CREATINE METABOLISM:

REGULATION AND NEONATAL ACCRETION

By

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ABSTRACT

A fraction of the body's creatine and creatine-phosphate spontaneously degrades to creatinine, which is excreted by the kidneys. In humans, this amounts to about 1-2 g per day and demands a comparable rate of *de novo* creatine synthesis. This is a two-step process in which L-arginine:glycine amidinotransferase (AGAT) catalyzes the conversion of glycine and arginine to ornithine and guanidinoacetate (GAA); guanidinoacetate methyltransferase (GAMT) then catalyzes the SAM-dependent methylation of GAA to creatine. AGAT is found in the kidney and GAMT in the liver, which implies an inter-organ movement of GAA from the kidney to the liver. We have studied the renal production of this metabolite in both rats and humans. In control rats, [GAA] was 5.9 μM in arterial plasma and 10.9 μM in renal venous plasma for a renal A-V difference of –5.0 μM. In the rat, infusion of arginine or citrulline markedly increased renal GAA production but infusion of glycine did not. Rats fed 0.4% creatine in their diet had decreased renal AGAT activity and mRNA, and arterial plasma [GAA] of 1.5 μM and a decreased renal A-V difference for GAA of –0.9 μM. In humans, [GAA] was 2.4 μM in arterial plasma, with a renal A-V difference of -1.1 μM. These studies show, for the first time, that GAA is produced by both rat and human kidneys *in vivo*.

Creatine is essential for normal neural development; children with inborn errors of creatine synthesis or transport exhibit neurological symptoms such as mental retardation, speech delay, and epilepsy. Creatine accretion may occur through dietary intake or *de novo* creatine synthesis. The objective of this study is to determine how much creatine an infant must synthesize *de novo*. We have calculated how much creatine an infant needs to

account for urinary creatinine excretion (creatine's breakdown product) and new muscle lay-down. To measure an infant's dietary creatine intake, we measured creatine in mother's milk and in various commercially available infant formulas. Knowing the amount of milk/formula ingested, we calculated the amount of creatine ingested. We have found that a breast-fed infant receives about 9% of the creatine needed in the diet and that infants fed cow's milk-based formula receive up to 36% of the creatine needed. However, infants fed a soy-based infant formula receive negligible dietary creatine and must rely solely on *de novo* creatine synthesis. This is the first time that it has been shown that neonatal creatine accretion is largely due to *de novo* synthesis and not through dietary intake of creatine. This has important implications both for infants suffering from creatine deficiency syndromes and for neonatal amino acid metabolism.

Approximately 1.7% of the body's creatine pool is spontaneously converted to creatinine each day and excreted in the urine. This loss must be replaced by a combination of diet and synthesis. During lactation there may be an additional requirement due to the provision of creatine to the milk. Our objectives were (i) to quantify milk creatine in lactating rats, (ii) to determine the origin of milk creatine, (iii) to determine the activities of the enzymes of creatine synthesis in lactating rats and suckling pups and (iv) to quantify the origin of the creatine that accumulates in suckling rat pups. The origin of milk creatine was determined by administering ^{14}C-creatine to lactating rats, followed by determination of its isotopic enrichment in milk and plasma. These experiments indicate that the mammary gland extracts creatine from the circulation, rather than synthesising it. This was confirmed by our failure to find significant activities of the enzymes of creatine

synthesis in mammary glands. The provision of milk creatine requires an additional 35-55% of creatine above the daily requirement by lactating mothers. However, there was no increased creatine synthesis by these dams, so that the additional creatine was largely provided by hyperphagia, as creatine is present in rat chow. There was substantial accumulation of creatine in the growing pups but only approximately 12 % was obtained from milk. The great bulk of creatine accretion was via *de novo* synthesis by the pups, which imposed a substantial metabolic burden on them.

DEDICATION

This thesis is dedicated to the memory of Mrs. Beatrice Hall, a strong woman who truly loved her job and could work "like a Bayman with two woodstoves" to make a batch of "happy hepatocytes" in record time. There was no problem too big for her to tackle. She may have "sputtered" about technical difficulties but she always finished every job with precision. She certainly had an influence on everyone she met; she was a devoted wife, a loving mother, a hard-working and dedicated scientist and a true friend. She was my "mom away from home" and I miss her. Bea, you will never be forgotten. I wish you were here to read through these pages.

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Last and certainly not least, I wish to extend a special thanks to my family and friends who have supported me in past endeavors and will continue to support me in the future. Russell, Samson and Emma, you have kept me grounded and in the moment. You have always been understanding when job demands are high. I appreciate our time together

and hope you know that I cherish every moment. My parents, Eric and Venus, you have supported me in every goal that I have set. You have watched me meet many of these goals and I know that you will be there to watch me meet many more. My brother, Adam, you have been a best friend to me and your down to earth opinion has always guided my decisions. Thank you all so much for your support.

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LIST OF ABBREVIATIONS:

A – arterial

AGAT – L-arginine:glycine amidinotransferase

ANOVA – analysis of variance

ASL – argininosuccinate lyase

ASS – argininosuccinate synthase

A-V – arteriovenous or arterial - renal venous difference

BBB – blood-brain barrier

BCSFB – blood-cerebrospinal fluid barrier

bp – basepairs

CK – creatine kinase

CNS – central nervous system

CPEC – choroid plexus epithelial cells

CPS 1 – carbamylphosphate synthetase 1

Cys - cysteine

EEG - electroencephalogram

FAD – flavin adenine dinucleotide

GAA – guanidinoacetic acid

GAMT – guanidinoacetate Methyltransferase

GBq - gigaBecquerel

GI - gastrointestinal

g - gram

Hcy – homocysteine

HPLC – high performance liquid chromatography

i.p. – intraperitoneal

i.v. – intravenous

kBq - kiloBecquerel

kDa – kiloDalton

K_{eq} – equilibrium constant

kJ – kilojoules

K_m – Michaelis-Menten constant

M – molar

mBq – milliBacquerel

MCEC – microcapillary endothelial cells

mL - milliliter

mM - millimolar

mol - mole

MS – methionine synthase

MTHFR – methylene tetrahydrofolate reductase

N – number of subjects

nm - nanometers

NADH – nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate

NCAA – National Collegiate Athletic Association

OD – optical density

PBS – phosphate buffered saline

PCA – perchloric acid

PCR – polymerase chain reaction

PEP – phosphoenolpyruvate

PITC – phenyl isothiocyanate

RT-PCR – reverse transcription-polymerase chain reaction

SAH – S-adenosylhomocysteine

SAM – S-adenosylmethionine

SHMT – serine hydroxymethyltransferase

TEA – triethanolamine

TFA – trifluoroacetic acid

uL – microlitre

uM – micromolar

um - micrometer

UV – ultraviolet

V – renal venous

Vol – volume

wt - weight

x g – times the gravitational force

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION AND OVERVIEW

1.1. FUNCTIONS OF CREATINE AND CREATINE PHOSPHATE

The name "creatine" stems from the Greek word *kreas*, meaning flesh, as creatine was first discovered in muscle tissue. Creatine is a small zwitterion that contains a guanidino group that can be phosphorylated to yield creatine-phosphate. The phosphorylation of creatine is catalyzed by creatine kinase (CK) and occurs via the transfer of the γ-phosphoryl group of ATP to creatine to yield creatine-phosphate and ADP [Walker, 1979; Wyss & Kaddurah-Daouk, 2000]. Creatine and creatine-phosphate occur in much higher cellular concentrations than ATP or ADP. The hydrolysis of creatine-phosphate to form creatine and ATP has a standard free energy change of -45 kJ/mol at pH 7.0. If we compare this standard free energy change to that of ATP hydrolysis (-31.8 kJ/mol) it is easy to see that the cytosolic phosphorylation potential of tissues containing the creatine/creatine-phosphate/CK system may be maintained at a higher level than in tissues devoid of the phosphagen system [Wyss & Kaddurah-Daouk, 2000].

Because the creatine/creatine-phosphate/CK phosphagen system plays an important role in energy storage and transmission [Balsom *et al*., 1994; Schulze, 2003; Walker, 1979; Wyss & Kaddurah-Daouk, 2000], it is understandable that tissues which contain high levels of creatine are those which have high or variable rates of ATP usage. For example, skeletal muscle, cardiac muscle, spermatozoa, and photoreceptors of the retina contain high amounts of creatine [Christensen *et al*., 1994; Kushmerick *et al*., 1992; Wallimann *et al*., 1986a; Wallimann *et al.*, 1986b]. Intermediate levels of creatine are found in brain, intestine, brown adipose tissue, endothelial cells, macrophages, seminal vesicles, and

seminal vesicular fluid [Berlet *et al*., 1976; Delanghe *et al*., 1989; Lee *et al*., 1988; Loike *et al*., 1992; Loike *et al*., 1986; Navon *et al*., 1985; Windischbauer *et al*., 1994]. Low levels are found in kidney, liver, lung, spleen, blood cells, serum, and white adipose tissue [Berlet *et al*., 1976; Delanghe *et al*., 1989; Wyss & Kaddurah-Daouk, 2000].

There are two proposed mechanisms by which the creatine/creatine-phosphate/CK phosphagen system acts to aid in energy storage and transmission. The first mechanism is known as the "temporal energy buffer" and the second, as the "spatial energy buffer", or the Energy Buffer Theory vs. the Phosphocreatine Energy Shuttle [Bessman & Geiger, 1981; Wallimann *et al*., 1992]. It is important to realize that these mechanisms are not mutually exclusive and both are physiologically relevant, though each may be more important in different cells.

1.1.1. The Energy Buffer Theory

Local ATP, ADP, and AMP concentrations, as well as the ATP/ADP ratio, act as key regulators that influence many fundamental metabolic processes. Therefore, it would not be appropriate to allow the intracellular concentrations of ATP to decrease during work [Wallimann *et al*., 1992]. To avoid this dilemma, many cells or tissues contain large quantities of phosphagens such as creatine/creatine-phosphate. CK works to replenish ATP from the large pools of creatine-phosphate through the following reaction:

$$\text{ADP} + \text{creatine-phosphate} \leftrightarrow \text{ATP} + \text{creatine} + H^+$$

During short activation of tissues which have high or variable rates of ATP usage, ATP levels do not significantly change even though cellular pools of ATP are quite small (2-5 mM) [Infante & Davies, 1965; Mommaerts & Wallner, 1967; Seraydarian, 1980]. Creatine-phosphate potentially plays an important role as an energy buffer in fast-twitch, glycolytic muscles. Because cytosolic CK activity is high in these muscles, its reaction can remain in a near-equilibrium state over several seconds, keeping ATP and ADP concentrations relatively constant. In this way, the creatine/creatine-phosphate/CK system "buffers" the cytosolic phosphorylation potential that is crucial for the proper function of a variety of cellular ATPases [Wyss and Kaddurah-Daouk, 2000].

1.1.2. The Energy Shuttle Theory

There are at least four isoforms of CK: muscle (M)-CK, brain (B)-CK, and two mitochondrial (Mi)-CK isoforms. These isoforms are expressed in a tissue-specific manner [Wallimann *et al*., 1992]. The presence of different isoforms and locations of the enzyme suggests that there are different metabolic roles for each isoform, leading to the energy shuttle theory of creatine-phosphate. The energy shuttle theory implies that creatine-phosphate acts as an energy carrier between sites of energy production (mitochondria) and sites of energy utilization in the cytoplasm [Greenhaff, 2001; Wallimann *et al*., 1992]. Bessman and Geiger [1981] suggest that the energy shuttle theory is central to aerobic muscle mechanics.

A key factor in the energy shuttle theory is the subcellular localization of the various isoforms of CK. For clarification, let us imagine an arbitrary aerobic muscle cell. The mitochondrial isoform of CK is located at contact points between the inner and outer mitochondrial membranes. ATP produced by oxidative phosphorylation is readily converted to creatine-phosphate in the presence of creatine. Creatine-phosphate can diffuse from the mitochondrion to the major cellular sites of ATP utilization, such as myofibrils, sarcoplasmic reticulum or plasma membranes [Wallimann *et al*., 1992]. Once ADP is phosphorylated to ATP by a cytosolic isoform of CK, creatine is free to diffuse back toward the mitochondrion. It has been postulated that creatine-phosphate (MW 211) and creatine (MW 131) would diffuse faster through the cytosol than their larger counterparts, ATP (MW 507) and ADP (MW 427) [Ellington, 2001]. However, the differences in free concentration in the cytosol likely play a more important role (ATP, 3-5 mM; ADP, 20-40 μM; creatine-phosphate 20-35 mM; creatine, 5-10 mM) [Wallimann et al., 1992]. In particular, the very low concentration of ADP may limit the rate of its diffusion, relative to that of creatine. **Figure 1.1** illustrates the concept of the energy shuttle theory. Slow-twitch, oxidative, or cardiac muscle would likely employ the energy shuttle function as this would allow higher endurance. It is worth mentioning again that the energy shuttle theory and the energy buffer theory are not mutually exclusive.

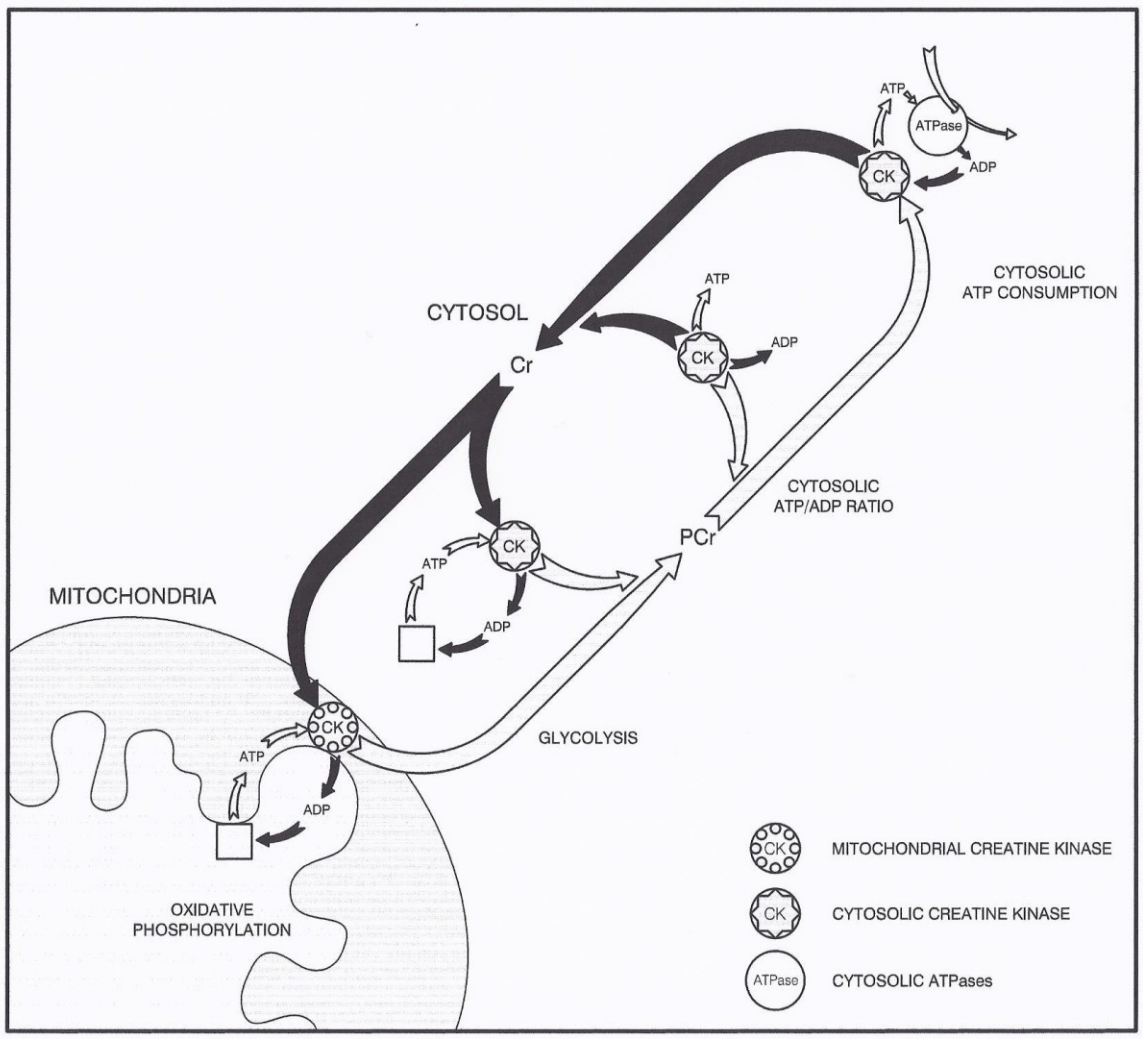

Figure 1.1: The Energy Shuttle Theory of Creatine Function. Creatine-phosphate acts as an energy carrier between sites of energy production (mitochondria) and sites of energy utilization in the cytoplasm. Produced for this thesis by Adam Samson.

1.2. CREATINE AS A SPORTS NUTRACEUTICAL

Upon commencement of high-intensity exercise the ATP demand of working muscles may increase to several hundred-fold higher than that at rest [Balsom *et al*., 1994]. Initially, ATP hydrolysis is buffered by creatine/creatine-phosphate but creatine-phosphate levels are depleted after about 10 seconds [Balsom *et al*., 1994; Wyss & Kaddurah-Daouk, 2000]. After the initial 10 seconds, other sources of ATP generation such as glycolysis and, then, mitochondrial oxidative phosphorylation are called into play [Balsom *et al*., 1994; Wyss & Kaddurah-Daouk, 2000]. Availability of creatine-phosphate may be a limiting factor in muscle force maintenance [Balsom *et al*., 1994]. It is this idea that has led to the popularity of creatine as a sports nutraceutical.

In 1992, Harris *et al.* first reported that dietary creatine is absorbed and that when creatine supplementation is carried over a period of time there are increases in muscle total creatine content. Since this time creatine has become one of the most popular of the nutraceutical supplements used by athletes. It has also proved to be profitable for the supplement industry [Volek & Rawson, 2004]. In 1996 it was estimated that 80% of the athletes competing in the Olympic Games were using or had been using creatine [Paddon-Jones *et al*., 2004]. In 1998 creatine sales in the United States were estimated at $100 million [Paddon-Jones *et al*., 2004]. In 1999, an estimated 2.5 million kg of creatine were consumed, and it was reported that 28% of NCAA Division I athletes used creatine [Racette, 2003]. A 2001 survey of 1349 high school football players indicated that 30% had used creatine [Racette, 2003]. These statistics reveal that creatine is an extremely

popular sports supplement and it is being used by a variety of athletes, from Olympic athletes to high school children. This last point warrants research into the long-term effects of creatine supplementation, especially in terms of adolescent development.

The typical supplement regimen followed by athletes involves a "loading phase" of 20-30 g of creatine/day (generally divided into 5-7.5 g doses taken 4 times daily) for 5 days followed by a "maintenance phase" of 2-5 g of creatine/day [Racette, 2003; Wyss & Kaddurah-Daouk, 2000]. Plasma creatine concentration peaks approximately 1 hour after a single 5 g dose. Intramuscular stores increase within 48 hours and at the end of the loading phase total intramuscular creatine content is increased by approximately 20%, depending on initial creatine levels [Rawson *et al*., 2002]. An alternative regimen for supplementation involves 3 g of creatine/day for 28 days. At the end of 28 days the same final creatine concentrations are reached as with the 5 day loading period. To aid in uptake of creatine, creatine monohydrate powder is dissolved in a carbohydrate solution. Green *et al*. [1996] showed that creatine dissolved in carbohydrate solution increased total muscle creatine stores by >25%, that is 60% more than when creatine was ingested alone. Steenge *et al*. [1998] reported that insulin, whose secretion was increased by carbohydrate ingestion, enhanced muscle creatine accumulation. In addition, they found that insulin's effect required quite high insulin concentrations. They suggested that an insulin-mediated augmentation of sodium-dependent creatine transport is responsible for the increased creatine uptake.

There seems to be no justification for taking higher doses of creatine because the capacity of muscle fibers to store creatine-phosphate is limited and excess creatine is excreted in the urine as either creatine or creatinine. Upon cessation of supplementation, total intramuscular creatine concentrations return to basal levels after approximately 4 weeks [Racette, 2003]. Creatine uptake is stimulated by exercise, as demonstrated by Harris *et al*. [1992] by single leg exercises. The exercised leg had appreciably higher muscle creatine levels than that of the unexercised, contralateral leg.

Since creatine-phosphate only buffers ATP levels for approximately 10 seconds after the onset of intense exercise [Balsom *et al*., 1994; Wyss & Kaddurah-Daouk, 2000], it seems likely that increasing creatine-phosphate stores in muscle would aid in short, high-intensity, repetitive exercise with little or no benefit for endurance-type exercise [Racette, 2003; Kreider, 2003]. Therefore, creatine's effects in athletic performance tend to be sport-specific with greatest benefit observed for bench press, leg press, squats, and jumping; minimal or no benefit was observed for swimming sprints, or tennis stroke power or precision [Racette, 2003]. For many types of exercise it is unclear what benefit creatine imposes upon athletic performance as results vary from one study to the next. For example, a meta-analysis of 96 creatine supplementation studies [Branch, 2003] found that creatine supplementation had minimal effectiveness in improving jumping, running and swimming performance. However, the findings supported the effectiveness of creatine supplementation in increasing total and lean body mass, and performance in high-intensity, short duration, repetitive exercises such as weight-lifting repetitions and

resistance. They concluded that the effect size is small and variable, highlighting the inconsistent effects of creatine supplementation for all variables and populations studied.

There are three proposed mechanisms of action of creatine supplementation: 1) greater ATP availability to meet the high ATP demand during anaerobic exercise; 2) faster creatine-phosphate resynthesis during recovery from intense exercise; and 3) depressed degradation of adenine nucleotides and depressed accumulation of lactic acid during exercise. These mechanisms may allow force or power to be maintained longer, thereby enhancing athletic performance. Faster creatine-phosphate resynthesis could be beneficial during repeated exercise bouts with little recovery time in between [Racette, 2003; Wyss & Kaddurah-Daouk, 2000].

The one side-effect that is common to almost all studies carried out on creatine supplementation is weight gain. Depending upon the sport, weight gain can be considered as either positive or negative. For example, a gymnast would consider weight gain a hindrance while a weightlifter/bodybuilder would value it as long as the weight represented muscle. A number of suggestions have been made as to how creatine supplementation causes weight gain but, as yet, the mechanism is largely unknown. Creatine is an osmolyte whose storage requires water [Paddon-Jones *et al*., 2004; Racette, 2003; Volek & Rawson, 2004; Wyss & Kaddurah-Daouk, 2000]. It has also been shown that creatine supplementation causes increases in glycogen storage in rat skeletal muscle [Eijnde *et al*., 2001]. In addition, increased water may cause cellular swelling which is known to enhance anabolism [Häussinger *et al*., 1999; Krause *et al*., 1996] and increase

cellular protein content. Creatine seems to have no effect on protein synthesis but has been reported to decrease whole-body protein breakdown [Louis *et al*., 2003; Volek & Rawson, 2004].

Other, less desirable side-effects that have occasionally been reported include mild asthmatic symptoms, gastrointestinal distress, muscle cramps, muscle strains, heat intolerance, rash, dyspnea, vomiting, diarrhea, nervousness, anxiety, fatigue, migraine, myopathy, polymyositis, seizures, and atrial fibrillation [Wyss & Kaddurah-Daouk, 2000]. A study by Greenwood *et al*. [2003] suggested that incidences of dehydration, muscle cramping or injury in athletes taking creatine were similar or lower than that among athletes not taking creatine. Kreider *et al*. [2003] studied long-term effects of creatine supplementation and its effect upon clinical markers of health. They found no adverse effect of creatine intake on markers of health status in athletes undergoing intensive training, in comparison to athletes who do not take creatine. It should be noted that the study only lasted 21 months and there were no follow-ups. It has also been postulated that creatine supplementation may improve brain performance, indicating that research should extend to other areas of performance outside of athletic performance [Rae *et al*., 2003].

Based upon these conflicting results on the effects of creatine supplementation, it is safe to say that more research needs to be conducted in this area. The basis for the conflicting results has been investigated by Greenhaff *et al*. [1994] and Syrotuik & Bell [2004]. Both studies suggest that non-responders may explain much of the variability in results. A

non-responder is defined as any person who ingests 20 g of creatine/day for 5 days and has an increase in total resting muscle creatine of less than 10 mmol/kg dry weight muscle [Syrotuik & Bell, 2004]. It is suggested that 20-30% of the population may be non-responders [Greenhaff *et al*., 1994]. Rawson *et al*. [2002] showed that subjects who have high initial muscle creatine levels respond less than those with low initial muscle creatine. This observation may account for many of the non-responders. More research needs to be conducted in the area of creatine supplementation; in particular, an examination with regard to any long-term effects on health is needed.

1.3. NEED FOR CREATINE SYNTHESIS

Creatine and creatine-phosphate spontaneously degrade to creatinine in an irreversible manner [Bloch & Schoenheimer, 1939; Borsook & Dubnoff, 1947]. At pH 7.0-7.2 and 38ºC, about 1.1% of creatine and 2.6% of creatine-phosphate are degraded per day, amounting to 1.7% of the total body creatine pool per day [Crim *et al*., 1976; Hoberman *et al*., 1948; Perrone *et al*., 1992]. The conversion of creatine to creatinine is favored by low pH and high temperature [Cannan & Shore, 1928; Edgar & Shiver, 1925]. Recently the turnover of the total creatine pool has been estimated by an entirely different approach and yielded a very similar result [Kan *et al*., 2006]. In this study, a healthy male volunteer ingested 20 g per day, for 5 days, of 11% ^{13}C-4-creatine. The signal intensity of the combined creatine and creatine-phosphate peak in the subject's calf muscle was then monitored over time by *in vivo* ^{13}C-NMR. The signal intensity decreased at a rate of 1.6 ± 0.5% per day, essentially the same as the previous estimate of 1.7% per day.

Creatinine has no known function in the body and is quantitatively excreted in the urine [Wyss & Kaddurah-Daouk, 2000]. Since approximately 95% of the total body pool of creatine in humans occurs in skeletal muscle [Balsom *et al*., 1994; Harris *et al*., 1974; Harris & Lowe, 1995], it is not surprising that urinary excretion of creatinine correlates with body muscle mass [Myers & Fine, 1913]. A 70 kg male, containing approximately 120 g of total creatine, excretes about 2 g per day of creatinine [Walker, 1979]. The creatine lost must be replaced either by diet, by *de novo* synthesis, or by a combination of both.

1.3.1. Creatine Balance

To estimate creatine synthesis, one must first calculate the amount of creatine degraded, and then subtract from this the amount of creatine ingested, taking into account bioavailability of orally ingested creatine. Harris *et al*. [2002] showed that dietary creatine is readily absorbed whether provided in solution, in meat or in solid (pill) form. Although ingestion of the creatine in meat and solid form gave lower peak concentrations, this was compensated for by an extended absorption time, such that the areas under the curve of plasma concentration over time were comparable. The bioavailability of oral creatine, determined in adults who ingested ^{2}H-creatine, has been estimated to be approximately 80% [MacNeil *et al*., 2005]. The mechanism for intestinal absorption of creatine is unclear, although mRNA for a creatine transporter has been identified in the GI tract [Persky *et al*., 2003]. Since creatine bears some similarity to basic amino acids, it

is possible that some creatine absorption may occur via the transporter for these amino acids [Persky *et al*., 2003].

Stead *et al*. [2006] have estimated that in 20-59 year-olds, dietary creatine provides approximately 50-60% of the daily requirement. If we go back to our example of the 70 kg male, this means that about 1-1.2 g of creatine is received via the diet. Therefore, 40-50%, or 0.8-1.0 g, must be acquired via *de novo* creatine biosynthesis. A more recent paper by Kasumov *et al*. [2009] examined the kinetics of creatine synthesis in a healthy adult using infusion of [1-^{13}C]glycine. The rate of creatine synthesis in a healthy 70 kg male was 1.85 g/day; this is of the same order of magnitude as previous estimates. We must bear in mind that this estimate was obtained using a single human subject who was placed on a weight maintenance diet containing at least 70 g of protein per day for 7 days prior to study initiation. It is unclear how much creatine was provided by this diet. Finally, it should be emphasized that creatine is only found in animal tissues. Vegetarians, and in particular vegans, will ingest little or no creatine and will need to provide all of their creatine by endogenous synthesis.

1.4. SYNTHESIS OF CREATINE

1.4.1. Pathway of Creatine Synthesis

The biological synthesis of creatine is a very simple process, involving just two enzymatic steps. The first step involves two amino acids: arginine and glycine. The

amidino group of arginine is transferred to glycine by L-arginine:glycine amidinotransferase (AGAT) to form ornithine and guanidinoacetic acid (GAA) [Bloch & Schoenheimer, 1941; Wyss & Kaddurah-Daouk, 2000].

Arginine + Glycine → Ornithine + GAA [Step 1]

GAA is methylated by guanidinoacetate methyltransferase (GAMT) using S-adenosylmethionine (SAM) as methyl donor to form creatine and S-adenosylhomocysteine (SAH) [du Vigneaud *et al*., 1940; Cantoni & Vignos, 1954].

