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EFFECTS OF SHORT-TERM CREATINE SUPPLEMENTATION ON WHOLE-BODY PROTEIN METABOLISM

EFFECTS OF SHORT-TERM CREATINE SUPPLEMENTATION ON WHOLE-BODY PROTEIN METABOLISM

By

Saša Mihić, B.Ph.E.

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AUTHOR:	Saša Mihić, B.Ph.E. (University of Novi Sad, Yugoslavia)
SUPERVISOR:	Dr. Mark A. Tarnopolsky, M.D., Ph.D., FRCP(C)
SUPERVISORY COMMITTEE:	Dr. Stephanie Atkinson, Ph.D. Dr. Duncan MacDougall, Ph.D. Dr. Stuart Phillips, Ph.D. Dr. Peter Lemon, Ph.D.
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Abstract

Creatine-monohydrate (CrM) supplementation has been shown to increase body mass and fat-free mass (FFM), however, the mechanism by which CrM affects body composition has not been determined. We investigated the effects of short-term CrM supplementation on whole-body protein turnover in 27 recreationally active male and female volunteers. Subjects underwent measurements prior to and following 9-10 days of CrM (20 g/d x 5 d, 5 g/d x 4-5 d) (n = 14), or placebo (PL) (n = 13) supplementation. Protein turnover was assessed using L-[1-^{13}C] leucine stable isotope tracer, urinary urea nitrogen (N) excretion, and N-balance (Nbal) techniques. Total body mass (TBM), leucine flux, leucine oxidation, non-oxidative leucine disposal (NOLD), 24-hr urinary urea N excretion, and Nbal were determined before and after treatment. Additionally, the effects of CrM supplementation on renal function and metabolite clearance were evaluated by measuring creatinine (CTN) excretion, plasma CTN concentration, and CTN clearance. There was no effect for CrM as compared to PL on TBM, leucine flux, leucine oxidation, or NOLD. However, leucine oxidation was lower for the CrM-treated males as compared to the PL-treated males following supplementation ($P < 0.05$). Leucine flux and NOLD were higher for the males vs. the females ($P < 0.05$). Neither urinary urea N excretion nor Nbal were affected by treatment. Plasma [CTN], CTN excretion, and CTN clearance were also unchanged for CrM vs. PL. These findings

suggested that CrM supplementation may have an effect upon leucine oxidation in males, yet there were no effects seen in females, nor were other indices of leucine turnover altered by CrM supplementation. Furthermore, short-term CrM supplementation did not have any adverse effects on renal function.

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In Serbian: hvala vama, Dušane i Ljiljana, jerbo bez vas i eurokrema mene ne bi bilo.

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ADP	adenosine diphosphate
AGAT	arginine:glycine amidinotransferase
ANOVA	analysis of variance
APE	atom percent excess
ATP	adenosine triphosphate
BIA	bioelectric impedance analysis
BM	body mass
BUN	blood urea nitrogen
cAMP	cyclic adenosine monophosphate
CHO	carbohydrate
CK	creatine-kinase
Cr	(free) creatine
[Cr]	creatine concentration
CrM	creatine-monohydrate
CrT	creatine transporter
CSA	cross-sectional area
CTN	creatinine
CV	coefficient of variation
CytCK	cytosolic creatine kinase
DM	dry mass
DXA	dual-energy x-ray absorptiometry
ETC	electron-transport chain
GC/MS	gas chromatograph/mass spectrometer
β-GPA	beta-guanidino propionic acid
FFM	fat-free mass
FSR	fractional synthetic rate
g	gram
H^+	hydrogen ion
K^+	potassium ion
Kcal	kilocalories
α-KIC	alpha-ketoisocaproic acid
L	liter
LBM	lean body mass
^{13}C leucine	stable isotope leucine tracer
ml	milliliter
mg	milligram
min	minute

List of Abbreviations (cont.'d)

mtCK	mitochondrial creatine kinase
µmol	micromol
mmol	millimol
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MVC	maximal voluntary contraction
MyHC	myosin heavy-chain
n	subject sample size
N	nitrogen
Na^{2+}	sodium ion
Nbal	nitrogen balance
NH_4^+	ammonium ion
NH_3	ammonia
NOLD	non-oxidative leucine disposal
O_{leu}	leucine tracer oxidation
PCr	phosphocreatine
[PCr]	phosphocreatine concentration
PL	placebo
Q_{leu}	leucine tracer flux
Ra	rate of appearance
Rd	rate of disappearance
1 RM	one maximal repetition
SAM	S-adenosyl methionine
SAM:GAMT	SAM:guanidinoacetate methyltransferase
SD	standard deviation
SIM	selected-ion monitoring
TBM	total body mass
TBW	total body water
TCr	total Cr
[TCr]	total Cr concentration
$\dot{V}CO_2$	volume of carbon-dioxide produced
$\dot{V}O_2$	volume of oxygen consumed
WBPS	whole-body protein synthesis

CHAPTER 1

Introduction

1.1 Creatine: metabolism and function

1.1.1 *Intake, synthesis, and regulation*

Creatine (Cr) is a nitrogen-containing compound found in most mammalian cells. In a 70-kg man, there is a total of ~120 g of Cr, stored primarily in muscle and nerve tissues as phosphocreatine (PCr), or as free Cr (Walker, 1979). Isotope decay studies with [^{14}C] Cr have shown that Cr turnover is rather slow, with a half-life of 38-42 days (Fitch and Sinton, 1964). Each day, approximately 1.7% (1.5-2 g/d) of the total Cr pool is being converted non-enzymatically into creatinine (CTN) in a non-reversible fashion, and is subsequently lost via urinary excretion (Chanutin and Guy, 1926; Crim et al., 1976; Walker, 1979). Tight regulation of Cr turnover is reflected by the constancy of CTN excretion within an individual, varying only during extreme changes of Cr intake (e.g., Cr supplementation, vegetarian diet), and/or due to defects in protein or Cr metabolism (Benedict and Osterberg, 1923; Fitch and Sinton, 1964).

The amount of Cr lost in the urine in the form of CTN and Cr, the latter being rather insignificant, is normally replenished by both exogenous dietary intake, and endogenous biosynthesis. Primary dietary sources of Cr are red meat, poultry and fish; ~200 g of raw, uncooked beef contains about 1 g of Cr (Harris et al., 1992). However, in

vegetarians, for instance, daily needs have to be met entirely by *de novo* biosynthesis of Cr. In this population, muscle [Cr], serum [CTN] and CTN excretion are lower than normal reference values (Delanghe et al., 1989), indicating that the lack of dietary Cr cannot be entirely replaced by endogenous synthesis. Cr synthesis occurs in the liver, kidneys and pancreas, which presumably are the primary and perhaps only sites of Cr synthesis in man (Sandberg et al., 1953; Walker, 1979; Wyss and Wallimann, 1994). The synthesis from the amino-acids arginine, glycine, and methionine occurs in two steps (Figure 1). In the first step, the amidino group from arginine is transferred to glycine to form guanidinoacetate and ornithine, a reaction that is catalyzed by the enzyme L-arginine:glycine amidinotransferase (transamidinase; AGAT). This enzyme is most abundant in the liver of humans and rabbits, but not in common laboratory animals (i.e., dog, rat), where AGAT expression is greatest in kidneys and pancreas (Walker, 1979; Wyss and Wallimann, 1994). After its formation, guanidinoacetate is methylated by S-adenosylmethionine (SAM) to produce Cr, via the catalytic action of SAM:guanidinoacetate methyltransferase (GAMT). Importantly, the AGAT step, but not that of GAMT, seems to be rate-limiting for Cr synthesis (Walker, 1979). It has been shown that there is end-product inhibition of AGAT expression and activity by extracellular Cr (Ramirez et al., 1970; McGuire et al., 1984). Fasting, severe muscle-wasting diseases, and Cr feeding/administration, have all been reported to raise plasma Cr levels, hence reducing AGAT levels in liver, pancreas and kidney (Wyss and Wallimann, 1994). Similar effects on kidney AGAT activity have been demonstrated by

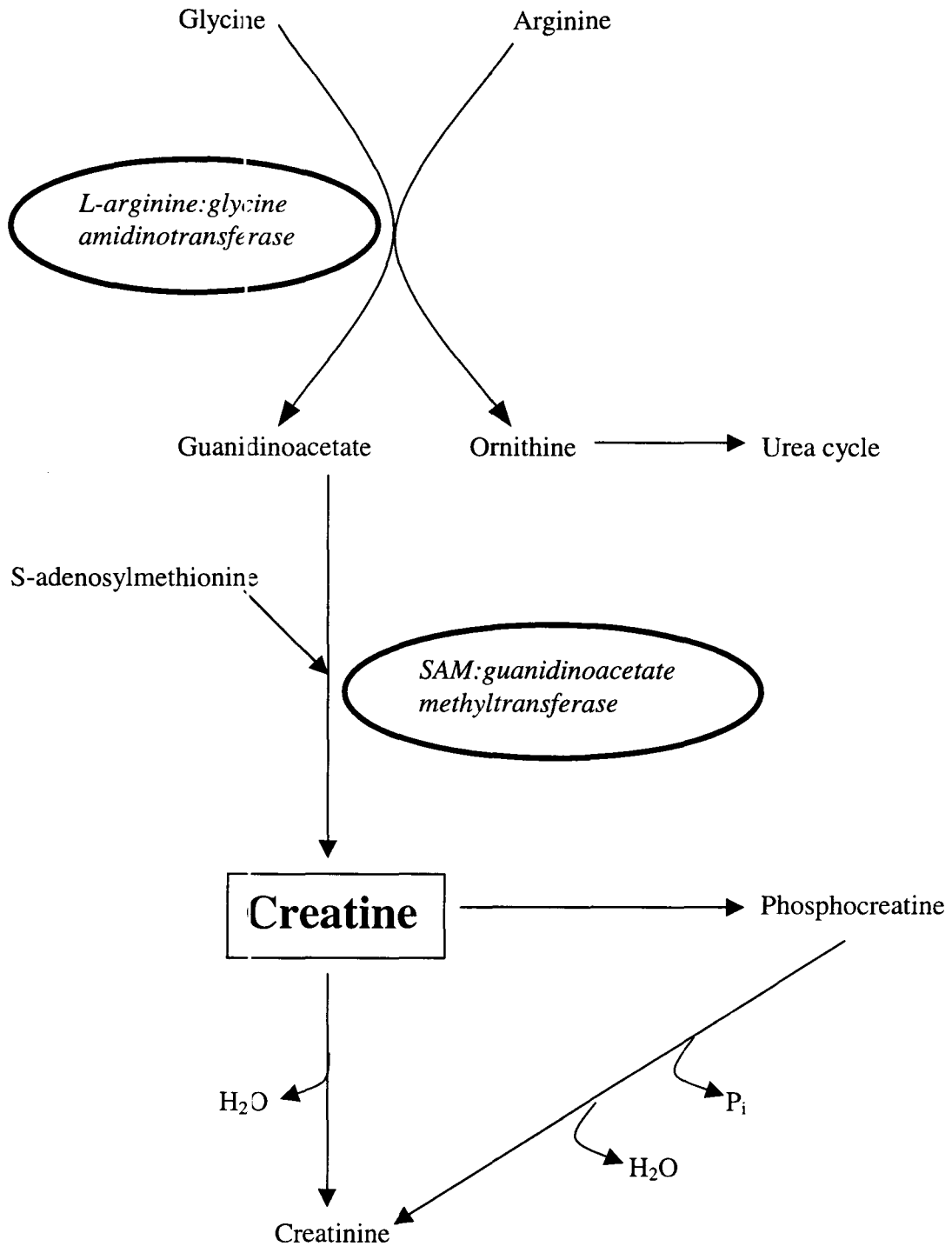


Figure 1. Creatine metabolism (modified from Wyss and Wallimann, 1994, Fig. 1).

hypophysectomy and thyroidectomy of rats, which are completely reversed by subsequent injection of growth hormone and thyroxine, respectively (Van Pilsum et al., 1970). Indeed, rat kidney AGAT concentration and activity are stimulated by growth hormone (McGuire et al., 1980), and the rate of Cr synthesis is stimulated by methyltestosterone (Hoberman et al., 1948).

1.1.2 *Transport and uptake*

From the sites of its synthesis, Cr is transported in the plasma (in physiological concentrations of ~40-100 μM; Delanghe, 1989; Harris et al., 1992) to tissues such as skeletal muscle, brain, heart, and peripheral nervous tissue (Wyss and Wallimann, 1994). The uptake and transport of Cr into the cell is a saturable, Na^+-dependent process, which likely utilizes the electro-chemical potential across cell membrane via the Na^+-K^+ pump (Fitch and Shields, 1966; Daly and Seifter, 1980; Loike et al., 1986; Loike et al., 1988), to overcome a large concentration gradient (200:1) of Cr between the intra- and extracellular spaces (Harris et al., 1992). Cr transport across plasma membrane is achieved by a Cr transporter protein (CrT), which is most abundant in Cr-utilizing tissues, i.e., skeletal muscle, heart, brain, nerve tissue, testes, and kidney (the site of Cr excretion and reabsorption; Guimbal and Kilimann, 1993; Sora et al., 1994). CrT expression and Cr uptake have been shown to be inhibited by high extracellular [Cr] (Loike et al., 1988; Guerrero-Ontiveros and Wallimann, 1998). Some experiments have suggested that high intracellular Cr concentration may also suppress Cr uptake by decreasing CrT expression (Berlet, 1975). On the other hand, insulin (Brivio Haugland and Chang, 1975; Steenge et al., 1998), muscle contraction (Harris et al., 1992), as well

as carbohydrate ingestion (Green et al., 1996), have all been reported to enhance Cr uptake by skeletal muscle both *in vitro* and *in vivo*.

1.1.3 *Storage and function*

Approximately 90-95% of Cr is stored in skeletal muscle, with a concentration of ~125 mmol/kg dry muscle mass[1] (total Cr, TCr; Forsberg et al., 1991; Harris et al., 1992). Of this, ~75-80 mmol/kg dm is found in its phosphorylated form (PCr), the rest (~45-50 mmol/kg dm) being stored as free Cr (Harris et al., 1992). These values are somewhat lower for vegetarians (Harris et al., 1992), elderly persons (Forsberg et al., 1991; Smith et al., 1998), and patients suffering from muscle-wasting diseases (Wyss et al., 1998). Gender does not appear to have an effect on intracellular concentrations, although females tend to have higher resting muscle [TCr] (Forsberg et al., 1991; Harris et al., 1992), in spite of well-documented lower dietary protein and/or Cr intake (Phillips et al., 1991; Tarnopolsky et al., 1997). Additionally, there seems to be an upper limit for muscle TCr concentration of ~160-180 mmol/kg dm (Harris et al., 1992; Hultman et al., 1996), likely due to the tight regulation of Cr transport and uptake that has been discussed above.

The creatine-kinase (CK) reaction is the only known enzymatically catalyzed reaction of Cr in the cell. CK catalyzes the reversible transfer of high-energy phosphate between PCr and Cr:

$$ADP + PCr + H^+ \leftrightarrow ATP + Cr \qquad \text{(Equation 1)}$$

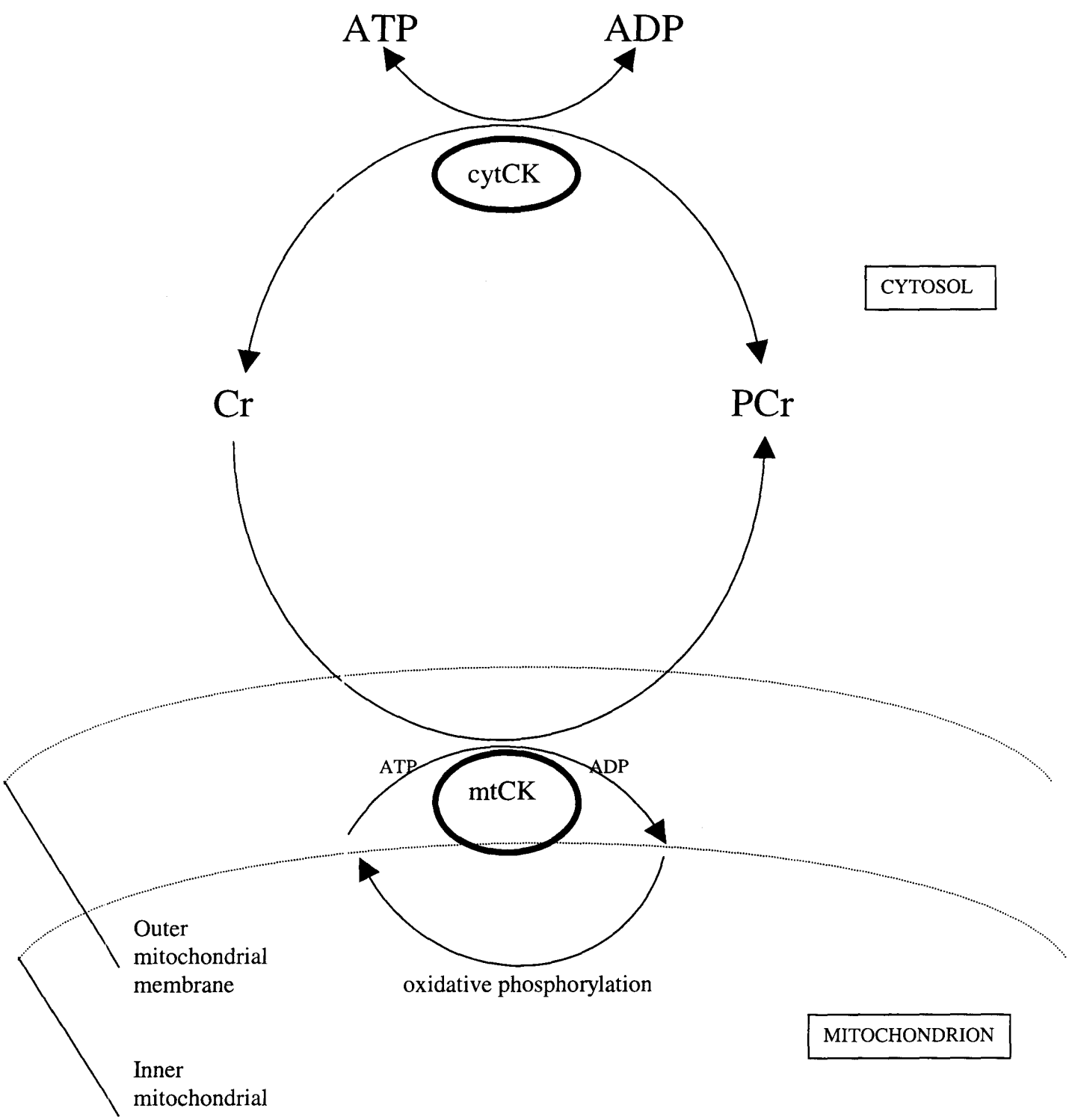
[1] Dry mass = mass after water extraction (approx. 20-25% of total muscle mass).

