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Cellular Morphology Associated with Creatine Monohydrate Supplementation with Concurrent Periodized Resistance Training

by

Lorraine Sim-Anderson 

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for
the degree of Master of Science

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Abstract

The purpose of this study was to examine cell morphology associated with creatine monohydrate supplementation, combined with periodized resistance training. Twenty-one male subjects were randomly assigned to one of three groups: five day load and 32 day maintenance dose (ALM), five day load and maintenance placebo (AL), or placebo group (PL). Creatine was consumed at a rate of 0.3 $g \cdot kg^{-1}d^{-1}$ for the acute phase and 0.03 $g \cdot kg^{-1}d^{-1}$ for the maintenance period. Skeletal muscle biopsies were used to determine fiber area, percentage, cell water and protein content. Significant increases in fiber areas of type I and type II subgroups, when collasped, occurred with training in all groups over time. No significant changes were noted in the percentage of cell water or protein content. These results suggest that 37 days of creatine supplementation does not have an effect on skeletal muscle morphology, protein content or cell water, compared with resistance training only.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Cellular Morphology Associated with Creatine Monohydrate Supplementation with Concurrent Periodized Resistance Training" submitted by Lorraine Sim-Anderson in partial fulfillment of the requirements for the degree of Master of Science.

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Dr. Gord Bell

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Chapter 1

Introduction

Background

The discovery of creatine dates back from as early as 1832 (Balsom et al., 1994). Only recently however, has creatine supplementation been a topic for many research studies involving human sport performance. Athletes participating in activities ranging from weight training to football have been interested in the performance enhancing benefits of creatine supplementation.

Creatine (Cr), in its phosphorylated form, phosphocreatine (PCr), is used as a source of energy in the production of adenosine triphosphate (ATP) through the creatine kinase reaction. It can be either synthesized within the body or it can be ingested through foods such as meat and fish (Balsom et al., 1994). Greenhaff et al. (1994) and Volek et al. (1997b) found that ingesting additional creatine, (ie. creatine loading) will enhance some exercise performance in activities that predominately utilize the phosphocreatine energy system. Improved performance during high intensity intermittent exercise with creatine supplementation has been attributed to increased initial creatine content, as well as accelerated rate of PCr resynthesis during the recovery phase (Greenhaff et al., 1994).

A number of researchers have examined and demonstrated the positive effects of creatine supplementation during various high intensity activities. In 1992, Harris et al. found subjects who received supplementation had faster sprinting times in both 300

and 1000 m distances. Earnest et al. (1995), Noonan (1998), and Volek et al. (1997b) reported increased one repetition maximum (1 RM) strength results with creatine supplementation. Similar results were found using a cycle ergometer. During repeated six second maximal bouts, supplemented subjects were able to perform greater total work (Balsom et al., 1993a; Kreider et al., 1998; Söderlund et al., 1994). In addition, increased peak power during 30 second wingate tests have been reported in studies by Birch et al. (1994) and Casey et al. (1996).

To date, the only side effect associated with creatine supplementation reported in the literature has been an increase in body mass. Studies have shown an average increase of 0.9 to 2.9 kilograms in body mass (Balsom et al., 1993a; Earnest et al., 1995; Greenhaff et al., 1994; Kreider et al., 1998; Söderlund et al., 1994; Volek et al., 1997a).

Despite numerous performance studies that have shown benefits associated creatine supplementation, little has been researched at the cellular level in regards to the body mass increase. The question of the composition of the increased body mass has not been answered. Many studies to date, which have reported acute body mass gains associated with creatine supplementation, have speculated that the increases reflect a water retention effect in the body. It has also been hypothesized that water retention, or cell swelling, may stimulate protein synthesis within the skeletal muscle (Volek et al., 1997a).

Significance of Study

The effects of creatine supplementation on enhancing exercise performance during maximal intermittent bouts of activity have captured the attention of many exercise physiologists and athletes who rely on the phosphocreatine energy system for their particular sport. The body mass increase resulting from creatine supplementation has been found in various research studies (Balsom et al., 1993a; Earnest et al., 1995; Greenhaff et al., 1994; Kreider et al., 1998; Söderlund et al., 1994; Volek et al., 1997a). This side effect of creatine supplementation also has many athletes interested and curious about this ergogenic aid. In sports that place a great emphasis on the athletes' size and mass, such as body building, football, and hockey, supplementation in conjunction with their training is thought to gain that extra advantage over their competitors.

With increasing popularity of this performance aid, many questions arise, such as the safety of supplementation, how much supplementation benefits athletes, and should creatine supplementation be banned in certain sporting events? Finally, and perhaps most applicable to exercise physiologists, what changes take place at the cellular level that make up the change in body weight? There is a need for further research in this area to identify the underlying changes associated with the acute mass gains reported with creatine supplementation. The present study attempts to examine these cellular changes.

Purpose

The purpose of this study was to investigate the cellular morphological changes associated with creatine monohydrate supplementation while undergoing periodized resistance training by investigating changes in muscle fiber area, changes in total cell water content, and changes in cell protein content.

