RENAL ARGININE METABOLISM

SRINIVAS NAGARAJ DHANAKOTI

RENAL ARGININE METABOLISM

by

© Srinivas Nagaraj Dhanakoti

A thesis submitted to the School of Graduate
Studies in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy

Department of Biochemistry
Memorial University of Newfoundland
July 1991

St. John's Newfoundland

ISBN 0-315-68253-1

DEDICATED TO MY LATE FATHER

NAGARAJ SRINIVASAIH DHANAKOTI

ABSTRACT

Kidneys of normal animals remove citrulline from blood and convert it, stoichiometrically to arginine. This citrulline arises from the intestinal metabolism of glutamine. This intestinal-renal pathway constitutes the major endogenous source of arginine. The objectives of the present studies are to determine the location of arginine synthesis in kidney and its response to citrulline concentrations *in vitro* and *in vivo* and to different arginine or protein intakes in rats.

Investigations on the localization of enzymes of arginine synthesis (argininosuccinate synthetase and argininosuccinate lyase) and of breakdown (arginase and ornithine aminotransferase), revealed that the enzymes of arginine synthesis are exclusively present in the cytosol of the cells of the proximal convoluted tubule and that of arginine degradation are enriched in other kidney regions.

Arginine synthesis from citrulline in isolated kidney cortical tubules was found to be highly sensitive to citrulline concentrations in the physiological plasma range (0.06 mM), suggesting that renal arginine synthesis *in vivo* could be regulated by circulating citrulline levels. Thus, in studies on renal arginine synthesis *in vivo*, it was found that kidneys of rats infused with citrulline (saline-infused, as controls) responded to the elevated plasma citrulline levels by increasing its uptake and producing increased quantities of arginine.

In studies with rats fed different levels of arginine (0.0, 0.5, 2.0%) or protein (5, 12 and 50%) for 1 week, it was found that the renal uptake of citrulline and release of arginine and also circulating citrulline levels were similar in all these animals. This suggested that renal arginine synthesis is independent of dietary arginine or protein intake. The results suggest that availability of citrulline is a limiting factor for renal arginine synthesis in rats.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. J. T. Brosnan for his supervision, guidance and encouragement throughout the course of this study. I would also like to thank Drs. G.R. Herzberg and M.E. Brosnan for acting both as co-supervisors and members of supervisory committee and for their helpful discussions and support.

Sincere thanks to Mr. Doug Hall and Mrs. Sonia Banfield for performing amino acid analysis. Thanks are due to Mrs. Bea Hall, Steve Ewart, Dalong Qian, Steve Squires, Susan Skanes, Vaughn Collett, Paul Warren, Dave Hall, Steve Day and Jane Qian for creating an excellent atmosphere in the laboratory.

A special thank you to Dr. Markandeya Jois and his family for their support and encouragement.

I would like to thank Dr. K.M.W. Keough (Deptartment Head) for giving me an opportunity to demonstrate biochemistry labs. Thanks are due to all members of the Biochemistry Department for creating a very freindly atmosphere.

I would like to thank Ms. Jackie Dalton and Ms. Betty-Ann Williams for typing the manuscript.

I would also like to thank my family members in India for their support and encouragement during the course of my studies.

I would like to thank the Dean of Graduate Studies for the financial assistance in the form of fellowship.

TABLE OF CONTENTS

Page

Abstract i

Acknowledgements..... iii

List of Tables..... vii

List of Figures..... ix

Chapter 1 Introduction..... 1

Arginine requirements in different species..... 2
Arginine requirements in physiological conditions.... 8
Species which do not require dietary arginine for growth or maintenance of nitrogen balance..... 9
Beneficial effects of arginine in different pathological conditions..... 10
Biochemical functions of arginine..... 17
Need for endogenous arginine synthesis..... 20
Minor drain on endogenous arginine pools..... 21
Enzymes involved in arginine utilization..... 22
Transport of arginine..... 28
Role of different organs and the importance of endogenous arginine synthesis..... 29
Sources of circulating citrulline..... 31
Importance of intestinal citrulline synthesis..... 34
What is known about renal arginine synthesis?..... 35
Unanswered questions..... 36
Objectives..... 36

Chapter 2 Materials and Methods..... 38

Animals..... 38
Diets and feeding protocol..... 38
Preparation of homogenates from different kidney regions..... 38
Preparation of cortex homogenate and subcellular fractionation..... 39
Preparation of kidney cortical tubule fractions..... 41
Fractionation of kidney cortical tubules on a Percoll^{R} gradient..... 43
Arginine production in kidney cortical tubules and its fractions..... 45
Determination of arginine by high-pressure liquid chromatography..... 46
Renal flux of citrulline and arginine (in vivo studies)..... 47

Enzyme assays..... 54
Protein and DNA measurement..... 61
Chemicals..... 61

Page

Calculations..... 61
Statistical analysis..... 62

Chapter 3 Cellular and subcellular localization of enzymes of arginine metabolism in rat kidney..... 64

Synopsis..... 65
Introduction..... 66
Objectives..... 67
Results..... 67
Distribution of enzymes of arginine metabolism in different kidney regions..... 67
Distribution of enzymes of arginine metabolism in kidney cortical tubule fractions..... 74
Arginine production in kidney cortical tubule fractions..... 80
Subcellular localization of enzymes of arginine synthesis in kidney cortex..... 80
Discussion..... 84
Concluding remarks..... 87

Chapter 4 Renal arginine synthesis: studies *in vitro* and *in vivo*..... 88

Synopsis..... 89
Introduction..... 89
Objectives..... 90
Arginine synthesis in isolated cortical tubules..... 91
Arginine synthesis as a function of time and tubule amount..... 91
Metabolism of newly synthesized arginine..... 92
Arginine synthesis in the presence of various substrates..... 95
Dependence on citrulline concentration..... 95
Arginine synthesis *in vivo*..... 99
Discussion..... 107

Chapter 5 Is renal arginine synthesis affected by dietary arginine or dietary protein intake?..... 112

Synopsis..... 113
Introduction..... 113
Objectives..... 115
Experimental protocol 115
Diets and feeding protocol 116
Results..... 119
Varying dietary arginine..... 119
Varying dietary protein..... 121
Discussion..... 128

Page

Chapter 6 Summary and general Discussion..... 135

Summary..... 136

General Discussion..... 137

References..... 143

Appendix A..... 157

LIST OF TABLES

		Page
Table 1.1	Arginine requirements for growth and minimal orotate excretion in different species.....	3
Table 1.2	Beneficial effects of arginine in different pathological conditions.....	11
Table 1.3	Biochemical functions of arginine.....	18
Table 1.4	Enzymes and reactions contributing to arginine removal.....	23
Table 1.5	Distribution of enzymes of arginine metabolism in adult male rat tissues.....	24
Table 1.6	Enzymes and reactions of net arginine synthesis..	30
Table 2.1	Plateau for ^{14}C-inulin and citrulline.....	52
Table 2.2	Recovery (%) of added citrulline, arginine and ornithine in whole blood or plasma.....	53
Table 2.3	Assay conditions for marker enzymes and enzymes of arginine metabolism.....	55
Table 3.1	Distribution of enzymes of arginine metabolism in different regions of the rat kidney.....	72
Table 3.2	Distribution of enzymes of arginine metabolism in rat kidney cortical tubule fractions.....	76
Table 4.1	Arginine production in kidney cortical tubules in the presence of various substrates.....	97
Table 4.2	Arteriovenous differences across the kidney in blood, plasma and blood cells for citrulline and arginine in normal and citrulline-infused rats..	100
Table 4.3	Basic physiological data for saline- and citrulline-infused rats.....	102
Table 4.4	Arterial plasma levels and net renal flux of citrulline and arginine in saline- and citrulline-infused rats.....	103
Table 5.1	Composition of arginine diets.....	117
Table 5.2	Composition of protein diets.....	118

Page

Table 5.3 Body weight, diet consumed and weight gain of adult Sprague-Dawley rats fed arginine diets.... 120

Table 5.4 Physiological data for rats fed arginine diets.. 122

Table 5.5 Arterial plasma levels and net renal flux of citrulline and arginine in rats fed arginine diets..... 123

Table 5.6 Body weight, diet consumed and weight gain of adult Sprague-Dawley rats fed protein diets..... 125

Table 5.7 Physiological data for rats fed protein diets... 126

Table 5.8 Arterial plasma levels and net renal flux of citrulline and arginine in rats fed protein diets..... 127

LIST OF FIGURES

		Page
Figure 2.1	Cross-section of the kidney (A) and different regions of kidney.....	40
Figure 2.2	Confirmation of arginine peak by spiking the sample with arginine (A) or treatment with arginase (B).....	48
Figure 2.3	Argininosuccinate synthetase activity in rat kidney homogenate as a function of time (A) and protein concentration.....	56
Figure 2.4	Argininosuccinate lyase activity in rat kidney homogenate as a function of time (A) and protein concentration (B).....	57
Figure 2.5	Arginase activity in rat kidney homogenate as a function of time (A) and protein concentration (B).....	58
Figure 2.6	Ornithine aminotransferase activity in rat kidney homogenate as a function of time (A) and protein concentration (B).....	59
Figure 3.1	The activity of argininosuccinate lyase activity in different kidney regions.....	69
Figure 3.2	Cross-section of the kidney (A) and structural features of a cortical nephron (B).....	70
Figure 3.3	Distribution of enzymes of arginine metabolism in different regions of the rat kidney.....	73
Figure 3.4	Isolation of cortical tubule fractions.....	75
Figure 3.5	Distribution of enzymes of arginine metabolism in rat kidney cortical tubule fractions.....	77
Figure 3.6	Arginine production in kidney cortical tubule fractions.....	81
Figure 3.7	Subcellular localization of enzymes of arginine synthesis in rat kidney cortex.....	83
Figure 4.1	Arginine synthesis in rat renal cortical tubules as a function of time (A) and tubule quantity (b).....	93
Figure 4.2	Reactions involved in the synthesis and breakdown of arginine.....	94

		Page
Figure 4.3	Metabolism of newly synthesized arginine.....	96
Figure 4.4	Dependence of arginine synthesis in rat renal cortical tubules on citrulline concentration..	98
Figure 4.5	Relationship between the A-V difference across the kidneys for citrulline and for arginine in saline- and citrulline-infused rats.....	105
Figure 4.6	Figure depicts citrulline removal in vivo: Dependence on filtered citrulline.....	106
Figure 5.1	The figure plots the circulating levels of citrulline (A) or arginine (B) against arginine ingested.....	129
Figure 5.2	The figure depicts the renal arginine release against renal citrulline uptake (A) and arginine ingested.....	130

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

The concept of essentiality of a nutrient was first introduced by Osborne and Mendel (1916). However, it was Rose and coworkers in the early 1930's who established the concept of essentiality of amino acids in the diet of animals and humans. Their approach primarily involved deleting a specific amino acid from the diet and determining the effect of this on the growth of weanling rats and on nitrogen (N) balance in humans. From such studies, it was concluded that arginine is a "dietary non-essential" amino acid for the maintenance of growth in weanling rats and nitrogen balance in adult humans (Scull & Rose, 1930; Rose et al, 1954). From these observations, it could be deduced that arginine is synthesized endogenously in rats and humans.

Borsook and Dubnoff (1941) were the first to report that arginine is formed from citrulline in rat kidney slices. However, it was not until 1973 that it became clear from the studies of Featherston and his associates (1973) that the kidney is a major endogenous source of arginine. An obvious question that arises is this: does the endogenous synthesis of arginine meet the dietary requirements within and across different animal species, and in different altered physiological and pathological states? Based on the evidence in the literature, it can be said that endogenously synthesized arginine does not meet the dietary requirement in all situations.

According to Rose (1937), if elimination of an amino acid from the diet results in growth failure and negative nitrogen balance, then it is considered as essential, otherwise as non-essential. These criteria alone may not be sufficient to define the essentiality of an amino acid. By employing other indicators such as orotic acid excretion and blood ammonia levels, arginine has been shown to be essential in a number of species and in different conditions. Increased orotic acid excretion is a unique indicator in that it is specific only to arginine deficiency (Milner and Visek, 1973). In arginine deficiency, impairment in urea cycle function not only results in increased blood ammonia levels but also causes accumulation of carbamoylphosphate in mitochondria followed by its leakage into the cytosol leading to the formation of orotic acid (Kesner, 1965). Arginine is known to have powerful secretogogue and thymotropic properties (Barbul, 1986). Therefore, it may be worthwhile to include indicators such as circulating hormone levels and immune responsiveness to assess arginine deficiency.

Arginine requirements in different species

The arginine requirement for growth or for minimal orotate excretion in different species is summarized in **Table 1.1.** In this section, I will discuss the studies carried out by various investigators on arginine requirements in different species. Also, its requirement in certain physiological conditions will be

Table 1.1

Arginine Requirements for Growth and Minimal Orotate Excretion in Different Species*

Animal Species	Dietary Arginine (% of diet, by weight)	Reference
Growth		
Rat	>0.26<0.56	(Milner & Visek,1974)
Dog	0.4	(Czarnecki & Baker, 1984)
Cat	0.8	(Costello et al, 1980)
Pig	0.48	(Southern & Baker, 1983)
Guinea Pig	1.7	(O'Dell & Regan, 1963)
Chicken	2.0	(Savage & O'Dell, 1960)
Rabbit	0.6	(Adamson & Fisher, 1976)
Turkey	1.35	(Kratzer et al, 1947)
Minimal Orotate Excretion		
Rat	>0.56<0.84	(Milner & Visek, 1974)
Dog	0.53	(Czarnecki & Baker, 1984)
Cat	1.05	(Costello et al, 1980)

* Ferret requires arginine (0.4%) to maintain low blood ammonia levels(Deshmukh & Shope, 1983).

Mouse does not require arginine for growth (Bauer & Berg, 1943).

Dietary arginine is not required for growth in human infants (Snyderman et al, 1959), children (Nakagawa et al, 1963) and for neither the maintenance of positive nitrogen balance (Rose et al, 1954) nor for minimal orotate excretion (Carey et al, 1987) in adults.

addressed.

Rat

Scull and Rose (1930) were the first to report that arginine is not an essential amino acid for weanling rats based solely on growth. In their study, rats which were fed diets (hydrolyzed casein treated with arginase and urease) containing low arginine (0.4%) gained body weight similar to those fed 1.3% arginine (hydrolyzed casein). Furthermore, their results showed that the increments in tissue arginine were 2 to 3 times as large as may be accounted for by the ingested arginine, indicating that arginine is synthesized endogenously. However, studies from the same laboratory (Borman et al, 1946) which involved feeding highly purified amino acid diets (with or without arginine) to weanling rats concluded that arginine is a necessary dietary component for optimum growth. These results from the same laboratory are contradictory. In the earlier studies (Scull & Rose, 1930), the presence of ornithine in the protein hydrolysates (generated by the action of arginase on arginine) was not taken into account. Therefore, it is possible that ornithine may have been converted to arginine and thus met the requirement for growth.

Dietary arginine is required for optimal mammary growth in rats (Pau & Milner, 1982). Immature (35-40 g) and mature rats (150-175 g) fed purified amino acid diets without arginine had depressed food intake, weight gain, lower positive nitrogen balance and showed marked increases in urinary excretion of

orotate and tricarboxylic acid cycle intermediates (Milner et al, 1974). Studies in rats have demonstrated the arginine requirements (% of diet) for maximal body weight gain and minimal orotate excretion to be >0.26<0.56 and >0.56<0.84, respectively (Milner & Visek, 1974).

Dog

The response of dogs to arginine deficiency is different from that of rats. Immature Labrador Retriever dogs (3 Kg) fed amino acid diets with no arginine (0%) and containing varying amount of dietary N (14, 21 or 28%) showed signs of emesis, excessive salivation and muscle tremors. Animals on arginine-devoid diets consumed less food, lost body weight and had elevated levels of blood orotate and ammonia compared to those fed 0.56 and 1.12% arginine (Ha et al, 1978). Mild hyperammonemia and increased orotate excretion occurred in young (7-wk) and old (17-wk) English Pointer dogs fed diets devoid of arginine (Czarnecki & Baker, 1984). The results from this study also suggested that young dogs need 0.4% arginine to maximize weight gain, while requiring 0.53% to minimize orotic acid excretion. On the contrary, earlier studies by Rose & Rice, 1939 demonstrated that for the adult dog, arginine is a dispensable amino acid as they were able to maintain body weight and nitrogen balance of mature dogs when fed arginine-free (purified amino acid) diets. Thus, the above two reports are apparently in conflict. This conflict may be largely due to experimental differences. Rose & Rice (1939) employed adult dogs

instead of young dogs and also they did not measure orotate excretion and blood ammonia levels. However, it has been shown that even in adult dogs, arginine is an indispensable amino acid based on orotate excretion and blood ammonia levels (Burns et al, 1981).

Cat

Arginine deficiency in the cat is the most rapidly induced nutrient deficiency observed in any mammal. The consumption by the near-adult cat of a single meal devoid of arginine led to serious clinical symptoms (frothing at the mouth, ataxia, emesis and tetanic spasms) or even death within a couple of hours from acute ammonia intoxication (Morris & Rogers, 1978). Costello et al (1980) have reported that the cat needs 0.8% arginine to support growth and 1.05% to minimize urinary orotic acid excretion.

Ferrets

Young, fasted ferrets develop hyperammonemia and encephalopathy soon after a single feeding of diet deficient in arginine (Deshmukh and Shope, 1983). Ferrets (8-wk old) develop hyperammonemia within 2-3 h when fed diets containing less than 0.3% arginine. These animals develop seizures and coma accompanied by shivering when their serum ammonia levels increase 10-fold. Ferrets require 0.4% arginine in their diet to maintain normal serum ammonia levels (250 μg/100 ml). This ammonia concentration is about 0.15 mM which would be very high in a rat or human.

Pigs

In young pigs (7-9 Kg) fed semipurified diets, supplemented with arginine to achieve final concentrations of 0.18-0.68%, body weight gain and efficiency of feed utilization were maximized at 0.48% dietary arginine. No improvement in these parameters was observed with more than 0.48% arginine. A modest increase in orotic acid excretion was observed in pigs when fed less than 0.48% arginine (Southern & Baker, 1983). The weanling pig resembles the rat in that it can synthesize arginine but needs a dietary supply of arginine for maximum growth (Mertz et al, 1952).

Guinea Pigs

The guinea pig, unlike the rat, has an unusually high requirement for arginine which is not clearly understood. The arginine requirement of the growing guinea pig is not met by 25% casein (Heinicke et al, 1955). Maximum growth was observed in these animals when fed 30% casein (1.2% arginine) supplemented with 0.5% arginine (O'Dell & Regan, 1963).

Rabbit

Mcward et al (1967) were the first to report the requirement of arginine for the young rabbit. The optimum arginine level for the growth of New Zealand rabbits (5-wk old) was found to be 0.98 and 1.25% of the diet for 2.75% and 3.75% nitrogen intakes respectively. Adamson and Fisher (1976) have reported a lower requirement (0.6% arginine) to be sufficient for optimal growth and for maintenance of serum arginine levels in young female

rabbits. These workers also reported that adult male rabbits do not require dietary arginine.

Chicken and **Turkey**

Although, the essentiality of arginine for the chick was established by Klose et al, (1938), it was Savage and O'Dell (1960) who reported that the newly hatched White Leghorn chick has a high requirement for arginine. The chick needs 2.0% arginine in the diet to support maximum growth. Bronze turkey poults (1-wk old) require approximately 1.35% arginine in the diet to support maximum growth (Kratzer et al, 1947). The reasons for the high arginine requirement in the chick and turkey poult are not known.

Arginine requirements in certain physiological conditions

Pregnancy and lactation

Arginine supplementation (1%) of casein diets fed to pregnant rats reduced their orotate excretion and increased the birth weight and weaning weight of the pups. It also reduced orotic acid excretion during late gestation (d 16-18) and early lactation. Failure of endogenous arginine synthesis to meet the requirement of the pregnant rat in reducing orotate excretion , suggested that dietary arginine is required during gestation and lactation for optimal reproductive response and nursing performance (Pau & Milner, 1981).

Spermatogenesis (reproduction)

The role of arginine in spermatogenesis is still unclear. In the studies of Holt et al (1942), in young men, there was a striking diminution in spermatozoa count after nine days of arginine deprivation. They also confirmed this finding in rats. Degenerative changes in the testes were noted within 3 weeks of arginine withdrawal. None of the men studied by Rose and his associates (1955) showed any reduction in sperm count when arginine was withheld for as long as 64 days. Although the effect of dietary arginine deficiency on spermatogenesis is at best unclear, there is a large body of clinical work reporting the use of supplemental arginine in various male infertility conditions, particularly oligospermia (Barbul, 1986).

Species which do not require dietary arginine for growth or maintenance of N-balance:

Mouse

Mice fed purified diets lacking in arginine grew comparably to those fed diets containing arginine. As there were no differences in the rate of weight gain, Bauer & Berg (1943) concluded that the mouse has the ability to synthesize arginine at a fairly rapid rate.

Humans

Human infants, (Snyderman et al, 1959) children (10-12 year old) (Nakagawa et al, 1963) and adults (Rose et al,1954) do not

require dietary arginine for the maintenance of positive nitrogen balance. Recently, Carey et al (1987) reported that an arginine-deficient diet in normal adult humans does not increase blood ammonia levels or orotic acid excretion over a short period of time (10 d). These authors concluded that the adult human has the capacity to synthesize arginine de novo, sufficient for the maintenance of normal cellular metabolism. Thus the above studies suggest that arginine is a dispensable amino acid for growth in infants, children and adults.

Beneficial effects of arginine in different pathological conditions

The beneficial effects of arginine in different pathological conditions are summarized in **Table 1.2.**

Urea cycle disorders

A number of genetic abnormalities of the urea cycle have been described (Walser, 1983), each involving a deficiency or defect of a particular enzyme in the cycle. The enzymopathies can lead to hyperammonemia, ornithinemia, citrullinemia, argininosuccinic aciduria and argininemia depending on the specific enzyme deficiency. Some of these urea cycle disorders have been treated by feeding low nitrogen diets, often supplemented with arginine or citrulline (Shih & Efron, 1972). Brusilow (1984) and coworkers have shown that arginine and sodium benzoate supplements can improve the clinical symptoms of

TABLE 1.2

Beneficial Effects of Arginine in Different Pathological Conditions

Pathological Condition	Reference
Urea cycle disorders	(Walser, 1983; Brusilow, 1984)
Chronic renal insufficiency	(Swenseid et al, 1975, Tizianello et al, 1980)
TPN solutions (Hospitalized patients)	(Fahey, 1957)
Loss of lean body mass (catabolic states as observed during surgery and prolonged hospitalization)	(Weinsier et al, 1979; Barbul et al, 1984; Barbul, 1986; Visek, 1986)
Protein malnutrition	(Visek et al, 1986)
Sepsis, Trauma and Injury (wound healing)	(Barbul, 1986; Kirk & Barbul, 1990)
Thermal burns	(Saito et al, 1987)
Reye's syndrome	(Sinatra et al, 1975; Delong & Glick, 1982)
Cancer (?)	(Levy et al, 1954; Milner & Stepanovich, 1979)

patients (children) with carbamoylphosphate synthetase-I (CPS-I), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) deficiency. These children developed hyperammonemia within 15-68 h after arginine deprivation. Nitrogen accumulated as ammonia or glutamine or both. Benzoic acid (sodium salt) supplementation helps in reducing nitrogen load often seen in patients with urea cycle disorders. Excess nitrogen in the form of glycine can conjugate with benzoic acid to form hippuric acid which can easily be excreted. Arginine intake (or citrulline in the case of OTC deficiency) proved effective in reversing acute hyperammonemic episodes. Ornithine was unable to prevent nitrogen accumulation in these enzyme-deficiency states. Thus, these workers concluded that arginine is an indispensable amino acid for children with urea cycle enzymopathies, whereas citrulline is indispensable for CPS-I or OTC deficiency.

Reye's syndrome

In children with this rare disease, there are abnormalities in cerebral and hepatic functions (Reye et al, 1963; Snodgross & DeLong, 1976). One of the abnormalities that is commonly seen in these children is elevated blood ammonia levels. This is mainly due to decreased activities of the mitochondrial enzymes carbamoyl phosphate synthetase-I and ornithine transcarbamoylase (Sinatra et al, 1975). Serum arginine and citrulline levels are markedly depressed in these children. Citrulline and arginine supplementation have been proven beneficial in reducing the

elevated blood ammonia levels (DeLong & Glick, 1982).

Patients on total parenteral nutrition (TPN)

In some hospitalized patients, there are situations in which their nutrition is provided by TPN solutions which usually contain either protein hydrolysates or pure amino acids. Protein hydrolysates frequently contain appreciable quantities of ammonia. If such solutions, with inadequate amounts of arginine, are administered to patients, hyperammonemia may ensue. Fahey (1957) demonstrated that infusions of large quantities of amino acids (casein or fibrin hydrolysates) in humans caused hyperammonemia and even coma. These effects were reversed by the addition of arginine to the amino acid solutions. Heird et al (1972) reported the occurrence of hyperammonemia and increased orotic aciduria in three infants who were infused with a solution of crystalline amino acids without arginine that was relatively free of ammonia. Infusion of large doses of essential amino acids without arginine led to depressed levels of urea cycle intermediates in the blood and hyperammonemia in two children with acute renal failure (Motil et al, 1980).

Repletion of lean body mass

One of the major clinical complications that arises during prolonged hospitalization of patients is the loss of lean body mass (Weinsier et al, 1979). This may be due to reduced protein intake due to loss of appetite and increased muscle proteolysis or decreased protein synthesis. It is known that endogenously synthesized arginine is utilized for muscle protein synthesis

(Featherston et al, 1973). It has been shown that rats fed a very low protein (0.5% lactalbumin) diet for 14 weeks lost 40% of their body weight and were in negative nitrogen balance (Kari et al, 1981). When these animals were refed amino acid diets containing varying amounts of arginine (0.0, 0.75 & 1.5%) for 14 d, they gained body weight and were in positive nitrogen balance. This increase in body weight gain and nitrogen balance was far higher in animals fed 0.75 and 1.5% arginine than in those fed 0% arginine. These results suggest that arginine is required for replenishment of lean body mass and for the maintenance of nitrogen balance. No attempts have been made to examine the effect of arginine in repleting lean body mass in hospitalized patients.

Chronic renal insufficiency

Impairment of endogenous arginine synthesis can occur in patients with chronic renal insufficiency. In adult rats with chronic renal insufficiency both a decrease in plasma arginine levels and a decrease in the incorporation of arginine in muscle protein have been observed (Chan et al, 1974; Swenseid et al, 1975; Wang et al, 1977). These results suggest that there is decreased endogenous arginine synthesis and thus provide the basis for essentiality of arginine in patients with chronic renal failure. In patients with chronic renal insufficiency, renal arginine release is greatly depressed (Tizianello et al, 1980). This suggests a need for dietary arginine.

Sepsis, trauma, thermal burns and enhancement of immune response

The beneficial effects of arginine supplementation in clinical situations such as surgery (pre- and post-operative), sepsis, trauma, thermal burns, and also in stimulating the response of the immune system have been recently described (Barbul, 1986; Kirk & Barbul, 1990; Saito et al, 1987; Daly et al, 1988).

The importance of arginine supplementation on recovery from trauma was first reported by Seifter et al (1978). These workers wounded rats (200-300 g) by making a 7-cm paravertebral incision through the skin and the panniculus carnosus. The rats were then placed on amino acid diets containing either 1% arginine or devoid of arginine. The controls were fed laboratory chow (1.8% arginine). Animals on arginine-deficient diet grew poorly (pre-(1.8 vs. 7.0 g/d) and post-(1 vs. 4.3 g/d)injury) and showed impaired wound healing compared to those fed arginine. Additional supplementation of arginine (1% arginine in drinking water) to injured animals, decreased the post-operative weight loss and increased hydroxyproline deposition (an index of collagen synthesis) at specific wound sites. From these observations, they concluded that arginine is essential for the synthesis of the increased quantity of collagen required for wound healing and it decreases some of the negative aspects of the metabolic responses to injury. Similarly, Pui and Fisher (1979) demonstrated the beneficial effect of arginine supplementation on

body weight gain and nitrogen retention in traumatized rats. Barbul and his associates (1977) have demonstrated that 1% arginine:HCl supplementation increased thymic weight and the number of thymic small lymphocytes in normal rats and prevented or reduced the thymic involution that occurs in mice and rats subjected to trauma.

The beneficial role of arginine in wound healing in humans has also been recently demonstrated by Barbul and his associates (1990). Human volunteers were subjected first to wounding (subcutaneous incision (5-cm) in the right deltoid region) and then were given daily supplements of 30 g arginine:aspartate (n=12) or 30 g arginine:HCl (n=12) or placebo (n=12) for 14 d. The deposition of hydroxyproline at the wounded site (on d 14) and also the immune response of peripheral blood lymphocytes (in vitro) to mitogenic stimuli (concanavalin A and phytohemagglutinin) was assessed on d 0, 7, and 14. Increased hydroxyproline deposition at the wounded site and enhanced lymphocyte mitogenesis were observed only in those given arginine supplements. It should be noted that in their studies (Barbul et al, 1990), arginine intake (30 g) can contribute significantly to nitrogen load. They did not employ proper controls and should have compared their results to those given an isonitrogenous load. Arginine supplements have proven effective in improving nitrogen balance, elevating plasma somatomedin C levels (an indicator of growth hormone secretion) and enhancing immune responses in surgical patients (Daly et al, 1988). Intakes of

arginine (2% of total energy intake) have been shown to improve metabolic and immune function in post-burn conditions (Saito et al, 1987).

Barbul and coworkers (1983) from their studies with hypophysectomized rats suggested that an intact hypothalamic-pituitary axis is necessary for the beneficial effects of arginine seen in traumatized animals. Studies of the effects of excess arginine on the immune system have indicated that arginine action may be mediated via the enhancement of natural killer activity and monocyte-mediated cytotoxicity in the host and its direct effect on the tumor itself (Moriguchi et al, 1987). In tumor-bearing mice, arginine supplementation (1%) enhanced interferon-induced natural killer cell activity, lymphokine-activated killer cell generation, macrophage cytotoxicity and extended the median survival time of these animals (Reynolds et al, 1990).

From the discussion thus far, it is clear that endogenously synthesized arginine does not meet the requirement for growth, for maintenance of nitrogen balance, and for minimal orotate excretion in various situations. Also, it is evident that arginine supplementation has a beneficial role in certain situations.

Biochemical functions of arginine

Arginine serves many important metabolic functions and these are summarized in **Table 1.3.**

TABLE 1.3

Biochemical Functions of Arginine

1.	Arginine is required for protein synthesis.
2.	Arginine is required for ammonia detoxification (an intermediate of urea cycle).
3.	Arginine is a precursor for creatine synthesis (role in energy metabolism).
4.	Arginine serves as a precursor for the synthesis of polyamines (role in cell division, tissue growth and differentiation).
5.	Arginine has thymotropic activity (role in injury or trauma, wound healing, sepis and thermal burns).
6.	Arginine mediates the cytotoxic effects of macrophages and polymorphonuclear neutrophils.
7.	Endothelium-derived relaxing factor is nitric oxide (NO) which is synthesized from arginine (NO activates soluble guanylate cyclase leading to a variety of functions in different cells).

Arginine is a normal constituent of proteins. Histones, which are involved with nucleic acid interactions, are highly basic proteins as they are enriched in arginine and lysine. Arginine is required for protein synthesis in both prokaryotic and eukaryotic cells.

Arginine also serves as an intermediate in the urea cycle and is involved in ammonia detoxification. The importance of arginine in ammonia detoxification is clearly evident in patients with inherited urea cycle disorders as previously discussed. Arginine serves as a amidino group donor for the synthesis of creatine, a major buffer reserve of high energy phosphate for regeneration of ATP in muscle. Arginine also functions as a precursor for the synthesis of polyamines, which appear to play an important role in cell division, tissue growth and differentiation (Pegg & McCann, 1982).

The endothelium-derived relaxing factor (EDRF) is NO and is synthesized from L-arginine (Ignarro et al, 1987; Palmer et al, 1987). Nitric oxide is known to have profound biological effects (Moncada et al, 1989; Ignarro, 1989). The various biological actions of NO include endothelium-dependent relaxation, cytotoxicity of macrophages (Hibbs et al, 1987), polymorphonuclear neutrophils (Rimele et al, 1988) and cell-to-cell communication in the central nervous system (Knowles et al, 1989; Bredt & Snyder, 1989). Nitric oxide is known to modulate vasodilation and arterial blood pressure and also to inhibit platelet aggregation and adhesion of platelets to endothelial

surface (Radomski et al, 1990). Thus, NO mimics the actions of vasodilators such as glyceryl trinitrate which has been in use clinically to decrease vascular resistance and blood pressure. Evidence that has been presented in the literature indicates that the L-arginine:NO pathway is a widespread transduction mechanism for the activation of the soluble guanylate cyclase leading to a variety of functions in different cells (vascular endothelial cells, neutrophils, macrophages, brain synaptosomes) (Moncada et al, 1989).

The focus of this review has been on the importance of arginine in different species and in different conditions and on its functions in metabolism. Therefore, it is clearly important to understand the need for endogenous arginine synthesis. Also, it is important to understand the metabolic fate of arginine. The remainder of this review will focus on the biosynthesis and breakdown of arginine.

Need for endogenous arginine synthesis

The synthesis of the precursor of polyamines, putrescine from ornithine (Pegg & McCann, 1982), does not have an overall effect on arginine utilization. Also, the urea cycle does not have a net effect on arginine degradation. One molecule of arginine is produced for every one removed in the operation of this cycle (Krebs & Henseleit, 1932). However, ornithine

generated by the action of arginase on arginine can be further metabolized via ornithine aminotransferase (OAT). In fact, the major metabolic fate of arginine in the liver is catabolism via arginase and ornithine aminotransferase. It has been suggested in the literature (McGivan et al, 1977; Henslee & Jones, 1982) that OAT is primarily involved in the catabolism of ornithine in the liver. Recently, Alonso & Rubio (1989) demonstrated that even under conditions of arginine deprivation, there is catabolism of ornithine in the liver, kidney and muscle of mice. In their studies, tissue ornithine concentrations were measured (on d 8, over a 10-h period) after the administration of gabaculine (a potential suicide inhibitor of OAT) to mice adapted (7 d) to amino acid diets with and without arginine. It was observed that the ornithine in the tissues accumulated, even in animals given arginine-devoid diets. They estimated that at least 45 μmoles of ornithine is synthesized and catabolized daily via OAT in the mouse deprived of arginine. This suggests that a net drain of the endogenous arginine pool can occur via the OAT reaction. Such a drain on the endogenous arginine pool may result in impairment in urea cycle function. Therefore, in order to replenish liver arginine pools, there is a need for endogenous arginine synthesis.

