver was calculated and the ornithine concentration at which half-maximal stimulation occurred was 4.5mM. It is clear that at the physiological portal vein concentration of ornithine (0.1mM) this process is not saturated. Thus, ornithine catabolism in the perfused rat liver responds to changes in the

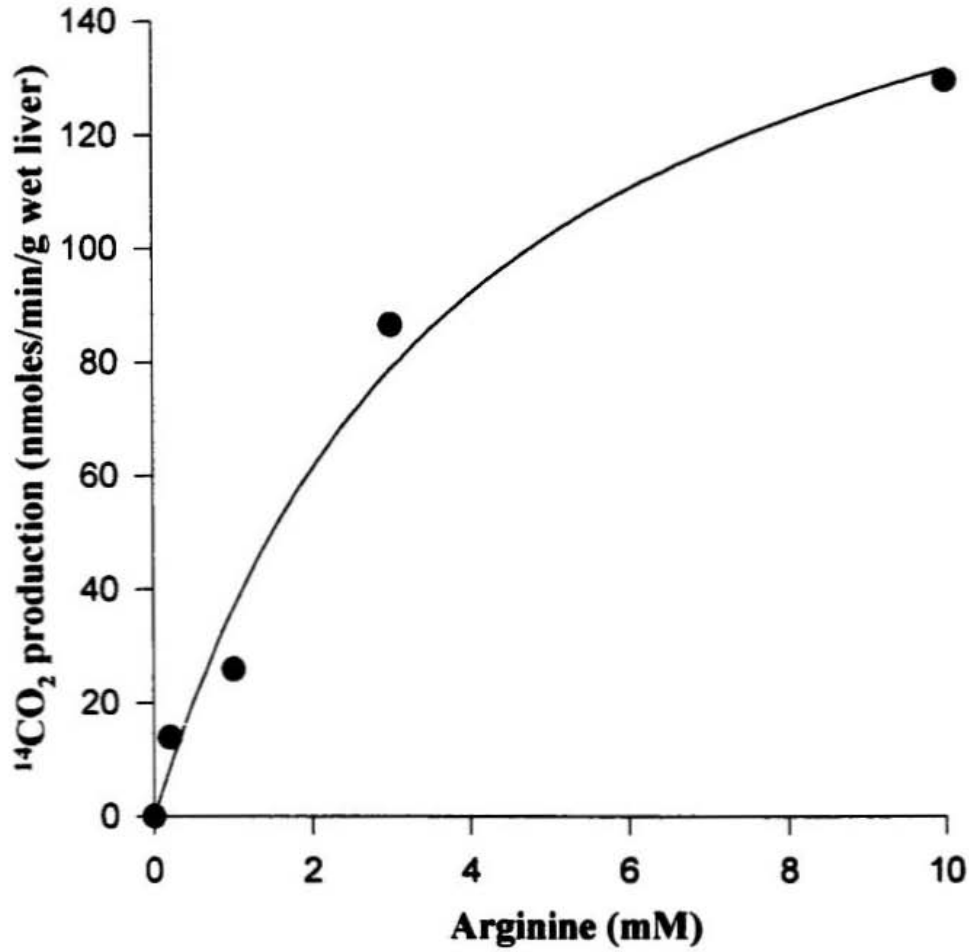
Figure 3.1. Substrate curve for the oxidation of ornithine in the isolated, nonrecirculating, perfused rat liver. Livers were perfused in the antegrade direction with different concentrations of [1-^{14}C]-ornithine, as described in **Materials and Methods**. Rats were fed a normal protein diet (15% casein) for a period of 3 days before the experiments. The data were used to construct a rectangular hyperbola using the GraphPad® program.


70
60
50
40
30
20
10
0
$^{14}CO_2$ production (nmoles/min/g wet liver)
0
2
4
6
8
10
Ornithine (mM)

concentration of this amino acid in the physiological range.

Figure 3.2 shows the data from similar experiments that were carried out with arginine. Once again, the data were fitted to a rectangular hyperbola (r^2=0.984). The V_{max} is 210 nmoles/min/g wet liver with the half-maximal velocity occurring at an arginine concentration of 5.25mM. Note that the higher V_{max} for the arginine experiments, *versus* ornithine, occurs because we measured the $^{14}CO_2$ released from [U-^{14}C]-arginine and the $^{14}CO_2$ released from [1-^{14}C]-ornithine; further experiments show substantial oxidation of the carbon backbone of ornithine and so the rate of release of $^{14}CO_2$ from [U-^{14}C]-ornithine is greater than that from [1-^{14}C]-ornithine (approximately 5 times, suggesting complete oxidation of carbon backbone of ornithine; see **Figure 3.5**). At similar concentrations of these amino acids, using [U-^{14}C] ornithine and [U-^{14}C] arginine, the rate of ornithine catabolism is higher than that of arginine. The key point is that the process for arginine catabolism is not saturated at the physiological concentration of this amino acid (0.2mM). Thus, arginine catabolism in the perfused rat liver responds to changes in the concentration of this amino acid within the physiological range. The next step was to determine the nature of the processes responsible for this catabolism. Specifically, the question as to the extent of ornithine aminotransferase's participation in this process was addressed.

Figure 3.2. Substrate curve for the oxidation of arginine in the isolated, nonrecirculating, perfused rat liver. Various concentrations of [U-^{14}C]-arginine were perfused across rat livers in the antegrade direction. Further details of this procedure are provided in **Materials and Methods**. Rats were fed a normal protein diet (15% casein) for a period of 3 days before the experiments. The results are the means for 2 independent experiments. The data were used to construct a rectangular hyperbola using the GraphPad® program.


140
120
100
80
60
40
20
0
$^{14}CO_2$ production (nmoles/min/g wet liver)
0
2
4
6
8
10
Arginine (mM)

The importance of OAT to the catabolism of arginine and ornithine

Figure 3.3 demonstrates, in rats fed a normal protein diet, that treatment with gabaculine, at the dose used by Alonso and Rubio in mice (50mg/kg body weight), reduces the catabolism of ornithine (as measured by $^{14}CO_2$ production from [1-^{14}C]-ornithine) at all time-points during the perfusions (verified by Student's unpaired t-test). At the 39 minute time-point the rate in the saline treated controls was 50.3 nmoles/min/g wet liver while in the gabaculine treated rats the rate was 4.5 nmoles/min/g wet liver, representing a 90% inhibition of the catabolism of ornithine by gabaculine. The OAT activity in livers from these rats were inhibited by ~80% (**inset Figure 3.3**). In the case of arginine catabolism (**Figure 3.4**) administration of gabaculine resulted in a substantial inhibition of the arginine catabolism. At the 39 minute time-point the rate of $^{14}CO_2$ production from [U-^{14}C]-arginine was 140 nmoles/min/g wet liver in the saline controls while in the gabaculine treated rats the rate at this time-point was 59 nmoles/min/g wet liver, representing a 60% inhibition of the $^{14}CO_2$ production by gabaculine. The inset in **Figure 3.4** shows that gabaculine treatment inhibited the OAT activity by 80%. The OAT activity determined in livers from rats fed the 15% casein diet are in close agreement with previous studies in rats fed diets similar in protein content (Herzfeld and Knox ,1968; Volpe et al, 1969).

The extent of catabolism of the carbon backbone of ornithine

The next experiments were carried out to determine the extent of catabolism of the

Figure 3.3. The effect of gabaculine administration on the oxidation of ornithine in the isolated, nonrecirculating, perfused rat liver. Gabaculine was administered to rats, by intraperitoneal injection, at a level of 50mg/kg body weight. 2 hours later, the rats were killed. Control rats were given the saline vehicle. Livers were perfused in the antegrade direction with 3mM [1-^{14}C]-ornithine as described in **Materials and Methods**. The arrow indicates the time at which the radio-labeled ornithine was added. Rats were fed a normal protein diet (15% casein) for 3 days before the experiments. (●) = control rats, (■) = gabaculine treated rats. Each point represents the mean ± S.D. for 3 independent experiments. * denotes a significant difference ($P<0.05$) from the gabaculine treated rats.

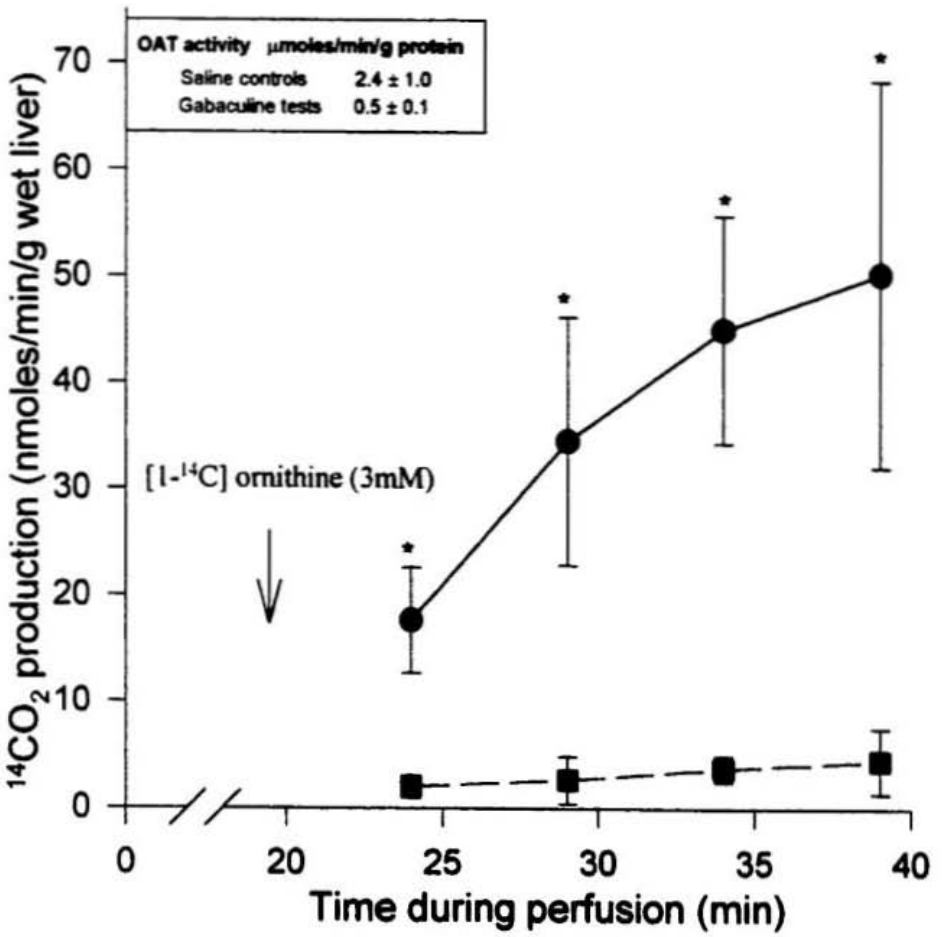
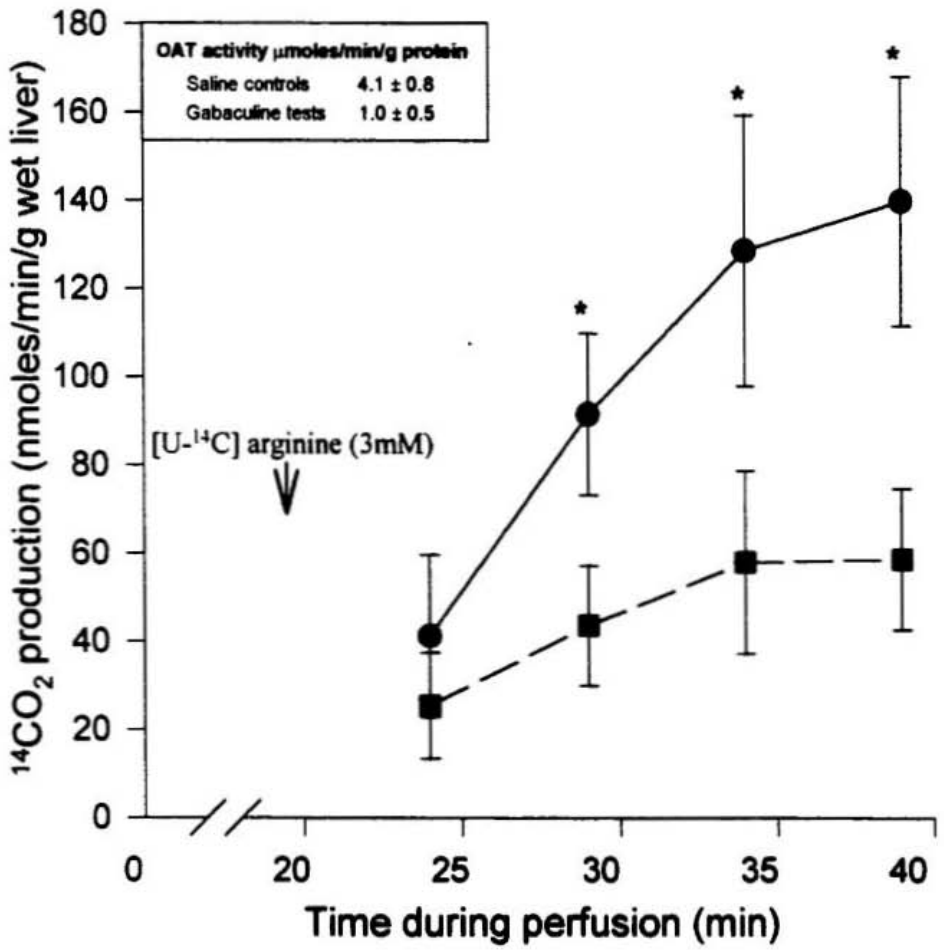

OAT activity µmoles/min/g protein
Saline controls 2.4 ± 1.0
Gabaculine tests 0.5 ± 0.1
[1-14C] ornithine (3mM)
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)
0
10
20
30
40
50
60
70
0
20
25
30
35
40

Figure 3.4. The effect of gabaculine administration on the oxidation of arginine in the isolated, nonrecirculating, perfused rat liver. Gabaculine was administered to rats, by intraperitoneal injection, at a level of 50mg/kg body weight. 2 hours later, the rats were killed. Control rats were given the saline vehicle. Livers were perfused in the antegrade direction with 3mM [U-^{14}C]-arginine as described in **Materials and Methods**. The arrow indicates the time at which the radio-labeled arginine was added. Rats were fed a normal protein diet (15% casein) for 3 days before the experiments. (●) = control rats, (■) = gabaculine treated rats. Each point represents the mean ± S.D. for 3 independent experiments. * denotes a significant difference ($P<0.05$) from the gabaculine treated rats.


OAT activity μmoles/min/g protein
Saline controls 4.1 ± 0.8
Gabaculine tests 1.0 ± 0.5
[U-14C] arginine (3mM)
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)
0
20
40
60
80
100
120
140
160
180
0
20
25
30
35
40
*
*
*

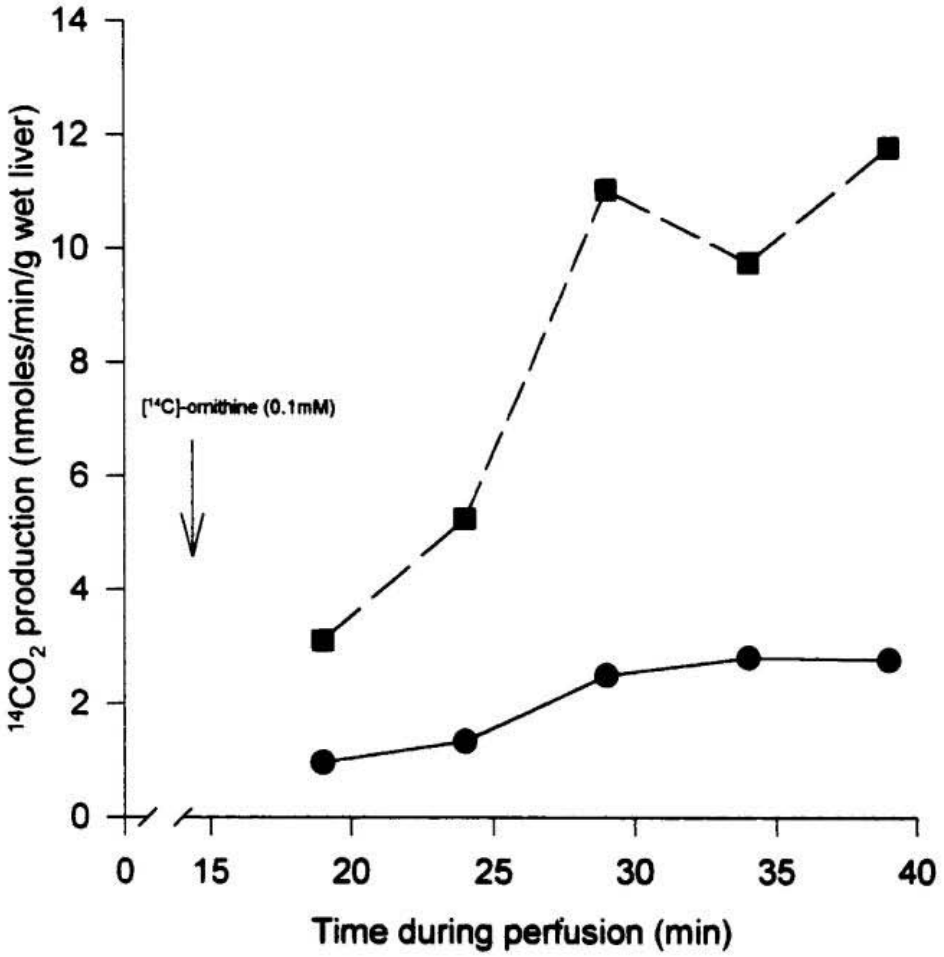
carbon backbone of ornithine (**Figure 3.5**). This was achieved using [1-^{14}C]-ornithine and [U-^{14}C]-ornithine; these radiolabeled tracers can be used to monitor the CO_2 released from the carbon at position 1, and that released from all five carbons of ornithine (see **Figure 1.1** for structure), respectively. The rates of $^{14}CO_2$ production are approximately 5 times greater from all five carbons of ornithine than they are from position one (12.4 ± 5.65 versus 2.58 ± 0.38 nmoles/min/g wet liver for [U-^{14}C]-ornithine and [1-^{14}C]-ornithine, respectively, at the 39 minute time-point, these are the means ± S.D. for 3 independent experiments). These results are similar to those of Alonso and Rubio who showed a substantial oxidation of the carbon backbone of ornithine in mice (Alonso and Rubio, 1989).