Hypothesis

If gains in body mass result from supplementing with creatine monohydrate, then it is hypothesized that there should be increases in intracellular water content, and thus an increase in muscle fiber area. The alternate hypotheses are that any observed gains in body mass reflect changes in extracellular water and therefore, no change in muscle area, or that there will be no changes in body mass at all.

Delimitations

The scope of this study was to investigate how creatine monohydrate supplementation affects skeletal muscle morphology in strength trained males, and was part of a larger research design that investigated performance, as well as biochemical changes related to creatine supplementation. As a result, some methods and procedures address these related research questions.

University aged males, with considerable weight training experience were recruited in order to compare the results to previous published work, which used similar subject selection criteria. In addition, recruitment of experienced trainees

helped to insure proper lifting mechanics, reduced the motor learning, or neurogenic phase associated with beginning a weight training program, and allowed for minimal supervision during training sessions.

The independent variables, or treatment conditions, involved the manipulation of creatine supplementation dose protocol and implementation of a common periodized strength training program. The dependent variables included the anthropometric and dietary changes, muscle fiber cross sectional area, fiber type percentage, and water and protein content changes of the muscle.

Limitations

The limitations of this study can be categorized into three main sections: the subjects, design, and analysis. Since all of the subjects were volunteers, attrition was a factor. To address the limitations of attrition, subjects were encouraged and motivated to complete the study. In addition, those subjects placed in the placebo group were provided with a one week post experimental load of creatine monohydrate so that every subject had the opportunity to trial the supplement. Limiting the subjects from performing additional or insufficient activity than those specified on the exercise program may have been a confounding factor. Subject effort during testing or training may have also affected the results. Improper recording of dietary logs, improper treatment and/or incorrect method of ingesting the supplementation may also have affected the results. To control for subject limitations, all subjects were informed with

both verbal and written instructions concerning the activity level, effort, supplementation schedule, and recording of dietary intake.

The major limitation surrounding the design was that this study was part of a larger project. Therefore, as a result, the method and procedures may not have been designed specifically for this particular investigation. These factors have been carefully examined and it was concluded that the design was sufficient to answer the questions associated with this thesis.

The final category for possible limitations was the analysis of the data. Histochemical and biochemical laboratory procedures and data entries were subject to human error. The investigators took measures to be educated and well practiced in the laboratory techniques prior to the study.

Definitions

Experienced recreational weight lifters: Subjects weight training for a minimum of one year, training at least six months out of the last 12 months, two or more times per week, with no sport specific goals or focus to their training.

24 Hour Urine Collection: The protocol involves collection of urine for a 24 hour period. Subjects were instructed to discard the first urination of the morning for the collection day. Following that first urination, subjects were instructed to collect all urine excreted in the bottle provided, until and including the following morning. The 24 hour collection period ends with the first urination on the second morning.

Chapter 2

Review of Literature

Introduction

A French scientist named Chevreul in 1832 first discovered creatine. It was found to be an organic compound present in meat (Balsom et al., 1994). In 1847, Lieberg confirmed this finding by extracting creatine from several kinds of muscle in mammals. He also observed the amount of creatine in wild foxes captured during a chase was greater than captive mammals and suggested that there was a relationship between creatine and muscle contraction. In addition, it was speculated that creatinine found in urine was a compound related to creatine stored in the human muscle (Balsom et al., 1994).

From studies of cat muscle by Fiske and Subboarow in 1927, it was reported that phosphocreatine diminished during electrical stimulation of skeletal muscle, and then regenerated during subsequent recovery periods (Balsom et al., 1994). Both free creatine and phosphorylated creatine, phosphocreatine, have since been acknowledged as a vital element in the regulation and homeostasis of skeletal muscle energy metabolism during high intensity exercise.

Only until recently has creatine supplementation in humans been examined. These studies have been sparked by the investigations done in 1992 by Harris et al. It was discovered that the phosphocreatine content found in human muscle tissue could be significantly increased following a period of oral ingestion of supplemental

creatine. Exercise physiologists have since been curious about the effect of creatine monohydrate supplementation on exercise performance.

Creatine Biosynthesis and Regulation

Creatine is a naturally occurring substance and is endogenously manufactured in the liver, kidneys and pancreas (Walker 1979). The three precursor amino acids involved in the synthesis of creatine are arginine, glycine, and methionine (Walker 1979). The formation of creatine occurs through two reactions (Walker 1960). The first reaction consists of arginine plus glycine to yield guanidinoacetate and ornithine. This reaction is referred to as transamidination and is reversible. The second irreversible reaction involves guanidinoacetate plus S-adenosylmethionine to yield S-adenosylhomocysteine and creatine. This second process is called transmethylation.

Creatine is found in small quantities in the brain, liver, kidney, and testes. Approximately 95-98% of the total creatine pool however, is found in skeletal muscle (Greenhaff, 1995; Heymsfield et al. 1983). Since there is a separation of biosynthesis from utilization, transportation must occur. Creatine is transported to the skeletal muscle via the bloodstream.