In the reaction, which occurs in the cytosol, PCr acts as a "phosphate donor" for ADP, thereby maintaining ATP levels as well as minimizing fluctuations of ATP/ADP ratio during muscle contraction. Interestingly, fast-twitch muscle fibers normally display greater resting [PCr] and ATP turnover rate during muscular work, thus indicating greater dependence on the CK reaction for ATP repletion (Katz et al., 1986; Wyss and Wallimann, 1994). The cytosolic CK (cytCK) is thought to be intimately linked to the sites of ATP utilization, such as next to the M-line of myofibrils, sarcoplasmic reticulum, sarcolemmal membrane, and ribosomes (Savabi et al., 1983; Meyer et al., 1984; Bessman and Savabi, 1988). In this way, the enzyme is readily available for effective buffering of changes in ATP and ADP concentrations during periods of increased demand for energy ("temporal buffering" role of CK, as proposed by Meyer et al., 1984). PCr has also been proposed to be a preferred substrate for myofibrillar high-energy phosphate transfer, as compared to externally supplied ATP (Meyer et al., 1984), providing further evidence for the role of PCr and Cr during muscle contraction.

In addition to this "classic view" of the role of Cr as a temporal buffer, it has also been proposed to function as a "spatial buffer" (Meyer et al., 1984). Spatial buffering, or "PCr energy shuttle" (Bessman and Geiger, 1981; Savabi et al., 1983; Kammermeier, 1987; Bessman and Savabi, 1988; Saks et al., 1993; Fedosov, 1994), refers to the transport of energy, i.e., high-energy phosphate, between mitochondria (sites of ATP production) and the cytosol (site of ATP utilization; Figure 2).

Two key findings support the existence of PCr energy shuttle; one is the discovery of a distinct mitochondrial CK isozyme (mtCK), located at the outer portion of

Figure 2. PCr energy shuttle (Modified from Juhn and Tarnopolsky, 1998).



the inner mitochondrial membrane (Bessman and Geiger, 1981; Meyer et al., 1984). MtCK may catalyze PCr resynthesis by utilizing ATP, produced by mitochondrial ETC (electron-transport chain), and Cr as substrates. From there, newly synthesized PCr can shuttle back into the cytosol to re-phosphorylate local ADP, generating a new ATP molecule (Meyer et al., 1984; Bessman and Savabi, 1988). The other important finding is that muscle contraction has been shown to cease following complete PCr depletion, even though [ATP] remains almost unchanged, indicating that PCr-mediated energy transfer from the sites of ATP synthesis to the sites of its utilization is essential in order membraneto sustain contraction (Bessman and Savabi, 1988). Furthermore, in the presence of externally supplied ADP, PCr can produce a faster, stronger contraction with a shorter relaxation time, than can the same amount of ATP alone (Savabi et al., 1983). Meyer et al. (1984) suggested that PCr shuttle can be particularly important in large cells with non-uniform distribution of mitochondria and large diffusion distances between sites of ATP synthesis and utilization, as is the case in fast-twitch, glycolytic muscle fibers and neuronal axons.

Cr/PCr and the CK reaction may also act as a proton (H^+) buffer (Volek and Kraemer, 1996), regulator of glycolysis (Storey and Hochachka, 1974; Meyer et al., 1984; Meyer et al., 1986), and a stimulator of protein synthesis (Ingwall et al., 1972; Young and Denome, 1984; Bessman and Savabi, 1988).

1.2 Effects of creatine on muscle protein synthesis

Cr may stimulate muscle protein synthesis both *in vitro* and *in vivo* (Ingwall et al., 1972; Ingwall et al., 1974; Zilber et al., 1975; Ingwall and Wildenthal, 1976; Young and

Denome, 1984). Ingwall et al. (1972) put forward a hypothesis that Cr, an end-product of muscular contraction (following PCr hydrolysis to Cr and ATP), could potentially increase the rates of contractile protein synthesis, thereby inducing muscle hypertrophy. Indeed, this and other experiments performed by this group using the amino acid isotope (^{3}H leucine) incorporation technique, demonstrated increased rates of myosin heavy-chain (MyHC) and actin synthesis by as much as twofold (Ingwall and Wildenthal, 1973; Ingwall et al., 1974; Ingwall and Wildenthal, 1976). These findings were later reproduced in similar conditions (Zilber et al., 1975; Young and Denome, 1984).

Specifically, in differentiating chick skeletal muscle cultures (Ingwall et al., 1972; Ingwall et al., 1974; Zilber et al., 1975; Ingwall and Wildenthal, 1976), steady-state chicken muscle-cell cultures (Young and Denome, 1984), and chick and mouse heart organ cultures formed *in vivo* (Ingwall and Wildenthal, 1973; Ingwall and Wildenthal, 1976), Cr has been shown to selectively stimulate ^{3}H leucine incorporation into myofibrillar proteins. However, Cr appears to have no effects on the mixed-muscle protein synthesis rates. Studies suggest that an increase in ^{3}H leucine incorporation into MyHC may be accompanied by a similar increase in MyHC content in Cr-treated skeletal muscle cultures (Young and Denome, 1984). Some Cr analogs have also been effective in stimulating the synthetic rates of MyHC and actin, but not those of mixed-muscle protein (Ingwall et al., 1974).

Protein degradation does not seem to be influenced by Cr. Half-life for MyHC was ~15 hr, however it was not different between the Cr-treated and control chick embryo skeletal muscle cultures (Ingwall et al., 1974).

An investigation by Fry and Morales (1980) found Cr to be ineffective in stimulating MyHC or actin synthesis *in vitro*, under very similar conditions that had previously proven to increase MyHC synthesis by others (Ingwall et al., 1972; Zilber et al., 1975; Young and Denome, 1984). Furthermore, CMIP (carboxy-methyl-iminohexahydro-pyrimidine), a Cr analog previously found to stimulate MyHC synthesis (Ingwall et al., 1974), depressed both total and MyHC protein synthesis (Fry and Morales, 1980). In another study, PCr was reported to lower the content of "water extractable" protein (Matiushichev et al., 1992). It is peculiar that no attempts were made on behalf of these two groups to account for the obvious discrepancies between their results and those obtained in several other studies (Ingwall et al., 1972; Ingwall et al., 1974; Zilber et al., 1975; Young and Denome, 1984).

These contradicting findings raise the question of the potential mechanism(s) by which Cr might influence protein synthesis in skeletal muscle. Cr may function either directly, at the molecular level, or via an indirect mechanism, i.e., by activating CK, and/or improving the cell's energetics. However, there is evidence which may rule out some of the indirect mechanisms of action. Ingwall et al. (1974) demonstrated that some Cr analogs successfully mimicked the effects of Cr on myofibrillar protein synthesis, irrespective of their reactivity as substrates for the CK reaction. This finding suggests that CK reaction may not be involved in the mechanism(s) of Cr action. Moreover, Cr action appears to be independent of its potential to increase the energy charge of the cell (Ingwall, 1976). Suggesting otherwise, inhibition of CK has been shown to suppress protein synthesis in extracted rat diaphragm (Carpenter et al., 1983), which prompted

Bessman and Savabi (1988) to hypothesize that the CK-mediated energy transport across cell might somehow regulate the rate of protein synthesis.

On the other hand, there are indications that Cr could stimulate MyHC and actin synthesis directly, at either the transcriptional or translational level, or perhaps both of them. Zilber et al. (1975) suggested that Cr's regulatory function may be linked to the nucleus itself, and is likely accomplished through "successive events at the transcriptional level". Indeed, an addition of 5 mM of Cr to growing cultures of chick embryo myoblasts increased isotope incorporation (^{14}C orotic acid) into total cellular RNA by 1.5-fold, and stimulated RNA-polymerase activity by ~2-fold (Zilber et al., 1975).

The content of MyHC mRNA was 15% higher in steady-state chicken muscle-cell cultures treated with Cr vs. controls (Young and Denome, 1984). It accounted only partially for the higher MyHC content (by ~60%), and ^{3}H leucine incorporation into MyHC (greater by ~30-40% vs. controls; Young and Denome, 1984). These findings have indicated that Cr may regulate post-transcriptional molecular events leading to embryonic skeletal muscle protein synthesis.

This brief review suggests that Cr may have the potential for stimulating contractile protein synthesis. However, the mechanism(s) for this effect remains unclear twenty-five years later, perhaps because very few studies have been done on the subject after the initial reports (Ingwall et al., 1972; Ingwall et al., 1974; Zilber et al., 1975; Ingwall and Wildenthal, 1976; Young and Denome, 1984). There is convincing evidence suggesting that extracellular Cr increases MyHC and actin synthesis rates by

approximately twofold (Ingwall et al., 1972, 1974, and 1976; Zilber et al., 1975; Young and Denome, 1984). Another important argument is that the externally supplied Cr maximally stimulated protein synthesis at >0.1 mM, the concentrations similar to those observed in Cr-supplemented humans (Harris et al., 1992). However, one of the major concerns have been the age of the cultured muscle tissue, considering that all the above experiments were conducted on the myoblasts undergoing differentiation. Cr seems to be a stimulant of on-going contractile protein synthesis, and we have still not answered the question whether Cr has a "growth" effect upon fully differentiated skeletal muscle.

1.3 Cellular hydration: role in protein turnover

Several studies in the early '90's provided strong evidence that protein synthesis and degradation were greatly dependent on the hydration state of the cell, i.e., its volume (Haussinger et al., 1991; Stoll et al., 1992; vom Dahl and Haussinger, 1996). Using a model of ^{3}H leucine isotope release from the perfused rat liver, Haussinger and colleagues (1991) showed that proteolysis was decreased following experimentally-induced hepatocyte swelling. This finding was consistent independent of the means used to increase cell volume, i.e., insulin, amino acids (glutamine and glycine), or simple changes of the osmotic pressure across cell membrane (hypoosmotic exposure). On the other hand, proteolysis was promoted by cell shrinkage, when either glucagon, cAMP, or hyperosmotic agents were introduced into the perfusate (Haussinger et al., 1991). A close positive relationship was found between the intracellular water content and the degree of proteolysis inhibition (Haussinger et al., 1991).

Swelling-induced inhibition of proteolysis in perfused rat livers was also demonstrated by vom Dahl and Haussinger (1996). Again, there was a significant relationship between cell volume increase and the extent of proteolysis inhibition. A ~1% increase in cell volume corresponded to a ≤2% inhibition of proteolysis in fed animals. Interestingly, this inhibition was even greater in livers from fasted (24 hr) rats (~2.2% for every ~1% increase in water space). Unlike the effect upon proteolysis, insulin-, glutamine-, or hypoosmolarity-induced cell swelling did not alter protein synthesis in isolated rat hepatocytes, as measured by ^{3}H leucine incorporation into protein (Stoll et al., 1992). However, when these hepatocytes were pre-shrunk, and the rate of protein synthesis became impaired, insulin, glutamine, and hypoosmotic exposure did indeed stimulate ^{3}H leucine incorporation into protein. This may suggest that in various pathological states, protein synthesis may be increased by an increased cellular hydration state, which seems unlikely when changing from euhydration to hyperhydration.

Gene expression may also be modified by cell volume (Haussinger, 1996). This seems to include not only osmoregulatory genes (e.g., osmolyte transporters in renal cells and astrocytes), but also those that are not coding for proteins coupled with osmotic function. Schulz et al. (1991) found increased levels of β-actin mRNA during hypoosmotically-induced cell swelling in perfused rat livers. This is especially relevant in relation to the work being presented in this thesis, for the whole-body protein metabolism has been shown to be partly dependent on myofibrillar protein (primarily actin and myosin) turnover (Lowry et al., 1985; Nair et al., 1988).

Poor hydration state of liver and skeletal muscle has been hypothesized to trigger the catabolic conditions commonly seen in malnutrition, severe trauma, sepsis, and various diseases (Haussinger et al., 1993). Decreased cellular water content is usually accompanied by a negative N-balance, severe nitrogen loss, and increased 3-methylhistidine excretion, an index of myofibrillar protein degradation (Long et al., 1981). Although a causal relationship between cellular hydration state and these outcomes has been proposed to exist from correlational data (Haussinger et al., 1993), no direct evidence has been presented to date to support this observation under experimentally manipulated conditions in humans.

1.4 Creatine supplementation

Oral Cr ingestion of 20-30 g/day for 5 days has been shown to increase skeletal muscle stores of total creatine (TCr) by 20-30% (Harris et al., 1992; Balsom et al., 1995; Hultman et al., 1996; Green et al., 1996). Additionally, Cr uptake and accumulation within skeletal muscle may be further enhanced by submaximal resistance exercise (Harris et al., 1992), simultaneous glucose ingestion (Green et al., 1996), and insulin (Steenge et al., 1998), suggesting that muscle contraction and/or circulating insulin may add to the effects of Cr supplementation on resting levels of TCr in human skeletal muscle. Following this acute elevation, muscle [TCr] can be maintained with only 2 g/d (approx. the rate of daily Cr loss through creatinine excretion in urine), for at least one month (Hultman et al., 1996). Similar increases in skeletal muscle [TCr] are also seen with Cr supplementation of 3 g/d for 28 d (Hultman et al., 1996). Individual responses to Cr supplementation can vary greatly, but Cr ingestion appears to be most effective in

increasing [TCr] in individuals with lowest muscle TCr content; accordingly, the increase is less marked in subjects with high endogenous muscle [TCr] (Harris et al., 1992; Green et al., 1996).

Considering that about 25-30% and ~70-75% of TCr accumulation following supplementary Cr ingestion can be attributed to increases in muscle [PCr] and [Cr], respectively (Harris et al., 1992; Balsom et al., 1995; Febbraio et al., 1995; Hultman et al., 1996; Green et al., 1996; Vandenberghe et al., 1997; Smith et al., 1998), it has been hypothesized that Cr supplementation could improve physical performance. The mechanism(s) behind Cr enhancing muscle performance is still unclear, but it is probably a combination of several factors. It has been demonstrated that ~20 g/d of Cr for 5-7 days can increase skeletal muscle contents of TCr, PCr, and Cr (Harris et al., 1992; Balsom et al., 1995; Febbraio et al., 1995; Hultman et al., 1996; Green et al., 1996; Vandenberghe et al., 1997). With this in mind, it has been speculated that during exercise, following Cr supplementation, elevated [PCr] would presumably accelerate ATP repletion, which in turn could improve average power output, and also better maintain power output during exercise. During recovery, increased availability of Cr could provide substrate for more rapid PCr resynthesis, and thus generating a higher peak power output at the beginning of the subsequent exercise bout(s). Yet, contemporary evidence for this is either unconvincing (Greenhaff, 1994), or is not supportive of the premise (Thompson et al., 1996; Smith et al., 1998).

Following another line of evidence, the CK reaction (Sec. 1.1.3) has been suggested to act as a proton buffer (Febbraio et al., 1995; Vandenberghe et al., 1996) by

decreasing post-exercise H^+ accumulation, and thus limiting the drop in pH (Balsom et al., 1995). Hence, the rate of post-exercise PCr resynthesis may be enhanced, since PCr resynthesis is significantly slowed by low pH (Arnold et al., 1984).

In activities such as weight-lifting, for instance, the beneficial effects of Cr supplementation upon muscular strength and power may also originate from the stimulation of FFM (Vandenberghe et al., 1997; Kreider et al., 1998; Mihic et al., 1998), as well as myofibrillar protein synthesis (Ingwall et al., 1972; Ingwall et al., 1974), two potential mechanisms which are being addressed in this literature review (Sec.'s 1.4.2 and 1.2, respectively).

Unless noted otherwise, a standard dosage of ~20 g/d x 5-7 d ("Cr loading"), with or without a subsequent maintenance dose of 2-5 g/d, has been used in the following studies.

1.4.1 Effects of Cr supplementation on muscle performance

1.4.1.1 *Cr supplementation, weight-lifting and isokinetic power of knee extensors*

Cr may have an effect upon muscular strength in high-intensity, short-lasting activities (Greenhaff et al., 1993; Earnest et al., 1995; Vandenberghe et al., 1996; Vandenberghe et al., 1997; Kreider et al., 1998). Following Cr supplementation, increased peak and average torque production have been reported during repeated isokinetic knee extensions (Greenhaff et al., 1993; Vandenberghe et al., 1996). Cr supplementation also has the potential for increasing 1RM (Earnest et al., 1995; Vandenberghe et al., 1997), and especially the number of repetitions until fatigue during weight-lifting (Earnest et al., 1995; Kreider et al., 1998). The latter finding is most likely

a result of an increased ATP turnover due to greater PCr and Cr stores in the Cr-supplemented muscle, and stimulated PCr-Cr shuttle. Theoretically, muscle hypertrophy should also result in a greater 1RM and repetitive muscle strength.

Short-term supplementation (up to 2 weeks). Twelve recreationally active males were supplemented with Cr+glucose for 5 days, performing a series of 5 x 30 maximal knee extensions, with bouts separated by short recovery periods (1 min; Greenhaff et al., 1993). Significantly greater total peak torque was observed for the Cr group vs. the placebo (PL) group during the exercise bouts 2 and 3, whereas the difference for the bout 4 almost reached significance. Dynamic torque production of the knee extensors has also been evaluated in healthy, untrained males before and after 6 days of supplementation with either Cr, Cr+caffeine, or PL supplementation (Vandenberghe et al., 1996). Three interval series of 90, 80, and 50 maximal voluntary contractions (MVC) were performed, with resting intervals of 2 min between the series. Torque production was significantly increased (+10-23%) for Cr as compared to PL, whilst treatment with Cr+caffeine had no effect on the outcome measure.

Long-term supplementation (>2 weeks). Earnest and colleagues (1995) tested the effects of a 28-day Cr-monohydrate (CrM) supplementation protocol on bench-press 1 maximal-repetition (1RM), and repetitive lifting power at 70% of 1RM until fatigue in weight-trained males. There were significant increases in bench-press 1RM (+6%), and the number of repetitions at 70% 1RM (+26%). Both absolute and relative (per kg body mass) total lifting volumes were significantly greater post-Cr treatment. Also evaluating well-trained male subjects (NCAA football players), who were taking either a Cr-

containing or a PL supplement for 28 days during pre-season training, Kreider et al. (1998) found significant improvements in bench-press summed lifting volume (+225 kg), but there were no differences in squat or power clean lifting volume for Cr vs. PL.