Antegrade/retrograde perfusions

The next experiments deal with the question of a possible localization of an arginase within the perivenous hepatocytes. There is a zonation of metabolism across the liver acinus (see **Introduction**). It has been shown that OAT is localized to the perivenous hepatocytes (Kuo et al, 1991). Thus, the catabolism of ornithine is a perivenous process (the ornithine enters the liver and may enter the urea cycle but this cycle cannot catabolize ornithine). Only when ornithine is taken up by the perivenous cells and metabolized does catabolism occur.

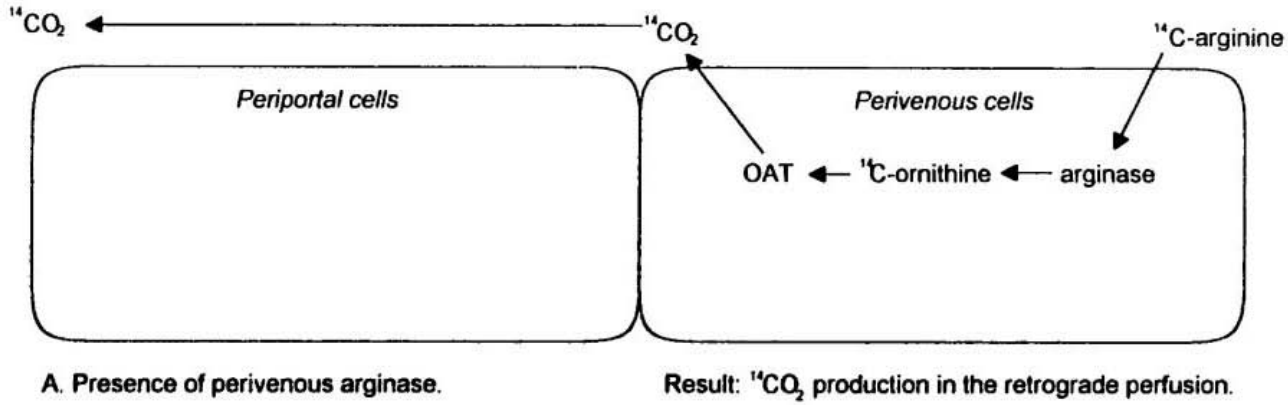
Whether or not arginine catabolism can occur in the perivenous hepatocytes depends on

Figure 3.5. The extent to which the carbon backbone of ornithine is catabolized. Rat livers were perfused , in the antegrade direction, with either [1-^{14}C]-ornithine (●) of [U-^{14}C]-ornithine (■) at a concentration of 0.1mM. The arrow indicates the time at which the radio-labeled ornithine was introduced. Rats were fed a normal protein diet (15% casein) for 3 days before the experiment. Results are from a typical experiment.


14
12
10
8
6
4
2
0
$^{14}CO_2$ production (nmoles/min/g wet liver)
[^{14}C]-ornithine (0.1mM)
0
15
20
25
30
35
40
Time during perfusion (min)

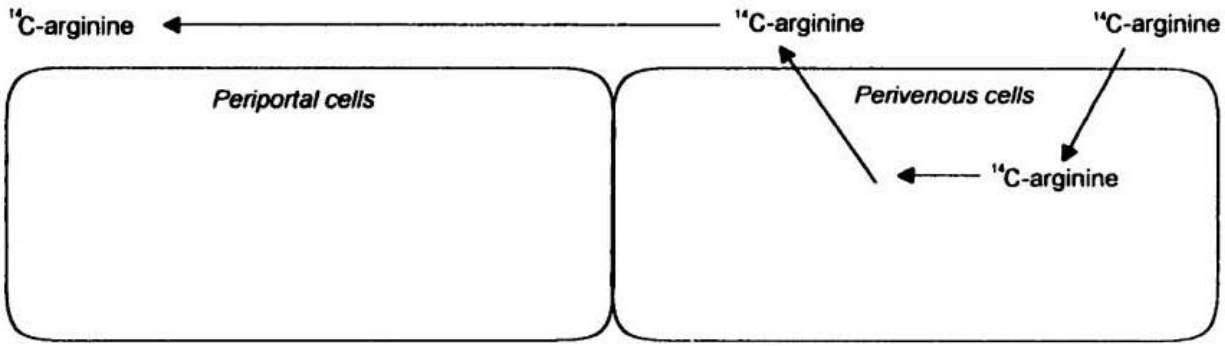
whether there is a perivenous arginase. In a retrograde perfusion, arginine catabolism would not occur unless there is a perivenous arginase. **Figure 3.6 (A)** illustrates that in the presence of a perivenous arginase, in a retrograde perfusion, the arginine would be converted to ornithine, and this would then be catabolized to CO_2 at a rate equal to that produced by arginine in the antegrade perfusion (**Figure 3.6. (B)**). This follows from the exclusive location of OAT and the fact that we used a nonrecirculating perfusion system. In order to carry out these experiments livers were perfused for 20-30 minutes in the antegrade direction with the ^{14}C-labelled amino acid and then the flow direction was reversed for a 20 minute wash-out period (the amino acid used for this period was non-radioactive). This reduced the production of $^{14}CO_2$ to low levels so that we could then continue the perfusion in the retrograde direction for a further 20-30 minutes with the labeled amino acid.

Having demonstrated previously that recirculation is not a feature of the perfusion system being used (**Materials and Methods, Figure 2.5**) the first set of experiments was carried out using ornithine. As shown in **Figure 3.7** the experiments began in the antegrade direction. The flow was then reversed to the retrograde and a wash-out period in which only cold amino acid was included lasted for 20 minutes. The ^{14}C tracer was reintroduced for a further 30 minutes after which the perfusion was terminated. The results show (1) that the wash-out period is sufficient to remove the bulk of the remaining radioactive label and (2) that there is no significant difference in the rates of $^{14}CO_2$



A. Presence of perivenous arginase.

Result: $^{14}CO_2$ production in the retrograde perfusion.

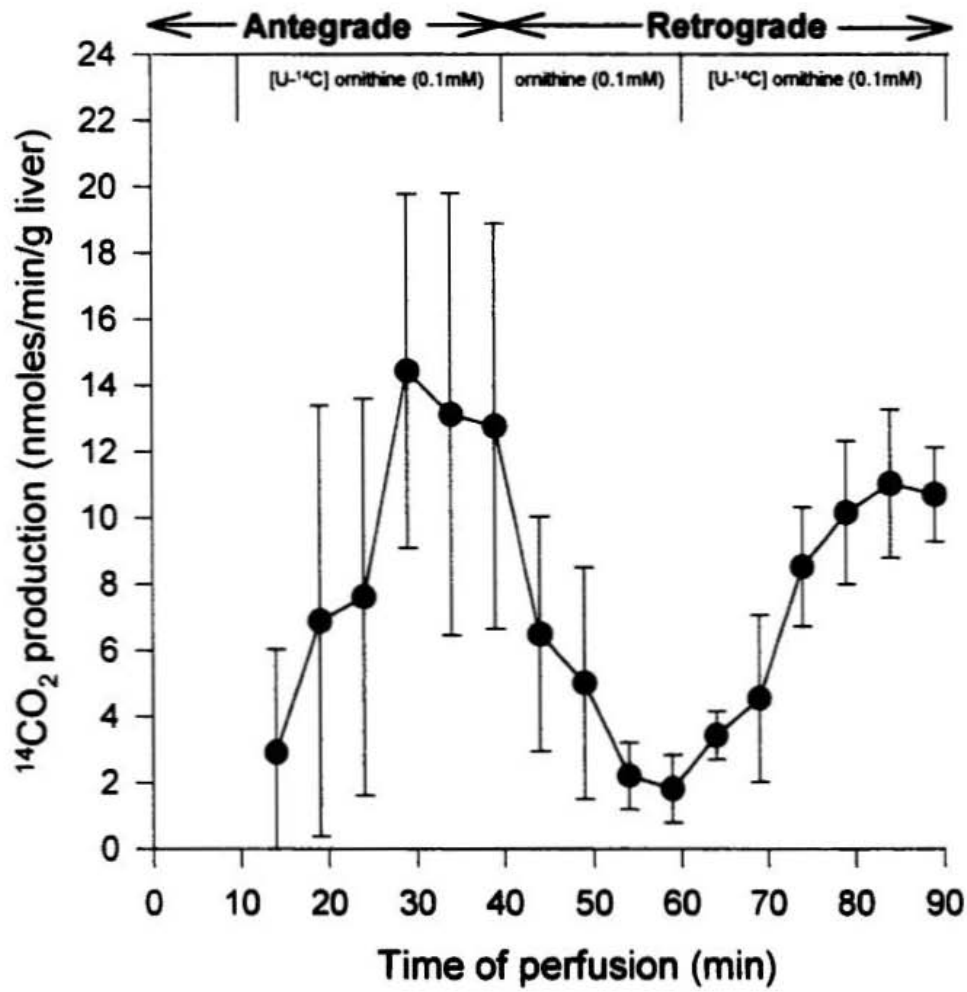


B. Absence of perivenous arginase.

Result: No $^{14}CO_2$ production in the retrograde perfusion

Figure 3.6. The effects of arginase localization on arginine catabolism through OAT.

Figure 3.7. The effect of the direction of perfusion on the oxidation of ornithine. A detailed account of the perfusion procedure is presented in Materials and Methods. Rat livers were perfused with 0.1mM [U-^{14}C]-ornithine between 10 and 40 minutes and between 60 and 90 minutes and with unlabelled ornithine between 40 and 60 minutes. Rats were a normal protein diet (15% casein) for a period of 3-5 days before the experiment. Each point represents the mean ± S.D. for 4 independent experiments.


Antegrade
Retrograde
[U-14C] ornithine (0.1mM)
ornithine (0.1mM)
[U-14C] ornithine (0.1mM)
14CO2 production (nmoles/min/g liver)
Time of perfusion (min)
0
2
4
6
8
10
12
14
16
18
20
22
24
0
10
20
30
40
50
60
70
80
90

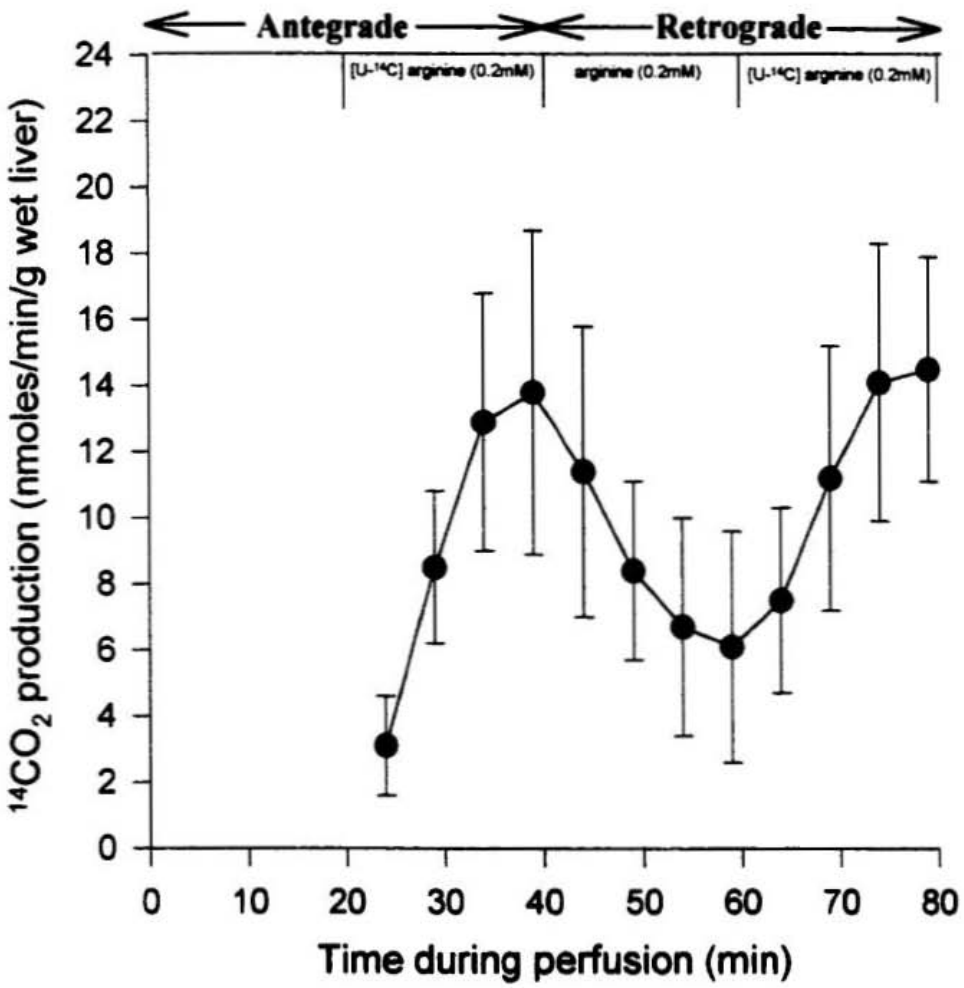
production from ornithine in the antegrade and retrograde directions.

The next step was to carry out these experiments using arginine. As can be seen from **Figure 3.8**, there is no significant difference in the rate of $^{14}CO_2$ production from arginine between the antegrade and retrograde directions. On the contrary, the rates are remarkably similar. Thus, there is a perivenous arginase.

Discussion

From the kinetic analysis, the concentration of amino acids required to produce half-maximal rates of catabolism are 4.5mM and 5.25mM for ornithine and arginine, respectively (**Figures 3.1 and 3.2**). Thus, any change in the portal vein concentration of arginine and ornithine within the physiological range (0.1-0.2mM) will produce a change in the rate of catabolism, making regulation possible at the level of substrate supply. It has been known for some time that arginine and ornithine transport into the liver is carried out by a low activity transporter (White, 1985; Christensen, 1984). This may be to avoid degradation of arginine by the large amounts of arginase in the liver. It may also limit the availability of arginine for the production of nitric oxide. The recently characterized transporter, MCAT2A, which is responsible for the transport of the basic amino acids into liver has a K_m between 2 and 5mM (Closs et al, 1993). Comparison with the K_ms for the catabolic processes suggests that transport could be a site of regulation, or rate limiting step, for the processes of arginine and ornithine catabolism.

Figure 3.8. The effect of the direction of perfusion on the oxidation of arginine. A detailed account of the perfusion procedure is presented in **Materials and Methods**. Rat livers were perfused with 0.2mM [U-^{14}C]-arginine between 10 and 30 minutes and between 50 and 70 minutes and with unlabelled arginine between 30 and 50 minutes. Rats were fed a normal protein diet (15% casein) for a period of 3-5 days before the experiment. Each point represents the mean ± S.D. for 3 independent experiments.


Antegrade
Retrograde
[U-14C] arginine (0.2mM)
arginine (0.2mM)
[U-14C] arginine (0.2mM)
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)
0
2
4
6
8
10
12
14
16
18
20
22
24
0
10
20
30
40
50
60
70
80

Teleologically, regulation at the first step (in this case, transport) is an efficient manner of controlling a metabolic process. Other examples of regulation of catabolic processes by transport exist in the field of amino acid transport (system A transport represents the rate limiting step in alanine catabolism in rat liver parenchymal cells (Sips et al, 1980) The distribution of the MCAT2A transporter within the liver has yet to be established. As the complete degradation of arginine can occur in the perivenous region, it is likely that a transporter is present in the perivenous region. *In situ* hybridization of the MCAT2A transcripts, complemented with an immunological study using a monoclonal antibody for the corresponding protein, could be used to determine the distribution of MCAT2A within mammalian liver.

The data in **Figure 3.3** show that OAT is responsible for the great bulk of ornithine catabolism. Thus, in the rat liver, the pathway responsible for the catabolism of ornithine is that shown in **Figure 1.3**. Any remaining activity may, in part, be due to residual uninhibited OAT activity. A minor ornithine decarboxylase (EC 4.1.1.17) activity exists in liver and will produce CO_2 form ornithine; gabaculine does not inhibit ornithine decarboxylase (Jung and Seiler, 1978; Rando and Bangerter, 1977).

The catabolism of arginine was not inhibited to the same extent by treatment with gabaculine (**Figure 3.4**). Two possible explanations exist:

(1) Alternative processes for the catabolism of arginine exist within the rat liver that do

not require the involvement of OAT and are capable of catabolizing a significant amount of arginine. As discussed in the **Introduction**, arginine consumption by the other processes, under normal physiological conditions, are quantitatively minor and/or result in the production of an intermediate that may be recycled back to arginine (e.g. citrulline in nitric oxide production). Relatively little is known concerning the production of agmatine (decarboxylated arginine) from arginine in mammalian tissues. A recent report indicates the presence of mRNA for the enzyme arginine decarboxylase (EC 4.1.1.19) within the liver (Morrissey et al, 1995). No arginase decarboxylase protein has been shown present in liver to date. The levels of agmatine in mammalian tissues studied, including liver, is in the nanomolar-picomolar range (Raasch et al, 1995). The measurement of $^{14}CO_2$ release from [1-^{14}C]-arginine in enriched liver mitochondrial fractions is not an appropriate way to determine arginine decarboxylase activity, as was done recently (Lortie et al, 1996). This is not merely a measure of any possible arginine decarboxylase enzyme within the tissue, but also a measure of the activity of the pathway shown in **Figure 1.3** of the **Introduction**. It is not a specific assay for arginine decarboxylase, and so its usefulness is limited. By virtue of the assay design used, the fact that no arginine decarboxylase protein has been shown in mammalian liver and the low levels of agmatine in tissues, it is unlikely that this pathway has a major rôle in arginine consumption. As was the case with ornithine, there will also be a contribution from ornithine decarboxylase to the rate of $^{14}CO_2$ production, albeit a minor one.