Uptake of Creatine

Creatine is absorbed from plasma to skeletal muscle through the cell membrane. This process of osmosis against a concentration gradient occurs at a rate of 200:1. It has been found that the uptake of creatine is through a sodium-dependent

carrier (Volek and Kraemer, 1996). In 1966, Fitch and Shields suggested that there was a special mechanism of cell membrane entry known as the saturable process. Intracellular water soaks through the membrane at a rate of 0.6 mmol per litre. Fitch and Shields also suggested that the intracellular trapping of creatine provide a plausible explanation for the high creatine content in skeletal muscle. The different synthesis sites and utilization allows for independent regulation of the processes involved (Walker 1979).

Functions of Creatine and Phosphocreatine

Creatine and phosphocreatine play an integral role in the regulation and homeostasis of skeletal muscle energy metabolism during high intensity exercise., There are four major functions of creatine and phosphocreatine (Volek and Kraemer 1996).

1. Temporal energy buffer:

Phosphocreatine serves as a donor of phosphate for energy production during high intensity activities. Since PCr and Cr are smaller in size and are less negatively charged than ATP and ADP, these compounds may be stored at a greater concentration (Plisk and Kreider, 1999). ATP can be quickly resynthesized anaerobically through the creatine kinase (CPK) reaction as demanded.

$$PCr + ADP + H^{+} \Leftarrow CPK \Rightarrow ATP + Cr$$

When the total muscle pool of creatine phosphate is high, the cell is considered to have a high energy state, allowing for the resynthesis of ATP from ADP. When the pool of PCr is low, the CPK reaction shifts to the left, resynthesizing PCr from ATP.

2. Spatial energy buffer or PCr energy shuttle:

Described by Bessman (1990), the phosphocreatine energy shuttle includes three distinct areas. First, at the peripheral terminus of the shuttle, the creatine kinase reaction occurs in which ADP is rephosphorylated to form ATP. The second part involves the intervening space, where the free creatine and phosphocreatine separate to travel in opposite directions within the cell. Finally, the creatine interacts with the mitochondrial isoenzyme of CPK at the energy-generating terminus. The PCr is shuttled back to the sites of utilization and the process continues to cycle.

3. Proton buffer:

During the creatine kinase reaction, PCr is used to maintain a functional intracellular pH by buffering the hydrogen ion.

4. Modulator of glycolysis:

Glycolysis may be stimulated during depressed levels of PCr. In vitro studies have shown that the glycolytic enzyme phosphofructokinase (PFK) is disinhibited when PCr levels decrease, and thus, increasing the rate of glycolysis (Volek and Kraemer, 1996).

Normal Values of Total Creatine and Resting Levels

Resting levels of ATP are approximately the same in both type I and type II muscle fibers. The resting levels of PCr however, are found to vary between the two fiber types. In human muscle, type I fibers have 5-15% less PCr levels than the type II fibers (Spriet, 1995). Total creatine content in skeletal muscle ranges from 115-140 mmol/kg of dry muscle (dm). Of the total creatine pool, approximately 60-

65% or 70-90 mmol/kg dm is found in the form of phosphocreatine (Balsom et al., 1994).

With creatine deplenishing at a rate of two grams per day (Greenhaff, 1995), creatine must be either endogenously formed, or obtained through dietary means. Creatine is predominantly found in foods such as meat, fish, and poultry. The concentration ranges from approximately three to nine grams per kilogram (Plisk and Kreider, 1999). The body creatine pool is influenced by dietary intake (Crim et al., 1976). Oral ingestion of creatine depresses its biosynthesis in the body. This response is reversible upon the cessation of supplementation (Walker 1960; Walker 1979). The fact that dietary creatine can enter the bloodsteam directly suggests that total muscle creatine concentration [TCr] and possibly PCr contents may be elevated through dietary means (Spriet, 1995).

Creatine Supplementation

The main premise whereby creatine supplementation may be beneficial is by increasing the total intramuscular creatine pool size, the availability of phosphocreatine will also be elevated and in turn, positively effect the anaerobic energy metabolism process. Harris et al. (1992) supplemented subjects with varying doses between 20-30 grams per day for three and a half to seven days. The result of this study was a mean increase in the total cellular creatine content ranging from 126.8-148.6 mmol/kg dm, or approximately 17.2% following creatine supplementation. Harris also found that total creatine content could be further augmented by 10% when exercise was performed during the supplementation period.

It was concluded that the initial resting levels of creatine determined the creatine absorption during supplementation.

Since Harris' study, numerous researchers have examined various dosing protocols and variables. Greenhaff et al. (1994) found creatine ingestion substantially increased total muscle creatine concentration and PCr resynthesis during recovery in five out of eight subjects. Hultman et al. (1996) found total muscle creatine concentration increased by 20% when subjects loaded for six days at a rate of 20 grams per day followed by a 30 day maintenance dose of two grams per day. It was found that a maintenance dose of three grams per day might be much more effective in maintaining the creatine levels (Hultman et al., 1996).

In 1996, Greenhaff found that 20-30% of individuals do not respond to creatine supplementation. These non-responders showed less than 10 mmol/kg dm increase in total muscle creatine. The magnitude of improvement in exercise performance following creatine supplementation is also closely related to the extent of muscle creatine accumulation during supplementation (Casey et al., 1996). Maximal total creatine uptake through dietary supplementation occurs in about 20% of subjects, with Vandenberghe et al. (1996) reporting muscular levels approaching 160 mmol/kg dm.