Minor drain on the endogenous arginine pools

A minor drain on the endogenous arginine pool can occur via the formation of metabolites such as creatine, nitric oxide and

polyamines. These metabolites have important metabolic functions. It should be noted that ornithine and citrulline generated in the formation of guanidinoacetic acid (precursor of creatine) and nitric oxide, respectively, can be recycled to arginine. Also, in the liver, the carbon skeleton of arginine can be either metabolized to glucose or completely oxidized to CO_2 and water. This depends on the fate of the glutamate that is generated by the combined actions of arginase and ornithine aminotransferase on arginine.

Enzymes involved in arginine utilization

It is appropriate at this point to discuss the reactions and the enzymes involved in the utilization of arginine (**Table 1.4**). I have discussed five enzymes: arginase, ornithine aminotransferase, L-arginine:glycine amidinotransferase (or transamidinase), ornithine decarboxylase and nitric oxide synthetase. The activities and the distribution of these enzymes are presented in **Table 1.5**).

Arginase [E.C. 3.5.3.1] catalyzes the conversion of arginine to urea and ornithine. The large negative free energy associated with this reaction makes it irreversible. The reported K_m values for arginase from rat liver and kidney range anywhere from 1-20 mM. The enzyme has an absolute requirement for Mn^{+2} (Garganta & Bond, 1986). Arginase is widely distributed (Herzfeld & Raper, 1976). It is abundant in the liver, primarily functioning in the

TABLE 1.4

Enzymes and Reactions Contributing to Arginine Removal

1. Arginase (A)

Arginine + H_2O ----------> Ornithine + Urea

2. Glycine amidinotransferase

Arginine + Glycine <----> Guanidinoacetic acid[a] + Ornithine

3. Ornithine aminotransferase (OAT)

Ornithine + α-ketoglutarate <--------> Glutamate + L-glutamic-γ-semialdehyde

4. Ornithine decarboxylase (ODC)

Ornithine --------> Putrescine[b] + CO_2

5. Nitric oxide synthetase (NO synthetase)

Arginine --> --> --> --> --> --> Nitric oxide[c] + Citrulline

[a] Converted to creatine by guanidinoacetate methyltransferase.

[b] Can be converted to spermidine and spermine (polyamines).

[c] Formation of nitric oxide from arginine involves many steps.

TABLE 1.5

Distribution of Enzymes of Arginine Metabolism in Adult Male Rat Tissues*

	Carbamoyl phosphate Synthetase-I	Ornithine Transcarbamoylase	Argininosuccinate Synthetase	Argininosuccinate Lyase	Arginase	Ornithine Aminotransferase
Reference	(Jones et al, 1961)	(Raijman, 1974)	(Kato et al, 1976)	(Ratner & Muraka-mi-Murofushi,1980)	(Herzfeld & Raper, 1976)	(Herzfeld & Knox, 1968)
Tissue	Activity expressed as μmoles/h/g wet weight of tissue*					
Liver	284	13,300	337.7	308	152,700	188
Intestine	10	2.60	0.46[b]	6.44	7740	117
Kidney	8	<0.22[c]	74.4	40.6	3390	166
Brain	4	0	3.88[b]	10.2	150	13
Spleen	0	<0.22[c]	1.49[b]	14.2	66	15
Pancreas	3	0.29	ND	ND	6180	40
Adrenal	0	0.67	ND	ND	42	12
Heart	0	0	ND	4.51	36	13
Lung	0	0	ND	9.98	324	15
Testis	0	0.32	ND	ND	ND	ND
Thymus	0	0	ND	ND	114	ND
Skeletal Muscle	0	<0.22[c]	0.59[b]	ND	24	ND
Salivary gland	10	<0.22[c]	ND	ND	ND	ND

*Glycine amidinotransferase is almost exclusively found in kidney and pancreas (Van Pilsum et al, 1972); Nitric oxide synthetase activity has been reported for endothelial cells, macrophages, neutrophils, Kupffer cells, kidney epithelial cells, adrenal gland, cerebellum and fore brain (Collier & Vallance, 1989). Determined in tissue homogenates and for clarity SD values have been excluded ; [b] Expressed as μmoles/h/g protein; [c] Below level of detection; ND: Not determined

formation of urea in ureotelic animals. However, it is also present in significant amounts in other tissues (Herzfeld & Raper, 1976; **Table 1.5**).

Recently Levillain and coworkers (1989) have shown that renal arginase is almost exclusively located in the rat proximal straight tubule (S_3 segment). They suggested a role for arginase in the maintenance of the medullary urea concentration gradient. Arginase exists in multiple forms in rat, mouse and human tissues (Skrzypek-Osiecka et al, 1983; Spolarics & Bond, 1988; Zamecka & Porembska, 1988). There are two main forms of arginase in rat kidney and liver. In kidney, arginase A_4 (acidic protein) is the main form, accounting for 95-98% of the activity while A_1 (alkaline protein) is present in trace quantities. The A_4 form of the enzyme is almost exclusively present in mitochondrial matrix and A_1 is localized in the cytosol (Skrzypek-Osiecka et al, 1983). In contrast to the kidney, arginase A_1 is the major form in the liver and is cytosolic. Immunological studies have demonstrated that the kidney major form (A_4) does not cross react with liver arginase A_1. The differences between the two forms of the enzyme in their properties and subcellular location may indicate different functions for the enzyme. The most likely metabolic role of arginase in kidney is to provide ornithine for the formation of proline or glutamate or polyamines or all three.

Ornithine aminotransferase (OAT) [E.C. 2.6.1.13] is involved in the reversible conversion of ornithine and α-ketoglutarate to L-glutamate-γ-semialdehyde and glutamate (**Table 1.4**).

L-glutamate-γ-semialdehyde is an unstable intermediate that undergoes spontaneous conversion to Δ^1 -L-pyrroline-5-carboxylate (P5C) which in turn can be converted to proline by P5C-reductase (Jones, 1983). The formation of ornithine, glutamate or proline in different tissues varies depending on a specific tissue requirement. In the liver, it is involved in the net catabolism of ornithine as discussed earlier. Synthesis of ornithine from glutamate occurs in the intestinal mucosa, while no synthesis occurs in liver or kidney homogenates (Henslee & Jones, 1982). The activity of OAT is highly dependent on pyridoxal phosphate and is inhibited by the general aminotransferase inhibitor aminooxyacetate. Activity is high in the kidney, liver, and small intestine of the rat (Herzfeld & Knox, 1968 ; **Table 1.5**). OAT is localized in the mitochondrial matrix in rat kidney (Passarella et al, 1989). However, its cellular location in the kidney has not been identified.

L-arginine:glycine amidinotransferase (or transamidinase) [EC 2.1.4.1] is involved in the formation of guanidinoacetic acid, a precursor for creatine synthesis, and ornithine from arginine and glycine in the kidney, and was first identified by Borsook & Dubnoff (1941a). Guanidinoacetic acid synthesized in kidney is then transported to the liver where it is methylated by guanidinoacetate methyltransferase [E.C. 2.1.1.2] to yield the final product creatine (Borsook & Dubnoff, 1940) which plays an important role in energy metabolism. It was mentioned earlier that creatinine excretion may serve as a drain on endogenous

arginine pool (Visek, 1986). This assumption is not correct in the sense that ornithine generated in the reaction catalyzed by glycine amidinotransferase can be recycled for arginine synthesis.

L-arginine:glycine amidinotransferase is predominantly found in kidney and pancreas of rat with some activity in other tissues (Van Pilsum et al, 1972). Multiple forms of the enzyme exist in rat and human kidney and they are similar in their properties (Gross et al, 1988). Glycine amidinotransferase is localized in a subpopulation of proximal convoluted tubules, probably proximal straight tubules (McGuire et al, 1986). Its subcellular location is in the inner mitochondrial membrane, probably on the external side (Magri et al, 1975). The K_m for arginine and V_{max} of purified kidney enzyme are 2.8 mM and 0.39 μmole/min/g protein, respectively (McGuire et al ,1980).

The first step in the formation of polyamines is catalyzed by ornithine decarboxylase (ODC) [E.C. 4.1.1.17]. It catalyzes the irreversible conversion of ornithine to putrescine. Putrescine formed in the reaction can be further converted to spermidine and spermine which are ubiquitously present (Pegg & McCann, 1982). The wide distribution of arginase and ODC (only in traces), suggests that the true precursor for polyamine formation is arginine. The arginine requirement for polyamine formation is very minimal. ODC has an absolute requirement for pyridoxal phosphate (Pegg & McCann, 1982). The rat liver ODC has a half-life of 10-15 min and the enzyme is located in the

cytosol. The cellular location of ODC in the kidney has not been determined. ODC can be inhibited irreversibly by its analog, DFMO (α-difluro-methylornithine) (Tabor & Tabor, 1984; Pegg & McCann, 1982).

The formation of nitric oxide (NO) and citrulline in a series of reactions from arginine is catalyzed by a complex enzyme, NO synthetase [E.C. 1.14.23] (**Table 1.4**). Nitric oxide formed in the reaction is highly unstable (half-life of only 3-5 s) and is rapidly inactivated by molecular oxygen leading to the formation of nitrite (NO_2^-) and nitrate (NO_3^-) (Ignarro, 1989). Nitrate excretion (Leaf et al, 1989) does not act as a drain on endogenous arginine pools, as citrulline formed in the reaction can be recycled to arginine (Sessa et al, 1990; Hecker etal, 1990).

Nitric oxide synthetase has an absolute requirement for NADPH and is activated by Ca^{+2}/calmodulin. Its activity has been reported in a wide number of cells and tissues (**Table 1.5**). The formation of nitric oxide is specifically inhibited by an arginine-analog, N^G-monomethyl-L-arginine (L-NMMA) which has been used as a tool to understand L-arginine:NO pathway in various biological systems.

Transport of arginine

Transport of arginine into cells of different tissues has been studied. It shares the transport system with other cationic

amino acids (lysine and ornithine) and the system involved is y^+, which is not dependent on sodium. This system has been characterized in hepatocytes and in the brush border membranes of the kidney and intestine (White, 1985). There exists a clinical condition in which transport of arginine is defective. Lysinuric-protein intolerence is a rare inherited disease with a defect in basic diamino acid transport, including intestinal, renal and hepatic transport of lysine, arginine and ornithine. Elevated blood ammonia and decreased plasma levels of arginine and ornithine and also an increased orotic acid excretion have been observed in these patients (Rajantie, 1981).

Role of different organs and the importance of endogenous arginine synthesis

From the discussion thus far, it is clear that in some animal species, arginine can be synthesized endogenously and that the liver does not release arginine for extra-hepatic utilization as arginine is catabolized by the actions of arginase and ornithine aminotransferase, respectively. The question that arises now is this: what is the importance of the net endogenous arginine synthesis and where does it occur? In this respect, I have discussed the role of kidneys and intestine. The enzymes and the reactions involved in arginine synthesis are presented in **Table 1.6.**

It is clear from the studies of Featherston and coworkers

TABLE 1.6

Enzymes and The Reactions Involved in Net Arginine Synthesis

Enzymes and Reactions of Net Arginine Synthesis

1. Carbamoyl phosphate synthetase-I [ammonia] (CPS-I)

Ammonia + Bicarbonate +2ATP--->Carbamoylphosphate +2ADP +P_i +$2H^+$

2. Ornithine transcarbamoylase (OTC)

Carbamoylphosphate + Ornithine <-----> Citrulline + P_i

3. Argininosuccinate synthetase (AS)

Citrulline + Aspartate + ATP ---> Argininosuccinate + AMP +PP_i

4. Argininosuccinate lyase (AL)

Argininosuccinate + H_2O -------> Arginine + Fumarate

(1973) that the kidney is a major endogenous source of arginine. In their experiments, animals were injected first with [^{14}C]Ureidocitrulline and then the kidneys or livers or both were ligated. They then measured the incorporation of [^{14}C]Guanidino-arginine into muscle protein. The incorporation of labelled arginine into muscle protein was greatly reduced in animals when kidneys were ligated while ligation of livers was without any effect. From these observations they concluded that the kidney is a major biosynthetic source of arginine. It is known that kidneys of normal animals remove citrulline from the blood and release it as arginine (Windmueller & Spaeth, 1981; Brosnan et al, 1983). The activities of carbamoylphospate synthetase-I [E.C. 6.3.4.16] (CPS-I) and ornithine carbamoyltransferase [E.C. 2.1.3.3] (OTC) are either low or absent in the kidney (Jones et al, 1961; Raijman, 1974; **Table 1.5**). This suggests that the kidneys cannot synthesize citrulline. An obvious question that arises is this: what is the source of circulating citrulline for renal arginine synthesis?

Sources of circulating citrulline

The evidence presented in the literature clearly suggests that the intestine plays an important role in the contribution to circulating citrulline. Windmueller & Spaeth (1974; 1978; 1980) have demonstrated in studies with isolated, vascularly perfused preparations of rat intestine and with autoperfused segments of

rat jejunum *in vivo*, that the small intestine releases citrulline (6.5 μmoles/h/100 g body weight) into the blood. This citrulline is not appreciably taken up by the liver. This conclusion is based on the studies by Windmueller & Spaeth (1981). In their perfusion experiments with rats, a continuous portal infusion of citrulline (38.0 nmoles/min, over a period of 150 min and about 40 passes), accumulated in the perfusate at a linear rate of 35.2 nmoles/min. Thus hepatic uptake was only about 7%. Although there is no data available in the literature on citrulline transport in the liver, it can be speculated that liver cells transport this amino acid poorly.

Kidneys readily take up citrulline. By employing the method of continuous tubular microperfusion (*in vivo* et *in situ*) of rat proximal convoluted tubules, Kettner and Silbernagl (1985) have shown the existence of two transport systems for citrulline in the brush border of the proximal tubule, one with a high capacity and low affinity, and the other with low capacity and high affinity. The cooperative interaction between these two systems provide for a virtually complete reabsorption of filtered citrulline at physiological as well as elevated levels of citrulline.

The question that arises now is this: what is the source of intestinal citrulline and how is it synthesized ? Studies by Windmueller and Spaeth (1974, 1978 & 1980) have demonstrated that citrulline is formed from glutamine, a major metabolic fuel for the intestine. In their studies, perfusion of isolated rat

small intestine with rat blood (containing glutamine, 150 μmoles of N), glutamine nitrogen was incorporated into citrulline (34%), alanine (32%), ammonia (23%) and proline (10%). From these observations, they concluded that the major metabolic fate of glutamine nitrogen in the intestine is the formation of citrulline.

The pathway of citrulline synthesis from glutamine has been described by Jones (1983). Glutamine is hydrolyzed to glutamate by glutaminase. Glutamate is then converted to γ-glutamate-γ-semialdehyde by the action of pyrroline-5-carboxylate synthase (Wakabayashi & Jones 1983). L-glutamate-γ-semialdehyde and glutamate are converted to ornithine and α-ketoglutarate, a reversible reaction catalyzed by ornithine aminotransferase (Henslee & Jones, 1982). Ornithine formed can be converted to citrulline by the action of ornithine transcarbamoylase (OTC) provided carbamoylphosphate is available. This is provided by carbamoylphophate synthetase-I. Both CPS-I and OTC are mitochondrial matrix enzymes and the reactions catalyzed by these enzymes are shown in **Table 1.6**. These enzymes are present in significant quantities in the intestine (**Table 1.5**). Cohen et al (1985) have demonstrated that the true substrate for CPS-I is ammonia and its K_m for the purified enzyme and for the isolated intact mitochondria (rat liver) are 0.038 and 0.013 mM, respectively. CPS-I has an absolute requirement for a cofactor, N-acetylglutamate (NAG). N-acetylglutamate is synthesized from acetyl CoA and glutamate catalyzed by another

mitochondrial matrix enzyme, NAG synthetase [E.C. 2.3.1.1]. Arginine allosterically regulates the synthesis of N-acetylglutamate (Walser, 1983). Unlike CPS-I, CPS-II utilizes glutamine as a substrate and is involved in the synthesis of pyrimidines (Kesner, 1965). The K_m of OTC for carbamoylphosphate and ornithine are 0.2 and 0.09 mM, respectively (Marshall & Cohen, 1972).

Importance of intestinal citrulline syntnesis

Having discussed the source and the pathway of intestinal citrulline synthesis, it is essential to understand the importance of its role in different metabolic and pathological situations. Evidence in the literature suggests an important role of the intestine in citrulline synthesis. Studies carried out by Hoogenraad and his associates (1985) demonstrated that the growth of rats was severely depressed when their intestinal citrulline synthesis was specifically inhibited. They brought about this inhibition by administering Gly-Gly-PALO (glycylglycine-δ-N-(phosphonacetyl)-L-ornithine), a powerful and specific inhibitor of intestinal ornithine transcarbamoylase, in the drinking water of rats fed diets containing either arginine or ornithine (arginine-deficient). In animals administered with the inhibitor, serum arginine and citrulline levels were also greatly reduced. The inhibition of growth was easily reversed by dietary citrulline. Carnivores like cats and ferrets, cannot

synthesize citrulline due to the low activities of the intestinal enzymes, pyrroline-5-carboxylate synthase (Rogers & Phang, 1985) and ornithine aminotransferase (Morris, 1985). Therefore, these animals strictly depend on dietary arginine. The importance of dietary citrulline in correcting hyperammonemia in certain enzymopathies (CPS-I & OTC), in Reye's syndrome and in lysinuric patients was discussed earlier. From the discussions so far, it can be concluded that the intestinal/renal system is essential for endogenous arginine synthesis.

What is known about renal arginine synthesis?

The conversion of citrulline to arginine in the kidney is due to the action of argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) (**Table 1.6**). Argininosuccinate synthetase [E.C. 6.3.4.5] catalyzes the conversion of citrulline, aspartate and ATP to argininosuccinate, AMP and pyrophosphate (PP_i). This reaction is driven effectively in the forward direction by the removal of PP_i by cytosolic inorganic pyrophosphatase. The conversion of argininosuccinate to arginine and fumarate is catalyzed by argininosuccinate lyase [E.C. 4.3.2.1] and the negative free energy change associated with this reaction drives it in the direction of arginine formation. The apparent K_m values of AS (hog kidney) for citrulline, aspartate are 0.044 and 0.038 mM, respectively (Rochovansky & Ratner, 1967) and that of AL (beef liver) for argininosuccinate is 0.15 mM

(Ratner et al, 1953). Both AS and AL are predominantly found in the liver but are also found in significant quantities in kidney. Some activity of these enzymes is also observed in brain and spleen (Kato et al, 1976; Ratner & Murakami-Murofushi, 1980; **Table 1.5**). Both AS and AL are present in the cytosol of liver cells, but their cellular and subcellular location in the kidney is not well characterized. Szepesi et al (1970) reported that arginine synthetase activity (measured as AS plus AL) was predominantly found within the renal medulla. Morris et al (1989) have recently shown that the mRNA's for AS and AL are localized in the renal cortex. These two reports are in conflict and thus definitive information is needed on their exact location in the kidney.

Unanswered questions

From the discussions so far, it can be seen that no previous attempts have been made to localize precisely the site of arginine synthesis in the kidney. No information is available on the regulation of renal arginine synthesis *in vivo* by citrulline concentrations or by dietary arginine.

Objectives

1. To determine the cellular and subcellular localization of the enzymes of arginine synthesis in the rat kidney.

2. To determine the dependence of arginine synthesis on citrulline concentrations *in vitro* and *in vivo*.
3. To determine whether renal arginine synthesis can be regulated either by the levels of dietary arginine or dietary protein.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats [Charles River, Montreal, Canada] initially weighing 300-450 g were used for all experiments. They were housed individually in cages in a room kept at 24 ± 1°C with a 12:12 h light:dark cycle. The lights were off between 2000 h and 0800 h. The animals had ad libitum access to tap water and food.

Diets and feeding protocol

Animals used for experiments in **CHAPTER 3** and **CHAPTER 4**, were fed Purina rat chow ad libitum. For the experiments in **CHAPTER 5**, the animals were fed for 7d, diets containing varying amounts of arginine or protein. The composition of the arginine (0, 0.5 and 2.0%) and protein (5, 12 and 50%) diets are presented in **Table 5.1** and **Table 5.2**, respectively. The preparation and the feeding of these diets to rats are explained in detail in **CHAPTER 5**.

Preparation of homogenates from different kidnet regions

Rats were sacrificed by cervical dislocation and the kidneys were rapidly removed, decapsulated and placed in ice-cold homogenization medium (0.33 M sucrose, 5 mM HEPES, 1 mM EGTA (pH

7.4), 1 mM DTT added just before use). The kidneys were bisected and further cut into cones which clearly displayed the cortex, medulla and papilla. Each cone was dissected into 5 regions (**Figure 2.1**) starting from the outside: outer cortex, inner cortex, outer medulla, inner medulla, and papilla (Lowry et al ,1986). Tissue regions from 2 to 3 animals were pooled, and 10% homogenates prepared in the above homogenization medium to which 0.5% (w/v) (final concentration) Triton X-100 was added. The tissue was homogenized using a Polytron (Brinkman Instruments, Toronto, Canada) for 10-20 s at setting #20. Aliquots of these homogenates were used for enzyme and protein assays.

Preparation of cortex homogenate and subcellular fractionation

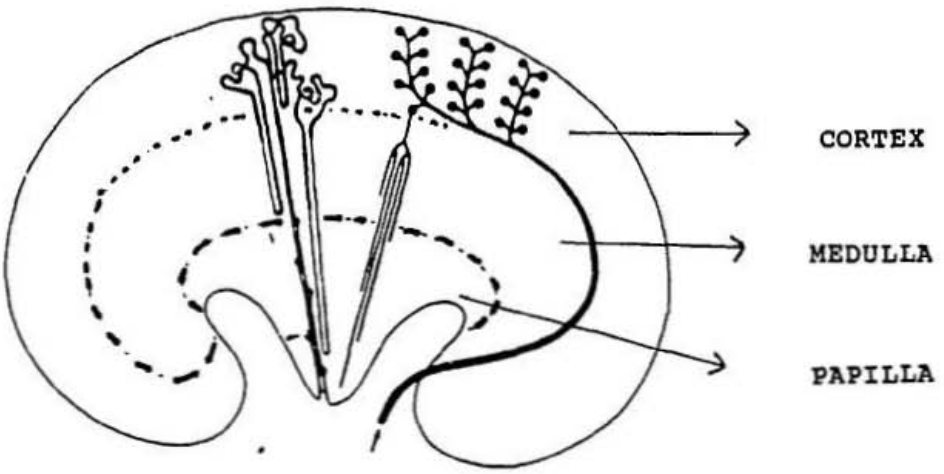
Animals were sacrificed by cervical dislocation and the kidneys were rapidly removed, decapsulated, and weighed after placing them in the above homogenization medium. Immediately after weighing, kidneys were rapidly demedullated, depapillated and then homogenized. Cortex homogenates (10%) were prepared in a Potter-Elvehjem homogenizer at 470 rpm (6 passes) with a loose-fitting Teflon pestle (clearance 0.3 mm). After filtration through two layers of cheese cloth, the cortex homogenate was fractionated by differential centrifugation. An aliquot of cortex homogenate was kept aside on ice after adding Triton X-100 (0.5% (w/v), final concentration). All procedures were carried out at 0-4^0 C.

The centrifugation procedures were essentially those of de

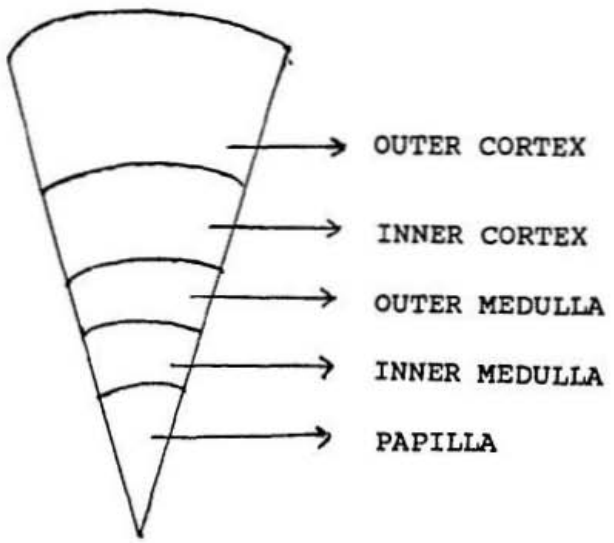
FIGURE 2.1

Cross-section of the kidney (A) and different regions of kidney (B)

A


CORTEX
MEDULLA
PAPILLA

B


OUTER CORTEX
INNER CORTEX
OUTER MEDULLA
INNER MEDULLA
PAPILLA

Duve et al (1955) with slight modifications as employed by Kalra and Brosnan (1974). The cortex homogenate was centrifuged in a JA-21 Beckman refrigerated centrifuge at 450xg for 2 min to give pellet and supernatant fractions. After decanting the supernatant, the pellet was washed once with the homogenization medium, and centrifuged as above to give the nuclear fraction. The supernatants were combined and centrifuged at 13,000xg for 10 min to give a lysosomal plus mitochondrial fraction which was also washed once. The resulting supernatant was then centrifuged in a Beckman L-50 ultracentrifuge at 105,000xg for 30 min to pellet the microsomal fraction. The final supernatant was the soluble cytosolic fraction. Each pelleted fraction was resuspended in 5 ml of homogenization medium containing 0.5% (w/v, final concentration) Triton X-100. The same concentration of Triton x-100 was added to the cytosolic fraction. All fractions including the original homogenate were subjected to Polytron homogenization for 20 s at setting #20.

Preparation of kidney cortical tubule fractions

Tubules were prepared from the kidney cortex of rats by the method of Guder et al (1971). Animals (3 to 4) were killed by cervical dislocation, the kidneys were rapidly removed, decapsulated and placed in about 25 ml of Krebs-Henseleit saline (final concentration :120 mM NaCl, 4.74 mM KCl, 0.84 mM KH_2PO_4, 0.84 mM $MgSO_4$, 24.9 mM $NaHCO_3$ and 1.27 mM $CaCl_2$), pH 7.4 (previously

gassed at 22^0 C with 95% O_2-5% CO_2 for 30 min). The kidneys were then cut in half with a knife and cortical slices (10 to 12/kidney) were obtained using a Stadie-Riggs microtome. The pooled slices (cortices) were washed 3 times with 25-30 ml ice-cold Krebs-Henseleit saline (KHS) solution and then resuspended in 20-30 ml KHS containing the following: 0.1% (w/v) of collagenase (obtained from Clostridium histolycum, grade II, Boehringer, Montreal, Canada), 0.2% bovine serum albumin (10% solution), 1 mM sodium pyruvate and 10 mM sodium lactate in a 250 ml Erlenmeyer flask. The contents of the flask were gassed for 2 to 3 min with 95%O_2-5%CO_2. The flasks were closed tightly with a rubber stopper, covered with Parafilm^R and placed in a Dubnoff shaking water bath (40 to 50 revolutions per min, setting #5) maintained at 37^0C for exactly 45 min. The digestion was terminated by adding approximately 30-ml of ice-cold KHS and the flask contents were gently shaken to disperse the fragments of tissue. The whole tubule suspension was filtered through a tea strainer to remove collagen fibres and the undigested tissue. The tubule suspension was spun in 50-ml polycarbonate tubes at 80 and 40xg for 20 and 40 s, respectively, in an International B-20 centrifuge (kept in cold room, 4°C). The supernatant was discarded and the tissue pellet rapidly suspended in 30 ml ice-cold KHS solution. This washing procedure was repeated three times. The final pellet was resuspended in 6 times its wet weight in ice-cold KHS solution. The viability of tubules during incubation was determined by measuring the leakage of the cytosolic enzyme, lactate

dehydrogenase (LDH) (Morrison et al, 1966). Aliquots of these suspensions were observed under a light microscope. The cortical suspension contained mainly proximal convoluted tubules and to a lesser extent, distal tubules and glomeruli as previously reported by Guder et al (1971). Aliquots of the tubule suspensions were used for protein and enzyme assays (for experiments in **CHAPTER 3**) and for dry weight and enzymatic or metabolic studies (**CHAPTER 4**).

Fractionation of kidney cortical tubules on a Percoll^{R}gradient

Kidney cortical tubules were prepared according to the procedure described above and were then fractionated on a Percoll^R (Pharmacia Fine Chemicals, Montreal, Canada) gradient as described by Vinay et al (1981). The cortical tubules were sedimented at 80xg for 1 min and the resulting pellet was mixed with 60 or 120 ml of previously gassed (95%O_2-5%CO_2 for 20 min at 22°C) ice-cold 50% Percoll solution. Percoll was diluted with water to give 50% solution and various salts were added to achieve a final concentration of the following: Sodium, 140 mM; chloride, 117 mM; HCO_3^-, 28mM; potassium, 4.9 mM; phosphate, 1.2 mM; and calcium, 2 mM. The pH and final osmolality of this solution were 7.4 and 300 mosmol/Kg, respectively. The Percoll solution, containing cortical tubules, was divided into 2 or 4, 30 ml portions in 50 ml polycarbonate tubes and after covering the tubes with Parafilm^R, were centrifuged at 12,500xg for 30 min at 4°C in a JA-21 Beckman centrifuge equipped with a fixed angle rotor head. This procedure

resulted in the separation of cortical tubules (F_t) into 4 distinct bands, F_1 (top) to F_4 (bottom). The bands were carefully removed from the Percoll gradient with a Pasteur pipette and transferred to 50-ml polycarbonate tubes. Each fraction was resuspended in ice-cold KHS and washed 3 times as described above. After the final centrifugation, the tubule fractions (F_1-F_4) and an aliquot of F_t, the original cortical tubule suspension, were resuspended in either 10 volumes of ice-cold homogenization medium (0.33 M sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4; 1 mM DTT and 0.5% (w/v) Triton X-100 were added just before use) for the determination of protein and enzyme activity (**CHAPTER 3**) or 6 volumes of ice-cold KHS solution for the determination of dry weight and measurement of arginine production (**CHAPTER 3** and **CHAPTER 4**). This allowed us to calculate the amount and the recovery (%) of tissue in each fraction and the total recovery (%) in terms of dry weight and protein. The tubules which were suspended in homogenization medium were homogenized using a Polytron for 10 to 20 s at setting #20.

The presence of different tubule fragments (proximal convoluted tubule, S_1 & S_2 segments; proximal straight tubule, S_3 segment; distal tubule) in the 4 fractions were determined by employing appropriate marker enzymes. The marker enzymes employed were: the glycolytic enzyme, hexokinase (distal tubule) (Schmidt et al, 1975); the brush border enzymes, γ-glutamyltranspeptidase (proximal straight tubule) and alkaline phosphatase (proximal convoluted and straight tubule) (Heinle etal, 1977; Schmidt & Dubach, 1971); and the gluconeogenic enzyme, phosphoenolpyruvete

carboxykinase (proximal convoluted and straight tubule) (Guder & Schmidt, 1974).

Arginine production in kidney cortical tubules and its fractions (metabolic studies)

Kidney cortical tubules were prepared and fractionated as described above. Cortical tubules and its fractions suspended in Krebs-Henseleit saline, KHS (pH 7.4) were used for determining arginine production (or synthesis) rates. About 1 mg dry weight of tubules were incubated with substrates (0.5 mM citrulline and 5 mM each of aspartate and lactate) in a total volume of 2 ml KHS solution (pH 7.4) equilibriated with 95%O_2-5%CO_2 for 30 min at 37^{0}C in a Dubnoff shaking water bath (40 strokes/min, setting #4). Incubations were started by the addition of tissue source, followed by gassing the contents of the flask (25-ml) with 95% O_2-5% CO_2 for 20 s and then the flask was stoppered with a rubber bung. Incubations were terminated by the addition of 0.2 ml 30% perchloric acid. Arginine formed was determined by high-pressure liquid chromatography which is described in the next section. Arginine synthesis as a function of time and tubule amount, and in the presence of different substrates (glutamine, glutamate, glycine, alanine, serine) and varying concentrations of citrulline (0 to 2.5 mM) were determined.

In one study (**CHAPTER 4**) the extent to which newly synthesized arginine was degraded by arginase in the cortical tubules was

determined. In this study [^{14}C]Ureidocitrulline was used as a substrate after purifying it (as supplied it contains 0.9 to 1.2% of [^{14}C]Urea as a contaminant) according to the method of Hurwitz and Kretchmer (1986). [^{14}C]Urea was determined in neutralized extracts as $^{14}CO_2$ after urease treatment. Briefly, an aliquot of PCA extract was neutralized with K_3PO_4 and the neutralized extract (pH 7.4) was incubated with 1 mg Jackbean urease (Sigma grade VI) at 37°C for 60 minutes. Incubations were terminated with 0.2 ml 30% PCA. $^{14}CO_2$ was collected in centre wells containing 0.2 ml NCS (Amersham) and counted in a scintillation counter.

Determination of Arginine by high-pressure liquid chromatography (HPLC)

Arginine formed in cortical tubules and its fractions was determined as follows. Following termination of incubation with perchloric acid (PCA), protein was removed by centrifugation, and an aliquot of the PCA extract was filtered through a 0.45 μm filter (Millipore). Arginine was determined by a modification of the HPLC procedure developed by Seiler et al (1985) and is described below. The modification used a gradient elution system instead of an isocratic procedure. Separation of arginine was achieved using a Beckman Ultrasphere ™I.P. column (4.6 mm i.d., 250 mm length) containing a 5 μm spherical silica core with chemically bonded C_{18}-groups. The separation column was guarded by a pre-column 2.1 mm i.d., 70 mm length) filled with pellicular ODS (C_{18}-groups bonded

to 37-53 μm particles; Whatman, Clifton, New Jersey). The gradient elution system consisted of 0.05 M sodium acetate, (pH 4.5), containing 10 mM octanesulfonic acid (OSA) and 10% methanol (buffer, A) and 0.2 M sodium acetate (pH 4.5), containing 10 mM OSA, 10:3 (v/v) acetonitrile and 10% methanol (buffer B). The column was eluted with 100% A for the first 5 minutes; then from 5-15 min 50% A + 50% B, and finally, for five minutes 100% B. Arginine eluted at about 17 min. After the run was completed, the column was washed for 10 min with 100% A prior to a new injection. The flow rate was maintained at 1 ml/min throughout. The identity of the arginine peak was confirmed both by spiking the PCA extract with arginine as well as by treatment with arginase, which eliminated the peak (**Figure 2.2**). Replicate analyses agreed within 3%. The recovery of arginine added to the samples was 99.0 ± 1.2%.