(2) The residual OAT activity after gabaculine could been higher in the experiments

with arginine than those with ornithine. This could be due to the presence of an initial higher OAT activity in the rats used in the arginine experiments compared with those used in the ornithine experiments. There is a higher level of OAT activity in the rats used in the arginine experiments treated with gabaculine when compared with the activity in the rats used in the ornithine experiments (1.0 μmoles/min/g liver protein *versus* 0.5 μmoles/min/g liver protein, respectively). This may account for the reduction in the inhibition of $^{14}CO_2$ production.

The extent of the catabolism of the carbon backbone (**Figure 3.5**) is instructive. The carbon backbone of ornithine enters the Krebs cycle as α-ketoglutarate. However, this alone cannot account for its complete oxidation. Our results show that complete catabolism of the carbon backbone of ornithine occurs; the rates of $^{14}CO_2$ production are approximately 5 times greater from all five carbons of ornithine than they are from position one (12.4 ± 5.65 versus 2.58 ± 0.38 nmoles/min/g wet liver for [U-^{14}C]-ornithine and [1-^{14}C]-ornithine, respectively, at the 39 minute time-point). An intermediate must leave the Krebs cycle and re-enter as pyruvate to allow the complete oxidation of the carbon backbone. This can be achieved by one of two mechanisms, or a combination of these. Malic enzyme (EC 4.1.1.40) can convert malate directly to pyruvate. Alternatively, oxaloacetate could be converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.29) which can then be converted to pyruvate by pyruvate kinase (EC 2.7.1.40). The resulting pyruvate will be completely

oxidized *via* pyruvate dehydrogenase and the Krebs cycle. Pyruvate kinase is present in the perivenous cells, as it is in all mammalian cells studied to date. A gradient exists in the distribution of the glycolytic enzymes increasing from periportal to perivenous cells (Jungermann and Katz, 1982). PEPCK protein (Andersen et al, 1982) by immunohistochemistry, and mRNA by *in situ* hybridization (Bartels et al, 1989) have been shown to be preferentially expressed in the periportal region, but the mRNA is certainly present in low levels in the perivenous cells (Bartels et al, 1989). Analysis of malic enzyme activity shows that it is present in both periportal and perivenous cells (Tosh et al, 1989).

Having established both the rate of catabolism of ornithine and arginine at the physiological concentrations of these amino acids, and that there is a substantial catabolism of the carbon backbone it is possible to estimate the total amount of the arginine load which can be catabolized through the liver by this process on a daily basis. The daily food intake for a rat on a normal protein diet (15% casein) is approximately 20g. Thus, the intake of protein is 3g. Arginine makes up 3% of the total amino acid composition of casein, therefore 0.09g of arginine is taken in daily (or 0.5 mmoles of arginine is taken in daily). At the physiological rat portal vein concentration of arginine (0.2mM) the rate of $^{14}CO_2$ production is 14 nmoles/min/g wet liver (**Figure 3.2**) from the entire backbone of the arginine molecule (since [U-^{14}C]-arginine was used as the tracer). As a substantial catabolism of the carbon backbone occurs from each arginine (if we

take the ornithine data, this represents a complete catabolism of the carbon backbone) the number of arginine molecules catabolized is lower than 14 nmoles/min/g wet liver. If we assume complete catabolism of the arginine molecule together with release of a urea molecule, each mole of arginine will produce 5 moles of CO_2 and 1mole of urea. Thus, the rate of arginine disposal is 2.8 nmoles/min/g wet liver. Over a 24 hour period and in a 300g rat with a 12g liver the total load of arginine catabolized is approximately 48μmoles or about 10% of the total daily arginine load. We know that the small intestine removes one third of the arginine in a single pass (Windmueller and Spaeth, 1976), thus liver can catabolize 15% of the total absorbed arginine. Data shown in **Chapter 4** suggests that the inclusion of the pancreas may increase this number by as much as 50%.

The antegrade/retrograde experiments show that there is a perivenous arginase. Thus, the catabolism of arginine, through OAT, can be carried out completely in the perivenous region. The periportal arginase could, in theory, produce ornithine for consumption in the perivenous region; however, the marked similarity in the antegrade/retrograde rates of catabolism (**Figure 3.8**) suggest that cooperation between periportal and perivenous cells is unlikely. As mentioned in the **Introduction**, the AI and AII account for the vast majority of the arginase activity in mammalian tissues. Experiments to date do not preclude the presence of still minor forms of arginase. Immunological studies, using anti-AI and anti-AII antibodies to pinpoint the location of these isozymes across the liver, would be useful. Cederbaum's groups have, for almost 20 years, investigated the tissue

distribution of arginases in humans and rats, principally by immunoprecipitation techniques. The results show that the anti-AI antibody can precipitate >95% of the total arginase activity in rat and human liver (Spector et al, 1994). The assumption which prevailed until recently was that all liver arginase was of the AI variety, and functioned in the urea cycle. Cederbaum's group showed that the anti-AI antibody also precipitated approximately 50% of the arginase activity in rat kidney; the remainder was precipitated by the anti-AII antibody. The mRNA transcript for the AI arginase has been found to be absent in kidney by groups (Morris et al, 1997), including Cederbaum's (Vockley et al, 1996). The presence of a specific mRNA does not guarantee the production of the corresponding protein, but no protein can exist without a corresponding mRNA transcript. Thus, Cederbaum's anti-AI antibody may not be specific enough.

Any form of arginase, other than the AI isozyme, present in rat liver will have substantially lower activity than AI. Raijman's group (Cheung and Raijman, 1981) demonstrated the presence of a minor activity of a mitochondrially associated arginase, possibly the AII isozyme. (Watford postulated that this low activity of arginase functions to complete the urea cycle as a 'metabolon' (Watford, 1991)). The number of cells containing OAT, and thus arginase, which is 2 to 3 cell layers surrounding the central vein (Kuo et al, 1991), accounts for <5% of the total hepatocyte population so that arginase activity here will be minor. Distinguishing the various isozymes of arginase within rat liver, on the basis of enzymatic activities, is, at best, a difficult task; it involves

accurate partition of a large activity (the arginase AI activity) from a much smaller activity (the AII activity and other possible isozymes). Immunoprecipitation studies, in this case, are not sensitive enough to determine the absence, or presence, of the minor forms of arginase within rat liver. *In situ* hybridization, using radioactively labeled antibody probes, could be used to good effect in this field.

CHAPTER 4

REGULATION OF THE CATABOLISM OF ORNITHINE AND ARGININE

SYNOPSIS

The rates of catabolism of arginine and ornithine, in isolated nonrecirculating rat liver perfusions, were established in rats fed a high protein diet (60% casein). Kinetic analysis shows that the processes are not saturated at the physiological concentrations of these amino acids. Thus, substrate supply can regulate these processes. Livers from rats fed a high protein diet (60% casein) catabolized ornithine (0.1mM) at rates four times higher than from rats fed a normal protein diet (15% casein). The rates of $^{14}CO_2$ production from [1-^{14}C]-ornithine were 14.4 ± 5.3 and 63.6 ± 22.6 nmoles/min/g wet liver for rats fed 15% casein and 60% casein diets, respectively. Thus, ornithine catabolism in the rat liver is subject to chronic regulation by the level of dietary protein. Rates of [U-^{14}C]-arginine (0.2mM) catabolism were 13.8 ± 4.9 and 74.5 ± 22.5 nmoles $^{14}CO_2$/min/g wet liver for 15% casein and 60% casein diets, respectively. Thus, arginine catabolism is also subject to regulation by the level of protein in the diet. Glucagon (10^{-7}M) acutely stimulated the catabolism of arginine by approximately 40% in rats fed a high protein diet. Similar experiments carried out with ornithine showed no such stimulation in the rate of its catabolism. Dibutyryl- cAMP (0.1mM) also stimulated the rate of catabolism of arginine by approximately 40%. In retrograde perfusions, glucagon stimulated the catabolism of arginine by approximately 2-fold; this means that glucagon exerts its effects in the perivenous region. Insulin infusion had no effect on either arginine or ornithine catabolism in rats fed the high protein diet.

INTRODUCTION

The catabolism of arginine, and ornithine, decrease under conditions of limited arginine supply, in effect conserving the supply of these amino acids. This has been shown to be the case in both rats (Dhanakoti et al, 1990) and humans (Castillo et al, 1994; Castillo et al, 1993). The rôles of the individual organs, indeed of individual processes, in these homeostatic mechanisms remains unclear.

Schimke (1962) demonstrated that increasing the level of dietary protein fed to rats resulted in a directly proportional increase in the level of urea excretion, and the activities of the individual enzymes of the urea cycle in rat livers. In this study, rats were fed diets containing either 15% or 60% casein, for a period of a week. With respect to short-term regulation, our group has shown that feeding rats a single high protein meal stimulates the glycine cleavage system (Ewart et al, 1992), and glutaminase activity (Ewart et al, 1993), in isolated rat liver mitochondria. In these examples, the agent of such changes in enzyme activity may be an alteration in the secretion of glucagon. Feeding rats a single high protein meal increases the circulating level of plasma glucagon (Robinson et al, 1981). Also, it has been shown that isolated hepatocyte preparations treated with glucagon exhibit stimulated activities of the glycine cleavage enzyme (Jois et al, 1989).

The experiments described in this chapter were designed to investigate whether the catabolism of arginine and ornithine in the perfused rat liver was subject to regulation by the level of dietary protein (chronic regulation) and/or acute regulation by pancreatic hormones such as glucagon and insulin.

OBJECTIVES

(1) To establish the kinetic characteristics of the catabolism of arginine and ornithine in the isolated perfused rat liver from rats fed a high protein diet.

(2) To determine the importance of the enzyme OAT to these processes.

(3) To compare the rates of catabolism of these amino acids in rats fed a high protein (60% casein) diet with those in rats fed a normal protein (15% casein) diet.

(4) To establish the extent to which the carbon backbone of ornithine is catabolized.

(5) To determine whether an infusion of glucagon (final concentration 10^{-7}M) alters the rate of catabolism of arginine, or ornithine, in rats fed a high protein diet.

(6) To establish whether an infusion of insulin (final concentration 10^{-7}M) alters the rate of catabolism of ornithine, or arginine, in rats fed a high protein diet.

As an effect of glucagon upon arginine catabolism was evident, further objectives included:

(7) To localize the site of glucagon action within the liver.

(8) To determine whether dibutyryl-cAMP could mimic the effects which glucagon exerted on the catabolism of arginine.

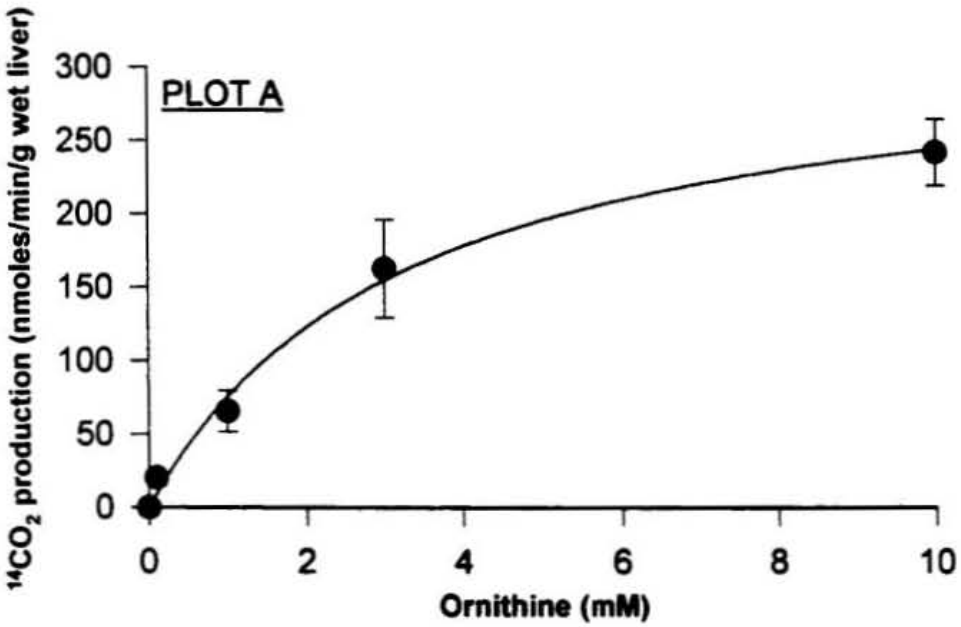
RESULTS

High protein diet fed rats; characteristics of the catabolism of ornithine and arginine, and dependence of these processes upon ornithine aminotransferase

Figure 4.1 (Plot A) shows the production of $^{14}CO_2$ from [1-^{14}C]-ornithine as a function of ornithine concentration. $^{14}CO_2$ production from [1-^{14}C]-ornithine was determined at a range of ornithine concentrations so as to determine the kinetic characteristics of the system. The data show that the V_{max} for this process is 324nmoles $^{14}CO_2$/min/g wet liver, and that the substrate concentration at which half-maximal catabolism occurs is 3.3mM. (To determine these kinetic characteristics the data were fit to a rectangular hyperbola (r^2=0.992). Similar experiments, which monitored the release of carbons from all positions of arginine (using [U-^{14}C]-arginine), are shown in **Figure 4.1, Plot B**. The data also fit well to a rectangular hyperbola (r^2=0.997). The V_{max} is 697 nmoles $^{14}CO_2$/min/g wet liver, and a concentration of 3.4mM is needed to give half-maximal rates of catabolism. Thus, at the physiological concentrations of these amino acids the processes are far removed from saturation.

Treatment with gabaculine inhibits the catabolism of ornithine by 80% when the last three time points of the perfusion were averaged (16.25 ± 0.12 *versus* 3.1 ± 0.45 nmoles/min/g wet liver, **Figure 4.2, Plot A**). In the livers from these rats OAT activity is inhibited by 92% (14.3± 5.91 *versus* 1.16 ± 0.31 μmoles/min/g liver protein).

Figure 4.1. Substrate curves for the oxidation of ornithine and arginine in the isolated, non-recirculating, perfused rat liver. Livers were perfused in the antegrade direction with different concentrations of [1-^{14}C]-ornithine (**Plot A**) and [U-^{14}C]-arginine (**Plot B**) as described in **Materials and Methods**. Rats were fed a high protein diet (60% casein) for a period of 3 days prior to the experiments. Each point represents the mean ± standard deviation for three independent experiments. The data were used to construct a rectangular hyperbola using the GraphPAD® program.


PLOT A
$^{14}CO_2$ production (nmoles/min/g wet liver)
300
250
200
150
100
50
0
0
2
4
6
8
10
Ornithine (mM)

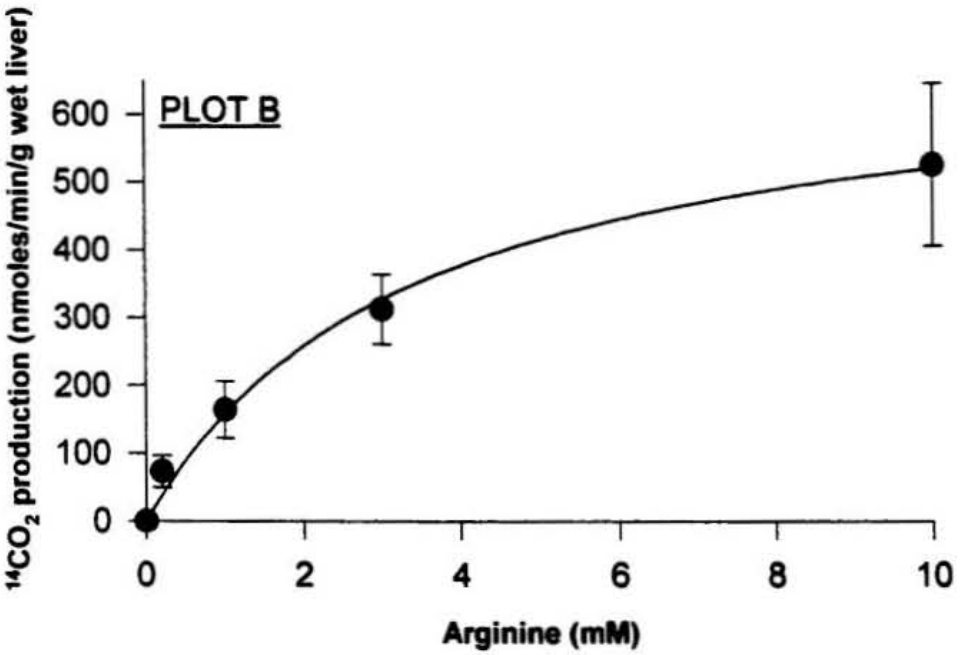
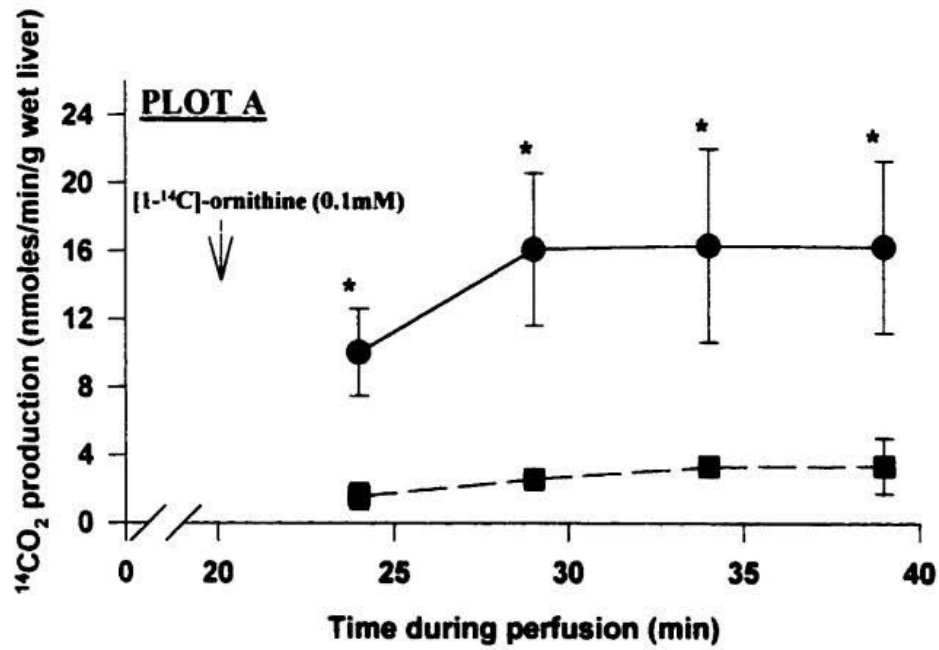
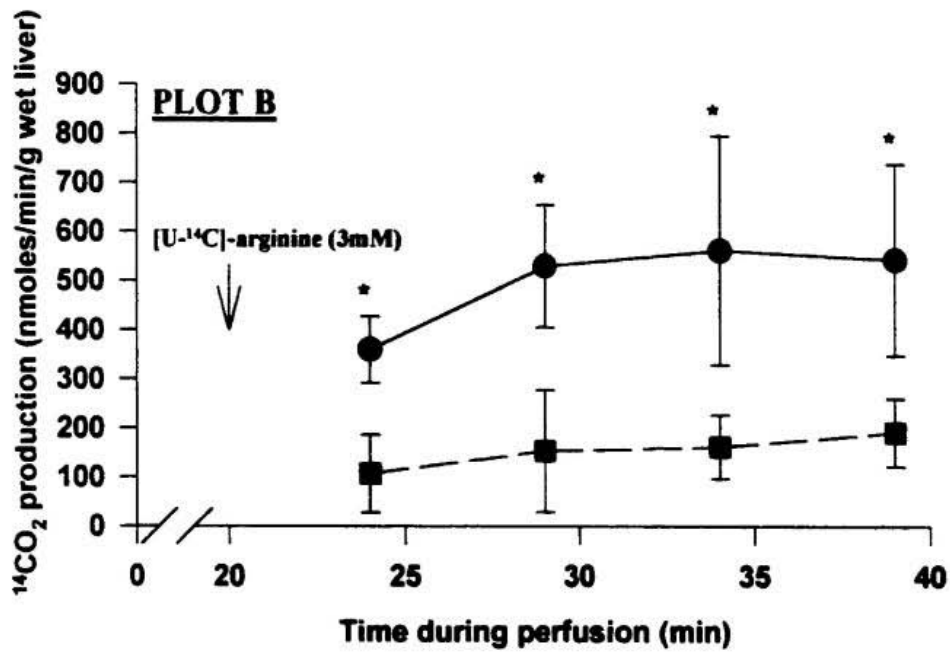