Renal Responses Associated with Creatine Supplementation

Poortmans et al. (1997) measured total protein and albumin urine excretion rates in order to determine the effect of creatine supplementation on renal responses. The major finding was that creatine supplementation, at a rate of 20 grams for five

days, had a significant incremental impact on arterial content (mean 3.7 fold) and urine excretion rate (mean 90 fold) of creatine as compared to the control group. Arterial and urine creatinine values were not affected by creatine supplementation while glomerular filtration rate and total protein and albumin excretion rates remained within normal ranges. This study concluded that short term supplementation of creatine does not have detrimental effects on the renal responses in healthy men but long term effects are not known.

Absorption Rate of Creatine with Carbohydrate Ingestion

In 1996, Green found total creatine accumulation could be further increased by an average of 60% when creatine is ingested in combination with a simple carbohydrate (CHO) solution. The mean concentration of cellular creatine in subjects ingesting creatine in a CHO solution reached closer to the maximum load of 160 mmol/kg dm. The loading increase was explained by the stimulatory effect of insulin on muscle creatine transport. Creatine retention was not further increased when exercise was performed prior to ingestion. In addition, contrary to other reported research (Harris et al., 1992), the initial muscle creatine concentration was found to have no significant effect on the extent of muscle creatine accumulation when creatine was ingested in combination with carbohydrate. It was also found that creatine supplementation alone had no effect on serum insulin concentration, but Cr and CHO ingested together dramatically elevated insulin concentration. For practical application of this finding, creatine should be ingested in combination with CHO to maximize creatine loading.

Absorption Rate of Creatine with Caffeine Ingestion

Vanakoski et al. (1998) showed that caffeine did not affect creatine supplementation when caffeine was taken at seven mg/kg·bm per day. In 1996, Vandenberghe et al. demonstrated that ingesting caffeine at five mg/kg·bm per day in a single dose with creatine can counteract the positive effect of creatine supplementation on performance during repeated bouts of high intensity exercise. Vandenberghe and researchers (1996) hypothesized that caffeine ingestion would augment muscle creatine accumulation through a direct and indirect, catecholamine mediated stimulation of sodium dependent muscle creatine transport, and thereby enhance exercise performance further. The results of these studies suggest that this benefit of caffeine can be negated when combined with creatine supplementation.

Diet and Creatine Accumulation

Since cellular creatine content can be effected by diet, especially through consuming natural sources such as fish, beef, and poultry, it has been shown that subjects with a vegetarian diet demonstrate lower resting levels of total cellular creatine concentration. The greatest amount of increase in total creatine levels following creatine supplementation has been shown by this group in comparison to non-vegetarians (Balsom et al., 1994).

Creatine Supplementation with Female Subjects

The majority of literature on creatine supplementation to date uses male subjects ages 20-30 years old. There are limited published works using female

subjects. Vandenberghe et al. (1996) studied the effect of creatine supplementation with female subjects. They found that PCr/ATP ratio remained elevated during prolonged creatine supplementation even though the dose of creatine was strongly reduced after an initial creatine loading procedure of four days at 20 grams per day with a maintenance load of five grams per day. It was suggested that the reported gains in high intensity intermittent exercise capacity as a result of concurrent strength training, was markedly enhanced by creatine supplementation in females.

Performance Effect of Creatine Supplementation

Creatine supplementation has been hypothesized to benefit activities that involve high intermittent energy, due to its vital role in the phosphocreatine energy system. Researchers have investigated the effects of creatine supplementation on activities such as isokinetic activity (Greenhaff et al., 1993; Ööpik et al., 1998), strength training (Earnest et al., 1995; Greenhaff et al., 1993; Kelly and Jenkins, 1998; Kreider et al., 1998; Noonan et al., 1998; Vandenberghe et al., 1997; Volek et al., 1997b), power events such as jumping (Balsom et al., 1993a; Balsom et al., 1995; Noonan et al., 1998; Volek et al., 1997b), single and repetitive sprint running (Balsom et al., 1993; Harris et al., 1993), sprint cycling (Balsom et al., 1993a; Balsom et al., 1995; Birch et al., 1994; Casey et al., 1996; Cooke et al., 1995; Earnest et al., 1995; Febbraio et al., 1995; Kreider et al., 1998; Odland et al., 1997; Söderlund et al., 1994), and endurance running (Balsom et al., 1993b; Ekblom, 1996; Stroud et al., 1994). The majority of these studies have supported creatine supplementation as an effective ergogenic aid during repeated high intensity, short duration activities.

Body Mass Increase Associated with Creatine Supplementation

The only known side effect associated with dietary creatine supplementation in the literature is an increase in body mass. The reported body mass changes associated with acute supplementation range from an increase of 0.9-3.8 kilograms. Table 1 summarizes body mass changes in studies with creatine supplementation.