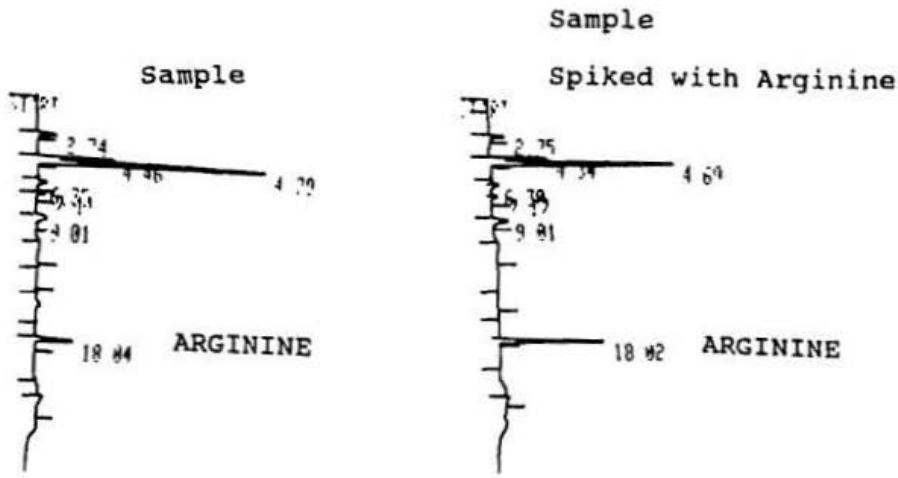
Renal flux of citrulline and arginine (*in vivo* studies)

Studies were carried out to measure renal citrulline removal and arginine synthesis in animals with elevated blood citrulline levels (citrulline infusion) and in control animals (saline infusion) [**CHAPTER 4**]. Also, the renal uptake of citrulline and release of arginine in animals fed different levels of dietary arginine or dietary protein were determined (**CHAPTER 5**). The question that arises is this: How is it possible to quantify the uptake or release of an amino acid across a particular organ ? What is the basis for such *in vivo* measurements ? One can measure the

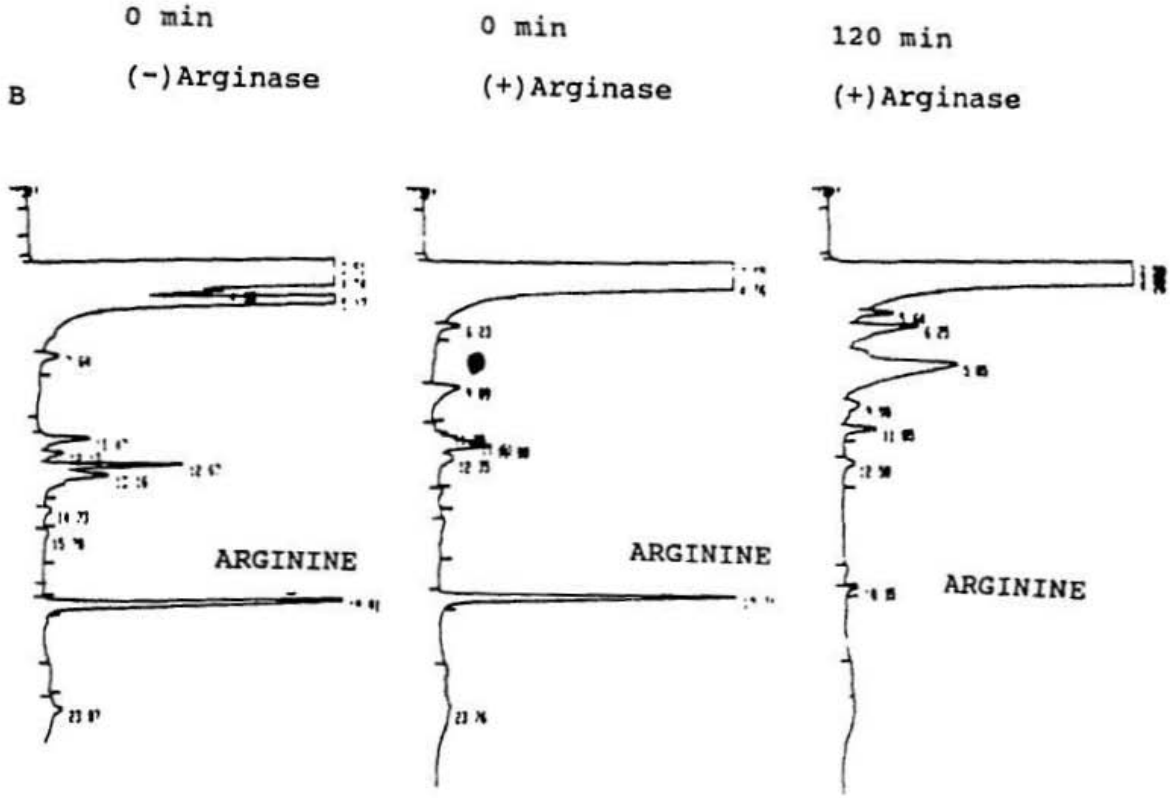
FIGURE 2.2

Confirmation of Arginine peak by spiking the sample with arginine (A) or treatment with arginase (B)

A


Sample
Sample
Spiked with Arginine
ARGININE
ARGININE

B


0 min
(-)Arginase
0 min
(+)Arginase
120 min
(+)Arginase
ARGININE
ARGININE
ARGININE

concentration of an amino acid in a small sample of the blood that enters (artery, A) or leaves (vein, V) the kidney. It should be borne in mind that redistribution of amino acids between blood cells and plasma can occur in its transit through the kidney. Thus, it is important to establish whether analysis of plasma samples alone would suffice in determining the uptake or release of an amino acid. The concentration difference (A-V) would indicate whether a particular amino acid is taken up (positive value) or released (negative value). The (A-V) difference for an amino acid may be very small and thus measurement requires great accuracy and precision. A simple (A-V) difference across kidneys for a particular amino acid gives only a qualitative picture and therefore requires quantification. When the (A-V) difference is multiplied by the blood flow one has a quantitative measure of the uptake or release of a particular amino acid by the kidney.

How does one measure renal blood flow? This is done by employing a substance that meets the following criteria: a) the mass should remain unaltered, b) it is neither synthesized nor metabolized by the kidney , c) it is not reabsorbed or secreted to any significant extent and finally, (d) it should not have any effect on renal function. Inulin, a fructose polysaccharide meets the above criteria and, therefore, was employed for the measurement of renal blood flow in the present studies. Inulin is also used for the measurement of glomerular filtration rate as it is freely filtered but is not reabsorbed or secreted to any significant

extent. By measuring the urine flow rate and the concentration of inulin in arterial and renal venous plasma and in urine, it is possible to calculate renal plasma flow (RPF) rate and glomerular filtration rate (GFR). If arterial hematocrit values are known, then one can calculate renal blood flow (RBF) from renal plasma flow. The calculations that are involved in determining RPF, RBF and GFR are explained elsewhere in this chapter.

The methodology used was similar to that previously employed in this laboratory (Lowry et al, 1987) for the measurement of renal blood flow. Briefly, after anaesthesia with sodium pentobarbital (60 mg/kg body weight, i.p.), the rat was placed on a heating pad and the trachea was cannulated with a small piece (2.5 cm long x 2.5 mm ID) of polyethylene tubing. The right jugular vein was then catheterized with PE-50 tubing (Clay-Adams, Parispany, New Jersey) for the infusion of inulin. For all the animals in this study, a priming dose of 1.75 μCi of [carboxyl-^{14}C]inulin (New England Nuclear, Lachine, Quebec) in 0.8 ml of 10% mannitol-0.45% NaCl was given through the saphenous vein and followed by the continuous infusion of the same solution at a rate of 0.037 ml/min using a Harvard Apparatus model 975 compact infusion pump. The mannitol present in the priming and infusion solution acts as a diuretic and thus helps in urine collection. The left ureter was then catheterized with a short piece of PE-10 tubing fitted inside a length of PE-50 tubing. Urine was collected between 20 and 40 min. At the end of the urine collection period, 1-ml blood samples were drawn from the left renal vein and the abdominal aorta. Blood from

abdominal aorta rather than the renal artery was drawn because it is easier to sample and there are no differences in the blood composition of any artery. The blood or plasma samples were deproteinized with sulfosalicylic acid containing 62.5 nmol of 2-Aminoethylcysteine (AEC) as an internal amino acid standard. This sample was used for the analysis of amino acids after removal of the precipitated proteins by centrifugation and adjustment of the supernatant to pH 2.2 ± 0.1 with lithium hydroxide. An aliquot (25 µl) of plasma was used for the determination of inulin radioactivity. Samples of the urine were treated in similar manner. Amino acid analysis was carried out by ion-exchange chromatography using a Beckman model 121 MB amino acid analyser (Brosnan et al, 1983). The experiments involving citrulline infusion were carried out identically to the controls except that citrulline was present at 100 mM in the priming solution and at 30 mM in the infusion solution. In these solutions, citrulline replaced an osmotically identical quantity of NaCl. Studies on renal arginine synthesis in animals fed different diets for 7 d (**CHAPTER 5**) were carried out identically to the controls above.

Preliminary experiments demonstrated that both ^{14}C-inulin and citrulline levels in blood were maintained at pleateau levels between 20 and 40 min (**Table 2.1**). Demonstration of plateau conditions in such experiments is essential because the calculation depends on a steady state. It was shown in preliminary studies that nearly 100% of citrulline, arginine and ornithine added to blood and plasma samples were recovered (**Table 2.2**).

TABLE 2.1

Plateau for ^{14}C-Inulin and Citrulline

	Inferior vena cava plasma (dpm/ml)		
Time (min)	0	20	40
^{14}C-Inulin	-	46886	46382

	Inferior vena cava blood (nmoles/ml)		
Time (min)	0	20	40
Citrulline	55.5	154.5	156.0

Animals were given a priming dose of a solution (0.8 ml) containing 1.75 μCi of ^{14}C-inulin, citrulline (100 mM), 10% mannitol-0.45% NaCl and infused with the same solution (citrulline, 30 mM) at the rate of 0.037 ml/min. Blood (inferior vena cava) samples were collected at the indicated times and analyzed for plasma inulin radioactivity or blood citrulline concentration. A representative experiment is shown.

TABLE 2.2

Recovery (%) of added citrulline, arginine and ornithine in whole blood or plasma[a]

Amino acid	Whole Blood	Plasma
Citrulline	104.0 ± 1.8	101.6 ± 13.1
Arginine	109.6 ± 13.1	100.2 ± 4.1
Ornithine	108.3 ± 12.3	96.9 ± 4.7

[a]Values are expressed as mean ± SD (n=3).

Enzyme assays

All enzyme assays were carried out on the same day on freshly isolated tissue, tubule fractions and sub-cellular fractions. The optimal amount of protein and incubation time for each enzyme (marker enzymes and enzymes of arginine metabolism) assay was chosen (**Table 2.3**) only after demonstrating linearity in enzyme activity with time and protein. The marker enzymes assayed were γ-glutamyltranspeptidase (Tate & Meister, 1974), alkaline phosphatase (Scholer & Edelman, 1979), phosphoenolpyruvate carboxykinase (Suebert & Huth, 1965), β-glucuronidase (Gianetto & de Duve, 1955) (measured at 37°C); succinate- and NADPH-cytochrome c reductase (Sottocassa et al, 1967), lactate dehydrogenase (Morrison et al, 1966) and hexokinase (Vinuela et al, 1963) all measured at room temperature (22 to 24°C).

The enzymes of arginine metabolism assayed were: argininosuccinate synthetase, argininosuccinate lyase, arginase and orthinine aminotransferase. The activities in kidney homogenates were demonstrated to be linear with time and protein for argininosuccinate synthetase (**Figure 2.3**), argininosuccinate lyase (**Figure 2.4**), arginase (**Figure 2.5**) and ornithine aminotransferase (**Figure 2.6**).

Argininosuccinate synthetase was assayed essentially according to the method of Nuzum and Snodgrass (1976) except that [^{14}C]Urea formed from [^{14}C]Ureidocitrulline was quantitated as $^{14}CO_2$ released after treatment with urease. The incubation mixture (pH 7.5)

TABLE 2.3

Assay conditions for marker enzymes and enzymes of arginine metabolism

	Protein (mg)	Incubation Time (min)
Marker Enzymes		
γ-glutamyltranspeptidase	0.08	4
Alkaline phosphatase	0.125	10
Phosphoenolpyruvate carboxykinse	0.5	10
Hexokinase	0.15	-
Succinate-cytochrome c reductase	0.128	-
β-glucuronidase	0.64	-
NADPH-cytochrome c reductase	0.32	-
Lactate dehydrogenase	0.065	-
Enzymes of Arginine Metabolism		
Argininosuccinate synthetase	0.25	30
Argininosuccinate lyase	1.0	15
Arginase	0.15	10
Orinithine aminotransferase	1.0	15

The enzyme activities as a function of time and protein concentration in whole kidney homogenates were determined. The indicated amount of protein and incubation time was chosen for the assay of the appropriate enzyme in tissue homogenates, in cortical tubule fractions or subcellular fractions of kidney cortex. When no incubation time is given, it indicates that these enzymes were continuously monitored with a recorder.

FIGURE 2.3

Argininosuccinate synthetase activity in rat kidney homogenate as a function of time (0-180 min with 0.26 mg protein) [A] and protein concentration (0-0.55 mg, 30 min incubation time) [B]. A representative experiment is shown.

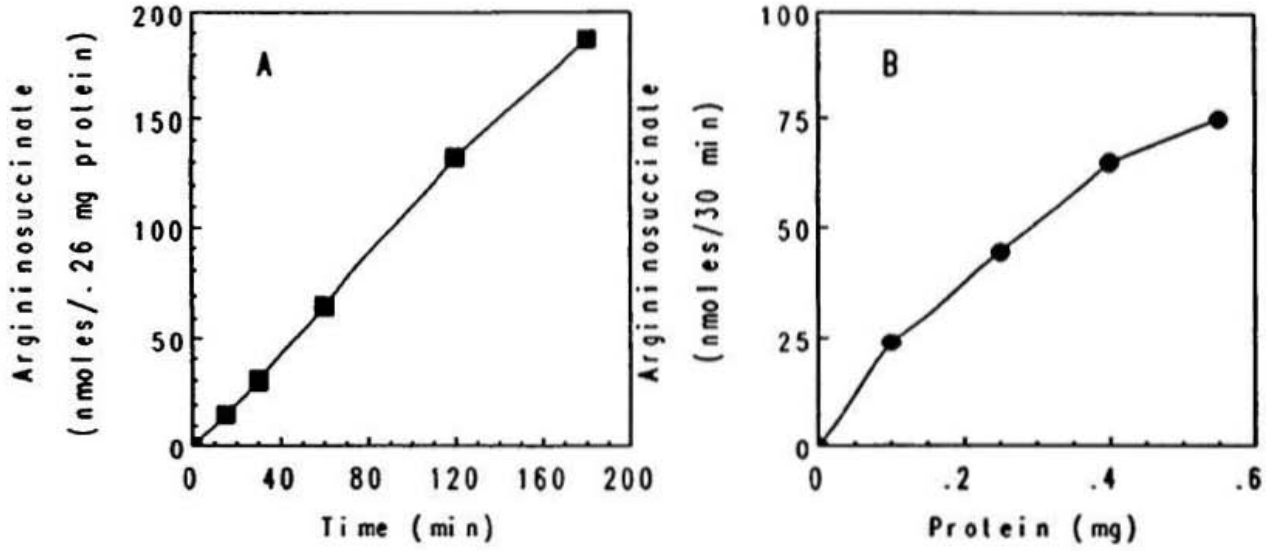

A
Argininosuccinate
(nmoles/.26 mg protein)
200
150
100
50
0
0
40
80
120
160
200
Time (min)
B
Argininosuccinate
(nmoles/30 min)
100
75
50
25
0
0
.2
.4
.6
Protein (mg)

FIGURE 2.4

Argininosuccinate lyase activity in rat kidney homogenate as a function of time (0-60 min with 1 mg protein) [A] and protein concentration (0-4 mg, 10 min incubation time) [B]. A representative experiment is shown.

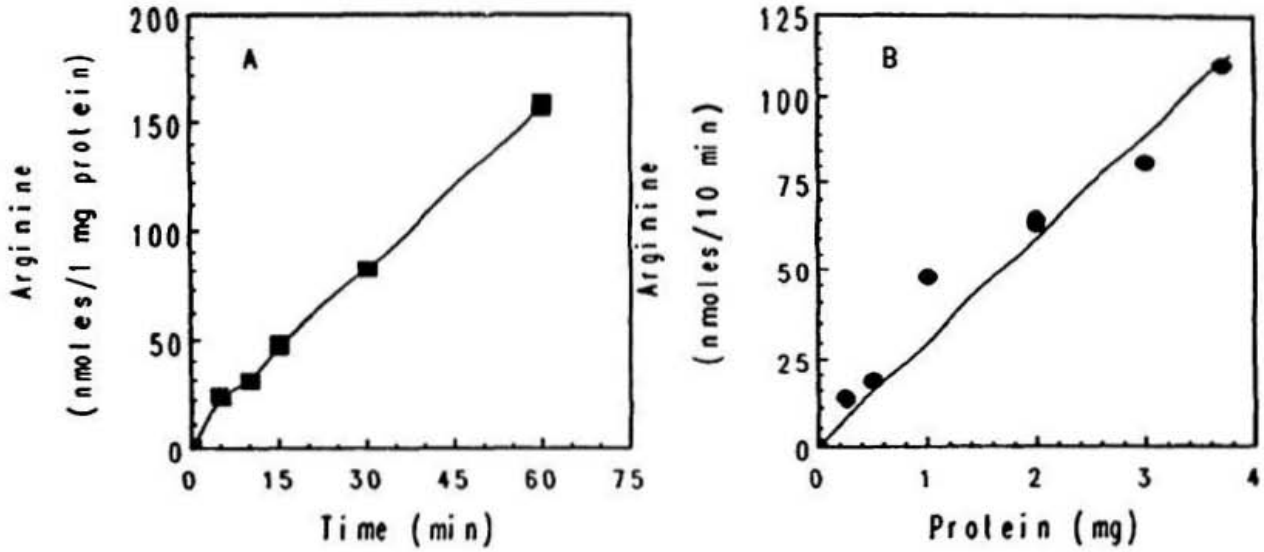

A
Arginine (nmoles/1 mg protein)
200
150
100
50
0
0
15
30
45
60
75
Time (min)
B
Arginine (nmoles/10 min)
125
100
75
50
25
0
0
1
2
3
4
Protein (mg)

FIGURE 2.5

Arginase activity in rat kidney homogenate as a function of time (0-60 min with 0.25 mg protein) [A] and protein concentration (0-0.75 mg, 10 min incubation time)[B]. A representative experiment is shown.

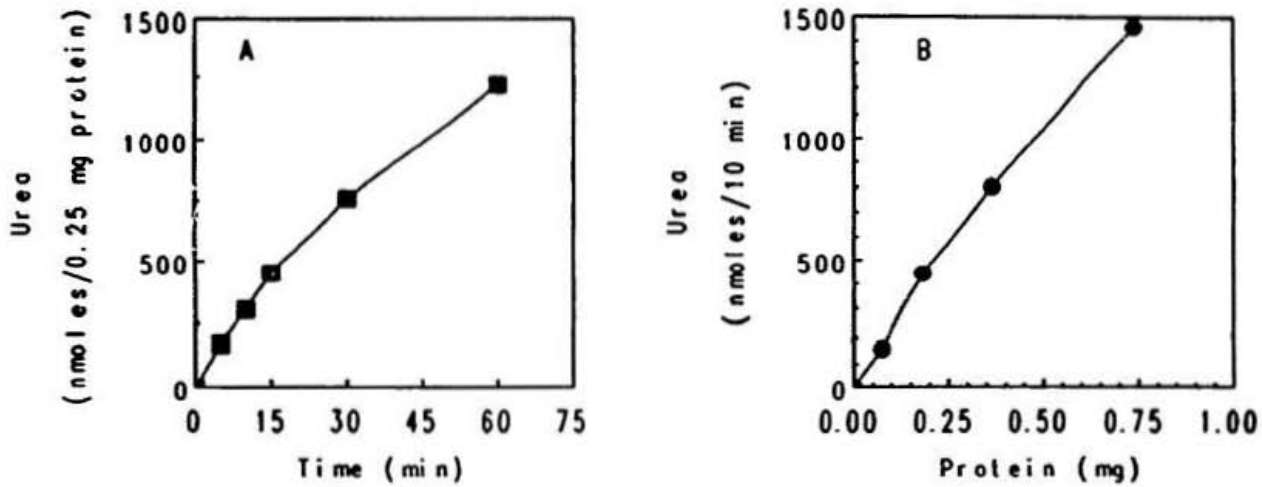
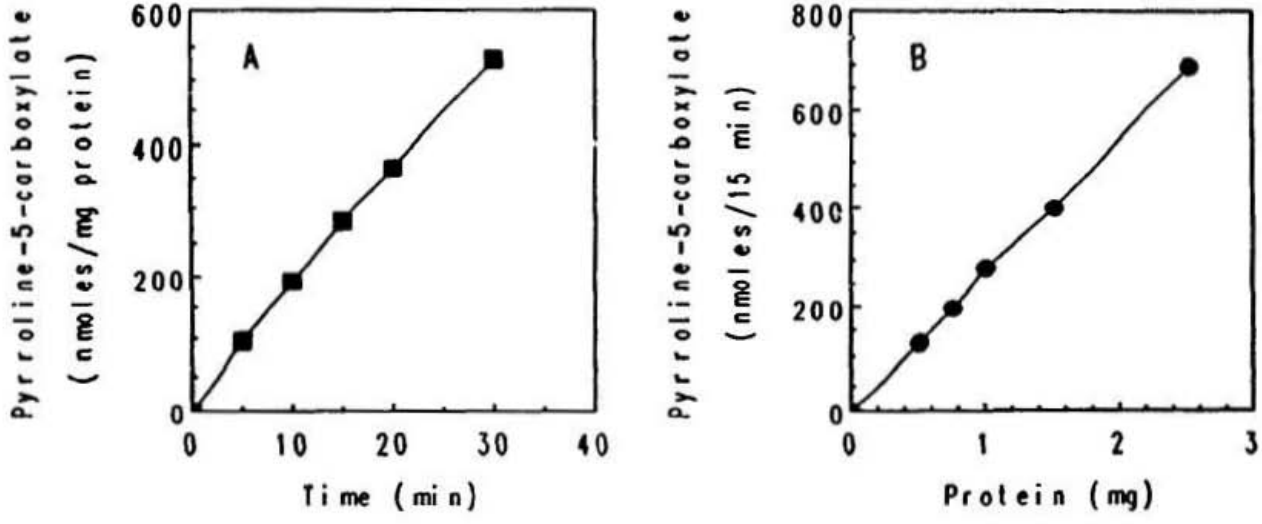

A
Urea
(nmoles/0.25 mg protein)
1500
1000
500
0
0
15
30
45
60
75
Time (min)
B
Urea
(nmoles/10 min)
1500
1000
500
0
0.00
0.25
0.50
0.75
1.00
Protein (mg)

FIGURE 2.6

Ornithine aminotransferase activity in rat kidney homogenate as a function of time (0-30 min with 1 mg protein [A] and protein concentration (0-2.5 mg, 15 min incubation time) [B]. A representative experiment is shown.


A
Pyrroline-5-carboxylate
(nmoles/mg protein)
600
400
200
0
0
10
20
30
40
Time (min)
B
Pyrroline-5-carboxylate
(nmoles/15 min)
800
600
400
200
0
0
1
2
3
Protein (mg)

(containing about 0.25 mg protein in a total volume 1.0 ml) contained the following at the indicated final concentrations: 50mM KH_2PO_4, 5mM potassium aspartate, 5mM [^{14}C]Ureidocitrulline, (370 KBq/mmole), 2mM ATP, 2mM $MgSO_4$, 2mM phosphoenolpyruvate, 3 units pyruvate kinase (Rabbit muscle, Type II Sigma), 0.8 units argininosuccinate lyase (Bovine liver type IV, Sigma), 5 units arginase (Bovine liver, type IV, Sigma), 14 units urease (Jack bean, Sigma type VI). The incubations were carried out for 0 and 30 min at 37°C in flasks fitted with center wells containing 0.2 ml NCS (Amersham) and terminated by injecting 0.2 ml 30% perchloric acid through the rubber cap. After 60 minutes, $^{14}CO_2$ collected in center wells was counted in a scintillation counter.

Argininosuccinate lyase was assayed according to the method of Nuzum and Snodgrass, (1976) with slight modifications. Briefly, the incubation mixture (pH 7.0) contained 20 mM argininosuccinate, 129 mM NaH_2PO_4, 64.5 mM EDTA and 1 mg protein in a total volume of 1.0 ml. Incubations were terminated with 0.5 ml 20% trichloroacetic acid after 0 and 15 min at 37°C. Aliquots of supernatants, after protein removal by centrifugation, were used for arginine determination by the Sakaguchi color reaction as described by Van Pilsum et al (1956).

Arginase was assayed using 0.15 mg protein at 37° for 10 minutes by the method of Herzfeld & Raper (1976) after heat activation of the various homogenates at 55°C for 5 min in the presence of 50 mM $MnCl_2$. Urea formed in the arginase assay was quantitated by the procedure of Geyer & Dabich (1971). Ornithine

aminotransferase activity was determined using 1.0 mg protein (15 min at 37°C) by measuring the formation of glutamic-γ-semialdehyde by the procedure of Periano & Pitot (1963) as modified by Pegg et al (1970).

Protein and DNA measurement

Protein was measured by the biuret method (Gornall et al, 1949), following solubilization with deoxycholate (Jacobs et al, 1956), using bovine serum albumin as standard. DNA was extracted by the method of Schneider (1945) and was determined with diphenylamine reagent (Burton, 1956) using calf thymus DNA as standard.

Chemiacals

All chemicals were obtained from Sigma Chemical (St. Louis, Mo) with the exception of [^{14}C]Ureidocitrulline and [^{14}C]Carboxylinulin (New England Nuclear, Lachine, Quebec).

Calculations

Glomerular filtration rate (GFR) was calculated from urinary inulin excretion in the 20 to 40 min clearance period. Renal plasma flow (RPF) was calculated using the expression derived by Wolf (1941). Renal plasma flow was calculated using the expression derived by Wolf (1941). The calculations that are involved in the measurement of renal plasma or blood flow and GFR is as follows:

$$\text{U/P (Inulin)} = \frac{\text{Inulin Counts in Urine (dpm/ml)}}{\text{Inulin Counts in arterial plasma (dpm/ml)}}$$

$$\text{GFR (ml/min)} = \text{U/P (Inulin)} \times \text{Urine flow rate (ml/min)}$$

Renal Plama Flow (RPF) Rate is calculated by determining the inulin counts in urine, in arterial and renal venous plasma and also urine flow rate (UFR) (Wolf, 1941).

$$\text{RPF} = \frac{\text{Urine (dpm/ml)} - \text{Renal venous plasma (dpm/ml)}}{\text{Arterial Plasma (dpm/ml)} - \text{Renal venous plasma (dpm/ml)}} \times \text{UFR}$$

$$\text{Renal Blood Flow Rate} = \frac{\text{Renal Plasma Flow Rate}}{1 - (\text{Arterial blood hematocrit}/100)}$$

The values of GFR, Renal plasma or blood flow rates are multiplied by 2 (for 2 kidneys) and are expressed as ml/min/100 g body weight.

Statistical analysis

All results are expressed as means $\pm$ SD. Significant differences between individual means were determined using paired or unpaired t test as appropriate (**CHAPTER 4**). Significant differences between individual treatments and the totals were determined by Dunnett's procedure (**Table 4.1**). Significant differences between individual means were determined by one-way

analysis of variance [ANOVA] followed by Tukey's test for multiple comparisons (**CHAPTER 5**) and differences were considered to be significant, if $P < 0.05$.

CHAPTER 3

CELLULAR AND SUBCELLULAR LOCALIZATION OF ENZYMES OF ARGININE METABOLISM IN RAT KIDNEY

Synopsis

Rat kidneys extract citrulline derived from the intestinal metabolism of glutamine, and convert it stoichiometrically to arginine. This pathway constitutes the major endogenous source of arginine. To identify the location of endogenous arginine synthesis, I have carried out studies to localize the enzymes of arginine synthesis (argininosuccinate synthetase and lyase) and of breakdown (arginase and ornithine aminotransferase) in 5 regions of rat kidney, in cortical tubule fractions and in subcellular fractions of cortex. Argininosuccinate synthetase and lyase were found almost exclusively in cortex. Arginase and ornithine aminotransferase were found in inner cortex and outer medulla. Since cortical tissue primarily consists of proximal convoluted and straight tubules, distal tubules and glomeruli, I prepared cortical tubule fragments by collagenase digestion of cortices and fractionated them on a Percoll^{R} gradient. Argininosuccinate synthetase and lyase were found to be markedly enriched in proximal convoluted tubules whereas less than 10% of arginase and ornithine aminotransferase were recovered in this fraction. Arginine production from citrulline was also enriched in proximal convoluted tubules. Subcellular fractionation of kidney cortex revealed that argininosuccinate synthetase and lyase are cytosolic. Thus, these studies led me to conclude that arginine synthesis occurs in the cytoplasm of the cells of the proximal convoluted tubule.

Introduction

Arginine is primarily synthesized in the kidney from citrulline which arises from the intestinal metabolism of glutamine (Windmueller and Spaeth, 1981). The enzymes of arginine synthesis from citrulline are argininosuccinate synthetase (L-citrulline: L-aspartate ligase, E.C. 6.3.4.5) and argininosuccinate lyase (L-argininosuccinate arginine-lyase, E.C. 4.3.2.1). Their location in the kidney is not well characterized. Szepesi et al (1970) reported that arginine synthetase activity (measured as argininosuccinate synthetase plus argininosuccinate lyase) was predominantly found within the renal medulla. Morris et al (1989) have recently shown that the mRNAs for argininosuccinate synthetase and argininosuccinate lyase are localized in the renal cortex. These two reports are in conflict. The kidney also contains enzymes that can catabolize arginine, specifically arginase (L-arginine aminohydrolase E.C. 3.5.3.1) and ornithine aminotransferase (E.C.2.6.1.13). Szepesi et al (1970) found arginase in both cortex and medulla. Levillain et al (1989) have shown by microdissecting the nephron that arginase is almost exclusively localized in the proximal straight tubule. It has been established that arginase is predominantly found in the mitochondrial matrix of rat kidney (Skrzypek-Osiecka & Porembska, 1983). Ornithine aminotransferase has been found in the renal mitochondrial fraction (Peraino & Pitot, 1963) but its cellular location has not been established.

Because of the importance of renal arginine synthesis,

definitive information on the location of the enzymes involved is required. Based on the distribution of specific markers employed for different kidney regions, cortical tubule fractions and subcellular fractions of kidney cortex, I have concluded that the enzymes of arginine synthesis are exclusively present in the cytoplasm of the cells of the proximal convoluted tubule and that the catabolic enzymes are enriched in other kidney regions.

Objective

To determine the location of enzymes of arginine synthesis (argininosuccinate synthetase and argininosuccinate lyase) and, of removal (arginase and ornithine aminotransferase) in rat kidney.

Animals

In all experiments, male Sprague-Dawley rats (Charles River, Montreal, Canada) weighing 350-450 g were used. They were allowed water and Purina rat chow ad libitum.

Results

Distribution of enzymes of arginine metabolism in different kidney regions

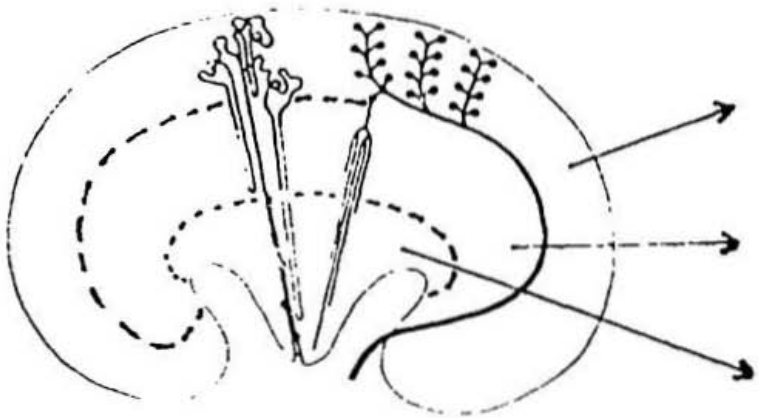
In a preliminary experiment, the activity of one of the enzymes of arginine synthesis, argininosuccinate lyase, was determined (**CHAPTER 2**) in the homogenates of cortex, medulla and

papilla of rat kidney. The activity of the enzyme was predominantly found in the cortex (**Figure 3.1**). This suggested to me that the enzymes of arginine synthesis are localized in the cortex.