PLOT B
$^{14}CO_2$ production (nmoles/min/g wet liver)
600
500
400
300
200
100
0
0
2
4
6
8
10
Arginine (mM)

Figure 4.2. The effect of gabaculine administration on the oxidation of ornithine and arginine in the isolated, non-recirculating, perfused rat liver. Gabaculine was administered to rats, by intraperitoneal injection, at a level of 50mg/kg body weight. 2 hours subsequent to this, the rats were killed. Control rats were given the saline vehicle. Livers were perfused in the antegrade direction with either 0.1mM [U-^{14}C]-ornithine (**Plot A**) or 3mM [U-^{14}C]-arginine (**Plot B**), as described in **Materials and Methods**. The arrow indicates the time at which the radiolabel was added. Rats were fed a high protein diet (60% casein) for a period of 3 days prior to the experiments. (●) = control rats, (■) = gabaculine treated rats. Each point represents the mean ± S.D. for 3 independent experiments. * denotes a significant difference (Student's unpaired t-test, $P<0.05$) from the gabaculine treated rats.


PLOT A
[1-14C]-ornithine (0.1mM)
14CO2 production (nmoles/min/g wet liver)
24
20
16
12
8
4
0
0
20
25
30
35
40
Time during perfusion (min)


PLOT B
[U-14C]-arginine (3mM)
14CO2 production (nmoles/min/g wet liver)
900
800
700
600
500
400
300
200
100
0
0
20
25
30
35
40
Time during perfusion (min)

Experiments carried out with arginine (**Figure 4.2, Plot B**) show that treatment of these rats with gabaculine inhibits the OAT activity by 82% (17.3 ± 3.45 *versus* 3.18 ± 1.17 μmoles/min/g liver protein, for saline treated and gabaculine treated rats, respectively) while reducing the rate of $^{14}CO_2$ production from [U-^{14}C]-arginine by 70% at the last three time points of the perfusion (545 ± 16.3 *versus* 168 ± 20.1 nmoles/min/g wet liver).

Comparison of the rates of catabolism of arginine and ornithine in rats fed the normal protein diet (15% casein) with those for rats fed the high protein diet (60% casein)

Figure 4.3 shows that, at the physiological portal vein concentration of ornithine (0.1mM), the livers from rats fed the high protein diet catabolize this amino acid at rates which are approximately four fold higher than in rats fed the normal protein diet (63.6 ± 22.6 *versus* 14.4 ± 5.3 nmoles $^{14}CO_2$/min/g wet liver from [1-^{14}C]-ornithine at the 29 minute time-point). In the case of arginine (**Figure 4.4**) the rates of catabolism were increased by approximately five fold in rats fed the high protein diet *versus* those fed the normal protein diet (74.5 ± 22.5 *versus* 13.8 ± 4.9 nmoles/min/g wet liver, at the 29 minute time-point). We also see differences in the rates of oxygen consumption; urea-N production and liver OAT activity between rats fed the high protein diet and those fed the normal protein diet. These are shown in **Figure 4.5**. The results are those from the saline groups from **Figure 3.3** and **Figure 4.2** (**Plot B**) (the only variable being the protein content of the diet). These show higher rates of oxygen consumption, urea-N

Figure 4.3. Chronic regulation of the catabolism of ornithine by dietary protein. Rats were fed either a normal protein diet (● 15% casein) or a high protein diet (■ 60% casein) for a period of 3-4 days prior to the experiment. Livers were perfused in the antegrade direction with 0.1mM [U-^{14}C]-ornithine as described in **Materials and Methods**. Each point represents the mean ± standard deviation for each time-point from 3 independent experiments. * denotes a significant difference ($P<0.05$) from the normal protein group (determined by Student's unpaired t-test).

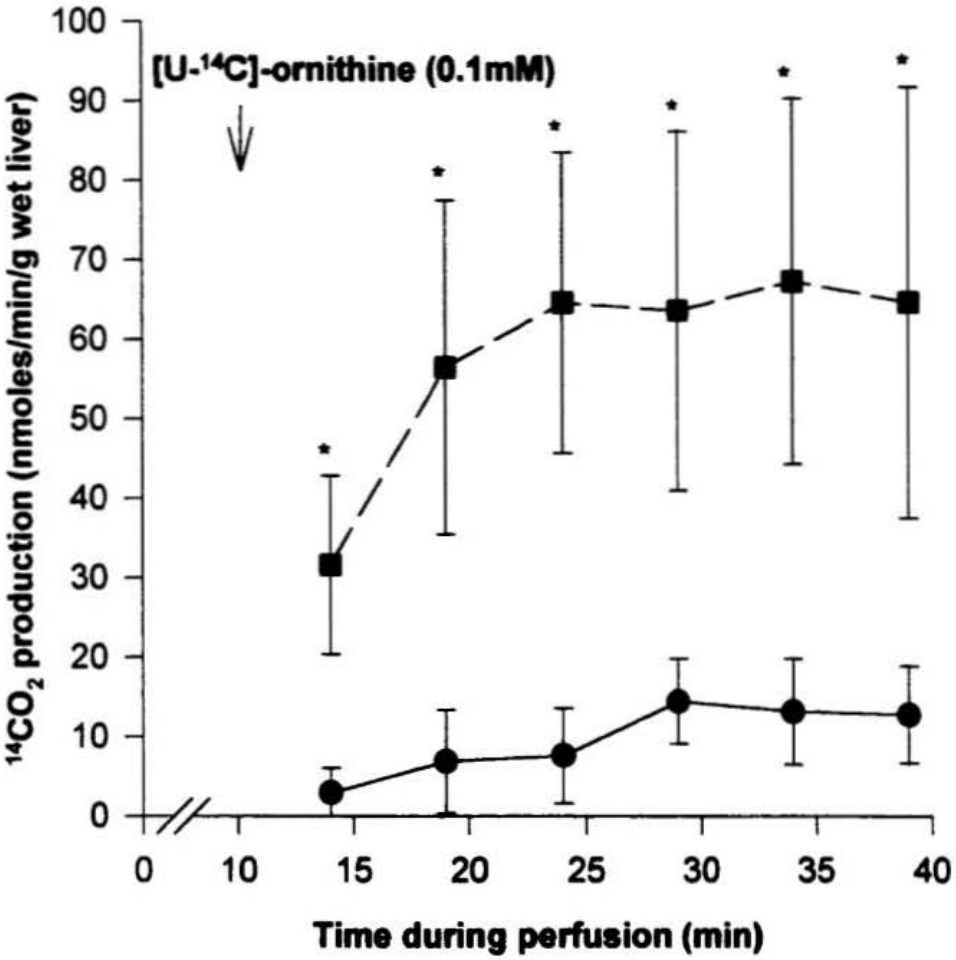

[U-14C]-ornithine (0.1mM)
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)
0
10
20
30
40
50
60
70
80
90
100
0
10
15
20
25
30
35
40

Figure 4.4. Chronic regulation of the catabolism of arginine by dietary protein. Rats were fed either a normal protein diet (● 15% casein) or a high protein diet (■ 60% casein) for a period of 3-4 days prior to the experiment. Livers were perfused in the antegrade direction with 0.2mM [U-^{14}C]-arginine as described in **Materials and Methods**. Each point represents the mean ± standard deviation for each time point for 3 independent experiments. * denotes a significant difference ($P<0.05$) from the normal protein group (determined by Student's unpaired t-test).

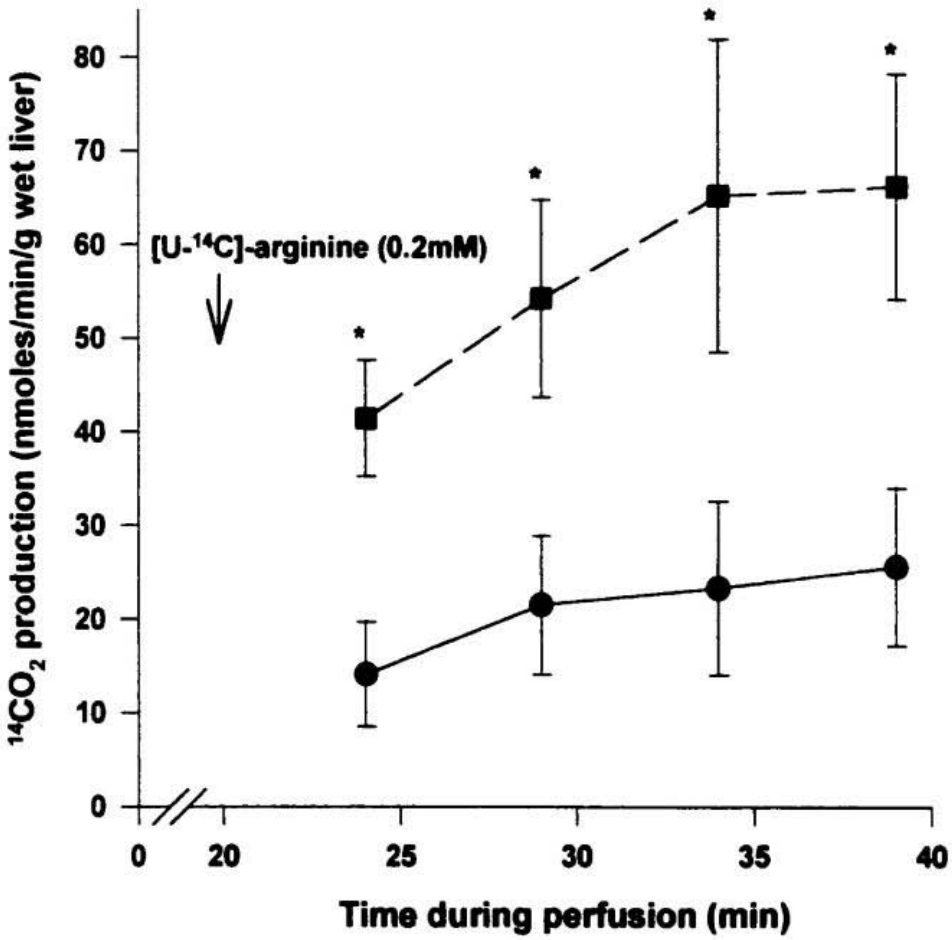
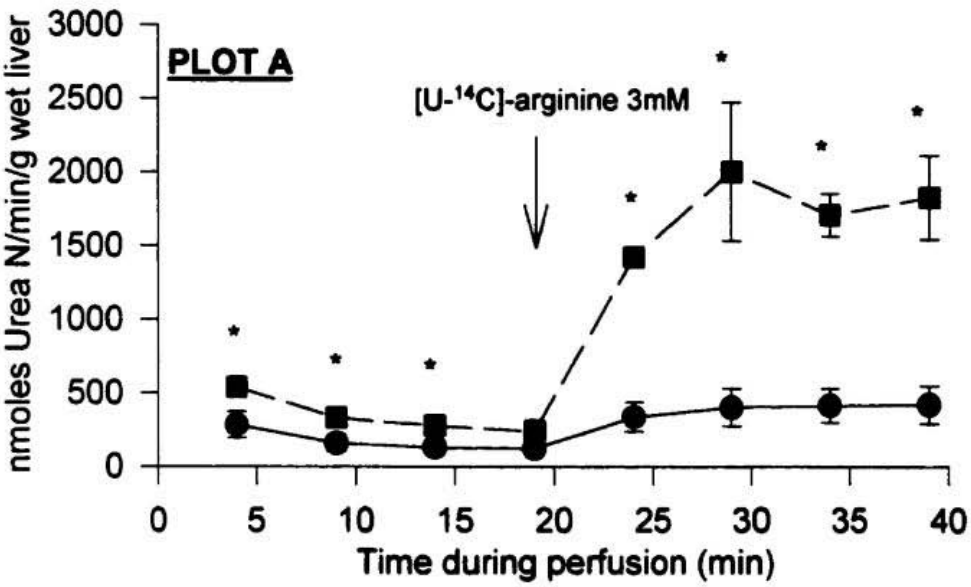
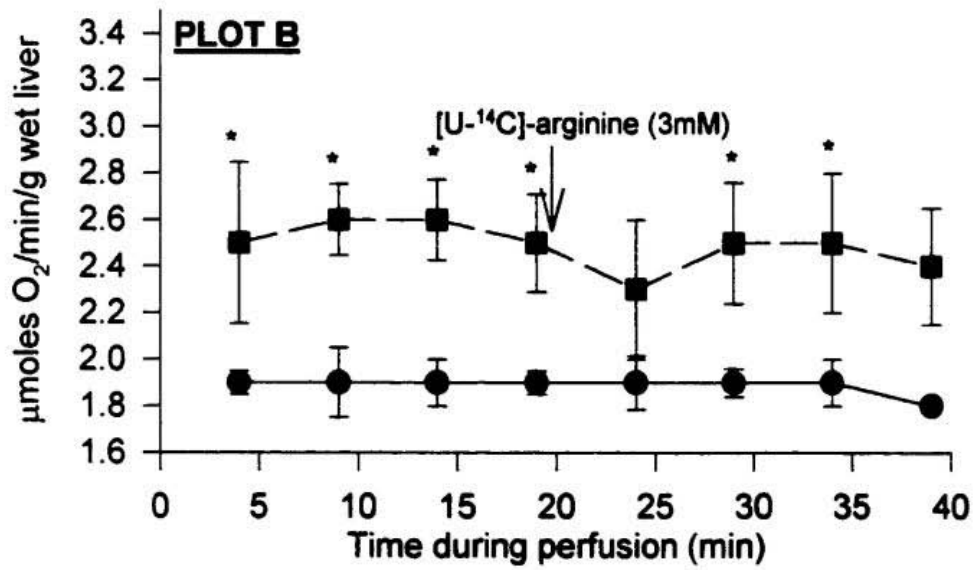

$^{14}CO_2$ production (nmoles/min/g wet liver)
80
70
60
50
40
30
20
10
0
[U-^{14}C]-arginine (0.2mM)
0
20
25
30
35
40
Time during perfusion (min)

Figure 4.5. Chronic regulation of urea-N production, oxygen consumption and OAT activity by dietary protein. Rats were fed either a normal protein diet (●15% casein) or a high protein diet (■60% casein) for a period of 3-4 days prior to the experiment. Livers were perfused in the antegrade direction with 3mM [U-^{14}C]-arginine. Urea N production (**Plot A**), oxygen consumption (**Plot B**) and OAT activities were measured as described in **Materials and Methods**. Each point represents the mean ± standard deviation for 3 independent experiments. * denotes a significant difference ($P<0.05$) from the normal protein group, determined by Student's unpaired t-test.



OAT activities	(μmoles/min/g liver protein)
Normal protein (15% casein)	4.12 ± 0.71
High protein (60% protein)	17.3 ± 3.45*



production and OAT activities in the rats fed the high protein diet, all consistent with previous studies in which rats have been fed high protein diets *versus* normal protein diets.

The extent of catabolism of the carbon backbone of ornithine

In the next experiments we perfused rat livers in the antegrade direction with [1-^{14}C]-ornithine (0.1mM) or [U-^{14}C]-ornithine (0.1mM); these radioactively labeled substrates were used to monitor the $^{14}CO_2$ release from the carbon at position 1 and from all 5 carbons of ornithine, respectively. The results (**Figure 4.6**) show that the rate of $^{14}CO_2$ production from all five positions of ornithine is approximately four times greater than that from position 1 of ornithine (79.4 ± 11.3 *versus* 19.6 ± 6.94 nmoles $^{14}CO_2$/min/g wet liver). Thus, in rats fed a high protein diet, there is a substantial oxidation of the carbon backbone of ornithine.