Proposed Theories or Mechanisms Associated with Body Mass Increase

The composition of the increased body mass and exact mechanism of how creatine supplementation results in these changes are not well known. Several studies have measured changes in body fat or lean body mass (Earnest et al., 1994; Kelly and Jenkins, 1998; Kreider et al., 1998; Noonan et al., 1998; Volek and Kraemer, 1996; Vandenberghe et al., 1997) and found no changes or a decrease in body fat.

There are several proposed theories on the composition of the increased mass including an increase in total body water, or retention of water, increase diameter in the fast twitch glycolytic muscle fibers, and increase in muscle synthesis of contractile protein (Balsom et al., 1993a). These proposed theories are related. Balsom et al. (1993a) speculated that in the short term, creatine supplementation would cause an increase in cell water, thus increasing the diameter of the cell, and in the long term, promote protein synthesis. The phosphocreatine energy shuttle, protein synthesis, and cellular hydration state have been examined in an attempt to explain the underlying mechanism behind the reported body mass increase.

Table 1: Summary of Body Mass Changes from Literature

Reference	Supplementation Duration (days)	N	Dose (g/day)	Average Increase (kg)
Balsom et al., 1993a	6	8	25	1.1
Balsom et al., 1993b	6	9	20	0.9
Balsom et al., 1995	6	7	20	1.1
Chanutin & Guy, 1962	29	1	10-20	3.2
	44	1	10	3.8
Earnest et al., 1995	28	4	20	1.7
Greenhaff et al., 1994	5	8	20	1.6
Kelly and Jenkins, 1998	26	18	20	2.9
Kreider et al., 1998	28	25	15.75	2.2
Peeters et al., 1999	3	11	20	1.7
Söderlund et al., 1994	6	8	20	1.5
Vandenberghe et al., 1997	4	19	20	1.8
Volek et al., 1997a	7	7	25	1.4

Phosphocreatine Energy Shuttle and Hypertrophy of Skeletal Muscle

The phosphocreatine energy shuttle acts a spatial energy buffering system that can increase the rate of protein synthesis, muscle contraction, and ion transport in the muscle (Bessman and Savabi, 1990). There is a close relationship between CPK isoenzymes and many sites of ATP utilization and production. The energy shuttle system involves three locations: peripheral terminus, energy-generating terminus, and intervening space. During contraction, creatine is liberated at the utilization sites and returns to the mitochondria to be rephosphorylated to phosphocreatine. In addition, creatine can also stimulate ATP sites in glycolysis, which would result in the stimulation of energy production anaerobically (Bessman and Savabi, 1990).

Exercise acts to stimulate protein synthesis by increasing the contractile activity, which in turn causes greater transport of phosphocreatine via the shuttle system. Creatine supplementation may cause an increase in the rate of the phosphocreatine energy shuttle and as a result, allow greater phosphocreatine to be free for protein synthesis (Balsom et al., 1993a). Approximately 70% of protein synthesis is dependent on the energy obtained from the phosphocreatine energy shuttle as a source of energy while the other 30% is dependent on insulin stimulation (Bessman and Savabi, 1990).

Protein Synthesis

Ingwall's (1976) study supports the theory of creatine in the role of protein synthesis by suggesting that creatine may be the chemical signal coupling increased muscular activity with increases in contractile protein synthesis during hypertrophy.

It was also suggested that creatine may act as a transcriptional factor, or it may alter charged t-ribonucleic acid (tRNA) levels, or amino acid pools that are responsible for muscle protein synthesis (Ingwall et al., 1976).

Based on studies done on mononucleated muscle cells obtained from 12 day old chick embryos, several speculations regarding the involvement of creatine on the rate of protein synthesis in differentiating skeletal muscle have been made (Ingwall et al., 1976). These studies conducted by Ingwall report that muscle specific protein synthesis in both skeletal and cardiac muscle is selectively stimulated by creatine. Increasing the intracellular creatine concentration lead to increased rates of synthesis of both myosin heavy chain and actin (major myofibrillar protein), and accumulation of the muscle specific isoenzymes of creatine phosphokinase (CPK). In contrast, the rate of total protein synthesis and the accumulation of the non-muscle isoenzyme of CPK and lactate dehydrogenase and its isoenzymes, were not affected (Ingwall et al., 1976). Creatine appeared to affect only cells that were already involved in synthesizing muscle protein, and did not affect cells involved in myoblast proliferation or the cell fusion process (Ingwall et al., 1976). In addition, creatine appeared to increase the overall synthesis of RNA and seemed to preferentially induce specific classes of RNA (Ingwall et al., 1976). Finally, the effect of creatine is manifested in different stages of the synthesis of muscle proteins (Ingwall et al., 1976) with the primary effect connected to the nucleus and accomplished at the transcription level.

In addition to these findings by Ingwall, Bessman and Savabi (1990), suggested that creatine stimulated the uptake of amino acids into contractile proteins.

It was suggested that the amount of body mass protein could cause hypertrophy in proportion to the rate of phosphocreatine hydrolysis. In contrast, unused muscle that does not react with phosphocreatine and liberate free creatine, could cause atrophy of the muscle. This point has been supported in studies that show rats that have been depleted of phosphocreatine have significantly decreased muscle diameter. Other studies such as those that provide long term, small dosages of creatine supplementation to those patients suffering from an eye disease, called gyrate atrophy, have shown an increase in the diameter of the type II, or fast twitch, muscle fibers in the vastus lateralis muscle (Sipilä et al., 1981). Research related to creatine supplementation on the rate of protein synthesis and regulating mechanisms require further investigation.