GAA + SAM → SAH + creatine [Step 2]

See **Figure 1.2** for a complete diagram of creatine synthesis from arginine, glycine and methionine.

1.4.1.1. L-Arginine:Glycine Amidinotransferase (AGAT)

AGAT (EC 2.1.4.1) catalyzes the reversible transfer of the amidino group of arginine to glycine to form ornithine and GAA. This reaction was first demonstrated by Bloch and Schoenheimer [1941] in rats fed diets containing various ^{15}N-labelled compounds, including glycine, arginine and GAA. Arginine and glycine were the only "natural" amino acids that produced a large amount of labeled creatine, each supplying nitrogen to different parts of the creatine molecule. Labeled GAA also produced significant amounts of labeled creatine but since GAA is not a normal constituent of food, but can be found in small quantities in normal urine and tissues, it was correctly suggested to be an intermediate in the creatine synthesis pathway.

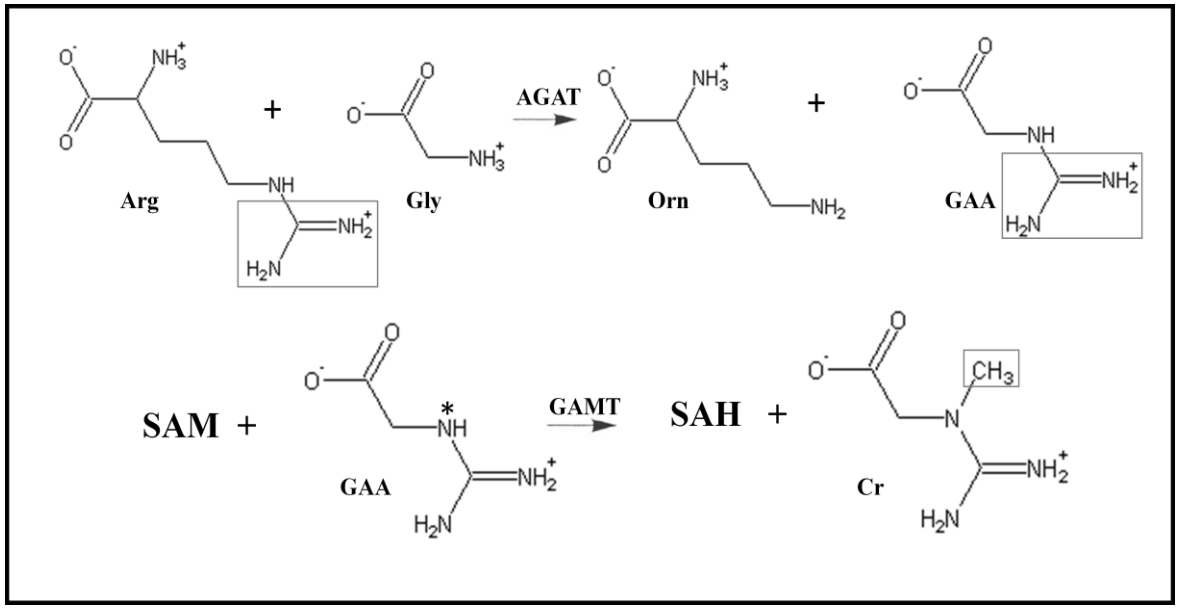

Figure 1.2: Creatine Synthesis. L-arginine:glycine amidinotransferase (AGAT) first catalyzes the transfer of an amidino group from arginine (Arg) to glycine (Gly), producing ornithine (Orn) and guanidinoacetate (GAA). Next, guanidinoacetate methyltransferase (GAMT) transfers a methyl group from S-adenosylmethionine (SAM) to GAA, producing S-adenosylhomocysteine (SAH) and creatine. The asterisk indicates point of methylation. Boxes are drawn to indicate transfer of common groups, as well as methylation.

AGAT has been localized to the outer surface of the inner mitochondrial membrane in the rat kidney and pancreas [McGuire *et al*., 1984; Walker, 1979]. There may also be some cytosolic AGAT [Tormanen, 1990], however, which could arise as a result of alternative splicing of AGAT mRNA [Humm *et al*., 1997a].

Gross *et al.* [1986] reported that there are at least two isoforms of AGAT, designated as α- and β-forms in rat and human tissues. These are known to exist as dimeric molecules with subunit molecular weights of 42,000-44,000 Daltons, giving a total molecular weight for the functioning enzyme between 82,600 and 89,000 Daltons [Gross *et al*., 1986; Gross *et al*., 1988; McGuire *et al*., 1980]. There is a 94-95% amino acid sequence homology between rat, pig and human AGAT [Humm *et al*., 1994].

Humm *et al*. [1997b] and Fritsche *et al*. [1999] used x-ray crystallography to gain insight into the mechanism of AGAT action. The enzyme appears to work using a double-displacement (Ping-Pong, bi-bi) mechanism whereby arginine binds first, ornithine is released and then glycine binds [Fritsche *et al*., 1999]. A conformational change takes place upon binding of substrate, arginine, which switches the enzyme from a closed to an open state. The amidino group of arginine is transiently attached to Cys 407 and the product, ornithine, leaves before glycine binds and accepts the amidino group to form GAA. The binding of glycine does not cause any structural change in the enzyme.

The optimum pH for GAA formation is 7.2-7.5 while that for arginine formation is 8.5. The Michaelis-Menten constant, K_m, for arginine was determined to be 1.3-2.8 mM, and

1.8-3.1 mM for glycine [Gross *et al*., 1986; McGuire *et al*., 1980; Walker, 1979] and the K_i for ornithine to be 0.25 mM [Fritsche *et al*., 1999].

Maximum velocity (V_{max}) values at 37ºC for human, rat α-, and rat β-AGAT were determined to be 0.5, 0.39, and 0.37 μmol ornithine formed per minute per mg protein, respectively [Gross *et al*., 1986; McGuire *et al*., 1980], while that of hog was determined to be 1.2 μmol ornithine formed per minute per mg protein [Conconi & Grazi, 1965].

Amino acids other than those involved in creatine synthesis may act as substrates for AGAT in the test tube. Other amidino group donors include homoarginine, 3-guanidinopropionate, 4-guanidinobutyrate, hydroxyguanidine and canavanine. Other amidino group acceptors include β-alanine, 3-aminopropionate, 4-aminobutyrate, 1,4-diaminobutylphosphonate, glycylglycine, hydroxylamine and canaline [Ennor & Morrison, 1958; Pisano *et al*., 1963; Walker, 1979]. No physiological role has been proposed for any of these compounds as alternative substrates for AGAT.

1.4.1.2. Guanidinoacetate Methyltransferase (GAMT)

GAMT (EC 2.1.1.2) catalyzes the irreversible methylation of GAA by SAM to produce creatine and SAH. The methylation occurs on the amidino group which originates from arginine [Wyss & Kaddurah-Daouk, 2000]. Borsook and Dubnoff [1940] were the first to show, *in vitro*, that the methyl group donated to GAA to form creatine was from methionine. It was later shown by Cantoni and Vignos [1954] that the immediate methyl

donor is SAM. Takata *et al*. [1994] have proposed that the GAMT reaction occurs via an ordered Bi Bi mechanism, in which SAM is the first reactant to bind.

In mammals, GAMT activity is highest in liver [Van Pilsum *et al*., 1972; Walker, 1979; Xue *et al*., 1988]. The pancreas, testis and kidney have intermediate levels of activity, while low levels have been found in skeletal and cardiac muscle, neuroblastoma cells, fibroblasts and spleen [Daly, 1985; Van Pilsum *et al*., 1972; Walker, 1979; Wyss & Kaddurah-Daouk, 2000; Xue *et al*., 1988].

Im *et al*. [1979] and Ogawa *et al*. [1983] have reported that GAMT from pig and rat liver functions as a monomeric protein with a molecular weight of 26,000-31,000 Daltons. It catalyzes an irreversible reaction with a K_m for SAM of 49 μM, and GAA of 23-27 μM [Im *et al*., 1979; Takata *et al*., 1991; Walker, 1979].

1.4.2. Creatine Transporter

The creatine transporter (SLC6A8) is a member of the SLC6 family of transporters. SLC6A8 activity has electrogenic properties because, in addition to creatine, it co-transports two sodium and one chloride ion [Chen *et al*., 2004; Guimbal & Kilimann, 1993; Valayannopoulos *et al*., 2013]. Because of inadequate methodologies for the extraction and purification of the creatine transporter, the exact biochemical nature of the protein remains elusive. There are no clear identification and sequence data currently available [Speer *et al*., 2004]. It is believed that, in addition to the high efficiency plasmalemma creatine transporter, there is a creatine uptake mechanism present in the

mitochondrion that has lower affinity and high K_m [Speer *et al*., 2004]. Speer *et al*. [2004] reported that mitochondrial creatine transport likely occurs by unspecific transport, possibly by an amino acid transporter.

Speer *et al.* [2004] speculate that the genuine creatine transporter protein is approximately 60-65 kDa in size, depending upon the tissue and the species. Creatine uptake appears to be regulated by a variety of mechanisms including changes in intracellular and extracellular creatine content, phosphorylation and glycosylation. Competitive inhibitors of the transporter include β-guanidinopropionic acid, arginine and lysine. The activity of the creatine transporter can also be modulated by the SH-modifying reagent, N-ethylmaleimide and the protonophoric uncoupler, p-trifluoromethoxyphenylhydrazone.

1.4.3. Tissues Responsible for Creatine Synthesis

Van Pilsum *et al.* [1972] found that in mammals, the highest AGAT activity was found in the kidney although appreciable activity was also found in the liver of some species. For example, livers of cow, pig, monkey and human seem to have high amounts of AGAT, whereas livers of rat, mouse, dog, cat and rabbit were reported to have low levels or no AGAT [Pisano *et al*., 1963; Van Pilsum *et al*., 1963; Van Pilsum *et al*., 1972; Walker, 1979]. Experiments were conducted by da Silva *et al.* [2009], using rat hepatocytes incubated with ^{15}N-ammonium chloride, as well as methionine and glycine. It was found that labeled urea was produced at a linear rate under all substrate conditions but not labeled creatine. Thus, it was concluded that rat hepatocytes are not capable of the entire

creatine synthetic pathway. It is not known whether the liver of humans is capable of creatine synthesis from arginine and glycine, but the presence of appreciable AGAT activity there would make it feasible.

Liver contained the highest activity of GAMT in all mammalian species examined [Van Pilsum *et al*., 1972]. Because of this and because of the fact that creatine biosynthesis is reduced in nephrectomized rats [Goldman & Moss, 1959; Levillain *et al*., 1995], it has been postulated that creatine biosynthesis is an interorgan process whereby GAA is synthesized from arginine and glycine by AGAT in the kidney and released into the bloodstream to be taken up by the liver and converted to creatine by GAMT. Creatine is then released into the bloodstream to be taken up by other tissues, such as skeletal and cardiac muscle, where it is required (see **Figure 1.3** for diagrammatic representation).

More recently, Braissant *et al*. [2007] have demonstrated creatine synthesis *in vitro* by various types of cerebral cells. Although cerebral creatine synthesis is not important for maintaining the total body creatine pool, it is probably necessary for CNS use. It is well-known that the brain is protected from various blood components via two main barriers, the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB is the largest barrier between the brain and surrounding periphery. It is made up of tightly connected, non-fenestrated, microcapillary endothelial cells (MCEC). There is tight regulation of exchange through the BBB. Exchange is regulated by 1) pericytes and

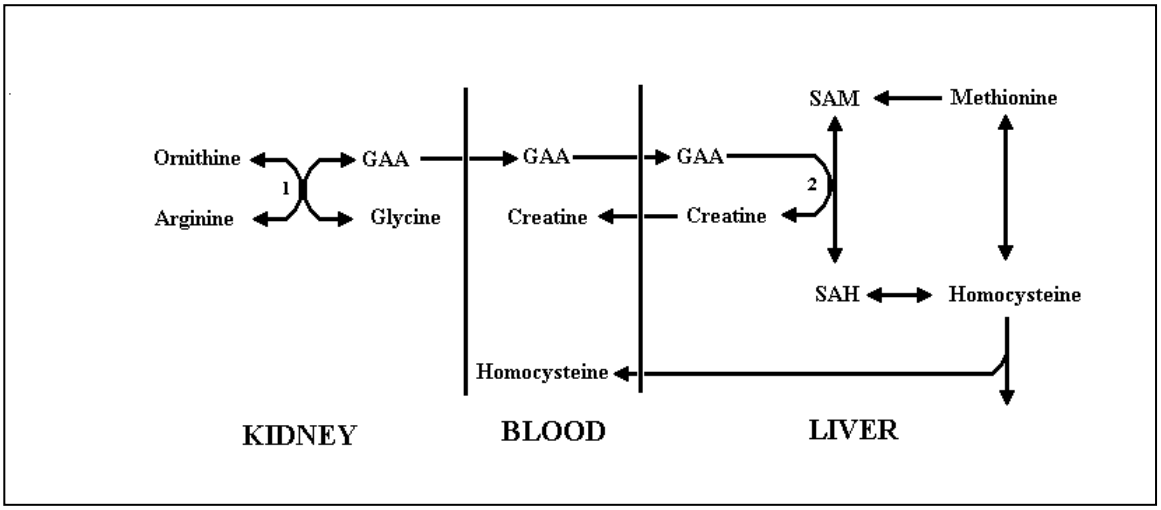

Figure 1.3: Creatine Biosynthesis and Proposed Interorgan Metabolism. GAA, guanidinoacetate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. 1. L-Arginine:glycine amidinotransferase (AGAT); 2. Guanidinoacetate methyltransferase (GAMT).

astrocytes on the abluminal side of MCEC and 2) pericytes and astrocytic feet that compose an extracellular matrix that sheaths the MCEC. The BCSFB is located in the lateral, third and fourth brain ventricles. It is composed of choroid plexus epithelial cells (CPEC) that form apical tight junctions between one another. The main functions of the BCSFB include the secretion of CSF and regulated exchanges between CSF and blood [Braissant, 2012].

The creatine transporter is expressed by neurons and oligodendrocytes, including MCEC at the BBB; it is not expressed by astrocytes, particularly the astrocytic feet that line the BBB [Braissant *et al*., 2001; Ohtsuki *et al*., 2002; Tachikawa *et al*., 2004]. This may account for the findings of inefficient transport of creatine from periphery to CNS as reported by Perasso *et al*. [2003]. Braissant [2012] suggests that the brain may be responsible for much of its own creatine supply through endogenous creatine synthesis. This synthesis appears to be an intercellular process, as the expression of AGAT and GAMT is dissociated in most regions of the brain. Therefore, a transporter would be needed for transport of GAA from AGAT- to GAMT-expressing cells to complete creatine synthesis [Braissant, 2012].

1.4.4. Regulation of Creatine Synthesis

The regulation of creatine biosynthesis is usually thought to occur during the production of GAA, at the level of AGAT [Walker, 1979]. It seems that GAMT does not become rate-limiting for creatine biosynthesis unless AGAT activity is highly stimulated [Wyss &

Kaddurah-Daouk, 2000]. AGAT activity has been shown to be regulated by circulating creatine levels, dietary deficiency, hormonal factors and disease [Derave *et al*., 2004; Funahashi *et al*., 1981; Guthmiller *et al*., 1994; Van Pilsum & Wahman, 1960]. An increase in serum levels of creatine results in a decrease in AGAT enzyme activity, enzyme protein amount and mRNA expression [Derave *et al*., 2004; Guthmiller *et al.*, 1994; Walker, 1979; Wyss & Kaddurah-Daouk, 2000]. Fasting may have a similar effect on creatine biosynthesis [Shindo *et al*., 1986]. During fasting, plasma creatine levels increase and this may be the cause of decreased GAA levels, observed in tissues (but not plasma) [Zhao *et al*., 2002]. Both thyroxine and growth hormone are believed to be involved in the maintenance of AGAT levels, as both thyroidectomy and hypophysectomy decrease rat kidney AGAT activity. AGAT activities are restored upon injection of thyroxine or growth hormone, respectively [Guthmiller *et al*., 1994; McGuire *et al*., 1980]. AGAT activity is also decreased by vitamin E deficiency, and by streptozotocin-induced diabetes [Funahashi *et al*., 1981; Van Pilsum & Wahman, 1960]. Fitch *et al*. [1961] have shown that vitamin E deficiency increases serum creatine levels, suggesting that the increased creatine is responsible for the decrease in AGAT activity. The effect of streptozotocin-induced diabetes on AGAT activity may not be a result of the altered hormonal state since administration of insulin does not restore original AGAT activity [Funahashi *et al*., 1981].

1.5. QUANTITATIVE ASPECTS OF CREATINE SYNTHESIS

A consideration of the quantitative aspects of creatine synthesis is revealing. This is best calculated from the rates of creatinine loss (about 1.7% of the total body creatine pool per day [Wyss & Kaddurah-Daouk, 2000]). Since most of the body's creatine and creatine-phosphate are found in muscle, creatinine excretion is directly related to lean body mass. Creatinine excretion is, therefore, dependent on age and sex (which are related to lean body mass). In general we excrete between 1.5 and 2 g of creatinine per day. This means that approximately 1.5 to 2 g of creatine needs to be acquired by the body each day.

Muscle meats, including fish, and dairy products are the sole sources of dietary creatine. Some creatine will be available from the diet to humans who ingest a typical mixed diet that includes meat and dairy foods. It has been calculated (using published data on the creatine content of cooked foods, as well as the USDA tables of food consumption in the US, and published data on the bioavailability of food creatine) that a maximum of about 0.5 g of creatine may be available in the diet [Brosnan & Brosnan, 2007]. This means that about 1.0 to 1.5 g of creatine per day will have to be synthesized to maintain creatine balance. Vegans would have to synthesize 1.5 to 2 g of creatine per day. These numbers are in agreement with Kasumov *et al.* [2009] who used a [1-^{13}C]glycine infusion to measure creatine synthesis in a healthy adult. They estimated a synthesis of 1.85 g of creatine per day in a single subject.

1.5.1. Demand for Amino Acids

Walser [1983] and Visek [1986] were the first to consider the burden that creatine synthesis in adults imposes on amino acid metabolism. They calculate that a typical, 70 kg, 18-29 year-old male, synthesizing about 1.65 g of creatine per day, requires 0.18 mmol of arginine per kg body weight per day (and also 0.18 mmol per kg body weight per day of glycine and of methyl groups) just for creatine synthesis. This amounts to 2.2 g of arginine which is about 50% of the arginine ingested by an individual with a typical North American protein intake of 80 g per day. Thus, creatine synthesis constitutes an enormous potential sink for arginine and the other amino acids required for its synthesis. Creatine synthesis was suggested by Mudd and Poole [1975] as the dominant user of methyl groups, amounting to about 70% of all SAM utilization in adults.

More recently, Brosnan *et al*. [2011] have re-examined the metabolic burden that creatine synthesis plays on each of the amino acids involved including glycine, arginine and methionine. They concluded that due to dietary intake and readily available endogenous synthesis, creatine synthesis does not make a great demand on glycine availability. In the neonatal piglet, they estimated that creatine synthesis required about 12% of dietary glycine [Brosnan *et al*., 2009]. These estimates are fairly straightforward because creatine synthesis utilizes the entire glycine molecule. Based on data accumulated by Wu *et al*. [2004] on the amino acid composition of piglets at term, Brosnan *et al*. [2009] have calculated that the glycine incorporated into creatine in the neonatal piglet is equivalent to about 2.7% of the net glycine incorporated into protein. Thus, they concluded that

creatine synthesis imposes a minor burden on glycine provision. However, Melendez-Hevia *et al*. [2009] have argued that humans may have limited capacity for glycine synthesis. As a result, the availability of glycine for creatine synthesis in humans clearly needs further investigation.

Ornithine, which is generated by AGAT during creatine synthesis, has one of two possible fates; 1) oxidation, as initiated by ornithine aminotransferase, or 2) reconversion to arginine. The latter process requires three enzymes: carbamoylphosphate synthetase 1 (CPS 1), argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). ASS and ASL are highly expressed in the kidney [Dhanakoti *et al*., 1990]. However, CPS 1 is more limited in distribution, mainly found in liver and small intestine and absent from the kidney [Goping *et al*., 1992]. Ornithine aminotransferase is expressed in significant amounts in the kidney, primarily in the proximal tubules [Levillain *et al*., 2000] where renal AGAT is also found [McGuire *et al*., 1986]. The issue may be debated, but it seems plausible that, in the kidney, the ornithine produced by AGAT may be readily catabolized rather than recycled back to arginine [Brosnan *et al*., 2011].

The synthesis of creatine also requires a methyl group, donated from SAM. Methyl groups may be obtained by SAM from dietary sources such as methionine, betaine, or choline via betaine, or produced endogenously via methylneogenesis. Methylneogenesis requires three successively active enzymes; serine hydroxymethyltransferase (SHMT), methylene tetrahydrofolate reductase (MTHFR), and methionine synthase (MS) and is dependent upon vitamin B status [Brosnan *et al*., 2011]. For example, MTHFR is

dependent upon NADPH (niacin/vitamin B_3) as a reductant, contains FAD (riboflavin/vitamin B_2) as a prosthetic group, and produces methylene-tetrahydrofolate (folate/vitamin B_9). MS also requires methylcobalamin (vitamin B_{12}) as a prosthetic group. Brosnan *et al*. [2011] have estimated that creatine synthesis may consume about 40% of the SAM available for all bodily methyltransferase reactions. Clearly this imposes an appreciable burden on methionine metabolism and methyl balance.

1.5.2. Creatine Synthesis Makes a Large Demand for Amino Acids in Infants

Because of the expanding tissue mass in infants, the burden of creatine synthesis on amino acid metabolism is expected to be greater than in adults. From 6-12 weeks of age, breastfed infants weigh an average of 5.7 kg [Motil *et al*., 1997] and excrete about 62 mg of creatinine per day [Coulthard *et al*., 2000]. This would total 434 mg of creatinine excreted per week or 3.8 mmol of creatinine. These same infants ingest an average of 143 ml of milk per kg per day and gain an average of 0.2 kg body weight per week [Motil *et al*., 1997]. Half of this weight gain is lean body mass and at least half of this is muscle [Motil *et al*., 1997]. This amounts to a gain of 50 g of muscle per week. According to Eriksson [1980], the skeletal muscle content of creatine-phosphate in infants of this age is between 12 and 22 mmol per kg wet wt, with a mean value of 17 mmol per kg wet wt. Data regarding creatine concentration or total creatine content of infant muscle are scarce. However, if a ratio of 3 is applied for the concentrations of creatine-phosphate:creatine, which is common in adult human muscle [Kemp *et al*., 2007], an estimate of creatine concentration in infant skeletal muscle of 6 mmol per kg wet wt is obtained. This implies

a total muscle creatine concentration of 23 mmol per kg wet wt for human infants aged 6-12 weeks.

Applying the above estimates of infant muscle total creatine content with an increase in muscle mass of 50 g per week, it can be calculated that 0.46 mmol of creatine is required each week for the expanding muscle mass. Therefore, creatine accretion should be about 0.46 mmol per week to account for muscle deposition and 3.8 mmol per week to account for creatinine loss, for a total creatine accretion of 4.26 mmol per week (0.48 g per week). To what extent this burden is borne by the infant or by the mother and its implication for amino acid metabolism was determined in this thesis.

1.6. MAMMARY GLAND PHYSIOLOGY

The mammary gland, characteristic of mammals, is an exocrine gland which produces milk for the sustenance of young offspring. During late pregnancy and immediately following birth, the mammary gland secretes colostrum (also known as first milk) which contains high concentrations of antibodies, carbohydrates and protein and low concentrations of fat. True milk secretion begins a few days after birth and contains higher concentrations of fat than does colostrum [Fetherston *et al*., 2001].

The number of mammary glands varies widely in different mammals. Humans have one pair of mammary glands whereas the rat has six ventrolateral pairs of mammary glands, each with its own nipple, that form subcutaneous sheets of tissue that extend from the

cervical to the inguinal regions [Howard & Gusterson, 2000; Masso-Welch *et al*., 2000]. During the reproductive cycle, the mammary gland undergoes many cycles of proliferation, differentiation and apoptosis. The mammary gland can be viewed as two compartments: 1) the adipose stroma, which is composed mainly of adipocytes and fibroblasts, and surrounds 2) the parenchyma, which is composed of a system of branching ducts and alveoli [Masso-Welch *et al*., 2000]. During the first half of pregnancy, the epithelial parenchymal cells proliferate causing the formation of branching ducts and alveolar buds, which is accompanied by increased tissue vascularization. The majority of differentiation occurs during the second half of pregnancy when the proportion of parenchymal ductal and alveolar cells increases dramatically [Masso-Welch *et al*., 2000].

1.6.1. Milk Production and Secretion

Milk is synthesized by the epithelial cells, secreted into the alveolar lumen and then transported by milk collecting ducts to the surface of the nipple. Mammary glands are fully capable of producing milk by about 20 weeks of gestation. Milk secretion is prevented until parturition by the high circulating levels of estrogen and progesterone during the latter half of gestation. Delivery of the placenta at parturition causes an abrupt decline in estrogen and progesterone and milk secretion is no longer inhibited [Sherwood, 2001].

The two main hormones responsible for maintaining lactation include: 1) prolactin, which promotes secretion of milk through its action on the alveolar epithelium, and 2) oxytocin, which aids in forced milk expulsion from the lumen of the alveoli into the ducts. Stimulation of sensory nerve endings in the nipple sends action potentials through the spinal cord and up to the hypothalamus. The hypothalamus then signals the posterior pituitary to release oxytocin. Stimulation of the nipple most often occurs through suckling by the infant [Sherwood, 2001].

Oxytocin causes contraction of the myoepithelial cells that surround each alveolus to bring about milk ejection or "let down". Milk ejection will continue as long as the suckling stimulus is present. Without the stimulation by oxytocin, the milk present in the alveoli will not be released. Conditioning can also result in oxytocin release. For example, an infant's cry can sometimes trigger milk ejection. On the other hand, hypothalamic stimulation through physiological stress can inhibit milk ejection [Dewey, 2001, Sherwood, 2001].

Suckling by the infant also triggers prolactin secretion. Prolactin is secreted by the anterior pituitary and is controlled by two hypothalamic molecules. The first is prolactin inhibiting hormone (PIH) which is now known to be dopamine, a neurotransmitter. PIH is the predominant influence on prolactin throughout a female's life, keeping levels of prolactin low. During lactation, a second hormone comes into play, prolactin releasing hormone (PRH) which causes prolactin to be secreted from the anterior pituitary. Prolactin is then available to stimulate the alveolar epithelium to secrete milk in order to

replenish what is lost through ejection [Sherwood, 2001]. In this way, suckling concurrently stimulates both milk production and ejection, ensuring that milk synthesis meets the needs of the infant. The more the infant suckles, the more milk is ejected and then the more milk is produced, preparing the mammary glands for the next feeding.

1.7. PATHOLOGY – CREATINE DEFICIENCY DISEASES

The synthesis of creatine requires two enzymes, AGAT and GAMT, and the uptake of creatine into tissues requires a creatine transporter, SLC6A8. The disorders of creatine metabolism can be divided into two main categories: disorders of creatine synthesis and disorders of cellular creatine transport [Stromberger *et al*., 2003]. The clinical characteristics of the disorders in creatine metabolism principally result from cerebral creatine depletion; these characteristics include mental retardation and speech delay and may include seizures. Surprisingly, although children with inborn errors in creatine metabolism may be hypotonic, cardiomyopathies or skeletal myopathies are not commonly reported although muscle tissue may be creatine-depleted [Stromberger *et al*., 2003].

1.7.1. L-Arginine:Glycine Amidinotransferase (AGAT) Deficiency

AGAT deficiency (McKusick 602360) has an autosomal recessive pattern of inheritance [Battini *et al*., 2006; Stromberger *et al*., 2003]. Mental retardation and speech delay are common clinical characteristics and epileptic episodes that are responsive to antiepileptic

drugs are also common [Stromberger *et al*., 2003]. Plasma creatine levels may be within the normal range but cerebral creatine levels are depleted. Levels of GAA in the plasma are characteristically low. Treatment with oral creatine monohydrate almost completely restores cerebral creatine levels and causes significant improvement in highly abnormal developmental scores [Stromberger *et al*., 2003]. In a case report by Battini *et al*. [2006], oral creatine supplementation begun early in life allowed normal growth patterns and normal developmental quotients at the age of 18 months, whereas untreated relatives had already begun to show symptoms of the disease at this age [Battini *et al*., 2006]. If treatment is administered early in development, AGAT deficiency may be considered a treatable metabolic disorder.