The effects of a glucagon infusion on the catabolism of arginine and ornithine in rats fed a high protein diet

We next investigated possible acute regulation of arginine and ornithine catabolism processes by the pancreatic hormone glucagon. **Figure 4.7** (**Panel A**) shows a typical experiment in which glucagon is infused during the perfusion procedure. Glucagon does not affect the rate of $^{14}CO_2$ production at any time point. The results of four independent experiments are shown in **Panel C**; these demonstrate that glucagon brings about an

Figure 4.6. The extent to which the carbon backbone of ornithine is oxidized. Rats, fed a high protein diet for 3 days, were perfused antegrade with either [U-^{14}C] ornithine (0.1mM) (●) or [1-^{14}C]-ornithine (0.1mM) (■). Perfusion procedures are described in **Materials and Methods**. Each point represents the mean ± standard deviation for each time-point from 3 independent experiments. * denotes a significant difference from the group perfused with ornithine (determined by Student's unpaired t-test).

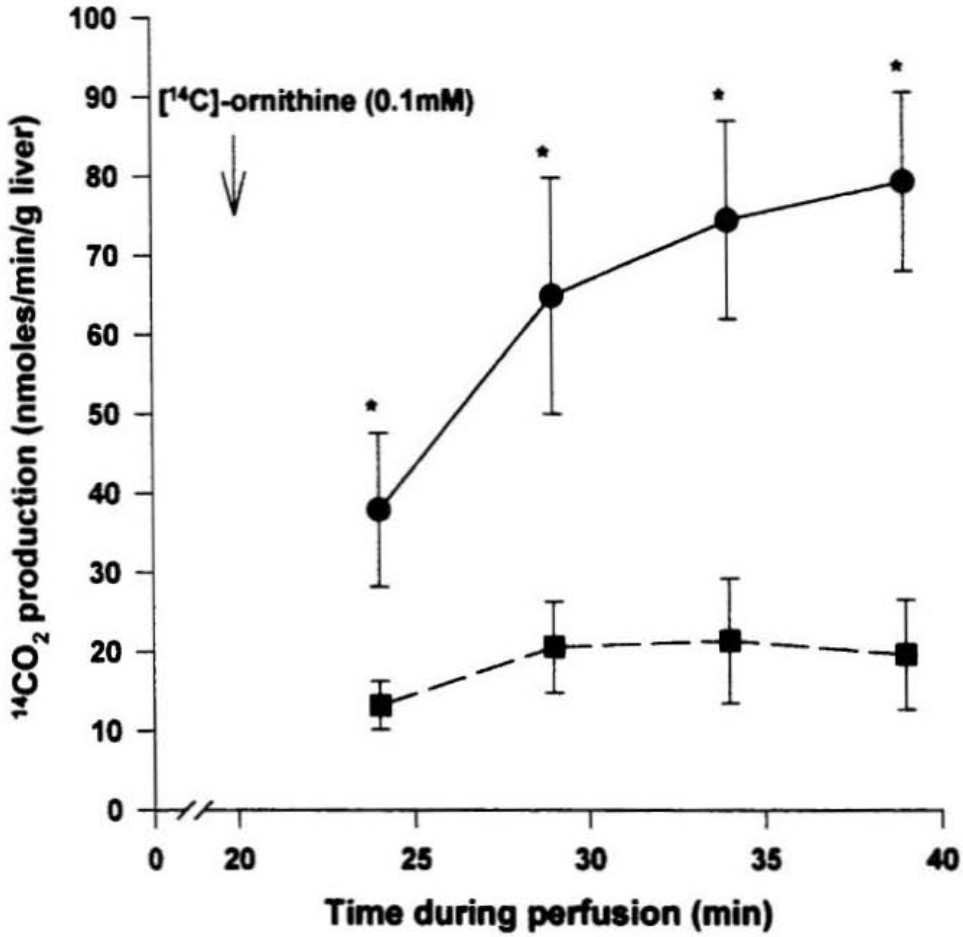
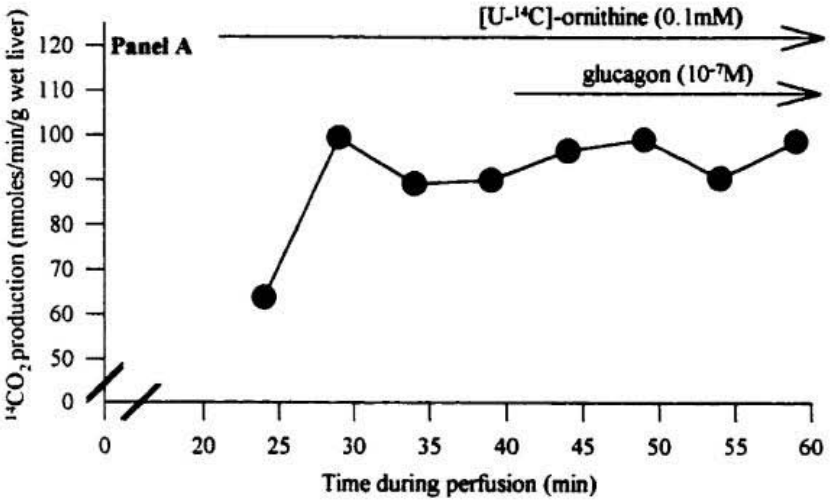
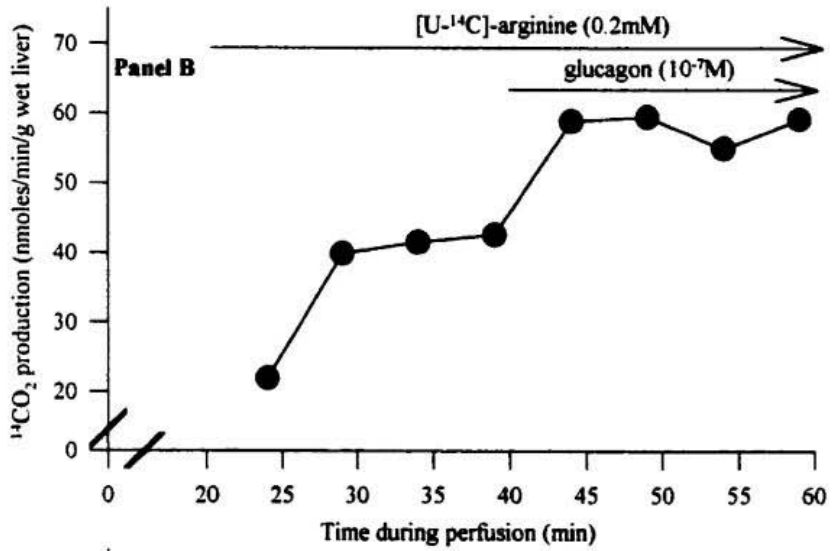
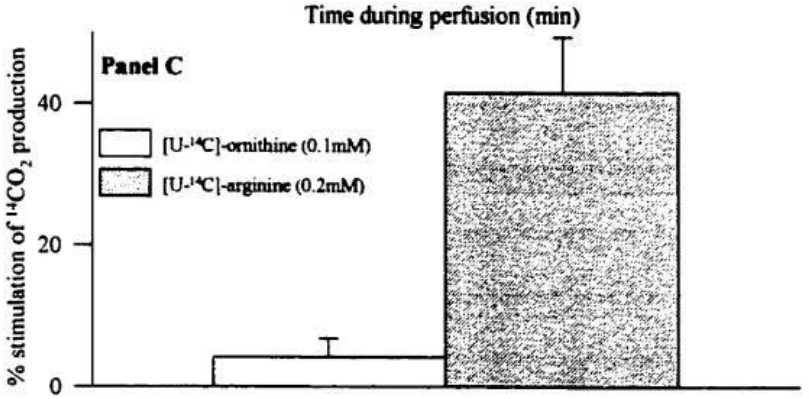

[14C]-ornithine (0.1mM)
14CO2 production (nmoles/min/g liver)
Time during perfusion (min)
100
90
80
70
60
50
40
30
20
10
0
0
20
25
30
35
40

Figure 4.7. The effect of glucagon infusion on the catabolism of ornithine and arginine. Rats, fed a high protein diet for 3 days, were perfused antegrade with [U-^{14}C]-arginine (0.2mM) or [U-^{14}C]-ornithine (0.1mM). Perfusion procedures are described in **Materials and Methods**. Glucagon (final concentration in the perfusate 10^{-7}M) was infused at the times indicated by the arrows. Typical experiments are shown for ornithine (**Panel A**) and arginine (**Panel B**) perfusions. **Panel C** shows the percentage stimulation in the rates of catabolism of arginine and ornithine by glucagon (expressed as the mean ± standard deviation for 3 independent experiments).


Panel A
[U-14C]-ornithine (0.1mM)
glucagon (10-7M)
14CO2 production (nmoles/min/g wet liver)
120
110
100
90
80
70
60
50
0
0
20
25
30
35
40
45
50
55
60
Time during perfusion (min)


Panel B
[U-14C]-arginine (0.2mM)
glucagon (10-7M)
14CO2 production (nmoles/min/g wet liver)
70
60
50
40
30
20
0
0
20
25
30
35
40
45
50
55
60
Time during perfusion (min)


Panel C
[U-14C]-ornithine (0.1mM)
[U-14C]-arginine (0.2mM)
% stimulation of 14CO2 production
40
20
0

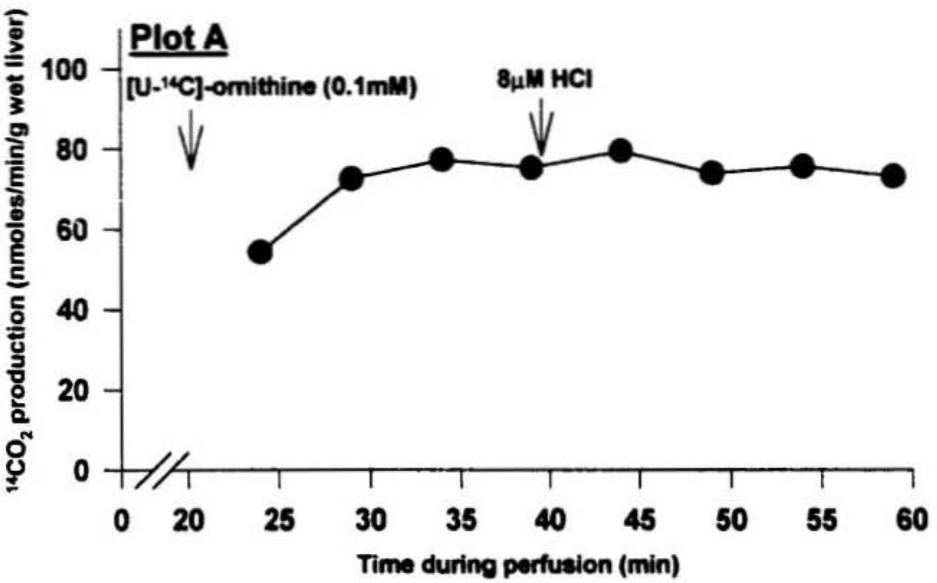
average increase of 4.2 ± 2.6% (calculated from the mean values for the 34 and 39 minute time points and the means for the 54 and 59 minute time points for each experiment). This was not statistically significant.

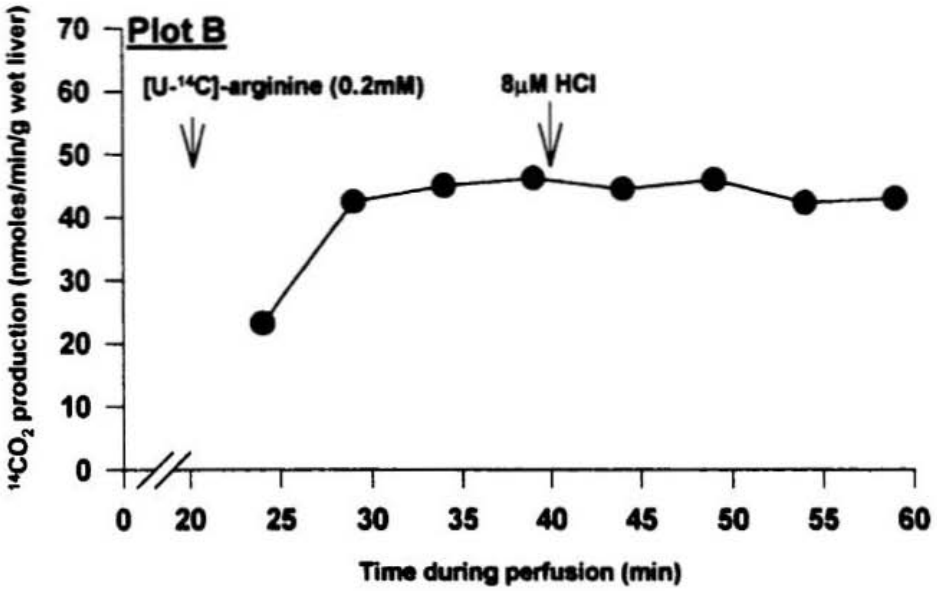
In **Panel B** we see that arginine catabolism is stimulated by glucagon; the stimulation for four independent experiments upon infusion of glucagon is 41.5 ± 7.9% (**Panel C**). This increase was statistically significant (Student's paired t-test, $P<0.05$) between the means of the two time points prior to the infusion of glucagon and the means of the 49, 54 and 59 minute time points; thus, glucagon elicits its effect within 10 minutes. Thus, in rats fed a high protein diet, the catabolism of arginine, but not that of ornithine is subject to acute regulation by glucagon. As glucagon was dissolved in 10mM HCl, control experiments in which 10mM HCl was infused (to give a final concentration in the perfusate of 8μM), under the same conditions, were also carried out. **Figure 4.8** shows that there is no difference in the catabolism of ornithine (**Plot A**) or arginine (**Plot B**) upon infusion of 10mM HCl.

The effects of dibutyryl-cAMP on the catabolism of arginine in rats fed a high protein diet

The next experiments were conducted to determine whether the effects of glucagon on arginine catabolism could be mimicked by infusion of dibutyryl-cAMP (0.1mM). **Figure 4.9 (Plot A)** shows the data from this study. The graph shows data from a typical

Figure 4.8. Controls for the effect of glucagon infusion on the catabolism of ornithine and arginine. Rats, fed a high protein diet for 3 days, were perfused antegrade with [U-^{14}C]-ornithine (0.1mM) (**Plot A**) or [U-^{14}C]-arginine (0.2mM) (**Plot B**). Perfusion procedures are described in **Materials and Methods**. Each point represents the mean for each time-point from 2 independent experiments.


Plot A
[U-14C]-ornithine (0.1mM)
8μM HCl
14CO2 production (nmoles/min/g wet liver)
100
80
60
40
20
0
0
20
25
30
35
40
45
50
55
60
Time during perfusion (min)


Plot B
[U-14C]-arginine (0.2mM)
8μM HCl
14CO2 production (nmoles/min/g wet liver)
70
60
50
40
30
20
10
0
0
20
25
30
35
40
45
50
55
60
Time during perfusion (min)

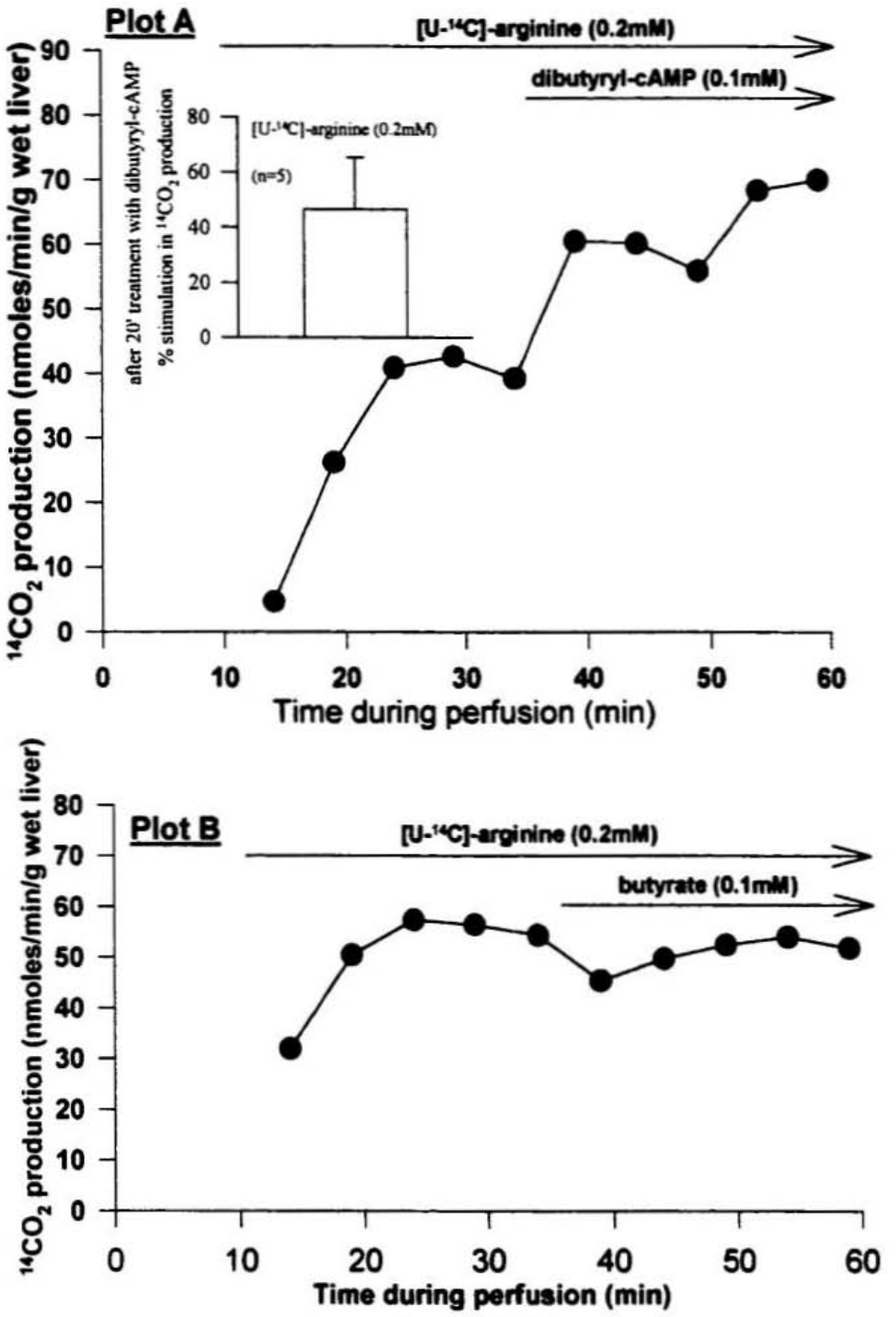
experiment in which dibutyryl-cAMP (0.1mM) was introduced at the 35 minute time-point. Dibutyryl-cAMP increases the rate of $^{14}CO_2$ production from [U-^{14}C] arginine. Statistical analysis of data from 5 independent experiments, using Student's paired t-test, showed a significant difference ($P<0.05$) in the rate of $^{14}CO_2$ production from [U-^{14}C]-arginine (0.2mM) within ten minutes of the inclusion dibutyryl-cAMP, and this difference increased steadily during the 35 minutes of treatment. After 20 minutes treatment with dibutyryl-cAMP, the mean % increase in the rate of $^{14}CO_2$ production from [U-^{14}C] arginine (the 34 minute time-point versus the 54 minute time-point) was $46.6 \pm 18.7\%$, for five independent experiments (inset). This difference was significant ($P< 0.05$, Student's paired t-test). Control perfusions were carried out in which butyrate (0.1mM) was added. These data are shown in **Plot B, Figure 4.9**. The results show that the inclusion of butyrate has no effect on the catabolism of arginine.