Cellular Hydration State

Since creatine is an osmotically active substance, cell swelling, water retention, or increase in total cell water content has been hypothesized to account for the increase in body mass associated with acute dietary creatine supplementation (Balsom et al., 1993a). Anecdotal reports suggest subjects supplemented with creatine may intake a greater amount of fluid than their control counterparts. Concentrative uptake of some amino acids like those transported by sodium dependent mechanisms into muscle, and in this case, creatine, would be expected to increase cellular hydration (Häussinger et al., 1993).

In 1991, Häussinger and Lang found that the degree of amino acid induced cell swelling appeared to be largely related to the steady state intra/extracellular

amino acid concentration gradient. Hormones and the nutritional state modify this gradient and the degree of cell swelling in a complex way. Creatine supplementation may affect the cell gradient, and in turn, the degree of cell water retention.

The cellular hydration status is an important factor for controlling cellular protein turnover, protein synthesis, and protein degradation (Häussinger et al., 1993; Häussinger et al., 1994). Hormones and amino acids can trigger those patterns simply by altering cell volume. An increase in cellular hydration, or cell swelling, may act as an anabolic proliferative signal, while cell shrinkage, or dehydration, may act as a catabolic antiproliferative signal (Häussinger et al., 1993). Alterations of cellular hydration may represent another important mechanism for metabolic control and act as another second or third messenger linking cell function to hormonal and environmental alterations (Häussinger et al., 1994). Increased cellular hydration is maintained as long as the amino acid is still present in the cell. If creatine supplementation serves to increase intracellular stores of creatine and phosphocreatine, then this may induce a state of cell swelling. Of the three hypothesized mechanisms, the one that appears to have the most credence would be that the increase in cellular fluid content.

Summary

Creatine appears to increase the muscle's ability to maintain power output during strenuous high intensity exercise in some research while concurrently increasing body mass (Balsom et al., 1993a; Earnest et al., 1995; Greenhaff et al., 1993; Söderlund et al., 1994). The causes of these performance enhancements are

attributed to increased intracellular storage of phosphocreatine and creatine, and greater rate of phosphocreatine resynthesis during rest periods. The associated body mass increases have been noted in several studies (Balsom et al., 1993a; Earnest et al., 1995; Greenhaff et al., 1994) but the components of this mass have not yet been adequately explained, and it may be speculated to be the result of an increase in total body water (Balsom et al., 1993a; Volek & Kraemer, 1996). If the reported gains in body mass are a reflection of increases in water within the muscle cell, then cell swelling and/or protein synthesis may be linked.

Chapter 3

Methods and Procedure

Subjects

Twenty-one healthy males from the University of Alberta's general student population participated in the study. All subjects had a minimum of one year strength training experience and had no prior history of creatine monohydrate supplementation. Written informed consent was obtained in compliance with the University's Ethics Review Committee for the use of human subjects in research (Refer to Appendix A). Prior to the experiment, subjects were briefed on the experimental procedures and associated risks (Refer to Appendix B). The subjects were informed that they were allowed to withdraw at any point of the study without consequence. The descriptive characteristics and previous weight training experience of the participants are shown in Table 2.

Table 2: Subject Characteristics

N=21	**Mean**	**±SE**
Age (yrs)	23.0	±0.4
Height (cm)	177.7	±2.0
Weight (kg)	76.4	±2.1
Resistance training experience (yrs)	5.1	±0.8
Training Frequency during previous 12 months (days/week)	3.3	±0.2

Experimental Design

The study consisted of three distinct phases. The first three weeks of the study represented the pre-load phase. The purpose of this phase was to familiarize and standardize the subjects with the testing and training procedures. This phase was also used to minimize any strength increases caused by neurological adaptations associated with initiating a resistance training program. An acute load phase followed, which consisted of five days of creatine supplementation or flavored placebo, combined with resistance training. The beginning of the acute load phase was referred to as time one (T1) and the end of the acute load phase is referred as time two (T2). The final 32 days was the maintenance phase of the study in which subjects continued to strength train while receiving either a maintenance dosage of creatine or a placebo. The end of this phase was referred to as time three (T3). One subject withdrew from the study during the maintenance phase. Figure 1 illustrates the experimental design, experiment groups, and loading protocol.