1.7.2. Guanidinoacetate Methyltransferase (GAMT) Deficiency

GAMT deficiency (McKusick 601240) follows an autosomal recessive inheritance pattern and leads to a lack of creatine and an accumulation of GAA [Schulze, 2003; Stromberger *et al*., 2003]. GAMT deficiency can be classified as mild, intermediate, or severe, with severe phenotypes exhibiting abnormal signal intensities of the basal ganglia on EEG, extrapyramidal movement disorder, untreatable epilepsy and early global developmental delay. The mild phenotype presents with mental retardation, autistic behavior and speech delay and the intermediate phenotype presents with symptoms on a severity level between the severe and mild phenotypes [Stromberger *et al*., 2003]. A number of mutations located on various exons of the GAMT gene have been reported to cause GAMT deficiency. GAMT-deficient patients have intracellular depletion of

creatine and creatine-phosphate and extracellular depletion of creatine and creatinine. Oral supplementation of creatine monohydrate has been found to alleviate depleted creatine concentrations, extrapyramidal symptoms, delayed developmental progress and epilepsy. Soon after GAMT deficiency was discovered, no patient had achieved a normal developmental level and all patients lacked active speech even with treatment [Schulze, 2003; Stromberger *et al*., 2003]. In 2006, Schulze *et al*. published a case study in which a child with GAMT deficiency was diagnosed early because of an older affected sibling. This child was provided with creatine, an arginine-restricted diet and benzoate to reduce glycine levels via the formation of hippuric acid. The arginine-restricted diet and benzoate work together to reduce the substrates of AGAT, consequently reducing the synthesis of GAA. Fourteen months following treatment, the child was normal developmentally and showed no symptoms of GAMT deficiency [Schulze *et al*., 2006].

1.7.3. Creatine Transporter (SLC6A8) Deficiency

Creatine transporter deficiency (SLC6A8, McKusick 300036) is an X-linked disorder that is clinically characterized by mental retardation, speech delay and antiepileptic-responsive epileptic episodes. Extracellular creatine concentrations may be low or elevated while intracellular creatine concentrations are depleted [Stromberger *et al*., 2003; Valayannopoulos *et al*., 2013]. Urinary creatinine excretion may be normal while creatine excretion is elevated thereby causing an increase in the creatine:creatinine excretion ratio, usually the first diagnostic marker for creatine transporter deficiency [Valayannopoulos *et al*., 2012]. Since the problem is with creatine transport and not with

synthesis, creatine supplementation is not an effective treatment of the disorder and does not replenish cerebral creatine levels [Stromberger *et al*., 2003]. Studies by Fons *et al.* [2008] found that oral supplementation with arginine as a precursor of creatine also failed to improve the neurological status in patients with creatine transporter deficiency. In 2010, Fons *et al.* tried supplementation with a lipophilic creatine-derived compound, creatine ethyl ester, which also failed to replenish brain creatine content or neurological status. Braissant *et al.* [2011] pointed out that even though the brain may be able to synthesize its own creatine, the synthesis appears to be an intercellular process and the creatine transporter is still needed to transport the precursors from one cell type to another. Therefore, the endogenous creatine synthesis pathway would remain deficient in the CNS of patients with creatine transporter deficiency syndromes because GAA cannot be transported from AGAT- to GAMT-expressing cells because of the lack of a functional creatine transporter.

1.8. STATEMENT OF PROBLEM

The overall problems addressed in this thesis concern (i) the pathway and regulation of creatine synthesis in rats and, to a limited extent, humans, and, (ii) the way in which neonatal animals, including humans, accumulate creatine, as well as the metabolic burden imposed by this accumulation.

1.8.1. Pathway and Regulation of Creatine Synthesis

One of the principal questions addressed concerns the inter-organ synthesis of creatine. Does the rat kidney, *in vivo*, produce guanidinoacetate and release it to the plasma? This is addressed, quantitatively, by means of measurements of arterio-venous differences together with renal blood flow. Response of renal GAA production to creatine ingestion is examined. As well, the extent that renal GAA production accounts for the need for creatine, as measured by renal creatinine excretion, is determined.

1.8.2. Creatine Synthesis in the Neonate

We have quantified creatine accretion in suckling rat pups and determined the extent to which this creatine is provided in mother's milk. We used an isotopic approach to characterize the origin of milk creatine, whether it be produced by the mammary gland or excreted from plasma.

We have measured creatine and guanidinoacetate in human milk and in commercial infant formulas and determined the total dietary intake of creatine by the infant. From these data, together with the estimates of total creatine accretion by the growing infant, we have determined the extent to which the infant and the mother bear the burden for providing creatine. We have then discussed the metabolic burden, in the context of other aspects of metabolism.

CO-AUTHORSHIP STATEMENT

The major intellectual and practical contributions to all work contained within this thesis were made by Erica Edison, B.Sc., M.D. She is first author of **Chapters 2** and **3** but listed as second author of **Chapter 4**.

The design and identification of the research proposal for **Chapter 2** was primarily conducted by E. Edison with guidance from J. Brosnan. All practical research and data analysis were conducted by E. Edison except for the collection of human serum samples, which was done by C. Meyer at the University of Rochester General Clinical Research Center in Rochester, NY. Manuscript preparation was done by E. Edison with guidance from J. Brosnan. All authors read and approved the final manuscript.

The design and identification of the research proposal for **Chapter 3** was primarily conducted by E. Edison with guidance from all co-authors. All practical research and data analysis were completed by E. Edison. Manuscript preparation was done by E. Edison with guidance from J. Brosnan. All authors read and approved the final manuscript.

E. Edison primarily designed and conducted much of the research of **Chapter 4** in conjunction with all co-authors. Because E. Edison was admitted to the MD program at Memorial University of Newfoundland and preparing for the birth of her second child,

both she and her supervisory committee felt it was inappropriate for her to conduct experiments that involved radioisotopes. Accordingly, the experimental work was completed by S. Lamarre. Data were analyzed primarily by E. Edison in conjunction with S. Lamarre. The manuscript was written primarily by S. Lamarre and J. Brosnan, in consultation with E. Edison. The contributions of S. Lamarre and E. Edison were equal and they should be considered to be joint first authors. All authors read and approved the final manuscript.

Appendix A was jointly written by the co-authors. All authors read and approved the final manuscript.

CHAPTER 2

CREATINE SYNTHESIS: PRODUCTION OF GUANIDINOACETATE BY THE RAT AND HUMAN KIDNEY *IN VIVO*

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Creatine synthesis: Production of Guanidinoacetate by the Rat and Human Kidney *in vivo*.

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Running head: Renal guanidinoacetate production

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2.1. ABSTRACT

A fraction of the body's creatine and creatine phosphate spontaneously degrades to creatinine, which is excreted by the kidneys. In humans, this amounts to about 1-2 g per day and demands a comparable rate of *de novo* creatine synthesis. This is a two-step process in which L-arginine:glycine amidinotransferase (AGAT) catalyzes the conversion of glycine and arginine to ornithine and guanidinoacetate (GAA); guanidinoacetate methyltransferase (GAMT) then catalyzes the SAM-dependent methylation of GAA to creatine. AGAT is found in the kidney and GAMT in the liver, which implies an interorgan movement of GAA from the kidney to the liver. We have studied the renal production of this metabolite in both rats and humans. In control rats, [GAA] was 5.9 μM in arterial plasma and 10.9 μM in renal venous plasma for a renal A-V difference of –5.0 μM. In the rat, infusion of arginine or citrulline markedly increased renal GAA production but infusion of glycine did not. Rats fed 0.4% creatine in their diet had decreased renal AGAT activity and mRNA, an arterial plasma [GAA] of 1.5 μM and a decreased renal A-V difference for GAA of –0.9 μM. In humans, [GAA] was 2.4 μM in arterial plasma, with a renal A-V difference of -1.1 μM. These studies show, for the first time, that GAA is produced by both rat and human kidneys *in vivo*.

Keywords: L-arginine:glycine amidinotransferase (AGAT), transamidinase, amino acid metabolism

2.2. INTRODUCTION

Creatine and creatine phosphate act to buffer the cytosolic ATP/ADP ratio in tissues that have high and variable rates of ATP usage (e.g. skeletal and cardiac muscle). Creatine kinase catalyzes the reversible transfer of the γ-phosphate group of ATP to the guanidino group of creatine to yield ADP and creatine phosphate.

ATP + Creatine ↔ ADP + Creatine Phosphate [Equation 1]

In skeletal muscle, creatine kinase activity is high, keeping its reaction at near-equilibrium. This keeps the ADP and ATP concentrations fairly constant and buffers the cytosolic phosphorylation potential (18, 27, 28). Energy storage and transmission by the creatine kinase system is hypothesized to work in two ways: as a temporal buffer and as a spatial buffer. The temporal energy buffer theory is best exemplified during episodes of high energy use, such as muscle contraction. As soon as ATP is hydrolyzed it must be replenished. The high-energy phosphate of creatine phosphate is transferred to ADP to regenerate ATP. This leads to an accumulation of creatine that must be rephosphorylated during recovery from exercise. The spatial energy buffer theory implies that creatine phosphate acts as an energy carrier, working to transport high-energy phosphate from sites of synthesis (mitochondria) to sites of ATP utilization in the cytosol (27).

There is a need for creatine replacement due to the spontaneous, irreversible conversion of creatine and creatine phosphate to creatinine (3). Creatinine is not used by the body and is excreted in the urine. Approximately 1.7% of the body's total creatine pool (creatine and creatine phosphate) is converted to creatinine per day (28). In a young 70

kg male, this amounts to a loss of about 2 g of creatine per day (21). This creatine has to be replenished by the dietary intake of creatine and by *de novo* creatine synthesis.

De novo creatine synthesis is a very simple process, involving just two enzymes. The first enzyme is L-arginine:glycine amidinotransferase (AGAT). AGAT catalyzes the transfer of a guanidino group from arginine to glycine to form ornithine and guanidinoacetate (GAA) (26). This is believed to be the regulated step of creatine biosynthesis. The second enzyme in the pathway is guanidinoacetate methyltransferase (GAMT). This enzyme catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to GAA to form S-adenosylhomocysteine (SAH) and creatine (26). There are high activities of AGAT in the kidneys and of GAMT in the livers of various species (26). Such tissue enrichment has suggested that creatine synthesis is an inter-organ process whereby GAA, produced by the kidney, is released into the circulation and is methylated to creatine in the liver. Creatine is then released from the liver and into the circulation where it can be taken up, via a specific transporter, by various tissues. However, this hypothesis has not been tested *in vivo*.

The regulation of creatine biosynthesis is usually thought to occur at the level of AGAT (26). An increase in serum levels of creatine results in a decrease in AGAT enzyme activity, enzyme level and mRNA expression in rat kidney (14).

In the current study we investigate the production of GAA, *in vivo*, by human and rat kidneys. We have also used the rat to study the regulation of renal GAA production. We

have investigated the effects of creatine supply as well as substrate supply on renal GAA production. This study has provided novel insights into the acute and chronic regulation of creatine synthesis.

2.3. MATERIALS AND METHODS

Chemicals and other reagents: Chemicals and the Hypersil ODS C18 reverse-phase HPLC column were purchased from Sigma (Oakville, ON, Canada). Diet components were purchased from MP Biomedicals (Montreal, QC, Canada). The Pico-Tag column for the measurement of free amino acids, Centricon 3000 molecular weight cut-off spin columns and other filters were supplied by Millipore (Barrerica, MA, USA). Creatine kinase was bought from Roche (QC, Canada) and Dulbecco's phosphate-buffered saline from Invitrogen Canada Inc. (Burlington, ON, Canada). A one-step RT-PCR kit was purchased from Qiagen (Mississauga, ON, Canada), and β-actin amplimer set primers were purchased from BD Biosciences Clontec (Palo Alto, CA, USA). Phenylisothiocyanate was provided by Pierce Biotechnology, Inc. (Rockford, IL, USA).

Human Subjects: Seven healthy subjects [44 years of age (SD 14), body mass index 27.9 kg/m^2 (SD 4.1)] were studied after the protocol had been approved by The University of Rochester Institutional Review Board and informed written consent had been obtained. None of the subjects were vegetarians or followed a creatine-restricted diet. Renal net balance measurements were performed as previously described (16). Briefly, subjects were admitted to the University of Rochester General Clinical Research Center between 6:00 and 7:00 pm on the evening prior to experimentation. A standard meal (10 kcal/kg;

50% carbohydrate, 35% fat, 15% protein) was consumed between 6:30 and 8:00 pm after which subjects were required to fast until completion of the experiment. At approximately 8:00 am the following morning, an infusion of para-aminohippuric acid was started for measurement of renal blood flow. Between 8:00 and 9:00 am, a renal vein was catheterized, under fluoroscopy, through the right femoral vein and the position of the catheter tip ascertained by injection of a small amount of iodinated contrast material. At approximately 9:00 am, a dorsal hand vein was cannulated and kept in a thermoregulated Plexiglas box at 65ºC for sampling arterialized venous blood. Starting at approximately 10:00 am, three blood samples were obtained simultaneously from the dorsal hand vein for determination of guanidinoacetate and creatinine concentrations.

Animals: The study was conducted in accordance with the Guidelines of the Canadian Council on Animal Care and was approved by Memorial University of Newfoundland's Institutional Animal Care Committee. Male Sprague-Dawley rats were purchased from Memorial University of Newfoundland's breeding colony and were fed *ad libitum* and had free access to water. The control diet, AIN-93G (17), is creatine-free while the creatine supplementation diet was a modified version of it. For the creatine supplementation diet, 4.0 g/kg creatine monohydrate replaced 4.0 g/kg cornstarch. Rats were placed on these diets for a period of two weeks. Prior to the initiation of each experimental period, animals were allowed to become accustomed to the AIN-93G powdered diet for a period of 3-5 days. At the beginning of the feeding study rats weighed about 200 g; at the end of the feeding period, control rats and creatine-fed rats weighed an average of about 400 g. All animals were exposed to a 12-hour light: 12-hour

dark cycle, with the light cycle commencing at 8 am. All procedures were conducted during the 12-hour light cycle.

Tissue preparation: Animals were anaesthetized with 65 mg/kg sodium pentobarbital (i.p.). Following a midline abdominal incision and opening of the abdominal cavity, the renal vein was cleaned of connective tissue and blood flow was measured using a T206 small animal blood flow meter (Transonic Systems Inc., Ithaca, NY, USA). Arterial and venous blood samples were taken across the kidney. Kidneys were removed and immediately placed in ice-cold 50 mM potassium phosphate buffer (pH 7.4), except for a small sample which was retained for RT-PCR analysis.

Infusion experiments: The femoral vein was cleaned to allow delivery of the 0.8 mL priming injection and the jugular vein was cannulated with PE50 tubing to allow delivery of infusate. A tongue restrainer was placed in the animal's mouth to prevent suffocation. The abdominal cavity was then opened, the T206 probe placed on the renal vein, the priming injection given and the infusion pump was set to deliver 0.037 mL/minute through the jugular vein for 20 minutes. Blood flow was measured during the entire 20 minute infusion. At 20 minutes, both arterial and venous blood samples were taken across the kidney. Priming injections for the different experimental conditions were 0.8 mL of either 0.9% NaCl, 200 mM arginine, 100 mM citrulline, 200 mM glycine, or 200 mM alanine. Infusions, for each condition, were either 0.9% NaCl, 60 mM arginine, 30 mM citrulline, 60 mM glycine, or 60 mM alanine, respectively. All amino acid solutions were made isotonic with saline.

Creatine, guanidinoacetate, creatinine, and L-arginine:glycine amidinotransferase assays: For creatine measurement, aliquots of the plasma samples were deproteinized by adding 50% vol/vol 0.6 M perchloric acid, kept on ice for 20 minutes and centrifuged at 20 000 x g for 5 minutes to sediment proteins. The supernatant was neutralized with 20% potassium hydroxide and creatine was measured as described by Lowry and Passonneau (12). For GAA measurement, the plasma samples were diluted 4-fold with PBS, and centrifuged through a Centricon spin filter at 10 000 x g for 30 minutes to remove large proteins. Filtered plasma was used for GAA analysis via HPLC according to the method of Carducci *et al.* (6). Creatinine was measured by Clinical Laboratories, Health Sciences Center (St. John's, NL, Canada). Renal AGAT activity was measured as described by Van Pilsum *et al.* (25).

Reverse Transcriptase/ Polymerase Chain Reaction (RT-PCR) analysis of AGAT mRNA: RNA was prepared from the kidney samples using a rapid guanidinium thiocyanate method as described by Chomczynski and Sacchi (7). 2 :g of RNA were reverse transcribed using a one-step reverse transcription kit and amplified by 21 cycles. An upstream primer (5'-ATGCTACGGGTGCGGTGT-3') and downstream primer (5'-ATAATTGACCTGTATGCGCGG-3') were designed from the rat kidney AGAT sequence. The primers were used to amplify a 599-bp PCR fragment. A 768-bp fragment of rat β-actin gene was co-amplified using amplimer set primers. PCR products

were separated on a 1.5% agarose gel. Ethidium bromide-stained gels were visualized by UV illumination and quantified by densitometry.

Amino acid analysis: Plasma samples were deproteinized by mixing 200 µL plasma, 200 µL internal standard (norleucine), and 1.0 mL 5% trifluoroacetic acid in methanol. Samples were centrifuged at 20 000 x g for 5 minutes so as to sediment proteins. The supernatant was poured off, frozen in liquid nitrogen and freeze-dried overnight. Amino acid analysis was as described by Bidlingmeyer *et al.* (2).

Statistical analysis: All data are presented as mean (standard deviation). In studies with only two groups, Student's paired or unpaired (depending upon appropriateness) t-test was used to identify significant differences. One-way ANOVA followed by Newman-Keuls post-hoc tests was used in studies involving three or more experimental groups. A P value of <0.05 was taken as significant. GraphPad Software Prism 3.0 was used for all statistical analyses.

2.4. RESULTS

Renal GAA Production and its Chronic Regulation. **Figure 1A** shows that rat kidneys produce GAA and release it into the renal vein; on the control diet, GAA concentration in renal venous plasma was about 9 µM, compared with 5 µM in arterial plasma. Creatine supplementation for two weeks massively decreased both the arterial GAA concentrations (by 72%) and renal A-V differences (by 81%). Renal AGAT activity was decreased by 86% upon creatine supplementation (**Figure 2.1B**). Densitometric quantitation of renal

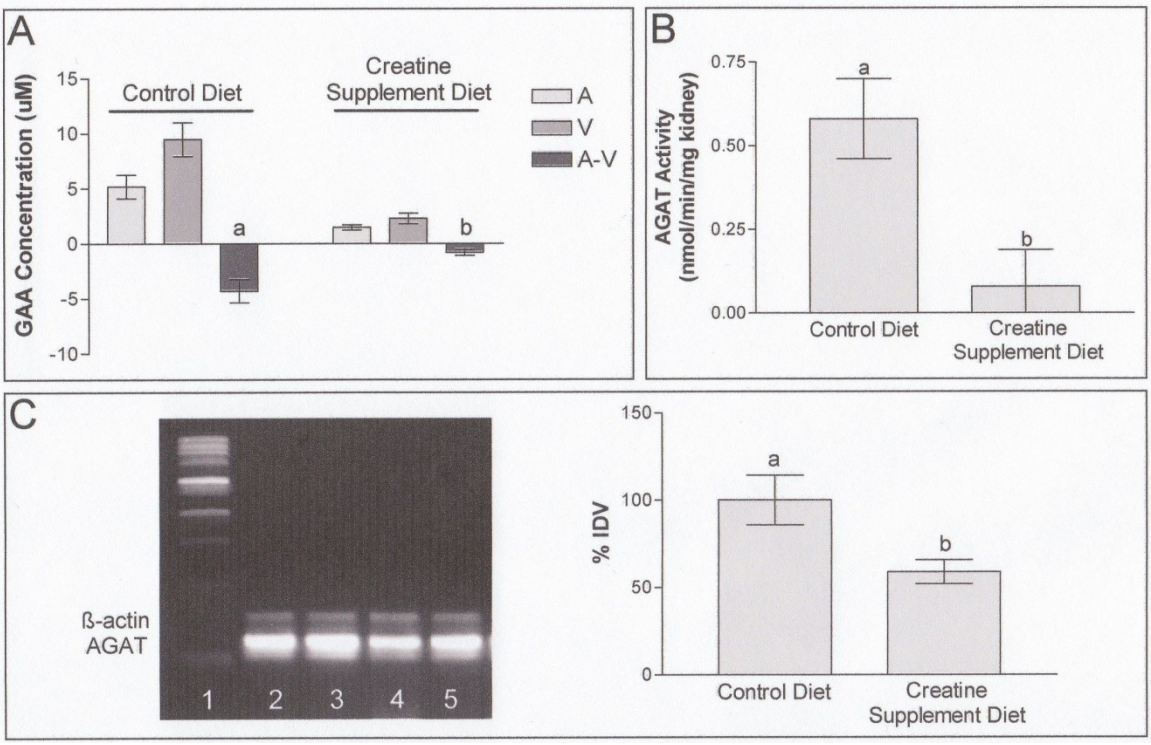

Figure 2.1: Effect of Creatine Feeding on Renal Guanidinoacetate (GAA) Production and L-Arginine:Glycine Amidinotransferase (AGAT) Activity and mRNA Expression in Rats. Bars represent means and vertical lines standard deviations of 6 observations. Different superscripts indicate statistical differences ($P<0.05$). *A*. Plasma GAA concentrations and renal A-V differences. A negative A-V difference represents renal output. *B*. Renal AGAT activity. *C*. Renal AGAT mRNA. *Lane 1*, MW standards; *lanes 2 and 3*, control diet; *lanes 4 and 5*, creatine supplementation. Densitometric quantitation of AGAT mRNA, normalized for β-actin.

AGAT mRNA, when normalized for β-actin, shows a 41% decrease in its abundance upon feeding creatine.

So as to determine the extent to which renal GAA production could account for total creatine synthesis, we measured, simultaneously, the A-V differences for GAA and creatinine across rat and human kidneys. This is based on the fact that creatinine clearance via the kidneys reflects creatine loss by conversion to creatinine and, therefore, of the need for creatine replacement. There was no significant difference between renal GAA production and creatinine loss in rats (**Figure 2.2**). In humans, however, renal GAA production only amounted to about 11-12% of renal creatinine excretion under both fed and fasted conditions (**Table 2.1**).

Acute Regulation of Renal GAA Production by Substrate Concentrations. We examined the effects of acute increases in the amino acid substrates for GAA synthesis. We employed two control groups, rats infused either with NaCl or with alanine. The experimental groups were infused with glycine or arginine. We also infused rats with citrulline, which is converted to arginine in kidneys (9). We measured renal GAA production, by measuring both renal plasma flow and A-V differences, after 20 minutes of infusion. Glycine infusion increased the circulating levels of this amino acid by about 50% (**Table 2.2**) but there was no increase in renal GAA production (**Figure 2.3**). Arginine infusion increased the plasma levels of this amino acid by about 300% (**Table 2.2**); renal GAA production was increased by about 150% (**Figure 2.3**). Citrulline

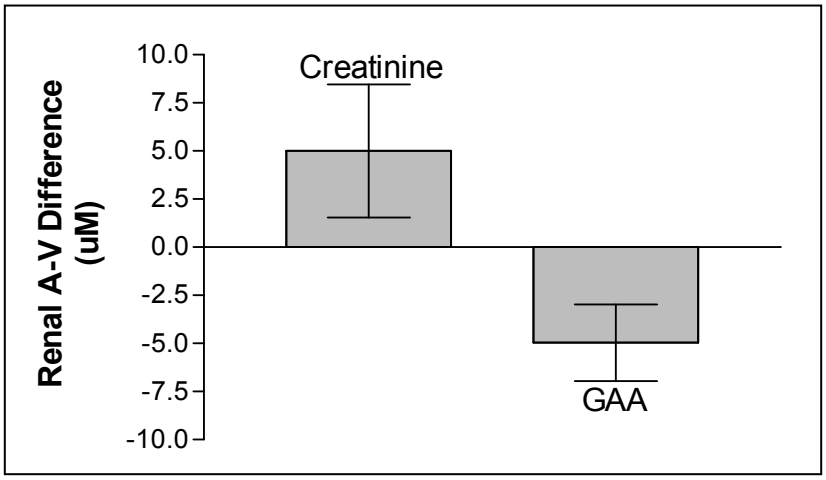

Figure 2.2: Creatinine Excretion vs. Guanidinoacetate (GAA) Production by the Kidneys of Rats fed the control (creatine-free) diet. Bars represent means and vertical lines standard deviations of 10 observations.

Table 2.1: Renal Metabolism of Guanidinoacetate (GAA) and Creatinine in Humans. All values are expressed as mean (standard deviation) of 7 observations. An asterisk indicates that the arteriovenous (A-V) difference is significantly different from zero ($P<0.01$).

	Guanidinoacetate		**Creatinine**	
	Fed	**Fasted**	**Fed**	**Fasted**
Arterial plasma concentration (μM)	2.4 (0.8)	2.5 (0.4)	76 (13)	75 (12)
Renal A-V difference (μM)	-1.5 (0.9)*	-1.5 (0.8)*	14 (8.1)*	11 (9.6)*
Renal flux (μmoles/minute)	-1.1 (0.5)	-1.0 (0.6)	10 (5.2)	8.3 (5.1)

Table 2.2: Plasma Amino Acid Levels following Infusion of Various Substrates in Rats. All values are expressed as mean (standard deviation) of 6 observations. An asterisk indicates statistical differences from the saline control in each vertical column ($P<0.05$).

Infusion	Glycine (µM)	Arginine (µM)	Citrulline (µM)	Alanine (µM)	Methionine (µM)	Ornithine (µM)
NaCl	268 (66)	111 (51)	85 (31)	566 (133)	42 (9)	93 (21)
Glycine	413 (29)*	79 (28)	71 (17)	482 (46)	44 (22)	95 (30)
Arginine	235 (44)	433 (219)*	92 (21)	516 (67)	40 (23)	315 (134)*
Citrulline	244 (50)	98 (77)	347 (201)*	424 (58)	33 (9)	154 (53)
Alanine	335 (84)*	93 (70)	101 (18)	925 (133)*	52 (10)	91 (33)

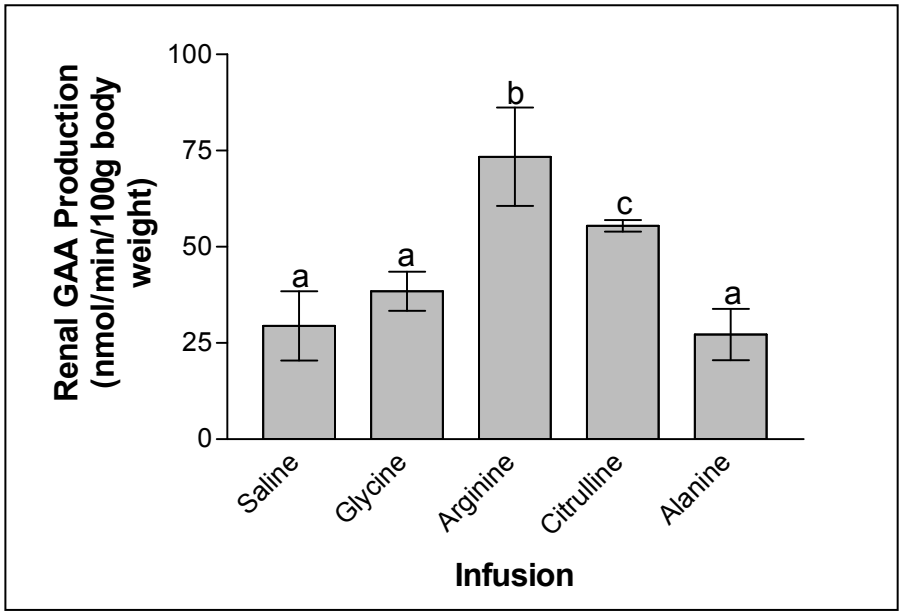

Figure 2.3: Total Renal Production of Guanidinoacetate (GAA) in Rats Infused with Various Substrates. Bars represent means and vertical lines standard deviations of 6 observations. Different superscripts indicate statistical differences ($P<0.05$).

infusion, which did not increase plasma arginine levels (**Table 2.2**), almost doubled renal GAA production (**Figure 2.3**).

2.5. DISCUSSION

The present study makes a number of novel contributions to our knowledge of creatine synthesis and its regulation:

(i) the rat kidney produces GAA, *in vivo*,

(ii) renal GAA production, *in vivo*, responds acutely to increased arginine levels but not to increased glycine levels,

(iii) citrulline, converted to arginine in the renal tubules, is immediately available for GAA synthesis; however, creatine ingestion does not down-regulate intestinal citrulline production,

(iv) creatine ingestion markedly decreases renal GAA production in the rat,

(v) in rats fed a creatine-free diet, renal GAA production *in vivo* equals creatinine loss in the urine,

(vi) human kidneys also produce GAA but, unlike in the rat, this is not the major source of GAA for creatine synthesis.

In addition, we confirm that creatine ingestion decreases both renal AGAT activity and mRNA level.

Since creatinine excretion is a measure of creatine and creatine phosphate breakdown and therefore, of the need for creatine replacement, our data on the equivalence of renal GAA production and creatinine excretion indicates that the kidney is the major organ for the

production of GAA in the rat. Borsook and Dubnoff (4) had pointed out that the activity of renal AGAT is more than sufficient to supply the body's creatine need. However, measurements of enzyme activity under optimal conditions cannot be extrapolated to the *in vivo* situation; the present data provide definitive information on the *in vivo* situation. It cannot, however, be concluded that the kidney is the sole site of GAA production in the rat. AGAT activity has been reported in rat kidney, pancreas, brain, spleen, and testes (24). Horner (11) studied transamidination in nephrectomized rats and found that GAA synthesis was not entirely ablated; indeed extrarenal transamidination, in this situation, may be considerable. Although we have shown that the rat kidney is the major organ involved in GAA production *in vivo*, extrarenal transamidination may be important in terms of local roles within tissues or in times of renal dysfunction.