Finally, a recent study tested the effects of chronic (10 weeks) Cr supplementation in combination with resistance training on muscular power indices of healthy, sedentary females (Vandenberghe et al., 1997). There were significant improvements for Cr vs. PL in 1RM of leg-press, leg extension, and squat, as well as in arm-flexion torque production in the females following the resistance training program.

1.4.1.2 *Cr supplementation and short-duration maximal cycling, swimming and running*

Ergogenic effects of Cr supplementation on bicycle ergometer sprinting in physically active males and females have been reported in some (Birch et al., 1994; Balsom et al., 1995; Prevost et al., 1997), but not all studies (Febbraio et al., 1995; Cooke et al., 1995; Cooke et al., 1997; Odland et al., 1997). It appears that Cr supplementation may enhance performance in repeated 6-30-second exercise bouts. Beneficial effects of Cr have included an increased mean power output (Birch et al., 1994; Balsom et al., 1995), peak power output (Birch et al., 1994), total work performed (Birch et al., 1994; Earnest et al., 1995; Kreider et al., 1998), and prolonged time to exhaustion (Prevost et al., 1997). However, Cr supplementation did not have any ergogenic effects on peak power or time to fatigue during two maximal, 6-second cycle sprints separated by 3-120-second recovery periods (Cooke et al., 1997), time to exhaustion (Febbraio, 1995), total work performed or time to peak power (Cooke et al., 1995; Odland et al., 1997).

In several studies on elite swimmers, no effects of short-term Cr supplementation were found on repeated sprints (25-100 m) interspersed with resting intervals (10-25 min; Burke et al., 1996; Mujika et al., 1996). Similarly, acute Cr ingestion did not have an effect on running velocity of male and female collegiate athletes during three consecutive 60-m sprints separated by 2-min recovery intervals (Redondo, 1996).

It is not clear why there is such discrepancy between these studies. One possibility lies in a great variability in individual response seen following Cr supplementation, especially in terms of Cr accumulation intracellularly (Harris et al., 1992; Hultman et al., 1996). There are also a lot of methodological flaws characteristic for most of these studies (e.g., small sample size, poor design, lack of dietary and/or other control, etc.), which could invalidate their findings. Additionally, in mass-dependent activities, such as running, negative results could also be a consequence of a significant weight gain associated with Cr supplementation.

1.4.1.3 *Cr supplementation, long-lasting maximal/submaximal exercise, and endurance exercise*

Cr has very little effect on mixed anaerobic-aerobic, and purely aerobic exercise, regardless of the duration of supplementation. Terrillion and others (1997) found no effects of acute Cr ingestion on mid-distance (700 m) running performance in highly-trained endurance athletes. Similarly, in a 25 km cycle-ergometer trial during which six 15 s sprints were performed, there was no improvement in time of cycling for Cr vs. PL in well-trained cyclists (Godly et al., 1997). No significant improvements in a treadmill

run to exhaustion (at 120% $\dot{V}O_{2max}$), or a 6 km terrain run were reported for habitually active and well-trained males supplemented with Cr for 6 days (Balsom et al., 1993).

These results are not surprising, considering that the contribution of the CK–mediated energy transfer to ATP repletion is rather insignificant in situations with sufficient oxygen availability. In aerobic conditions, muscle relies heavily on substrates other than PCr and Cr for fuel. An additional problem that may diminish the effects of Cr ingestion on endurance exercise, is an increased body mass commonly observed following Cr supplementation (Greenhaff et al., 1994; Earnest et al., 1995; Becque et al., 1997; Kirksey et al., 1997; Kreider et al., 1997). In activities such as running, in which weight is not supported, there is little doubt that additional weight could negatively affect exercise performance.

1.4.1.4 *Cr supplementation and muscle performance in the elderly*

The declines in skeletal muscle strength and exercise performance associated with aging (Rogers and Evans, 1993) may reflect decreased muscle [TCr] (Forsberg et al., 1991) and [PCr] (Moller et al., 1980). Therefore, the aging muscle could potentially benefit from Cr supplementation, for the magnitudes of increase in muscle [TCr] and [PCr] during Cr ingestion have been shown to be inversely correlated with the initial levels of the two (Harris et al., 1992). To date, only a handful of studies have examined the effects of Cr supplementation on muscle performance in middle-aged (Smith et al., 1998) and elderly individuals (Bermon et al., 1998; Rawson et al., 1998). Acute Cr ingestion has failed to produce ergogenic effects on isometric strength of the forearm flexors, or the isokinetic MVC of the knee extensors of men and women aged >60 y

(Rawson et al., 1998). However, Cr supplementation had a significant effect on time to exhaustion in middle-aged men and women (mean age 58 years) performing single-leg knee extension exercise (Smith et al., 1998). Finally, following a 52-day supplementation program, neither Cr ingestion-alone nor Cr ingestion in combination with resistance training had any effects on leg-press, chest-press, or two-leg extension of elderly males and females aged 67-80 y, compared to training without the supplement (Bermon et al., 1998).

1.4.1.5 *Creatine supplementation and muscle performance: clinical studies*

Lower [TCr], [Cr], and [PCr] are common findings in the diseased human skeletal muscle (Wyss et al., 1998) and myocardium (Ingwall et al., 1985). Cr supplementation, with its potential to elevate the intramuscular stores of TCr, Cr, and PCr (Harris et al., 1992; Hultman et al., 1996), may improve the impaired muscle energy metabolism, thus enhancing muscle strength and exercise performance in these patients. In patients suffering from chronic heart failure, Gordon and others (1995) found significant improvements in two-legged strength (~10%) and two-legged endurance exercise (~21%) in Cr-supplemented subjects. The combined peak torque of the two exercises increased for Cr as compared to PL. These improvements in muscle strength and endurance were accompanied by an increase of 17% and 12% in skeletal muscle [TCr] and [PCr], respectively. Following a 30-day rehabilitation treatment with exercise+intravenous Cr-phosphate supplementation (1 g/d), there was a significant increase in peak torque during various movements of the lower extremities in patients with muscle hypotonotrophy of the lower extremity (Satolli and Marchesi, 1989). At the

end of treatment, the difference in strength for the Cr group vs. the control group was 13% and 18% in knee flexion and extension, respectively.

Experimental data derived from a clinical trial on patients suffering from various forms of neuromuscular diseases have suggested that oral CrM supplementation may favorably affect muscle strength and resistance to fatigue (Tarnopolsky et al., 1997). In patients with mitochondrial cytopathies, a 19% increase in the isometric handgrip strength, and an 11% increase in dorsiflexion torque was observed (Tarnopolsky et al., 1997).

1.4.2 Effects of Cr supplementation on body mass, fat-free mass, and protein turnover

There have been a number of studies indicating that both acute supplementation with Cr of ~20 g per day for <2 weeks (Balsom et al., 1993; Greenhaff et al., 1994; Stroud et al., 1994; Green et al., 1996), and chronic Cr ingestion for >2 weeks (~20 g/d x 5-7 d; 3-5 g/d thereafter; Earnest et al., 1995; Becque et al., 1997; Kirksey et al., 1997; Kreider et al., 1997) may increase total body mass (TBM) in healthy, young males and females. Since an initial report of a decreased 24-hr urinary output following supplemental Cr ingestion (Hultman et al., 1996), there has been speculation that the observed mass gains may be related to an increased body water retention (Volek and Kraemer, 1996; Williams and Branch, 1998). The net water retention is thought to be intracellular, due to the amino acid-like osmolyte effects associated with Cr uptake and transport (Loike et al., 1988; Ziegenfuss et al., 1998; Oopik et al., 1998). However, skeletal muscle hypertrophy and increased protein synthesis have also been implicated in

mass changes accompanying Cr supplementation (Sipila et al., 1981; Flisinska-Bojanowska, 1996).

In recent years (1995-present), the effects of Cr supplementation on body composition, and especially fat-free mass (FFM), have been extensively explored (Earnest et al., 1995; Becque et al., 1997; Kirksey et al., 1997; Stout et al., 1997; Vandenberghe et al., 1997; Kreider et al., 1998; Mihic, 1998). Data obtained in these studies have suggested that both acute and chronic Cr supplementation may increase FFM, without affecting the fat mass/fat percent, in healthy, young individuals. Additionally, a hypothesis has been tested that resistance training, when combined with supplemental Cr ingestion, could increase FFM to a significantly greater extent than would resistance training alone (Vandenberghe et al., 1997; Kreider et al., 1998).

Doses of Cr or Cr-containing supplements used in the experiments discussed below are all similar (15-20 g/d x 3-7 days, 3-5 g/d thereafter), and have previously proven to be effective in increasing muscle TCr, PCr, and Cr stores (Harris et al., 1992; Hultman et al., 1996).

1.4.2.1 *Short-term Cr supplementation and FFM*

Following a 3-day Cr supplementation trial with weight-trained male and female participants, a 6.6% increase in magnetic resonance imaging (MRI)-measured muscle size of the thigh was found in "five of six Cr subjects" (Ziegenfuss et al., 1997). In a recent randomized, double-blind, placebo-controlled experiment, our group demonstrated a significant increase in FFM (+0.8 kg), as determined by dual-energy x-ray absorptiometry (DXA) analysis after Cr-monohydrate (CrM) ingestion of 20 g/d for 5

days in recreationally active males and females (Mihic, 1998). Interestingly, the FFM changes associated with CrM ingestion tended to be greater for males (+2.0%) than for females (+1.0%; Mihic, 1998).

1.4.2.2 *Long-term Cr supplementation and FFM*

In association with resistance training, chronic Cr supplementation was effective in promoting greater gains in FFM as compared to training alone (Becque et al., 1997; Kirksey et al., 1997; Stout et al., 1997; Vandenberghe et al., 1997; Kreider et al., 1998). Following a 28 day-protocol, DXA analysis detected significant increases in FFM during pre-season training (weight-training, agility/sprint training) for college football players supplemented with a mixture containing Cr (Kreider et al., 1998) as compared to a protein-glucose supplement (PL; 99 g/d of glucose, 3 g/d of taurine, 1.1 g/d of disodium phosphate, and 1.2 g/d of potassium phosphate). Changes in FFM for the Cr group were almost twofold greater than for the weight-matched controls ingesting the PL supplement while training (Cr, +2.4 kg; PL +1.3 kg). This difference could not be explained by increased net water retention, because the bioelectric impedance analysis (BIA) revealed no inter-group difference in total body water (TBW) after treatment, when TBW was expressed relatively, as a percentage of body mass (Kreider et al., 1998).

A long-term Cr supplementation trial by Vandenberghe and colleagues (1997) appears to have been well-controlled. In this study, 19 sedentary females underwent 5 weeks of resistance training while taking Cr-monohydrate (CrM) or a PL supplement. During this time, training workloads were identical in the two groups (70% of 1RM). At 5 weeks, workloads were adjusted to a new 1RM, and both supplements and training

were continued for another 5 weeks. Importantly, FFM was found to be increased more for the Cr group than the PL group both after 5 (Cr, +4.5%; PL, +2.5%) and 10 weeks (Cr, +5.8%; PL, +3.7%) of training. In addition, FFM remained increased for Cr as compared to PL during 10 weeks of detraining while supplements were continued.

Additional benefits of Cr-containing supplements on body composition after 5-12 weeks of (mostly resistance) training have also been reported in weight-trained males (Becque et al., 1997; Stout et al., 1997), and male and female track athletes (Kirksey et al., 1997). Importantly, all of the relevant techniques for body composition assessment have appeared to be sensitive enough to detect Cr-induced changes in FFM, i.e., 7-site skin-fold analysis (Kirksey et al., 1997), DXA scanning (Stout et al., 1997; Kreider et al., 1998; Mihic, 1998), and hydrostatic weighing (Becque et al., 1997; Vandenberghe et al., 1997).

Earnest et al. (1995) have shown that chronic Cr ingestion alone may also affect body composition in resistance-trained men, whose FFM was calculated via hydrostatic weighing before and after 28 days of CrM supplementation. The observed increase in FFM for the Cr group tended to be significant (+1.6 kg).

1.4.2.3 *Cr supplementation and FFM in the elderly*

A study by Rawson et al. (1998) showed a small, but significant increase in TBM for healthy males aged 60-78 years after only 5 days of supplementation with Cr (20 g/d). However, there were no treatment effects on TBM, FFM (calculated from skin-fold thickness), or lower limb muscle volume following long-term Cr supplementation (Bermon et al., 1998). In this double-blind, placebo-controlled trial, 32 elderly males and

females (67-80 y) underwent an 8-week protocol of either placebo, CrM supplementation, CrM+strength training, or strength training-alone. It was concluded that oral CrM supplementation did not provide additional benefits for body composition in this group of older adults.

1.4.2.4 *Cr supplementation and FFM: clinical populations*

Few studies have examined the effects of Cr supplementation on diseased muscle. Although Cr supplementation has been proposed to have the therapeutic potential for increasing muscle mass (Wyss et al., 1998), Tarnopolsky and colleagues (1997) were unable to find any differences in FFM (DXA analysis) in patients with mitochondrial cytopathies after the Cr trial, as compared to the PL trial. However, there was an absolute increase in FFM (+0.6 kg), which may have been significant, for instance, had a larger sample size been employed. The duration of treatment (3 weeks), and/or the dosage supplied (10 g/d x 14 d, 4 g/d x 7 d; as opposed to 20 g/d, used in most studies), may also have influenced the results.

The findings of Sipila et al. (1981), and Vannas-Sulonen et al. (1985), in patients with gyrate atrophy of the choroid and retina, are encouraging. After a one-year supplementation period with Cr (1.5 g/d), all of the seven patients displayed a remarkable enlargement of the type II muscle fiber diameter of ~46% (pre-treatment mean, 34.1 μm; post-treatment, 49.9 μm). No differences were seen in the type I muscle fibers of the same muscle (*m. vastus lateralis*), or the fiber distribution. A follow-up on the same patient population (Vannas-Sulonen et al., 1985) demonstrated the maintenance of the improved type II fiber diameter 5 years after treatment with 1.5 g of Cr per day.

1.4.2.5 *Cr supplementation and protein turnover*

In rats, an investigation of the effects of acute Cr ingestion (6-9 days) on total and myofibrillar protein contents of rat gastrocnemius muscle, has come from the work of Flisinska-Bojanowska (1996). She found that both Cr alone, and Cr combined with muscle electrostimulation, enlarged both total and myofibrillar protein contents in the isolated muscle. The former finding is peculiar considering that Cr ingestion alone did not increase Cr accumulation in muscle, causing one to question the signal for the stimulation of contractile protein synthesis in those rats.

Finally, there has been only one abstract report on the effectiveness of Cr supplementation on improving net protein retention in humans (Ziegenfuss et al., 1997). The preliminary data (n = 3), generated from a ^{15}N glycine isotope study, suggested that acute, 5-day Cr ingestion may improve net nitrogen status by stimulating whole-body protein synthesis and/or decreasing protein breakdown. However, urinary urea N excretion, a marker of protein catabolism, has been found to be unchanged following Cr supplementation (Earnest et al., 1996).

In summary, some of the research evidence presented above supports the concept that both acute (Milnic et al., 1998), and chronic Cr supplementation for 2-10 weeks (Earnest et al., 1995; Becque et al., 1997; Kirksey et al., 1997; Stout et al., 1997; Vandenberghe et al., 1997; Kreider et al., 1998), may have stimulating effects on FFM, without affecting fat mass/fat percent in healthy young humans. The effects seem to be more apparent in association with resistance training, possibly through a combination of an increased training volume (see Sec. 1.4.1) and Cr action *per se* (Becque et al., 1997;

Kirksey et al., 1997; Stout et al., 1997; Vandenberghe et al., 1997; Kreider et al., 1998). Additionally, muscle contraction has been shown to enhance Cr uptake by skeletal muscle, thereby maximizing intracellular Cr accumulation (Harris et al., 1992).

Increased total body water and intracellular water is associated with Cr supplementation (Ziegenfuss et al., 1998; Kreider et al., 1998; Oopik et al., 1998). These are important findings in light of the hypothesis that net water retention could account for increases in FFM following chronic Cr supplementation (Volek and Kraemer, 1996; Williams and Branch, 1998). However, we can also speculate that muscle hypertrophy may also account for the effects of Cr on body mass *in vivo*. Only once to date has the latter assumption been experimentally tested (Flisinska-Bojanowska, 1996). In this study, total and myofibrillar protein contents were determined in the gastrocnemius muscle of the rat following 6 or 9 days of Cr feeding. There was a significant increase in contents of both total and myofibrillar protein, yet Cr accumulation was not affected by the feeding. Thus, it is not clear why or how a Cr-enriched diet stimulated protein synthesis, without having affected Cr content in the muscle.

Gender does not appear to alter the effects of Cr on FFM. However, several studies have demonstrated that Cr-induced mass changes might not be as remarkable for females as they are for males (Thompson et al., 1996; Hamilton-Ward et al., 1997; Terrillion et al., 1997; Mihic, 1998). This could indicate that gender differences, primarily in muscle contents of TCr, PCr, and Cr, as well as possible differences in Cr uptake and retention by skeletal muscle, may alter the magnitude of Cr effects on FFM in

males as compared to females. This observation is only preliminary, but requires further attention.

Data on elderly individuals are scarce and inconclusive, and require more extensive work. The mass changes commonly found for young men and women taking Cr have not been observed in older adults over 60 years of age (Bermon et al., 1998). This may be attributed to an impaired Cr uptake and/or retention by muscle, possibly due to: 1) a decreased CrT expression, 2) a reduction in lean tissue, a major storage of Cr, with advancing age, or, 3) an increased type I/type II fiber distribution ratio in the elderly (Rogers and Evans, 1993).

On the other hand, lower skeletal muscle [TCr] (Forsberg et al., 1991) and [PCr] (Moller et al., 1980), associated with aging, could theoretically allow for a greater (relative) increase in muscle [TCr] and [PCr] following Cr loading (Harris et al., 1992), thereby accentuating the potential for Cr to increase FFM. I believe that further research is warranted, and that chronic Cr supplementation, especially in association with exercise, may prove to be a useful strategy for the prevention of lean tissue loss in the elderly. The same can be argued for the clinical populations with severe muscle wasting diseases.

1.4.2.6 *Creatine depletion studies: effects on muscle mass and myosin heavy-chain isoform expression*

Cr depletion is an exciting model for exploring the physiological significance of Cr in muscle biochemistry and metabolism. The initial studies were reported several decades ago (Fitch et al., 1974; Shields et al., 1975), and have since provided very useful

information on structural and biochemical consequences of experimentally-induced Cr depletion in skeletal muscle.