Since heterogeneity exists within cortex itself, the strategy for establishing the cellular and subcellular localization of the renal enzymes of arginine metabolism relied on the use of defined marker enzymes. This is especially important with respect to the kidney where such terms as inner cortex, outer medulla, etc. seem to mean different things in different laboratories. The discrepancies in the location of these enzymes mentioned in the **Introd''tion** may relate to this problem. Accordingly, I have used the terminology recommended by Kriz and Bankir (1988) for the different parts of the nephron as is shown in **Figure 3.2**. The main cortical components of the nephron are glomerulus, proximal convoluted, straight tubule and distal tubule. Accordingly, I have employed, as markers, enzymes whose locations have been unambiguously established. Two enzymes are known to be localized in the brush border membrane of the proximal tubule. Alkaline phosphatase is found throughout the length of the proximal tubule (S_1, S_2 and S_3), (Schmidt & Dubach, 1971), while γ-glutamyltranspeptidase is highly enriched in the straight segment (S_3) of this tubule (Heinle et al, 1977). Phosphoenolpyruvate carboxykinase , a gluconeogenic enzyme, is found throughout the proximal tubule, with somewhat higher activities being found in the early convoluted (S_1, S_2) portions (Guder & Schmidt, 1974). Hexokinase, a glycolytic enzyme, is essentially absent from the proximal tubule but is present in all later tubular segments (Schmidt et al, 1975). The principal utility of hexokinase in the

FIGURE 3.1

The activity of argininosuccinate lyase
in different kidney regions

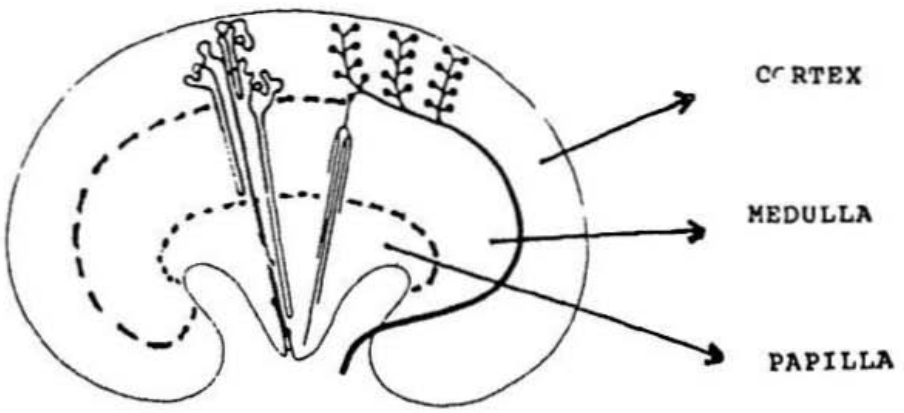


Kidney Region	Activity (μmoles/h/g wet wt. of kidney)
CORTEX	30.2
MEDULLA	0.4
PAPILLA	0.2

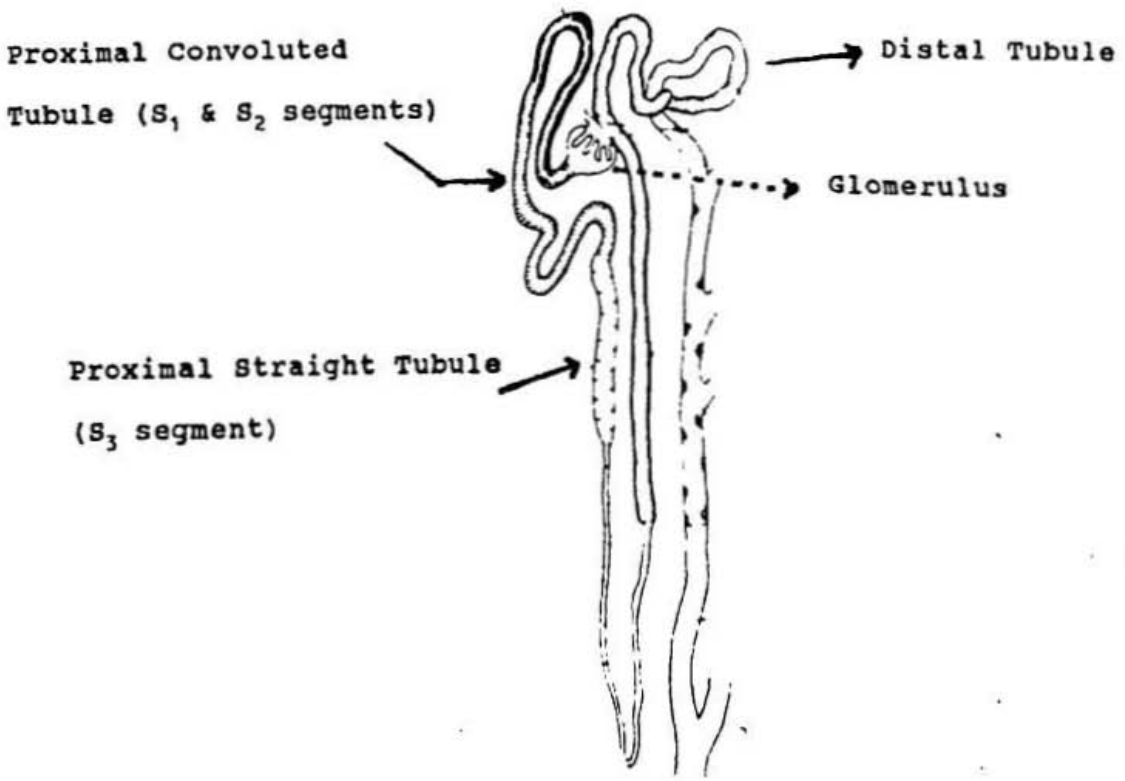
FIGURE 3.2

A cross section of the kidney is shown (**A**). The three distinct regions are cortex, medulla and papilla. The main components of cortical nephron are , glomerulus, proximal convoluted tubule (S_1 and S_2 segments), proximal straight tubule (S_3 segment) and distal tubule and are shown in **B**. The figure was taken from "Metabolic Compartmentation", Ross and Guder, pp. 363-409, 1982 (edited by H. Sies Academic Press, NY).

A


CORTEX
MEDULLA
PAPILLA

B


Proximal Convoluted
Tubule (S_1 & S_2 segments)
Distal Tubule
Glomerulus
Proximal Straight Tubule
(S_3 segment)

present context is that very low activities are diagnostic of proximal tubules. One of the enzymes of arginine utilization, arginase, has been established (reported when the present studies were in progress), by microdissection studies, to be highly enriched in the proximal straight tubule (S_3) (Levillain et al, 1989) and thus it should closely follow the distribution of γ-glutamyltranspeptidase.

The specific and total activities and recoveries for the marker enzymes and enzymes of arginine metabolism in different zones of rat kidney are presented in **Table 3.1.** I have also presented the data in the form of histograms (**Figure 3.3**) which depict the specific actvity of an enzyme plotted against protein content in a particular zone of kidney. The protein content in cortex (outer and inner), medulla (outer and inner) and papilla, accounted for about 83%, 15% and 2.6%, respectively.

The specific activities of the brush border enzymes γ-glutamyltranspeptidase and alkaline phosphatase were highest in the inner cortex but high activities were also evident in the outer cortex and medulla. About 85% of γ-glutamyltranspeptidase and alkaline phosphatase were found in the cortex with 41 and 48%, respectively, being recovered in outer cortex alone. These results are in agreement with those reported by Lowry et al (1985 & 1986).

Phosphoenolpyruvate carboxykinase was essentially restricted to the cortex and was predominantly found in the outer cortex which also had the highest specific activity. The distribution of argininosuccinate synthetase and argininosuccinate lyase was very similar to that of phosphoenolpyruvate carboxykinase in that the cortex contained about 90-95% of each enzyme with the greatest

Table 3.1 Distribution of enzymes of arginine metabolism in different regions of the rat kidney

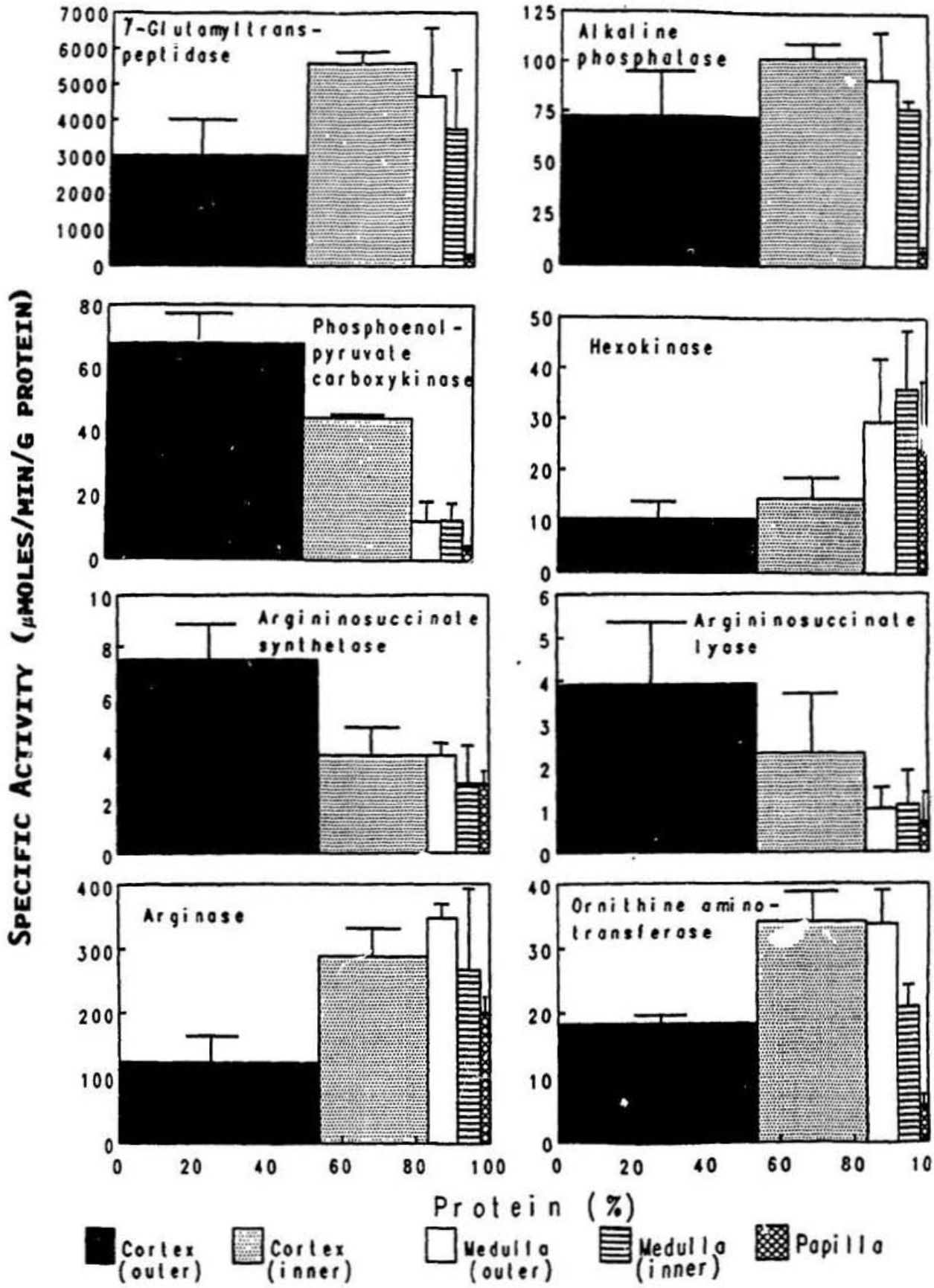
The enzyme activities were assayed as described under "Materials and Methods". All specific activities are expressed as μ moles/min/g protein. Total activity is expressed as μ moles/min/region. Values are mean ±SD for 3 experiments. Values in parentheses represent % recovery.

KIDNEY REGION		CORTEX (Outer)	CORTEX (Inner)	MEDULLA (Outer)	MEDULLA (Inner)	PAPILLA
Protein	(mg/g Wet weight of whole kidney)	628 ± 53.5 (53.9)	343 ± 36.9 (29.4)	95.2 ± 11.6 (8.2)	69.7 ± 2.1 (6.0)	29.6 ± 11.7 (2.6)
γ-glutamyl transpeptidase	Specific Activity	2992 ± 894	5566 ± 338	4638 ± 1936	3735 ± 1653	274 ± 39
	Total Activity	1846 ± 332 (41.2)	1921 ± 260 (43.3)	447 ± 182 (9.7)	260 ± 92 (5.6)	8 ± 3 (0.2)
Alkaline phosphatase	Specific Activity	72.6 ± 18.8	102.4 ± 7.6	91.3 ± 24.4	77.1 ± 4.1	7.4 ± 2.1
	Total Activity	44.9 ± 6.6 (48.1)	34.9 ± 1.2 (37.9)	8.8 ± 2.6 (9.3)	4.2 ± 0.9 (4.5)	0.2 ± 0.1 (0.2)
Phosphoenol pyruvate carboxykinase	Specific Activity	67.7 ± 9.9	44.1 ± 1.1	12.6 ± 6.3	12.0 ± 5.3	3.9 ± 0.8
	Total Activity	42.3 ± 4.6 (70.9)	15.1 ± 1.1 (25.4)	1.2 ± 0.6 (2.1)	0.8 ± 0.3 (1.4)	0.1 ± 0.1 (0.2)
Hexokinase	Specific Activity	10.4 ± 3.2	14.4 ± 4.1	29.5 ± 12.4	35.9 ± 11.7	24.1 ± 13.3
	Total Activity	6.6 ± 1.9 (37.6)	4.9 ± 1.0 (28.7)	2.7 ± 0.7 (15.7)	2.5 ± 0.7 (14.4)	0.6 ± 0.1 (3.6)
Arginino-succinate synthetase	Specific Activity	7.5 ± 1.9	3.8 ± 1.1	3.8 ± 0.5	2.7 ± 1.5	2.7 ± 0.5
	Total Activity	4.7 ± 1.3 (70.6)	1.3 ± 0.4 (19.9)	0.4 ± 0.1 (5.6)	0.2 ± 0.1 (2.7)	0.1 ± 0.1 (1.2)
Arginino-succinate lyase	Specific Activity	3.9 ± 1.6	2.3 ± 1.4	1.0 ± 0.5	1.1 ± 0.8	0.7 ± 0.7
	Total Activity	2.4 ± 0.7 (73.0)	0.8 ± 0.4 (21.5)	0.1 ± 0.1 (2.9)	0.1 ± 0.1 (2.1)	0.1 ± 0.1 (0.5)
Arginase	Specific Activity	124 ± 26.5	289 ± 43	348 ± 22	267 ± 125.5	201 ± 24.5
	Total Activity	77.2 ± 9.9 (33.0)	99.6 ± 18.0 (42.3)	33.3 ± 4.7 (14.2)	18.6 ± 7.0 (7.9)	6.0 ± 2.1 (2.7)
Ornithine aminotrans-ferase	Specific Activity	18.2 ± 1.7	34.1 ± 4.6	33.7 ± 5.2	20.8 ± 3.4	5.5 ± 1.7
	Total Activity	11.4 ± 0.2 (41.1)	11.8 ± 2.1 (41.7)	3.2 ± 0.7 (11.5)	1.4 ± 0.2 (5.2)	0.2 ± 0.1 (0.5)

FIGURE 3.3

Distribution of enzymes of arginine metabolism
in different regions of the rat kidney

The results presented in **Table 3.1** are shown here in the form of histograms. The figure shows the specific activity of enzyme plotted against protein content in different zones of kidney.


γ-Glutamyltrans-peptidase
Alkaline phosphatase
Phosphoenol-pyruvate carboxykinase
Hexokinase
Argininosuccinate synthetase
Argininosuccinate lyase
Arginase
Ornithine amino-transferase
Specific Activity (μMOLES/MIN/G PROTEIN)
Protein (%)
Cortex (outer)
Cortex (inner)
Medulla (outer)
Medulla (inner)
Papilla

amount and the highest specific activity being found in the outer cortex.

The specific activity of hexokinase was lower in outer cortex than in medulla and papilla. About 38% of hexokinase was found in outer cortex. From previous studies (Vinay et al, 1981) it is known that cortical hexokinase is associated with distal tubules. Arginase and ornithine aminotransferase were similarly distributed between the regions. In each case the bulk of the enzyme was found in cortex and the highest enrichments in the inner cortex and outer medulla.

The predominant location of argininosuccinate synthetase, argininosuccinate lyase, and of arginase and ornithine aminotransferase in the kidney cortex prompted me to determine the abundance of these enzymes in different tubule elements of the cortical nephron.

Distribution of enzymes of arginine metabolism in kidney cortical tubule fractions

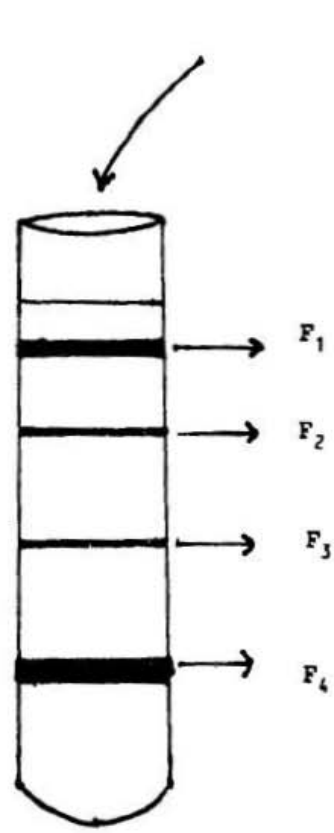
Experiments aimed at locating the enzymes of arginine metabolism in different tubule elements of the cortical nephron (**Figure 3.2**) were carried out. Separation of these structural elements of the nephron was attempted by fractionating cortical tubules (obtained by collagenase digestion of kidney cortices) on a Percoll^{R} gradient (**CHAPTER 2**). Fractionation of cortical tubules (F_t) yielded four different fractions (F_1-F_4) (**FIGURE 3.4**). The activities of enzymes of arginine metabolism were assayed in these fractions, including F_t, the original tubule suspension.

FIGURE 3.4

Isolation of cortical tubule fractions

Cortical tubule suspension (F_t) prepared by the collagenase digestion of kidney cortices and was fractionated on a Percoll^R gradient (Vinay et al, 1981). Four different fractions (F_1-F_4) were obtained.

Cortical Tubule Suspension



The data are shown in **Table 3.2.** The data are also presented in the form of histograms (**Figure 3.5**) which show the relative specific activity of an enzyme (specific activity in tubule fraction divided by the specific activity in F_t) against protein content in a particular tubule fraction. These histograms offer a great variety of distribution patterns. The area of each block is proportional to the percentage of activity recovered in the corresponding fraction and its height to the degree of purification achieved over the original tubule suspension.

The total recovery of protein in tubule fractions F_1 to F_4 from the original cortical tubule suspension (F_t) was about 60 to 63% and slightly lower than that obtained (70%) by Vinay et al, (1981). Loss of protein was attributed by these authors to the washing procedures employed for the removal of Percoll^R. The specific activity (μmoles/min/g protein) of γ-glutamyltranspeptidase was higher (3167 vs. 1319) in F_1 than in F_4 and that of alkaline phosphatase (370 vs 230) in F_4 than in F_1 fractions. The recovery of γ-glutamyltranspeptidase in F_1 alone was 23 % and that in F_4, 9%, whereas for alkaline phosphatase the recoveries in F_1 and F_4 were 17 and 27%, respectively. The total recoveries for γ-glutamyltranspeptidase and alkaline phosphatase were 58 and 73%, respectively and were in agreement with the data of Vinay et al (1981).

A 1.5-fold increase in specific activity was observed for the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (marker for proximal convoluted and straight tubules) in the F_4 fraction. The ratio of F_4/F_1 was 1.88 and the total recovery of the enzyme was about 72%. The recovery of the enzyme in F_4 was almost twice that

Table 3.2 Distribution of enzymes of arginine metabolism in rat kidney cortical tubule fractions

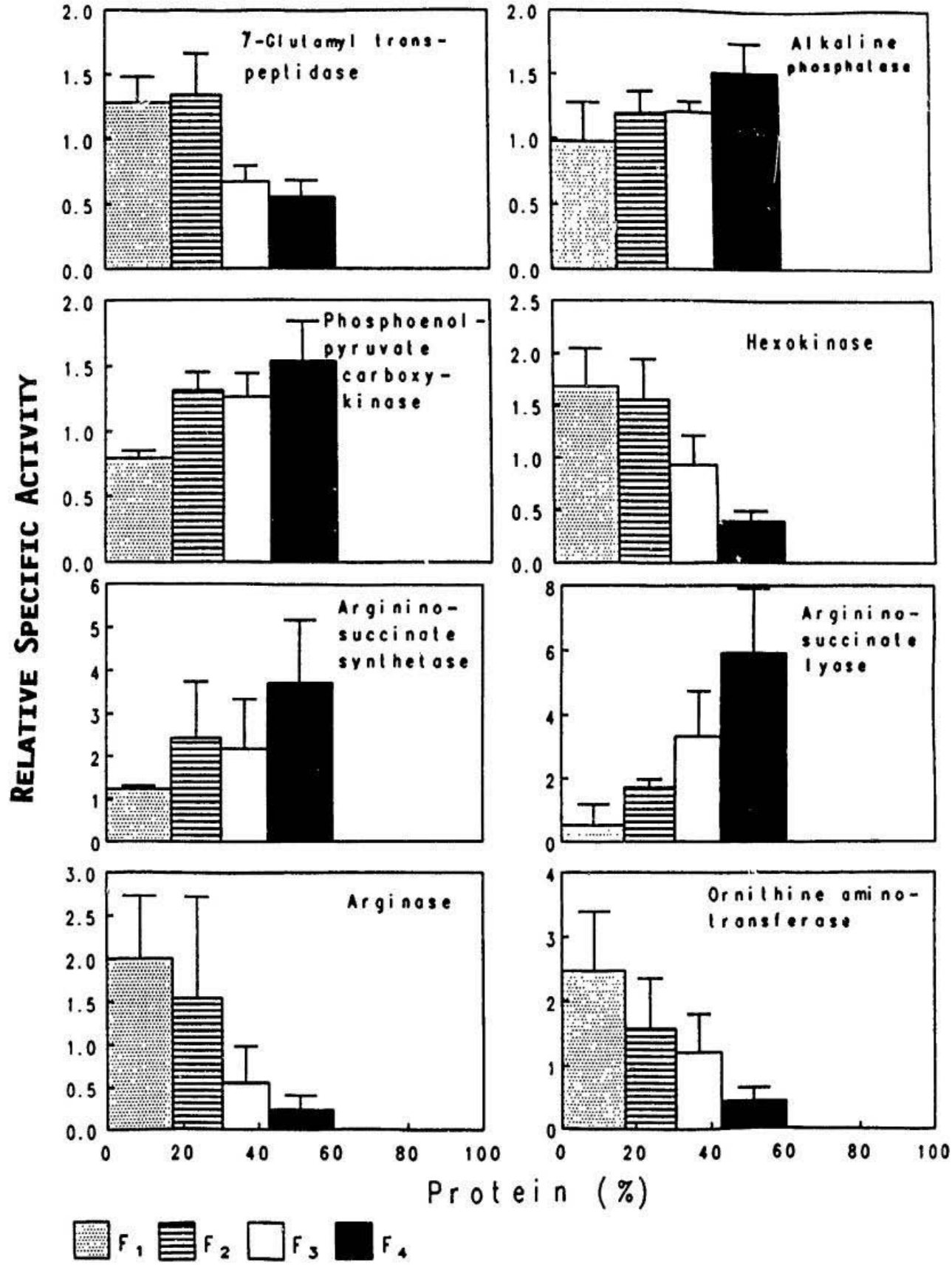
Kidney cortical tubules were prepared by collagenase digestion and then fractionated on Percoll® gradient. F_t is the fraction before fractionation. F_1, F_2, F_3 and F_4 are the fractions obtained after fractionation. The enzyme activities were assayed as described under "Materials and Methods". All specific activities are expressed as μ moles/min/g protein. Total activity is expressed as μ moles/min/fraction. Values are mean $\pm$SD for 3 experiments. Values in parentheses represent % recovery.

TUBULE FRACTION		F_t	F_1	F_2	F_3	F_4	F_4/F_1
Protein	(mg)	83.9 ± 24.6 (100)	14.4 ± 2.1 (17.1)	11.3 ± 2.9 (13.5)	9.0 ± 2.3 (12.1)	14.6 ± 4.6 (17.4)	
γ-glutamyl transpeptidase	Specific Activity	2469 ± 928	3167 ± 692	3311 ± 1884	1586 ± 276	1319 ± 458	0.42
	Total Activity	191.9 ± 17.5 (100)	44.6 ± 3.5 (23.3)	34.3 ± 10.7 (18.1)	14.0 ± 2.6 (7.2)	17.8 ± 1.0 (9.4)	
Alkaline phosphatase	Specific Activity	252.4 ± 94.9	230.2 ± 11.8	295.2 ± 89.3	300 ± 93.3	370.1 ± 81.8	1.61
	Total Activity	19.8 ± 3.4 (100)	3.31 ± 0.50 (16.9)	3.20 ± 0.36 (16.3)	2.56 ± 0.20 (13.1)	5.14 ± 0.82 (26.5)	
Phospho-enolpyruvate carboxylinase	Specific Activity	47.5 ± 11.8	37.4 ± 10.2	61.8 ± 14.3	58.8 ± 7.9	70.2 ± 5.5	1.88
	Total Activity	3.88 ± 1.21 (100)	0.52 ± 0.1 (14.0)	0.67 ± 0.07 (18.0)	0.53 ± 0.14 (13.7)	1.02 ± 0.33 (26.4)	
Hexokinase	Specific Activity	9.0 ± 0.06	15.2 ± 3.2	14.0 ± 3.6	8.4 ± 2.5	3.5 ± 0.9	0.23
	Total Activity	0.76 ± 0.22 (100)	0.22 ± 0.08 (29.4)	0.16 ± 0.06 (21.2)	0.08 ± 0.03 (9.8)	0.05 ± 0.02 (6.7)	
Argininosuccinate synthetase	Specific Activity	3.20 ± 0.74	3.97 ± 1.09	7.9 ± 4.84	7.42 ± 5.41	12.34 ± 7.19	3.11
	Total Activity	0.256 ± 0.026 (100)	0.056 ± 0.006 (2.21)	0.086 ± 0.05 (34.1)	0.058 ± 0.03 (23.5)	0.158 ± 0.05 (62.8)	
Argininosuccinate lyase	Specific Activity	2.36 ± 1.94	1.26 ± 0.56	3.88 ± 2.82	9.02 ± 8.0	13.62 ± 9.60	10.81
	Total Activity	0.17 ± 0.1 (100)	0.019 ± 0.01 (14.4)	0.041 ± 0.02 (23.3)	0.069 ± 0.05 (35.4)	0.178 ± 0.12 (101.7)	
Arginase	Specific Activity	71.2 ± 38.5	132.9 ± 65 0	79.8 ± 31.9	29.7 ± 7.3	12.0 ± 4.0	0.09
	Total Activity	5.96 ± 4.0 (100)	1.93 ± 1.0 (34.6)	0.90 ± 0.46 (21.6)	0.27 ± 0.11 (5.7)	0.17 ± 0.08 (3.8)	
Ornithine amino-transferase	Specific Activity	9.3 ± 3.9	20.7 ± 5.5	13.2 ± 4.8	11.1 ± 7.9	3.9 ± 2.6	0.19
	Total Activity	0.71 ± 0.14 (100)	0.30 ± 0.08 (41.9)	0.15 ± 0.04 (22.0)	0.09 ± 0.04 (12.6)	0.05 ± 0.02 (7.5)	

FIGURE 3.5

Distribution of enzymes of arginine metabolism
in rat kidney cortical tubule fractions

The results presented in **Table 3.2** are shown here in the form of histograms. The figure shows the relative specific activity plotted against protein content (%) in tubule fractions. Protein (%) is the recovery of the protein of the original tubule suspension. The relative specific activity is calculated as the specific activity in the fraction divided by that in the original tubule suspension. The specific activities (μmoles/min/g protein) of the marker enzymes in the original suspension were, 2469 ± 928 (γ-glutamyltranspeptidase), 252 ± 95 (alkaline phosphatase), 47.5 ± 11.8 (phosphoenolpyruvate carboxykinase) and 9.0 ± 0.06 (hexokinase). The specific activities of the enzymes of arginine metabolism in the original tubule suspension were, 3.20 ± 0.74 (argininosuccinate synthetase), 2.36 ± 1.94 (argininosuccinate lyase), 71.2 ± 38.5 (arginase) and 9.3 ± 3.9 (ornithine aminotransferase).


Relative Specific Activity
7-Glutamyl transpeptidase
Alkaline phosphatase
Phosphoenolpyruvate carboxykinase
Hexokinase
Arginino-succinate synthetase
Arginino-succinate lyase
Arginase
Ornithine aminotransferase
Protein (%)
F1
F2
F3
F4

recovered in F_1. On the other hand, the specific activity of the glycolytic enzyme hexokinase (marker for distal tubules) was about 1.7-fold higher in F_1, the ratio of F_4/F_1 was 0.23 and the total recovery was about 67%. Almost a 5-fold increase in the recovery of hexokinase was observed in F_1 (29.4 vs. 6.7 in F_4). These results are in agreement with the data of Vinay et al (1981). The specific activities of argininosuccinate synthetase and lyase were 3- to 10-fold higher in F_4 than in F_1. Similar observations were made for their recoveries in these fractions.

From the distribution of these marker enzymes I conclude that F_4 consists primarily of proximal convoluted tubules (S_1 & S_2 segments). This conclusion is supported by the enrichment in this fraction of both phosphoenolpyruvate carboxykinase and alkaline phosphatase (both present throughout the proximal tubule) together with the enrichment in F_1 of the two enzymes (γ-glutamyl transpeptidase and arginase) known to be found in the proximal straight (S_3) segments. The very low activity of hexokinase attests to the minimal presence of distal tubules in F_4. Since both of the enzymes of arginine synthesis (argininosuccinate synthetase and lyase) were found predominantly in F_4 I concluded that these, too, primarily occur in the proximal convoluted tubule. This is consistent with their occurrence in the outer cortex (**Table 3.1**).

It should be noted that the total recoveries of argininosuccinate synthetase and lyase were 143 and 175%, respectively. These results raised the possibility that there might be an inhibitor(s) of arginine synthesis in the original tubule suspension. If there was an inhibitor in the original

tubule suspension, then the enrichment of these two enzymes in F_4 could be attributed to the loss of an inhibitor from this fraction. On the other hand, if the fractionation procedure has resulted in the complete loss of the inhibitor, then the results would be reliable. To address this question, I separated cortical tubules and then recombined them in appropriate proportions to give a reconstituted fraction (F_r). I then determined argininosuccinate synthetase and argininosuccinate lyase in this reconstituted fraction and compared it to the activity in the original tubule suspension (F_t). Again, I observed an increase in the activities of the two enzymes in the sum of the four fractions (F_{1-4}). The mean total enzyme activity (nmoles/min/fraction) of argininosuccinate synthetase and lyase in F_r and F_t were, 460 and 176 and 433 and 134, respectively. This represented a 6 and 31% increase in the amount of argininosuccinate synthetase and lyase recovered in F_r. Although the increase in these enzyme activities seen in these experiments is less than in **Table 3.2**, it still contrasts markedly with the recovery of the other enzymes in this separation procedure, which were in the region of 60-70%. Most importantly, the total activities and recoveries of these enzymes in the reconstituted fraction were not different from that in the sum of the fractions. I can therefore be confident that the enrichment of the enzymes of arginine synthesis in F_4 is not an artifact due to a retention of inhibitor in one of the fractions.

Ornithine aminotransferase is enriched in F_1, (**Table 3.2**) but I was unable to determine whether it is localized in distal or in proximal straight tubules. It does follow the distribution of arginase closely and it would be reasonable to find it in the

proximal straight tubule together with this enzyme since it could metabolize any ornithine produced by the action of arginase.

Arginine production in kidney cortical tubule fractions

Since arginino-succinate synthetase and arginino-succinate lyase were enriched in the F_4 fraction (proximal convoluted tubules), I then determined whether the metabolic production of arginine from citrulline would also be enriched here. Arginine production in different tubule fractions followed closely the distribution of phosphoenolpyruvate carboxykinase (**Figure 3.5**). The recoveries for hexokinase, phosphoenolpyruvate carboxykinase and arginine production were about 50, 75 and 102%, respectively. The rates of arginine production were 0.57 and 5.36 μmoles/min/g protein in fractions F_1 and F_4 (F_4/F_1 = 9.4), respectively, in comparison to 2.36 μmoles/min/g protein in the original cortical tubule suspension. This confirms the data indicating that arginine synthesis occurs in proximal convoluted tubules.