The situation in humans, however, is quite different as renal GAA production accounts for only about 11-12% of daily creatine loss as measured by creatinine excretion. Thus, almost 90% of daily creatine replenishment occurs without renal involvement. As our human subjects were not on a creatine-free diet, this creatine replenishment occurs via a combination of diet and *de novo* synthesis. Stead *et al.* (21) have estimated that in 20-59 year-olds on a typical western diet, about 50% of the creatine need can be provided by the diet. If we take this into consideration, renal GAA production in a human may account for approximately 20% of total GAA synthesis. The kidney, therefore, does not appear to be the major organ involved in GAA production in humans although it clearly plays a role. An earlier study by Sandberg *et al*. (19) also reported GAA production by human kidneys, *in vivo*. Again, GAA production amounted to only 25% of the renal creatinine

loss. Although these data agree, in general, with our results, it should be appreciated that the GAA concentrations reported in this 1953 study are probably not reliable as they are 5-fold those found by modern analytical techniques (8).

We report that feeding a 0.4% creatine diet for a period of two weeks causes a down-regulation of renal AGAT activity and mRNA expression as well as a decrease in *in vivo* renal GAA production in the rat. The down-regulation of AGAT activity by creatine and GAA feeding has been previously shown by Van Pilsum (23) and in our lab by Stead *et al.* (20). The effect of GAA is believed to be due to the conversion of GAA to creatine and the action of creatine to down-regulate the enzyme. Our findings on the decreased levels of renal AGAT mRNA brought about by creatine feeding, confirm those of McGuire *et al.* (14). These data suggest that AGAT activity may be regulated at the pretranslational level. However, the decrease in renal AGAT mRNA expression is not as large as the decrease in renal AGAT activity (41% vs. 86%, respectively) suggesting that an additional mechanism may play a role.

We also report, for the first time, that feeding a creatine-containing diet decreases GAA production by rat kidneys, *in vivo*. Intake of a diet containing 0.4% creatine decreases renal GAA production by 81% and lowers plasma GAA values by 72%. A recent study conducted by Derave *et al.* (8) showed that feeding creatine to humans for a period of 20 weeks lowered plasma GAA levels in a dose-dependant manner: by 50% during the loading phase (20 g creatine per day) and by 30% during the maintenance phase (5 g creatine per day).

Our experiments also shed light on the acute regulation of renal GAA synthesis by increased provision of the amino acid substrates, glycine and arginine. Increased provision of glycine had no effect on GAA production whereas provision of arginine did. These experiments are best interpreted in terms of the kinetic properties of AGAT and the renal concentrations of the substrates. The Km of the rat kidney AGAT for glycine is reported to be 2.4-2.8 mM; that for arginine is 3.0-3.1 mM (15). We have reported the rat renal levels of glycine and arginine to be 1320 and 170 nmol/g, respectively (5); this translates to average intracellular concentrations of about 2.5 mM and 0.32 mM, respectively for glycine and arginine. Given that the cell glycine concentration is close to AGAT's Km and that the infusion only raised the circulating glycine concentration by about 50% one may expect, at most, an increase of about 25% in GAA production. An increase of this magnitude was observed but was not statistically significant. On the other hand, cellular arginine concentration is well below the Km value of AGAT for this amino acid. The infusion increased circulating arginine concentration four-fold so that, assuming that changes in cellular arginine concentration parallel the plasma levels and applying the Michaelis-Menten equation, we may anticipate an increased GAA production of about 3-fold. This is remarkably close to the 2.7-fold increase that we observed. The close agreement between the observed and calculated rates may be, to some degree, fortuituous given the nature of the assumptions we were obliged to make. Nevertheless, it does serve to highlight the sensitivity of renal GAA synthesis to ambient arginine concentration. That GAA synthesis in humans is also markedly sensitive to

arginine concentration is suggested by a report of low plasma GAA concentration in children with urea cycle defects that may be normalized by the provision of arginine (1).

Finally, we draw attention to our finding that renal GAA production was doubled by the infusion of citrulline even though there was no increase in circulating arginine concentration. This result is attributable to the conversion of citrulline to arginine by the renal tubules. We have already shown that this process is very sensitive to circulating citrulline concentration in the rat (9). Renal arginine synthesis occurs in the cells of the proximal tubule (10) as does GAA synthesis (13, 22). Therefore, arginine synthesized in these cells becomes immediately available as a substrate for GAA synthesis. This indicates that citrulline synthesis (which occurs in the gut) may contribute in part to creatine synthesis. It also suggests that gut citrulline synthesis may be regulated by ingestion of creatine. To test this hypothesis, we measured gut citrulline flux in rats fed the 0.4% creatine diet. We found, however, that the need for creatine synthesis does not drive intestinal citrulline synthesis, which was not altered in the creatine-fed rats (data not shown).

In summary, this study has shown, for the first time, that GAA is produced by rat kidneys, *in vivo*. We have also shown that the human kidney produces GAA. Creatine-feeding downregulates renal AGAT activity and mRNA expression as well as *in vivo* production of GAA by the rat kidney. Provision of the substrate glycine was without effect on renal GAA production *in vivo*; however, provision of the substrate arginine or citrulline, which is converted to arginine in the kidney, increased renal GAA production in the rat.

2.6. ACKNOWLEDGEMENTS

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2.7. GRANTS

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CHAPTER 3

CREATINE AND GUANIDINOACETATE CONTENT OF HUMAN MILK AND INFANT FORMULAS: IMPLICATIONS FOR CREATINE DEFICIENCY SYNDROMES AND AMINO ACID METABOLISM

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Creatine and Guanidinoacetate Content of Human Milk and Infant Formulas: Implications for Creatine Deficiency Syndromes and Amino Acid Metabolism

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Key words: methionine, arginine, methylation reactions

3.1. ABSTRACT

Creatine is essential for normal neural development; children with inborn errors of creatine synthesis or transport exhibit neurological symptoms such as mental retardation, speech delay, and epilepsy. Creatine accretion may occur through dietary intake or *de novo* creatine synthesis. The objective of this study is to determine how much creatine an infant must synthesize *de novo*. We have calculated how much creatine an infant needs to account for urinary creatinine excretion (creatine's breakdown product) and new muscle lay-down. To measure an infant's dietary creatine intake, we measured creatine in mother's milk and in various commercially available infant formulas. Knowing the amount of milk/formula ingested, we calculated the amount of creatine ingested. We have found that a breast-fed infant receives about 9% of the creatine needed in the diet and that infants fed cow's milk-based formula receive up to 36% of the creatine needed. However, infants fed a soy-based infant formula receive negligible dietary creatine and must rely solely on *de novo* creatine synthesis. This is the first time that it has been shown that neonatal creatine accretion is largely due to *de novo* synthesis and not through dietary intake of creatine. This has important implications both for infants suffering from creatine deficiency syndromes and for neonatal amino acid metabolism.

Key Words: arginine, methionine, methylation reactions

3.2. INTRODUCTION

Creatine is essential for normal brain development. We know this because infants with inborn errors in either creatine synthesis or transport develop a consistent series of neurological symptoms, including speech delay, mental retardation and epilepsy[1,2,3]. Nevertheless, the great bulk of the body's creatine and creatine phosphate occurs in muscle, and it is clear that these molecules play critical roles in the buffering of ATP levels, the vectorial transport of high-energy phosphates and in the control of the production of reactive oxygen species by mitochondria[4,5,6]. The skeletal muscle content of creatine plus creatine phosphate is of the order of 25-30 μmol/g wet weight. The brain content of these molecules is perhaps half that of muscle[7]. The total body content of creatine plus creatine phosphate is quite high, approximately 100 g in an adult human[8]. Creatine and creatine phosphate are spontaneously and irreversibly broken down to creatinine at a rate of 1.7% per day. Creatinine is excreted in the urine. Therefore, adults have a continuous need to replace the creatine, usually by a combination of diet and endogenous synthesis[9]. In addition to the replacement of this creatine lost as creatinine, infants must also supply creatine to their expanding tissue mass. There are only two ways in which a growing infant may acquire creatine; it is either provided in mother's milk (or in infant formula) or is synthesized *de novo* by the infant. The situation with formula-fed infants is more complex and depends upon the type of formula used. The present study examines the creatine and guanidinoacetate (a metabolic precursor of creatine) content of mother's milk and various infant formulas, and estimates the degree to which diet or endogenous synthesis provides creatine to the growing infant. The implications of the quantity of dietary creatine available to infants suffering from creatine-deficiency

syndromes as well as the burden that creatine synthesis places on an infant's amino acid metabolism are discussed.

3.3. EXPERIMENTAL METHODS

Twenty healthy lactating women (31 ± 5 years of age) donated milk at either 1-2 weeks or 5-6 weeks post-partum (10 subjects at each time-period). The milk was collected at the Janeway Children's Health and Rehabilitation Centre, St. John's, using electronic breast pumps. Blood was taken from these subjects by nurses at the Janeway Blood Collection Services. None of the subjects were vegetarian. All protocols were approved by both the Memorial University Human Investigation Committee and the Research Approval Committee of the Health Care Corporation of St. John's. Informed written consent was obtained before the collection of any human samples.

Eight different types of infant formula were purchased from various department stores in St. John's. Formulas from six different lots of each type were purchased, except for Isomil, for which samples from only five different lots were available. Each formula was reconstituted according to the manufacturers' instructions and an aliquot of each infant formula, or human milk sample, was centrifuged at 4ºC at 150,000 g for 60 minutes so as to separate the aqueous phase. Blood samples were collected with lithium-heparin and centrifuged at 4ºC at 3,000 g for 10 minutes to separate the plasma. Samples of the aqueous phase of the milk and formula samples, and of the plasma samples, were deproteinized by the addition of 50% vol/vol of 0.6 M perchloric acid, kept on ice for 20 minutes and centrifuged at 20,000 g for 5 minutes to sediment the proteins. To 1.5 ml

aliquots of the resulting supernatants were added 25 µl of 50% potassium carbonate and 20 µl of universal indicator before neutralization with 20% potassium hydroxide. The deproteinized, neutralized samples were used for the measurement of creatine by the enzymatic method of Lowry and Passonneau[10] and guanidinoacetate by the HPLC method of Carducci *et al.*[11].

Data are represented as means ± standard deviations. Statistical significance was determined by means of a one-way ANOVA followed by Newman-Keuls post-hoc test. A p value of <0.05 was taken to indicate statistical significance.

3.4. RESULTS AND DISCUSSION

Figure 3.1 shows the concentrations of creatine and guanidinoacetate in human milk and plasma. Since there was no difference between the concentrations measured at 1-2 weeks or at 5-6 weeks post-partum, these data were combined. Both plasma and milk contained appreciable concentrations (60-70 µM) of creatine but creatine was not more concentrated in milk than in plasma. The circulating concentration of guanidinoacetate was much lower than that of creatine and was even lower still in milk. It is clear, therefore, that milk is a source of creatine for the nursing infant but that guanidinoacetate is not supplied in significant quantities by this route.

Table 3.1 shows the creatine and guanidinoacetate content of the infant formulas. There were appreciable concentrations of creatine in all of the cows' milk-based formulas; indeed, these were substantially higher than the concentrations found in human milk,

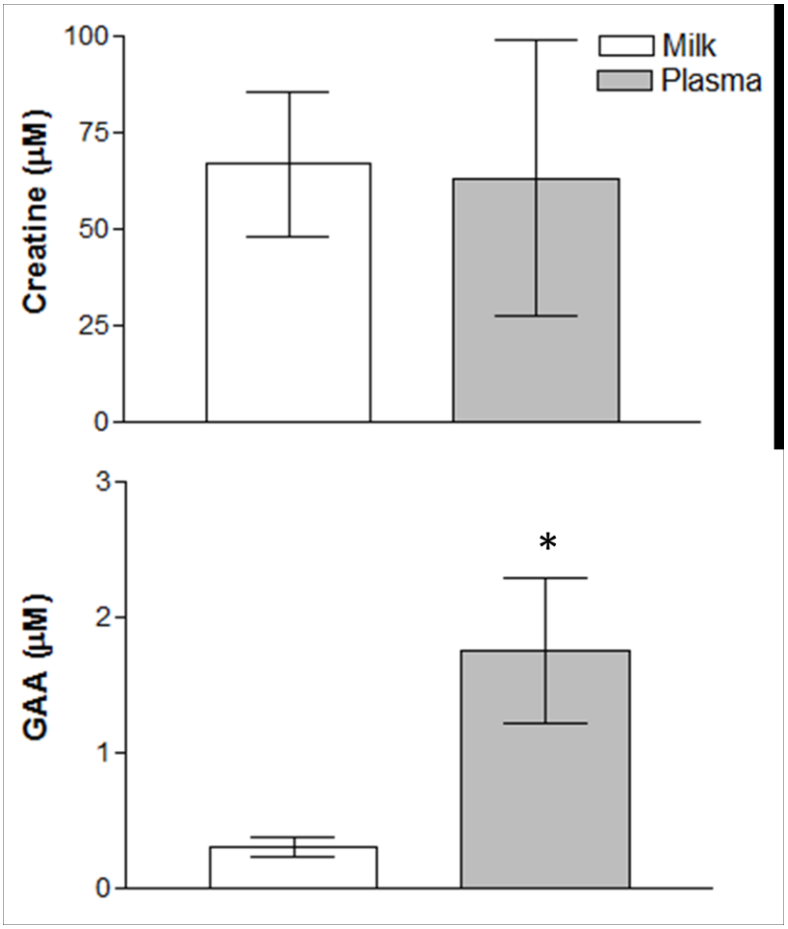

Figure 3.1: Concentrations of Creatine and Guanidinoacetate (GAA) in Human Milk and Plasma. Bars represent means and vertical lines standard deviations of 5-20 observations. Asterisk indicates statistical significance (P=0.004).

Table 3.1: Creatine and guanidinoacetate concentrations in various infant formulas.

N=6 (except N=5 for Nestle Alsoy 1).

	Creatine (uM)		Guanidinoacetate (uM)	
	Mean	SD	Mean	SD
Similac (ready-to-use)	114	3	1.1	1.9
Similac (concentrated)	119	7	3.0	4.3
Isomil	13	2	1.9	1.2
Enfalac	334	10	3.7	1.1
Enfamil A+	311	18	3.5	0.4
Lactofree	10	2	ND	
Nestle Goodstart	159	5	ND	
Nestle Alsoy 1	11	6	0.3	0.4

SD, standard deviation. ND, values which could not be determined.

which is consistent with the very high creatine concentration (550 μM) that we measured in cow's milk. On the other hand, the creatine concentration of Isomil, Lactofree and Nestle Alsoy 1 were very low (10-13 μM). Isomil and Alsoy 1 are soy-based. Lactofree is made with modified cow's milk ingredients and is intended for infants that suffer from lactose intolerance. The concentration of guanidinoacetate in the infant formulas was very low.

It is of interest to estimate the extent to which mother's milk provides the nursing infant with the necessary creatine. So as to do this we need to calculate creatine intake in mother's milk as well as the accretion of creatine by the infant. We have selected as our reference, breast-fed infants between 6 and 12 weeks of age. These infants weigh an average of 5.7 kg, ingest an average of 143 ml of milk·kg^{-1}·day^{-1} and gain an average of 0.2 kg body weight per week[12]. Since the muscle mass of infants of this age is about 25% of body mass[13], this amounts to a gain of 50 g of muscle per week. These infants excrete an average of 62 mg of creatinine per day, or 3.8 mmol per week[14]. From the data in **Figure 3.1** we can calculate that these breast-fed infants ingest about 0.4 mmol of creatine per week. There is no data on the bioavailability of creatine in infants. However, creatine bioavailability has been determined to be 79% in adult humans[15]. In addition, it is known that the transporter responsible for creatine absorption is elevated in suckling rat pups[16]. It is likely, therefore, that the bioavailability of milk creatine is very high in suckling infants.

The accretion of creatine by these infants can also be estimated. First of all, it is necessary to replace the creatine lost to creatinine formation and excretion, 3.8 mmol per week. In addition, it is necessary to provide creatine to the expanding muscle mass. According to Eriksson[17], the skeletal muscle content of creatine phosphate in infants of this age is between 12 and 22 mmol·kg wet weight^{-1}. This gives a mean value of 17 mmol·kg wet weight^{-1}. We were unable to find any recently published data on the creatine concentration or total creatine content of infant muscle. However, applying a ratio of 3 for [creatine phosphate]/[creatine], which is common in adult human muscle[18], we estimate [creatine] in infant skeletal muscle to be approximately 6 mmol·kg wet weight^{-1}, for a total muscle creatine concentration of 23 mmol·kg wet weight^{-1}. This is somewhat lower than concentrations found in adult muscle, which is consistent with measurements of creatine in adult and infant muscle reported in the early 20th Century[19]. However, since the methodology used in these early studies is not nowadays considered to be very reliable we have not employed them in our calculations. Applying our new estimates of infant muscle creatine content with an increase in muscle mass of 50 g per week we calculate that 0.46 mmol of creatine per week is required for the expanded muscle mass. Together with the 3.8 mmol of creatine lost as creatinine, we estimate that infants of this age require 4.26 mmol of creatine per week. Since they only receive about 0.4 mmol per week via milk, almost 90% of their creatine must be provided by *de novo* synthesis by the infant. These findings are in accord with our study of creatine accretion in rat pups[20] and piglets[21] where we showed that milk provided, respectively, only 12% and 25% of total creatine accretion, the balance being provided by endogenous synthesis by the neonatal animal. The high rate of endogenous creatine synthesis has important

implications both for infants suffering from creatine deficiency syndromes and for the infant's amino acid metabolism.

The fact that infants receive almost all of their creatine by endogenous synthesis is entirely consistent with the speed with which symptoms become apparent in infants suffering from genetic defects in creatine synthesis. Symptoms become apparent at an early age, regardless of whether the defect is due to an enzyme or a transporter deficiency. These symptoms include highly abnormal developmental scores, mental retardation, speech impairment and epileptic seizures[1,2,3].

It is important to analyze the metabolic burden that the synthesis of approximately 3.9 mmol of creatine per week places on these infants. Creatine synthesis takes place by means of a very simple pathway, employing only two enzymes, L-arginine:glycine amidinotransferase (AGAT), and guanidinoacetate methyltransferase (GAMT) [see **Figure 3.2**]. AGAT catalyzes a reaction between arginine and glycine, to produce guanidinoacetate (GAA) and ornithine:

$$\text{arginine} + \text{glycine} \rightarrow \text{ornithine} + \text{GAA}.$$

GAMT uses S-adenosylmethionine (SAM) to methylate GAA to creatine and S-adenosylhomocysteine (SAH):

$$\text{GAA} + \text{SAM} \rightarrow \text{creatine} + \text{SAH}.$$

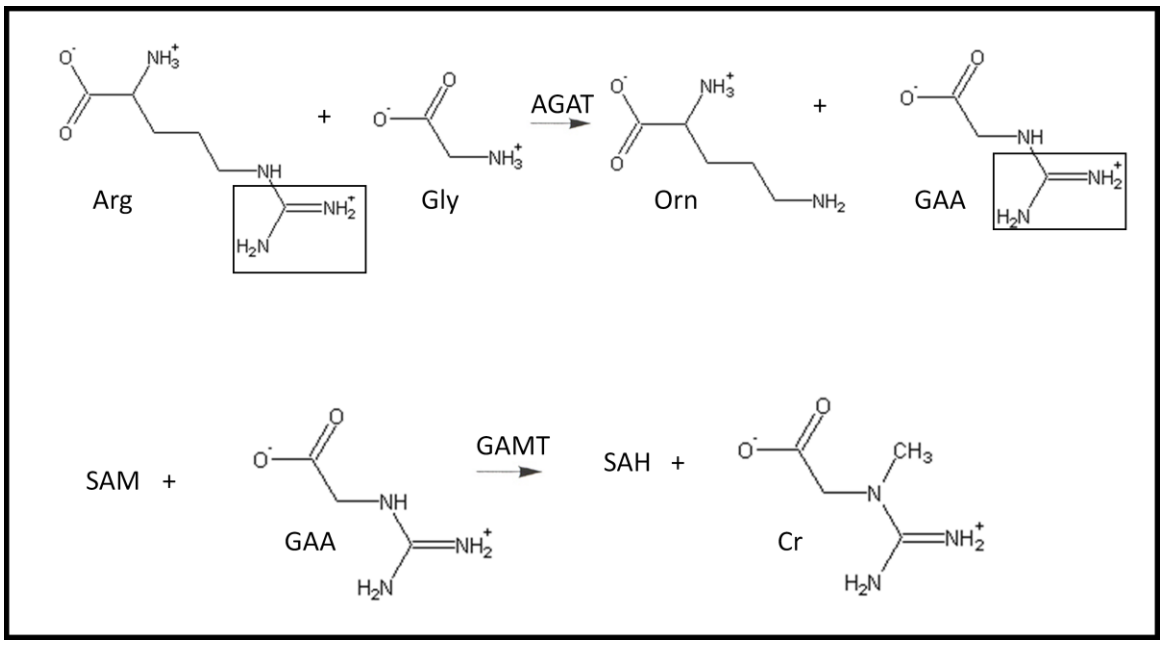

Figure 3.2: Creatine (Cr) Synthesis. L-Arginine:glycine amidinotransferase (AGAT) first catalyzes the transfer of an amidino group from arginine (Arg) to glycine (Gly) to produce ornithine (Orn) and guanidinoacetate (GAA). Guanidinoacetate methyltransferase (GAMT) then catalyzes the methylation of GAA from S-adenosylmethionine (SAM) to produce S-adenosylhomocysteine (SAH) and Cr.

In this pathway, the entire glycine molecule is incorporated into creatine as well as a guanidino group from arginine and a methyl group from SAM.

Using the data provided by Davis *et al.*[22] on the amino acid composition of human milk, we calculate that 6-12 week old infants ingest an average of 14.3, 10, and 5.2 mmol per week respectively, of glycine, arginine, and methionine. Therefore, creatine synthesis consumes 27% of dietary glycine. However, glycine is a nonessential amino acid and may be produced endogenously by the infant. Creatine synthesis also consumes guanidino groups corresponding to 39% of those in dietary arginine and methyl groups corresponding to 75% of those in dietary methionine. AGAT converts arginine to ornithine and this ornithine may be reconverted to arginine by the combined actions of carbamoyl phosphate synthetase I (CPS-I), argininosuccinate synthetase and argininosuccinate lyase. We are aware of no data on the rate of this reconversion in infants. SAH, produced by GAMT, is hydrolyzed by SAH hydrolase to adenosine and homocysteine; the homocysteine may be remethylated to methionine either by methionine synthase or betaine:homocysteine methyltransferase. We are aware of no data on the rate of remethylation in 6-12 week old infants. However, Thomas *et al.*[23] have shown that remethylation is quite active in newborn infants; therefore, it is likely that much of the SAM required for creatine synthesis is regenerated via the remethylation pathway.

In sum, these calculations reveal that the great bulk of the nursing infant's need for creatine must be met by endogenous synthesis and that this imposes a considerable metabolic burden on the growing infant, particularly with regard to amino acid

metabolism. Formula-fed infants receiving cow's milk-based formula receive 2-4 times as much creatine as nursing infants depending upon the formula of choice. Therefore, the metabolic burden of endogenous creatine synthesis may be reduced yet remains an appreciable burden on amino acid requirements even in these infants.

3.5. ACKNOWLEDGMENTS

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CHAPTER 4

SUCKLING RAT PUPS ACCUMULATE CREATINE PRIMARILY VIA *DE NOVO* SYNTHESIS RATHER THAN FROM DAM MILK

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Suckling Rat Pups Accumulate Creatine Primarily via de Novo Synthesis Rather Than from Dam Milk[1],[2]

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Running title: Provision of creatine to lactating rat pups

4.1. ABSTRACT

During lactation, there may be a higher need for creatine replacement due to the provision of creatine to the milk. Our objectives were to: *1*) quantify the creatine concentration in rat milk; *2*) determine the origin of milk creatine; *3*) determine the activities of the enzymes of creatine synthesis in lactating rats and pups; and *4*) quantify the origin of the creatine that accumulates in rat pups. The origin of milk creatine was determined in 4 dams following the administration of ^{14}C-creatine by measuring the isotopic enrichment of creatine in the milk and plasma. The activities of the 2 enzymes involved in creatine synthesis were compared in lactating and virgin females (n = 7). For all experiments, the litter size was standardized to 8 pups. The data indicated that the mammary gland extracts creatine from the circulation rather than synthesizing it. This was confirmed by our failure to find substantial activities of the enzymes of creatine synthesis in mammary glands. The provision of milk creatine requires an additional 35–55% of creatine above the daily requirement by lactating rat dams. However, there was no increased creatine synthesis by these dams; the additional creatine was largely provided by hyperphagia, because creatine is present in commercial rat diet. There was a substantial accumulation of creatine in the growing pups, but only ~12% was obtained from milk. The great bulk of creatine accretion was via de novo synthesis by the pups, which imposed a substantial metabolic burden on them.

4.2. INTRODUCTION

The creatine and creatine phosphate system serves as an important energy buffer and shuttle of high-energy phosphates from mitochondria to the cytoplasmic sites of ATP utilization. This system is confined to tissues that have a high and variable energy demand such as muscles and brain. The functions of creatine have recently been reviewed (1, 2). The synthesis of creatine is a very simple process, involving only 2 reactions and requiring 3 amino acids: arginine, glycine, and methionine (3). In the first reaction, arginine:glycine amidinotransferase (AGAT;[4] EC 2.1.4.1) catalyses the transfer of the amidino group from arginine to the amino group of glycine to form guanidinoacetic acid (GAA) and ornithine. In the second reaction, GAA methyltransferase (GAMT; EC 2.1.1.2) catalyses the transfer of a methyl group from *S*-adenosylmethionine to GAA, producing creatine and *S*-adenosylhomocysteine. The highest activities of AGAT are found in the kidney and pancreas, although lesser activities are found in the brain, testes, lung and spleen. The highest activities of GAMT are found in the liver, with lesser activities in pancreas and testes. We have shown that, in rats, the bulk of creatine is synthesized via an inter-organ process where GAA produced by the kidney is methylated in the liver (1) to form creatine that is released to the circulation, where it can be taken up by tissues via a specific transporter. Methylation of GAA by GAMT is a major consumer of labile methyl groups. As revealed by analyses of transmethylation fluxes, creatine synthesis is a major consumer of labile methyl groups and is thought to account for up to 45% of the methyl flux in humans (4, 5). Creatine synthesis is regulated by pretranslational control of AGAT expression in kidney. Rats fed a creatine-supplemented

diet have a marked decrease in AGAT activity, AGAT protein amount, and mRNA expression in their kidneys (6–9).

Under physiological conditions, creatine and creatine phosphate spontaneously and irreversibly break down to creatinine, which is subsequently lost in the urine (10). The daily loss of creatine to creatinine is estimated to be 1.7% of the total creatine pool (2). This creatine loss is replaced both by dietary creatine and de novo synthesis. Humans consuming a typical Western diet obtain ~50% of their daily creatine provision from their diet and synthesize the other one-half (5). During lactation, there is an additional requirement for creatine due to the provision of creatine in the milk. Accordingly, the objectives of this study were to: *1*) quantify the creatine content in rat milk and the additional burden it imposes on creatine synthesis; *2*) determine the origin of creatine in milk; *3*) determine the impact of lactation on the activities of the enzymes of creatine synthesis in rats; and *4*) quantify the sources of creatine in suckling rat pups (milk or de novo synthesis).

4.3. MATERIALS AND METHODS

Chemicals and other supplies. HPLC-grade methanol, Hypercarb HPLC columns, and Scintiverse E were purchased from Fisher Scientific. ^{14}C-creatine (creatine [4-^{14}C], with a specific radioactivity of 1.96 GBq·mmol^{-1}) was obtained from American Radiolabeled Chemicals. Oxytocin was obtained from Hospira Healthcare. Ion exchange resin (AG 50W-X8 200–400 mesh) was obtained from Bio-Rad. Unless otherwise stated, all other chemicals were purchased from Sigma.

Rats. All animal experimentation was approved by Memorial University's Institutional Animal Care Committee in accordance with the Guidelines of the Canadian Council on Animal Care. Lactating Sprague-Dawley rats (337.4 ± 16.5 g) with their pups were provided by Memorial University's breeding colony. At d 10 postpartum, each litter was standardized to 8 pups; extra pups were randomly selected and killed by inhalation of halothane (Isoflo, Abbott Laboratories). Each litter was individually housed on a 12:12-h light-dark cycle and consumed Purina Rat Diet 5012[5] ad libitum. Milk, blood, and various tissue samplings were conducted 14 d after parturition, which coincides with the peak of lactation (11). When required, size-matched virgin females were used as control. Rats were anesthetized with an intraperitoneal injection of 6 mg of sodium pentobarbital·100 g body weight^{-1}.