Initial studies demonstrated that feeding rats diets containing 1% β-guanidinopropionic acid (β-GPA), a Cr analog, could reduce muscle [PCr] and [Cr] after 6 weeks to <10% of the control values seen in rats fed normal diet (Fitch et al., 1974; Shields et al., 1975). β-GPA is similar to Cr structurally, and is known to compete with it for transport into skeletal muscle, thereby inhibiting Cr uptake *in vitro* (Fitch et al., 1968). It has been demonstrated that β-GPA-fed rats may (Adams et al., 1994) or may not (Shields et al., 1975; Shoubridge et al., 1985) exhibit significant loss of body mass. However, muscle wet-weight has been commonly found to be reduced by as much as ~30% following treatment with β-GPA (Shields et al., 1975; Petrofsky and Fitch, 1980; Shoubridge et al., 1985; Adams and Baldwin, 1995) . Importantly, the weight loss has commonly been observed in fast-twitch muscles with high type II/ type I fiber ratios, such as *Mm. plantaris* (Petrofsky and Fitch, 1980; Shoubridge et al., 1985), *gastrocnemius* (Shields et al., 1975; Shoubridge et al., 1985), and *tibialis anterior* (Adams and Baldwin, 1995). It is not surprising that highly glycolytic, fast-twitch muscle groups are more affected by the lack of PCr and Cr than are slow-twitch muscle groups, for PCr content (but not that of Cr) is higher in type IIa and IIb, as compared to type I muscle fibers (Soderlund et al., 1992; Greenhaff et al., 1994; Wyss and Wallimann, 1994).

Decreased cross-sectional area (CSA) *of m. gastrocnemius* type IIb and IIa muscle fibers by ~30 and 25%, respectively, was found in exercised rats vs. matched controls

after 4 weeks of treatment with dietary β-GPA (Shields et al., 1975). In a more recent investigation, similar changes in CSA (-33%) were seen after 16 weeks of β-GPA-feeding (Levine et al , 1996).

The observed decrease in muscle mass is most likely a result of several related structural adaptations of skeletal muscle to severe Cr depletion. Several experiments have implied that these adaptations may be linked to alterations of MyHC isoform expression in skeletal muscle (Moerland et al., 1989; Wiseman and Kushmerick, 1994; Adams et al., 1994; Adams and Baldwin, 1995; Levine et al., 1996). In rats, MyHC is believed to be expressed in 4 isoforms: type I (slow), type IIa (intermediate), type IIx (transitional), and IIb (fast; Termin et al., 1990). There is evidence (Moerland et al., 1989; Wiseman and Kushmerick, 1994; Adams et al., 1994; Adams and Baldwin, 1995; Levine et al., 1996) to suggest that Cr-depleted fast-twitch rat muscle can display a consistent pattern of change in MyHC isoform expression: 1) from type IIb toward type IIx, and, 2) from types IIx and IIa, to type I isoforms. Moreover, type IIb MyHC mRNA was significantly decreased in the plantaris and medial gastrocnemius muscles, whereas in the soleus, type I MyHC mRNA increased after Cr depletion (Adams et al., 1994). Moerland et al. (1989) found similar adaptations in *m. soleus*, where treatment with β-GPA initiated a 50% reduction of the intermediate MyHC isoform expression, simultaneously doubling that of the slow isoform.

1.5 Techniques used for studying whole-body protein and amino acid turnover

1.5.1 *Urinary urea nitrogen excretion*

Measurement of urinary urea nitrogen (N) excretion is a commonly employed method for studying whole-body protein metabolism in humans (Tarnopolsky et al., 1988; Lemon et al., 1992; Tarnopolsky et al., 1992; Phillips et al., 1993). It is a reasonably convenient procedure for both subjects and experimenters, requiring only daily urine collections. However, the accuracy of 24 hr urine collections is the major source of error.

Urinary urea is the route of excretion of practically all the N released by tissues undergoing various processes of protein catabolism (e.g., deamination, irreversible amino-acid oxidation; Groff et al., 1995). Upon the delivery of N-groups (i.e., NH_4^+) to the liver, chiefly via glutamine, alanine, and ammonia (NH_3), and subsequent ureagenesis in the liver mitochondria and cytosol, urea is transported via blood to the kidneys, where it is eventually excreted in the urine (Abumrad et al., 1989). Urinary urea serves as a carrier for ~90-95% of total N excreted in the urine (Lemon et al., 1992; Groff et al., 1995), the rest being excreted via creatinine, amino acids, ammonia, uric acid, and trace amounts of creatine and protein (Groff et al., 1995). Other routes of N excretion are feces, skin, and other miscellaneous routes (i.e., sweat, mucous, saliva, semen, etc.; Calloway et al., 1971)

Urinary N excretion has been considered to be an indicator of the overall protein catabolism when protein intake is constant. However, a change in protein consumption (Tarnopolsky et al., 1988; Tarnopolsky et al., 1992), energy intake (Chiang and Huang, 1988; Hoffer and Forse, 1992), or diet composition (Richardson et al., 1979), can

significantly alter urea N excretion; thus particular attention has been given in the present study to control for these factors (see Methods).

1.5.2 *Nitrogen balance*

Nitrogen balance (Nbal) indicates the overall state of all synthetic and catabolic processes in the body over a given period of time. It can be considered to be the difference between dietary N intake and N excretion via urine, feces, sweat and other routes of excretion (miscellaneous losses). Mathematically, Nbal can be expressed as follows:

$$Nbal = N_{in} - (N_{urinary\ urea} + N_{feces} + N_{sweat\ and\ misc.losses}) \qquad \text{(Equation 2)}$$

A zero net balance could then be said to be a state in which N intake equals total N excretion. The fact that Nbal can also be positive, normally resulting in protein and lean body mass accretion, or negative (N wasting and loss of lean body mass), displays an unfortunate use of terminology, which does not appear to be the only limitation of this technique. Among the main concerns regarding the interpretation of Nbal results is an unrealistically positive Nbal evident at very high protein intakes, without any lean body mass accretion (Hegsted, 1976). The most popular explanation of this "artifact" is that Nbal studies involve a systematic error of overestimating N intake, while underestimating N excretion (Hegsted 1976). Some authors, however, have disputed this opinion saying that such errors cannot be inherent to this method, for they only occur at random (Oddoye and Margen, 1979). Whatever the case, the root of this discrepancy is still unclear.

Another limitation of the Nbal methodology may become apparent during sub-optimal protein intakes. It has been documented that a person consuming amounts of protein that are well below recommended levels ("accommodation state", as proposed by Young, 1986 and 1987) may in fact be in zero Nbal, but at the same time undergoing a physiological compromise to achieve it (e.g., decreased protein synthesis). This is obviously not beneficial to the person, and that is why one must be extremely careful when interpreting Nbal data, especially when dietary intake changes over the course of an experiment.

In spite of the problems discussed above, the Nbal method provides invaluable information about the overall status of protein turnover, particularly when used with other techniques, such as stable isotope tracer methodology. In this study, a rigorous dietary control was exercised such that our subjects' total energy and protein intake did not change during the study, allowing for accurate and valid interpretation of the Nbal results.

1.5.3 *Tracer methodology*

This technique involves the administration of "labeled" isotope tracer (e.g., ^{13}C glucose) in order to quantitatively describe the kinetics of a molecule being traced (tracee; e.g., ^{12}C glucose), with the assumption that "the labeled molecule will not be discriminated from the unlabeled molecule, and that the labeled molecule will trace the movement of the unlabeled molecules". [2] By accessing certain body pools (blood,

[2] Cited from Wolfe (1992), p. 14.

expired air, urine or muscle) for sample collection, tracer enrichment can be measured, and the flux of the tracee calculated.

The ^{13}C stable isotope has been widely used to study carbohydrate (e.g., ^{13}C glucose, ^{13}C pyruvate), fat (e.g., ^{13}C glycerol), and amino acid and protein metabolism (e.g., ^{13}C leucine; Wolfe, 1992). Although its natural occurrence is physiologically insignificant (~1.1% of total C pool, as in Wolfe, 1992), the background enrichment must be accounted for when isotope specific activity is calculated.

In the present study, a "primed-continuous" infusion of L-[1-^{13}C] leucine has been employed as described elsewhere (Matthews et al., 1980). A priming dose of ^{13}C leucine tracer designed to rapidly raise isotope levels is intravenously administered into the sampling site (venous blood), followed by a continuous infusion of the labeled isotope until an "isotopic equilibrium" has been reached (Matthews et al., 1980; Wolfe, 1992). Isotopic equilibrium is also known as isotopic steady-state, or isotopic plateau, and it has been defined as a state in which the rate of appearance (Ra) of unlabeled tracee equals the rate of its disappearance (Rd) from the same pool (Matthews et al., 1980; Wolfe et al., 1992). One may also think of it as a state in which isotopic enrichments, or the dilution of the tracer in the sampling pool and intracellular space, are equal and do not change over time, at least for the duration of experiment (Wolfe, 1992). Statistically, isotopic plateau has been achieved if the regression equation slope, generated by isotopic enrichments at all the time-points during infusion, is not significantly different from zero (Hoerr et al., 1991), or when their coefficient of variation (CV) is $\bullet$10% (Thompson et al., 1988).

The single-pool model, described by Waterlow and others (1978), was used in this investigation to estimate the overall protein kinetics, or whole-body protein turnover. This model is only concerned with the "overall in and out of the system" (Waterlow et al., 1978), and it does not include the analysis of separate compartments of the body (Waterlow et al., 1978; Wolfe, 1992). The approach has been criticized for being over-simplistic, and not reflective of the true kinetics of body protein; on the other hand, the advantage is that it reduces the number of sampling sites, which is particularly important when dealing with human subjects.

Providing that isotopic steady-state exists at the time of sampling, the single-pool model can be described by the following equation (Matthews et al., 1980):

$$Q = S + C = B + I \qquad \text{(Equation 3)}$$

Where, Q is the total flux (turnover of tracer); S (synthesis) is the rate of incorporation of tracer into protein (also referred to as non-oxidative leucine disposal; NOLD); C is catabolism, or, in this particular case, leucine oxidation; B refers to the rate of endogenous protein breakdown (Ra, as described above); and, I represents the exogenous or dietary protein intake (normally equals zero).

When there is no dietary intake of leucine during isotope infusion, total flux (Q) must equal the breakdown of endogenous protein (Ra), and is calculated as follows (Matthews et al., 1980):

$$Q\ (\mu mol \cdot kg^{-1} \cdot h^{-1}) = i \cdot [\ (E_i/E_p) - 1] \qquad \text{(Equation 4)}$$

Where, i is the rate of infusion of L-[1-^{13}C] leucine tracer (expressed in $\mu mol \cdot kg^{-1} \cdot h^{-1}$); Ei equals the enrichment of the infused leucine in APE (atom % excess); Ep is the

plasma enrichment of α-KIC, an estimate of the intracellular leucine precursor pool (in APE); the term -1 corrects for the contribution of tracer to flux.

α-KIC (alpha-ketoisocaproic acid) is the product of the intracellular transamination of leucine, a reaction that is readily reversible in muscle (Abumrad et al., 1989; Wagenmakers, 1998). The use of plasma α-KIC or leucine enrichment to estimate the intracellular leucine precursor pool has been referred to as the "reciprocal pool model", which has been validated previously (Matthews et al., 1982; Wolfe et al., 1982; Schwenk et al., 1985; Vazquez et al., 1986). However, as the leucine↔α-KIC ↔leucine inter-conversion is probably not rate-limiting in muscle (Wagenmakers, 1998), and since it appears that intracellular α-KIC is in "rapid equilibrium" across the plasma membrane (Wolfe, 1992), the enrichment of plasma α-KIC is thought to more closely predict the intracellular leucine tracer enrichment than does plasma leucine enrichment (Wolfe, 1992). For this reason, plasma α-KIC enrichment was used in all the tracer calculations in this thesis.

Finally, according to Equation 4, NOLD (an estimate of whole-body protein synthesis) is calculated as the difference between tracer flux and its oxidation, and is represented by the following formula (Wolfe, 1992):

$$S = Q - O \qquad \text{(Equation 5)}$$

Where, S is whole-body protein synthesis, as estimated by NOLD; Q is leucine flux; and O is leucine oxidation. Leucine oxidation is calculated as follows:

$$\text{Oxid. } (\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}) = [\, (E_{CO2} \cdot \dot{V}CO_2) \div (E_p \cdot c) \,] \qquad \text{(Equation 6)}$$

Where, E_{CO2} is the isotopic enrichment of the breath CO_2 (in APE); $\dot{V}CO_2$ is the volume of CO_2 produced (in $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{hr}^{-1}$); E_p is the plasma α-KIC enrichment (in APE); and, c is the "bicarbonate retention factor" (the value of 0.81 has been accepted as the correction factor at rest; Wolfe, 1992).

1.6 Rationale for the present study

Cr supplementation has been shown to increase both total body mass (TBM) and fat-free mass (FFM) after short- (1-2 weeks) and long-term (>2 weeks) protocols (Earnest et al., 1995; Becque et al., 1997; Kirksey et al., 1997; Stout et al., 1997; Vandenberghe et al., 1997; Kreider et al., 1998; Mihic, 1998). These mass gains have most often been attributed to net water retention, since Cr presumably acts as an osmotic agent, drawing water into skeletal muscle where approximately 95% of Cr is stored (Loike et al., 1988; Ziegenfuss et al., 1998; Oopik et al., 1998).

An increased hydration state, or cell swelling, thought to occur as a result of Cr supplementation (Ziegenfuss et al., 1998; Oopik et al., 1998), has been recognized as a potent inhibitor of proteolysis (Haussinger et al., 1991; Schulz et al., 1991; vom Dahl and Haussinger, 1996), and, under certain conditions, a stimulator of protein synthesis (Stoll et al., 1992).

Cr may also stimulate skeletal muscle hypertrophy, primarily by regulating the synthesis rates of the myofibrillar proteins, myosin heavy-chain (MyHC) and actin (Ingwall et al., 1972; Ingwall et al., 1974; Young and Denome, 1984; Flisinska-

Bojanowska, 1996) which comprise ~80-90% of total muscle protein content (Devlin, 1997).

Short-term supplementation with Cr-monohydrate (CrM) employed in this study (20 g/d x 5 d, 5 g/d x 4-5 d), has been reported to be effective in increasing the skeletal muscle TCr stores by ~20% in healthy young humans (Harris et al., 1992; Hultman et al., 1996). Thus, it was thought that an increased Cr accumulation might provide two distinct or possibly coordinated signals, 1) action of Cr *per se*, and, 2) Cr-induced cell swelling, for the stimulation of muscle protein synthesis and/or inhibition of protein degradation and irreversible catabolism.

An earlier investigation by our group demonstrated that the increases in TBM and FFM may be greater for males than females following a 5-day CrM supplementation protocol, suggesting that there may be a gender difference associated with mass changes resulting from CrM ingestion (Mihic, 1998). We speculated that greater muscle [TCr] in females vs. males (Forsberg et al., 1991) may have diminished the capacity of skeletal muscle to Cr-load, as demonstrated previously (Harris et al., 1992).

1.7 Objectives and hypotheses

We believed that it was important to pursue this research primarily for the reason that Cr supplementation has been shown to be an effective method of enhancing athletic performance, as well as safe practice among the athletic community. It has also been successfully used in treatment of disorders such as gyrate atrophy of the choroid and retina (Sipila et al., 1981; Vannas-Sulonnen et al., 1985), and various mitochondrial cytopathies (Tarnopolsky et al., 1997). Our goal was to account for the effects of Cr supplementation

on FFM, by investigating the relationship between Cr and whole-body protein metabolism. This work had never been undertaken before, and it was the first attempt to duplicate the work *in vitro*, that demonstrated significant stimulation of myofibrillar protein synthesis by Cr (Ingwall et al., 1972, 1974, 1976; Young and Denome, 1984), in humans.

With this study, we wanted to determine whether short-term (9-10 d) CrM supplementation would affect whole-body protein turnover and net protein accretion in recreationally active healthy males and females (n = 27). To do this, we utilized the ^{13}C leucine stable isotope tracer, urinary urea N excretion, and Nbal techniques. Our primary objectives were to determine whether CrM supplementation would affect:

1. leucine flux (an index of protein breakdown),
2. leucine oxidation,
3. non-oxidative leucine disposal (NOLD; an estimate of whole-body protein synthesis),
4. urinary urea N excretion, and
5. Nbal.
6. We were also interested in investigating whether there would be any gender-based differences in response to CrM supplementation.

A secondary objective was to assess the efficacy of the renal system to excrete extra creatinine (CTN) resulting from the supplementary Cr ingestion. To do this, plasma [CTN], urinary [CTN], and CTN clearance were measured pre- and post-treatment.

Our *a priori* hypotheses were that, following CrM supplementation,

1. body mass would increase,
2. NOLD would increase,
3. leucine flux and leucine oxidation would decrease,
4. 24-hr urinary output and urinary urea N excretion would decrease, and Nbal would increase, becoming either more positive or less negative,
5. all the effects would be significantly greater for males than females, and,
6. neither plasma [CTN], urinary [CTN], nor CTN clearance would change.

CHAPTER 2

Methods

2.1 Experimental protocol

2.1.1 *Study population*

Twenty-seven young (mean age 23 ± 4 y) males (n = 13) and females (n = 14) volunteered for the study. They were all physically active (exercised 3-5 times per week), and were not taking any medications, excluding vitamin supplements. They had not taken Cr as a supplement for at least six months before the study. Their weight, training intensity and volume had been reported to be unchanged ~3-4 weeks prior to the study. The study was approved by the McMaster University Ethics Committee, and all the subjects gave their informed written consent.