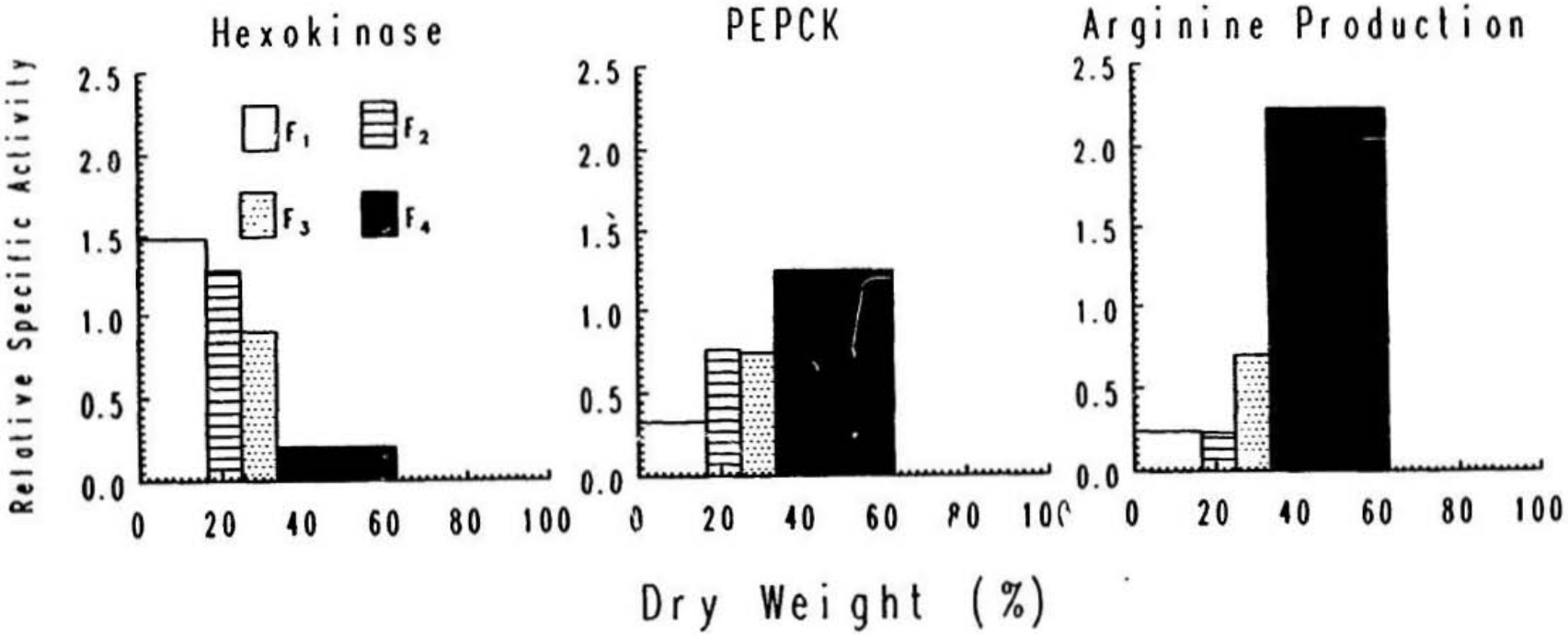
Subcellular localization of enzymes of arginine synthesis in cortex

Having localized the enzymes of arginine synthesis in the proximal convoluted tubule, experiments were carried out to further localize them with respect to their subcellular organelle localization in the cortical cells. This objective was achieved by the fractionation of cortex homogenate to yield different subcellular components by the classical differential centrifugation method of de Duve et al (1955), as modified by Kalra and Brosnan

FIGURE 3.6

Arginine production in kidney cortical tubule fractions

The figure shows a de Duve plot of mean values obtained from three separate experiments. Kidney cortical tubules were prepared by collagenase digestion and then fractionated on Percoll^{R} gradient. F_1, F_2, F_3, F_4 are the fractions obtained after fractionation. The relative specific activity is plotted against dry weight (%). Dry weight (%) is the % recovery of the dry weight of the original tubule suspension in each fraction. The relative specific activity is calculated as the specific activity in the fraction divided by that in the original tubule suspension. The specific activities of hexokinase, phosphoenolpyruvate carboxykinase and arginine production in F_t, the original tubule suspension were 8.6, 32.4 and 1.68 μmoles/min/g dry weight, respectively.


Hexokinase
PEPCK
Arginine Production
Relative Specific Activity
F1
F2
F3
F4
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Dry Weight (%)

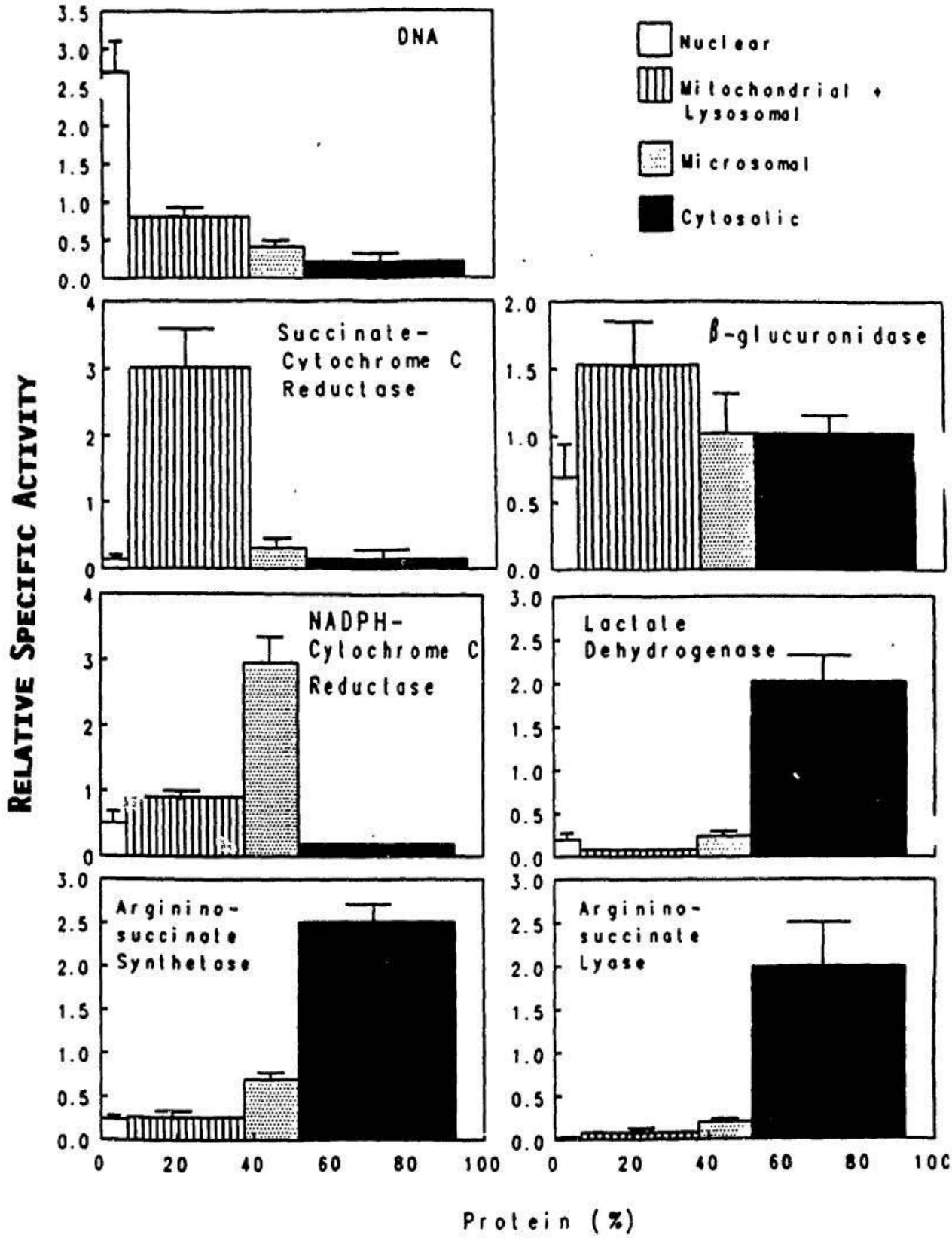
(1974). The identity of subcellular organelles (nuclei, mitochondria, lysosomes, endoplasmic reticulum, cytosol) in different fractions was demonstrated by determining the activity of marker enzymes or of DNA (nuclei) that are known to have a specific subcellular location. The activities of enzymes of arginine synthesis were determined and their distribution was compared to that of the marker enzymes. The data are presented in the form of histograms in **Figure 3.7** which show the relative specific activity (activity in fraction/activity in homogenate) plotted against protein content in a particular subcellualar fraction.

The recoveries of protein and DNA were 93 and 94%, respectively. A 2.7-fold increase in the enrichment of DNA was observed for the nuclear fraction. The specific activities (μmoles/min/g protein) and the total recoveries (%) of the markers were, 86.9 & 99.6 (succinate-cytochrome c reductase), 2.85 & 106 (β-glucuronidase) and 10.5 & 80 (NADPH-cytochrome c reductase) and 2687 & 89.4 (lactate dehydrogenase). The specific activities and the total recoveries for the marker enzymes succinate-cytochrome c reductase, β-glucuronidase, NADPH-cytochrome c reductase, lactate dehydrogenase, DNA and protein were in agreement with those reported by de Duve et al (1955) and Kalra and Brosnan (1974). It was noted that β-glucuronidase was also present in microsomal and cytosolic fractions which is attributable to damage of lysosomes during the fractionation procedure. Similar results were reported by Kalra and Brosnan (1974). The marker for cytosol, lactate dehydrogenase, was exclusively found in the 105,000xg supernatant (cytosolic fraction). The data presented in **Figure 3.7.** clearly

FIGURE 3.7

Subcellular localization of enzymes of arginine synthesis in rat kidney cortex

The figure depicts a de Duve plot of relative specific activity versus protein (%). Kidney cortex homogenate was fractionated and the enzymes, protein and DNA were assayed as described under "Materials and Methods". Results are presented as the mean ± SD (n=3). Protein (%) is the % recovery of protein of the cortex homogenate. The relative specific activity is calculated as the specific activity in the fraction divided by that in the cortex homogenate. The DNA content in the homogenate was 23.2 ± 2.2 μg/mg protein. The specific activities (μmoles/min/g protein) of the marker enzymes in the homogenate were, 29.3 ± 4.8 (succinate-cytochrome c reductase), 1.56 ± 0.55 (β-glucuronidase), 1.79 ± 0.60 (NADPH-cytochrome c reductase) and 1344 ± 187 (lactate dehydrogenase). The specific activities of argininosuccinate synthetase and argininosuccinate lyase in the cortex homogenate were 9.44 ± 0.34 and 4.16 ± 0.61, respectively.


DNA
Nuclear
Mitochondrial + Lysosomal
Microsomal
Cytosolic
Succinate-Cytochrome C Reductase
β-glucuronidase
NADPH-Cytochrome C Reductase
Lactate Dehydrogenase
Argininosuccinate Synthetase
Argininosuccinate Lyase
Relative Specific Activity
Protein (%)

demonstrate that argininosuccinate synthetase and argininosuccinate lyase are located in the cytosolic fraction as they followed the distribution of the cytosolic enzyme lactate dehydrogenase. The specific activities in the cytosolic fraction and the total recoveries for argininosuccinate synthetase and argininosuccinate lyase were 23.6 and 8.04 μmoles/min/g protein, and 122 and 86%, respectively. Once again, the higher recovery observed for argininosuccinate synthetase might be attributed to the presence of an inhibitor which may have disappeared during the fractionation procedure.

Discussion

The localization of enzymes of arginine metabolism in rat kidney was investigated. In assessing the distribution of the enzymes of arginine metabolism in kidney regions, I found that the enzymes of arginine synthesis followed closely the distribution of phosphoenolpyruvate carboxykinase. The enzymes of arginine catabolism followed γ-glutamyltranspeptidase and hexokinase.

Since cortical tissue is heterogeneous, I examined the distribution of enzymes of arginine metabolism in different tubule elements. This was achieved by determining their activities in different tubule fractions obtained after fractionation of kidney cortical tubules. The data in **Table 3.2** and **Figure 3.5** clearly demonstrate that the enzymes of arginine synthesis closely follow the distribution of phosphoenolpyruvate carboxykinase and were enriched predominantly in F_4 fraction. This led me to conclude that the enzymes involved in arginine synthesis are predominantly

localized in proximal convoluted tubule. Surprisingly, the recoveries of these enzymes were more than 100% which led me to postulate that there may be an inhibitor(s) of arginine synthesis in the original tubule suspension. The reconstitution experiments confirmed the presence of this postulated inhibitor. However, I have not determined the location of this inhibitor. More definitive information is required.

Arginine production from citrulline also followed the distribution of phosphoenolpyruvate carboxykinase (**Figure 3.6**), thus supports the fact that the enzymes of arginine synthesis are located in the proximal convoluted tubule. Again, the enrichment of arginine production rates was higher than that of phosphoenolpyruvate carboxykinase. This suggests that the inhibitor that decreased the activities of the enzymes of arginine synthesis in homogenates (**TABLE 3.2**) was also active in inhibiting arginine synthesis in intact cells. Again, this inhibition was lost upon fractionation.

The finding that arginine synthesis occurred mainly in proximal convoluted tubule is further supported by two recent independent reports. Levillain and coworkers (1990) demonstrated, by microdissecting rat nephron, that arginine production from citrulline occurs mainly in the proximal convoluted tubule. Morris et al (1991) presented evidence that the mRNAs for argininosuccinate synthetase and argininosuccinate lyase are predominantly localized in the proximal tubule.

In studies on the subcellular location of the enzymes of arginine synthesis, it was observed that they are present in the cytosol as they followed the distribution of the cytosolic marker,

lactate dehydrogenase (**Figure 3.7**). Our data is in agreement with those reported by Kato et al (1976) who localized argininosuccinate synthetase and argininosuccinate lyase in the soluble fractions of the whole kidney homogenate although no marker enzymes were employed in their study. These authors also observed an increase in total enzyme activity after fractionation. This observation is consistent with the present study in indicating the presence of an inhibitor of these enzymes.

The distribution of the catabolic enzymes arginase and ornithine aminotransferase followed closely the distribution of γ-glutamyltranspeptidase and hexokinase suggesting their location in the proximal straight or distal tubule. However, it is known from the microdissection studies of Levillain et al (1989) that arginase is exclusively present in the proximal straight tubule. I cannot make firm conclusions about the location of ornithine aminotransferase based on the present study. Definitive experiments need to be carried out to establish the nephron location of ornithine aminotransferase. It is clear that the enzymes of arginine synthesis and of arginine degradation are present in different cells as is apparent from the very low arginase activity in F_4. Thus, newly synthesized arginine is not immediately subjected to catabolism but may be released for extra-renal utilization. In this connection I have demonstrated a mole for mole relationship between renal citrulline uptake and arginine release *in vivo* (**CHAPTER 4**).

Concluding remarks

The results of this study clearly establishes that arginine synthesis occurs in the cytosol of the cells of the proximal tubule in the rat kidney. It is known that urine contains virtually no citrulline so that essentially all of the filtered citrulline is reabsorbed (**CHAPTER 4**). Since the proximal convoluted tubule is the predominant site of renal amino acid reabsorption (Silbernagl, 1988)the localization of the enzymes of arginine synthesis in these cells would ensure that filtered citrulline would be available for arginine synthesis.

CHAPTER 4

RENAL ARGININE SYNTHESIS: STUDIES *IN VITRO* AND *IN VIVO*

Synopsis

Renal arginine synthesis is a major endogenous source of arginine. The enzymes of arginine synthesis are predominantly found in kidney cortex. In studies with isolated renal cortical tubules, I observed rapid rates of arginine synthesis from citrulline provided a source of the "N" atom of the guanidino group of arginine was supplied. Aspartate, glutamate or glutamine were effective whereas glycine, alanine, serine or NH_4Cl were ineffective as this second substrate. Arginine synthesis was found to be highly sensitive to citrulline concentrations in the physiological range (60 μm), suggesting that renal arginine synthesis *in vivo* could be regulated by circulating citrulline levels. Therefore, arginine synthesis by the kidney was investigated *in vivo* by measuring the net flux of citrulline and arginine in saline-infused and citrulline-infused rats. In normal animals uptake of citrulline was 60.5 ± 20.7 nmoles/min/100 g body weight, and a similar arginine release was observed. Citrulline infusion that increased circulating citrulline levels four-fold resulted in a similar increase in renal citrulline uptake (224 ± 33 nmoles/min/100 g) and a similar increase in renal production of arginine. The results suggest that the availability of citrulline is a limiting factor for renal arginine synthesis in rats.

Introduction

It is clear from the discussion in **CHAPTER 1** that the kidney

synthesizes arginine from citrulline and that this is a major endogenous source of arginine. Studies on the subcellular localization of enzymes of arginine metabolism clearly demonstrated that the enzymes of arginine synthesis (argininosuccinate synthetase and argininosuccinate lyase) are predominantly found in the cytosol of the cells of the proximal convoluted tubule and of removal (arginase and ornithine aminotransferase) in other kidney regions (**CHAPTER 3**). It was observed that metabolic production of arginine from citrulline occurred mainly in the proximal convoluted tubule (**CHAPTER 3**). However, the regulation of this process has not been examined. In the experiments reported in this Chapter, I examined the regulation of arginine synthesis in isolated tubules and *in vivo*.

Objectives

The main objectives of the present study were (1) to investigate arginine synthesis from citrulline in isolated cortical tubules and (2) to examine the renal release of arginine *in vivo* in normal rats and in animals whose blood citrulline levels have been increased by citrulline infusion.

Experimental protocol

The methodology involved in studying arginine synthesis *in vitro* (isolated cortical tubules) and *in vivo* (renal fluxes of

citrulline and arginine) has been described in detail in **MATERIALS AND METHODS (CHAPTER 2)** section.

Animals

Male Sprague-Dawley rats (Charles River, Montreal) weighing 300-425 g were used for all experiments. They were allowed water and Purina rat chow ad libitum.

Results

Arginine Synthesis in Isolated Cortical Tubules.

Arginine synthesis as a function of time and tubule amount

Tubules were prepared from the kidney cortex of rats by the method of Guder et al (1971) as described in detail in **CHAPTER 2**. The tubules were viable (>95%) over a 60 min incubation period as measured by the leakage of the cytosolic enzyme, lactate dehydrogenase. The activity of lactate dehydrogenase was determined in the pellet and supernatant fractions obtained after incubating cortical tubules (approximately 1 mg dry weight) for 0 and 60 min. In a representative experiment, it was observed that the activity (nmoles/min/mg dry weight) of lactate dehydrogenase in the 0 and 60 min pellets were 1250 and 1200 and correspondingly, an activity of 48 and 64 was obtained in the supernatant fractions. Thus, the leakage of lactate dehydrogenase during 60 min incubation period

was only of the order of about 5%.

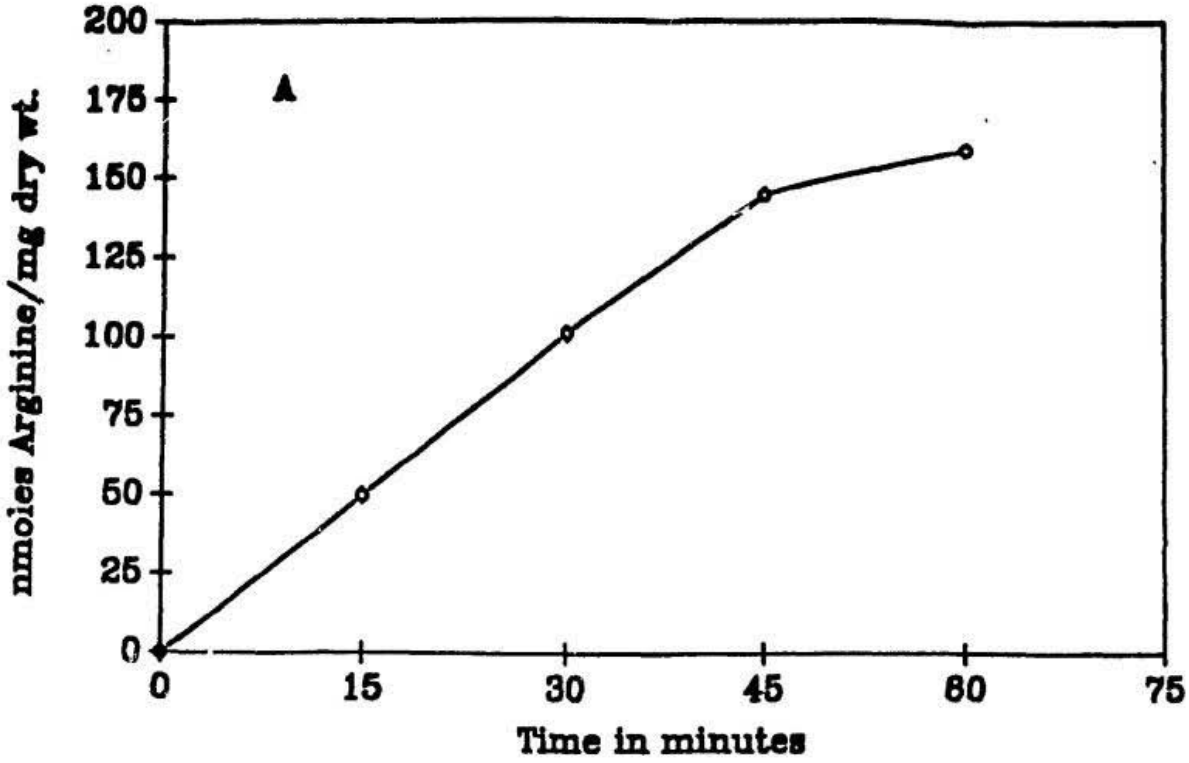
Arginine synthesis was strictly dependent on added citrulline. In its absence the rate was minimal (1 to 2 nmoles/30 min/mg dry weight). Arginine synthesis during incubation with citrulline, aspartate and lactate was linear with time for up to 45 min and with increasing quantity of tubules up to 2 mg dry wt. (**Figure 4.1 A & 4.1 B**). This linearity indicates the absence of any inhibition by the end products or any other factors generated during the course of the reaction. Therefore, I chose 30 min incubation time and a tubule quantity equivalent to 1 mg dry weight in the subsequent experiments. In the absence of lactate the rates of arginine synthesis were reduced by about 34% (81 vs. 54 nmoles/30 min/mg dry weight). This is probably due to lactate serving as an energy source since ATP is required in the argininosuccinate synthetase reaction (**Table 1.6**).

Metabolism of newly synthesized arginine

Since kidney is known to contain an arginase (Kaysen & Strecker, 1973; Levillain et al, 1989; **Table 3.1 & 3.2**), it was important to determine the extent to which arginine synthesized in these experiments was further metabolized to ornithine and urea. Accordingly, experiments were performed in which [^{14}C]Ureido-citrulline was used as substrate so that [^{14}C]Guanidinoarginine would be produced (**Figure 4.2**). Any metabolism of this arginine via arginase would result in the production of [^{14}C]Urea which was detected as $^{14}CO_2$ after urease treatment. With such experiments I

FIGURE 4.1

Arginine synthesis in rat renal cortical tubules as a function of time (A) and tubule quantity (B). Tubules were incubated with 0.5 mM citrulline and 5 mM each of aspartate and lactate. In Panel A 0.925 mg of tubules were used; in Panel B, tubules were incubated for 30 min. A representative experiment is shown.


A
nmoles Arginine/mg dry wt.
200
175
150
125
100
75
50
25
0
0
15
30
45
60
75
Time in minutes

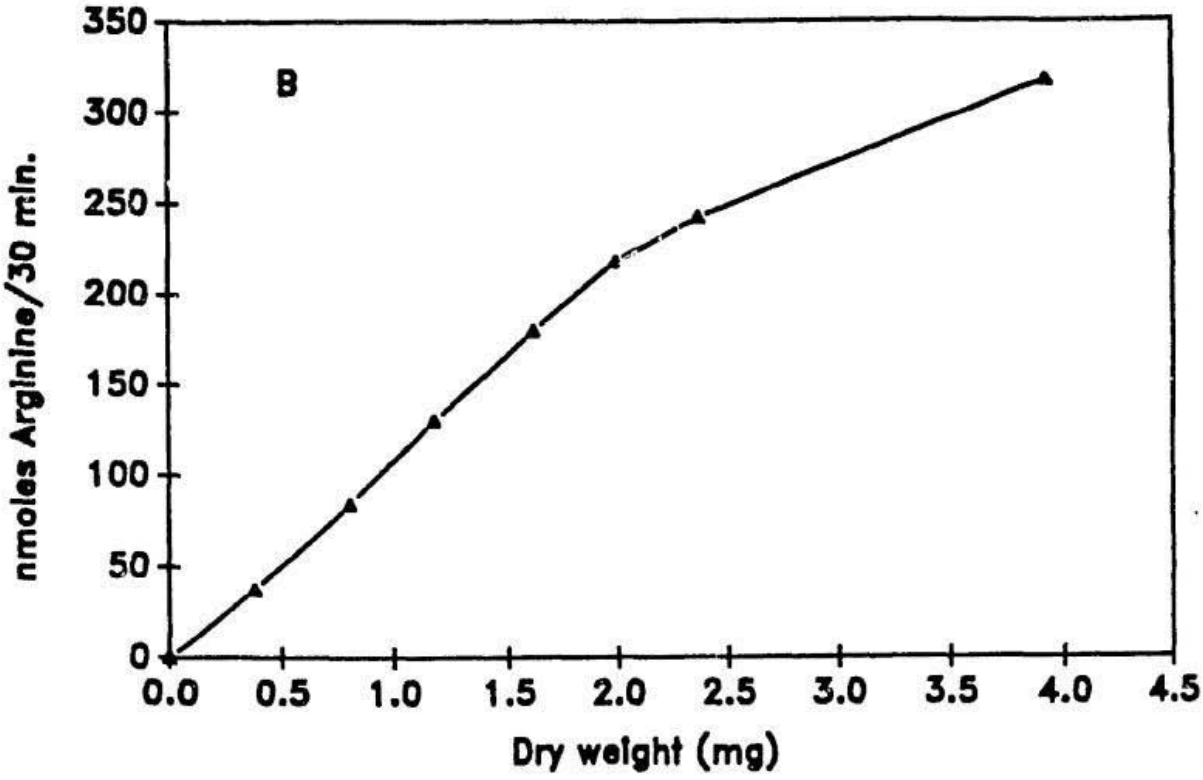
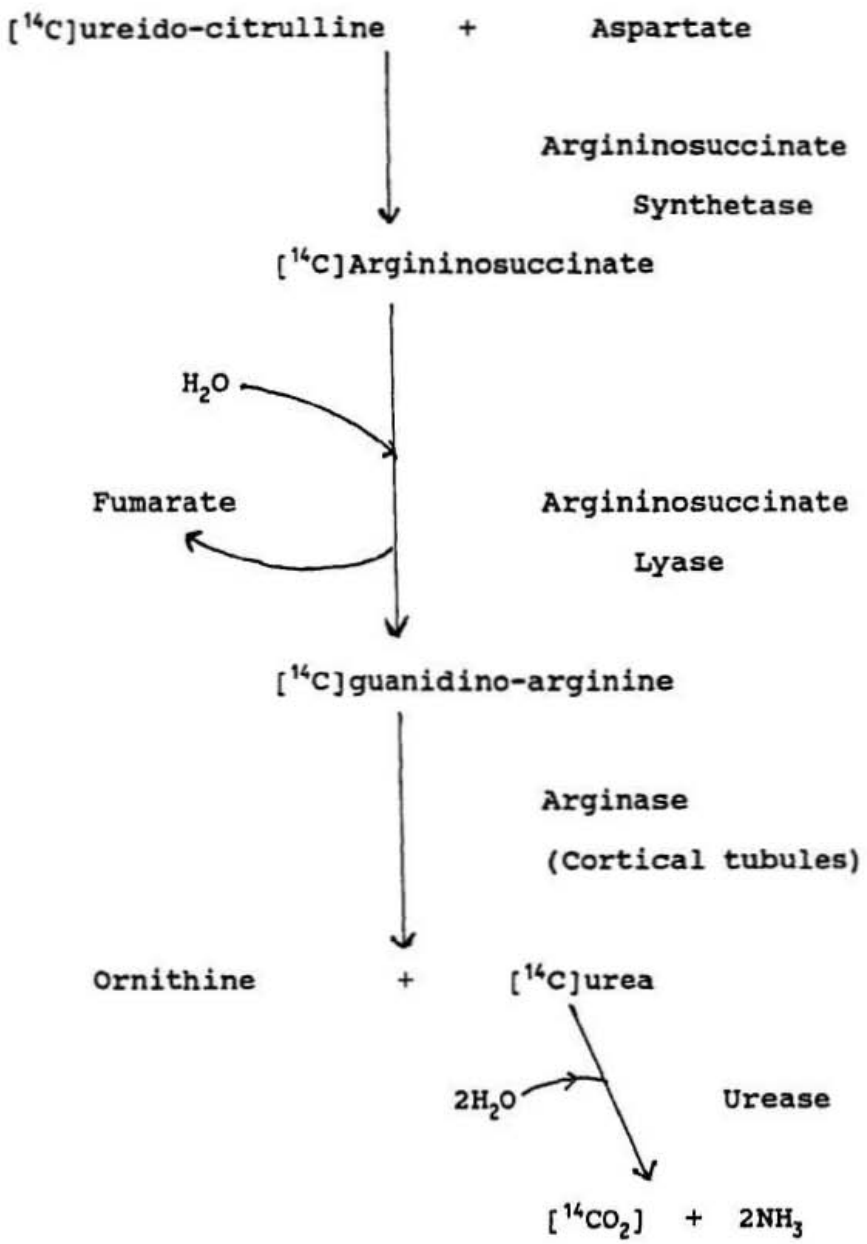

B
nmoles Arginine/30 min.
350
300
250
200
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Dry weight (mg)

FIGURE 4.2

The reactions involved in the synthesis and breakdown of arginine are shown. [^{14}C]Guanidinoarginine is synthesized from [^{14}C]Ureidocitrulline by the actions of argininosuccinate synthetase and argininosuccinate lyase present in the tubules. If this newly synthesized [^{14}C]Guanidinoarginine is broken down by tubule arginase, it will yield [^{14}C]Urea and ornithine. [^{14}C]Urea which can be quantitated by treatment with urease which generates $^{14}CO_2$, that can be collected and counted.


[14C]ureido-citrulline
+
Aspartate
Argininosuccinate
Synthetase
[14C]Argininosuccinate
H2O
Fumarate
Argininosuccinate
Lyase
[14C]guanidino-arginine
Arginase
(Cortical tubules)
Ornithine
+
[14C]urea
2H2O
Urease
[14CO2]
+
2NH3

observed that only 6-7% of [^{14}C]Guanidinoarginine was metabolized by this route over 60-min incubation perid (**Figure 4.3**). Thus, the newly synthesized arginine is not appreciably metabolized to ornithine and urea. This conclusion is supported by the fact that arginase is not localized in the same cells where arginine synthesis occurs (**Table 3.2, CHAPTER 3**).

Arginine synthesis in the presence of various substrates

In the presence of lactate, arginine synthesis from citrulline alone was about 20 nmol/30 min/mg dry weight and this was increased about four-fold by addition of aspartate, glutamate or glutamine whereas glycine, alanine, serine or NH_4Cl did not stimulate arginine synthesis (**Table 4.1**). These substrates (potential sources of second "N" atom of the guanidino group of arginine) were chosen because they are actively metabolized in the kidney. The enzyme argininosuccinate synthetase uses aspartate as a N donor for the second N atom in the guanidino group of arginine. Thus glutamate and glutamine were effective, due presumably to the high renal activities of glutaminase and aspartate aminotransferase specially in proximal convoluted tubule. On the other hand, alanine, serine and glycine were ineffective. This may be due to the inability of the kidneys to convert these amino acids to aspartate.

Dependence on citrulline concentration

The data in (**Figure 4.4**) show the rate of arginine synthesis

FIGURE 4.3

Metabolism of Newly Synthesized Arginine

Tubules (1 mg dry weight) were incubated with ^{14}C-ureido-citrulline (0.5 mM, 25 dpm/nmole), aspartate and lactate, 5 mM each in a total volume of 2-ml, at 37^0 C for 0-60 min. Neutralized extracts were treated with urease and the amount of [^{14}C]Urea formed was quantitated as $^{14}CO_2$. Arginine was determined by HPLC method. Figure shows mean values obtained from 3 separate experiments.

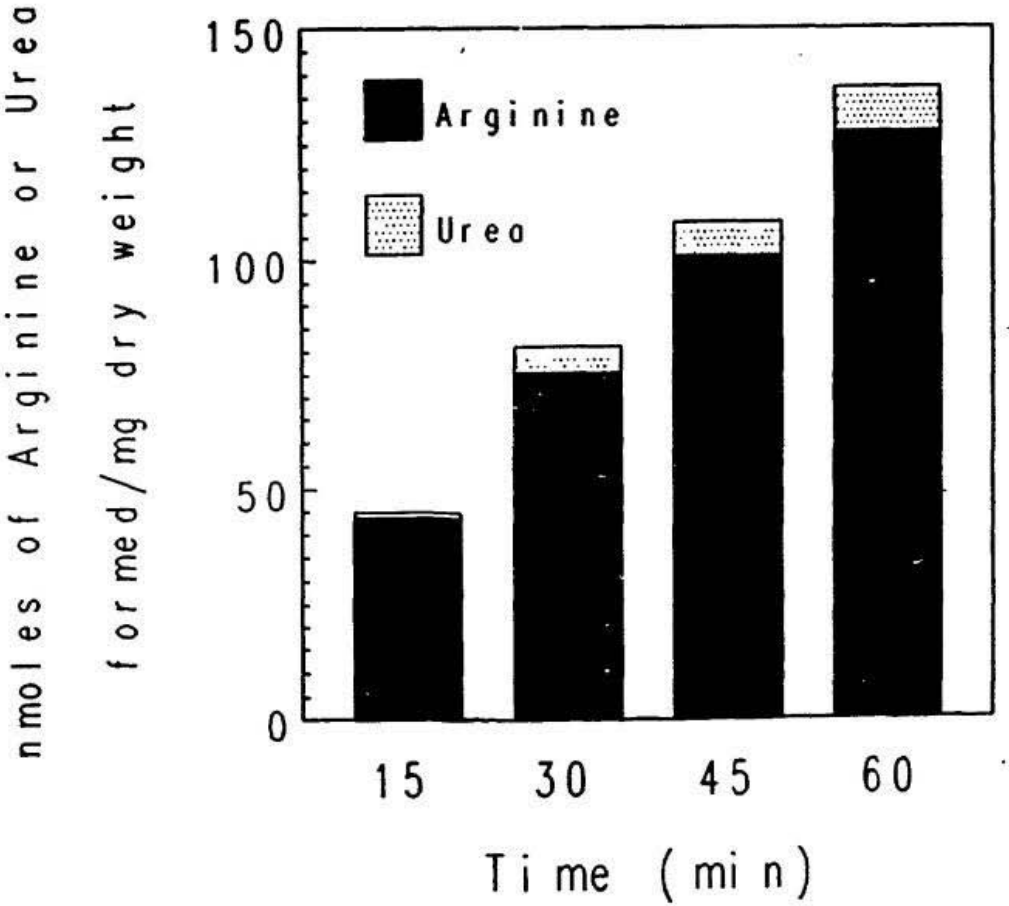

nmoles of Arginine or Urea formed/mg dry weight
150
100
50
0
Arginine
Urea
15
30
45
60
Time (min)

TABLE 4.1.