The localization of the site of action of glucagon on arginine catabolism

Having established that glucagon stimulates the catabolism of arginine in the perfused rat liver, experiments were designed to determine the site at which this stimulation occurs. The previous chapter showed that the catabolism of arginine could be carried out, in its entirety, in the perivenous region. Glucagon may exert its effect by:

(1) stimulating some enzyme or signal in the periportal region (e.g. arginase, or a secondary messenger), an effect which would alter the rate of $^{14}CO_2$ from [U-^{14}C] in the antegrade, but not a retrograde perfusion (see **Figure 4.10**).

Figure 4.9. The effect of dibutyryl-cAMP infusion on the catabolism of arginine. Rats, fed a high protein diet for 3 days, were perfused antegrade with [U-^{14}C]-arginine (0.2mM). Perfusion procedures are described in **Materials and Methods**. At the time indicated by the arrow, dibutyryl-cAMP (0.1mM) was introduced. A typical experiment is shown in **Plot A**. The inset shows the % stimulation in the rate of $^{14}CO_2$ production from [U-^{14}C]-arginine (expressed as the mean ± standard deviations for each time point from 5 independent experiments). **Plot B** represents data from control experiments in which butyrate (0.1mM) was introduced into the perfusate (data are expressed as the mean of two independent experiments).


Plot A
[U-14C]-arginine (0.2mM)
dibutyryl-cAMP (0.1mM)
[U-14C]-arginine (0.2mM)
(n=5)
after 20' treatment with dibutyryl-cAMP
% stimulation in 14CO2 production
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)
Plot B
[U-14C]-arginine (0.2mM)
butyrate (0.1mM)
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)

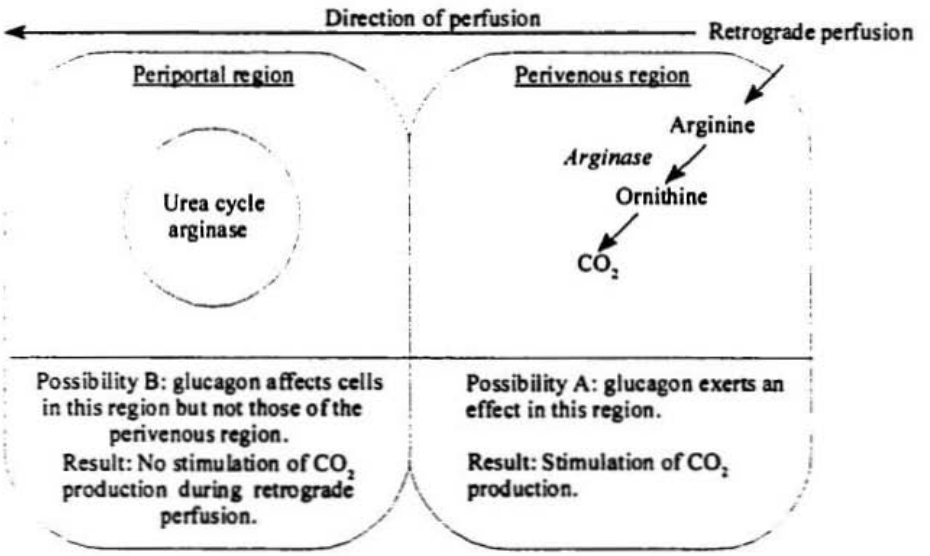


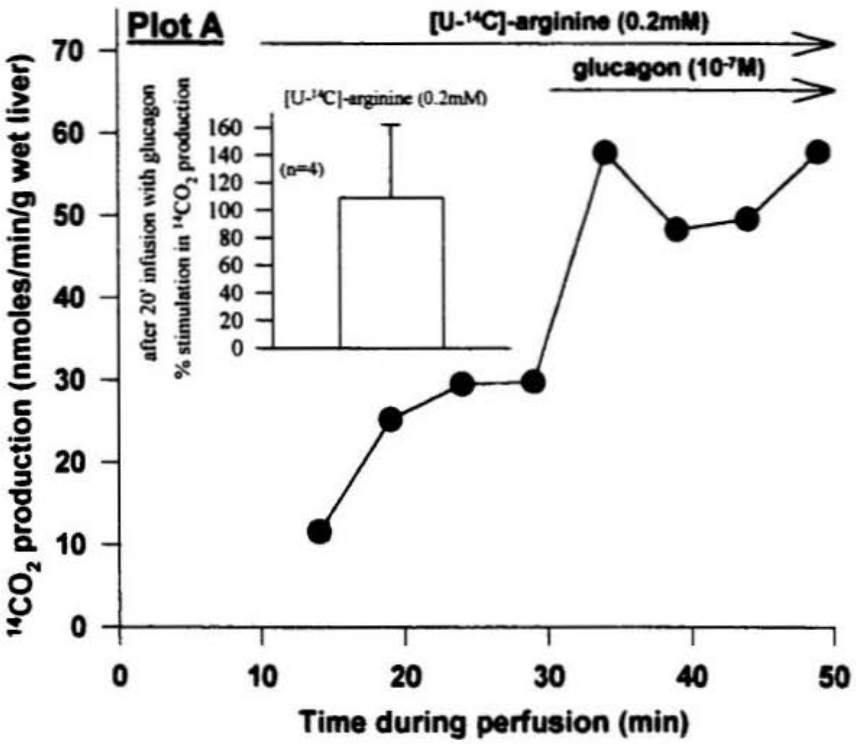
Figure 4.10. Determining the localization of the glucagon effect.

(2) by directly affecting the process in the perivenous region, which would result in. the stimulation of the $^{14}CO_2$ production from [U-^{14}C] arginine in the retrograde perfusion (see **Figure 4.10**). **Figure 4.11** shows that in a typical retrograde perfusion (**Plot A**), an infusion of glucagon (10^{-7}M) after 30 minutes in the perfusion causes an increase in the $^{14}CO_2$ production from [U-^{14}C] arginine (0.2mM) of almost 2 fold (29.8 and 57.8 nmoles CO_2/min/g wet liver at the 29 and 49 minute time-points, respectively). The inset shows the results from 4 independent experiments, and represents the mean percentage increase in the production of $^{14}CO_2$ from [U-^{14}C]-arginine from the 29 minute to the 49 minute time-point. Thus, glucagon can exert its effect directly on the perivenous region. Control experiments (**Plot B, Figure 4.11**) demonstrated that infusion of 10mM HCl to give a final concentration in the perfusate of 8μM (the vehicle for the glucagon infusion) does not alter the rate of $^{14}CO_2$ production from [U-^{14}C]-arginine (0.2mM).

The effects of insulin infusion on the catabolism of ornithine and arginine catabolism in rats fed a high protein diet

Similar experiments to those carried out to investigate the action of glucagon in antegrade perfusions were carried out using insulin. **Figure 4.12** shows data for experiments carried out with [U-^{14}C]-ornithine (**Plot A**) and [U-^{14}C]-arginine (**Plot B**). The results show that an infusion of insulin (10^{-7}M) has no effect on the rate of $^{14}CO_2$ production from either [U-^{14}C]-arginine (0.2mM) or [U-^{14}C]-ornithine (0.1mM).

Figure 4.11. The effect of glucagon infusion on the catabolism of arginine in the retrograde perfusion. Rats, fed a high protein diet for 3 days, were perfused retrograde with [U-^{14}C]-arginine (0.2mM). Perfusion procedures are described in **Materials and Methods**. Glucagon (final concentration in the perfusate 10^{-7}M) was infused at the times indicated by the arrow in **Plot A**. **Plot A** represents a typical experiment. The inset shows the % stimulation in the rates of catabolism due to glucagon (expressed as the mean ± standard deviation for each time-point from 4 independent experiments). **Plot B** represents data from control experiments in which the vehicle for glucagon (8μM HCl, final concentration in the perfusate) was added (indicated by the arrow).


Plot A
[U-14C]-arginine (0.2mM)
glucagon (10-7M)
[U-14C]-arginine (0.2mM)
(n=4)
after 20' infusion with glucagon
% stimulation in 14CO2 production
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)

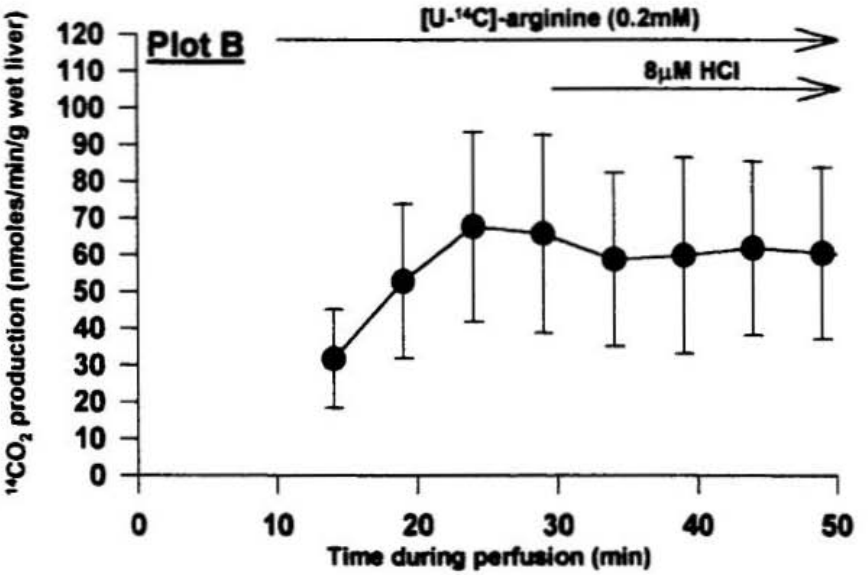
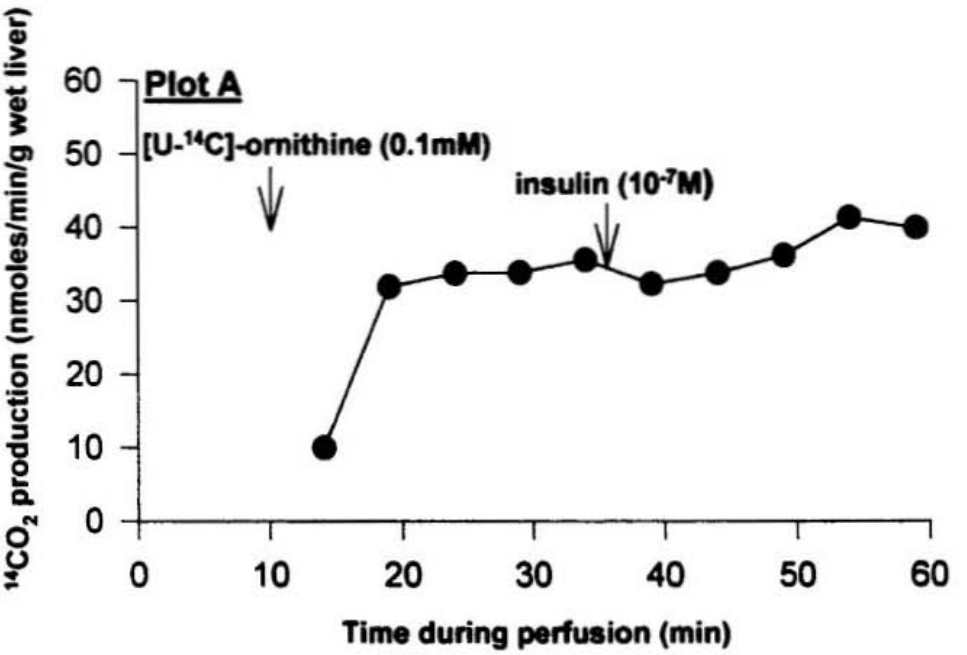
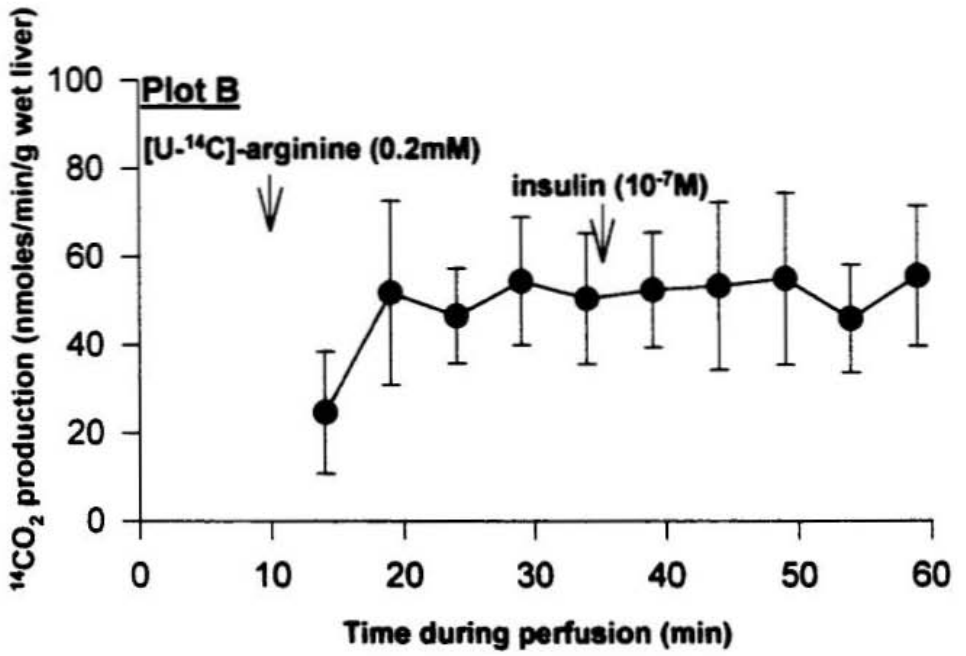

Plot B
[U-14C]-arginine (0.2mM)
8μM HCl
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)

Figure 4.12. The effect of insulin infusion on the catabolism of arginine and ornithine. Rats, fed a high protein diet for 3 days, were perfused antegrade with either [U-^{14}C]-arginine (0.2mM) or [U-^{14}C]-ornithine (0.1mM). Perfusion procedures are described in **Materials and Methods**. At the times indicated by the arrows, insulin (10^{-7}M) was introduced. **Plot A** shows the means for two independent experiments carried out with [U-^{14}C]-ornithine (0.1mM). **Plot B** shows the mean ± standard deviation for each time-point from three independent experiments carried out with [U-^{14}C]-arginine (0.2mM).


Plot A
[U-14C]-ornithine (0.1mM)
insulin (10-7M)
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)
0
10
20
30
40
50
60


Plot B
[U-14C]-arginine (0.2mM)
insulin (10-7M)
14CO2 production (nmoles/min/g wet liver)
Time during perfusion (min)
0
20
40
60
80
100
10
30
50

DISCUSSION

The results from the saturation curves show that at the physiological concentrations of these amino acids, under the chosen dietary conditions, the processes involved in the release of CO_2 from position 1 of ornithine (**Figure 4.1, Plot A**) are not saturated; the concentration at which half-maximal velocity occurs is 3.3mM. Similarly, the processes involved in the release of the entire carbon backbone of arginine (monitored using [U-^{14}C]-arginine) are not saturated at the rat portal vein concentrations of arginine under conditions of high dietary protein. The concentration at which half-maximal velocity occurs is 3.4mM (**Figure 4.1, Plot B**). In rats fed a 50% casein diet *versus* those fed a 13% casein diet, the portal vein concentration of arginine rises from 0.14mM to 0.27mM (Remesy et al, 1978). The markedly higher rate of catabolism of arginine V_{max} (697nmoles/min/g wet liver), when compared with ornithine (324nmoles/min/g wet liver), is a function of the radiolabels used rather than a higher rate of catabolism for arginine *per se*; [U-^{14}C]-arginine monitors the release from the entire carbon backbone while [1-^{14}C] ornithine monitors the carbon, released as $^{14}CO_2$, from the carbon at position 1 of ornithine. In this respect, it has been shown (**Figure 4.6**), and is discussed later, that there is a substantial oxidation of the carbon backbone of ornithine. Note that these concentrations are in the region of the estimated values for the K_m of the MCAT2A transporter in liver i.e. 2-5mM (Closs et al, 1993); it is possible that transport is the rate limiting step for both diet groups. In rats fed as much as 51% protein in the diet for a period of 15 days, rat liver arginine levels were undetectable (Colombo et al, 1992). This

is most likely due to a combination of poor supply of this amino acid to the liver and the very high levels of arginase in this organ. Possibly, under conditions of high dietary protein the spectrum of MCAT transporters differs from that present in livers from rats fed a normal protein diet. As was mentioned in the **Introduction**, the expression of the individual MCAT transporters is regulated independently. For example, in rat brain astroglial cultures, only the MCAT2B transporter responds to treatment with interferon-γ and endotoxin (Stevens et al, 1996) while the expression of MCAT2 and MCAT1 remain unchanged. An approach that could be used to good effect would be to establish the kinetics of the transport of arginine into hepatocytes under conditions of varying protein (for instance in isolated hepatic plasma membrane vesicles). A single low affinity transporter should be seen in rats fed the normal protein diet (MCAT2A). The induction of other transporters (in this case of higher affinity; MCAT2B and MCAT1) would be evident by Eadie-Hoffstee analysis. This type of analysis was not possible in the case of the data reported here, as the entire process of the catabolism of arginine was measured and not the individual steps. Alternatively, cDNA probes for the transporters could be used to determine their expression under different dietary conditions.