In vivo GAA production, creatine concentration, AGAT, and GAMT assay. The renal GAA output was measured in lactating and virgin rats (n = 7) as described previously (7). Briefly, the renal vein was cleaned of connective tissue and blood flow was measured using a T206 small animal blood flowmeter (Transonic Systems). Flow rate was measured for 10 min, then arterial and venous blood samples were taken across the kidney. Kidney samples were immediately freeze-clamped and stored at −80°C. AGAT was assayed in kidney samples that were frozen for <1 wk, as previously described (12). GAMT was measured on fresh liver samples using the method of Ogawa et al. (13), except that creatine production was quantified by HPLC (14). Enzyme activities were expressed in nmol·min^{-1}·g tissue^{-1}.

We examined the origin of milk creatine (whether it is synthesized within the mammary gland or extracted by it from circulating plasma) by comparing the specific radioactivity of creatine in arterial blood and milk. This required the injection of lactating rats with ^{14}C-creatine and sampling blood and milk after an equilibration period. The equilibration period was determined by pilot experiments (n = 3) in which the excretion of ^{14}C in urine was monitored. Urinary ^{14}C reached a plateau after 3 d; this plateau extended for at least 6 d (not shown). The dams (n = 4) were injected i.v. in the caudal vein with 740.9 kBq ^{14}C-creatine prepared in 0.5 mL saline at d 11 and immediately returned to their pups. At d 14, milk and blood samples were taken. One hour prior to milk collection, the pups were removed from the dam who was anesthetized and injected intraperitoneally with oxytocin (4 iu·kg body weight^{-1}) to stimulate milk release. The nipples and surrounding fur were then thoroughly cleaned by rinsing with distilled water. Milk was collected using a vacuum milking system adapted from Rodgers (11). Typically, 3–4 mL of milk was collected from each dam in <10 min. The collected milk was immediately frozen in liquid nitrogen and kept at −80°C until further analysis. Immediately following milk collection, arterial blood was drawn from the dam's abdominal aorta into a heparinized syringe and centrifuged at 6000 × g at 4°C for 10 min. Plasma was removed and stored at −80°C until analysis.

Determination of creatine specific radioactivity. We used a modification of the HPLC method of Gu and Lim (15) to measure the creatine concentration; the eluate from the HPLC column was collected by fraction collection and the creatine peak counted in a liquid scintillation spectrometer. Milk and plasma samples (1 mL) were deproteinized by

the addition of 100 μL ice-cold trifluoroacetic acid, placed on ice for 10 min, and the precipitated protein pelleted at 10,000 × *g* for 10 min at 4°C. The supernatant was immediately applied to a Bond-Elut C18 solid-phase extraction cartridge (100 mg, 1 mL; Varian) and the eluate collected. The cartridge was washed with 1 mL dH_2O and eluate was again collected. The pooled eluate was applied to a disposable chromatography column containing 2 mL of cation-exchange resin using sodium as a counterion. It was followed by two 3-mL water washes and this eluate was discarded. Creatine bound to the resin was eluted with 1 mL of 2 $mol \cdot L^{-1}$ ammonium hydroxide. This eluate was freeze-dried and dissolved in 250 μL of the mobile phase immediately prior to HPLC analysis. A 100-μL volume of the dissolved eluate was injected onto a porous graphitized carbon column (Hypercarb, 100.0 × 4.6 mm). The column was eluted isocratically with 1% trifluoroacetic acid and 2% methanol; the absorbance was monitored at 210 nm. Fractions were collected every 0.5 min for 15 min. All fractions were mixed with 10 mL of Scintiverse E and counted for 10 min on a LKB 1214 Rackbeta liquid scintillation counter (LKB). Creatine eluted at ~4 min and the corresponding fractions were the only ones containing substantial quantities of radioactivity. The creatine concentration was referenced to a set of creatine standards (0–1 $mmol \cdot L^{-1}$) that was processed in parallel to the samples (including solid phase extraction and ion exchange chromatography). The creatine specific radioactivity was expressed as $mBq \cdot nmol^{-1}$.

Creatine content of adult tissues and pups. To determine whether rats' total creatine pool becomes depleted during lactation, we measured the creatine concentration of muscle and brain (2 major pools of creatine) in lactating and virgin females (n = 4). Tissues were

homogenized in 150 mmol·L^{-1} sodium phosphate (pH 7.4). Rat pups were killed at d 10, 15, and 20 (n = 6) by halothane inhalation and each pup was homogenized. The homogenates were allowed to stand for 30 min at room temperature to allow for the conversion of creatine phosphate to creatine. The total creatine content was measured by HPLC (15) and was reported as nmol·g^{-1} for adult tissues and μmol·$animal^{-1}$ for the pups.

Statistical analysis. All data are presented as means ± SD. Lactating and virgin female rats were compared using Student's t test, or, when variances were heterogeneous, the Mann-Whitney U test. Plasma and milk samples of lactating rats were compared using paired t tests, and 1-way ANOVA and Tukey's post hoc test were used to compare enzyme activities among lactating rats, virgin rats, and pups. Differences were considered significant at $P < 0.05$. GraphPad Prism 3.0 was used for statistical analyses.

4.4. RESULTS

The creatine concentrations in milk and plasma from lactating rats were 273.7 ± 47.7 μmol·L^{-1} and 150.0 ± 48.8 μmol·L^{-1}, respectively. The isotopic enrichments of creatine in plasma and in milk did not differ (paired t test, P = 0.37) (**Fig. 4.1**), indicating that the bulk, if not all, of milk creatine is derived from the plasma. To further explore this, we measured the activities of the enzymes of creatine synthesis in the mammary gland. The activity of AGAT was below our limit of detection (<10 nmol·min^{-1}·g^{-1}) and the activity of GAMT was very low (0.19 ± 0.08 nmol·min^{-1}·g^{-1}) compared with that in liver (13.10 ± 4.11 nmol·min^{-1}·g^{-1}).

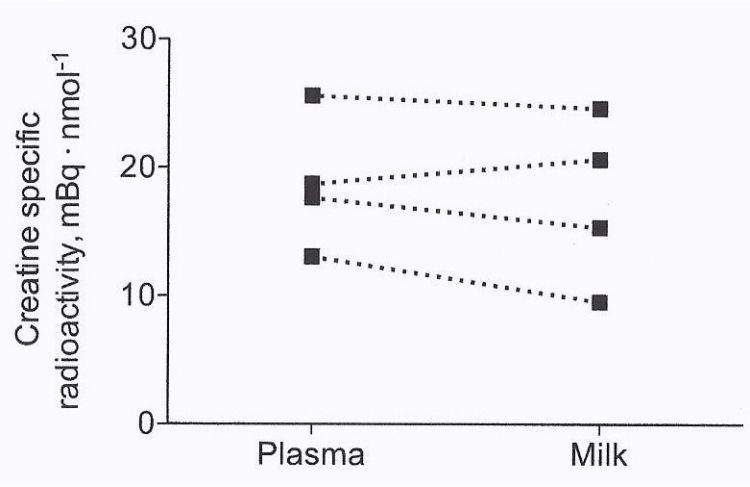

Figure 4.1: Isotopic enrichment of creatine in plasma and milk of 4 lactating rats.

Means did not differ.

The arterial plasma concentration of GAA was significantly lower in the lactating females than in the virgin controls; however, the renal arteriovenous difference did not differ between lactating and virgin females (**Fig. 4.2**). A quantitative analysis of renal GAA output (**Table 4.1**) confirmed that lactation was not accompanied by an increased renal GAA output. It is noteworthy that the plasma creatine concentration was significantly lower in the lactating females (**Table 4.1**). Renal AGAT (0.24 ± 0.04 nmol·min^{-1}·g^{-1}) and GAMT (12.47 ± 4.66 nmol·min^{-1}·g^{-1}) did not differ between lactating and virgin rats.

The limb muscle (biceps femoris) creatine concentration did not differ between the groups (19.51 ± 0.79 μmol·g^{-1}). Unexpectedly, however, the brain creatine concentration was higher in lactating females (15.24 ± 1.13 μmol·g^{-1}) than in virgin females (12.85 ± 1.12 μmol·g^{-1}) (P = 0.02). The pups accumulated an average of 20.7 μmol·d^{-1} between d 10 and 15 and 42.0 μmol·d^{-1} between d 15 and 20, resulting in total body creatine contents that increased progressively (**Fig. 4.3**). On d 14, the hepatic GAMT activity of pups (15.94 ± 3.50 nmol·min^{-1}·g^{-1}) did not differ from virgin or lactating rats, but renal AGAT activity was higher in pups (0.50 ± 0.16 nmol·min^{-1}·g^{-1}) compared with both groups of adult females (0.24 ± 0.04 nmol·min^{-1}·g^{-1}) (P = 0.03).

4.5. DISCUSSION

The importance of the provision of creatine to neonatal animals has been emphasized by the discovery of inborn errors of creatine synthesis and of the creatine transporter. These diseases result in a common spectrum of neurological disorders, including epilepsy,

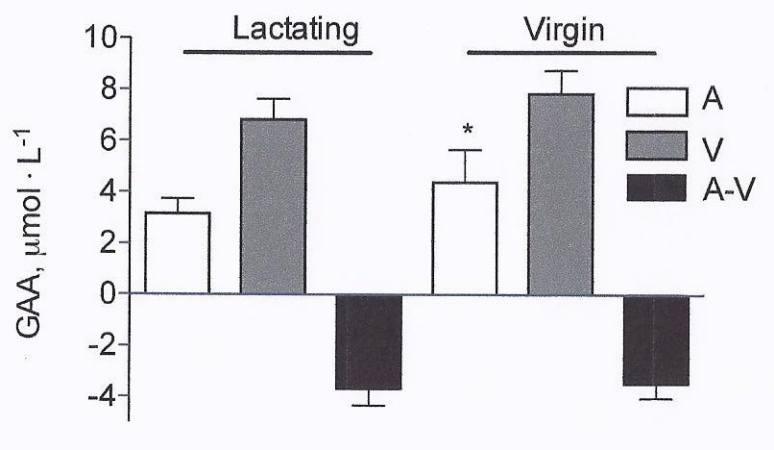

Figure 4.2: Arterial (A) and venous (V) plasma GAA concentrations and renal A-V differences in lactating and virgin females. Values are means ± SD, *n*=7. *Different from lactating, *P*<0.05.

Table 4.1: Renal GAA output in lactating and virgin rats. Values are expressed as means ± SD, n=7. *Different from virgin, P = 0.005.

	Lactating rats	**Virgin rats**
Number of animals	7	7
Total mass (g)	337.4 ± 15.2	355.3 ± 37.6
Kidney mass (g)	3.1 ± 0.2	3.0 ± 0.1
Hematocrit	0.42 ± 0.01	0.45 ± 0.02
Blood flow ($mL \cdot min^{-1}$)	7.0 ± 1.0	8.7 ± 2.9
Total renal blood flow ($mL \cdot min^{-1} \cdot 100$ g body mass)	4.2 ± 0.8	5.0 ± 1.8
Renal GAA output ($nmol \cdot min^{-1} \cdot 100$ g body mass)	9.45 ± 3.59	8.41 ± 3.40
Plasma creatine concentration ($\mu mol \cdot L^{-1}$)	160.0 ± 15.6*	231.3 ± 62.2

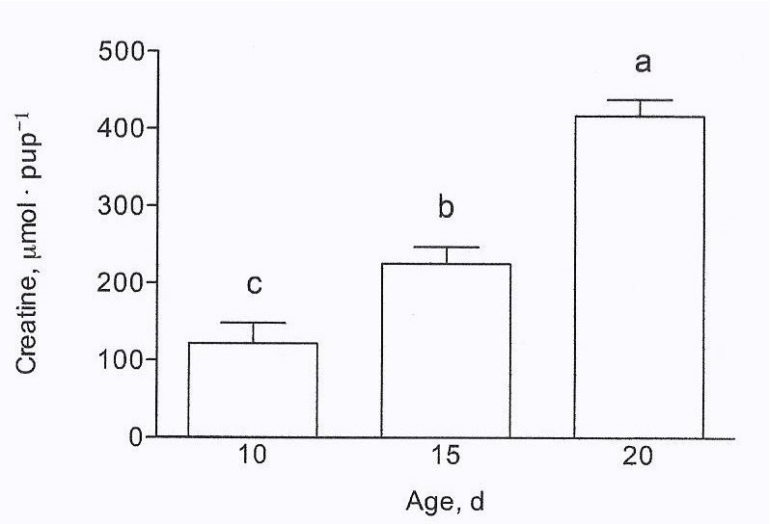

Figure 4.3: Total body Creatine content in rat pups at d 10, 15 and 20 of age. Bars are means ± SD, n=6. Means with different letters differ, P<0.05.

speech delay, and mental retardation (1). Neonatal animals can obtain creatine by a combination of diet (milk) and de novo synthesis. There is little information on the origin of milk creatine. To address this question, we compared the simultaneous isotopic enrichment of ^{14}C-creatine in the plasma and milk of lactating rats. We also assayed the activities of the enzymes of creatine synthesis in mammary gland. The data clearly demonstrate that mammary glands extract creatine from the circulating plasma and have little or no capacity for creatine synthesis. Furthermore, the lower plasma concentrations of creatine and GAA in the lactating mother compared with virgin females are consistent with a larger extraction of creatine from plasma. However, a portion of this decrease may be attributable to the increased plasma volume, from 8.1 to 9.6 mL·100 g^{-1} (16), in lactating compared with nonlactating rats. The creatine concentration in milk was almost twice that of the plasma, indicating that the mammary gland is capable of concentrating creatine.

The creatine concentration of rat milk averaged 274 μmol·L^{-1} and the milk production of a female during the peak production period (13–16 d postpartum) is estimated to be ~40 mL·d^{-1} (17). Therefore, the daily output of creatine at peak lactation is ~11 μmol. We can calculate the total creatine pool (creatine + creatine phosphate) of a 335 g female rat to be ~1.67 mmol, given a total muscle creatine concentration of 20 mmol·kg^{-1} and a relative muscle mass of 25%. The daily creatine loss to creatinine (1.7%·d^{-1}) can be calculated to be ~28 μmol·d^{-1}. Therefore, during lactation, the need for creatine replacement would be ~40% higher than in the nonlactating rat. However, we found no evidence for increased endogenous creatine synthesis in lactation; the renal activity of AGAT, the renal

production of GAA, and the hepatic activity of GAMT were unchanged during lactation. In lactating rats, food consumption is 2–3 times that of nonlactating, nonpregnant females (18). We fed the rats a commercial diet that by our measurements contained 0.9 μmol creatine·g^{-1} . We suggest that hyperphagia alone is adequate to provide the creatine required to cover the additional needs of lactation.

Our data show that the pup accumulates an average of 20.7 μmol creatine·d^{-1} between d 10 and 15. Between these ages, milk intake increases from 7.46 to 10.31 g·pup^{-1}·d^{-1} (19). We have employed an average milk intake of 8.9 g·pup^{-1}·d^{-1}. We therefore calculate a creatine intake of ~2.4 μmol·d^{-1} of creatine between d 10 and 15 (this calculation is based on the assumptions that 1 g of milk = 1 mL, that the milk creatine concentration is 274 μmol·L^{-1}, and that milk creatine is 100% bioavailable). Accordingly, milk provides only a small fraction (~12%) of the creatine needs of rat pups between d 10 and 15. This figure is even lower when creatine accretion is measured between d 15 and 20. Therefore, the bulk of creatine accretion must come from de novo synthesis. To determine whether this is feasible, we measured the activities of the enzymes of creatine synthesis in 15-d-old pups. Renal AGAT activity was higher in pups compared with the activity in adult animals. This increase in renal AGAT activity may be brought about by growth hormone, which induces the synthesis of AGAT by a pretranslational mechanism (20). The lack of increase of GAMT activity is consistent with previous findings that this enzyme is not limiting for the synthesis of creatine (1).

It is also instructive to compare the rates of creatine synthesis in the pups with their intake of amino acids, using the data of Davis et al. (19) on the amino acid composition of rat milk protein. Between d 10 and 15, the mean intakes of methionine, glycine, and arginine are calculated to be 87, 146, and 153 μmol·pup^{-1}·d^{-1}, respectively. In the same time period, creatine synthesis, calculated as the difference between creatine accretion and creatine intake, was 19.2 (20.7–1.5) μmol·d^{-1}. Creatine synthesis requires a full molecule of glycine, the amidino group of arginine, and the methyl group of methionine. Accordingly, creatine synthesis consumes 13, 13, and 22% of dietary glycine, arginine, and methionine, respectively. We obtained very similar numbers in a previous study on piglets (1).

In summary, our study showed that milk's creatine is extracted from circulating plasma by the mammary gland. We also showed that there is no apparent capacity for creatine synthesis in the rat mammary gland and that during lactation, the increased demand for creatine is primarily supported by hyperphagia when creatine is present in the food. In neonatal rats, the provision of creatine in the milk is not the primary source of creatine and pups must rely mostly on de novo synthesis. This finding highlights the burden that creatine synthesis imposes on methyl group metabolism and arginine metabolism in neonatal rats.

4.6. ACKNOWLEDGMENTS

J.T.B., E.E.E., M.E.B., and S.G.L. designed research; S.G.L., E.E.E., and E.P.W. conducted research; S.G.L. and E.E.E. analyzed data; and S.G.L. and J.T.B. wrote the

paper and have primary responsibility for the final content. All authors read and approved the final manuscript.

4.7. FOOTNOTES

1 Supported by the Canadian Institute of Health Research (grant no. 97851 to J.T. Brosnan and postdoctoral fellowship to S.G. Lamarre) and by the Industrial Research and Innovation Fund of Newfoundland (postdoctoral fellowship to S.G. Lamarre).

2 Author disclosures: S. G. Lamarre, E. E. Edison, E. P. Wijekoon, M. E. Brosnan, and J. T. Brosnan, no conflicts of interest.

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4 Abbreviations used: AGAT, arginine:glycine amidinotransferase; GAA, guanidinoacetic acid; GAMT, guanidinoacetic acid methyltransferase.

5 Composition: ground corn, dehulled soybean meal, fish meal, wheat middlings, cane molasses, dehydrated alfalfa meal, soybean oil, brewers dried yeast, wheat germ, dried beet pulp, ground oats, dicalcium phosphate, monocalcium phosphate, salt, dl-methionine, choline chloride, vitamin A acetate, cholecalciferol, menadione dimethylpyrimidinol bisulfite, pyroxine hydrochloride, biotin, thiamine mononitrate, vitamin B-12, dl- alpha -tocopheryl acetate, nicotinic acid, calcium pantothenate, riboflavin, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, and sodium selenite.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1. RENAL GUANIDINOACETATE PRODUCTION *IN VIVO*

A number of novel contributions to our knowledge of creatine synthesis and its regulation have been made through the course of the present research. This is the first time that renal production of guanidinoacetate (GAA) has been shown *in vivo* in rodents. We have also shown that in rats fed a creatine-free diet, renal GAA production *in vivo* equals creatinine loss in the urine. We have shown that human kidneys also produce GAA but, unlike the rat, this is not the major source of GAA for creatine synthesis.

Creatinine excretion is a measure of creatine and creatine-phosphate breakdown, and therefore, represents the need for creatine replacement in the adult animal. Since it has been shown that renal GAA production was equivalent to renal creatinine excretion in the rat, we can conclude that the kidney is the major organ involved in the production of GAA in the rat. In 1940, Borsook and Dubnoff pointed out that the activity of renal AGAT was more than sufficient to supply the body's creatine need. However, measurements of enzyme activity under optimal conditions cannot be extrapolated to the *in vivo* situation; the present data provide definitive information on the *in vivo* situation. It cannot, however, be concluded that the kidney is the sole site of GAA production in the rat. AGAT activity has been reported in rat kidney, pancreas, brain, spleen, and testes [Van Pilsum *et al.*, 1972]. In 1959, Horner studied GAA synthesis in nephrectomized rats. He found that synthesis was not entirely ablated; and indeed extrarenal transamidination, in that situation, may be considerable. Although we have shown that the rat kidney is the major organ involved in GAA production *in vivo*, extrarenal

transamidination may be important in terms of local roles within tissues or in times of renal dysfunction.

Renal GAA production in humans was quite different from that found in rats. In humans, the kidney's production of GAA could only account for about 11-12% of daily creatine loss as measured by urinary creatinine excretion. Thus, almost 90% of daily creatine replenishment occurs without renal involvement. The human subjects used in the current research were not on a creatine-free diet and we know that creatine replenishment occurs via a combination of diet and *de novo* synthesis. Stead *et al.* [2006] have estimated that in 20-59 year-olds on a typical western diet, about 50% of the creatine need can be provided by the diet. If this is taken into consideration, renal GAA production in humans may account for approximately 20% of total GAA synthesis. The kidney, therefore, does not appear to be the major organ involved in GAA production in humans although it clearly plays a role. This is supported by reports of AGAT immunofluorescence in liver and pancreas [McGuire *et al*., 1986].

In 1953, Sandberg *et al*. reported *in vivo* renal GAA production in humans. This GAA production amounted to 25% of creatinine loss through renal excretion. These data are in agreement with results obtained during the present studies. However, it should be noted that concentrations of GAA reported in this 1953 study are 5-fold those found by modern analytical techniques and may not be reliable [Derave *et al*., 2004].

5.2. Effect of Creatine Feeding on GAA Production

This is the first time that renal GAA production has been shown to occur in the rat, *in vivo*. Ingestion of a 0.4% creatine diet decreased renal GAA production by 81% and lowered plasma GAA concentrations by 72%. Thus the present study also shows, for the first time, that creatine ingestion markedly decreases renal GAA production *in vivo* in the rat.

A 0.4% creatine diet was fed to Sprague-Dawley rats for a period of two weeks. After this two week period, there was a marked down-regulation of renal AGAT activity and mRNA expression as well as a decrease in renal GAA production *in vivo*, as compared to controls. The down-regulation of AGAT activity by creatine and GAA feeding has been shown previously [Stead *et al*., 2001; Van Pilsum, 1971]. It is believed that the conversion of GAA to creatine, and therefore, an increase in creatine concentration, is what actually causes the down-regulation of AGAT during GAA feeding. The present study has confirmed findings by McGuire *et al*. [1984], that creatine feeding can decrease levels of renal AGAT mRNA. These data suggest that AGAT activity may be regulated at the pretranslational level. However, the decrease in renal AGAT mRNA expression is not as large as the decrease in renal AGAT activity (41% vs. 86%, respectively) suggesting that there may be an additional postranslational mechanism(s) involved (e.g. feedback inhibition through allosteric regulation, etc.).

Derave *et al.* [2004] showed that feeding creatine to humans for a period of 20 weeks lowered plasma GAA levels in a dose-dependent manner: by 50% during the loading phase (20 g creatine per day) and by 30% during the maintenance phase (5 g creatine per day). They did not determine the source of the GAA or the reason for the decrease in its concentration. However, these data suggest that creatine ingestion may also down-regulate creatine synthesis in humans.

5.3. Role of Substrate Supply in GAA Synthesis

Our experiments also addressed the acute regulation of renal GAA synthesis by increased provision of the amino acid substrates, glycine and arginine. Increased provision of glycine had no effect on GAA production whereas provision of arginine did. This is best interpreted in terms of the kinetic properties of AGAT and the renal concentrations of the substrates. The K_m of rat kidney AGAT for glycine is reported to be 2.4-2.8 mM; that for arginine is 3.0-3.1 mM [McGuire *et al*., 1980]. Brosnan *et al*. [1983] have reported the rat renal contents of glycine and arginine to be 1320 and 170 nmol/g, respectively. Assuming that these amino acids are evenly distributed in the kidney, we can estimate intracellular concentrations of about 2.5 mM and 0.32 mM, respectively for glycine and arginine. Renal cellular glycine concentration would be close to the Km of that for AGAT. The infusion of glycine, in the present study, only raised the circulating glycine concentration by about 50%. Keeping both of these facts in mind, an increased glycine concentration of 50% under these conditions would, at most, increase renal GAA

production by 25%. An increase in the mean GAA production of this magnitude was observed but was not statistically significant.

Our estimated renal cellular arginine concentration is well below the K_m value of AGAT for this amino acid. In the present study, the circulating arginine concentration increased four-fold during infusion so that, assuming that changes in cellular arginine concentration parallel the plasma levels and applying the Michaelis-Menten equation, we may anticipate an increased GAA production of about 3-fold. This is remarkably close to the 2.7-fold increase that was observed. Given the nature of the assumptions made, the close agreement between the observed and calculated rates may be somewhat fortuitous. Regardless, the current experiments have highlighted the sensitivity of renal GAA synthesis to the ambient arginine concentration. GAA synthesis in humans may also be markedly sensitive to arginine concentration, since Arias *et al*. [2004] have reported that provision of arginine to children with urea cycle defects normalized low plasma GAA concentrations.

Renal GAA production was doubled by the infusion of citrulline, even though there was no increase in the circulating arginine concentration. This result is attributable to the conversion of citrulline to arginine by the renal tubules. Dhanakoti *et al*. [1990] have previously reported that arginine production in the rat is very sensitive to circulating citrulline concentrations. Renal arginine synthesis occurs in the cells of the proximal tubule [Dhanakoti *et al*., 1992] as does GAA synthesis [McGuire *et al*., 1986; Takeda *et al*., 1992]. Therefore, arginine synthesized in these cells becomes immediately available

as a substrate for GAA synthesis. This indicates that citrulline synthesis (which occurs in the gut) may contribute in part to creatine synthesis. To test whether gut citrulline synthesis could be regulated by ingestion of creatine, gut citrulline flux was measured in rats fed a 0.4% creatine diet and compared to that of aged-matched AIN-93G-fed controls as described in **Section B.3**. At the end of the 2 week feeding period, animals were anaesthetized, portal vein blood flow was measured and plasma was prepared from samples taken across the gut as described previously. Plasma amino acids were analyzed as described in **Section B.5.4**. and gut citrulline production was calculated. Intestinal flux of citrulline did not differ significantly between the groups (control 117 nmol/min/100 g body wt, creatine-treated 93 nmol/min/100 g body wt, n=12, P = 0.26). Therefore, the need for creatine synthesis does not drive intestinal citrulline synthesis, as this was not altered in the creatine-fed rats

.

In 2011, Brosnan *et al*. examined the metabolic burden that creatine synthesis plays on each of the amino acids involved including glycine, arginine and methionine. They concluded that creatine synthesis only imposes a minor burden on glycine provision. However, the availability of glycine for creatine synthesis in humans does need further investigation, as Melendez-Hevia *et al*. [2009] questioned the capacity for glycine synthesis in humans.

Brosnan *et al*. [2011] estimated that dietary arginine availability averaged between 20-45 mmol/day in young adults and that endogenous synthesis may represent an important supply of arginine for creatine synthesis. The most revealing aspect of their calculations

was that creatine synthesis may consume about 40% of the S-adenosylmethionine available for all bodily methyltransferase reactions. Taking this into consideration, clearly, creatine biosynthesis imposes an appreciable burden on methionine metabolism and methyl balance.

5.4. IMPORTANCE OF CREATINE INTAKE TO INFANTS

In contrast to the abundance of information on adults, we know very little about human neonatal creatine metabolism other than that it is a crucial issue. There are examples of genetic defects in each of the three proteins responsible for creatine synthesis and distribution - AGAT, GAMT and the creatine transporter [Schulze, 2003; Stromberger *et al*., 2003]. The extraordinary finding is that, in each case, the principal symptoms are neurological, even though approximately 90 - 95% of the body's creatine is found in muscles. Regardless of which of these proteins is defective, the affected children displayed mental retardation, epilepsy and speech delay [Schulze, 2003; Stromberger *et al*., 2003]. The symptoms tend to be more severe in the children with GAMT deficiency, which has been attributed to an additional, specific effect of the accumulated GAA. Early diagnosis of infants with the synthetic enzyme deficiencies is critical. Case studies of the diagnoses of AGAT and GAMT deficiencies at birth and then subsequent supplementation with creatine showed remarkable success. All developmental milestones were normal at 18 months [Battini *et al*., 2006; Schulze *et al*., 2006]. In the case of GAMT deficiency, arginine-restriction and treatment with benzoate to remove glycine as hippuric acid was employed, in addition to creatine supplementation, to prevent

accumulation of neurotoxic levels of GAA. Clearly, creatine plays an unanticipated role in brain development.

5.5. CREATINE BALANCE IN INFANTS

In addition to creatine replacement to account for urinary creatinine loss, growing infants need to deposit substantial quantities of creatine in muscle and brain during the course of normal growth and development of these tissues [Wyss & Kaddurah-Daouk, 2000]. In order to derive estimates of loss and accretion, a number of assumptions had to be made. First, an average creatine-phosphate concentration in infant skeletal muscle was used; this was obtained from Eriksson [1980]. Second, we could find no recently published data on either the creatine concentration or total creatine content of infant skeletal muscle; we were obliged to use the common ratio of creatine-phosphate:creatine concentrations for adult skeletal muscle [Kemp *et al*., 2007].

Briefly, we calculated that 3.8 mmol of creatine are lost per week to creatinine excretion and 0.46 mmol of creatine is needed per week for expanding muscle mass. Therefore, we estimated that an infant, 6-12 weeks of age, would need a total of 4.26 mmol of creatine per week (see **Chapter 3**).