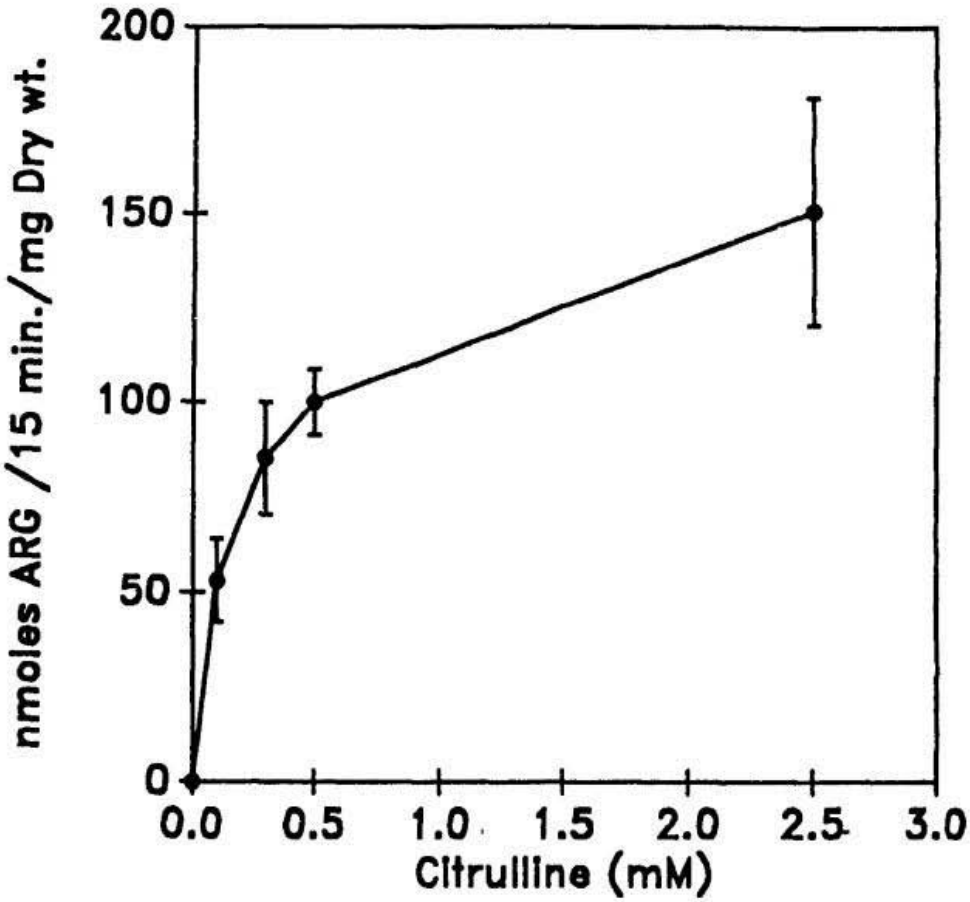
Arginine Production in Kidney cortical tubules in the presence of various substrates.

Substrate(s)	Arginine Production Rate (nmoles/30 min/mg dry wt.)
Citrulline + Aspartate (Control)	85.0 ± 10.9
Lactate (alone)	1.4 ± 0.3*
Citrulline	21.4 ± 2.6*
Citrulline + Glutamate	92.4 ± 16.5
Citrulline + Glutamine	88.7 ± 17.9
Citrulline + Glycine	21.8 ± 5.8*
Citrulline + Alanine	16.7 ± 1.1*
Citrulline + Serine	14.5 ± 3.3*
Citrulline + NH_4Cl	32.5 ± 3.5*

Values are means ± SD (n = 3 for all except control, n = 8). *Significantly different from control by Dunnett's procedure ($P < 0.05$). Tubules equivalent to approximately 1.0 mg dry wt. were incubated for 30 min. All substrates were initially present at 5 mM except citrulline and NH_4Cl, which were present at 0.5 mM and 2 mM respectively. Lactate (5 mM) was present in all incubations.

FIGURE 4.4

Dependence of arginine synthesis in rat renal cortical tubules on citrulline concentration. Tubules (approximately 1 mg dry weight) were incubated for 15 min with aspartate and lactate, 5 mM each, at 37°C. Values are means ± SD (n = 4).


200
150
100
50
0
nmoles ARG /15 min./mg Dry wt.
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Citrulline (mM)

as a function of citrulline concentration. In this experiment incubations were carried out for 15 min so as to minimize substrate depletion at low substrate concentrations. The synthesis of arginine from citrulline is very sensitive to citrulline concentrations up to 0.5 mM and was not saturated even at 2.5 mM. Most important, arginine synthesis was very sensitive to citrulline concentration in the region of circulating citrulline concentration (0.06 mM) suggesting that renal arginine synthesis in vivo could be regulated by circulating citrulline levels.

Arginine synthesis In Vivo

The dependence of renal arginine synthesis in vivo on citrulline concentration was examined. Experiments were carried out to quantify the renal uptake of citrulline and release of arginine in animals infused with saline or citrulline (to elevate blood citrulline levels). The arteriovenous differences across the kidneys for both citrulline and arginine were determined, Renal hemodynamic parameters such as GFR and renal blood flow rates were also determined in this study as described in detail in **CHAPTER 2.**

The initial experiments examined whether citrulline uptake and arginine release by the kidney occurred from plasma, blood cells or both since substantial redistribution of amino acids between blood cells and plasma can occur as the blood passes through the kidney. The data in **Table 4.2**, shows that A-V differences for citrulline and arginine measured in plasma can account for all of the

TABLE 4.2

Arteriovenous differences across the kidney in blood, plasma and blood cells for citrulline and arginine in normal and citrulline-infused rats.

Parameter	Normal (n = 5)	Citrulline -Infused (n = 3)
Arterial Hematocrit	45.3 ± 2.4	47.0 ±2.6
Renal Venous Hematocrit	45.6 ± 2.1	47.3±2.1
(A-V) Blood Citrulline (nmoles/ml)	11.2 ± 6.2[a]	42.1 ±7.6[a,b]
(A-V) Blood Arginine (nmoles/ml)	-13.7 ± 7.4[a]	-38.6 ± 4.8[a,b]
(A-V) Plasma Citrulline (nmoles in 1 ml Blood)	13.2 ± 3.2[a]	45.6 ± 4.9[a,b]
(A-V) Plasma Arginine (nmoles in 1 ml Blood)	-10.6 ± 3.6[a]	-42.4 ± 7.5[a,b]
(A-V) Blood cell Citrulline (nmoles in 1 ml Blood)	- 2.0 ± 3.3	-3.5 ± 3.5
(A-V) Blood cell Arginine (nmoles in 1 ml Blood)	- 3.1 ± 8.8	3.8 ± 4.1

Values are means ± SD. [a]$P < 0.05$ for values significantly different from zero. The values for (A-V) for plasma, for both citrulline and arginine, were not significantly different from the respective blood (A-V) values ($P > 0.05$).

[b]Significantly different from normal rats ($P < 0.05$)

differences seen in whole blood and that there is no contribution from blood cells. It is also evident that in normal and citrulline-infused rats there is a 1:1 molar relationship betwen citrulline uptake and arginine release. Such a relationship has also been shown in rats (Windmueller & Spaeth, 1981) and in humans (Tizianello et al, 1980). I then measured total renal fluxes for these two amino acids in normal rats and in rats infused with citrulline to increase circulating concentrations. Since the data in **Table 4.2** show that exchange from plasma accounted for all of the flux, I quantified the renal flux by multiplying the plasma A-V by the plasma flow rate. The basic physiological parameters of renal function in these experiments are presented in **Table 4.3**. The basic values are in agreement with those reported earlier in our laboratory (Lowry et al, 1987). There were no significant differences for any of the measured parameters between control and citrulline-infused rats.

The arterial levels, A-V differences and renal fluxes of all the amino acids in saline- and citrulline-infused rats are presented in **APPENDIX A**. The data in **Table 4.4** focus on citrulline and arginine. These data show that citrulline infusion increased plasma citrulline four-fold (62 vs. 242 nmoles/ml) and also caused comparable increases in renal fluxes of citrulline and arginine. There was also an increase in arterial plasma arginine (174.6 $\pm$ 26.2 vs. 244.1 $\pm$ 35.0 nmoles/ml), presumably as a result of an increased rate of renal arginine synthesis. Further, the renal uptake of citrulline was not significantly different from the

TABLE 4.3

Basic physiological data for saline and citrulline-infused rats.

Parameter	Saline (n=5)	Citrulline (n=5)
Body Weight (g)	425 ± 37	403 ± 38
U/P inulin	237 ± 36	216 ± 44
Glomerular filtration rate (ml/min/100 g body wt.)	0.70 ± 0.06	0.93 ± 0.28
Renal plasma flow rate (ml/min/100 g body wt.)	1.97 ± 0.32	2.42 ± 0.70
Filtration fraction	0.36 ± 0.04	0.39 ± 0.02
Urine flow rate (µl/min/100 g body wt.)	3.0 ± 0.28	4.4 ± 1.96

Values are means ± SD. There were no significant differences between the saline-infused and citrulline-infused rats for any of the parameters measured.

TABLE 4.4

Arterial plasma levels and net renal flux of citrulline and arginine in saline and citrulline-infused rats.

	Arterial plasma concentration nmoles/ml plasma		Renal Flux nmoles/min/100 g	
Infusion				
	Citrulline	Arginine	Citrulline	Arginine
Saline	62.1 ± 7.8	174.6±26.2	60.5±20.7	-78.9 ± 24.5
Citrulline	241.9±38.0[a]	244.1±35.0[a]	223.6±33.2[a]	-264.6 ± 82.7[a]

Values are means ± SD (n=5 for each treatment). [a] $P < 0.05$ for comparison with saline infusion. A positive value for the flux indicates uptake and a negative value indicates output.

arginine output in either the saline/or the citrulline-infused rats. The urinary excretion of citrulline and arginine in saline and citrulline-infused rats was 0.01 ± 0.01 and 0.09 ± 0.05 vs. 0.06 ± 0.02 and 0.15 ± 0.08 nmoles/min/100 g body weight, respectively. Thus urinary excretion of these amino acids was not a significant factor. The renal output of ornithine was 4.0 ± 14.5 and 26.7 ± 15.5 nmoles/min/100 g body weight, respectively, thus accounting for 5 to 10% of arginine release in saline- and citrulline infused rats. However, ornithine release by the kidneys of these animals was not statistically different. **Figure 4.5** plots the relationship of citrulline uptake to arginine release. It is clear from **Figure 4.5**, that for every mole of citrulline taken up there is a mole of arginine released (slope = 1.086) in normal, saline- and citrulline-infused rats.

Citrulline removal by the kidneys may depend on the route of citrulline delivery. Delivery can be either via its filtration at the glomeruli or by its uptake from the peritubular capillaries. Therefore, I made some calculations to establish the relationship between filtered citrulline and citrulline uptake. Filtered citrulline was calculated by multiplying the arterial plasma concentration by the glomerular filtration rate. **Figure 4.6** shows the relationship of filtered citrulline to citrulline uptake in saline- and citrulline-infused rats. An excellent correlation ($r^2 = 0.96$) was observed. The calculated slope value (0.89) indicated that approximately one mole of citrulline was taken up for every mole of citrulline filtered.

FIGURE 4.5

Relationship between the A-V difference across the kidneys for citrulline and for arginine in saline- and citrulline-infused rats. Each point represents the values obtained from individual rats.

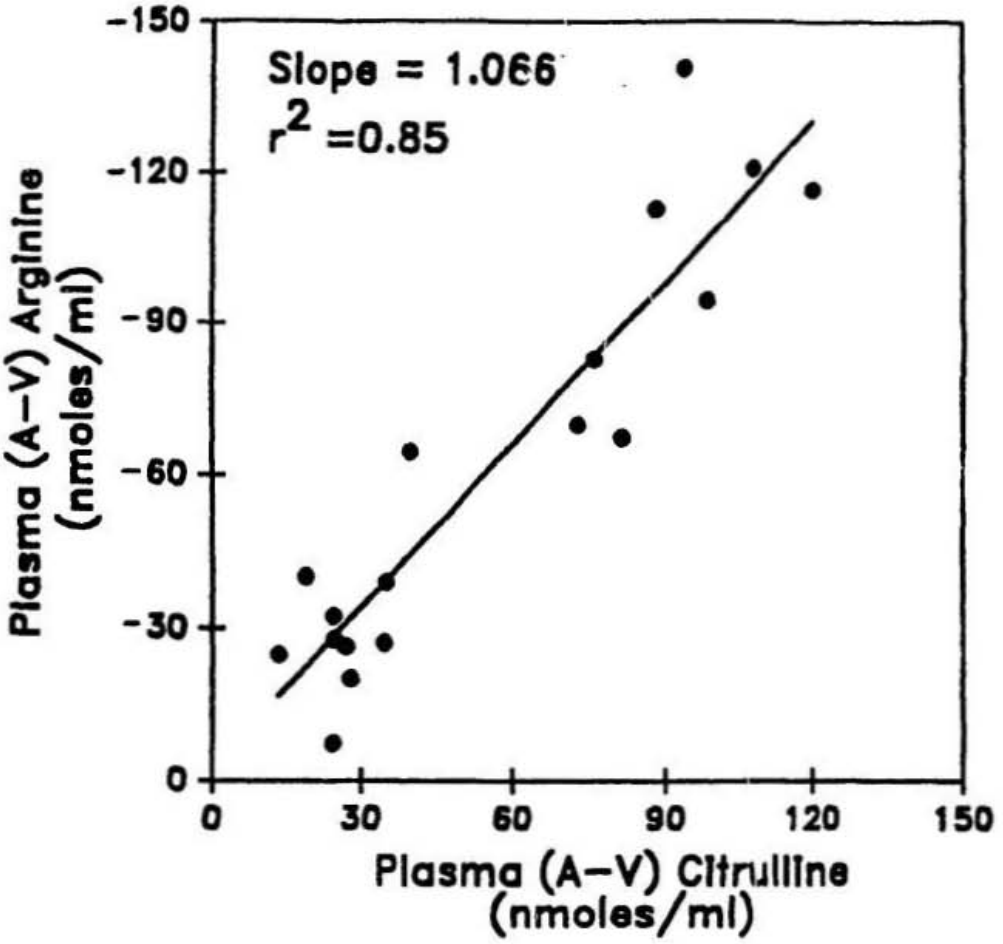
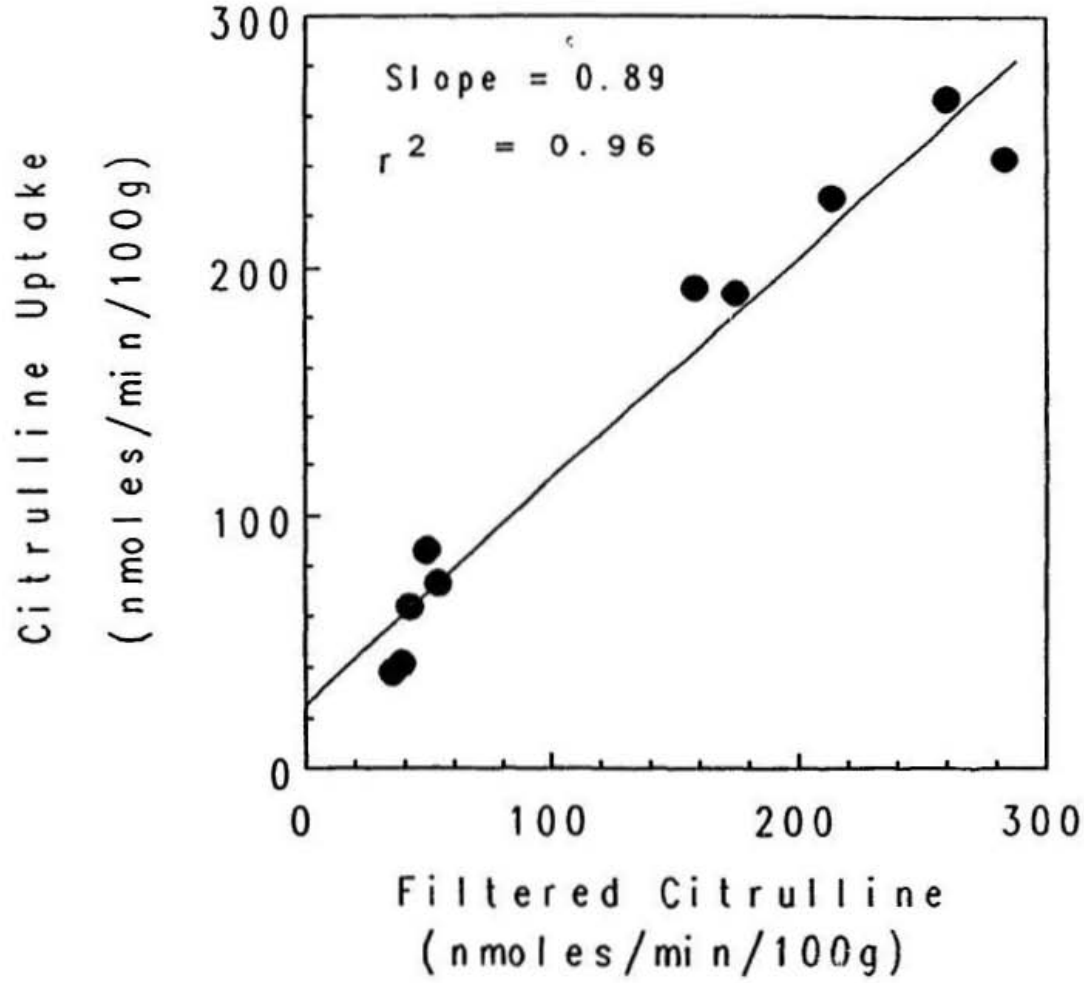

Slope = 1.066
r^2 =0.85
Plasma (A–V) Arginine (nmoles/ml)
Plasma (A–V) Citrulline (nmoles/ml)
-150
-120
-90
-60
-30
0
0
30
60
90
120
150

FIGURE 4.6

Figure depicts Citrulline removal *in vivo*:
Dependence on filtered citrulline

Arterial Plasma Citrulline x GFR (Filtered Citrulline) is plotted against (A-V) Citrulline x Renal Plasma Flow (Citrulline Uptake) Each point represents the values obtained from individual rats.


Slope = 0.89
r 2 = 0.96
300
200
100
0
0
100
200
300
Citrulline Uptake
(nmoles/min/100g)
Filtered Citrulline
(nmoles/min/100g)

Discussion

Arginine production in renal cortical tubules

Since the enzymes of arginine synthesis were found in the cortex, I carried out metabolic studies with isolated cortical tubules. The results clearly show that high rates of arginine synthesis from citrulline depend upon the presence of a source of the second N atom in the guanidino group of arginine. Aspartate, glutamate or glutamine served as an excellent source whereas glycine, alanine or serine did not. Higher rates of arginine production were observed in the presence of an oxidizable substrate (lactate). Very recently, Levillain and coworkers (1990) have shown in experiments in which microdissected portions of the nephron were examined that arginine synthesis from citrulline occurs predominantly in the proximal convoluted tubule. In the present study it was observed that the arginine which was newly synthesized from citrulline was not appreciably metabolized (6-7%) to urea and ornithine in cortical tubules, suggesting either that it was not readily available to renal arginase or that the effective renal arginase activity is low. The former possibility is supported by the presence of arginase in different cells (proximal straight tubule) than the arginine synthesising enzymes (Levillain et al, 1989; **Table 3.1 & Table 3.2**). The observation of minimal conversion of arginine to ornithine and urea in cortical tubules is also consistent with the data of Perez et al (1978). In their study with isolated perfused rat kidney, when

[^{14}C]Ureidocitrulline was added to the perfusate, the label predominantly (>95%) appeared as [^{14}C-guanidino]arginine and less than 5% of the label appeared as [^{14}C-urea] during the 90-min recirculating perfusion period.

Arginine synthesis in vivo

There was good stoichiometry between citrulline removal and arginine production in vivo, indicating that renal arginine synthesis is for export to extra-renal tissues. It also suggests that arginine synthesis was not subjected to breakdown by arginase in the kidney. No significant differences were observed in ornithine output by the kidneys of animals infused with saline or citrulline and ornithine accounted for only 5-10% of arginine release (**APPENDIX A, Table 6.1**).

A comparison of the rates of arginine synthesis in vivo and in vitro is of interest. In renal cortical tubules, arginine synthesis was very sensitive to citrulline concentration, up to 0.5 mM, and did not appear to be saturated even at 2.5 mM. In Vitro rates of 867 and 1450 nmoles/min/g wet weight of kidney were found at 0.1 and 0.3 mM citrulline, respectively (**Figure 4.4**) whereas, in vivo rates of 98 and 331 nmoles/min/g wet weight of kidney were found at 0.06 and 0.24 mM citrulline, (**Table 4.4**), respectively. Thus, the rates in vitro were about 4 to 5-fold higher at comparable citrulline concentrations than in vivo. A partial explanation for this discrepancy is that the rates in vivo refer to the whole kidney whereas the studies in vitro were carried out with a cortex preparation, in which arginine synthesis is enriched. However, since cortex comprises 83% of the

kidney in the rat (**Table 3.1**) such enrichment cannot account for a 4-5 fold discrepancy. It is not likely that the second "N" source is limiting *in vivo* as there was a linear response in arginine output to increased citrulline concentration. A more likely explanation relates to the different modes of delivery of citrulline to the tubules *in vivo* and *in vitro*. The tubules (*in vitro*) are open ended and thus have a continuous supply of citrulline available to them. The total delivery of citrulline *in vivo* depends on external factors such as GFR and RPF and offers a limit to the extent to which citrulline can be converted to arginine.

The increased rate of arginine synthesis with increasing arterial citrulline concentrations during citrulline infusion could be due to the increased delivery of citrulline in the plasma or in the filtrate. The increased citrulline uptake upon infusion of citrulline closely paralleled the increase in the filtered load (**Figure 4.6**) and indeed, was not significantly different from it, suggesting that this may explain the increased citrulline uptake. This suggestion requires further experimentation before firm conclusions can be drawn.

A most striking observation in the present study was the marked sensitivity of renal arginine synthesis to citrulline concentrations in the physiological range. A similar dependence is evident from the data obtained by Tizianello (1980) in humans. These findings imply that the intestinal production of citrulline may be the principal site of regulation of net endogenous arginine synthesis in kidney. This is consistent with the data from Windmueller and Spaeth (1981)

that most (83%) of the citrulline produced in the intestine is converted to arginine in the kidney. In my experiments citrulline uptake by the kidney during citrulline infusion was 223 ± 33 nmoles/min/100 g, so that the extra citrulline metabolized was 163 ± 39 nmoles/min/100 g after deducting the uptake in saline-infused rats (60.5 ± 20.7 nmoles/min/100 g). This accounted for 58% of the infused citrulline (281 ± 29 nmoles/min/100 g) and thus indicates that the kidney remains the principal organ for citrulline metabolism even when citrulline levels are elevated.

It is known that weanling rats (Borman et al, 1946) or rats whose intestinal citrulline synthesis is specifically inhibited (Hoogenraad et al. 1985) require exogenous arginine. However, in those studies it was demonstrated that citrulline can effectively substitute for arginine. This implies that the body contains a system for the conversion of citrulline to arginine that is not saturated (i.e., can accomodate an increased delivery of citrulline). The kinetic properties of the renal system for synthesizing arginine reported here strongly suggest that it could serve as this system. It could also serve as the system responsible for the metabolism of any dietary citrulline. This is made use of in patients suffering from lysinuric protein intolerance (Rajantie et al, 1980 & 1983). In this condition, there is defective intestinal absorption and renal reabsorption of dibasic amino acids such as lysine, arginine and ornithine. Oral arginine supplementation cannot, therefore, be used to correct the hyperammonemia arising from deficiency of the urea cycle intermediates arginine and ornithine. Oral citrulline

administration (2 to 3 g) prevented hyperammonemia as indicated by a marked decrease in orotic acid excretion when compared to arginine supplementation. However, there was a great increase in urinary arginine excretion after citrulline supplementation indicating both a conversion of citrulline to arginine and confirming the defect in the reabsorption of arginine. It has been observed in children suffering from Reye's syndrome that there is abnormal urea cycle function due to lowered activities of carbamoyl phosphate synthetase-I and ornithine aminotransferase (Sinatra et al, 1975). These children have elevated blood ammmonia levels and cerebral dysfunction and, if untreated, may die. Their serum arginine levels are low and serum citrulline levels are either low or absent. Treatment with citrulline has proven beneficial in these children (De Long & Glick, 1982). Also, it has been shown by Brusilow (1984) that citrulline is an indispensable amino acid for children with urea cycle enzymopathies, specifically that of carbamoyl phosphate synthetase-I and ornithine transcarbamoylase. The correction of hyperammonemia by citrulline in these children suggests the existence of a system for endogenous arginine synthesis.

Although citrulline is not found in protein (with very minor exceptions) considerable quantities of free citrulline occur in some foods. For example, water melon, Citrullus vulgaris, contains about 100 mg/100 g. Therefore, it is possible that the kidney can initiate the metabolism of such exogenous citrulline by converting it to arginine which can be subsequently metabolized in the liver or extra-hepatic tissues.

CHAPTER 5

Is renal arginine synthesis affected by dietary arginine or dietary protein intake ?

Synopsis

Kidneys of adult animals serve as a major biosynthetic source of arginine. In the previous chapter I demonstrated that kidneys of rats infused with citrulline responded to the elevated plasma citrulline levels by increasing its uptake and producing increased quantities of arginine. The objective of the present study was to determine whether feeding adult male rats different levels of arginine or protein would affect renal arginine synthesis. Feeding arginine (0, 0.5 and 2.0%) for 1 week increased circulating plasma levels of arginine 2.5-fold in the 2.0% arginine group compared to 0 and 0.5% arginine groups. However the plasma citrulline concentration was unchanged. The renal uptake of citrulline and release of arginine were similar in all the three groups on different intakes of arginine. Feeding diets containing 5, 12 and 50% protein for 1 week did not alter the circulating plasma levels of either citrulline or arginine and there were no significant differences in the renal release of arginine. These results suggest that renal arginine synthesis is independent of dietary arginine or protein intake.

Introduction

Arginine has been considered to be a dietary dispensable amino acid in adults. This conclusion was based on the observations that arginine is neither required for the maintenance of growth in adult rats (Scull & Rose, 1930) nor for the maintenance of positive

nitrogen balance in adult humans (Rose et al, 1954). However, based on a more sensitive indicator, orotic acid excretion, it has been established that arginine is indispensable in a variety of animals and in different physiological situations (**CHAPTER 1**). The role of the kidney as a major biosynthetic source of arginine and that of the intestine as a source of citrulline has been discussed earlier. The enzymes of arginine synthesis from citrulline are argininosuccinate synthetase and argininosuccinate lyase and these enzymes are predominantly found in the cytosol of the cells of the proximal convoluted tubule (**CHAPTER 3**). It was demonstrated that arginine synthesis in isolated kidney cortical tubules is highly sensitive to the citrulline concentration in the physiological plasma range (**CHAPTER 4**). Further, kidneys responded linearly to elevated plasma citrulline levels by increasing its uptake and producing increased quantities of arginine in citrulline-infused adult rats (**CHAPTER 4**). No previous attempts have been made to quantify renal arginine release in rats subjected to different dietary treatments.

The present study addresses whether renal arginine synthesis can be regulated either by the levels of dietary arginine or dietary protein. I have measured arterial and renal venous plasma flows and quantitated the net renal uptake of citrulline and release of arginine in rats consuming different amounts of arginine or protein in the diet. The results reported in this study suggest that arginine production in the rat kidney is independent of arginine or protein intakes.

Objective

Is renal arginine synthesis *in vivo* affected by dietary arginine or dietary protein intake ?

Experimental protocol

In this study the animals (adult rats) were fed diets containing either varying amounts of arginine or of protein for 7 days. Three different levels of dietary arginine (0.0, 0.5 and 2.0%) and three different levels of protein (5, 12 and 50%) were used. Body weight and food consumption were monitored on a daily basis. On day 7, the measurements of citrulline uptake and release of arginine by the kidneys of rats fed different diets were completed before 12 noon. These measurements were carried out according to the procedure described in **CHAPTER 2 (MATERIALS** and **METHODS)**. The principles behind quantifying renal fluxes of citrulline and arginine have been discussed earlier (**CHAPTER 2**).

Animals

Adult male Sprague-Dawley rats [Charles River, Montreal, Canada] initially weighing 320-390 g were used. They were housed individually in cages in a room kept at 24 $\pm$ 1°C with a 12:12 h light:dark cycle. The lights were on between 2000 h and 0800 h. The animals had *ad libitum* access to tap water and food.

Diets and feeding protocol

The composition of the arginine diets is presented in **Table 5.1.** Arginine-containing crystalline amino acid diets were prepared as described by Rogers and Harper (1965) with slight modifications as described below. The diets were prepared in gel form using 2% agar. These diets were made isonitrogenous and isocaloric. Various investigators who have worked on arginine nutrition in rats (**CHAPTER 1**) have fed them diets in which arginine was replaced by glycine. Since arginine contains four nitrogen atoms, its replacement by glycine requires large amounts of glycine. It has been reported that excess of glycine causes hyperammonemia in dogs (Harper et al, 1956) and in humans (Fahey et al, 1957). Therefore, in the present study the total amount of amino acid N (18.86 g/kg) was maintained by replacing arginine with a mixture of non-essential gluconeogenic amino acids (alanine, aspartic acid, asparagine, cysteine, proline, serine and tyrosine). These agar-gel based diets contained 60 ± 0.8% (mean ± SD) water and were stored in a refrigerator (+4°C). They were fed to rats (5 animals per treatment) ad libitum for 7 d. Food consumption and body weights were monitored on a daily basis. The dry weight of food consumed was determined by drying in an oven maintained at 50°C for 48 h.

The composition of the protein diets is given in **Table 5.2.** These diets were isocaloric as casein was replaced by sucrose and corn starch. A group of 5 rats was fed protein diets ad libitum

TABLE 5.1

Composition of arginine diets[1]

Arginine in diet (%)	0	0.5	2.0
Ingredient		g/kg	
Corn starch[2]	464.2	468.3	482.6
Sucrose[3]	238.1	234.2	240.9
Corn Oil[4]	100.0	100.0	100.0
Vitamin mix[5]	5.0	5.0	5.0
Mineral mix[5]	50.0	50.0	50.0
Choline bitartrate[5]	2.0	2.0	2.0
Amino acids[2]			
Histidine	2.6	2.6	2.6
Isoleucine	5.3	5.3	5.3
Leucine	7.1	7.1	7.1
Lysine (Free base)	11.6	11.6	11.6
Methionine	5.3	5.3	5.3
Phenylalanine	7.5	7.5	7.5
Threonine	5.3	5.3	5.3
Valine	5.3	5.3	5.3
Glutamic acid (Mono-K salt)	22.5	22.5	22.5
Glycine	7.5	7.5	7.5
Tryptophan	1.1	1.1	1.1
Alanine	12.6	10.0	2.55
Aspartic acid (Free acid)	6.6	6.6	2.2
Proline	8.1	6.6	2.2
Cysteine	7.55	6.6	2.2
Serine	12.6	10.0	2.5
Tyrosine	8.1	6.6	2.2
Asparagine	10.1	8.0	4.2
Arginine (Free base)	0.0	5.0	20.0
Total amino acids (g/kg)	146.75	140.50	119.15
Total amino acids N (g/kg)	18.86	18.86	18.86

[1]Diets were prepared in agar-gel form (20 g/kg diet; Rogers & Harper, 1965).
[2]Sigma Chemical, St. Louis, MO.
[3]U.S. Biochemicals, Cleveland, OH.
[4]Best Foods Canada Inc., Etobicoke, ON.
[5]ICN Biochemical, Cleveland, OH. AIN-76 mineral mix and AIN-76A vitamin mix are given in J. Nutr. 107:1340-1348 (1977) and J. Nutr. 110:1726 (1980).

TABLE 5.2

Composition of protein diets[1]

Protein in diet (%)	5	12	50
Ingredient		g/kg	
Casein[2]	50.0	120.0	500.0
Sucrose[3]	601.1	549.0	264.0
Corn starch[4]	200.4	182.5	87.5
Alpha cellulose[2]	50.0	50.0	50.0
Corn oil[5]	50.0	50.0	50.0
Vitamin mix[2]	10.0	10.0	10.0
Mineral mix[2]	35.0	35.0	35.0
Choline bitartrate[2]	2.0	2.0	2.0
L-Methionine[4]	1.5	1.5	1.5

[1]Metabolizable energy content of diet was calculated to be 16 kJ/g.

[2]ICN Biochemicals, Cleveland, OH. AIN-76 mineral mix and AIN-76A vitamin mix are given in J. Nutr. 107: 1340-1348 (1977) and J. Nutr. 1101726 (1980).

[3]U.S. Biochemicals, Cleveland, OH.

[4]Sigma Chemical, St. Louis, MO.

[5]Best Foods Canada Inc., Etobicoke, ON.

for 7 d and food consumption and body weights were monitored daily. On day 7, the measurements of citrulline uptake and release of arginine by the kidneys of rats fed different amounts of dietary arginine or protein were carried out and were completed before 12 noon.

Results

Varying dietary arginine

Animals were fed diets devoid of arginine (0%) or diets containing arginine (0.5 and 2%) for 7 d. Body weight and food consumption were monitored in those animals. On d 7, renal fluxes of citrulline and arginine were determined.

The data for body weight and food consumed during the study period are presented in **Table 5.3**. Adult rats consuming a diet containing no arginine failed to gain weight. However, the animals which were receiving 0.5 and 2.0% dietary arginine gained about 5g/day. There were no significant differences in either food consumption or the gain in body weight of animals fed 0.5 and 2.0% arginine. Rats given arginine-devoid diets ate significantly less than those fed 2.0% arginine. However, there were no significant differences in the amount of food consumed by the animals receiving 0.0 and 0.5% arginine. The failure to gain body weight in adult animals fed a diet lacking in arginine suggests a dietary arginine requirement for growth.

TABLE 5.3

Body weight, diet consumed and weight gain of adult Sprague-Dawley rats fed arginine diets[1].

Parameter (n)	Arginine in diet (%) 0.0 (4)	0.5 (5)	2.0 (5)
Body weight (g) Day 0	368 ± 29	357 ± 18	391 ±15
Body weight (g) Day 7	372 ± 26[a]	395 ± 17	428 ±23
Diet consumed[2] (g/d)	27.1 ± 2.6[b]	30.1 ± 4.6	37.1 ±4.1
Weight gain (g/d)	0.6 ± 1.1[c]	5.3 ± 1.9	5.2 ±1.6

[1]Values expressed as mean ± SD. The statistical significance of means assessed by ANOVA and Tukey's test.

[2]Determined as dry weight (48 h at 50°C).