2.1.2 *Study design*

Four-day dietary records, that included one weekend day, were obtained within a week of commencement of the experiment, and complementary check-list diets were developed for the subjects (Nutritionist IV, First Data Bank, San Bruno, CA; Figure 3). The mean energy and protein intakes were 3216 ± 587 Kcal/d, and 14.8 ± 2.6% of total energy intake, respectively, for males, and 2287 ± 497 Kcal/d, and 15.5 ± 4.7%, respectively, for females (Table 1). Isoenergetic, isonitrogenous, and flesh-free diet was designed from the individual's habitual intake, and was followed for three days prior to the

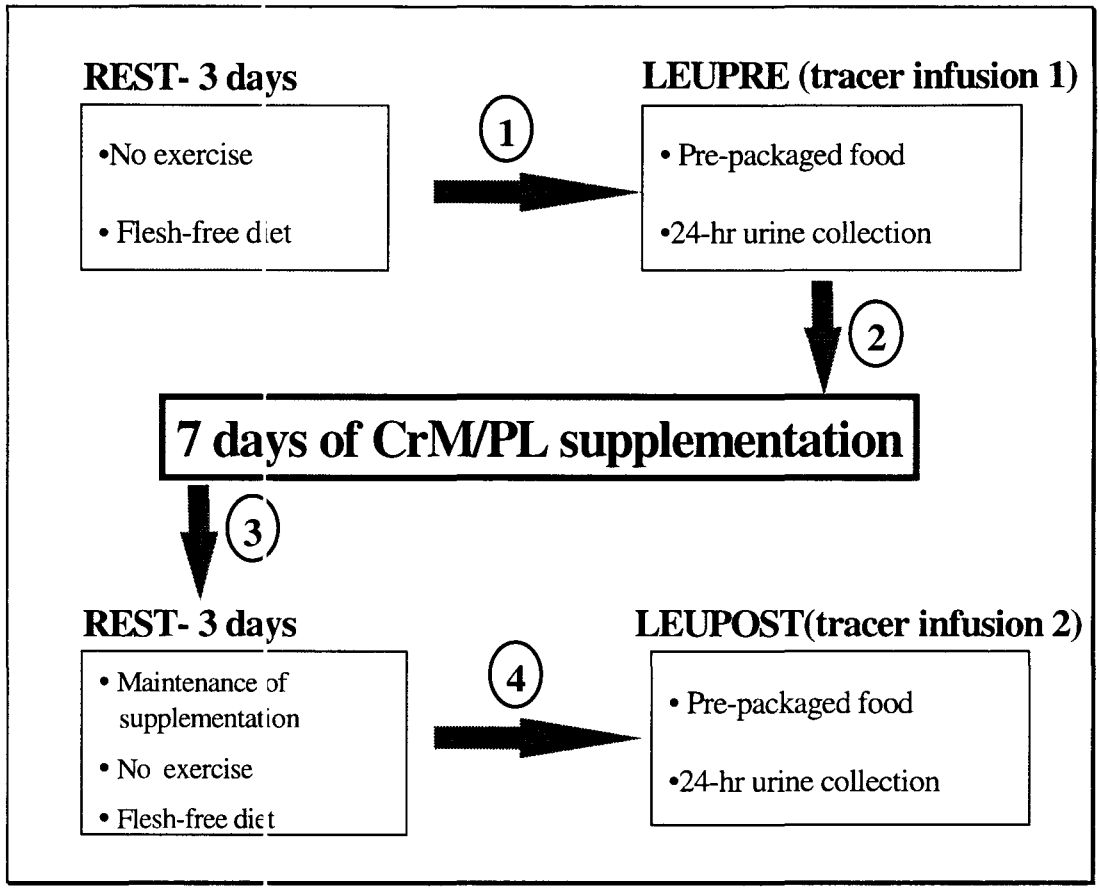


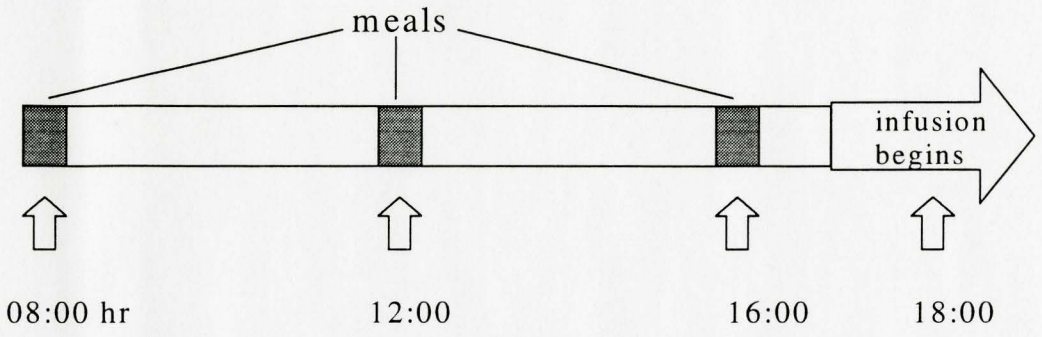
Figure 3. Study design.

day of tracer infusion (see below). Subjects were asked to refrain from any strenuous exercise with the legs three days before the day of infusion, and from any form of exercise for 48 hr prior to tracer infusion.

On the day of infusion (Figure 4), pre-packaged food was provided that precisely matched the individual's habitual diet in terms of total energy intake and proportion of fat, protein, and carbohydrate. This isoenergetic, isonitrogenous, and flesh-free diet was consumed in three meals (at ~0800 hr, ~1200 hr, and ~1600 hr), after which subjects reported (at ~1700 hr) to the laboratory for a stable isotope infusion (LEUPRE). In addition, a 24-hr urine sample was collected on the day of LEUPRE.

2.1.3 *Tracer infusion*

After a background breath sample was obtained for $^{13}CO_2/^{12}CO_2$ isotopic analysis, a 22-ga plastic catheter was inserted into an antecubital vein, and a blood sample taken for determination of plasma isotopic alpha-ketoisocaproate (α-KIC) enrichment (t = -120 min). Subsequent breath and "arterialized" blood samples (electrical heating pad at ~65°C) were taken at +4 hr, +1175 hr, and +1200 hr (all time-points are relative to the time of first muscle biopsy that was performed as a part of another student's project) after an isotopic plateau was established (Wolfe, 1992; Figure 8). Another catheter was placed into a forearm vein of the other arm for primed- (0.99 ± 0.02 mg · kg^{-1} body mass) constant (0.98 ± 0.04 mg · kg^{-1} · hr^{-1}) infusion of L-[1-^{13}C] leucine tracer (99% isotopic enrichment, Cambridge Isotope Laboratories, Andover, MA), as previously described (Tarnopolsky et al., 1991; Yarasheski et al., 1992; Phillips et al., 1993; Roy et al., 1997). The isotope was mixed with sterile saline on the day of infusion under aseptic conditions, and was passed



* Subjects were also collecting a 24-hr urine sample until end of infusion.

1 - infusion
2 - breath sample
3 - blood sample
4 - breath sample (for VCO_2)
5 - leucine-free snack

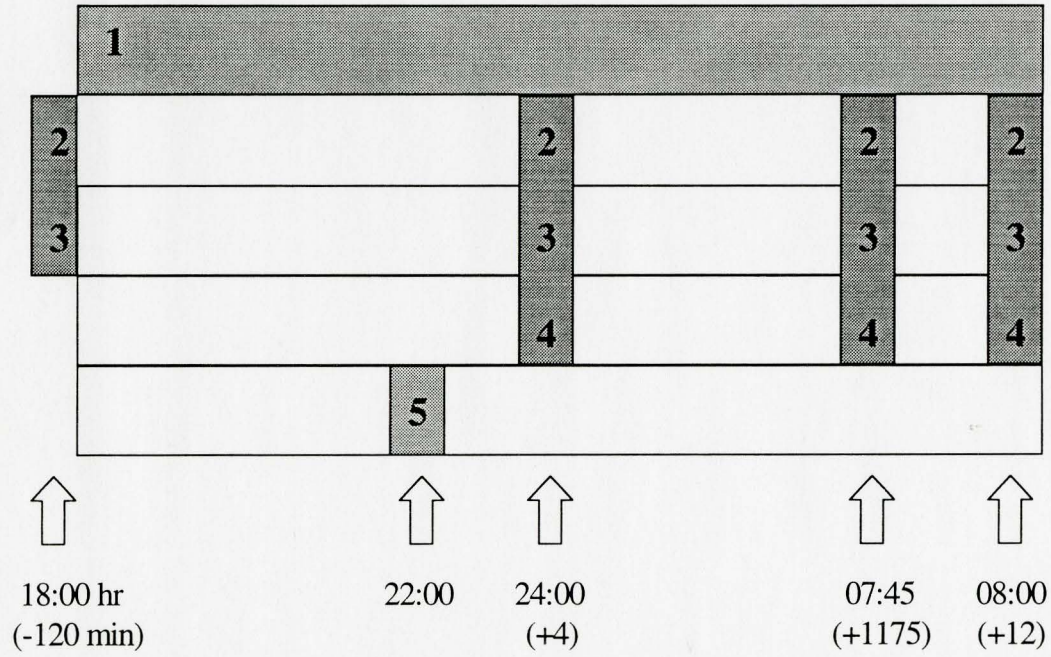


Figure 4: Testing day – tracer infusion.

through an antibacterial filter (0.2 μm; Acrodisc) immediately before the infusion. Additionally, a single dose of [^{13}C] sodium bicarbonate (0.292 ± 0.01 mg · kg^{-1}) was administered prior to tracer infusion. Subjects were then placed in a resting supine position until the next morning. They also consumed a very light snack (at 2100 hr) that contained minimal amounts of amino acids (i.e., potato rings, diet coke). At +1200 hr, the infusion was discontinued and subjects were free to leave the lab after the collection of the last urine specimen.

2.1.4 *Supplementation*

LEUPRE was followed by 3-5 days of rest to ensure sufficient time for a full recovery from invasive procedures, especially muscle biopsies. Subjects were then randomly assigned to take either creatine monohydrate (CrM; 7 males, 7 females) (99% pure, ISA, Hamilton, ON) or placebo (PL; 6 males, 7 females) (Polycose, Ross Laboratories, Montreal, Quebec). The supplement was consumed at 20 g/day for 5 days, followed by a maintenance dosage of 5 g/day for 4-5 days. Subjects were instructed to take the substance with chocolate milk, sweetened pop, or orange juice.

On Day 6 of supplementation, a performance testing was done as a part of another student's project. Following this, diet and exercise were controlled for as described above (see prior to LEUPRE), and another tracer infusion (LEUPOST) took place on Day 9/10 of supplementation in an identical manner as for LEUPRE.

2.2 Measurements and analysis

2.2.1 *Breath*

Expired air was collected into a 150 L meteorological balloon, and immediately injected into 10 ml evacuated tubes (two per sample) for subsequent determination of $^{13}CO_2/^{12}CO_2$ isotopic ratio by an isotope ratio mass spectrometer (Breath-MAT Plus, Finnigan, Bremen, Germany). The intra-assay coefficient of variation (CV) of this method was 2.2% (n = 6), and the inter-test CV was 2.6% (n = 6). Additionally, the expired CO_2 was recorded by a CO_2 analyzer (HP 78356, Hewlett-Packard, Avondale, PA) for subsequent calculations of $\dot{V}CO_2$.

2.2.2 *Blood*

6-8 ml blood samples were collected into chilled heparinized tubes and spun for 5 min at 1,200 g. Plasma was then extracted, stored at -50°C, and later analyzed for creatinine (Kit 555, Sigma Diagnostics, St. Louis, MO) (CV = 7.6%), as well as for α-KIC enrichment (CV_{PRE} = 3.9%; CV_{POST} = 2.0%). The o-quinoxalinol-trimethylsilyl derivative of plasma α-KIC was prepared as described previously (Tarnopolsky et al., 1991; Wolfe, 1992). Its isotopic enrichment was analyzed by electron-impact ionization capillary gas chromatograph/mass spectrometer (GC = HP 6890, MS = HP 5973, Hewlett-Packard, Wilmington, DE). The derivatized sample (1 μl) was injected into a 15 m fused silica capillary column with the oven program set at an initial temperature of 120°C, ramped to 160°C (8°C per min), and then ramped to 300°C (20°C per min), and held at 300°C for 3 min. Helium was used as the carrier gas (32 cm per s). The column exited directly into the MS ion source, and electron impact was used to ionize the sample (70 eV, trap current 170 μA, source current 1.6 mA). Its enrichment was monitored by selected-ion

monitoring (SIM) of 233/232 mass-to-charge (m/z) ratio as described previously (Wolfe, 1992).

2.2.3 *Urine*

24-hr urinary urea N (CV = 16.4%) and creatinine excretion (CV = 14.5%) were determined by colorimetric methods (Kit 640 and 555, respectively, Sigma Diagnostics, St. Louis, MO). Creatinine clearance was calculated according to the following formula (Bishop et al., 1992):

$$Creatinine\ clearance = (U_{Cr} \cdot V/P_{Cr}) \cdot 1.73/A \qquad \text{(Equation 7)}$$

Where, U_{Cr} = concentration of creatinine in urine (mg/dl), P_{Cr} = concentration of creatinine in plasma (mg/dl), V = volume of urine flow (ml/min), A = body surface area (m^2), and, 1.73 = average-size body surface area. Nbal was calculated as the difference between dietary N intake - N excretion (urinary urea + urinary Cr + feces + sweat + miscellaneous losses; Calloway et al., 1971; Tarnopolsky et al., 1988; Lemon et al., 1992; Phillips et al., 1993; Hultman et al., 1996; Vandenberghe et al., 1997).

2.3 Tracer kinetics calculations

The calculations for leucine flux, oxidation, and non-oxidative leucine disposal are presented in Chapter 1, Section 1.5.3 (Equations 3-6). Also see Appendices B and C for sample calculations.

2.4 Statistical analysis

STATISTICA 5.1 (StatSoft, Inc., Tulsa, OK), and Prism 2.01 (GraphPad Software, Inc.) stats and graph software packages were used for all analyses. Leucine tracer, nitrogen, and creatinine data were analyzed by the three-way between-within analysis of

variance (ANOVA), with Treatment (2 levels: CrM and PL) and Gender as the between-subject variables, and Time (2 levels: pre- and post-treatment) as the within-subject variable. Separate two-way ANOVA's were employed for comparisons within the same gender (i.e., the CrM group males were compared to those in the PL group, and the CrM group females were compared to those in the PL group), with Treatment and Time as the between-subject and within-subject variable, respectively. Tukey HSD post-hoc test was used for paired comparisons when significant differences were found. Differences in subject characteristics were analyzed using a two-way between-ANOVA, with Treatment and Gender as the between-subject variables. Linear regression was used to determine the linearity of plasma α-KIC enrichment over time. A confidence level of $P < 0.05$ was considered to be statistically significant. All results are presented as means $\pm$ SD.

CHAPTER 3

Results

3.1 Subjects' descriptive characteristics

There were no reports of any adverse side-effects associated with the ingestion of supplementation. When CrM and PL groups were compared, there were no significant differences in age, height, mass, daily absolute or relative energy intake, or composition of the diet (Table 1). As expected, males had greater height, mass, and daily total energy intake vs. females ($P < 0.05$). Males had greater fat intake as compared to females, whereas female subjects consumed more carbohydrates than males ($P < 0.05$).

3.2 Body mass changes

Total body mass did not change for the CrM group as compared to the PL group (CrM, +0.5 ± 1.1 kg; PL, +0.6 ± 1.0 kg; Appendix E).

3.3 24-hr creatinine excretion, creatinine clearance, and total 24-hr urinary output

There was no difference in 24-hr creatinine excretion for CrM as compared to PL (Table 2). Absolute creatinine excretion showed a trend for the males to be higher vs. females (1.8 ± 0.6 g/d vs. 1.4 ± 0.7 g/d; $P = 0.055$). No change in creatinine clearance was observed for CrM vs. PL group following treatment. Total 24-hr urinary output decreased slightly following either CrM or PL treatment, but was not different for the CrM as compared to the PL group (Figure 5).

3.4 24-hr urinary urea N excretion and Nbal

24-hr urinary urea N excretion remained unchanged for CrM when compared to PL (Table 3; Figure 6). Similarly, Nbal did not change for CrM as compared to PL (Figure 7).

3.5 [^{13}C] leucine flux, oxidation, and non-oxidative disposal (NOLD)

Isotopic steady-state was established throughout the two infusions (LEUPRE and LEUPOST), as demonstrated by constant α-KIC enrichments over time (Figure 8). CrM supplementation did not have an effect on tracer kinetics, and neither of the tracer measures changed significantly for CrM vs. PL following treatment (Table 4; Figures 9-11). However, leucine oxidation was decreased for the CrM-treated males as compared to the PL-treated males following treatment ($P < 0.05$; Figure 12). The males had higher leucine flux (114.1 ± 13.7 vs. 96.9 ± 11.7 μmol/kg/hr), and NOLD (89.7 ± 9.1 vs. 76.8 ± 11.1 μmol/kg/hr) as compared to the females (Figure 13).

Table 1. *Subject characteristics.*

	PL		CrM	
	Females (n=7)	Males (n=6)	Females (n=7)	Males (n=7)
Mass, kg *	63.0 ± 6.4	76.4 ± 6.5	63.3 ± 7.4	87.3 ± 16.0
Height, cm *	167.6 ± 4.3	183.3 ± 6.8	165.0 ± 5.4	183.0 ± 10.3
Energy intake:				
Kcal/day *	2444 ± 669	3273 ± 705	2105 ± 414	3167 ± 701
Kcal/kg/day	38.9 ± 10.9	43.2 ± 9.7	32.9 ± 5.4	37.5 ± 10.1
% PRO	14 ± 5	14 ± 3	16 ± 4	15 ± 2
% CHO **	67 ± 8	56 ± 4	62 ± 4	58 ± 6
% Fat *	18 ± 5	29 ± 5	21 ± 3	26 ± 6

Values are means ± SD. % PRO, relative protein intake; % CHO, relative carbohydrate intake; % fat, relative fat intake. *Males had greater mass, height, total energy intake, and % fat intake ($P < 0.05$). **Females had a greater relative CHO intake ($P < 0.05$).

Table 2. *24-hr creatinine excretion, plasma [creatinine], total 24-hr urinary output, and creatinine clearance.*

	PL		CrM	
	Pre	Post	Pre	Post
Creatinine excretion (n=26), g/24 hr	1.8 ± 0.6	1.6 ± 0.6	1.5 ± 0.7	1.5 ± 0.8
Plasma creatinine (n=21), μmol/L	81.6 ± 13.6	86.8 ± 15.7	79.7 ± 14.3	85.5 ± 17.7
Urine volume (n=27), ml/24 hr	1740 ± 813	1595 ± 696	2310 ± 1137	1838 ± 671
Creatinine clearance (n=21), ml/min/1.73 m^2	124.8 ± 44.9	104.3 ± 31.2	99.7 ± 49.0	111.2 ± 71.1

Values are means ± SD. No significant differences were observed for any of the measures.