5.6. How much Creatine is Provided to the Infant via the Diet?

As stated earlier, neonatal creatine accretion can occur in two ways, through dietary intake of creatine, *de novo* creatine biosynthesis, or both. This thesis has provided data on the provision of creatine in the infant diet. The provision of creatine in mother's milk or various infant formulas has been quantified. We propose that accretion of creatine in the infant through biosynthesis creates an appreciable metabolic burden on amino acid metabolism.

5.6.1. Role of the Mammary Gland in Provision of Neonatal Creatine

It is of note that renal AGAT activity, renal GAA production and hepatic GAMT activity were not increased during lactation in the rat (**Table 4.1**). However, during lactation, the need for creatine replacement would be 40% higher than in the nonlactating rat (see detailed calculations in **Chapter 4**). In lactating rats, food consumption is 2–3 times that of nonlactating, nonpregnant females [Cripps & Williams, 1975]. Hyperphagia alone was adequate to provide the creatine required to cover the additional needs of lactation, as the commercial diet used in the current study contained 0.9 μmol of creatine per g. However, it is important to understand that the creatine content of commercial rat chow may vary from batch to batch, as the amount of fish and meat meal used to prepare the Purina Rat Chow 5012 changes from one lot to another depending upon availability and cost of the ingredients.

Previous studies showed that creatine occurs in milk, including human milk [Hülsemann *et al*., 1987]. However, there is little information on the origin of milk creatine. To address this question, we compared the simultaneous isotopic enrichment of ^{14}C-creatine in the plasma and milk of lactating rats. Activities of the enzymes involved in creatine synthesis were also measured in the mammary gland. The isotopic enrichments of creatine in plasma and in milk of lactating rats did not differ. In addition, activities of the enzymes required for creatine synthesis were negligible in the rat mammary gland as compared to the activities of these enzymes in organs known for creatine synthesis (e.g. AGAT activity in the rat kidney and GAMT activity in the rat liver). Combined, these data demonstrate that the mammary gland must extract creatine from the circulating plasma and has little or no capacity for creatine synthesis.

Plasma concentrations of creatine and GAA in the lactating rat were lower than those in virgin female controls. This is consistent with the extraction of creatine from the plasma by mammary gland. The creatine concentration in rat milk was almost twice that of the plasma, indicating that the mammary gland of the rat is capable of concentrating creatine in the milk. A minor component of the decreased plasma creatine and GAA in lactating rats may be attributable to the increased plasma volume, from 8.1 to 9.6 mL per 100 g body wt, in lactating compared with nonlactating rats [Bond, 1958].

The mean creatine concentration of rat milk was 274 μM. The milk production of a female rat during the peak production period (between days 13–16 postpartum) is estimated to be 40 mL per day [Flint & Vernon, 1998]. From these data the daily output

of creatine at peak lactation can be calculated to be 11 μmol to be distributed to the entire litter. From **Figure 4.3** we can deduce that an average of 20.7 µmol of creatine is needed each day to account for the total creatine accumulation in the rat pup during peak lactation. In **Chapter 4** it was calculated that the 10-15 day old rat pup receives on average about 2.4 µmol of creatine per day from mother's milk. This is only a small fraction, about 12%, of the creatine need. Therefore, the bulk of the rat pup's creatine need must be accounted for by *de novo* synthesis.

The role of the human mammary gland in providing creatine is not as well studied. Human milk contains a lower concentration of creatine than rat milk, about 70 µM. The findings from **Chapter 3** show that there is no difference between the milk and plasma creatine concentrations in lactating humans. We assume that, like the rat, the human mammary gland does not have a significant role in the production of creatine but derives milk creatine from the plasma; however, we have no experimental data on the matter. GAA was also measured in mother's milk because its presence would spare the infants' glycine and arginine requirements for creatine synthesis. Very little GAA was found in human milk. In fact, the GAA concentration of human milk was lower than that of plasma.

As discussed earlier, between 6-12 weeks of age, a human infant requires approximately 4.26 mmol of creatine each week to account for the expanding muscle mass and creatine lost as creatinine. In **Chapter 3** it was calculated that these same infants receive only 0.4 mmol of creatine per week from mother's milk. This means that *de novo* synthesis of

creatine must account for approximately 90% of the accreted creatine in human neonates. This high rate of endogenous creatine synthesis has important implications for the neonate's amino acid metabolism, posing a substantial metabolic burden. In conclusion, the mammary gland of both the human and the rat have a very limited role with regards to supplying the neonate with the creatine required for normal development. It should be pointed out that a study of creatine metabolism in neonatal piglets came to the same conclusion (Brosnan *et al*., 2009) so it is likely to be a general feature of mammalian metabolism that neonatal animals are required to synthesize most of their creatine.

Dickinson *et al*. [2013] reported that maternal dietary creatine supplementation during pregnancy increases the total creatine content of mouse fetal tissues but does not cause downregulation of AGAT expression in the neonatal mouse kidney. However, AGAT gene expression was decreased in the brain. They concluded that maternal creatine supplementation does not impair endogenous creatine synthesis in the key creatine synthesizing organs (kidney and liver) of the mouse neonate but local sites of creatine synthesis (e.g. brain) may be affected.

5.6.2. Role of Dietary Intake in Provision of Creatine in Formula-Fed Infants

Table 3.1 lists the creatine and GAA concentrations of the various infant formulas. The creatine concentrations vary from 334 μM to 10 μM, depending upon the brand and the type of milk base. For example, cow's milk-based formulas had much higher creatine

concentrations than did soy-based formulas. Formulas that claimed to be most similar to mother's milk (Similac) had moderate levels of creatine.

Using tables of recommended formula intake [Dietitians of Canada, 2008], a formula-fed infant could potentially receive, through the diet, up to 63% of the creatine needed (based on the creatine concentration of Enfalac and the maximum recommended intake for 12 week old formula-fed infants). Infants fed a soy-based infant formula would have to rely solely upon *de novo* creatine synthesis to meet their body's need, as these formula-fed infants would be provided with less than 2% of the creatine needed for accretion. Therefore, the amount of creatine provided in the diet varies widely, depending upon the dietary source. However, regardless of the dietary source, there is a large metabolic burden placed on a neonate by *de novo* synthesis of creatine to meet accretion requirements.

GAA was measured in each of the infant formulas because its presence would spare the neonates' glycine and arginine requirements for creatine synthesis. The GAA concentration of each of the formulas tested was negligible. Creatinine concentration was also measured in the various infant formulas. It was significantly higher for both Enfalac (156 μM) and Enfamil A+ (176 μM) in comparison with the other infant formulas (from "not detectable" to 8 μM). Creatinine concentrations of Similac Ready To Use, Similac Concentrated, and Lactofree were below the limits of detection. These results are somewhat surprising because it may be expected that the creatinine content would reflect the creatine content, since creatinine is simply the non-enzymatic breakdown product of

creatine. It is possible that the creatinine content of the various formulas reflects the processing procedures. Either acidic conditions or high temperatures during the canning and sterilization process could speed up the degradation of creatine to form creatinine [Wyss & Kaddurah-Daouk, 2000]. This may explain the variations in both creatine and creatinine concentrations in milk-based formulas. Infants ingesting Enfalac or Enfamil A+ might be expected to show elevated creatinine levels in plasma and urine, relative to infants on other formulas or on breast milk due to the increased intake of creatinine. Such increased creatinine would not reflect on renal function.

5.7. METABOLIC BURDEN OF *DE NOVO* CREATINE BIOSYNTHESIS IN INFANTS

Creatine is synthesized, *de novo*, by the neonate from the amino acids glycine, arginine and methionine, as described above. The quantities that need to be synthesized could become a very large drain on these three key amino acids. In particular, it would represent an enormous drain on "labile methyl groups" and on arginine.

Perhaps the supply of methyl groups for S-adenosylmethionine is of even more importance. Creatine synthesis in adults uses about 40% of all S-adenosylmethionine-derived methyl groups [Mudd *et al*., 2007; Stead *et al*., 2006]. Does it also use such a large proportion in infants? Our study is an important step in understanding this question. We are just beginning to appreciate the importance of methyl group metabolism in developmental biology. DNA methylation plays a crucial role in the epigenetic silencing of one of the X-chromosomes in females [Bestor, 2000]. Impairment of such

methylations, whether as a result of a deficiency of specific proteins required for DNA methylation [Neul & Zoghbi, 2004] or of folate deficiency [Scott *et al*., 1994] results in developmental disorders such as Rett syndrome and neural tube defects. There has been a recent report of trials of "pro-methylation" dietary supplements in mice to ensure that the dietary supply of methyl groups is not limiting [Waterland, 2006; Waterland *et al*., 2006]. Such dietary supplements have been successful in correcting two separate developmental anomalies in mice that are known to be caused by hypomethylation of epigenetic regions of specific genes.

Infants suffering from genetic defects in creatine synthesis display symptoms at a very early age, consistent with our data that creatine accretion in neonates is almost entirely brought about by endogenous synthesis. The symptoms of creatine deficiency appear early regardless of the manifestation of the disorder, be it an enzyme or transporter defect. These symptoms include abnormal developmental scores, mental retardation, speech impairment and epileptic seizures.

In **Chapter 3** it was calculated that neonates must synthesize approximately 3.9 mmol of creatine per week. Since creatine synthesis requires the entire glycine molecule, a guanidino group from arginine and a methyl group from S-adenosylmethionine, creatine synthesis potentially consumes about 27% of dietary glycine, 39% of dietary arginine and up to 75% of dietary methionine. These percentages are based on the amino acid composition of human milk as reported by Davis *et al*. [1994]. Infants fed cow's milk-based formulas may receive 2-4 times as much creatine in the diet as does the nursing

infant, depending upon the formula of choice. In this case the metabolic burden of endogenous creatine synthesis may be reduced but yet an appreciable burden on amino acid metabolism remains even in these infants.

Creatine synthesis is not thought to place an appreciable burden on glycine metabolism as this amino acid is synthesized in significant quantities [Brosnan *et al*., 2011]. The same is not true of arginine and methionine. AGAT converts arginine to ornithine; through the combined actions of carbamoyl phosphate synthetase I, argininosuccinate synthetase and argininosuccinate lyase, this ornithine may be reconverted to arginine. We are aware of no data on the magnitude of this reconversion in neonates. However, to the extent that it occurs, it will minimize the potential net arginine loss brought about by creatine synthesis. The GAMT reaction produces S-adenosylhomocysteine as well as creatine. This S-adenosylhomocysteine is hydrolyzed by S-adenosylhomocysteine hydrolase to produce adenosine and homocysteine. Homocysteine may either be irreversibly catabolised to cysteine by the transsulphuration pathway or it may be remethylated to methionine by either methionine synthase or betaine:homocysteine methyltransferase. Using [1-^{13}C]methionine and [C^2H_3]methionine tracers, Thomas *et al*. [2008] measured remethylation in newborn infants, only days old. They reported that remethylation was even more active in infants than in adults, and they attributed this to a high rate of methyltransferase activity. We agree with this characterization and suggest that creatine synthesis represents one of the major users of S-adenosylmethionine-derived methyl groups in this situation. To our knowledge, rates of remethylation in 6-12 week old infants have not been reported in the current literature.

To add to our calculated metabolic burden of creatine synthesis, we must consider possible urinary loss of creatine. The current literature does not seem to address urinary creatine excretion in the neonate. Our own data involving 24 hour urine collection in adult rats found negligible amounts of urinary creatine excretion in comparison to creatinine excretion. However, we did not measure urinary creatine excretion in our rat pups, nor in human neonates. In 2004, Volongo *et al*. measured urinary creatine and GAA excretion in children and adolescents. They analyzed 4 study groups based on age: < 1 year, 1-4 years, 5-11 years and 12-16 years. They did not find any significant differences between the creatine and GAA excretion based on gender or upon these initial age groups. Subsequently, they combined male and female results and analyzed the data based on the following age ranges: ≤4 years, 5-11 years and 12-16 years. Unfortunately, there were few demographic details outlined in the paper. The age ranges of the subjects ranged from 6 days to 16 years but it was not stated how many subjects were less than 1 year of age and, more importantly for this study, how many neonatal subjects were recruited. There were 70 subjects in the age group ≤4 years. They found that GAA excretion ranged from 18-159 μmol per mmol of creatinine in children ≤4 years. This amount is negligible when considering the effect it would have on the need for creatine replacement in neonates. However, they found urinary creatine excretion to be 0.04-1.51 mmol per mmol creatinine in these same children. These numbers, if confirmed, would have significant implications for the rate of creatine replacement. In fact, in some cases, the estimates calculated in **Chapter 3** of the need for creatine replacement in neonates may be substantially underestimated. These considerations, however, only strengthen one

of our principal conclusions, that creatine biosynthesis places a substantial burden on an infants' amino acid metabolism. Indeed, the burden may be appreciably greater than our data suggest.

When creatine synthesis rates were compared to the intake of amino acids in the rat pup, it was calculated that creatine synthesis could potentially consume 13, 13 and 22% of dietary glycine, arginine, and methionine, respectively. These numbers were calculated based on data provided by Davis *et al.* [1993] regarding the amino acid composition of rat milk protein. These numbers are very similar to those found in piglets [Brosnan & Brosnan, 2007]. It is clear that creatine biosynthesis places a significant burden on methyl group metabolism and arginine metabolism in neonatal rats.

5.8. CONCLUSIONS

This study has shown, for the first time, that GAA is produced by rat kidneys, *in vivo*. It was also shown that the human kidney produces GAA. Creatine feeding downregulates renal AGAT activity and mRNA expression as well as the *in vivo* production of GAA by the rat kidney. Acute provision of additional glycine was without effect on renal GAA production *in vivo*; however, acute provision of arginine or citrulline, which is converted to arginine in the kidney, increased renal GAA production in the rat.

The investigation of creatine balance in the adult human indicated that creatine synthesis imposes a considerable metabolic burden on each of the amino acids involved [Brosnan *et*

al., 2011]. Our analysis of the situation in neonates suggests that this metabolic burden may be greater still, because the need for creatine accretion in neonates is larger because of the expanding muscle mass. The current study has shed light on both the provision of creatine to neonates via the diet and the impact that creatine synthesis has on amino acid metabolism in the neonate.

The role of the mammary gland in the provision of creatine to rat pups was investigated. It was found that milk creatine was extracted by the rat mammary gland from the circulation, rather than being synthesized within the gland, since there was no apparent capacity for creatine synthesis in the rat mammary gland. During lactation, the increased demand for creatine in the lactating mother can be largely supported by hyperphagia when creatine is present in the food.

This is the first time it has been shown that neonatal creatine accretion is largely due to *de novo* synthesis in both the human and the rat. Very little creatine is supplied to the neonate from mother's milk or from commercially available formulas. Since there is a large need for creatine in the neonate due to creatine lost as creatinine and new muscle lay-down, creatine biosynthesis places a significant drain on the available amino acids, arginine, glycine and methionine. Of particular importance is the burden that creatine synthesis imposes on methionine metabolism and methyl balance. This study has provided a firm foundation for our understanding of the regulation of creatine synthesis and the burden that creatine synthesis places on amino acid metabolism, particularly in the developing neonate.

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APPENDIX A

NEW INSIGHTS INTO CREATINE FUNCTION AND SYNTHESIS

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New Insights into Creatine Function and Synthesis

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A.1. INTRODUCTION

This paper reviews new aspects of the function and synthesis of creatine and creatine phosphate. We examine creatine synthesis, which has emerged as a quantitatively major pathway in amino acid metabolism. We emphasize the inter-organ aspects of creatine synthesis, involving both the kidney and liver. Finally, though interest in creatine has traditionally been concerned with its role in muscle, we highlight the critical role that creatine plays in brain function, both in the developing brain as well as in the adult.

A.1.1. Creatine Function

The role of creatine and creatine phosphate in energy metabolism has long been recognized. The creatine kinase (EC 2.7.3.2) reaction, (ATP+creatine↔creatine phosphate+ADP) provides the basis for this role. However, in recent years, our view of the function served by this reaction has changed from one in which the primary role is that of buffering ATP levels or the ATP/ADP ratio to one of rapid, intracellular translocation of high-energy phosphate compounds from their mitochondrial sites of production to their cytosolic sites of utilization. This latter view is supported by the subcellular location of different creatine kinase isoenzymes, in the mitochondria as well as in myofibrils, sarcoplasmic reticulum and plasma membranes (Schlattner *et al*., 2006).

This view of the creatine/creatine phosphate system is illustrated in **Figure A.1**. It is often suggested that the major advantage provided by this energy translocation model over the more conventional system, where ATP and ADP are thought to diffuse between sites of production and utilization, is the higher diffusivity of creatine phosphate compared to

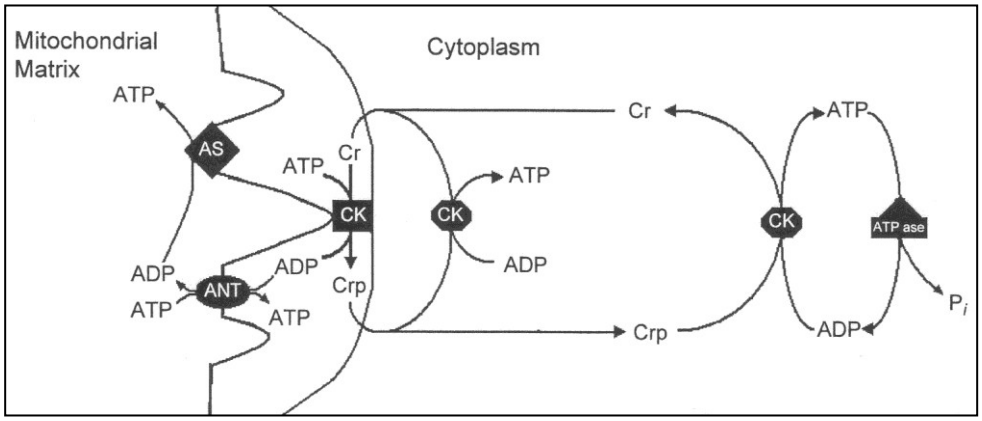

Figure A.1. Mitochondrial–cytoplasmic interactions in creatine function. ATP is produced by ATP synthase (AS) on the mitochondrial inner membrane. It exits to the intermembrane space, in exchange for ADP, via the adenine nucleotide transporter (ANT). This ATP phosphorylates creatine (Cr), catalyzed by the mitochondrial isoform of creatine kinase (CK), at contact points between the inner and outer mitochondrial membranes. Creatine phosphate (CrP) may be used to buffer ATP levels (or ATP:ADP ratios) in the cytosol and to rephosphorylate ADP at sites of ATP utilization (ATPase).

ATP, as the molecular weight of creatine phosphate is less than half that of ATP. In fact, the diffusion coefficient for creatine phosphate is only about 30% higher than that for ATP (Ellington, 2001). It is much more likely that the key difference is in the diffusivity of creatine compared to ADP and is provided less by the difference in their molecular weights (ADP=427; creatine=131) than by very great differences in their cytosolic concentrations (Wallimann *et al*., 1992). Free cytosolic concentrations of ATP, creatine phosphate and creatine in skeletal muscle are, respectively, about 8, 27 and 13 mM (Veech *et al*., 1979). The free cytosolic ADP concentration is about 20 μM (Nioka *et al*., 1992). It should, of course, be emphasized that the two postulated roles of the creatine/creatine phosphate system are not mutually exclusive and it is likely that both temporal energy buffering and spatial energy translocation occur, to different degrees, in different cell types. It appears that temporal buffering is more important in fast-twitch muscles whereas the energy translocation function predominates in slow-twitch muscles (Wallimann *et al*., 1992).

The creatine/creatine phosphate system is not equally important in every cell. Indeed, some cells with high rates of oxidative metabolism (e.g. hepatocytes) do not employ this system. It is apparent that it is primarily present in cells with high and intermittent energy demands, such as skeletal muscle (where energy output can vary by a factor of 100), heart (where there are substantial differences in energy demand from systole to diastole) and the brain (where certain tasks require some neurons to fire very quickly) (Wallimann *et al*., 1992).

In addition to these physiological roles of creatine, this substance is also consumed in quite substantial amounts both by athletes, as an ergogenic supplement (Kraemer and Volek, 1999) and by patients suffering from a variety of neurological disorders (Baker and Tarnopolsky, 2003). Annual consumption of creatine is estimated at about four million kilograms per annum with a value, in the United States alone, of more than $200 million.

A.1.2. Creatine Loss and Replacement

The whole body pool of creatine plus creatine phosphate in 70 kg young adults is of the order of 120 g. Both creatine and phosphocreatine are non-enzymatically and irreversibly degraded to creatinine at a rate of about 1.7% of the total body pool per day (Wyss and Kaddurah-Daouk, 2000). This creatinine is lost in the urine. The amount of this loss is proportional to muscle mass and is, therefore, greater in males than in females and in young adults than in the elderly. Daily creatinine excretion (and creatine loss) varies from about 1.7 g to about 0.9 g in 70 kg males of ages 18–29 and 70–79, respectively (Stead *et al*., 2006). In vegetarians, all of this creatine needs to be replaced via synthesis. However, for individuals on a typical North American omnivorous diet, we have calculated that approximately half of the replacement can be provided via food creatine and the other half requires endogenous synthesis. Thus, we have calculated that men, aged 20–39, need to synthesize about 1.0 g creatine/day; women of the same age group synthesize about 0.75 g/day (Stead *et al*., 2006).

A.1.3. Creatine Synthesis and Transport

Creatine synthesis occurs via an exceedingly simple metabolic pathway, consisting of only two enzymes (**Figure A.2**). The first enzyme, l-arginine:glycine amidinotransferase (AGAT) (EC 2.1.4.1) catalyzes the formation of guanidinoacetate and ornithine from arginine and glycine. The second enzyme, guanidinoacetate N-methyltransferase (EC 2.1.1.2), employs S-adenosylmethionine to methylate guanidinoacetate, producing creatine and S-adenosylhomocysteine. Although most of the body's creatine is found in skeletal muscle, there is very little evidence for appreciable creatine synthesis in this tissue. Rather, creatine, whether of dietary origin or synthesized endogenously, is taken up from the plasma via a specific transporter (CreaT). CreaT is found in a variety of tissues, including skeletal muscle, kidney, heart, forebrain, cerebellum and liver (Snow and Murphy, 2001). It transports creatine, together with sodium and chloride ions, against a considerable concentration gradient. Additional complexity is provided by the occurrence of at least two different isoforms of CreaT, most likely as a result of alternative mRNA splicing (Guerrero-Ontiveras and Wallimann, 1998).

Creatine synthesis represents a considerable metabolic load, in particular with regard to amino acid utilization. A comparison of rates of creatine synthesis with the dietary intake of glycine, arginine and methionine is revealing. The synthesis of 1.0 g creatine/day amounts to 7.6 mmol. This compares to a dietary intake of about 78 mmol glycine, 37 mmol of arginine and 19 mmol of methionine (these figures are based on an intake of 80 g protein/day with the amino acid composition of beef protein). Therefore, creatine

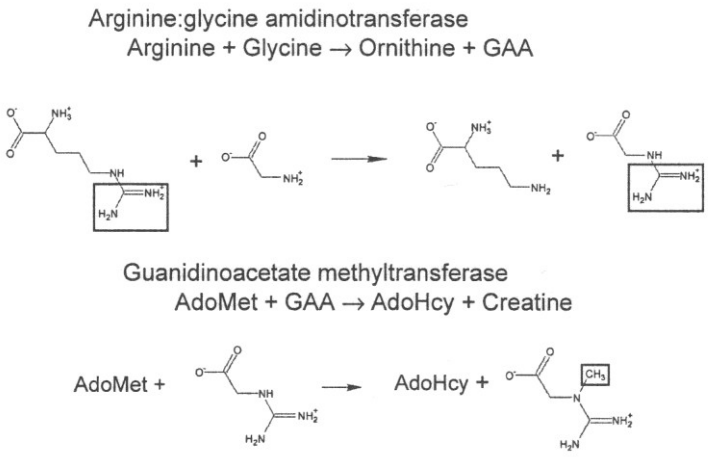

Figure A.2. Pathway of creatine synthesis. The boxes indicate the groups transferred by these transferase reactions. Abbreviation: GAA, guanidinoacetate; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine.

synthesis consumes about 10% of dietary glycine as the entire glycine molecule is incorporated into creatine. It is not certain whether similar arguments can be made for methionine and arginine since only the methyl group of methionine and the amidino group of arginine are incorporated into creatine. In addition to methionine, the diet provides labile methyl groups in the form of choline and betaine and new methyl groups (methylneogenesis) can be produced via the remethylation of homocysteine (Stead *et al*., 2006). Comparison of rates of creatine synthesis with isotopically measured rates of transmethylation reveal that the synthesis of this molecule makes very great demands on S-adenosylmethionine. Approximately 40% of S-adenosylmethionine's methyl groups are consumed by creatine synthesis (Stead *et al.*, 2006).

The ornithine produced by AGAT could, in theory, be reconverted to arginine by the reactions of the urea cycle (Brosnan and Brosnan, 2004). However, there is, at present, no compelling evidence that this actually occurs. Therefore, it is possible that creatine synthesis could consume as much as 20% of dietary arginine. Regardless of the uncertainties inherent in these calculations, it is apparent that creatine synthesis places a considerable demand on amino acid metabolism. This demand is even greater in vegetarians where creatine synthesis is double that in omnivores and where protein intake is, typically, less.

A.1.4. Inborn Errors of Creatine Synthesis and Transport

Inborn errors involving mutations in each of the three proteins required for creatine synthesis and transport are now known. Perhaps the most remarkable feature of these

disorders is that they present, in children, with a very similar constellation of neurological symptoms, i.e. mental retardation, epilepsy and difficulties in language development. The symptoms are invariably more severe in the GAMT-deficient children and this has been attributed to the accumulation of guandinoacetate, which is thought to exert specific neurotoxic actions. Because of its methyl group, creatine is readily detected non-invasively by ^{1}H-MRS. Children who present with these inborn errors display a massive depletion of brain creatine. In addition, those with AGAT deficiency have decreased plasma creatine and guanidinoacetate while those with GAMT deficiency have markedly increased plasma guanidinoacetate and lowered creatine. In the case of patients with creatine transporter deficiency, plasma guanidinoacetate is in the normal range while creatine is elevated (Stromberger *et al*., 2003).

Treatment of children with these enzyme defects with creatine largely or completely restores brain creatine over time and results in a marked clinical improvement. The general experience with creatine supplementation, however, has been that the children never fully recover; learning and language functions remain impaired. However, until recently, it has never been clear whether the neurological deficits could have been avoided if creatine supplementation had begun at birth. One recent case-study gives encouraging information on this point. The affected child was homozygous for the W149X mutation in AGAT that also affected two older siblings. A very early diagnosis could, therefore, be made. Treatment with creatine monohydrate was begun at 4 months, upon weaning. This child's growth and development was entirely normal at the age of 18 months (Battini *et al*., 2006). In contrast to the improvement found upon creatine

supplementation of children with the enzyme deficiencies, the transporter-deficient patients are refractory to treatment with creatine. There is no accumulation of creatine in the brain and no clinical improvement (Stromberger *et al*., 2003).

A.1.5. Creatine Synthesis in the Rat

The creatine-deficiency syndromes clearly emphasize the importance of creatine synthesis. We have, therefore, conducted a series of studies on creatine synthesis, employing the rat as a model. Previous work has indicated that the two enzymes of creatine synthesis occur in a variety of tissues. In particular, AGAT is most highly active in the rat kidney but this enzyme is also found in pancreas, spleen, brain, testis and thymus (Wyss and Kaddurah-Daouk, 2000). A particularly vexing question concerns the possible occurrence of AGAT in the liver. McGuire *et al*. (1986) using immunofluorescence microscopy reported AGAT protein in hepatocytes. However, we have been unable to detect enzyme activity in the liver. The highest activity of GAMT is found in the rat liver but activity is also reported in pancreas, spleen and testis (Wyss and Kaddurah-Daouk, 2000). Based on the fact that the highest abundance of AGAT is in the kidney whereas that of GAMT is in the liver, we have postulated the occurrence of an inter-organ pathway for creatine synthesis (**Figure A.3**) by which guanidinoacetate is released by the kidney, taken up by the liver and methylated there to produce creatine.

We have tested this inter-organ hypothesis in a variety of ways. Direct measurements of arterio-venous (A-V) differences across rat kidneys reveal guanidinoacetate production (arterial concentration 5 μM, renal venous concentration 9 μM, $p<0.01$). When these

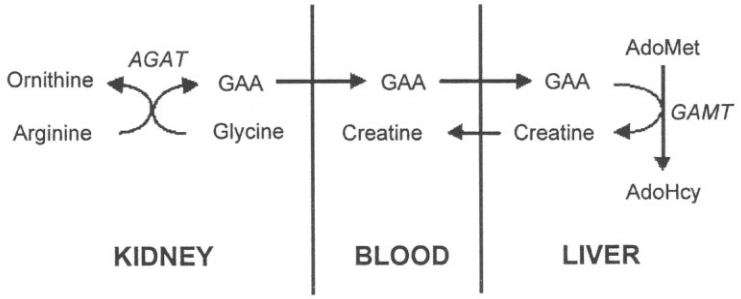

Figure A.3. Inter-organ pathway for creatine synthesis in the rat. Abbreviations: AGAT, l-arginine:glycine amidinotransferase; GAA, guanidinoacetate; GAMT, guanidinoacetate *N*-methyltransferase; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine.

studies are carried out on rats fed purified diets (i.e. no dietary creatine), we found that guanidinoacetate production by the kidney was not significantly different from creatinine loss across the same kidney. This implies that the kidney is the organ responsible for the great bulk of guanidinoacetate production in the rat. We have also examined the production of creatine, from guanidinoacetate in isolated hepatocytes. These cells produced creatine from guanidinoacetate (half maximal rates at [guanidinoacetate]=14 μM). The rate of creatine production was doubled by the provision of a source of methyl groups (e.g. methionine or betaine in the presence of homocysteine). Maximal rates of creatine synthesis by rat hepatocytes are about 25 nmol/h/mg dry weight.