[a,b]Significantly different from 2.0% arginine fed rats ($P < 0.05$).

[c]Significantly different from 0.5 and 2.0% arginine fed rats ($P < 0.05$).

Basic renal physiological data for rats on different intakes of arginine are shown in **Table 5.4**. There were no significant differences in wet or dry weight of kidneys, arterial or renal venous hematocrit and urine flow rate of animals fed 3 different levels of arginine. Renal hemodynamic parameters such as glomerular filtration rate and plasma flow rate were similar in all 3 groups of animals. From these observations, it was concluded that dietary arginine does not influence these physiological parameters of renal function.

The effects of dietary arginine on arterial levels of citrulline and arginine and renal uptake of citrulline and release of arginine were examined. Feeding rats diets containing varying amounts of arginine caused a significant increase (2.5-fold) in circulating levels of plasma arginine (196 μmol/L) in those animals fed 2.0% arginine (**Table 5.5**). The arterial plasma levels of arginine in rats fed 0 and 0.5% arginine were 73 and 81 μmol/L, respectively. There were no significant differences in circulating plasma levels of citrulline in any of the three groups. The net renal uptake of citrulline and release of arginine were similar in all the three groups.

Varying dietary protein. In this study, the effects of dietary protein on citrulline uptake and arginine release by the kidneys were examined. Animals were fed three different levels of protein for 7 d. Body weight and food consumption were monitored on a daily basis. On d 7, the renal fluxes of citrulline and arginine were

TABLE 5.4

Physiological data for rats fed arginine diets[1]

Parameter	Arginine in diet (%)		
(n)	0.0* (4)	0.5 (5)	2.0 (5)
Wet weight of kidneys (g)	2.44 ± 0.40	2.61 ± 0.26	2.81 ± 0.31
Dry weight of kidneys[2] (g)	0.63 ± 0.11	0.68 ± 0.04	0.75 ± 0.08
Arterial hematocrit	46.5 ± 1.7	45.2 ± 1.9	46.4 ± 2.1
Renal venous hematocrit	46.5 ± 1.7	45.6 ± 1.5	46.6 ± 2.2
U/P-inulin[3]	126.0 ± 20.5	155.6 ± 50.8	173.3 ± 45.0
Glomerular filtration rate[4]	0.66 ± 0.17	0.65 ± 0.26	0.66 ± 0.20
Renal plasma flow rate[4]	1.62 ± 0.42	1.90 ± 0.85	2.25 ± 0.46
Filtration fraction	0.41 ± 0.08	0.36 ± 0.10	0.29 ± 0.05
Urine flow rate[5]	5.3 ± 1.2	4.5 ± 2.0	4.3 ± 2.6

[1]Values expressed as mean ± SD. There were no significant differences ($P > 0.05$) in any of the parameters in rats fed different amounts of protein as assessed by ANOVA.

[2]Determined after 48 h at 50°C.

[3]U/P-urine-to-plasma.

[4]Glomerular filtration rate and renal plasma flow rate expressed as mL/min/100 g body weight.

[5]Urine flow rate expressed as μL/min/100 g body weight.

*One animal was lost during surgery.

TABLE 5.5

Arterial plasma levels and net renal flux of citrulline and arginine in rats fed arginine diets[1]

Arginine in diet (%)	(n)	Arterial Plasma concentration		Net Renal Flux[2]	
		Citrulline	Arginine	Citrulline	Arginine
		nmoles/ml		nmoles/min/100 g body weight	
0.0	(4)	59.2 ± 9.0[a]	72.7 ± 4.3[a]	50.9 ± 16.6[a]	-31.6 ± 32.6[a]
0.5	(5)	48.2 ± 3.4[a]	80.8 ± 11.3[a]	37.6 ± 13.4[a]	-39.3 ± 31.8[a]
2.0	(5)	54.1 ± 6.0[a]	195.9 ± 37.2[b]	55.5 ± 11.8[a]	-54.6 ± 13.2[a]

[1]Values expressed as mean ± SD. Values within a column not sharing a common superscript are significantly different ($P < 0.05$) as assessed by ANOVA and Tukey's test.

[2]Positive value for flux indicates uptake and negative value indicates output. No significant differences ($P > 0.05$) between citrulline uptake and arginine release.

determined. The data in **Table 5.6** shows the results of weight gain and food consumed by these rats. The rats fed a low protein (5%) diet lost body weight although they consumed a similar quantity of food compared to those fed 12% and 50% protein. There were no significant differences in either food consumption or gain in body weight of animals fed 12 and 50% protein.

The basic parameters of renal function in rats fed different amounts of protein are presented in **Table 5.7.** There were no significant differences in any of the measured parameters amongst the three groups except for glomerular filtration and renal plasma flow rates which were significantly elevated in those animals fed 50% protein.

The effects of dietary protein on arterial levels of citrulline and arginine and on the renal citrulline uptake and arginine release were examined. The results are presented in **Table 5.8.** The level of dietary protein (5, 12 and 50%) did not influence the arterial plasma concentrations of citrulline or arginine. There was a significant increase in the renal uptake of citrulline (84.2 ± 25.1 nmoles/min/100 g body wt.) in rats fed 50% protein. Mean renal arginine release was also higher in these animals (81.4 ± 37.4 nmoles/min/100 g body weight) but was not significantly different from those fed 5 and 12% protein. The net renal citrulline uptake matched the renal arginine release in all the three groups.

The circulating plasma levels of citrulline [A] and arginine [B] in rats on different intakes of arginine (protein and arginine

TABLE 5.6

Body weight, diet consumed and weight gain of adult Sprague-Dawley rats fed protein diets[1].

Parameter (n)	Protein in diet (%) 5 (6)	12 (5)	50 (5)
Body weight (g) Day 0	370 ± 69	319 ± 39	342 ±12
Body weight (g) Day 7	368 ± 54	356 ± 33	365 ±18
Diet consumed (g/d)	23.2 ± 2.2	21.5 ± 0.7	20.0 ±2.1
Weight gain (g/d)	-0.4 ± 2.6[a]	5.6 ± 2.2[b]	3.8±1.1[b]

[1]Values expressed as mean ± SD. The statistical significance of means assessed by ANOVA and Tukey's test.

[a,b] Values with a different superscript are significantly different ($P < 0.05$).

TABLE 5.7

Physiological data for rats fed protein diets[1]

Parameter	Protein in diet (%)[6]		
(n)	5 (6)	12 (5)	50 (5)
Wet weight of kidneys (g)	2.43 ± 0.4[ab]	2.05 ± 0.1[b]	2.58 ± 0.3[b]
Dry weight of kidneys (g)[2]	0.58 ± 0.11[ab]	0.53 ±0.03[b]	0.69 ± 0.07[b]
Arterial hematocrit	46.5 ± 1.7	45.4 ± 1.5	46.2 ± 1.6
Renal venous hematocrit	46.0 ± 1.3	45.8 ± 1.3	46.6 ± 1.3
U/P-inulin[3]	136.0 ± 59.0	122.8 ± 24.5	161.2 ± 47.0
Glomerular filtration rate[4]	0.57 ± 0.26[b]	0.60 ± 0.1[b]	1.07 ± 0.03[a]
Renal plasma flow rate[4]	1.75 ± 0.92[ab]	1.44 ± 0.16[b]	2.68 ± 0.39[a]
Filtration fraction	0.36 ± 0.08	0.42 ± 0.06	0.40 ± 0.05
Urine flow rate[5]	4.3 ± 1.5	5.1 ± 1.4	7.1 ± 1.9

[1]Values expressed as mean ± SD. The statistical significance of means assessed by ANOVA and Tukey's test.

[2]Determined after 48 hr at 50°C.

[3]U/P-Urine-to-plasma.

[4]Glomerular filtration rate and renal plasma flow rate expressed as mL/min/100 g body weight.

[5]Urine flow rate expressed as μL/min/100 g body weight.

[6]Values with a different superscript are significantly different ($P < 0.05$).

TABLE 5.8

Arterial plasma levels and net renal flux of citrulline and arginine in rats fed protein diets[1]

Protein in diet (%)	(n)	Arterial Plasma Concentration		Net Renal Flux[2]	
		Citrulline	Arginine	Citrulline	Arginine
		nmoles/ml		nmoles/min/100 g body weight	
5	(6)	57.3 ± 16.1[1]	96.5 ± 30.7[1]	52.9 ± 22.1[1]	-60.4 ± 30.6[1]
12	(5)	75.3 ± 9.4[1]	95.0 ± 20.1[1]	51.8 ± 7.3[1]	-46.8 ± 25.1[1]
50	(5)	60.7 ± 11.3[1]	113.8 ± 18.9[1]	84.2 ± 25.1*	-81.4 ± 37.4[1]

[1]Values expressed as mean ± SD. The statistical significance of means assessed by ANOVA and Tukey's test.

*Significantly different ($P < 0.05$) from rats fed 12% protein.

[2]Positive value for flux indicates uptake and negative value indicates output. No significant differences ($P > 0.05$) were observed between citrulline uptake and arginine release.

diets) are shown in **Figure 5.1.** I have calculated the amount of arginine consumed (g/d) by each rat from the diet consumed and the arginine content of the diet. The arginine content of the 5, 12 and 50% protein diets were 2.1, 5.0 and 20.6 mg/g, respectively. It can be seen in **Figure 5.1 A** that the arterial plasma citrulline levels were unchanged in animals on different intakes of arginine. On the other hand, circulating plasma arginine levels (**Figure 5.1 B**) increased with an increase in the intake of arginine and an excellent correlation ($r = 0.88$) was observed.

It can be seen from **Figure 5.2 A** that there is a 1:1 molar relationship (slope = 0.996, $r = 0.70$) between citrulline uptake and arginine release in animals fed different levels of arginine or protein. The net renal release of arginine was plotted against arginine ingested by each rat and the results are presented in **Figure 5.2 B.** The figure clearly demonstrates that the release of arginine by the kidneys is independent of arginine intake.

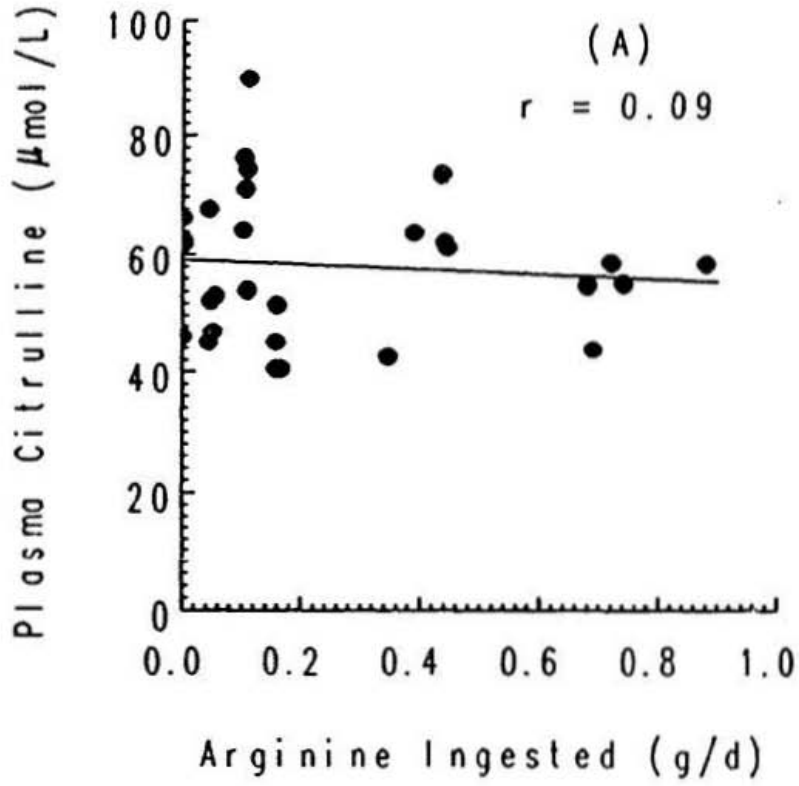
Discussion

The kidneys of normal animals remove citrulline from the blood and release arginine (Windmueller and Spaeth, 1981; Brosnan et al, 1983). It was demonstrated that a quantitative relationship between citrulline uptake and arginine release by kidneys exists in saline- and citrulline-infused rats (**CHAPTER 4**). The activity of "arginine synthetase" (measured as argininosuccinate synthetase plus argininosuccinate lyase) in the kidney has been reported to

FIGURE 5.1

Circulating levels of citrulline and arginine in rats on different intakes of arginine

The figure plots the circulating levels of citrulline (**A**) or arginine (**B**) against arginine ingested. Each point represents an individual animal.


(A)
r = 0.09
Plasma Citrulline (μmol/L)
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
Arginine Ingested (g/d)

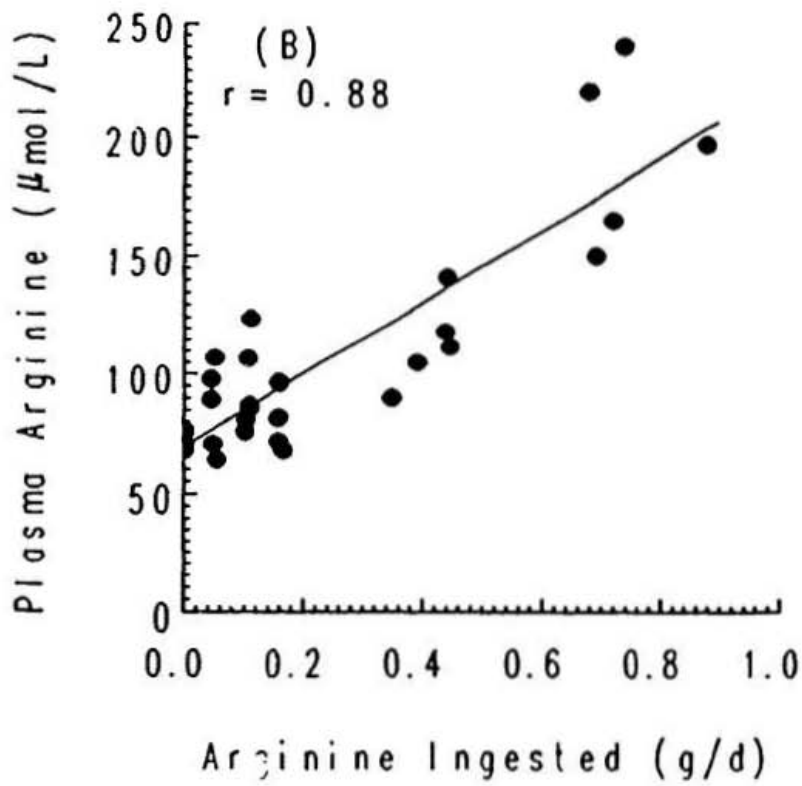
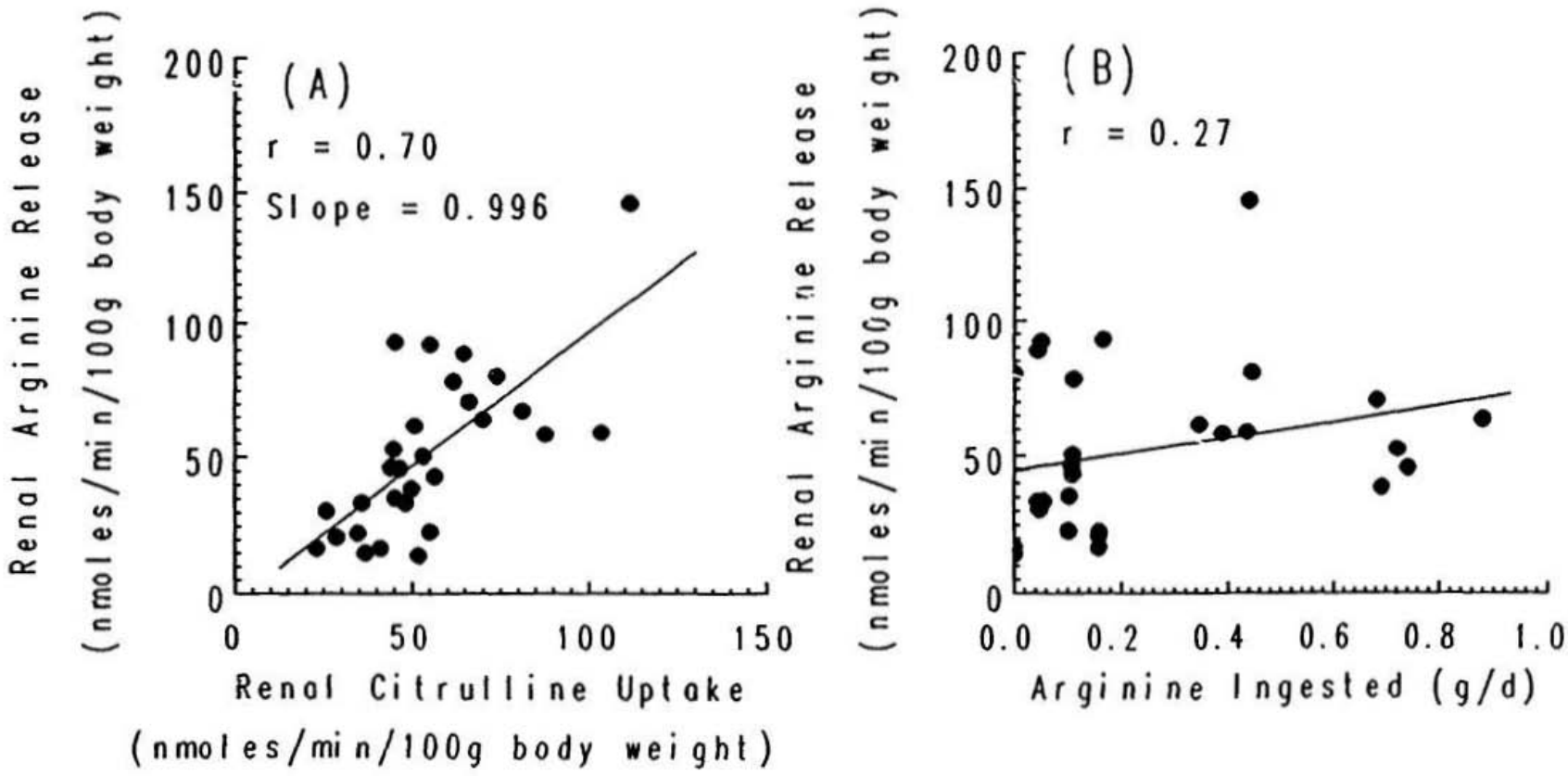

(B)
r = 0.88
Plasma Arginine (μmol/L)
250
200
150
100
50
0
0.0
0.2
0.4
0.6
0.8
1.0
Arginine Ingested (g/d)

Figure 5.2

Renal uptake of citrulline and release of arginine in rats on different intakes of arginine

The figure plots the renal arginine release against renal citrulline uptake (**A**) and arginine ingested (**B**). Each point represents an individual animal.


(A)
r = 0.70
Slope = 0.996
Renal Arginine Release
(nmoles/min/100g body weight)
Renal Citrulline Uptake
(nmoles/min/100g body weight)
0
50
100
150
200
(B)
r = 0.27
Renal Arginine Release
(nmoles/min/100g body weight)
Arginine Ingested (g/d)
0.0
0.2
0.4
0.6
0.8
1.0

increase by 2-fold in rats fed 90% protein vs. 25% protein (Szepesi et al, 1970). Also, the relative abundance of mRNAs for the enzymes of arginine synthesis in the kidney are reported to be increased by 2-fold in animals fed high protein (60%) vs. low protein (27%) (Morris et al, 1989). However, Aperia et al (1979) reported no changes in the activities of arginine synthesizing enzymes in kidneys of rats fed 6 and 21% protein. From studies involving measurement of enzymatic activities, one cannot obtain definitive information about the actual metabolism of a substrate in an organ. No previous attempts have been made to quantify renal citrulline uptake and arginine release in animals subjected to different dietary treatments. Therefore, systematic studies were carried out to examine the possibility of renal adaptation of arginine synthesis in rats fed diets containing varying amounts of arginine or protein.

Adult rats fed a diet devoid of arginine did not gain body weight and the food intake of those animals was significantly less than those fed 2% arginine (**Table 5.3**). The failure to gain weight is probably due to the insufficiency of endogenously synthesized arginine. Depressed feed intake and gain in body weight has been observed in animals which were fed diets without arginine (Milner et al, 1974; Milner & Visek, 1974). Animals fed a low protein (5%) diet lost body weight (**Table 5.6**). This loss in body weight was not due to insufficient food intake but due to protein malnutrition.

The basic parameters of renal function (glomerular filtration rate, renal plasma flow, filtration fraction and urine flow rate)

were unaffected by arginine intake (**Table 5.4**) and were in agreement with those reported in chow fed rats (**CHAPTER 4**; Lowry et al, 1987; Brosnan & Hall, 1989). Glomerular filtration rate and renal plasma flow were increased in those animals fed 50% casein (**Table 5.7**). It has been recognized that high protein feeding causes increased glomerular filtration rate and renal blood flow in rats (Shannon et al, 1932; Van Slyke et al, 1934; Pitts, 1935) and in humans (Pullman et al, 1954). However, it is not clearly understood how dietary protein affects glomerular filtration and renal plasma flow rates.

There was a 2.5-fold increase in arterial levels of arginine in those animals receiving 2.0% arginine. Excess dietary arginine might have been catabolized by arginase and ornithine aminotransferase in the liver where their activities are reported to be highest (Herzfeld & Raper, 1976; Herzfeld & Knox, 1968). An important observation in this study was that plasma arginine levels were maintained in animals even though they were consuming arginine-free diets (**Table 5.5**). Regardless of the arginine content of the diet, there was a similar uptake of citrulline and release of arginine by the kidneys.

Protein-deprivation (5%), moderate (12%) and high (50%) protein feeding caused no significant changes in the arterial plasma concentrations of citrulline and arginine. Strikingly, neither protein-deprivation nor high-protein feeding influenced plasma arginine levels. High protein feeding increased renal citrulline uptake (**Table 5.8**). Renal arginine release matched

citrulline uptake in these animals but was not significantly different from the other animals.

The plot of arginine ingested by animals given different amounts of arginine or protein versus renal arginine release (**Figure 5.2 B**) illustrates that renal arginine production is independent of dietary arginine. A probable explanation for this is that in all these animals the circulating level of citrulline remained unchanged (59 $\pm$ 9 μmol/L) (**Figure 5.1 A**). This suggests that the availability of citrulline is the limiting factor for renal arginine synthesis. Thus, it is possible that in the present study the intestinal release of citrulline may have been tightly regulated. It is known that the intestine is the principal organ that releases citrulline which is utilized by the kidney (Windmueller & Spaeth, 1981). The importance of intestinal citrulline synthesis in arginine nutrition was demonstrated in the studies of Hoogenraad et al (1985). In their studies, when intestinal citrulline synthesis was specifically inhibited, a severe depression in the growth of rats was observed. They also demonstrated that dietary citrulline can effectively substitute for arginine in meeting growth requirements of rats. The utilization of dietary citrulline for renal arginine synthesis can occur. Therefore, it would be interesting to carry out studies to examine the effect of dietary citrulline on the release of arginine by the kidneys. Although citrulline is not found in proteins, it is present in substantial quantities (100 mg/100 g) in watermelon, Citrullis vulgaris. It is possible that citrulline supplementation

by dietary means may elevate its plasma level which the kidney can metabolize to arginine.

In conclusion, the response of adult rat kidney arginine synthesis is independent of arginine and protein intakes and is probably limited by availability of citrulline in blood.

CHAPTER 6

SUMMARY AND GENERAL DISCUSSION

SUMMARY AND GENERAL DISCUSSION

Summary

The investigations conducted for the thesis revealed the following major findings:

1. The enzymes of arginine synthesis, argininosuccinate synthetase and argininosuccinate lyase are exclusively found in the cytosol of the cells of the proximal convoluted tubule.
2. Arginine production from citrulline was also confined to proximal convoluted tubule and thus confirmed the enzyme localization data that arginine synthesis occurs in proximal convoluted tubule.
3. The enzymes of arginine utilization, arginase and ornithine aminotransferase, are located in other kidney regions.
4. The highest rates of arginine production from citrulline in isolated kidney cortical tubules required a source of the second "N" atom of the guanidino group of arginine. In this respect, aspartate, glutamate and glutamine were effective whereas glycine, serine and alanine were ineffective.
5. The newly synthesized arginine is not metabolized to a great extent (only 6%) to ornithine and urea by kidney cortical tubules. Accordingly, in vivo, renal arginine production equalled citrulline uptake.

6. Arginine synthesis in kidney cortical tubules was found to be highly sensitive to citrulline concentrations in the physiological plasma range (60 μM), suggesting that citrulline availability could be a limiting factor for renal arginine synthesis *in vivo*.

7. When plasma citrulline levels were elevated by infusing citrulline into adult rats, their kidneys responded linearly by increasing citrulline uptake and producing increased quantities of arginine.

8. Adult animals, when given an arginine-deficient diet for 7 d, did not gain weight. This suggested that arginine is essential for growth even in adult animals.

9. The renal uptake of citrulline and release of arginine were similar and the circulating levels of citrulline (60 μM) remained unchanged in animals given different amounts of dietary arginine or dietary protein. This suggested that renal arginine synthesis is independent of dietary arginine or protein intake and is probably limited by availability of citrulline in blood.

General Discussion

It is important to understand why the kidney, and in particular the proximal convoluted tubule, is the site of arginine biosynthesis. It is well established (Krebs & Henseleit, 1932) that liver plays a pivotal role in nitrogen disposal via the formation of urea. Although arginine is synthesized in the liver, it is catabolized by the combined actions of arginase (urea cycle) and

ornithine aminotransferase. One molecule of arginine is removed for every one produced in the operation of the urea cycle. Thus, the urea cycle does not contribute new arginine.

Recently, Alonso & Rubio (1989) demonstrated that even under conditions of arginine deprivation there is catabolism of ornithine in the liver, kidney and muscle of mice. In their studies tissue ornithine concentrations were measured (on d 8, over a 10 h period) after the administration of gabaculine (a potential suicide inhibitor of ornithine aminotransferase) to mice adapted (7 d) to amino acid diets with and without arginine. It was observed that the tissue ornithine accumulated, even in animals given arginine-devoid diets. They estimated that at least 45 μmoles of ornithine is synthesized and catabolized daily via ornithine aminotransferase in the mouse deprived of arginine. This suggests that the net drain of the endogenous arginine pool can occur via the OAT reaction. The anatomical location of the liver ensures that newly absorbed arginine must pass through the liver before being distributed to the periphery. The liver can metabolize dietary arginine by the combined actions of arginase and ornithine aminotransferase. Although these enzymes are widely distributed (Herzfeld & Raper, 1976; Herzfeld & Knox, 1968; **Table 1.5**), they are abundantly present in the liver. The L-glutamate-γ-semialdehyde produced by ornithine aminotransferase may be converted to glutamate and either oxidized or converted to glucose. It is clearly important to separate the site of arginine synthesis from that of arginine breakdown.

The kidney is an appropriate site for arginine synthesis because of its high blood flow and ready supply of substrates. The renal blood supply amounts to approximately 20% of cardiac output. The major functions of kidneys are to maintain electrolyte balance and to reabsorb important metabolites (glucose, amino acids, etc.). This occurs largely by glomerular filtration and tubular reabsorption. The major site of reabsorption of glucose, amino acids and sodium is the proximal convoluted tubule. The reabsorption of glucose and amino acids exceeds 99%. Filtration and reabsorption of amino acids involves their passage through the tubular cells, being transported into the cells at the luminal side and out of the cells at the basolateral side. Citrulline and other amino acids (aspartate, glutamate and glutamine) which serve as donors of the second "N" atom of guanidino group of arginine will, therefore be available to the arginine synthetic enzymes in the cytoplasm of these cells. Kettner and Silbernagl (1986) have suggested that in the rat kidney proximal tubule there exist two transport systems which can account for the complete reabsorption of citrulline, one with a high capacity and low affinity and the other a high affinity and low capacity. Therefore, it is advantageous to have the arginine synthesizing machinery in the cells of the proximal convoluted tubule presumably because of the availability of large quantities of the substrates.

It was clear from the discussions in **CHAPTER 1** that blood citrulline arises mainly from the intestinal metabolism of glutamine. This citrulline is not appreciably taken up by the liver

(Windmueller and Spaeth, 1981). This may be due to lack of a specific transport system for citrulline in the liver. Studies are required to establish unambiguously the nature and extent of citrulline transport into hepatocytes. On the other hand, the kidneys do have a system to take up citrulline (Kettner and Silbernagl, 1986) and convert it to arginine and this system does not appear to be saturated in vivo (**CHAPTER 4**). Thus, the kidneys have the capacity to metabolize any dietary citrulline or endogenous citrulline that is generated either in the intestine or in the reaction catalyzed by nitric oxide synthetase. It would be interesting to examine the effect of feeding dietary citrulline on renal arginine synthesis in rats.

It is important to understand the quantitative aspects of arginine consumption, production and utilization. In this respect, I have calculated on a daily basis the quantity of arginine that is available for various metabolic processes in a rat and the values have been normalized to a 300 g rat. A normal rat consumes approximately 21 g Chow/d (98 μmoles arginine/g of Purina Chow, as measured in this laboratory), such that the arginine intake corresponds to 2000 μmoles/d. The endogenous renal arginine synthesis is about 340 μmoles/d (**CHAPTER 4**). Thus, exogenous and endogenous arginine availability amounts to 2340 μmoles/d for utilization in vivo. Since protein synthesis and breakdown is in balance over the 24-h period, it is not factored into this calculation. Arginine is utilized for the formation of some important metabolites with major functions such as creatine, nitric

oxide and polyamines. Creatinine (a nonenzymatic breakdown product of creatine in muscle) excretion in a 300 g rat is about 11 μmoles/d (Visek, 1986). Creatine synthesis must balance creatinine excretion. Nitrate (a stable end product produced from nitric oxide) excretion is about 5 μmoles/d (Wagner et al, 1983). The utilization of arginine for polyamine synthesis is minimal (Pegg & McCann, 1982). Thus, the above metabolites accounts for only a small drain on arginine pools in the rat. However, the major drain on arginine pools can occur via the reactions of arginase and ornithine aminotransferase. In fact, it has been demonstrated in the mouse that arginine is catabolized primarily via the reaction catalyzed by ornithine aminotransferase (Alonso & Rubio, 1989). This drain can amount to 45 μmoles/d in a 30-g mouse. This value was estimated by Alonso & Rubio (1989), based on ornithine accumalation in liver, kidney, muscle, brain and blood in arginine-deficient mice administered gabaculine (a powerful inhibitor of ornithine aminotransferase).

The studies conducted in this thesis suggest that renal arginine synthesis is probably limited by the availability of blood citrulline. In situations such as infections, citrulline production increases as a result of the increased nitric oxide synthetase reaction in macrophages. It has been shown that nitrate excretion is increased in rats treated with endotoxin. Although, citrulline is not commonly found in protein it is present in substantial amount in water melon (Citrullus vulgaris). Finally, under the conditions where intestinal citrulline production may be increased,

renal citrulline uptake and arginine output may also increase. It appears that the intestinal citrulline output may be a key regulator of renal arginine synthesis. Therefore, it is important to determine the factors that are involved in the regulation of intestinal citrulline synthesis.

One possible regulator of intestinal citrulline synthesis may be the availability of glutamine. It would be interesting to examine the effect of glutamine infusion on intestinal citrulline production. Further studies are needed to understand the regulation of intestinal citrulline synthesis.

REFERENCES

Adamson, I. & Fisher, H. (1976) Further studies on the arinine requirement of the rabbit. J. Nutr. 106:717-723.

Alonso, E. & Rubio, V. (1989) Participation of ornithine aminotransferase in the synthesis and catabolism of ornithine in mice. Biochem. J. 259:131-138.

Aperia, A., Broberger, O., Larsson, A. & Snellman, K. (1979) Studies of renal urea cycle enzymes. I. Renal concentrating ability and urea cycle enzymes in the rat during protein deprivation. Scan. J. Clin. Lab. Invest. 39: 329-336.

Barbul, A., Rettura, G. & Levenson, S.M. & Seifter, E. (1977) Arginine: Thymotropic and wound healing promoting agent. Surg. Forum 28:101-103.

Barbul, A., Rettura, G., Levenson, S.M. & Seifter, E. (1983) Wound healing and thymotropic effects of arginine: a pituitary mechanism of action. Am. J. Clin. Nutr. 37: 786-794.

Barbul, A., Wasserkrug, H.L., Yoshimura, N., Tao, R. & Efron, G. (1984) High arginine levels in intravenous hyperalimentation abrogate post-traumatic immune suppresion. J. Surg. Res. 36: 620-624.

Barbul, A. (1986) Arginine: Biochemistry, physiology and therapeutic implications. JPEN 10:227-238.

Barbul, A., Lazarou, S.A., Efron, D.T., Wasserkrug, H.L. & Efron, G. (1990) Arginine enhances wound healing and lymphocyte immune responses in humans. Surgery 108:331-337.

Bauer, C.D. & Berg, C.P. (1943) The amino acids required for growth in mice and the availability of their optical isomers. J. Nutr. 26:51-63.

Borman, A., Wood, T.R., Black, H.C., Anderson, E.G., Oesterling, M.J., Womack, M. & Rose, W.C. (1946) The role of arginine in growth with some observations on the effects of argininic acid. J. Biol. Chem. 166:585-594.

Borsook, H. & Dubnoff, J.W. (1940) The formation of creatine from glycocyamine in the liver. J. Biol. Chem. 132:559-574.

Borsook, H. & Dubnoff, J.W. (1941) The conversion of citrulline to arginine in kidney. J. Biol. Chem. 141:717-738.

Borsook, H. & Dubnoff, J.W. (1941a) The formation of glycocyamine

in animal tissues. J. Biol. Chem. 138:389-403.

Bredt, D.S. & Snyder, S.H. (1989) Nitric oxide mediates glutamate-linked enhancement of c-GMP levels in the cerebellum. Proc. Natl. Acad. Sci. USA 86:9030-9033.

Brosnan, J.T. & Hall, B. (1989) Renal serine production in vivo: effects of dietary manipulation of serine status. Can. J. Physiol. Pharmacol. 67:1058-1061.