Table 3. *Urinary urea nitrogen (N) excretion and Nbal.*

	PL		CrM	
	Pre	Post	Pre	Post
Urinary urea N excretion, g/d:				
females (n=10)	13.0 ± 3.1	12.5 ± 3.2	12.0 ± 3.1	11.5 ± 3.6
males (n=9)	13.4 ± 4.4	12.5 ± 1.9	12.3 ± 6.0	9.7 ± 2.6
total (n=19)	13.2 ± 3.6	12.5 ± 2.5	12.1 ± 4.3	10.6 ± 3.1
Nbal, g/d:				
females (n=8)	-0.9 ± 7.9	-0.2 ± 5.1	1.1 ± 2.7	1.6 ± 2.8
males (n=8)	3.7 ± 5.9	2.8 ± 2.4	5.9 ± 10.8	8.8 ± 7.0
total (n=16)	1.4 ± 6.9	1.3 ± 4.0	3.5 ± 7.7	5.2 ± 6.3

Values are means ± SD. No significant differences were observed for any of the measures.

Table 4. *Leucine flux, oxidation, and NOLD.*

	PL		CrM	
	Pre	Post	Pre	Post
Leucine flux, μmol/kg/hr:				
females (n=12)	97.2 ± 14.7	100.0 ± 10.1	95.7 ± 10.5	94.8 ± 13.8
males (n=13)	117.9 ± 7.0	122.4 ± 17.3	111.5 ± 13.6	104.6 ± 10.4
total (n=25)	107.5 ± 15.1	111.2 ± 18.1	103.6 ± 14.3	99.7 ± 12.8
Leucine oxidation, μmol/kg/hr:				
females (n=12)	19.5 ± 2.1	21.8 ± 6.1	19.0 ± 4.8	20.4 ± 3.6
males (n=12)	21.5 ± 3.2	24.2 ± 7.3	24.2 ± 3.6	19.6 ± 3.8 *
total (n=24)	20.5 ± 2.8	23.0 ± 6.7	21.6 ± 4.9	20.0 ± 3.6
NOLD, μmol/kg/hr:				
females (n=12)	77.8 ± 13.7	78.2 ± 10.6	76.7 ± 11.1	74.4 ± 11.8
males (n=12)	94.2 ± 2.5	92.6 ± 6.5	87.2 ± 11.6	84.9 ± 9.8
total (n=24)	86.0 ± 12.7	85.4 ± 11.2	82.0 ± 12.2	79.7 ± 11.8

NOLD, non-oxidative leucine disposal. * Significantly different from the PL-treated males after treatment ($P < 0.05$).

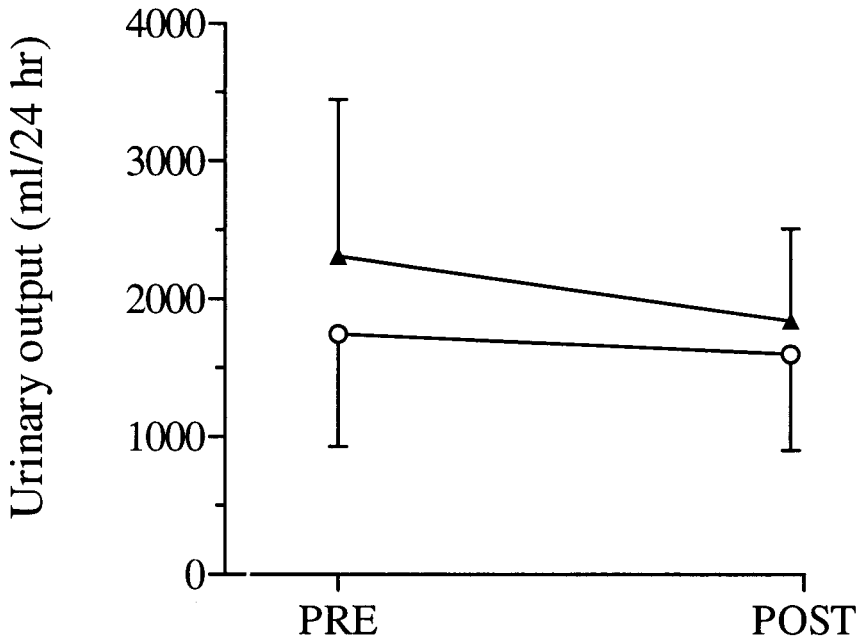


Figure 5. 24-hr total urinary output pre- and post-treatment. There were no differences for CrM as compared to PL. Open circle, PL; filled triangle, CrM.

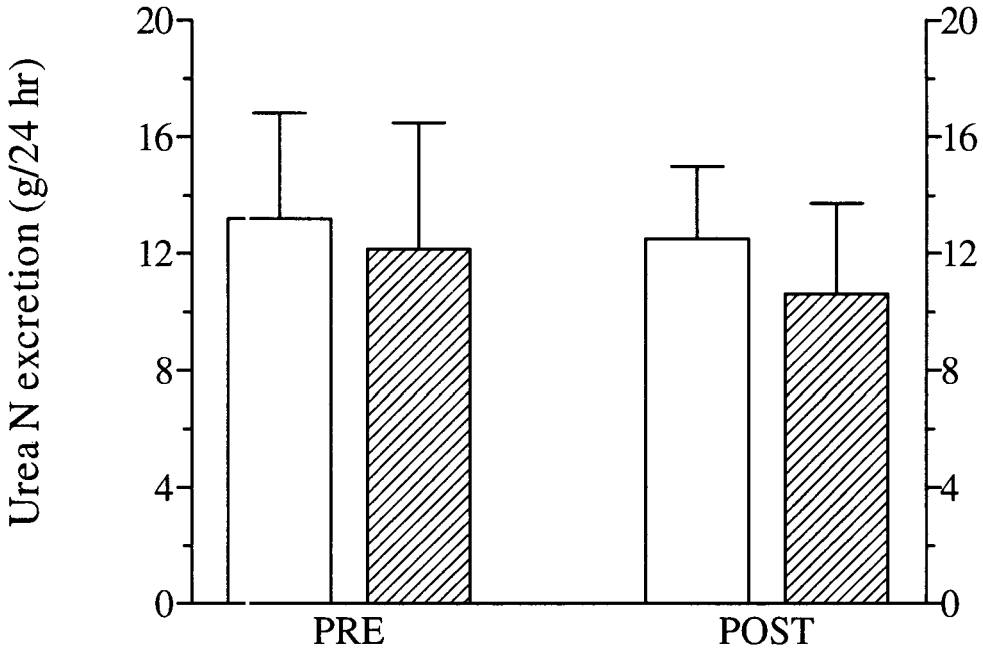


Figure 6. 24-hr urinary urea N excretion pre- and post-treatment. There were no differences for CrM vs. PL. Open bar, PL; hatched bar, CrM.

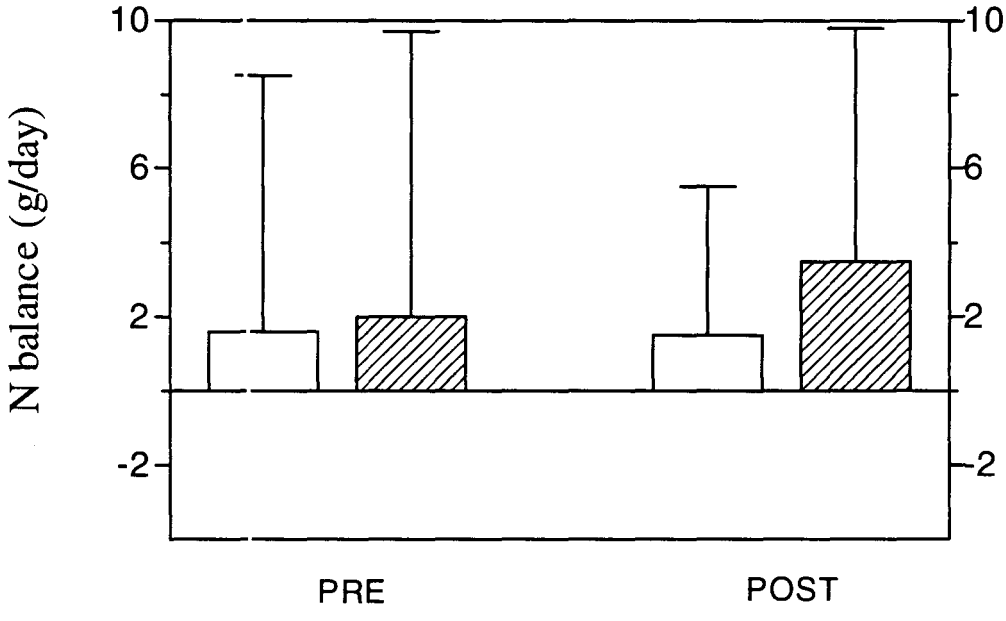


Figure 7. N-balance pre- and post-treatment. There were no differences for CrM vs. PL. Open bar, PL; hatched bar, CrM.

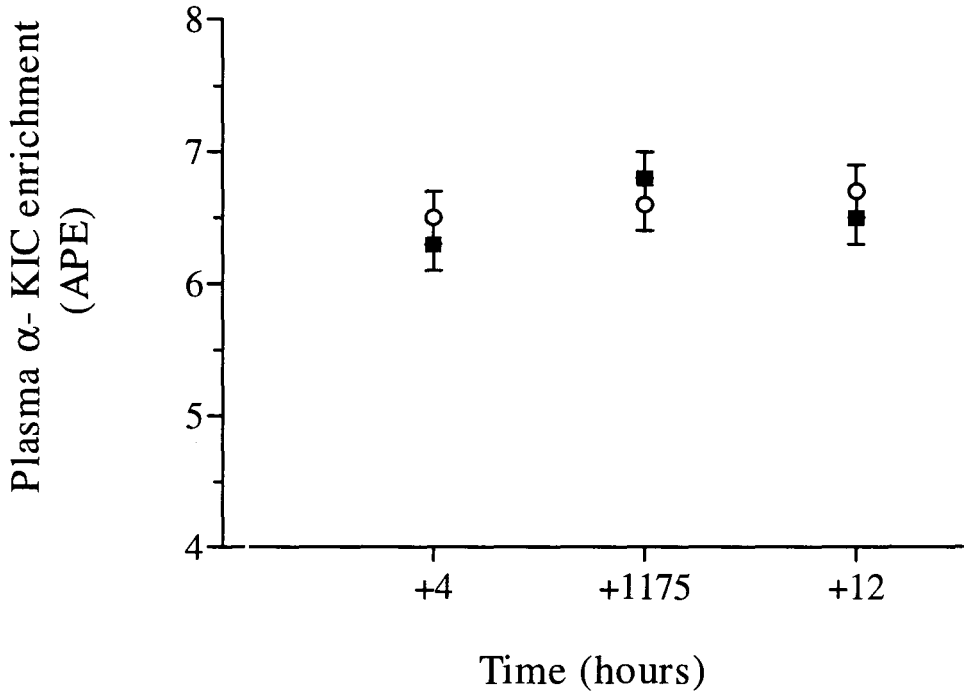


Figure 8. Plasma α-KIC enrichment across time. APE, atom % excess. Open circle, before treatment; filled circle, after treatment (all subjects).

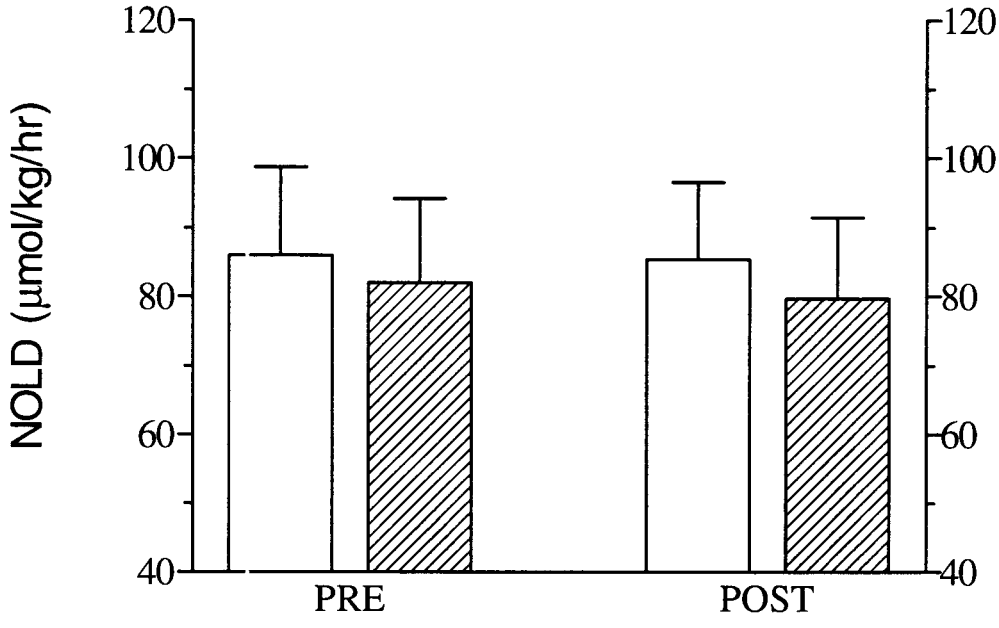


Figure 9. Non-oxidative leucine disposal (NOLD) pre- and post-treatment. There were no differences for CrM as compared to PL. Open bar, PL; hatched bar, CrM.

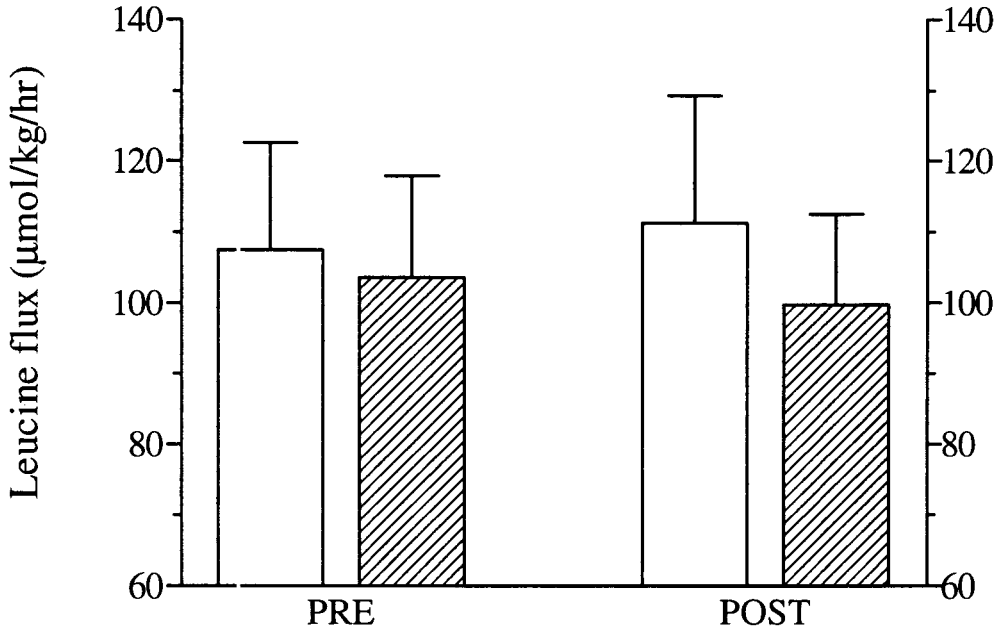


Figure 10. Leucine flux pre- and post-treatment. There were no differences for CrM as compared to PL. Open bar, PL; hatched bar, CrM.

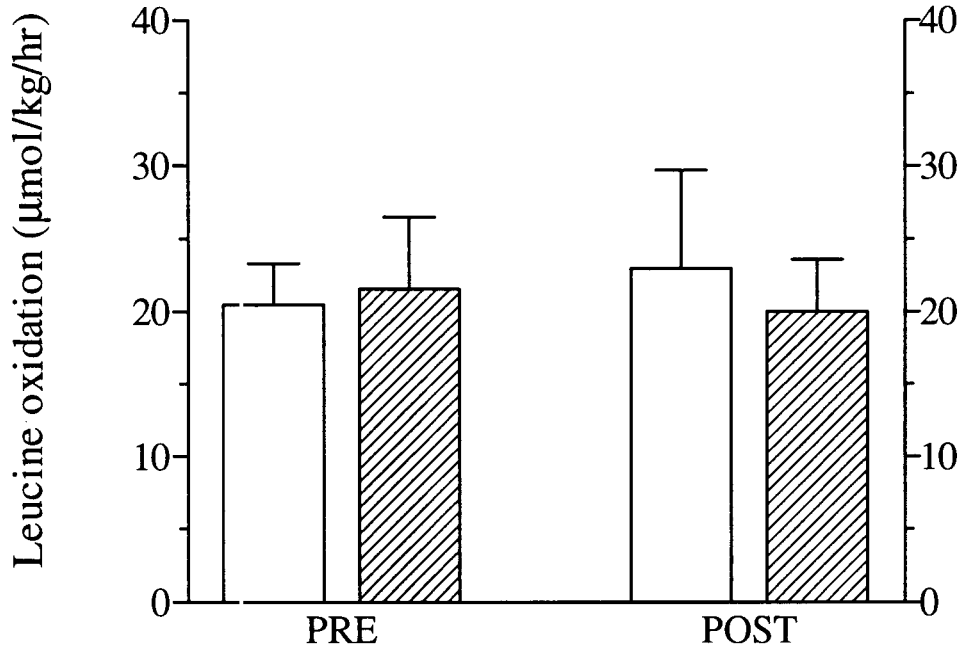


Figure 11. Leucine oxidation pre- and post-treatment. There were no significant differences for CrM as compared to PL. Open bar, PL; hatched bar, CrM.