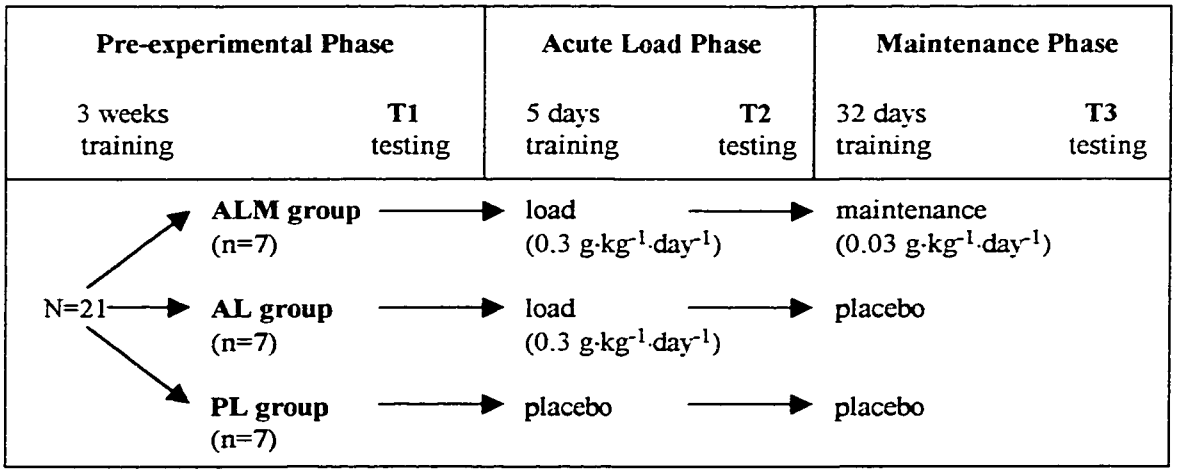

Figure 1: Experimental Design, Experiment Groups, and Loading Protocol

Experiment Groups

The 21 subjects were matched on absolute leg press scores and randomly assigned to one of three experimental groups.

1. Acute creatine load / placebo maintenance (AL)
2. Acute creatine load / creatine maintenance (ALM)
3. Placebo load / placebo maintenance (PL)

Table 3 illustrates the descriptive characteristics of the three experimental groups.

Table 3: Characteristics of Experimental Groups

Experimental Group	**AL N= 7**	**ALM N= 7**	**PL N= 7**
	Mean ± SE	**Mean ± SE**	**Mean ± SE**
Age (yrs)	22.7 ±0.2	22.7 ±0.4	23.6 ±0.5
Height (cm)	180.2 ±4.0	132.0 ±2.6	172.2 ±1.9
Weight (kg)	79.4 ±3.4	77.4 ±4.6	71.8 ±1.6*
Experience (yrs)	5.4 ±1.3	5.5 ±1.7	4.8 ±1.3

* significantly lighter, $P<0.05$

Loading Protocol

Creatine supplementation was administered at a rate of 0.3 $g \cdot kg^{-1} \cdot day^{-1}$ during the acute load and 0.03 $g \cdot kg^{-1} \cdot day^{-1}$ during the maintenance phase. The creatine supplementation was dissolved with one thousand milliliters of heated water (40-50°C) along with a powdered glucose mix and then cooled prior to distribution. An equal

amount of powdered glucose mix and water was administered to the placebo group. Preparation and distribution of the solutions were conducted in a double blind format.

The acute dose phase consisted of five days of loading. Subjects were instructed to consume the one thousand milliliter mixture in four equal parts to be ingested over the course of the day with a minimum of three hours between each dose. The solution was consumed on an empty stomach; either half an hour prior to a meal or one hour after a meal. During the 32 day maintenance phase, subjects ingested 250 milliliters of creatine or placebo solution once per day (Refer to Appendix C).

Physiological Training

Subjects did not participate in additional training or physical activity beyond the periodized resistance training program stipulated by the investigators of the study. The results obtained from eight repetition maximum (8RM) tests were used to determine the starting intensity for each of the exercises (Refer to Appendix D). The loading of each exercise was the same relative load for each individual.

A two day split routine program was used throughout the study. Subjects were encouraged to perform adequate warm up, cool down, stretching, and abdominal exercises as part of the training session. Weekly training cycles consisted of two days of consecutive workouts, one day of rest, followed by another two days of consecutive workouts, ending with two days of rest. Day one of the split routine targeted the chest, back, and shoulders using eight exercises including bench press, inclined bench press, shoulder press, dumbbell lateral raise, upright rowing, latissimus pulldowns, and

dumbbell flys. Day two of the split routine focussed on the arms and legs using six exercises including bilateral inclined leg press, leg extensions, hamstring curls, calf raises, bicep curls, and tricep pressdown.

All training intensities and exercises were printed out for the subjects with the assistance of a computer software program (B.E. Software. Lincoln, NE). During the pre-load phase, core exercises, which included bench press, inclined bench press, latissimus pulldowns, and inclined leg press, were performed with three sets of eight to ten repetitions at 65-80% of one repetition maximum (1RM). The remaining peripheral exercises were performed with two sets of eight to twelve repetitions at 65-75% of 1RM. During the loading week, training volume and intensity increased to four to five sets of two to ten repetitions at 75-95% of 1RM for the core exercises, and three sets of eight to ten repetitions at 65-75% for the peripheral exercises. During the final maintenance phase of the study, subjects lifted four to five sets of two to ten repetitions at 70-95% of 1RM for the core exercises and two to three sets of eight to ten repetitions for the peripheral exercises at 65-85% of 1RM. Rest periods of at least one to three minutes between sets were encouraged to ensure a high quality of work was performed. For strength results, refer to Appendix E.