The dependence of these catabolic processes upon OAT is demonstrated in **Figure 4.2**. As was the case in rats fed a normal protein diet, there is a marked reduction in the rate of $^{14}CO_2$ production from [1-^{14}C] ornithine (0.1mM) and [U-^{14}C] arginine (0.2mM). Any residual production of $^{14}CO_2$ occurs as a result of the remaining OAT activity, CO_2

production from ornithine decarboxylase (rate limiting enzyme in polyamine synthesis), and, in the case of arginine catabolism, in agmatine synthesis. It means that in rats fed a high protein diet, the major route for the disposal of arginine and ornithine occurs *via* the enzyme OAT and , therefore through the pathway outlined in **Figure 1.3** (**Introduction**).

Comparison of the rates of CO_2 production from ornithine (**Figure 4.3**) show a marked increase in rats fed a high protein diet, when compared to rats fed a normal protein diet. There is a direct proportionality between the % protein in the diet and the rates of $^{14}CO_2$ production from ornithine; a fourfold increase in the % of dietary protein (15%-60%) caused a fourfold increase in the rates of $^{14}CO_2$ production from ornithine. The rate of $^{14}CO_2$ production from arginine is also chronically regulated by the level of dietary protein. **Figure 4.4** shows that the rates are increased by over threefold in the rats fed a high protein diet. Schimke showed a similar linear relationship between the level of dietary protein and the enzymes of the urea cycle, including arginase (Schimke, 1962). **Figure 4.5** shows that the increase in dietary protein also increases urea production and OAT activity. These results are in agreement with previous studies (Schimke, 1962; Volpe et al, 1969).

As outlined in the **Introduction (Chapter 1)**, the catabolic processes are the ones that have been reported to fluctuate under conditions of limiting arginine. This is the case for both humans (Castillo et al, 1994) and rats (Dhanakhoti et al, 1990). The results

presented here show that decreasing the level of dietary protein (and, thus, arginine) results in a reduced capacity to catabolize this amino acid in the rat liver. Therefore, rat liver can contribute to maintaining arginine levels under conditions in which the supply of this amino acid is reduced.

Although these studies do show altered rates of catabolism, they do not show the site(s) at which these changes are brought about. We may speculate that changes may be effected through (1) increased enzyme activity (of one of the enzymes shown in **Figure 1.3, Introduction**) or (2) increase in the y^+ transporter activity. In relation to enzyme activity, it is known that both P5C-dehydrogenase and OAT are subject to induction upon feeding a high protein diet. A previous study showed that OAT activities in rats fed a 15% protein diet *versus* those fed a 60% casein diet were 2.08 μmoles/min/g liver protein and 12.17 μmoles/min/g liver protein, respectively (Volpe et al, 1969). Our results show a similar induction of OAT. With respect to the P5C-dehydrogenase activities, feeding rats diets containing 70% protein versus 5% protein, for a period of a week, results in a 3 fold increase P5C-dehydrogenase activity (Matzuzawa et al, 1994). Arginase is induced by increasing dietary protein (Schimke, 1962); it is not known whether the arginase AII is subject to such stimulation.

With respect to transport, feeding specialized diets enriched in arginine and glutamine, for a period of 3 days, has been shown to increase the y^+ transporter activity in isolated

hepatic plasma membrane vesicles by as much as fourfold (Espat et al, 1996). As mentioned in **Chapter 1** feeding a high protein diet over a period of days is known to increase the circulating levels of glucagon (Peret et al,1981) and the glucagon/insulin ratio. The change in rate of catabolism may be brought about indirectly by the action of glucagon. In rats, an i.p. injection of glucagon (2mg/100g body weight) increases the activity of the y^+ transporter activity in primary hepatocyte cultures isolated from these rats (Handlogten and Kilberg, 1984).

As a result of the availability of the cDNAs for the MCAT transporters (MCAT1, 2A and 2B), arginase AI and AII, OAT and P5C-dehydrogenase, the effects of feeding rats different diets on the level of transcription of these enzymes (northern blotting) within the liver could be established. Monitoring the expression levels of the individual proteins (immunohistochemistry) and their activity (enzymatic analyses) would have to be carried in conjunction with the mRNA *in situ* hybridization study. This type of study, if carried out, would show which enzymes are changing and at which step they are changing (possibilities exist for change at a number of levels e.g. transcription, translation or post-translational etc.).

In **Figure 4.6** we see that a substantial catabolism of the carbon backbone of ornithine occurs. This result can be interpreted as in **Chapter 3** to mean that the enzymes PEPCK/pyruvate kinase and/or malic enzyme occur in the same site as the catabolic

processes of ornithine to allow removal of certain Krebs cycle intermediates, and their subsequent reintroduction as pyruvate allowing further catabolism of the carbon backbone of this molecule (see **Discussion, Chapter 3**).

Pancreatic hormones, such as glucagon (produced by the α-cells) and insulin (synthesized in the B-cells), are secreted into the hepatic portal vein bloodstream, reach the liver and are known to affect a number of metabolic processes within this organ. Glucagon regulates a number of metabolic processes in amino acid metabolism. For instance, glucagon is known to stimulate acutely flux through the glycine cleavage system *in vitro* in isolated rat hepatocytes (Jois et al, 1989). Other experiments in this study involved administration of glucagon to rats (0.1mg/100g body weight delivered by i.p. injection 25 minutes prior to sacrifice) and subsequent isolation of liver mitochondria. The results showed that the glycine cleavage system was activated, relative to saline injected control rats, in these mitochondria. No attempt was made in these experiments to maintain the phosphorylation state, in fact mitochondria were washed three times. This is an interesting example of a short-term regulatory process that persists for some time in the absence of the initial stimulus.

In many metabolic situations, glucagon's action is opposite to that of insulin. After a typical carbohydrate-containing meal the plasma insulin concentration is high while the plasma glucagon concentration is low. Insulin is responsible for activating those

processes responsible for the storage of fuels in times of excess. In the fasted state the levels of insulin are low while those of glucagon are high. Glucagon activates the processes concerned with the mobilization and utilization of fuel in order to maintain an energy supply. **Figure 4.7** shows that the production of $^{14}CO_2$ from [U-^{14}C]-arginine is subject to acute regulation (within a matter of minutes) by administration of glucagon. Thus, the arginine catabolic rate can respond in the short-term to fluctuations in glucagon. *In vivo* the level of arginine catabolism in the rat liver may alter from minute to minute accordingly as the levels of glucagon change. These experiments were not designed to determine the location of the site(s) at which the alteration is effected. However, the fact that there is no such stimulation in the production of $^{14}CO_2$ from [U-^{14}C]-ornithine suggests that regulation by glucagon may occur at the level of the arginase. This assuming that the transport of arginine and ornithine into hepatocytes and mitochondria occurs *via* common transporters. As the pathway outlined in **Figure 1.3** (**Introduction**) shows, the catabolism of arginine through OAT differs from that of ornithine at one step i.e. the step involving arginase. As previously stated in **Chapter 3**, it remains to be established which arginase isozyme is involved in catabolism of arginine through OAT. Experiments were carried out to study the effects of glucagon and insulin in rats which were fed a normal protein diet (15% casein) but persistent problems were faced with rats on this diet (i.e. rats developed fatty livers); eventually, these studies had to be abandoned.

In many cases the effects of glucagon are exerted through an increase in the second messenger cAMP. This cAMP can in turn stimulate cAMP-dependent protein kinases to phosphorylate various target enzymes, which in turn can increase or decrease the activity of these enzymes. The experiments represented in **Figure 4.9** were carried out to determine whether increased cAMP (provided as dibutyryl-cAMP (0.1mM)) could stimulate CO_2 production from arginine. The increased catabolism (which is of a magnitude similar to that caused by glucagon) shows that glucagon may be increasing the catabolism of arginine by increasing intracellular cAMP.

The next piece of work using retrograde perfusions showed that the stimulation of arginine catabolism caused by glucagon occurs in the perivenous cells of the liver **Figure 4.11**. The results suggest the presence of glucagon receptors in the cells of the perivenous region. Further studies in this area would best be carried out with preparations of perivenous cells. The procedures to separate the perivenous cells from the periportal vary from selective cell damage of cells from one region to isolation on gradients (Jungermann and Katz, 1989); the key problem lies with the consistency of the preparations. Another potential problem is the phenomenon of "dynamic zonation" (discussed in the **Introduction**). Currently, marker enzymes are used to determine the enrichment of a cell preparation in either perivenous or periportal cells. These are enzymes which are known to be preferentially, or exclusively expressed, in either the perivenous region or the periportal region. It must be ensured that the under the

conditions of a given experiment the marker enzyme itself does not alter its expression within the different regions e.g. the processes for long chain fatty acid oxidation switch from being primarily periportal to being preferentially carried out in the perivenous region when the rats are subjected to cold exposure (Guzman et al, 1995).

CHAPTER 5

SUMMARY AND GENERAL DISCUSSION

SUMMARY

1. In the nonrecirculating isolated rat liver perfusion, the majority of the catabolism of arginine and ornithine is carried out through the metabolic pathway involving ornithine aminotransferase (OAT). This is true for rats fed either the normal protein diet (15% casein) or the high protein diet (60% casein).

2. The rates of catabolism are arginine are the same in rat livers whether they are perfused in the antegrade of retrograde direction. Thus, the catabolism of arginine through OAT, in the nonrecirculating isolated perfused rat liver, can be carried out in its entirety in the perivenous hepatocytes. This in turn means that there is a perivenous arginase.

3. There is substantial catabolism of the backbone of ornithine. This was shown to be the case whether the rats were fed normal or hihg protein diets. Experiments indicate complete catbolism of the ornithine backbone; this requires the presence of either malic enzyme or a combination of phosphoenolpyruvate carboxykinase and pyruvate kinase in the perivenous hepatocytes.

4. The rates of catabolism of arginine and ornithine were stimulated in nonrecirculating isolated rat liver perfusions in rats fed a high protein diet (60% casein) when compared with rates in animals fed a nornmal protein diet (15% casein).

5. Glucagon, but not insulin, stimulated the rate of catabolism of arginine in isolated nonrecirculating rat liver perfusions. This stimulation (50% increase when compared with controls) occurred within ten minutes of the introduction of the hormone. Glucagon did not affect the rate of ornithine catabolism. Retrograde liver perfusions showed that the glucagon effect does not require the participation of the periportal region; contact of the hormone with the perivenous cells is all that is required for stimulation to occur. The effect of glucagon on arginine catabolism could be mimicked by inclusion of dibuyryl-cAMP in the perfusion medium showing the second messenger cAMP can elicit this effect.

GENERAL DISCUSSION

The liver is important to the catabolism of excess (excess over that required for protein synthesis) amino acids apart from the branched chain amino acids. The liver is the first organ to encounter the amino acids as they enter the circulation from the intestine and so palys a key rôle in determining amino acid levels throghout the body. With respect to arginine and ornithine, it is the catabolic processes that regulate arginine homeostasis (Castillo et al, 1994a; Castillo et al, 1994b; Dhanakoti et al, 1990). Thus, a fuller understanding of these processes will aid in the deveopment of plans for maintaining adequate nutritional status. Achieving this understanding has been the aim of this thesis. There are however a number of question that remain unanswered and these are addressed in the following discussion.

Having established whether or not a process is retricted to a particular part of the liver, the question arises as to how this localization is achieved. With recent advances in moleculr techniques, genetic sequences (including promoter regions) are known for many enzymes and transporters. Closer inspection of the sequences indicate commom genetic elements, both in promoter regions, within the coding sequence itself, among genes whose protein products are localized to the same cells of the liver. Furthermore, the inclusion of certain genetic elements into sequences can direct the expression of the gene product (the protein) to a particular set of cells within the liver. Sequence elements from the glutamine synthetase gene (including promoter region) can direct expression of the

chloramphenicol-acetyltransferase gene to the same set of perivenous cells as the glutamine synthetase (Lie-Venema, 1995). In the case of our study the distribution of P5C-dehydrogense needs to be established. In addition, the arginase present in the perivenous cells has to be fully characterized (with respect to the isozyme type, sequence etc.), and its localization established. If these enzymes are specifally localized then they may share common genetic elements that can direct their expression. Also, the distribution of the MCAT transporters across the liver reamins unknown; as the members of this group differ in their affinity for arginine, a particular transporter(s) present in the perivenous cells will affect the rate of supply of this amino acid, abd possibly the rate of catabolism.

The localization of different metabolic processes within the same cells also makes competition for substrates and intermediates between these more likely. For instance, arginine is the sole substate for the NO synthases. If NO bisynthesis occurs in the perivenous cells (NO production occurs in hepatocyte cultures which are treated with combination of insulin and glucagon to produce cells with perivenous attributes (Ohno et al, 1995)) then it is possible that affecting the catabolism of arginine through OAT may also effect the rate of NO biosynthesis in these cells. This would be the case if they draw from the same pool of arginine and the K_ms of the enzymes for arginine as substare were not appreciably different. In addition, L-OH-arginine (an intermediate of the NO biosynthetic pathway is the most potent arginase inhibitor known (Boucher et al, 1994)).

Thus, NO biosynthesis may be able to switch off arginase in the perivenous cells, and thus arginine catabolism, without affecting the urea cycle. NO is a potent radiacl capable of causing significant cellular damage. The possibility exists that the process characterized herein plays an important role in controlling the NO production in the perivenous cells. Isolation of pure cell preparations of perivenous cells would allow a study that could determine whethr the ate of catabolism of arginine affect NO biosynthesis.

The details of the mechanism(s) at play in the stimulation of arginine and ornithine catabolism caused by increasing dietary protein have yet to be fully elucidated. Previous studies provide us with some plausible mechanisms. Schimke (1962) studied the increases in the urea cycle enzymes with increased dietary protein and found a direct, proportional, and coordinated increase in the levels of the urea cycle enzymes with dietary protein. Several hepatic amino acid catabolizing enzymes increase in concentration in animals fed chronically with high protein diets (Krebs, 1972). To increase the rate of catabolism it is necessary to stimulate an enzyme or transporter which is rate-limiting for the process. Increasing the activity of those enzymes whose forward and abckward reaction rates are kept far from equilibrium (the irreversible enzymes) is the most effective way of regulating a metabolic process. The first reaction catalyzed by an irreversible enzyme which is involved in both arginine and ornithine catabolism (see **Figure 1.3**) is the conversion of pyrroline-5-carboxylate to glutamate catalyzed by P5C-

dehydrogenase. It is known that P5C-dehydrogenase is elevated in livers from rats chronically fed a high protein diet (Matsuzawa et al, 1994); this is a alikely target site for regulation. Transport may also be rate-limiting for this process. As mentioned earlier, the specific transpoters fro arginine in the perivenous cells have yet to be determined. Once this is known it would be possible to carry out northern blotting experiments to determine the rates of transcription of the relevant transporter genes in livers from rats fed differing amounts of dietary protein. These studies would need to be complemented by a determination of the level of the protein product (the transporter protein itself). It should be mentioned that feeding high protein diets increases the activity of the rat liver branched chain oxo-acid dehydrogense without increasing the level of this enzyme (Miller et al, 1998). This is a type of covalent modification, a reversible phosphorylation-dephosphorylation mechanism. Thus, increased enzyme amount is not the only way of regulating enzymes of amino acid metabolism.

With respect to the effects of glucagon on the catabolim of arginine the mechanism of signal transduction remains unknown. We have shown that the glucagon effect can be mimicked by cAMP but this does not preclude the possibility that another classical 'secondary messenger' is involved. For example, it is known that both glucagon and cAMP can increase cytoplasmic calcium levels (Charest et al, 1983), so it could be that these effects are caused by calcium. Cahnges in cell volume caused by glucagon may also be responsible for regulating metabolism (Häussinger et al, 1997). Routine isolation

of pure preparations of perivenous cells would be of benefit to future studies which investigate this glucagon effect.

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APPENDIX A.