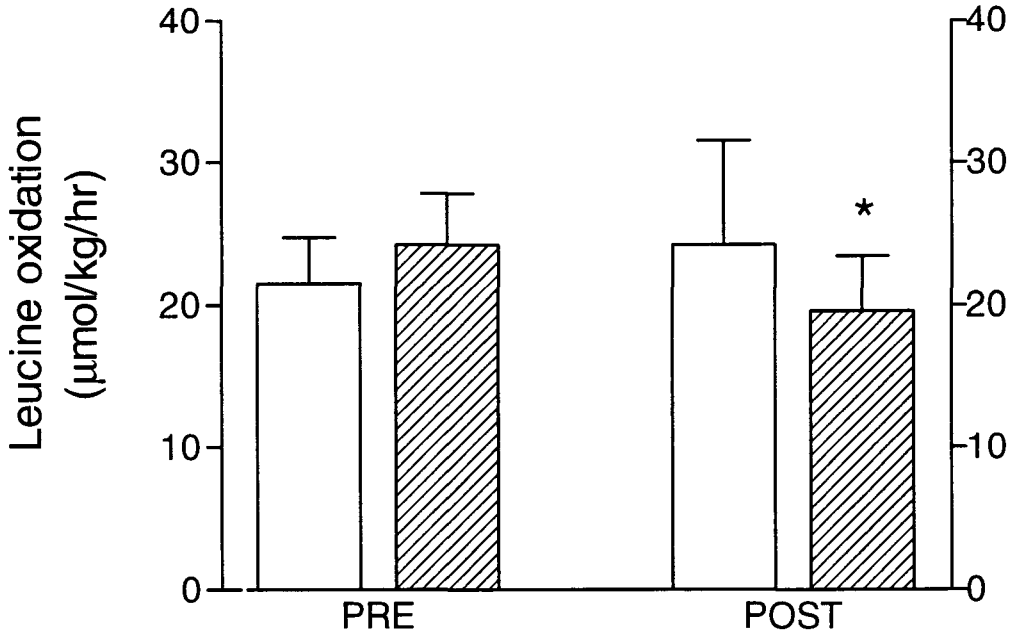


Figure 12. Leucine oxidation for males alone pre- and post-treatment. Open bar, PL-treated males; CrM-treated males, hatched bar. * Significantly different following treatment ($P < 0.05$).

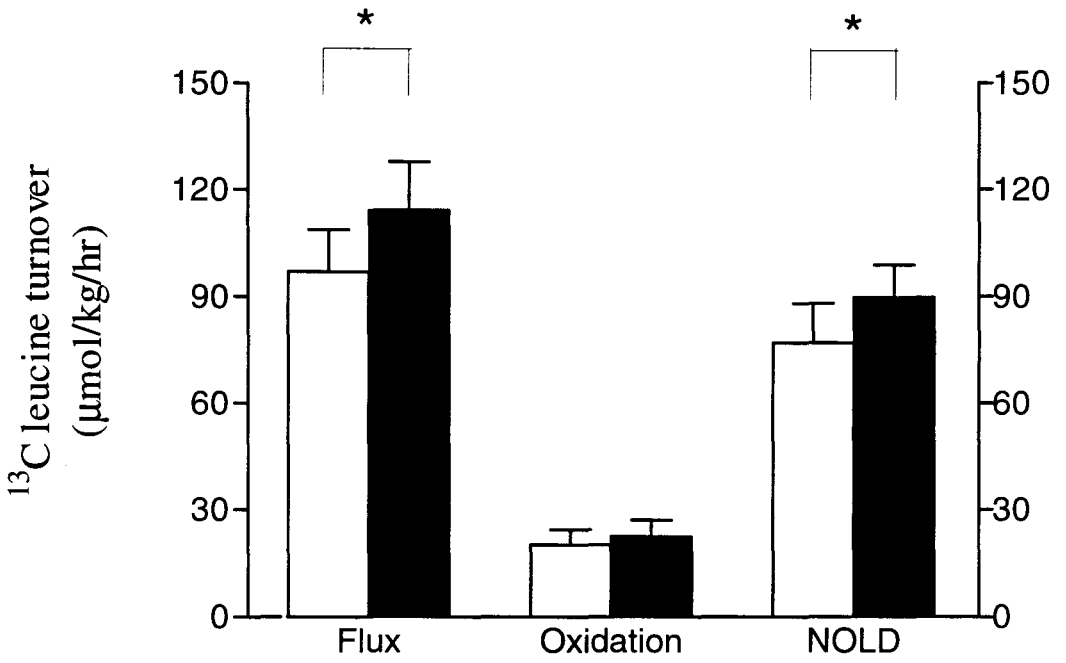


Figure 13. ^{13}C leucine turnover - effects of gender. Open bar, females; solid bar, males. * Significantly different at $P < 0.05$.

CHAPTER 4

Discussion

To my knowledge, this is the first double-blind, randomized, placebo-controlled study to examine the effects of Cr-monohydrate (CrM) supplementation on whole-body protein turnover. It was hypothesized that CrM supplementation would increase body mass and positively affect indices of whole-body net protein accretion in healthy young humans at rest. Moreover, we also expected that there might be gender differences in response to CrM supplementation similar to those that we have recently reported (Mihic et al., 2000). However, the results, obtained by the ^{13}C leucine tracer and N-balance (Nbal) techniques, indicate that the short-term (9-10 d) CrM supplementation protocol affected neither non-oxidative leucine disposal (NOLD), leucine flux (Q_{Leu}), urinary urea N excretion, nor Nbal. Leucine oxidation (O_{Leu}) was significantly decreased for the CrM-treated males as compared to the PL-treated males following treatment, whereas it did not change for the CrM-treated females. This suggests that there may be a gender difference associated with the effects of CrM supplementation on whole-body amino acid oxidation.

One of the *a priori* hypotheses was that CrM would stimulate myofibrillar, and potentially mixed-muscle protein synthesis, as had been demonstrated previously *in vitro* (Ingwall et al., 1972; Ingwall et al., 1974; Young and Denome, 1984), and *in vivo* (Flisinska-Bojanowska, 1996). We also hypothesized that this would be detectable at the

whole-body level, that is, that the magnitude of an increase in skeletal muscle protein synthesis would be sufficient to influence whole-body protein synthesis (WBPS), which represents the sum of all synthetic processes in the body at the time of measurement. This was based on the fact that approximately 25-30% of whole-body protein turnover is accounted for by skeletal muscle protein turnover (Lowry et al., 1985; Nair et al., 1988), or a little less, were the CrM effect strictly specific to myosin heavy-chain (MyHC) and actin synthetic rates.

However, short-term CrM supplementation did not have an effect on whole-body, or mixed-muscle protein synthesis, as indicated by NOLD and FSR (fractional synthetic rate; Parise, 1999), respectively. Knowing that muscle TCr stores were increased following CrM supplementation (Parise, 1999), there may be several explanations for the lack of CrM effect on protein synthesis. Although substantial, the observed increase in muscle [TCr] (~13%), and free [Cr] (~15%; Parise, 1999), may not have reached the threshold for an increased net protein accretion. *In vitro* experiments demonstrated that MyHC and actin synthesis were maximally stimulated at extracellular Cr concentrations of 50–900 μmol/L (Ingwall et al., 1972; Ingwall et al., 1974; Young and Denome, 1984). These concentrations are very similar to those observed in Cr-supplemented humans *in vivo* (Harris et al., 1992). However, the main difference between an *in vivo* physiological system and the differentiating skeletal muscle *in vitro*, is the intracellular Cr concentration, and its relative increase following Cr addition/supplementation. In the chick embryo myotubes, there is virtually no Cr nor PCr prior to Cr addition to the medium in which the cells are incubated. In the grown, mature skeletal muscle, however,

Cr and PCr are abundant, and thus a relative increase in intracellular Cr following Cr supplementation are not nearly as great as that observed *in vitro*. This may have diminished CrM's potential for stimulating muscle-specific protein synthesis.

We had anticipated increases in muscle [TCr] and [Cr] of this magnitude following 9-10 days of CrM supplementation, having hypothesized that they would be sufficient to promote increments in both FSR and NOLD. However, acute CrM supplementation affected neither FSR nor NOLD, suggesting that a ~13-15% increase in muscle Cr concentration had no effect on transcriptional and/or translational events in skeletal muscle.

It is possible that CrM supplementation did promote myofibrillar protein synthesis, that could not be detected either as a higher FSR or NOLD. There may be a mechanism by which a supplementation-induced increase in muscle TCr stores can regulate the rates of MyHC and actin synthesis, probably in a similar way that they are regulated in chick skeletal muscle cultures treated with Cr (Ingwall et al., 1972; Ingwall et al., 1974; Young and Denome, 1984). Unfortunately, we did not measure myofibrillar protein or myofibrillar mRNA content in the muscle, nor did we measure tracer incorporation into the myofibrillar proteins. Therefore, CrM ingestion may have stimulated MyHC and actin synthetic rates, yet they would <u>not</u> necessarily have shown as a greater whole-body protein synthesis, as indicated by an unchanged NOLD.

We speculated that net water retention accompanying CrM uptake would affect protein breakdown to a greater extent than would a direct action of Cr on proteolytic pathways. This assumption resulted from the body of research suggesting that an increase

in cell volume may inhibit proteolysis (Haussinger et al., 1991; Schulz et al., 1991; vom Dahl and Haussinger, 1996), and, under specific conditions, stimulate protein synthesis *in vitro* (Stoll et al., 1992). However, Q_{Leu} remained unchanged following CrM supplementation, suggesting that, like WBPS, protein breakdown was not affected by short-term supplementary CrM ingestion.

A possible gender-specific response was observed for O_{Leu}, which was significantly lower following supplementation for the CrM-treated males as compared to the PL-treated males, while there was no difference in O_{Leu} between treatment groups in females. Considering that the relative increase in muscle TCr content was very similar for the CrM-treated females (~12.5%) as compared to the CrM-treated males (~13.5%), it is unlikely that differences in Cr accumulation produced different effects on O_{Leu} in males and females. There may have been gender differences in the magnitude of cell swelling. A recent investigation by Berneis et al. (1999) with the ^{13}C leucine tracer demonstrated that both Q_{Leu} and O_{Leu} were decreased in young men during experimentally-induced moderate cell swelling. In the present study, a similar decrease in O_{Leu} has been found in the males following CrM supplementation, whereas in the CrM-treated females O_{Leu} did not change. It is possible that cell swelling was an important mechanism by which CrM altered O_{Leu} in the males.

Since 1920's, Cr has been thought to have a N-sparing effect (Benedict and Osterberg, 1923; Chanutin and Guy, 1926). Yet, in the present study, urinary urea N excretion did not change following CrM supplementation. Earlier studies demonstrated no effect of Cr supplementaton on the urinary (Kreider et al., 1998) or blood urea N

concentration (BUN; Earnest et al., 1996; Ransom et al., 1999), another marker of catabolism. These studies, however, were not controlled for dietary intake and composition (Earnest et al., 1996), and included only male athletes undergoing pre-season training work-outs (Kreider et al., 1998; Ransom, 1999). Our findings indicate that CrM supplementation did not slow protein catabolism under the conditions of the study. In addition, Nbal was also unchanged by treatment. This is expected considering that, with the provision of an isoenergetic diet and little variability in creatinine excretion, only changes in urinary urea N excretion could have altered Nbal. Based on these results, it appears that whole-body protein turnover and net N status remained intact during supplementation with CrM. Importantly, urinary urea N excretion and Nbal results are in agreement with those obtained by the tracer methodology (i.e., unchanged Q_{Leu}, O_{Leu}, and NOLD), most likely for the reasons explored earlier in the discussion.

We hypothesized that plasma [creatinine] (CTN), CTN excretion, and CTN clearance, would not change following CrM supplementation, as has been demonstrated both after short- and long-term protocols (Poortmans et al., 1997; Poortmans and Francaux, 1999; Mihic et al., 2000). Indeed, all of these variables remained constant over the course of ten days for both the CrM and PL groups. This provided further evidence that acute, short-term CrM supplementation, does not alter blood chemistry or renal function in healthy young men and women, and that it is a safe practice when set guidelines and recommended doses are followed.

In conclusion, ~125 g of CrM was orally administered over 9-10 days in 27 recreationally active males and females. Treatment with CrM did not have significant

effects on Q_{Leu}, O_{Leu}, NOLD, Nbal, urinary urea N excretion, plasma and urine [CTN], or CTN clearance. However, O_{Leu} was significantly lower for the CrM-treated males as compared to the PL-treated males. It is possible that CrM may have affected the rate of the myofibrillar protein synthesis, but the effects were not great enough to demonstrate protein accretion at the whole-body level. Thus, to be able to measure the true effects of CrM ingestion on whole-body protein metabolism, several methodological recommendations could be made for future research in the field:

1. It would be critical to measure the contents, as well as the synthesis rates of the myofibrillar proteins, MyHC and actin, and their respective mRNA content, in order to determine the effects of Cr supplementation, if any, at the level of contractile protein.

2. Some kind of resistance training should accompany Cr supplementation, since resistive exercise has been shown to maximize Cr uptake and intracellular accumulation, thereby increasing the proposed signal for muscle protein accretion.

3. Males and females should be tested separately, because of potential differences in response to Cr supplementation.

4. Some trends that were observed in this study could reach statistical significance, were we to improve statistical power by using a cross-over design, and/or subjects of the same gender, to reduce the inter-subject variability.

5. The stimulation of Cr uptake and accumulation in skeletal muscle is known to be the greatest in individuals with low muscle Cr and PCr stores (also with impaired cellular energetics ?), so theoretically, neuromuscular patients and older persons are those who could benefit from Cr supplementation the most.

This work was intended to resolve some of the uncertainties regarding the role of Cr in resting protein metabolism. The lack of significant effects of CrM supplementation on whole-body protein turnover might have been a physiological fact, however, arguments provided above were meant to encourage further work in the field. It is my belief that it is indeed desired, not only on behalf of the athletic community, but especially for the implications this work may have in the clinical field.

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APPENDIX A:

Information and consent form

CREATINE SUPPLEMENTATION: EFFECTS ON MUSCLE PROTEIN SYNTHESIS AND MUSCULAR PERFORMANCE

INFORMATION AND CONSENT FORM

INVESTIGATORS	DEPARTMENT:	CONTACT:
Dr. Mark Tarnopolsky	Medicine and Kinesiology	x24465
Mr. Sasa Mihic	Kinesiology	x27037
Mr. Dan MacLennan	Kinesiology	x27037
Mr. Gianni Parise	Kinesiology	x27037

PURPOSE:

Oral creatine supplementation has been a popular practice among athletes involved in anaerobic, short-duration, high-intensity activities (e.g. jumping, sprinting, weight-lifting etc.). It has been suggested that oral ingestion of creatine monohydrate of 20g per day for 5-6 days may benefit physical performance by enhancing volitional strength and anaerobic power. It has also been shown that this protocol is usually accompanied by an increase in body mass, and that males tend to gain significantly more weight than females. These changes in mass are likely due to greater water retention by the muscles, which suggests that lean tissue (fat-free mass) is affected. Indeed, a recent work in our lab (Mihic and colleagues, unpublished data, 1997) has demonstrated that the increase in whole body weight was primarily due to increased lean body mass. Body fat was not affected by the supplementation. Therefore, this study will try to address the following questions:

1. Does creatine supplementation have an effect on muscle protein synthesis?
2. Will creatine supplementation benefit muscle performance in short-term, high-intensity activities?

OUTLINE:

You will be one of the 28 male and female volunteers participating in the study. After having recorded your 4-day dietary intake, you will first be familiarized with the performance testing. You will also be asked to abstain from any form of leg exercise during the trial.

Following an overnight fast, you will report to the Human Performance Laboratory in IWC for the performance protocol (PPRE). You will undergo the following tests: a) 60- sec. handgrip strength test b) a 2-min strength test by the m.tibialis anterior (front of

your shin - lifts your toes off the ground) c) knee extension fatigue protocol (Cybex apparatus), and d) two consecutive 30 sec. all-out bike sprints (Wingate), interspersed with a 4 min. recovery. Prior to and following exercise, small amounts of blood will be sampled (~10ml) through a plastic catheter. The catheter will have been inserted into the antecubital vein of your dominant arm by a trained, certified lab member. In addition, you will collect urine over the 24hr of the testing day.

After a three-day rest, you will undergo a stable isotope infusion protocol (ISOPRE). Prior to and on the day of infusion, it will be required that you eat pre-packaged food designed to mach your habitual intake. A prime (1mg/kg body mass) dose of sterile amino acid (leucine) tracer will then be infused via a plastic catheter (see above), followed by a constant infusion (1mg/kg/hr) for the next several hours. Another catheter will be inserted into the other arm for blood collection at different time-points (total amount ~120ml). ~90 min. after the onset of the infusion, Dr. Tarnopolsky will perform a muscle biopsy on the outer portion of your leg (m. vastus lateralis). As well, expired gas will be collected a number of times. The infusion protocol will be completed following another muscle biopsy of the contralateral m.vastus lateralis. A 24hr urine collection will again take place on the day of testing.

On the following day, you will have your body composition (fat-free mass, body fat %, bone mineral density) assessed by DEXA (x-ray scan). At this point you will be supplemented with either creatine (CR) or placebo (PL; sugar-like substance), as assigned in a randomized, double-blind fashion. The substance will be ingested 4 x 5g/d x 5d, and 3 x 1g/d for the subsequent 4 days (total of 9 days). To control for dietary creatine intake, the consumption of food will be pre-determined (check-list diet) for the duration of the supplementation.

On Day 6 of the protocol, you will again perform a performance test, identical to that previously described. Also, you will collect 24hr urine samples on the day of testing. You will undergo another DEXA scan on Day 9. On Day 10, supplementation will cease, and a leucine infusion will take place in the same way as ISOPRE (see above). Like for the ISOPRE, pre-packaged diet will be consumed, and 24hr urinary sample collected.

DETAILS OF THE PROCEDURES AND POSSIBLE RISKS:

1. Blood sampling. There may be slight bruising at the site of insertion of the plastic venous catheter. The catheter itself is designed to allow for blood to be drawn safely with minimal discomfort to you. It will be inserted by a trained physician or by a trained and certified member of the lab. The total amount of blood taken will be up to 120ml per testing (1/3 cup).
2. Needle biopsy procedure. Involves the local injection of an anesthetic ("freezing") into the skin of the outer thigh area, after which a small (4mm) incision will be made and a small (50-100mg) piece of muscle will be removed using a sterile hollow needle. After the procedure, a suture will be used to close the skin, and ice and pressure will be applied to minimize bruising. The procedure will be performed by Dr. Tarnopolsky who has done it more than 7,000 times. Complications with the procedure are rare. However, in our

experience with athletes, 4/7,000 experience a local skin infection, 6/7,000 have a small lump at the site of biopsy (all disappear with massage after ~1 week), 1/400 have temporary (up to 4 months) localized loss of sensation in the skin at the site of incision, and a few subjects have mild bruising around the incision for 4-5 days. In theory, one could damage a small motor branch of the m. vastus lateralis, which should not affect function (knee extension). Nevertheless, this has not been seen in any of the patients/subjects biopsied by Dr. Tarnopolsky.

3. Stable isotope infusion. ^{13}C leucine has been widely used to examine whole body protein metabolism, as well as muscle protein synthetic rates. The isotope is stable (i.e. non-radioactive), with the natural abundance of ~1.11% of the total body carbon pool. The slight increase in your isotopic enrichment will return to baseline after 24hr. The infusion solution is guaranteed sterile by the manufacturer.