We have also examined the regulation of creatine synthesis. It is known that renal AGAT activity is down-regulated by dietary creatine (McGuire *et al*., 1984). We have confirmed this in rats that were fed a diet containing 0.4% creatine for 2 weeks—renal AGAT activity was decreased by 85%; renal AGAT mRNA abundance also fell, though not as much. In turn, this decreased renal AGAT activity translated into a reduced A-V difference for guanidinoacetate across the kidney (1 vs. 4 μM) and a markedly reduced circulating guanidinoacetate concentration (1.5 vs. 5 μM). We suggest that the primary regulation of creatine synthesis occurs at the level of renal AGAT expression and its modulation by dietary creatine. We suggest that little active regulation occurs in the liver. Half-maximal creatine production by hepatocytes is found at about 14 μM guanidinoacetate, which is appreciably higher than circulating levels (about 5 μM). Therefore, we suggest that hepatic creatine synthesis can respond to increased or

decreased renal guanidinoacetate production in a simple concentration-dependent manner. This is not to suggest, however, that our knowledge of the hepatic aspect of creatine synthesis is complete. In particular, we need to understand the transport mechanisms whereby guanidinoacetate enters hepatocytes and by which creatine exits. There is the well-described creatine transporter in tissues such as muscle, which takes up creatine against a considerable gradient. However, creatine transport in hepatocytes occurs in the opposite direction and may require a unique transporter.

A.1.6. Creatine Synthesis and Homocysteine

Creatine synthesis accounts for approximately 40% of all S-adenosylmethionine-derived methyl groups (Stead *et al*., 2006). This, in turn, implies that it is responsible for the production of about 40% of the body's homocysteine. Elevated plasma homocysteine has been implicated as a risk-factor for a number of chronic illnesses, including cardiovascular disease (Stampfer *et al*., 1992), Alzheimer's disease (Seshadri *et al*., 2002) and fractures (van Meurs *et al*., 2004). There is, therefore, considerable interest in ways of reducing plasma homocysteine levels. Given that we have shown that dietary creatine down-regulates endogenous creatine synthesis, it was important to determine whether dietary creatine could decrease plasma homocysteine levels. We, therefore, measured plasma homocysteine in rats that had been fed a diet containing 0.4% creatine for 2 weeks (Stead *et al*., 2001). **Figure A.4** shows that plasma homocysteine decreased by approximately 25%. There was no effect on methionine levels.

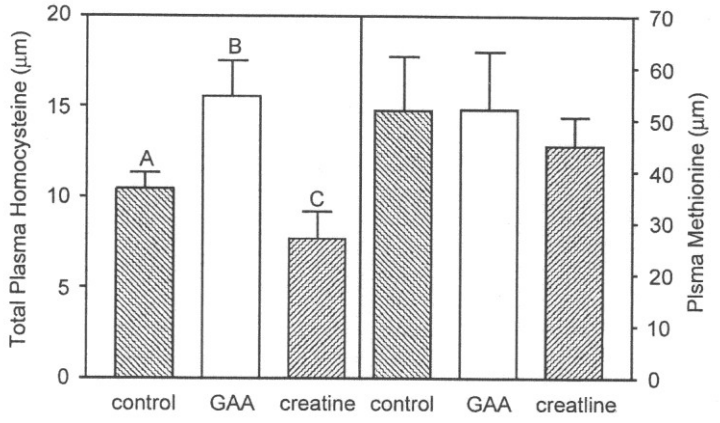

Figure A.4. Effect of dietary GAA and creatine supplementation on total plasma homocysteine and methionine levels. Rats were fed the diets for 2 weeks. From Stead *et al*. (2001), with permission. Abbreviation: GAA, guanidinoacetate.

Does dietary creatine decrease plasma homocysteine in humans? Certainly, there is evidence that it down-regulates guanidinoacetate production as the plasma concentration of this compound decreases in subjects who ingested creatine (Derave *et al*., 2004). Two groups have examined plasma homocysteine with discordant results. Steenge *et al*. (2001) found no decrease in plasma homocysteine in subjects who ingested 3 g creatine monohydrate/day for 61 days. On the other hand, Korzun (2004) found a 10% decrease in subjects who, each day, ingested an amount of creatine equal to twice their daily creatinine excretion for 28 weeks. We consider it likely that the decrease in homocysteine that we observed in rats also occurs in humans although its magnitude may be somewhat less.

A.1.7. Perspective

As the great bulk of the body's total creatine is found in muscle, attention has primarily centered on its function in this tissue. However, the fact that children with inborn errors in creatine synthesis or transport present with profound neurological impairments obliges us to focus both on how the brain acquires its creatine as well as its function in this organ. The occurrence of the neurological symptoms implies that dietary creatine is insufficient in these children. However, the almost complete absence of brain creatine is somewhat puzzling since infant formulas that are based on cow's milk contain substantial quantities of creatine. However, our own analyses show that soy-based formulas are devoid of creatine. It is possible, therefore, that these children's diet may play some role in the severity of their symptoms. The fact that creatine supplements are effective in restoring

brain creatine in these children indicates that the brain can take up blood-borne creatine. Nevertheless, both AGAT and GAMT have been reported to be ubiquitously located, in neuronal and glial cells, throughout the rat brain (Braissant *et al*., 2001). The possible role of brain creatine synthesis, vis-à-vis creatine uptake from the circulation, needs to be clarified.

What role(s) might creatine play in the brain? At the moment, we have no reason not to suppose that the traditional roles of creatine phosphate and creatine kinase are paramount. The brain has a very high rate of oxygen consumption; the adult human brain weighs less than 1% of body weight but accounts for about 10% of the total oxygen consumption (Burton, 1966). In addition, individual rapidly firing neurons have very high energy requirements.

An intriguing study from Australia examined the effect of creatine supplementation on performance in a number of cognitive tests (Rae *et al*., 2003). The subjects, students from the University of Sydney, were either vegetarians or vegans because it was reasoned that their creatine status might be impaired due to little or no dietary creatine ingestion. A double-blind, placebo-controlled, cross-over trial was carried out in which subjects ingested a daily dose of either 5 g of creatine monohydrate or placebo for 6 weeks, Then, after a 6-week wash-out period, the doses were reversed. A number of cognitive tests that require speed of processing were administered at the beginning and after each 6-week period. Creatine ingestion resulted in remarkably improved performance in these tests. This remarkable result emphasizes the importance of creatine in non-muscle tissues and

suggests that brain creatine acquisition and function is an important field for future research.

A.2. SUMMARY

The text-book view of the role of the creatine/creatine phosphate system as an energy buffer has been expanded to include functions such as energy shuttling, proton buffering and regulating cytosolic ADP levels. There is continuous need for creatine replacement due to creatinine formation. Replacement involves a combination of diet and *de novo* synthesis. Creatine synthesis makes very significant demands on amino acid metabolism, in particular that of glycine, arginine and methionine. It uses about 40% of all methyl groups transferred from *S*-adenosylmethionine. Although the traditional view of the function of the creatine/creatine phosphate system is largely concerned with its role in skeletal and cardiac muscle, recent work obliges us to take a broader view. In particular, its role in the brain is brought into sharp focus by the neurological symptoms displayed by children suffering from inborn errors of creatine synthesis and transport, as well as by suggestions that brain creatine status may play a role in cognitive performance in adults.

A.3. ACKNOWLEDGEMENTS

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APPENDIX B

MATERIALS AND METHODS

B.1. CHEMICALS

Only chemicals of the highest quality available were used in the following experiments. All chemicals were purchased from Sigma (Oakville, ON, Canada), except where mentioned in the text.

B.2. ANIMAL HANDLING

Male Sprague-Dawley rats weighing between 200-300g were purchased from Memorial University of Newfoundland's breeding colony and were exposed to a 12-hour light: 12-hour dark cycle. The light cycle commenced at 0800 h. All experiments were performed during the light cycle. Rats were fed *ad libitum* either laboratory chow, AIN-93 growth diet, or 0.4% creatine-supplemented AIN-93 growth diet. All rats had free access to water. All procedures were conducted in accordance with the guidelines and principles of the Canadian Council on Animal Care and were approved by Memorial University of Newfoundland's Institutional Animal Care Committee.

B.2.1. Urine and Feces Collection

Urine and feces were collected from animals placed on the recommended AIN-93G diet for 24 hours prior to sacrifice. Urine and feces were collected approximately every 2 hours (except overnight it was left at room temp for about 8 hours) and frozen at -20°C until analysis.

B.3. EXPERIMENTAL DIETS

When animals were fed purified diets instead of laboratory chow, the diets were based upon the American Institute of Nutrition AIN-93G diet for growth. Prior to the initiation of each experimental period, animals were allowed to become accustomed to the AIN-93G powdered diet for a period of 3 days. Some experiments involved the feeding of a 0.4% creatine diet for a period of 2 weeks. When creatine was fed, an equivalent mass of cornstarch was omitted. The control diet was the recommended AIN-93G diet (20% casein). For experimental diet composition, refer to **Table B-1**.

B.4. TISSUE PREPARATION

Animals were anaesthetized with 65 mg/kg sodium pentobarbital (i.p.). Following a midline abdominal incision and opening of the abdominal cavity, the renal vein was cleaned and blood flow was measured using a T206 small animal blood flow meter by Transonic Systems Inc. (Ithaca, NY, USA). Arterial and venous blood was taken across the kidney. Kidneys were removed and immediately placed in ice-cold 50 mM potassium phosphate buffer (pH 7.4), except for a small sample which was retained for RT-PCR analysis.

For infusion experiments, before opening of the abdominal cavity, the femoral vein was cleaned to allow delivery of the 0.8 mL prime injection and the jugular vein was cannulated with PE50 tubing to allow delivery of infusate. A restrainer was placed in the animal's mouth to prevent suffocation by the tongue. Once the abdominal cavity was opened and the T206 probe placed on the renal vein, the prime was given and the infusion

Table B.1: Experimental diet composition. All quantities are listed as g/kg diet. Diet conditions are written in the text. Vitamin Mix and Mineral Mix were purchased from MP Biomedicals (Irving, California, USA).

Component	Amount in g/kg	
	20% Protein	0.4% Creatine
Cornstarch	397.486	393.486
Casein	200.000	200.000
Dextrinized Cornstarch	132.000	132.000
Sucrose	100.000	100.000
Soybean Oil	70.000	70.000
Alphacel	50.000	50.000
Mineral Mix	35.000	35.000
Vitamin Mix	10.000	10.000
L-cystine	3.000	3.000
Choline Bitartrate	2.500	2.500
t-Butylhydroquinone	0.014	0.014
Creatine	0.000	4.000

pump was set to deliver 0.037 mL/minute into the jugular vein for 20 minutes. Blood flow was measured during the entire 20 minute infusion. After infusion, both arterial and renal venous blood was sampled. For prime and infusate concentrations, refer to **Table B-2**.

B.4.1. Plasma Preparation

Arterial and renal venous blood samples were centrifuged for 10 minutes at 6000 x g for plasma separation. Plasma samples were frozen at -20°C for later analyses.

B.4.2. Kidney Preparation

Upon removal, kidneys were immediately placed in 50 mM potassium phosphate buffer as previously described (**Section B.4**). After being rinsed of blood, kidneys were diluted 4 mL phosphate buffer/g kidney, chopped with scissors and homogenized with a Polytron homogenizer (Brinkman Instruments, Toronto, ON, Canada) for 25 seconds at 50% output. The homogenate was diluted 19:1 vol/vol with phosphate buffer to prepare a 1% kidney homogenate for AGAT analysis, as described in **Section B.6.1**.

B.5. PLASMA ANALYSES

Frozen plasma samples were prepared as described above and used for all analyses. To calculate arteriovenous (A-V) differences, analyses were performed on both the arterial and renal venous plasma samples and the values obtained for renal venous samples were subtracted from those values obtained for arterial samples. A positive value

Table B.2: Prime and infusate concentrations. Infusion conditions are found in **Section B.4.** All solutions were made isotonic with sodium chloride. 0.9% sodium chloride served as a control.

Amino Acid	Prime	Infusate
Glycine	200 mM	60 mM
Arginine	200 mM	60 mM
Citrulline	100 mM	30 mM
Alanine	200 mM	60 mM

for an A-V difference is indicative of an uptake of the given metabolite by the kidney while a negative value for an A-V difference would indicate that there was an output of the given metabolite by the kidney. If the A-V difference is zero, this would imply that the kidney does not take-up nor release the given metabolite unless the uptake rate is the same as the output rate.

B.5.1. Plasma Guanidinoacetate Analysis

Plasma was diluted 3:1 vol/vol with PBS (Dulbecco's phosphate buffered saline, Invitrogen Canada Inc., Burlington, Ontario, Canada) and placed in a Centricon 3000 molecular weight cutoff spin column (Millipore, Barrerica, Massachusetts, USA) to remove large proteins. The spin columns were centrifuged at 10 000 x g for 30 minutes. Filtered plasma was used for GAA analysis via HPLC according to the method of Carducci *et al*. (2001). Plasma samples were derivatized with benzoin under basic conditions at 100ºC for 15 minutes, neutralized and loaded onto a Hypersil ODS 5 µm 150 mm x 4.6 mm C18 reverse-phase HPLC column. Buffer A consisted of 20 mM sodium acetate, pH 7.35 and Buffer B was 20% vol/vol 100 mM sodium acetate buffer, pH 7.35 and 80% vol/vol methanol. Both solutions were filtered through a 0.45 µm Millipore filter (Millipore, Barrerica, Massachusetts, USA) prior to use. Separation of GAA was obtained at approximately 22 minutes using a flow rate of 0.8 mL/minute under the following gradient conditions: a linear increase in Buffer B from 40% to 70% over 15 minutes, maintained constant at 70% for 10 minutes, increased to 100% over 5 minutes, maintained at 100% for 5 minutes, decreased to 40% over 1 minute, and maintained at 40% for 9 minutes. The column was maintained at 38ºC. Excitation at 325 nm and

emission detection at 435 nm allowed fluorescence detection of the benzoin-derivatized guanidino compounds. An example chromatogram for standards and plasma samples is shown in **Figure B-1**. The peaks obtained before 3.5 minutes and at approximately 23.5 minutes are common to all chromatograms regardless of the sample being derivatized. A standard curve for GAA is shown in **Figure B-2**.

B.5.2. Plasma Creatine Analysis

Before creatine analysis, plasma was deproteinized by adding 50% vol/vol 0.6 M perchloric acid, kept on ice for 20 minutes, and centrifuged at 20 000 x g for 5 minutes to sediment proteins. To 1.5 mL supernatant, 25 µL 50% potassium carbonate, and 20 µL universal indicator were added before neutralization with 20% potassium hydroxide. The deproteinized, neutralized plasma was used for the creatine assay following the spectrophotometric method as described by Lowry & Passonneau [1972]. The reaction mixture consisted of imidazole cocktail, pH 8.0 (50 mM imidazole, 5 mM magnesium chloride, 30 mM potassium chloride), 0.17 mM NADH, 0.2 mM PEP, 1 mM ATP, pyruvate kinase (rabbit muscle), 5 µg/mL (0.75 U/mL), lactate dehydrogenase (beef heart), 1 µg/mL (0.2 U/mL), and 0.1 mL of neutralized plasma to a final volume of 3.0 mL. The optical density (OD) was read at 340 nm every 2 minutes until steady (about 10 minutes). The reaction was started by the addition of 5 µL creatine kinase (rabbit muscle), 10 mg/mL (Roche, Quebec, Canada). The OD was read every 2 minutes until steady and the difference in OD (from before the creatine kinase was added until it leveled out) was used to calculate the amount of creatine present in the sample as

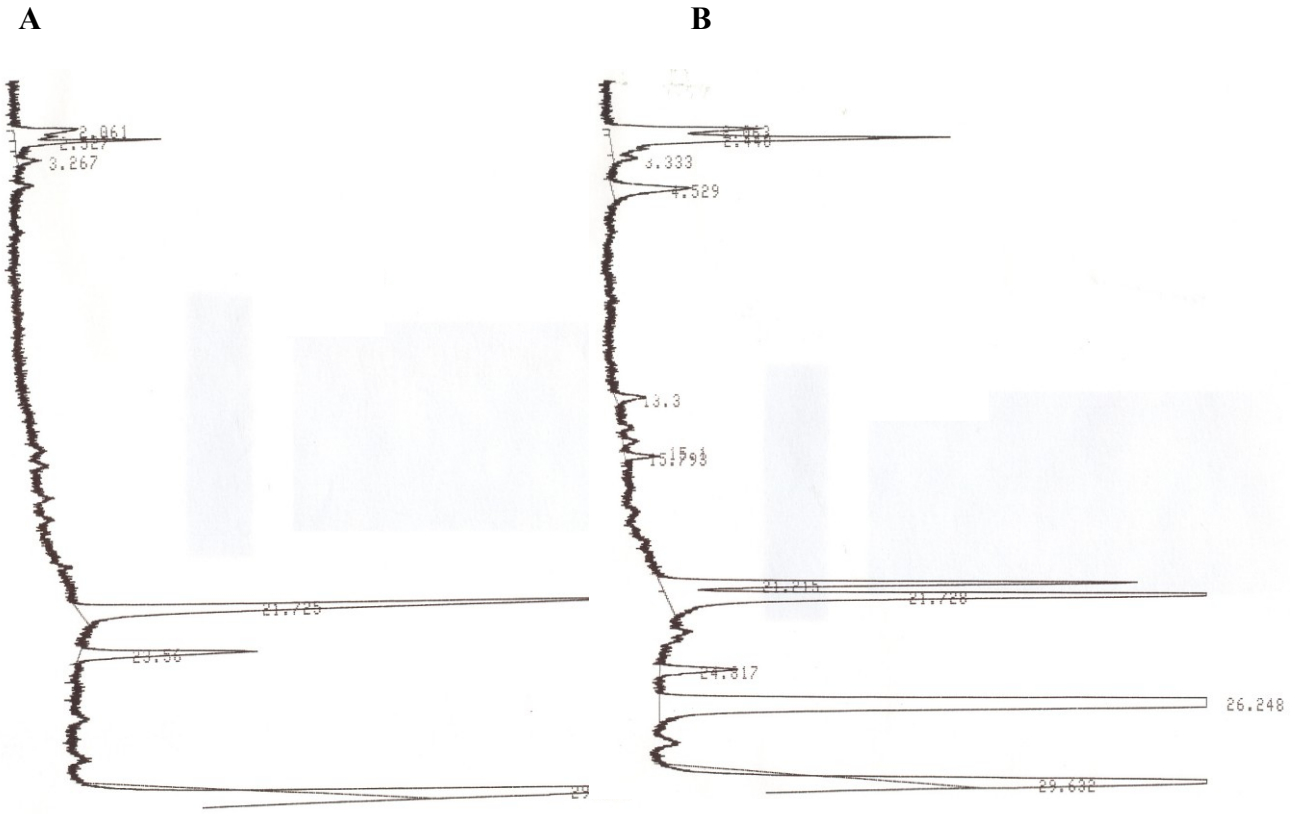


Figure B.1: Sample chromatogram of GAA as measured via HPLC. The peak at 21.7 minutes corresponds to GAA. **A**. Known standard derivatized with benzoin. **B**. Plasma sample derivatized with benzoin. See text for preparation, HPLC apparatus, and run conditions (**Section B.5.1**).

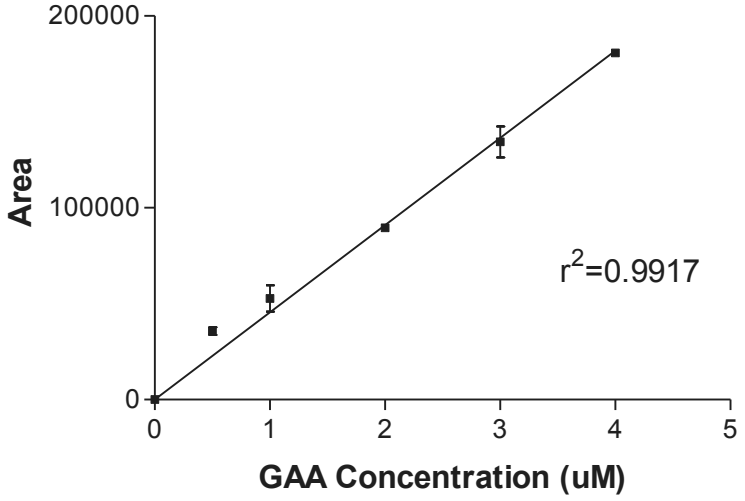


Figure B.2: Standard curve of GAA detection via HPLC. Linearity was shown by derivatization of known concentrations of GAA with benzoin as described in **Section B.5.1**. Areas obtained for the plasma samples were within this linear range.

compared to a known standard concentration. A creatine standard curve is shown in **Figure B-3**.

B.5.3. Plasma Creatinine Analysis

Plasma creatinine was determined in one of three ways. First, a creatinine kit from Sigma Diagnostics was used, procedure number 555. The Sigma kit became discontinued so samples were sent to Dr. Edward Randell who determined creatinine levels using a Synchron LX System (Beckman Coulter Inc., Buckinghamshire, UK). Finally, another creatinine assay kit (500701) became available from Cayman Chemical (Michigan, USA). All kits/methods employed the Jaffé reaction [Jaffé, 1886]. When creatinine is added to a mixture of picric acid and subjected to alkali conditions, a creatinine-picrate complex forms which is red in color and can be measured at 495 nm. A standard curve for creatinine via the Cayman Chemical kit number 500701 is shown in **Figure B-4**.

B.5.4. Plasma Amino Acid Analysis

For amino acid analysis, plasma samples were deproteinized by mixing 200 µL plasma, 200 µL internal standard (norleucine), and 1.0 mL 5% trifluoroacetic acid in methanol. Samples were frozen in liquid nitrogen and placed on a freeze-dryer overnight. Dried plasma samples were used for amino acid analysis via HPLC according to a modified method by Bidlingmeyer *et al.* [1984]. Plasma samples were derivatized with phenyl isothiocyanate (PITC) under basic conditions (10% triethanolamine (TEA), 70% methanol and 10% PITC) for 35 minutes at room temperature, frozen in liquid nitrogen

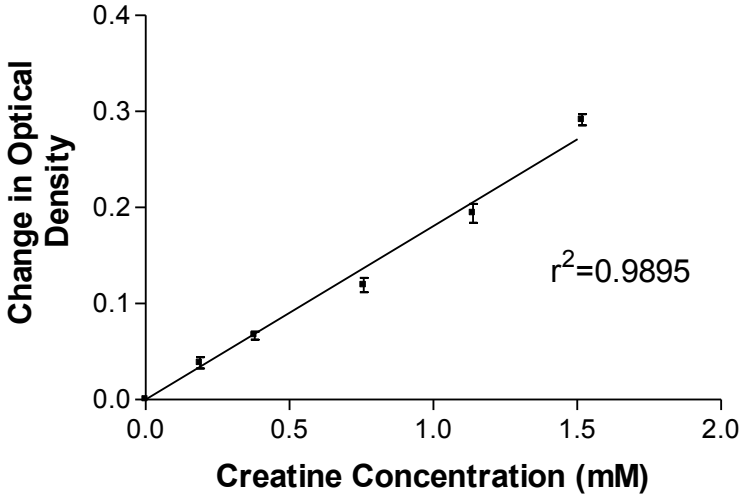


Figure B.3: Standard curve of creatine detection via spectrophotometry. Linearity was shown by spectrophotometric detection of known concentrations of creatine at 340 nm as described in **Section B.5.2**. Changes in optical density for each of the samples were within this linear range.

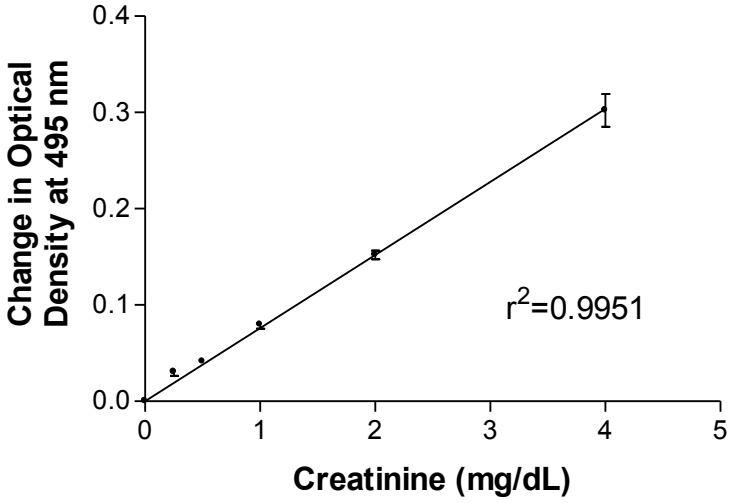


Figure B.4: Standard curve of creatinine detection via Cayman Chemical kit. Linearity was shown by spectrophotometric detection of known concentrations of creatinine at 495 nm as described in **Section B.5.3**. Changes in optical density for each of the samples were within this linear range.

and placed on a freeze dryer overnight. Dried amino acids were resuspended in 2.65 mM sodium phosphate (pH 7.4) in 5% acetonitrile and loaded onto a Waters Pico-Tag® 3.9 x 300 mm C18 column (Millipore Corporation, Waters Chromatography Division, Milford, Massachusetts). Buffer A consisted of 70 mM sodium acetate in 2.5% acetonitrile, pH 6.55, and Buffer B was 45% vol/vol acetonitrile, 15% vol/vol methanol. All solutions and samples were filtered through a 0.45 um Millipore filter (Millipore, Barrerica, Massachusetts, USA) prior to use. Separations of various amino acids were obtained using a flow rate of 1.0 mL/min under the following gradient conditions: a hyperbolic increase (curve 11) in Buffer B from 0% to 3% over 13.5 minutes, increased to 6% over 10 minutes (curve 8), increased to 9% over 6 minutes (curve 5), a linear increase to 34% over 20 minutes, maintained at 34% for 12 minutes, increased linearly to 100% over 30 seconds, maintained at 100% for 12 minutes, decreased to 0% over 30 seconds, and maintained at 0% for 12 minutes. The column was maintained at 46ºC and the samples were stored at 4ºC prior to loading. Detection of the various amino acids occurred at 254 nm using a Waters Lambda Max LC Spectrophotometer model 481 (Millipore Corporation, Waters Chromatography Division, Milford, Massachusetts). An example chromatogram for standards and plasma samples is shown in **Figure B.5**. A standard curve for each measured amino acid is shown in **Figure B.6**.

B.6. KIDNEY ANALYSES

All analyses were performed on kidney samples prepared as previously described (**Section B.4.2**).