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

Brusilow, S.W. (1984) Arginine, an indispensable amino acid for patients with inborn errors of urea synthesis J. Clin. Invest. 74:2144-2148.

Burns, R.A., Milner, J.A. & Corbin, J.E. (1981) Arginine: An indispensable amino acid for mature dogs. J. Nutr. 111: 1020-1024.

Burton, K. (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-323.

Carey, G.P, KIme, Z., Rogers, Q.R., Morris, J.G., Hargrove, D., Buffington, C.A. & Brusilow, S.W. (1987) An arginine-deficient diet in humans does not evoke hyperammonemia or orotic aciduria. J. Nutr. 117:1734-1739.

Chan, W., Wang, M., Kopple, J.D. & Swenseid, M.E. (1974) Citrulline levels and urea cycle enzymes in uremic rats. J. Nutr. 104:678-683.

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

Collier, J. & Vallance, P. (1989) Second messenger role for NO widens to nervous and immune systems. TiPS 10:427-431.

Costello, M.J., Morris, J.G. & Rogers, Q.R. (1980) Effect of dietary arginine level on urinary orotate and citrate excretion in growing kittens. J. Nutr. 110:1204-1208.

Czarnecki, G.L. & Baker, D.H. (1984) Urea cycle function in the dog with emphasis on the role of arginine. J. Nutr. 114:581-590.

Daly, J.M., Reynolds, J., Thom, A., Kinsley, L., Dietrick-Gallagher, M., Shou, J. & Ruggieri, B. (1988) Immune and

metabolic effects of arginine in the surgical patient. Ann. Surg. 208:512-523.

de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. & Applemans, F. (1955) Tissue Fractionation Studies 6. Intracellualr distribution patterns of enzymes in rat-liver tissue. Biochem. J. 60:604-617.

DeLong, G.R. & Glick, T.M. (1982) Ammonia metabolism in Reye's syndrome and the effect of citrulline. Ar- Neurol. 11:53-58.

Deshmukh, G.R. & Shope, T.C. (1983) Arginine requirement and ammonia toxicity in ferrets. J. Nutr. 113:1664-1667.

Fahey, J.L. (1957) Toxicity and blood ammonia rise resulting from intravenous amino acid admi tration in man: The protective effect of L-arginine. J. Cl. .. Invest. 36:1647-1655.

Featherston, W.R., Rogers, Q.R. & Freedland, R.A. (1973) Relative importance of kidney and liver in the synthesis of arginine by the rat. Am. J. Physiol. 224:127-12^

Garganta, C.L. & Bond, J.S. (1986) Assay and kinetics of arginase. Anal. Biochem. 154:388-394.

Geyer, J.W. & Dabiach, D. (1971) Rapid method for the determination of arginase activity in tissue homogenates. Anal. Biochem. 39:412-417.

Gianetto, R. & de Duve, C. (1955) Tissue Fractionation Studies 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by liver particles. Biochem. J. 59:433-438.

Gornall, A.G., Bardawill, C.J. & David, M.M. (1949) Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751-7766.

Gross, M.D., Simon, A.M., Jenny, R.J., Gray, E.D., McGuire, D.M. & Van Pilsum. J.F. (1988) Multiple forms of rat kidney L-arginine:glycine amidinotransferase. J. Nutr. 118:1403-1409.

Guder, W., Wiesner, W., Stukowski, B. & Wieland, O. (1971) Metabolism of Isolated kidney cortical tubules. Hoppe-Seyler's Z. Physiol. Che. 352:1319-1328.

Guder, W.G. & Schmidt, U. (1974) The localization of gluconeogenesis in rat nephron. Determination of phosphoenolpyruvate carboxykinase in microdissected tubules. Hoppe-Seyler's Z. Physiol. Chem. 355:273-278.

Ha, Y., Milner, J.A. & Corbin, J.E. (1978) Arginine requirements

in immature dogs. J. Nutr. 108:203-210.

Harper, H.A., Najarian, J.S. & Silen, W. (1956) Effect of intravenously administered amino acids on blood ammonia. Proc. Soc. Exp. Biol. Med. 92:558-560.

Hecker, M., Sessa, W.C., Harris, H.J., Anggard, E.E. & Vane, J.R. (1990) The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: Cultured endothelial cells recycle L-citrulline to L-arginine. Proc. Natl. Acad. Sci. USA 87:8612-8616.

Heinicke, H.R., Harper, A.E. & Elvehjem, C.A. (1955) Protein and amino acid requirements of the guinea pig. J. Nutr. 57:483-496.

Heinle, H., Wendel, A. & Schmidt, U. (1977) The activities of the key enzymes of the γ-glutamyl cycle in microdissected segments of the rat nephron. FEBS Lett. 73:220-224.

Heird, W.C., Nicholson, J.F., Driscoll, Jr., J.M., Schullinger, J.N. & Wimters, R.W. (1972) Hyperammonemia resulting from intravenous alimentation using a mixture of synthetic amino acids: a prelimianry report. J. Pediatr. 81:162-165.

Henslee, J.G. & Jones, M.E. (1982) Ornithine synthesis from glutamate in rat small intestinal mucosa. Arch. Biochem. Biophys. 219:186-197.

Herzfeld, A. & Knox, W.E. (1968) The properties, developmental formation and estrogen induction of ornithine aminotransferase in rat tissues. J. Biol. Chem. 243:3327-3322.

Herzfeld, A. & Raper, S.M. (1976) The heterogeneity of arginases in rat tissues. Biochem. J. 153:469-478.

Hibbs, Jr., J.B., Taintor, R.R. & Vavrin, Z. (1987) Macrophage cytotoxicity: Role of for L-arginine deiminase and immuno nitrogen oxidation to nitrite. Science 235:473-476.

Holt, L.E., Jr., Albanese, A.A., Shettles, L.B., Kajdi, C. & Wangerin,P.M. (1942)Studies of experimental amino acid deficiency in man. I. Nitrogen balance. Fed. Proc. 1:116-.

Hoogenraad, N., Totino, N., Elmer, H., Wraight, C., Alewood, P. & Johns, R.B. (1985) Inhibition of intestinal citrulline synthesis causes severe growth retardation in rats. Am. J. Physiol. 249:G792-G799.

Hurwitz, R. & Kretchmer, N. (1986) Development of arginine-synthesizing enzymes in mouse intestine. Am. J. Physiol. 251:G103-110.

Ignarro, L.J. (1989) Endothelium-derived nitric oxide: actions and properties. FASEB J. 3:31-36.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. & Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. USA 84:9265-9269.

Jacobs. E.E., Jacob, J., Sanadi, D.R. & Bradley, L.B. (1956) Uncoupling of oxidative phosphorylation by cadmium ion. J. Biol. Chem. 223:147-156.

Jones, M.E., Anderson, A.D., Anderson, C. & Hodes, S. (1961) Citrulline synthesis in rat tissues. Arch. Biochem. Biophys. 95:499-507.

Jones, M.E. (1983) Catalysts of the urea cycle. Trans. New York Acad. Sci. 45:77-82.

Kalra, J. & Brosnan, J.T. (1974) The subcellular localization of glutaminase isoenzymes in rat kidney cortex. J. Biol. Chem. 249:3255-3260.

Kari, F.W., Ulman, E.A., Mulloy, A.L. & Visek, W.J. (1981) Arginine requirement of mature protein-malnourished rats for maximal rate of repletion. J. Nutr. 111:1489-1493.

Kato, H. Oyamada, I. Mitzutani-Funahashi & Nakagawa, H. (1976) New radioisotopic assays of argininosuccinate synthase and argininosuccinase. J. Biochem. 79:945-953.

Kaysen, G. A. & Strecker, H.J. (1973) Purification and properties of arginase of rat kidney. Biochem. J. 133:779-788.

Kesner (1965) The effect of ammonia administration on orotic acid excretion in rats. J. Biol. Chem. 240:1722-1724.

Kettner, A. & Silbernagl, S. (1985) Renal handling of citrulline. In: Kidney Metabolism and Function (eds. Dzurik, R., Lichardus, B. and Guder, W.) Martinus Nijhoff Publishers, Boston, pp. 51-60.

Kirk, S.J. & Barbul, A. (1990) Role of arginine in trauma, sepsis and immunity. JPEN 14:226S-229S.

Klose, A.A., Stokstad, E.L.R. & Almquist, H.J. (1938) The essential nature of arginine in the diet of the chick. J. Biol. Chem. 123:691-698

Knowles, R.G., Palacios, M., Palmer, R.M.J. & Moncada, S. (1989) Formation of nitric oxide from L-arginine in the central nervous system. A transduction mechanism for the stimulation

of the soluble guanylate cyclase. Proc. Natl. Acad. Sci. USA 86:5159-5162.

Kratzer, F.H., Bird, F.H., Asmundson, V.S. & Lepkovosky, S. (1947) The arginine requirement of young turkey poults. J. Nutr. 34:167-171.

Krebs, H.A. & Henseleit, K. 1932) Utersuchungen uber die Harnstoffbildung im Tierkorper. Hoppe-Seyler's Z. Physiol. Chem. 210:33-

Kriz, W. & Bankir, L. (1988) A standard nomenclature for structures of the kidney. Am. J.Physiol. 254:F1-F8.

Leaf, C.D., Wishnok, J.S. & Tannenbaum, S.R. (1989) L-arginine is a precursor for nitrate biosynthesis in humans. Biochem. Biophys. Res. Commun. 163:1032-1037.

Levillain, O., Hus-Citharel, A., Morel, F. & Bankir, L. (1989) Production of urea from arginine in pars recta and collecting duct of rat kidney. Renal Physiol. Biochem. 12:302-312.

Levillain, O., Hus-Citharel, A., Morel, F. & Bankir, L. (1990) Localization of arginine synthesis along rat nephron. Am. J. Physiol. 259:F916-F923.

Levy, H.M., Montanez, G., Feaver, E.R., Murphy, E.A. & Dunn, M.S. (1954) Effect of arginine in tumor growth in rats. Cancer Res. 14:198-200.

Lowry, M., Hall, D.E. & Brosnan, J.T. (1985) Hydroxyproline metabolism by the rat kidney: Distribution of renal enzymes of hydroxyproline catabolism and renal conversion of hydroxyproline to glycine and serine. Metabolism 34:955-961.

Lowry, M., Hall, D.E. & Brosnan, J.T. (1986) Serine synthesis in rat kidney: studies with isolated perfused kidney and cortical tubules. Am. J. Physiol. 250:F649-F658.

Lowry, M., Hall, D.F., Hall, M.S. & Brosnan, J.T. (1987) Renal metabolism of amino acids <u>in vivo</u>: studies on serine and glycine fluxes. Am. J. Physiol. F304-F309.

Magri, E., Baldoni, G. & Grazi, E. (1975) On the biosynthesis of creatine. Intra-mitochondrial localization of tranamidinase from rat kidney. FEBS Lett. 55:91-93.

Marshall, M. & Cohen, P.P (1972) Ornithine transcarbamylase from <u>Streptococcus faecalis</u> I. Isolation and subunit structure. J. Biol. Chem. 247:1641-1653.

McGivan, J.D., Bradford, N.M. & Beavis, A.D. (1977) Factors

influencing the activity of ornithine aminotransferase in isolated rat mitochondria. Biochem. J. 162:147-156.

McGuire, D.M., Tormanen, C.D., Segal, I.S. & Van Pilsum, J.F. (1980) The effect of growth hormone and thyroxine on the amount of L-arginine:glycine amidinotransferase in kidneys of hypophysectomized rats. J. Biol. Chem. 255:1152-1159.

McGuire, D.M., Gross, M.D., Elde, R.P. & Van Pilsum, J.F. (1986) Localization of L-arginine-glycine amidinotransferase protein in rat tissues by immunofluorescence microscopy. J. Histochem. Cytochem. 34:429-435.

McWard, G.W., Nicholson, L.B. & Poulton, B.R. (1967) Arginine requirement of the young rabbit. J. Nutr. 92:118-120.

Mertz, E.T., Beeson, W.M. & Jackson, H.D. (1952) Classification of essential amino acids for the weanling pig. Arch. Biochem. Biophys. 38:121-128.

Milner, J.A. & Visek, W.J. (1973) Orotic aciduria and arginine deficiency. Nature (London) 245:211-213.

Milner, J.A. & Visek, W.J. (1974) Urinary metabolites characteristic of urea-cycle amino acid deficiency. Metabolism 24: 643-651.

Milner, J.A. & Visek, W.J. (1974) Orotate, citrate, and urea production in rats fed various levels of arginine. Proc. Soc. Exp. Biol. Med. 147:754-759.

Milner, J.A., Wakeling, A.E. & Visek, W.J. (1974) Effect of arginine deficiency on growth and intermediary metabolism in rats. J. Nutr. 104:1681-1689.

Milner, J.A. & Stepanovich, L.V. (1979) Inhibitory effect of dietary arginine on growth of Ehrlich Ascites tumor cells in mice. J. Nutr. 109:489-494.

Moncada, S., Palmer, R.M.J. & Higgs, E.A. (1989) Biosynthesis of nitric oxidee from L-arginine. Biochem. Pharmacol. 38:1709-1715.

Moriguchi, S., Mukai, K., Hiraoka, I. & Kishino, Y. (1987) Functional changes in human lymphocytes and monocytes after *in vitro* incubation with arginine. Nutr. Res. 7:719-729.

Morris, J.G. & Rogers, Q.R. (1978) Arginine: An essential amino acid for the cat. J. Nutr. 108:1944-1953.

Morris, J.G. (1985) Nutritional and metabolic responses to arginine deficiency in carnivores. J. Nutr. 115:524-531.

Morris, Jr., S.M., Moncman, C.L., Holub, J.S. & Hod, Y. (1989) Nutritional and hormonal regulation of mRNA abundance for arginine biosynthetic enzymes in kidney. Arch. Biochem. Biophys. 273:1-8.

Morris, Jr., S.M., Sweeney, Jr., W.E., Kepka, D.M., O'Brien, W.E. & Avner, E.D. (1991) Localization of arginine biosynthetic enzymes in renal proximal tubules and abundance of mRNA during development. Pediatr. Res. 29:151-154.

Morrison, G.R., Brock, F.E., Sorbal, D.T. & Shank, R.E. (1966) Cold-acclimatization and intermediary metabolism of carbohydrates. Arch. Biochem. Biophys. 114:494-501.

Motil, K.J., Harmon, W.E. & Grupe, W.E. (1980) Complications of essential amino acid hyperalimentation in children with acute renal failure. JPEN 4:32-35.

Nakagawa, I., Takahashi, T., Suzuki, T. & Kobayashi, K. (1963) Amino acid requirements of children: Minimal needs of tryptophan, arginine and histidine based on nitrogen balance method. J. Nutr. 80:305-310.

Nuzum, C.T. & Snodgrass, P.J. (1976) Multiple assays of the five urea-cycle enzymes in human liver homogenates. In: The Urea Cycle (Grisolia, S., Baguena, R. & Mayor, F., eds.) pp.325-349, John Wiley & Sons, New York.

O'Dell, B.L. & Regan, W.O. (1963) Effect of lysine and glycine upon arginine requirement of guinea pigs. Proc. Soc. Exp. Biol. Med. 112:336-337.

Osborne & Mendel (1916) In: Mendel, L.B. Harvey Lecture series. Vol. 10: 111 (1914-1915).

Palmer, R.M.J., Ferrige, A.G. & Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature (London) 327:524-526.

Passarella, S., Atlante, A. & Quagliariello, E. (1989) Metabolite transport in rat kidney mitochondria: Ornithine/phosphate translocator. Biochem. Biophys. Res. Commun. 158:87-879.

Pau, M.Y. & Milner, J.A. (1981) Arginine deficiency during gestation and lactation in the rat. J. Nutr. 111:184-193.

Pau, M.Y. & Milner, J.A. (1982) Effect of arginine deficiency on mammary gland development in the rat. J. Nutr. 112:1827-1833.

Pegg, A.E., Lockwood, D.H. & Williams-Ashman, H.G. (1970) Concentration of putrescine and polyamines and their enzymic synthesis during androgen-induced prostatic growth. Biochem.

J. 117:17-31.

Pegg, A.E. & McCann, P.P. (1982) Polyamine metabolism and function. Am. J. Physiol. 243:C212-C221.

Periano, C. & Pitot, H.C. (1963) Ornithine-δ-transaminase in the rat. I. Assay and some general properties. Biochim. Biophys. Acta. 73:222-231.

Perez, G.O., Epstein, M., Rietberg, B. & Louzenhiser, R. (1978) Metabolism of arginine by the isolated perfused rat kidney. Am. J. Physiol. 235:F376-F380.

Pitts, R.F. (1935) The effect of protein and amino acid metabolism on the urea and xylose clearance. J. Nutr. 9:657-666.

Pui, Y.M.L. & Fisher, H. (1979) Factorial supplementation with arginine and glycine on nitrogen retention and body weight gain in traumatized rat. J. Nutr. 109:240-246.

Pullman, T.N., Alving, A.S., Dern, R.J. & Landowne, M. (1954) The influence of dietary protein on specific renal functions in normal man. J. Lab. Clin. Med. 44:320-332.

Radomski, M.W., Palmer, R.M.J. & Moncada, S. (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc. Natl. Acad. Sci. USA 87:5193-5197.

Raijman, L. (1974) Citrulline synthesis in rat tissues and liver content of carbamoyl phosphate and ornithine. Biochem. J. 138:225-232.

Rajantie, J. Simell, O., Rapola, J. & Perheentupa, J. (1980) Lysinuric protein intolerance: A two-year trial of dietary supplementation therapy with citrulline and lysine. J. Pediatr. 97:927-932.

Rajantie, J. (1981) Orotic aciduria in lysinuric protein intolerence: Dependence on the urea cycle intermediates. Pediatr. Res. 15:115-119.

Rajantie, J. Simell, O. & Perheentupa, J. (1983) Oral administration of urea cycle intermediates in lysinuric protein intolerance: Effect on plasma and urinary arginine and ornithine. Metabolism 32:49-51.

Ratner, S., Anslow, W.P. & Petrack, B. (1953) Biosynthesis of urea. VI. Enzymatic cleavage of argininosuccinic acid to arginine and fumaric acid. J. Biol. Chem. 204:115-125.

Ratner, S. & Murakami-Murofushi, K. (1980) A new radiochemical

assay for argininosuccinase with purified [^{14}C]argininosuccinate. Anal. Biochem. 106:134-147.

Reye, R.D.K., Morgan, G. & Barol, J. (1963) Encephalopathy and fatty degeneration of the viscera: a disease entity of childhood. Lancet 2:749-752.

Reynolds, J.V., Daly, J.M., Shou, J., Sigal, R., Ziegler, M.M. & Naji, A. (1990) Immunologic effects of arginine supplementation in tumor-bearing and non-tumor bearing hosts. Ann. Surg. 211:202-210.

Rimele, T.J., Sturm, R.J., Adams, L.M., Henry, D.E., Heaslip, R.J., Weichman, B.M. & Grimes, D. (1988) Interaction of neutrophils with vascular smooth muscle: Identification of a neutrophil-derived relaxing factor. J. Pharmacol. Exp. Ther. 245:102-111.

Rochovansky, O. & Ratner, S. (1967) Biosynthesis of urea XII. Further studies on argininosuccinate synthetase: substrate specificity and mechanism of action. J. Biol. Chem. 242:3839-3849.

Rogers, Q.R. & Harper, A.E. (1965) Amino acid diets and maximal growth in the rat. J. Nutr. 87:267-273.

Rogers, Q.R. & Phang, J.M. (1985) Deficiency of pyrroline-5-carboxylate synthase in the intestinal mucosa of the cat. J. Nutr. 115:146-150.

Rose, W.C. (1937) The nutritive significance of the amino acids and certain related compounds. Science (Washington, D.C.) 86:298-300

Rose, W.C. & Rice, E.E. (1939) The significance of amino acids in canine nutrition. Science (Washington, D.C.) 90:186-187.

Rose, W.C., Haines, W.J. & Warner, D.T. (1954) The amino acid requirements of man. V. The role of lysine, arginine and tryptophan. J. Biol. Chem. 206:421-430.

Rose, W.C., Wixom, R.L., Lockhart, H.B. & Lambert, G.F. (1955) The amino acid requirements of man XV. The valine requirement: summary and final observations. J. Biol. Chem. 217:987-995.

Saito, H., Trocki, O., Wang, S.L, Gance, S., Joffe, S.N. & Alexander, W. (1987) Metabolic and immune effects of dietary arginine supplementation after burn. Ann. Surg. 122:784-789.

Savage, J.E. & O'Dell, B.L. (1960) Arginine requirement of the chick and the arginine sparing value of related compounds. J. Nutr. 70:129-134.

Schmidt, U. & Dubach, U. C. (1971) Quantitative Histochemic am Nephron. Prog. Histochem. Cytochem. 2:185-298.

Schmidt, U., Marosvari, J. & Dubach, U.C. (1975) Renal metabolism of glucose: Anatomical sites of hexokinase activity in the rat nephron. FEBS Lett. 53:26-28.

Scholer, D.E. & Edelman, I.S. (1979) Isolation of rat kidney cortical tubules enriched in proximal and distal tubules. Am. J. Physiol. 237:F350-F359.

Schneider, W.C. (1945) Phosphorous compounds in animal tissues I. Extraction and estimation of deoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. 161:293-303.

Scull, C.W. & Rose, W.C. (1930) Arginine metabolism. I. The realtion of the arginine content of the diet to the increments in tissue arginine during growth. J. Biol. Chem. 89:109-123.

Seifter, E., Rettura, G., Barbul, A. & Levenson, S.M. (1978) Arginine: An essential amino acid for injured rats. Surgery 84:224-230.

Seiler, N., Sarhan, S. & Knodgen, B. (1985) Developmental changes of putreanine in vertebrate brains. Int. J. Dev. Neurosci. 3:317-322.

Sessa, W.C., Hecker, M., Mitchell, J.A. & Vane, J.R. (1990) The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor:L-glutamine inhibits the generation of L-arginine by cultured endothelial cells. Proc. Natl. Acad. Sci. USA 87:8607-8611.

Shanon, J.A., Jolliffe, N. & Smith, H.W. (1932) The excretion of urine in the dog. IV. The effect of maintenance diet, feeding etc., upon the quantity of glomerular filtrate. Am. J. Physiol. 101:625-638.

Shih, V.E. & Efron, M.L. (1972) Urea cycle disorders. In: The Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B. & Fredrickson, D.S. eds.) pp. 370-392, McGraw-Hill Book Co., New York.

Silbernagl, S. (1988) The renal handling of amino acids and oligopeptides. Physiol. Rev. 68:911-1007.

Sinatra, F., Yoshida, T. Applebaum, M., Masion-Hoosenraad, N.J. & Sunshine, P. (1975) Abnormalities of carbamoylphosphate synthetase and ornithine transcarbamoylase in liver of patients with Reye's syndrome. Pediatr. Res. 9:829-832.

Skrzypek-Osiecka, I., Robin, Y. & Porembska, Z. (1983) Purification

of rat kidney arginases A_1 and A_4 and their subcellular distribution. Acta Biochem. Polon. 30:83-92.

Snodgross, P.J. & DeLong, G.R. (1976) Urea cycle enzyme deficiencies and an increased nitrogen load producing hyperammonemia in Reye's syndrome. New Engl. J. Med. 294:855-860.

Snyderman, S.E., Boyer, A. & Holt, L.E. (1959) The arginine requirement of the infant. Am. J. Dis. Child. 97:192-195.

Sottocassa, G.L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) An electron-transport system associated with the outer membrane of liver mitochondria. J. Cell. Biol. 32:415-438.

Southern, L.L. & Baker, D.H. (1983) Arginine requirement of the young pig. J. Anim. Sci. 57:402-412.

Spolarics, Z. & Bond, J.S. (1988) Multiple molecular forms of mouse liver arginase. Arch. Biochem. Biophys. 260:469-479.

Suebert, W. & Huth, W. (1965) On the mechanism of gluconeogenesis and its regulation. II. The mechanism of gluconeogenesis from pyruvate and fumarate. Biochem Z. 343:176-191.

Swendseid, M.E., Wang, M., Chan, W. & Kopple, J.D. (1975) The urea cycle in uremia. Kidney Int. 7:S280-S284.

Szepesi, B., Avery, E.H. & Freedland, R.A. (1970) Role of kidney in gluconeogenesis and amino acid catabolism. Am. J. Physiol. 219:1627-1631.

Tabor, C.W. & Tabor, H. (1984) Polyamines. Ann. Rev. Biochem. 53:749-790.

Tate, S.S. & Meuster, A. (1974) Interaction of γ-glutamyltranspeptidase with amino acids, dipeptides and derivatives of glutathione. J. Biol. Chem. 249:7593-7602.

Tizianello, A., De Ferrari, G., Garibotto, G., Guerreri, G. & Robaudo, C. (1980) Renal metabolism of amino acids and ammonia in subjects with normal renal function and in patients with chronic renal insufficiency. J. Clin. Invest. 65:1162-1173.

Van Pilsum, J.F., Martin, P., Kito, M.E. & Hess, J. (1956) Determination of creatine, creatinine, arginine, guanidinoacetic acid, guanidine and methylguanidine in biological fluids. J. Biol. Chem. 222:225-236.

Van Pilsum, J.F., Stephens, G.C. & Taylor, D. (1972) Distribution of creatine, guanidinoacetic acid and the enzymes for their

biosynthesis in the animal kingdom. Biochem. J. 126:325-345.

Van Slyke, D.D., Rhoads, C.P., Hiller, A. & Alving, A. (1934) The relationship of the urea clearance to the renal blood flow. Am. J. Physiol. 110:387-391.

Vinay,P., Gougoux, A. & Lemieux, G. (1981) Isolation of a pure suspension of rat proximal tubules. Am. J. Physiol. 241:F403-F411.

Vinuela, E., Salas, M. & Sols, A. (1963) Glucokinase and hexokinase in liver in relation to glycogen synthesis. J. Biol. Chem. 238:1175-1177.

Visek, W.J. (1986) Arginine needs, physiological state and usual diets. A reevaluation. J. Nutr. 116:36-46.

Wagner, D.A., Young, V.R. & Tannenbaum, S.R. (1983) Mammalian nitrate biosynthesis: Incorporation of $^{15}NH_3$ into nitrate is enhanced by endotoxin treatment. Proc. Natl. Acad. Sci. USA 80:4518-4521.

Wakabayashi, Y. & Jones, M.E. (1983) Pyrroline-5-carboxylate synthesis from glutamate by rat intestinal mucosa. J. Biol. Chem. 258:3865-3872.

Walser, M. (1983) Urea cycle disorders and other hereditary hyperammonemic syndromes. In: The Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B. & Fredrickson, D.S. eds.) pp. 402-438, McGraw-Hill Book Co., New York.

Wang, M., Kopple, J.D. & Swendseid, M.E. (1977) Effect of arginine-devoid diets in chronically uremic rats. J. Nutr. 107:495-501.

Weinsier, R.L., Hunker, E.M., Krumdieck, C.L. & Butterworth, C.E. (1979) Hospital malnutrition. A prospective evaluation of general medical patients during the course of hospitalization. Am. J. Clin. Nutr. 32:418-429.

White, M.F. (1985) The transport of cationic amino acids across the plasma membrane of mammalian cells. Biochem. Biophys. Acta 822:355-374.

Windmueller, H. G. & Spaeth, A.E. (1974) Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood. Arch. Biochem. Biophys. 171:662-672.

Windmueller, H. G. & Spaeth, A.E. (1978) Identification of ketone bodies and glutamine as the major respiratory fuels in vivo for postabsorptive rat small intestine. J. Biol. Chem.

253:69-76.

Windmueller, H. G. & Spaeth, A.E. (1980) Respiratory fuels and nitrogen metabolism *in vivo* in small intestine of fed rats: quantitative importance of glutamine, glutamate and aspartate. J. Biol. Chem. 255:107-112.

Windmueller, H. G. & Spaeth, A.E. (1981) Sources and fate of circulating citrulline. Am. J. Physiol. 241:E473-E480.

Wolf, A.V. (1941) Total renal blood flow at any urine flow or extraction fraction. Am. J. Physiol. 133:496-497.

Zamecka, E. & Porembska, Z. (1988) Five forms of arginase in human tissues. Biochem. Med. Metabol. Biol. 39: 258-266.

APPENDIX A

Arterial Plasma Levels, Arteriovenous (A-V) Differences, Renal Fluxes and Urinary Excretion of Amino Acids in Saline- and Citrulline-infused Rats

	Arterial Plasma Conc. (nmoles/ml)		Arteriovenous Differences (nmoles/ml Plasma)		Renal Flux (nmoles/min/100 g)		Urinary Excretion (nmoles/min/100g)	
Infusion	Saline	Citrulline	Saline	Citrulline	Saline	Citrulline	Saline	Citrulline
TAU	153.0 ± 56.7	130.0 ± 41.1	5.2 ± 12.4	1.0 ± 4.3	10.2 ± 23.1	4.2 ± 10.1	20.03 ± 18.45	15.35 ± 6.32
ASP	15.5 ± 4.1	13.0 ± 1.7	4.3 ± 3.0	5.4 ± 2.4	8.9 ± 6.7	13.3 ± 7.1	0.23 ± 0.06	0.29 ± 0.07
HYPRO	33.4 ± 15.4	33.2 ± 14.7	-1.0 ± 3.4	-0.1 ± 7.0	-1.6 ± 6.0	-0.6 ± 21.2	0.08 ± 0.14	0.03 ± 0.05
THR	227.9 ± 34.9	221.1 ± 45.9	-12.6 ± 14.2	-13.9 ± 9.5	-24.9 ± 28.3	-33.8 ± 29.6	0.25 ± 0.14	0.25 ± 0.08
SER	178.6 ± 27.4	166.2 ± 18.4	-96.1 ± 19.4	-86.4 ± 14.8	-191.9 ± 55.6	-206.1 ± 62.1	0.14 ± 0.09	0.13 ± 0.03
ASN	56.6 ± 7.4	58.5 ± 8.1	-8.4 ± 7.8	-7.7 ± 5.9	-16.9 ± 14.3	-19.0 ± 14.7	0.07 ± 0.04	0.08 ± 0.08
GLU	75.3 ± 24.8	59.6 ± 16.1	24.6 ± 16.7	20.9 ± 9.2	52.1 ± 45.1	47.7 ± 14.5	0.07 ± 0.04	0.06 ± 0.02
GLN	583.5 ± 56.7	531.2 ± 50.3	42.4 ± 45.2	82.4 ± 31.3	74.2 ± 77.3	184.6 ± 37.7	0.34 ± 0.25	0.32 ± 0.08
PRO	154.8 ± 10.7	152.4 ± 20.3	6.6 ± 11.7	10.5 ± 8.5	13.9 ± 26.4	29.9 ± 26.7	0.05 ± 0.07	0.03 ± 0.04
GLY	210.5 ± 55.3	198.8 ± 30.3	21.0 ± 18.3	22.1 ± 5.2	39.6 ± 32.4	54.2 ± 22.0	0.31 ± 0.12	0.35 ± 0.09
ALA	322.9 ± 29.7	327.3 ± 50.7	-21.9 ± 15.1	-11.9 ± 13.2	-43.7 ± 30.1	-33.5 ± 40.5	0.19 ± 0.08	0.21 ± 0.06
CIT	62.1 ± 7.8	241.9 ± 38.0	30.5 ± 8.6	96.5 ± 18.0	60.5 ± 20.7	223.6 ± 33.2	0.01 ± 0.01	0.06 ± 0.02
VAL	197.7 ± 14.0	193.8 ± 21.4	-0.6 ± 14.4	6.9 ± 15.8	0.6 ± 30.6	16.6 ± 31.8	0.05 ± 0.04	0.02 ± 0.03
CYT	70.1 ± 10.3	69.0 ± 18.2	-17.5 ± 2.8	-19.7 ± 8.4	-34.7 ± 8.8	-49.1 ± 31.2	0.05 ± 0.02	0.05 ± 0.01
MET	50.4 ± 6.1	48.8 ± 3.0	-4.0 ± 2.4	-3.0 ± 2.1	-8.0 ± 5.2	-7.9 ± 6.9	0.03 ± 0.03	0.02 ± 0.01
ILE	77.5 ± 6.5	78.8 ± 6.6	-6.3 ± 4.9	-6.0 ± 1.4	-13.3 ± 11.1	-14.9 ± 6.3	0.02 ± 0.02	0.01 ± 0.01
LEU	136.6 ± 8.9	137.0 ± 11.5	-10.5 ± 7.1	-9.9 ± 3.9	-21.6 ± 15.6	-25.1 ± 15.3	0.05 ± 0.04	0.05 ± 0.02
TYR	88.7 ± 9.8	81.8 ± 11.4	-8.9 ± 3.1	-12.3 ± 4.4	-17.7 ± 7.0	-29.8 ± 12.7	0.04 ± 0.04	0.02 ± 0.02
PHE	65.0 ± 4.3	65.6 ± 6.3	1.1 ± 6.9	3.4 ± 3.1	3.0 ± 14.3	8.1 ± 7.8	0.04 ± 0.02	0.02 ± 0.03
TRP	101.8 ± 16.9	89.7 ± 11.2	-9.3 ± 14.8	-2.7 ± 5.9	-18.9 ± 28.1	-5.0 ± 15.4	0.05 ± 0.06	0.08 ± 0.09
ORN	39.9 ± 8.3	47.3 ± 9.1	-1.9 ± 7.7	-10.3 ± 3.9	-4.0 ± 14.5	-26.7 ± 15.5	0.04 ± 0.02	0.05 ± 0.04
LYS	361.3 ± 71.9	342.9 ± 43.1	-15.5 ± 13.3	-19.0 ± 10.3	31.7 ± 28.0	-49.3 ± 35.8	0.21 ± 0.19	0.17 ± 0.06
HIS	73.1 ± 10.2	72.8 ± 17.2	-10.2 ± 8.6	-1.0 ± 8.2	19.5 ± 14.3	-2.6 ± 21.9	0.09 ± 0.03	0.08 ± 0.03
ARG	174.6 ± 26.2	244.1 ± 35.0	-40.7 ± 14.4	-112.2 ± 25.9	-78.9 ± 24.5	-264.6 ± 82.7	0.09 ± 0.05	0.15 ± 0.08

[1]Values expressed as Mean ± SD (n=5 for each treatment).