4. Creatine monohydrate supplementation. Administration of creatine similar to that of this study's has been used in a number of experiments (Harris et al., 1992; Hultman et al., 1996). No side-effects have been observed, except for a transient increase in total body mass (~1kg). This weight gain is mostly due to water retention by the lean tissue, and will return to baseline shortly after the cessation of the supplementation. Anecdotal reports of increased blood pressure and altered kidney function have not been supported by a recent well-controlled, randomized, double-blind trial done by our lab (Mihic et al., unpublished data, 1997). However, it is not known what impact creatine ingestion may have in conditions of dramatic changes in body mass, typical for some combat sports (wrestling, judo, boxing etc.). The press recently reported deaths of two elite wrestlers who had been losing weight in an extremely short period of time. The autopsies revealed severe cases of dehydration and it was stated that both athletes were taking creatine as they were losing weight. This report suggests that caution should be exercised when major weight reduction programs are combined with creatine supplementation.

BENEFITS:

You should be aware that the results of this study will be made available to the scientific community, although neither your name nor any reference to you will be used in compiling or publishing these results. You may withdraw from the study at any time without any adverse repercussions, even after signing this form.

You will receive an honorarium of $175 upon the completion of the study to compensate for your time commitment. Additionally, you will have access to your own data (body composition, average dietary intake etc.), as well as the group data when it becomes available.

You will be able to contact student investigators at 525-9140 (x27037) and/or Dr. Mark Tarnopolsky at any time regarding your questions or concerns about the study. Dr. Tarnopolsky can be contacted at: W- 521-2100 (x6593, or x6443 pager: 2888); H- 527-1295.

I,_____, HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT, AND AGREE TO PARTICIPATE AS A SUBJECT.

_____ _____

SIGNATURE DATE

_____ _____

WITNESS DATE

APPENDIX B:

Leucine flux: sample calculation

Calculation for leucine flux (Q; Matthews et al., 1980), with a sample calculation given below:

$$Q\ (\mu mol \cdot kg^{-1} \cdot h^{-1}) = i \cdot [\ (E_i/E_p) - 1]$$

Given:

i - the rate of infusion of L-[1-^{13}C] leucine tracer ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)

Ei - enrichment of the infused leucine, atom % excess (APE)

Ep - plasma enrichment of α-KIC (APE)

-1 - corrects for the contribution of tracer to flux

Sample calculation:

$$Q = 7.57 \cdot [\ (99 / 5.8) - 1] = 121.64\ \mu mol \cdot kg^{-1} \cdot h^{-1}$$

APPENDIX C:

Leucine oxidation: sample calculation

Calculation for leucine oxidation (O; Wolfe, 1992), with a sample calculation:

$$O\ (\mu mol \cdot kg^{-1} \cdot hr^{-1}) = [\ (E_{CO2} \cdot \dot{V}CO_2) \div (E_p \cdot c)\ ]$$

Given:

E_{CO2} - isotopic enrichment of the expired breath CO_2 (APE)

$\dot{V}CO_2$ - the volume of CO_2 produced ($\mu mol \cdot kg^{-1} \cdot hr^{-1}$)

E_p - plasma α-KIC enrichment (APE)

c - bicarbonate retention factor (0.81 was used at rest)

Sample calculation:

$$\text{Oxid.} = [\ (0.0128 \cdot 8053.33) \div (5.8 \cdot 0.81)\ ]$$
$$= 21.94\ \mu mol \cdot kg^{-1} \cdot hr^{-1}$$

APPENDIX D:

Sample size calculation

Sample size estimate equation was based upon the data on changes in FFM following CrM supplementation (Mihic et al., 1998). FFM was used because the main objective of this study was to evaluate the source of Cr supplementation effects on FFM.

$$N = 2[(Z_\alpha + Z_\beta) * SD/\Delta\ ]^2$$

where,

$\alpha = 0.05$ (level of significance), $1-\beta = 0.8$ (statistical power);

$\Delta = 0.8$ kg FFM (effect size); SD = 0.8

APPENDIX E:

Muscle water content, body mass, and fat-free mass

Table 5. *Total body mass, fat-free mass, and muscle water content.*

	PL		CrM	
	Pre	Post	Pre	Post
Total body mass, kg:				
females	60.8 ± 2.9	61.2 ± 2.9	63.3 ± 7.4	63.5 ± 7.4
males	76.4 ± 6.5	77.1 ± 6.0	87.3 ± 16.0	88.0 ± 15.7
total	68.6 ± 9.5	69.2 ± 9.4	75.3 ± 17.2	75.8 ± 17.3
Fat-free mass, kg: *				
females	46.0 ± 4.1	46.5 ± 4.8	44.5 ± 2.9	45.0 ± 3.8
males	63.0 ± 3.6	63.8 ± 3.4	66.9 ± 8.0	67.9 ± 9.0
Muscle water, % of total muscle mass:				
females	74.9 ± 1.8	77.0 ± 2.1	76.5 ± 4.3	76.8 ± 0.1
males	75.6 ± 3.5	76.1 ± 1.9	75.9 ± 1.3	78.1 ± 3.6
total	75.3 ± 2.6	76.6 ± 2.0	76.2 ± 2.3	77.4 ± 2.7

*Data taken from Parise (1999).

APPENDIX F:

Raw data

Table 1a: Tracer kinetics.

			INFUSION RATE (umol/kg/hr)		13C KIC ENRICHMENT(APE)	
Subject #	**Condition**	**Gender**	**PRE**	**POST**	**PRE**	**POST**
3	pla	F	7.20	7.64	6.2	6.2
5	pla	F	6.98	7.63	6.1	7.5
20	pla	F	7.44	7.61	8.3	7.3
22	pla	F	7.47	7.24	6.3	6.3
23	pla	F	7.60	7.74	8.5	7.8
9	pla	M	7.57	7.48	6.1	6
10	pla	M	7.40	7.59	6.1	6.2
14	pla	M	7.43	7.53	5.8	5.3
16	pla	M	7.67	7.52	6.5	6.9
27	pla	M	7.59	7.67	5.8	5.9
4	cr	F	7.22	7.57	6.7	7.8
6	cr	F	6.01	6.06	6.2	5.8
7	cr	F	6.98	7.05	7.4	6.4
8	cr	F	7.52	7.61	6	5.9
18	cr	F	7.51	7.65	7.5	7.5
19	cr	F	7.47	7.47	7.8	8
21	cr	F	7.37	6.96	6.9	8.2
11	cr	M	7.29	7.56	6.6	7.3
12	cr	M	7.54	7.29	7	6.6
13	cr	M	7.57	7.51	5.1	5.9
15	cr	M	7.43	7.54	6.5	7.6
24	cr	M	7.61	7.41	6.2	5.9
25	cr	M	7.63	7.64	6.7	6.9
26	cr	M	7.42	7.39	6	6.4

Table 1b: Tracer kinetics (cont.'d).

			LEUCINE FLUX (umol/kg/hr)		LEUCINE OXIDATION (umol/kg/hr)		NOLD (umol/kg/hr)	
Subject #	**Condition**	**Gender**	**PRE**	**POST**	**PRE**	**POST**	**PRE**	**POST**
3	pla	F	107.8	114.4	21.7	30.0	86.1	84.4
5	pla	F	106.2	93.1	20.2	21.2	86.1	71.9
20	pla	F	81.4	95.6	20.2	21.2	61.2	74.4
22	pla	F	109.9	106.5	19.2	13.0	90.7	93.5
23	pla	F	81.0	90.5	16.1	23.5	64.8	67.0
9	pla	M	115.3	116.0	20.9	20.8	94.4	95.2
10	pla	M	112.6	113.6	19.0	18.0	93.7	95.6
14	pla	M	119.4	133.1	22.1	35.3	97.2	97.7
16	pla	M	109.2	100.4	18.7	19.1	90.4	81.3
27	pla	M	121.9	121.0	26.7	27.8	95.2	93.2
4	cr	F	99.4	88.5	18.2	15.6	81.3	72.9
6	cr	F	90.0	97.4	26.7	21.3	63.3	76.1
7	cr	F	86.3	102.1	14.5	19.8	71.8	82.3
8	cr	F	116.6	120.0	18.4	26.7	98.2	93.3
18	cr	F	91.7	93.3	13.5	20.4	78.2	72.9
19	cr	F	87.4	84.9	17.6	17.1	69.8	67.9
21	cr	F	98.4	77.1	23.8	21.8	74.5	55.4
11	cr	M	102.1	94.9	27.3	23.9	74.8	71.0
12	cr	M	99.1	102.1	23.3	20.8	75.8	81.3
13	cr	M	139.4	118.5	29.9	21.8	109.6	96.7
15	cr	M	105.8	90.7	19.8	13.6	86.0	77.0
24	cr	M	113.9	117.0	25.6	19.1	88.3	97.8
25	cr	M	105.1	102.0	20.7	15.5	84.4	86.5
26	cr	M	115.0	106.9	23.3	22.8	91.7	84.1

Table 2a: Urinary urea N excretion, and N-balance.

			N in (g/d) [1]		Total urinary N out (g/d)		Urinary urea N out (g/d) [2]	
Subject #	**Condition**	**Gender**	**PRE**	**POST**	**PRE**	**POST**	**PRE**	**POST**
1	pla	F	15.7	15.7	12.1	9.7	11.5	9.2
2	pla	F	20.8	20.8	11.9	18.4	11.3	17.5
3	pla	F	17.0	17.0	15.1	12.7	14.4	12.0
5	pla	F	8.8	8.8	18.7	14.3	17.8	13.6
10	pla	M	16.7	16.7	10.9	13.8	10.4	13.1
14	pla	M	19.7	19.7	16.7	14.6	15.9	13.9
17	pla	M	18.5	18.5	17.0	14.0	16.1	13.3
27	pla	M	22.3	22.3	7.7	13.6	7.3	12.9
4	cr	F	15.1	16.8	12.8	13.6	12.2	12.9
6	cr	F	21.8	23.5	15.9	14.1	15.1	13.4
7	cr	F	14.2	15.9	14.4	12.0	13.7	11.4
8	cr	F	17.7	19.4	13.1	15.2	12.4	14.5
11	cr	M	13.4	15.1	19.9	12.8	18.9	12.2
12	cr	M	23.6	25.3	16.4	8.3	15.6	7.9
15	cr	M	21.4	23.1	5.8	7.4	5.5	7.0
24	cr	M	26.9	28.6	9.6	12.2	9.1	11.6

[1] 16% of PRO intake (Tarnopolsky et al., 1992).

[2] Urinary urea N was assumed to be 95% of total urinary N excretion (Lemon et al., 1992).

Table 2b: Urinary urea N excretion, and N-balance (cont.'d).

			N out-Cr (g/d) [3]		N out-feces (g/d) [4]	N out-sweat (g/d) [5]	N out-misc. (g/d) [6]	N balance (g/d)	
Subject #	**Condition**	**Gender**	**PRE**	**POST**				**PRE**	**POST**
1	pla	F	0.0	0.0	1.4	0.5	0.12	1.6	4.0
2	pla	F	0.0	0.0	1.4	0.5	0.12	6.8	0.4
3	pla	F	0.0	0.0	1.4	0.5	0.12	-0.1	2.3
5	pla	F	0.0	0.0	1.4	0.5	0.12	-11.9	-7.5
10	pla	M	0.0	0.0	1.6	0.75	0.12	3.3	0.5
14	pla	M	0.0	0.0	1.6	0.75	0.12	0.5	2.6
17	pla	M	0.0	0.0	1.6	0.75	0.12	-1.0	2.0
27	pla	M	0.0	0.0	1.6	0.75	0.12	12.1	6.2
4	cr	F	0.0	1.5	1.4	0.5	0.12	0.3	-0.3
6	cr	F	0.0	1.5	1.4	0.5	0.12	3.8	5.8
7	cr	F	0.0	1.5	1.4	0.5	0.12	-2.2	0.4
8	cr	F	0.0	1.5	1.4	0.5	0.12	2.6	0.6
11	cr	M	0.0	1.5	1.6	0.75	0.12	-8.9	-1.7
12	cr	M	0.0	1.5	1.6	0.75	0.12	4.7	13.0
15	cr	M	0.0	1.5	1.6	0.75	0.12	13.1	11.7
24	cr	M	0.0	1.5	1.6	0.75	0.12	14.9	12.5

[3] Based on: 1) molecular weight of N=15, and Cr=131; 2) there are 3 N in a Cr molecule, and, Cr retention on day 10 of supplementation is assumed to be ~12 % (4.4/5 g is excreted; Hultman et al., 1996).

[4, 5] Estimates from Phillips, 1991.

[6] Estimate from Calloway et al., 1971.

APPENDIX G:

Statistical summary tables

ANOVA Table 1: Height.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	14.175	23	50.567	.28	.6
Gender	1	1914.975	23	50.567	37.87	.00

ANOVA Table 2: Mass.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	211.73	23	100.57	2.1	.16
Gender	1	2353.81	23	100.57	23.4	.00

ANOVA Table 3: Total daily energy intake.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	319525.5	22	408203.4	.78	.38
Gender	1	5785191.0	22	408203.4	14.17	.00

ANOVA Table 4: Relative daily energy intake (per kg body mass).

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	222.22	22	88.78	2.5	.13
Gender	1	126.02	22	88.78	1.42	.25

ANOVA Table 5: % PRO intake.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	12.81	20	15.03	.85	.37
Gender	1	4.06	20	15.03	.27	.61

ANOVA Table 6: % CHO intake.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	13.39	20	33.0	.4	.53
Gender	1	360.34	20	33.0	10.92	.00

ANOVA Table 7: % Fat intake.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	.03	20	24.29	.00	.97
Gender	1	382.66	20	24.29	15.75	.00

ANOVA Table 8: Creatinine excretion.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	.4355	22	.4944	.8808	.3581
Gender	1	2.029	22	.4944	4.1033	.055
Time	1	.08	22	.4107	.1947	.6632
Condition x Gender	1	.0309	22	.4944	.0625	.8048
Condition x Time	1	.1683	22	.4107	.4098	.5286
Gender x Time	1	.755	22	.4107	1.8383	.1888
Condition x Gender x Time	1	.9826	22	.4107	2.3923	.1361

ANOVA Table 9: Creatinine clearance.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	847.603	17	3064.881	.2765	.6057
Gender	1	6.493	17	3064.881	.0021	.9638
Time	1	208.719	17	2299.776	.0907	.7668
Condition x Gender	1	76.076	17	3064.881	.0248	.8766
Condition x Time	1	2615.331	17	2299.776	1.1372	.3011
Gender x Time	1	6279.079	17	2299.776	2.7303	.1168
Condition x Gender x Time	1	3290.131	17	2299.776	1.4306	.248

ANOVA Table 10: 24-hr urinary output.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	2225932	23	1052975.0	2.1139	.1594
Gender	1	204634.7	23	1052975.0	.19433	.6634
Time	1	1279214	23	510387.5	2.5063	.127
Condition x Gender	1	7131.857	23	1052975.0	.0067	.9351
Condition x Time	1	359857.2	23	510387.5	.7051	.4097
Gender x Time	1	158253.8	23	510387.5	.31	.583
Condition x Gender x Time	1	56888.05	23	510387.5	.1114	.7415

ANOVA Table 11: Mass changes.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	571.491	22	187.8272	3.0426	.095
Gender	1	5163.694	22	187.8272	27.4917	.0000
Time	1	3.594	22	.6327	5.6805	.0262
Condition x Gender	1	232.248	22	187.8272	1.2365	.2781
Condition x Time	1	.04	22	.6327	.0639	.8027
Gender x Time	1	.423	22	.6327	.6687	.4222
Condition x Gender x Time	1	.029	22	.6327	.0463	.8315

ANOVA Table 12: Urinary urea N excretion.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	20.6225	15	16.87212	1.222282	.2863
Gender	1	.8598	15	16.8721	.0509	.8244
Time	1	11.917	15	9.3666	1.2722	.277
Condition x Gender	1	2.431	15	16.8721	.144	.7096
Condition x Time	1	1.686	15	9.3666	.18	.6774
Gender x Time	1	4.09	15	9.3666	.4367	.5187
Condition x Gender x Time	1	1.339	15	9.3666	.143	.7106

ANOVA Table 13: N-balance.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	73.2092	12	68.2121	1.0732	.3206
Gender	1	194.3004	12	68.2121	2.848	.1172
Time	1	5.1057	12	9.6373	.5298	.4807
Condition x Gender	1	9.5794	12	68.2121	.1404	.7144
Condition x Time	1	6.6162	12	9.6373	.6865	.4235
Gender x Time	1	.2852	12	9.6373	.0296	.8663
Condition x Gender x Time	1	8.2013	12	9.6373	.851	.3744

ANOVA Table 14: Non-oxidative leucine disposal.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	277.65	20	177.9381	1.5604	.226
Gender	1	1961.206	20	177.9381	11.0218	.0034
Time	1	24.349	20	40.165	.6062	.4453
Condition x Gender	1	69.208	20	177.9381	.3889	.5399
Condition x Time	1	8.767	20	40.165	.2183	.6454
Gender x Time	1	2.928	20	40.165	.0729	.7899
Condition x Gender x Time	1	3.139	20	40.165	.0782	.7827

ANOVA Table 15: Leucine flux.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	736.105	21	255.7302	2.8784	.1045
Gender	1	3609.083	21	255.7302	14.1128	.0012
Time	1	.215	21	58.4427	.0037	.9522
Condition x Gender	1	231.323	21	255.7302	.9046	.3524
Condition x Time	1	174.393	21	58.4427	2.984	.0988
Gender x Time	1	13.838	21	58.4427	.2368	.6316
Condition x Gender x Time	1	45.592	21	58.4427	.7801	.3871

ANOVA Table 16: Leucine oxidation.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	10.0495	20	27.6807	.363	.5536
Gender	1	58.9991	20	27.6807	2.1314	.1598
Time	1	2.5138	20	12.2433	.2053	.6553
Condition x Gender	1	.0132	20	27.6807	.0004	.9828
Condition x Time	1	49.3205	20	12.2433	4.0283	.0584
Gender x Time	1	23.1786	20	12.2433	1.8931	.1841
Condition x Gender x Time	1	29.8194	20	12.2433	2.4356	.1343

ANOVA Table 17: Leucine flux - males.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	944.6278	11	270.0257	3.4983	.0882
Time	1	9.2205	11	49.2472	.1872	.6735
Condition x Time	1	209.8856	11	49.2472	4.2619	.0634

ANOVA Table 18: Leucine oxidation - males.

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Condition	1	4.6675	10	32.9086	.1418	.7143
Time	1	5.213	10	9.0534	.5758	.4655
Condition x Time	1	77.9198	10	9.0534	8.6067	.0149