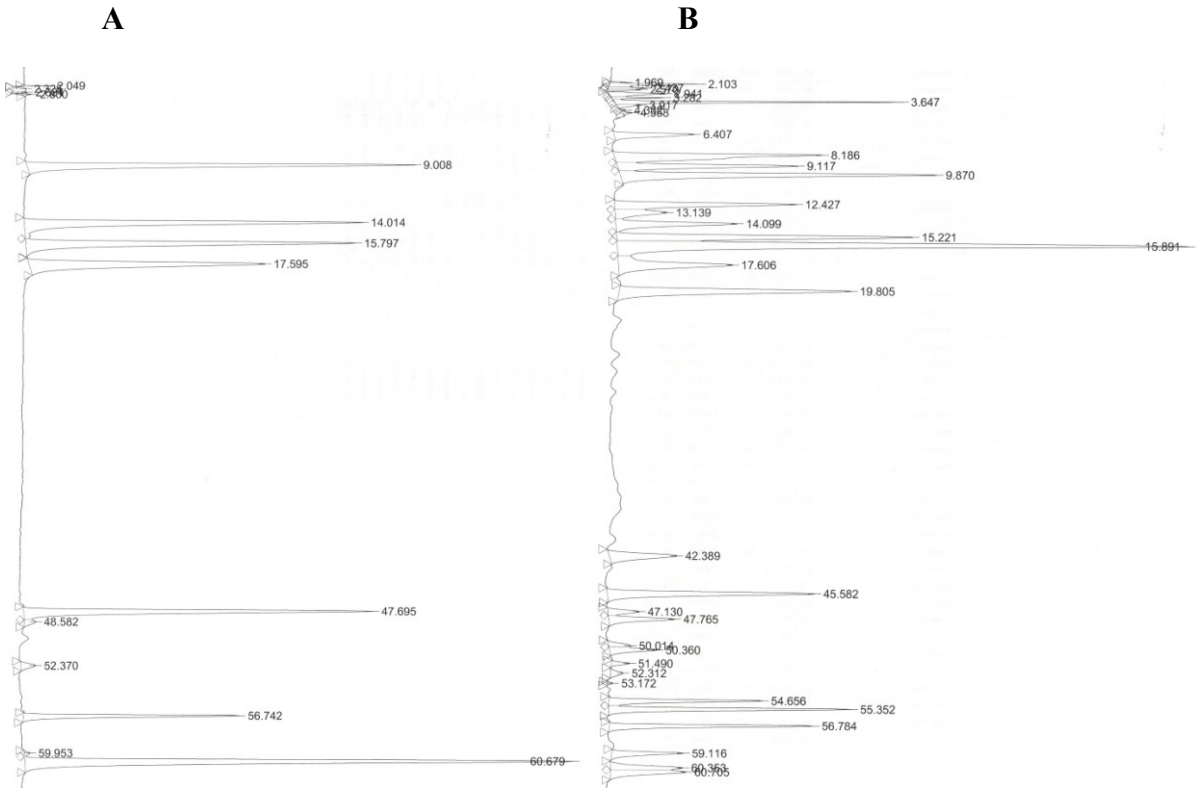
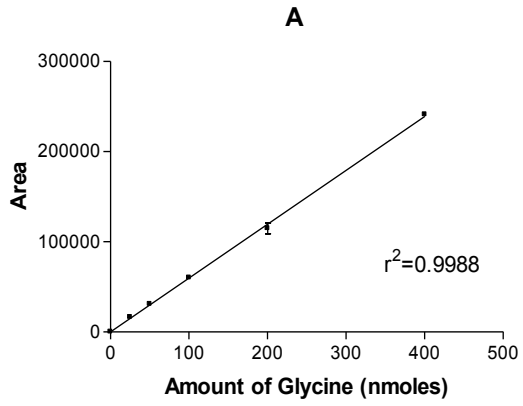


Figure B.5: Sample chromatograms of amino acids as measured via HPLC. Glycine separates at 9 minutes, citrulline at 14, alanine at 15.8, arginine at 17.6, methionine at 47.7, norleucine (internal standard) at 56.7 and ornithine at 60.7 minutes. **A**. Known standards derivatized with PITC. **B**. Plasma sample derivatized with PITC. See text for preparation, HPLC apparatus, and run conditions (**Section B.5.4**).

Figure B.6: Amino acid standard curves. Linearity was shown by derivatization of known concentrations of amino acids with PITC as described in **Section B.5.4**. Areas obtained for the plasma samples were within these linear ranges. **A.** Glycine standard curve. **B.** Citrulline standard curve. **C.** Alanine standard curve. **D.** Arginine standard curve. **E.** Methionine standard curve. **F.** Ornithine standard curve.


A
Area
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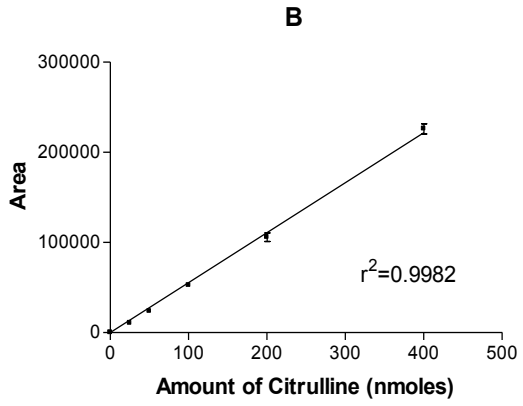
200000
100000
0
0
100
200
300
400
500
r2=0.9988
Amount of Glycine (nmoles)


B
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100000
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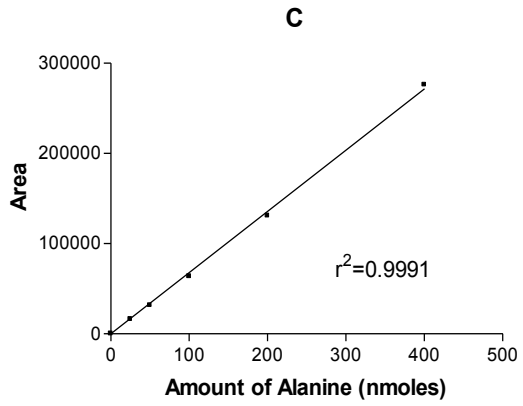
200
300
400
500
r2=0.9982
Amount of Citrulline (nmoles)


C
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300
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500
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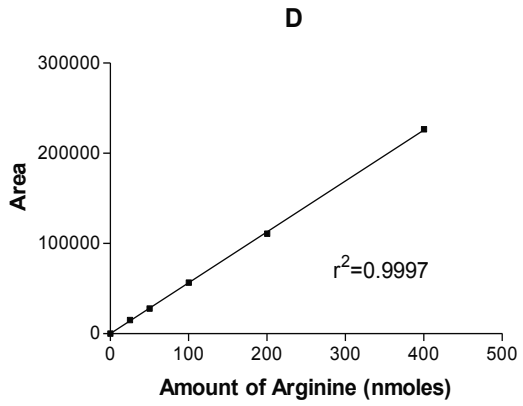
Amount of Alanine (nmoles)


D
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100000
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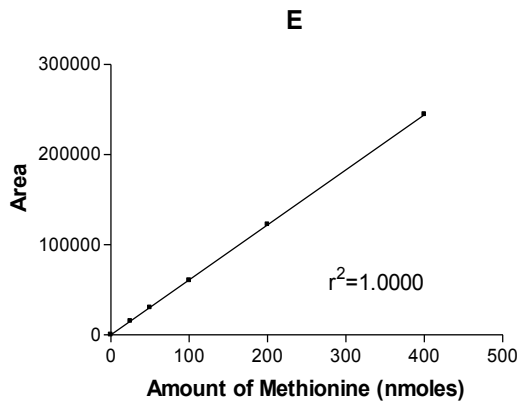
100
200
300
400
500
r2=0.9997
Amount of Arginine (nmoles)


E
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100000
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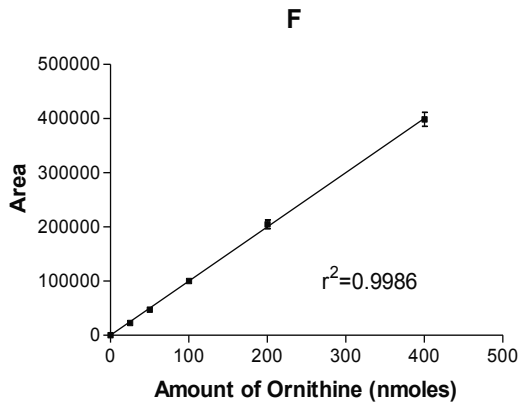
100
200
300
400
500
r2=1.0000
Amount of Methionine (nmoles)


F
Area
500000
400000
300000
200000
100000
0
0
100
200
300
400
500
r2=0.9986
Amount of Ornithine (nmoles)

B.6.1. L-Arginine:Glycine Amidinotransferase (AGAT) Activity

L-Arginine:glycine amidinotransferase (AGAT) was assayed in the 1% kidney homogenate (prepared as described previously; **Section B.4.2**) using a modified method of Van Pilsum *et al*. [1970], in which ornithine is detected by ninhydrin. The reaction mixture consisted of 100 mM potassium phosphate (pH 7.4) with 33 mM sodium fluoride (an arginase inhibitor), and 30 mM L-arginine and 45 mM glycine, as appropriate. Blanks for the assay involved omitting arginine and glycine separately and concurrently from the potassium phosphate-sodium fluoride reaction mixture. 0.5 mL of the appropriate incubation mixture was added to each flask and maintained at 37ºC in a shaking water bath (Precision Scientific, Winchester, Virginia, USA). To start the reaction, 0.5 mL of the 1% kidney homogenate was added to each flask and incubated at 37ºC for 60 minutes. The reaction was terminated by the addition of 1.5 mL 0.6 M perchloric acid (PCA). Tissue blanks for 0 time incubations had PCA added before the addition of homogenate. After addition of PCA, a portion of the resulting mixture was added to a microfuge tube and centrifuged at 10 000 x g for 5 minutes to sediment the precipitated proteins. To assay for ornithine, 0.5 mL of the resulting supernatant was added to 1.5 mL of the ninhydrin color reagent (6 grams of ninhydrin in 100 mL of ethylene glycol monomethyl ether) and incubated at 100ºC for 15 minutes. Tubes were cooled to room temperature and the optical density recorded at 505 nm. The blank for this assay consisted of 100 µL water, 300 µL PCA and 100 µL of the potassium phosphate-sodium fluoride buffer incubated with 1.5 mL of the ninhydrin color reagent under the same assay conditions. To determine the amount of ornithine produced by AGAT, the optical density of each of the blanks (arginine alone, glycine alone, and the

colorimetric blank) was subtracted from the optical density of the arginine-glycine incubation and compared with the optical density of a known concentration of ornithine. AGAT activity was shown to be directly proportional to the amount of kidney in the incubation mixture and to the incubation time (**Figure B-7**). Also, the optical density values from the assay were all within the linear portion of the ornithine standard curve (**Figure B-8**).

B.6.2. RT-PCR Analysis of L-Arginine:Glycine Amidinotransferase (AGAT) mRNA

RNA was prepared from the kidney samples using a rapid guanidinium thiocyanate method as described by Chomczynski & Sacchi [1987]. RT-PCR analysis of AGAT mRNA was performed by Dr. Shobhitha Ratnam using a one-step reverse transcription kit from Qiagen (Mississauga, Ontario, Canada). 2 µg of RNA was reverse transcribed for 30 minutes at 50ºC and denatured at 95ºC for 10 minutes. PCR amplification consisted of 21 cycles of 3 steps: denaturation (1 minute at 95ºC), annealing (1 minute 30 seconds at 56ºC), and elongation (2 minutes at 72ºC). An upstream primer (5'-ATGCTACGGGTGCGGTGT-3') and a downstream primer (5'-ATAATTGACCTGTATGCGCG-3') were designed from a rat kidney AGAT sequence (Genbank #U07971). The primers were used to amplify a 599-basepair (bp) PCR fragment. A 768-bp fragment of the rat ß-actin gene was coamplified using amplimer set primers from BD Biosciences Clontec (Palo Alto, California, USA). PCR products were separated on a 1.5% agarose gel. Ethidium bromide stains were visualized by UV

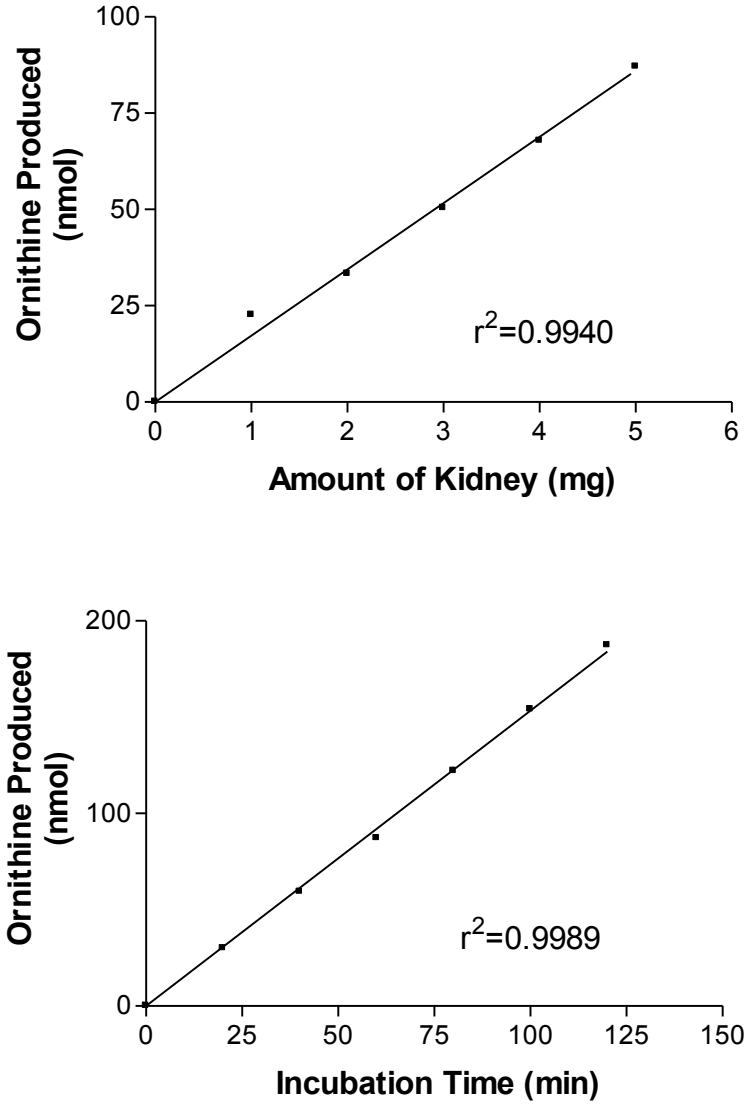


Figure B.7: Dependence of L-arginine:glycine amidinotransferase activity on amount of kidney and incubation time. Linearity with respect to protein was determined using a 60 minute incubation. The time curve was obtained using 5 mg of kidney.

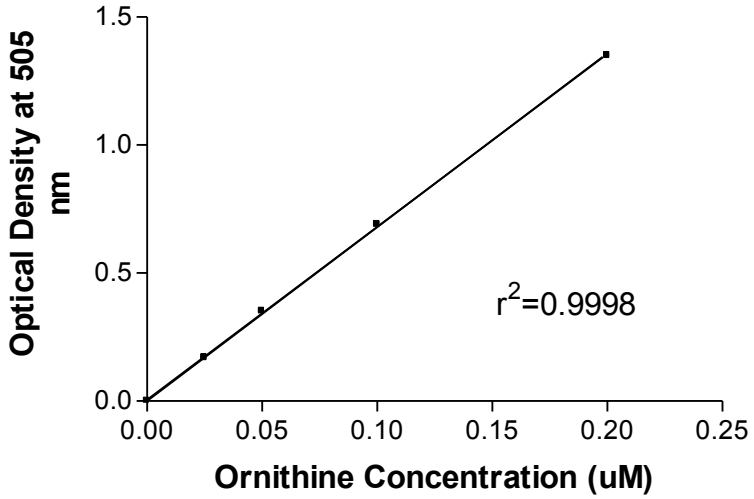


Figure B.8: Standard curve of ornithine detection by 6% ninhydrin. Linearity was shown by spectrophotometric detection of known concentrations of ornithine at 505 nm as described in **Section B.6.1**. Changes in optical density for each of the samples were within this linear range.

illumination and quantified by densitometry.

B.7. URINE ANALYSIS

Urine creatine levels were measured in previously frozen urine samples (collected as previously described; **Section B.2.2**) using a spectrophotometric method as described by Lowry & Passonneau [1972] (see **Section B.5.2**). Urine creatinine levels were measured by Dr. Edward Randell as previously described for plasma (**Section B.5.3**).

B.8. INFANT FORMULA ANALYSES

Eight different types of infant formula were purchased from various Wal-Mart stores (St. John's, Newfoundland and Labrador, Canada). Six different lot numbers of each type were purchased, except for Isomil for which only five different lot numbers were available. For details on each type of formula see **Table B-3**.

A fraction of each infant formula was centrifuged at 150 000 x g for 60 minutes to separate the aqueous phase. The aqueous phase was taken for creatinine analysis using a kit from Cayman Chemical (see **Section B.5.3** for kit details).

A separate fraction of the various formulas was deproteinized and neutralized as described for plasma in **Section B.5.2**. The deproteinized, neutralized formula was used for creatine and GAA determination as described in **Sections B.5.2** and **B.5.1**, respectively.

Table B.3: Infant Formula Information. There were 6 different lot numbers purchased for each formula type, except for Similac Advance Isomil (only 5 lot numbers were available). Preparation and analyses are explained in the text.

Formula Name	Number of Samples	Type of Formula	Mixing Directions
Similac Advance Concentrate	6	Cow's milk-based, Iron fortified	Equal amounts of formula and cooled, previously boiled, water
Similac Advance Ready to Use	6	Cow's milk-based, Iron fortified	Do not dilute with water
Similac Advance Isomil	5	Soy-based, Lactose-free	Do not dilute with water
Nestle Goodstart	6	Cow's milk-based, Iron fortified	Equal amounts of formula and cooled, previously boiled, water
Nestle Alsoy 1	6	Soy-based, Iron fortified, lactose-free	Equal amounts of formula and cooled, previously boiled, water
Enfalac with Iron	6	Cow's milk-based, Iron fortified	Do not dilute with water
Enfamil A+	6	Cow's milk-based, DHA, ARA and iron fortified	Equal amounts of formula and cooled, previously boiled, water
Enfalac Lactofree	6	Soy-based, Lactose-free	Equal amounts of formula and cooled, previously boiled, water

B.9. HUMAN MILK COLLECTION AND ANALYSIS

Twenty healthy subjects were studied after the protocol had been approved by Memorial University of Newfoundland Human Investigation Committee and the Research Approval Committee for The Health Care Corporation of St. John's. Informed written consent was obtained before collecting 5 mL of milk at either 1-2 weeks or 5-6 weeks postpartum (10 subjects for each time-period). Milk was collected at the Janeway Children's Health and Rehabilitation Centre's breastfeeding clinic using an electronic breast pump. 2 mL of blood was also collected from the subjects by nurses at the Janeway Children's Health and Rehabilitation Centre Blood Collection at these times (1-2 weeks and 5-6 weeks postpartum; consent and time permitting) to obtain about 1 mL of plasma by centrifugation as previously described in **Section B.4.1**. Milk samples were centrifuged at 100 000 x g for 60 minutes to separate the aqueous phase. Both the aqueous phase of the milk samples and the plasma samples were analyzed for GAA, creatine and creatinine as described in **Section B.5.1**, **Section B.5.2** and **Section B.5.3**, respectively.

B.10. HUMAN UMBILICAL CORD BLOOD COLLECTION AND ANALYSIS

Eighteen healthy subjects were studied after the protocol had been approved by Memorial University of Newfoundland Human Investigation Committee and the Research Approval Committee for The Health Care Corporation of St. John's. Informed written consent was obtained before collecting 2 mL of arterial and 2 mL of venous umbilical cord blood from discarded placentas after elective Caesarian birth. 2 mL of arterial blood was obtained from each mother from samples already drawn and stored for other purposes at the blood bank at the Health Science Centre. Blood samples were centrifuged as described in

Section B.4.1 to obtain about 1 mL of plasma. Plasma samples were analyzed for GAA, creatine and creatinine as described in **Section B.5.1**, **Section B.5.2** and **Section B.5.3**, respectively.

B.11. LACTATING RAT STUDIES

HPLC-grade methanol, Hypercarb HPLC columns and Scintiverse E were purchased from Fisher Scientific (Ottawa, ON, Canada). ^{14}C-creatine (creatine [4-^{14}C], with a specific radioactivity of 1.96 GBq·mmol^{-1}) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). Oxytocin was obtained from Hospira Healthcare Corporation (Montréal, QC, Canada). Ion exchange resin (AG 50W-X8 200-400 mesh) was obtained from Biorad (Mississauga, ON, Canada).

Lactating Sprague-Dawley rats (337.4 ± 16.5 g) with their pups were provided by Memorial University's breeding colony. At day 10 postpartum, each litter was standardized to 8 pups; extra pups were randomly selected and killed by inhalation of halothane (Isoflo, Abbott Laboratories, Saint-Laurent, QC, Canada). Each litter was individually housed on a 12:12-hour light-dark cycle and consumed Purina Rat Diet 5012 *ad libitum*. Milk, blood and various tissue samplings were conducted 14 days after parturition which coincides with the peak of lactation [Rodgers, 1995]. When required, size-matched virgin females were used as controls. One hour prior to milk collection, the pups were removed from the dam who was anaesthetized with an i.p. injection of 6 mg of sodium pentobarbital per 100 g body weight and injected i.p. with oxytocin (4 IU·kg body wt^{-1}) to stimulate milk release. The nipples and surrounding fur were then thoroughly

cleaned by rinsing with distilled water. Milk was collected using a vacuum milking system adapted from Rodgers [1995]. Typically, 3-4 mL of milk were collected from each dam in less than 10 minutes. The collected milk was immediately frozen in liquid nitrogen and kept at -80ºC until further analysis. Immediately following milk collection, arterial blood was drawn from the dam's abdominal aorta into a heparinised syringe and centrifuged at 6000 X g at 4ºC for 10 minutes. Plasma was removed and stored at -80ºC until further analysis.

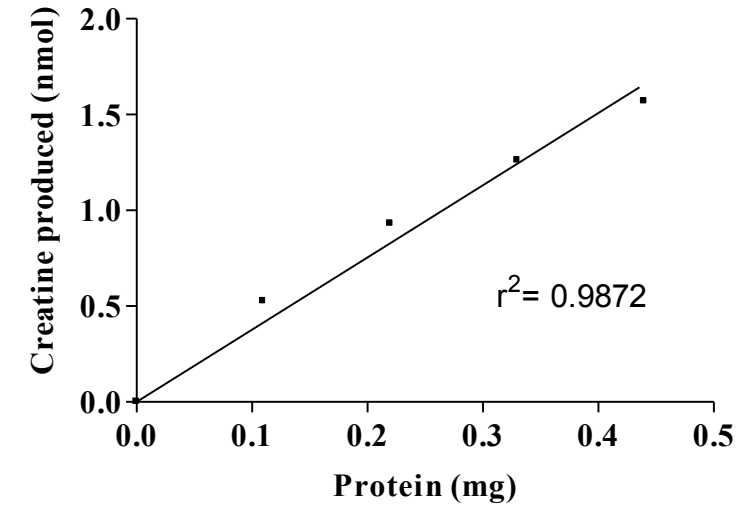
B.11.1. *In vivo* GAA production, milk and plasma creatine concentration, and renal AGAT activity

The renal GAA output was measured in plasma from lactating and virgin rats (n=7) as described previously in **Section B.4** and **Section B.5.1**. Kidney samples were immediately freeze-clamped and stored at -80°C. AGAT was assayed in kidney samples that were frozen for less than a week, as previously described in **Section B.6.1**.

B.11.2. Hepatic GAMT activity

GAMT was measured on fresh liver samples using the method of Ogawa *et al.* [1983], except that creatine production was quantified by HPLC [Buchberger & Ferdig, 2004]. Fresh rat liver was homogenized in 4 volumes of a buffer solution consisting of 0.25 M sucrose, 10 mM HEPES and 1 mM EDTA. Homogenates were centrifuged at 100,000 x g at 4ºC for 1 hour and supernatant assayed for GAMT activity. Basically, the supernatant was incubated at 37ºC for 10 minutes in 35 mM Tris buffer (pH 7.4), 7 mM 2-mercaptoethanol and 50 μM S-adenosylmethionine. The assay was started by addition

of 0.2 mM GAA. Blanks for the assay did not contain GAA. After incubation, the assay was stopped by addition of 75 µL of 15% (w/v) TCA. The assay mixture was immediately neutralized by the addition of 72 µL of 1 M Tris (pH 7.4) and centrifuged at 10,000 x g for 5 minutes at room temperature to pellet the precipitated protein. Supernatant samples were derivatized by mixing 400 uL of the ultra-filtrated sample solution with 300 uL of 1.3 M potassium hydroxide and 150 uL of 0.9% aqueous ninhydrin solution. The samples were allowed to sit for 15 minutes at room temperature and then 100 uL of 5% ascorbic acid and 100 uL of 5 M phosphoric acid were added. Ascorbic acid was added to the reaction solution prior to the acidification with phosphoric acid to act as an antioxidant and minimize oxidative decomposition. The closed vials were then incubated at 90ºC for 30 minutes. Samples were allowed to cool at room temperature for 5 minutes, filtered, and 50 uL was injected into the HPLC column (Hypercarb YMC 150 mm X 4.6 mm from Fisher Scientific, Ottawa, ON, Canada). The mobile phases consisted of 50 mM formic acid (Buffer A) and 100% methanol (Buffer B). At time 0, Buffer A was 95% and Buffer B was 5%. Over 15 minutes Buffer A changed to 10% and Buffer B, 90%. This remained constant for 5 minutes. Over 2 minutes Buffer A went back to 95% and Buffer B, 5%. This was maintained for 10 minutes. The flow rate was 1 mL per minute. Excitation was at 390 nm and emission was at 470 nm. Dependence of GAMT activity on both the amount of protein and on time is shown in **Figure B.9**. Total protein was measured using the Biuret method.



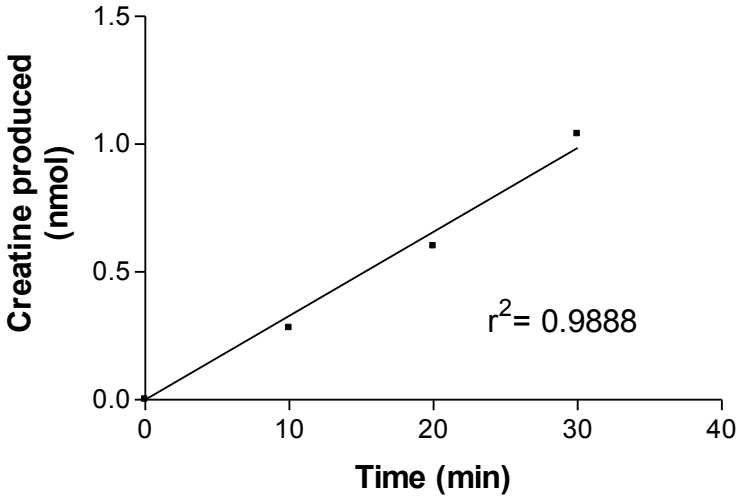


Figure B.9: Dependence of guanidinoacetate methyltransferase activity on amount of protein and incubation time. Linearity with respect to protein was determined using a 20 minute incubation. The time curve was obtained using 5 mg of liver. Both curves were produced from work completed by Dr. Simon LaMarre.

B.11.3. Determination of creatine specific radioactivity

This involved the determination of both the creatine concentration and its associated radioactivity. Lactating rats were administered 740.9 kBq ^{14}C-creatine (prepared in 0.5 mL saline) via the caudal vein on day 11 postpartum and immediately returned to their pups. After a 3 day equilibration period, milk and blood samples were drawn.

We used a modification of the HPLC method of Gu & Lim [1990] to measure the creatine concentration; the eluate from the HPLC column was collected by fraction collection and the creatine peak counted in a liquid scintillation spectrometer. Milk and plasma samples (1 mL) were deproteinized by the addition of 100 µL of ice-cold trifluoroacetic acid (TFA), placed on ice for 10 minutes and the precipitated protein pelleted at 10,000 X *g* for 10 minutes at 4 °C. The supernatant was immediately applied to a Bond-Elut C18 solid-phase extraction cartridge (100 mg, 1 mL; Varian Inc., Paulo Alto, CA, USA) and the eluate collected. The cartridge was washed with 1 mL dH_2O and eluate was again collected. The pooled eluate was applied to a disposable chromatography column containing 2 mL of cation-exchange resin using sodium as a counterion. It was followed by two 3 mL water washes and this eluate was discarded. Creatine bound to the resin was eluted with 1 mL of 2 M ammonium hydroxide. This eluate was freeze-dried and dissolved in 250 µL of the mobile phase immediately prior to HPLC analysis. A 100 µL volume of the dissolved eluate was injected onto a porous graphitized carbon column (Hypercarb, 100.0 x 4.6 mm). The column was eluted isocratically with 1% TFA and 2% methanol; the absorbance was monitored at 210 nm. Fractions were collected every 30 seconds for 15 minutes. All fractions were mixed with 10 mL of Scintiverse E and

counted for 10 minutes on a LKB 1214 Rackbeta liquid scintillation counter (LKB, Gaithersburg, MD, USA). Creatine eluted at approximately 4 minutes and the corresponding fractions were the only ones containing substantial quantities of radioactivity. The creatine concentration was referenced to a set of creatine standards (0 to 1 mM) that was processed in parallel to the samples (including solid phase extraction and ion exchange chromatography). The creatine specific radioactivity was expressed as $mBq \cdot nmol^{-1}$.

B.11.4. Creatine content of adult tissues and pups

To determine whether rats' total creatine pool becomes depleted during lactation, we measured the creatine concentration of muscle and brain (2 major pools of creatine) in lactating and virgin females (n=4). Tissues were homogenized in 150 mM sodium phosphate (pH 7.4). Rat pups were killed at days 10, 15, and 20 (n=6) by halothane inhalation and each pup was homogenized. The homogenates were allowed to stand for 30 minutes at room temperature to allow for the conversion of creatine-phosphate to creatine. The total creatine content was measured by HPLC as described in **Section B.11.2.** and **Section B.11.3.** and was reported as $nmol \cdot g^{-1}$ for adult tissues and $\mu mol \cdot g^{-1}$ for the pups.

B.12. STATISTICAL ANALYSES

In studies with only two groups, a student's unpaired t-test was used to determine significant differences. When variances were heterogeneous, the Mann-Whitney U test was used. One-way ANOVA followed by Newman-Keuls post-hoc tests were used in

studies involving three or more experimental groups. Tukey's post hoc test was used for the lactating rat experiments. A P value of <0.05 was taken as significant.