THE OXIDATION OF PROLINE IN ISOLATED RAT LIVER MITOCHONDRIA

INTRODUCTION

In addition to the studies concerning the catabolism of arginine and ornithine, a smaller project was undertaken to determine whether proline oxidation was affected by various dietary and hormonal stimuli. It has been shown, in isolated liver mitochondria, that the oxidation of glycine, *via* the glycine cleavage system, is stimulated by intraperitoneal administration of glucagon to rats (Jois et al, 1992). The glucagon-mediated activation of the glycine cleavage system is long-lived in that the activation persists in mitochondria isolated 25 minutes subsequent to the glucagon administration. Also, glutaminase activity is stimulated in isolated rat liver mitochondria by feeding rats a single meal which is high in protein (60% casein) after a 3-4 day period of feeding a 15% casein diet (Ewart and Brosnan, 1993). Circulating levels of glucagon increase in rat plasma after a high protein meal (Robinson et al, 1981) and this is thought to be a key stimulus. *In vitro*, incubation of mitochondria in a hypotonic medium (hormones may elicit responses through increases in the mitochondrial matrix volume (see Halestrap, 1989; Häussinger et al, 1997)) increases the flux through the glycine cleavage system (Jois et al, 1992). Proline oxidation, like glutaminase and the glycine cleavage system, occurs within mitochondria (the steps involved in its oxidation are outlined in **Figure A1**). Feeding rats a high protein diet over a period of days also results in the stimulation of hepatic processes such as the urea cycle (Schimke, 1962). Whether or not proline oxidation in isolated rat liver mitochondria is subject to similar dietary and hormonal regulation is not known. To determine proline oxidation rates, we measured the

PROLINE OXIDATION IN RAT LIVER MITOCHONDRIA

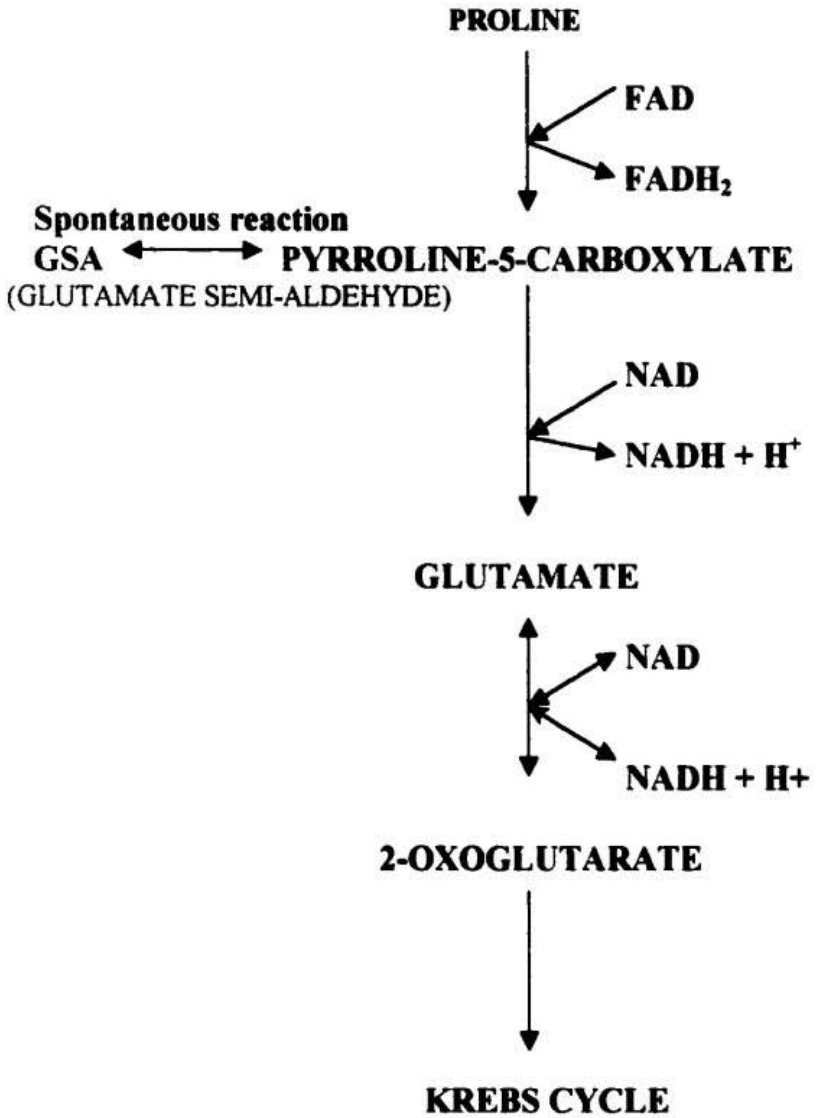


Figure A1. The metabolic pathway for the oxidation of proline.

oxygen consumption (in a Clark oxygen electrode) in isolated rat liver mitochondria, under state 3 conditions, with proline serving as the oxidizable substrate.

OBJECTIVES

(1) To determine whether the oxidation of proline, in liver mitochondria isolated from rats which were administered an i.p. injection of glucagon, is regulated by this hormone.

(2) To determine the effects of feeding rats a single high protein meal on the oxidation of proline in isolated rat liver mitochondria.

(3) To determine whether feeding rats a high protein diet over a period of a week affects proline oxidation in isolated rat liver mitochondria.

(4) To determine the effect on proline oxidation of incubating isolated rat liver mitochondria in a hypotonic medium.

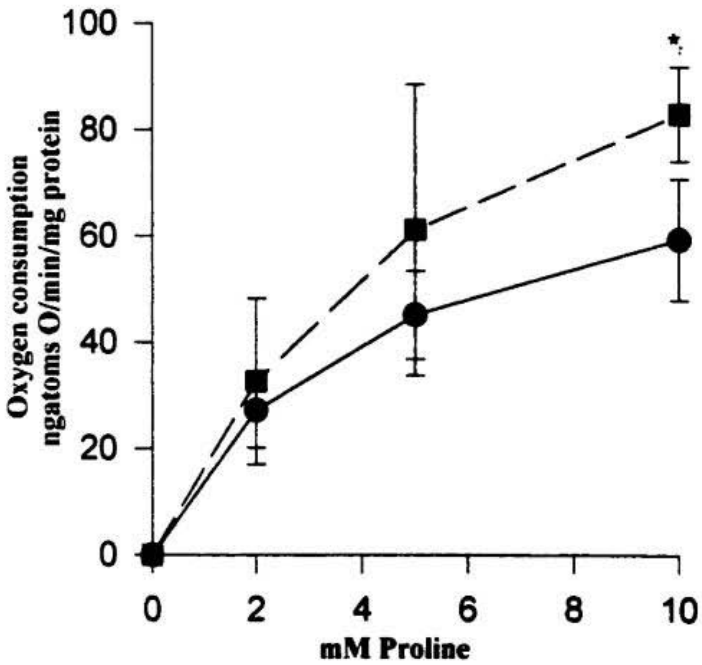
MATERIALS AND METHODS

These are as described in full in **Chapter 2** of this thesis.

RESULTS

Figure A2 shows the effect on proline oxidation of administering an i.p. injection of glucagon to rats (1mg/kg) 25 minutes prior to beginning the isolation of liver mitochondria. Only at the highest concentration of proline (10mM) is there a statistically significant effect of glucagon on the oxygen consumption due to the addition

Figure A2. The effect of intraperitoneal glucagon injection on proline oxidation. Glucagon was administered as described in **Materials and Methods**. Rats were fed a normal protein diet (15% casein) for 7 days. Each point in the upper plot represents the means ± the standard deviation for 4 independent experiments using glucagon treated rats (■) and saline treated controls (●). Control experiments in which flux through glycine cleavage system was assayed are shown below the plot (see Materials and Methods for details). The data represent the mean ± standard deviation from four independent experiments. * denotes a statistically significant difference ($P < 0.05$) from the control group, determined by Student's unpaired t-test.



GLYCINE OXIDATION IN ISOLATED RAT LIVER MITOCHONDRIA:

	nmol/min/mg/protein
CONTROL	**0.18 +/- 0.04**
GLUCAGON TREATED	**0.46 +/- 0.08***

of proline (59.4 ± 11.4 and 83.1 ± 8.8 ngatoms O/min/mg protein for controls and glucagon treated rats, respectively). The effects shown on the glycine cleavage system are shown in the table in **Figure A2**; glucagon administration results in an almost threefold increase in the activity at physiological concentrations of this amino acid (in agreement with previous studies carried out in this laboratory). Thus, glucagon has only a minor effect on the oxidation of proline in isolated rat liver mitochondria.

Feeding rats a high protein diet (60% casein) results in approximately a 2 fold increase in proline oxidation at all proline concentrations tested (**Figure A3**). This effect could occur through increased concentration of one of the enzymes involved. It is known that proline oxidase is increased in livers from rats fed a high protein diet (Kawabata et al, 1980). Increased P5C-dehydrogenase could be responsible as this activity increases in livers from rats fed high protein diets *versus* low protein diets (Matsuzawa et al, 1994). Both of these enzymes catalyze irreversible reactions and so could be rate limiting steps for this process. However, in isolated rat liver cells the oxidation through glutamate has been shown to be limiting for the oxidation of proline (Hensgens et al, 1978). In this respect regulation of α-keto-glutarate dehydrogenase (EC 1.2.4.2) by raised circulating levels of glucagon could be responsible for the stimulation.

Figure A4 demonstrates that feeding rats a high protein meal (60% casein), *versus* a normal protein meal (15% casein), does not alter the rate of proline oxidation in isolated

Figure A3. The effects of dietary protein on proline oxidation. Rats were fed either a high protein diet (■) (60%casein) or a normal protein diet (●) (15% casein) for a period of a week. Data represent the mean ± standard deviation from 4 independent experiments. * denotes a statistically significant difference ($P<0.05$) from the normal protein group.

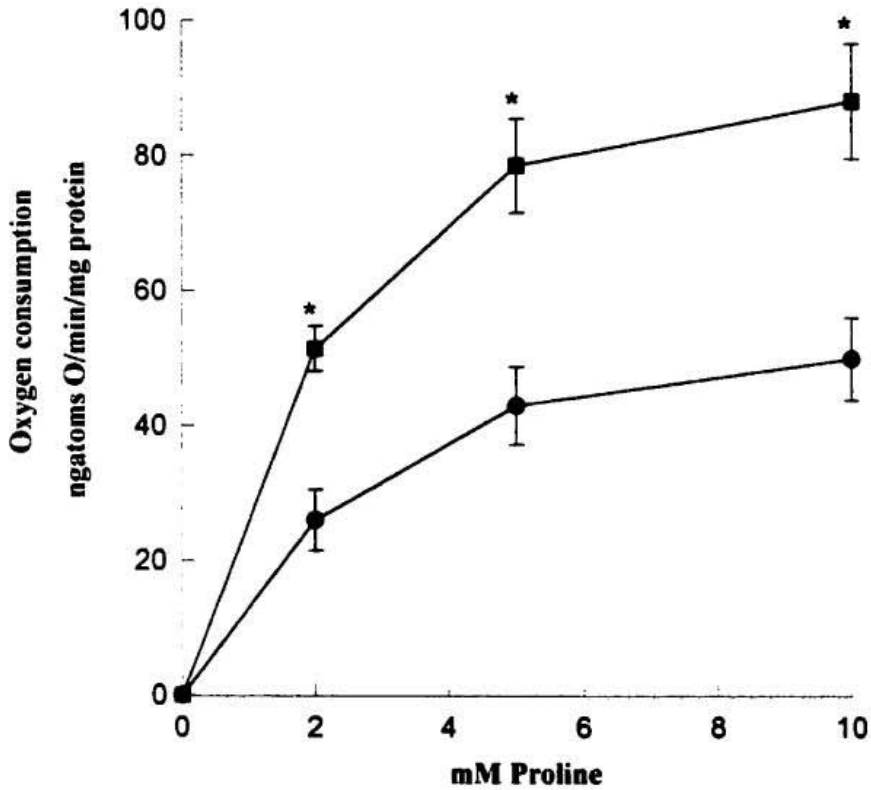
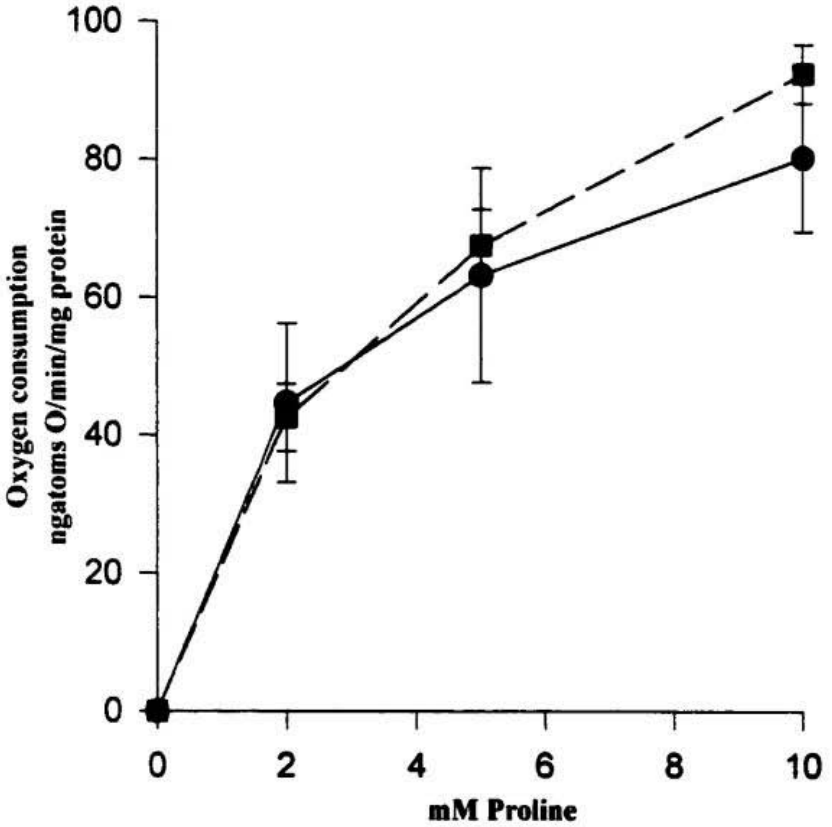

Oxygen consumption
ngatoms O/min/mg protein
100
80
60
40
20
0
0
2
4
6
8
10
mM Proline
*
*
*

Figure A4. The effects of a single high protein meal on the oxidation of proline. Experimental details are provided in **Materials and Methods**. Rats were fed either a single high protein meal (■) or a single normal protein meal (●). Each point on the plot represents the mean ± standard deviation from 4 independent experiments. The corresponding data for flux through the glycine cleavage system are shown in the lower section of the diagram. * denotes a statistically significant difference ($P<0.05$) from the normal protein group, determined by Student's unpaired t-test.



GLYCINE OXIDATION IN ISOLATED RAT LIVER MITOCHONDRIA :	nmol/min/mg protein
NORMAL PROTEIN	0.13 +/- 0.02
HIGH PROTEIN	0.45 +/- 0.06*

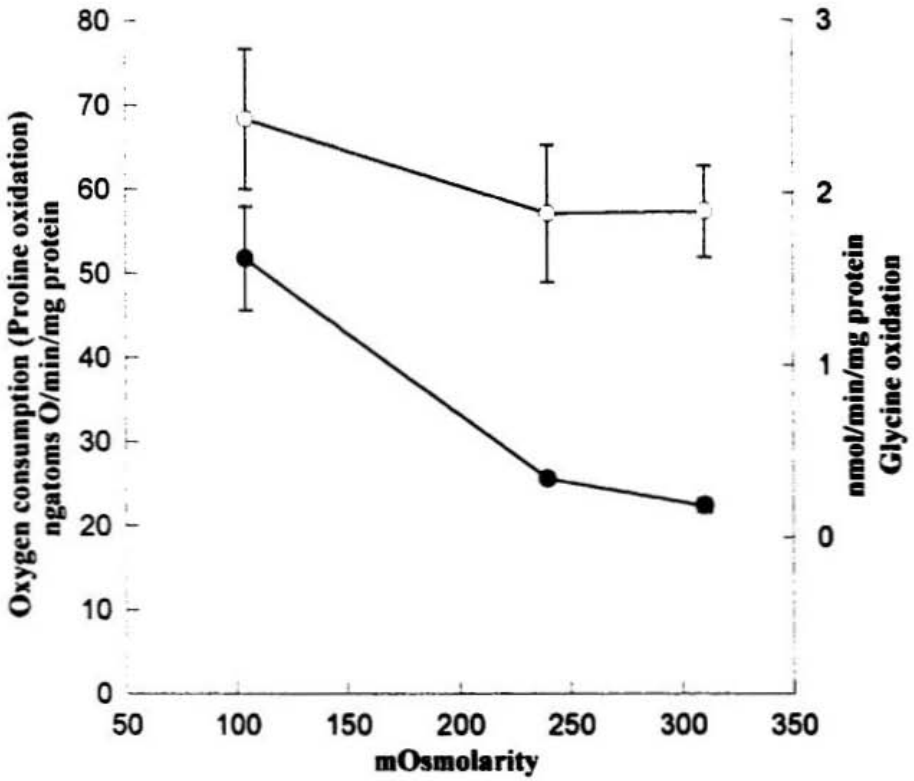
This contrasts with glycine oxidation; flux through the glycine cleavage enzyme is increased by 3 fold in these experiments (in agreement with previous results from this laboratory (Jois et al, 1992)).

Finally, **Figure A5** demonstrates that there is no effect *in vitro* of incubating rat liver mitochondria in a hypotonic medium, whereas glycine oxidation is stimulated in a hypotonic medium almost 9 fold (0.19 ± 0.04 and 1.62 ± 0.30 nmoles/min/mg protein for the 310 mOsmol and 100mOsmol media, respectively).

CONCLUSION

We conclude that proline oxidation (as measured by oxygen consumption under state 3 conditions) is affected in a minor way by glucagon treatment of the animals. Incubating mitochondria in hypotonic media or feeding a singl high protei meal has no effect on proline oxidation. This is in marked contrast to the mitochondrial oxidation of glutamine and glycine (Ewart and Brosnan, 1993; Jois et al, 1992) Chronic feeding of rats with a high protein diet does increase the oxidation of proline in isolated rat liver mitochondria. Our studies show that there is a slow adaptation (presumably by enzyme induction) of proline oxidation on feeding a high protein diet, but provides little support for the acute regulation that is evident in the catabolism of glutamine and glycine.

Figure A5. The effect of hypotonicity on the oxidation of proline. Isolated rat liver mitochondria were incubated in media of varying osmolarity (see **Materials and Methods**). (●) proline oxidation experiments, (○) corresponding flux through glycine cleavage system experiments. Each point represents the mean ± standard deviation from 4 independent experiments.


Oxygen consumption (Proline oxidation)
ngatoms O/min/mg protein
nmol/min/mg protein
Glycine oxidation
mOsmolarity
80
70
60
50
40
30
20
10
0
3
2
1
0
50
100
150
200
250
300
350