Data Collection and Assessment

1. Anthropometric Assessment

Anthropometric tests were conducted at T1, T2, and T3. Subjects were assessed on their height in centimeters, weight in kilograms, and skinfolds at five sites

in millimeters. The five sites assessed included: triceps, biceps, iliac crest, subscapular, and medial calf. Percent body fat was calculated from the mean of two sets of skinfold measures that were within 0.4 mm using the Durnin-Womersley (1974) method. This calculation used the measurements from the triceps, biceps, subscapular and iliac crest.

Anthropometric measures were assessed prior to physical testing. The Canadian Physical Activity and Lifestyle Appraisal (CPAFLA) protocol was used. Height was measured using a metric wall tape and a set-square. The subject stood with no footwear, erect with arms at their sides, feet together, and heels and shoulder blades against the wall. As the subject looked directly forward and took a deep breath in, the measurement was recorded with the set-square placed on top of their head and square to the wall. Height measurements were recorded to the nearest 0.5 centimeter. Body weight was recorded using a spring beam scale to the nearest 0.1 kilogram. Subjects were instructed to remove their footwear and to dress in light clothing (ie. tee shirt and shorts).

The five sites were landmarked prior to measuring skinfolds with Harpenden calipers. Skinfolds were measured at each of the five sites once, followed by a second measurement at each of the sites. Skinfolds scores were recorded to the nearest 0.2 millimeters. An average of two scores was calculated except when the two measurements did not fall within 0.4 millimeters of one another. In that case, a third reading was conducted, and the average of all three measurements was used to calculate the value, unless there were two measurements that were within 0.4 millimeters. In the case of two out of three readings falling within 0.4 millimeters of

one another, those two readings were averaged for the score and the third score was deleted. All measurements were conducted on the right side of the body. Calipers were applied one centimeter below the point where the skinfold was raised. The following illustrates the landmark sites used for the five skinfolds.

Skinfolds:

a. Triceps: a vertical fold on the posterior surface of the upper arm, half-way between the acromion of the shoulder and the olecranon process of the elbow.

b. Biceps: a vertical fold on the anterior surface of the upper arm at the level of the triceps skinfold.

c. Subscapular: a diagonal fold running downward and laterally at a 45 degree angle from the vertebral border of the scapula, to a point one centimeter beneath the inferior angle.

d. Iliac crest: a diagonal fold running downward and anteriorly, raised three centimeters above the crest of the ilium on the mid-axillary line.

e. Medial Calf: a vertical fold on the medial aspect of the calf at the level of the greatest circumference, with the foot placed on a box to flex the knee 90 degrees in order to facilitate a relaxed muscle.

2. Three Day Dietary Log

Subjects were required to record their food and fluid intake in a three day dietary intake booklet. Subjects were briefed on how to accurately record items such as serving sizes and complete food descriptions, to limit human error (Refer to Appendix F). The booklets provided additional written instructions and directed

subjects to fill in the menu item, unit of measurement, number of units, brand name of item, type of flavour, and method of preparation. The recording process began with the morning meal, then repeated for the mid-morning snack, mid-day meal, afternoon snack, evening meal, and evening snack. Recording of diet occurred during the last three consecutive days at the beginning of the pre-load week, and at T1, T2, and T3. The three days selected were always Thursday, Friday, and Saturday to ensure the recording of a weekend day, as peoples' eating habits generally change during this period. The logs were used to determine any significant alterations in dietary macronutients (proteins, fats, carbohydrates, and water), as well as total ingested daily calories. A computer software program, Food Processor (ESHA Research, Salem, OR) was used to analyze raw data from the dietary logs.

3. 24 Hour Urine Volume

Subjects were provided with a four liter container to collect a urine sample for a 24 hour period. Urine sample collection began following the first urination on the first day and ended with the first urination on the second day. The samples were obtained at T1, T2, and T3. Urine volumes were measured to the nearest milliliter. Urine collected was measured for total urine output.

4. Muscle Biopsies

Muscle biopsies from the vastus lateralis were obtained at T1, T2, and T3 following all strength performance tests. The tissue was analyzed for muscle cross sectional area, determination of muscle fiber type, calculation of total cellular fluid,

and total protein content of the tissue. The site of the biopsy was the lateral aspect of the vastus lateralis muscle of the right leg. All muscle samples were stored at –80°C freezer until analyzed.

A physician, who was experienced in the needle muscle biopsy technique, performed the procedure with suction (Evans, 1982). Extreme caution was used to ensure sterilization of the biopsy site and of the entire laboratory. Biopsy needles were placed in the autoclave unit for at least 20 minutes for sterilization purposes. The thigh region was cleansed with alcohol, then a local anesthetic was injected under the skin at the area of the biopsy. A five millimeter incision was made and the biopsy needle was then inserted into the incision through the iliotibial band, to a depth of approximately 2.5 centimeters. Two tissue plugs were then obtained from the same site. A steristrip was used to close the surgical opening and pressure and ice were applied to the area for a minimum of 10 minutes while the subjects remained resting in a supine position (Refer to Appendices G and H).

The first biopsy sample used for biochemical procedures was snap frozen using liquid nitrogen within three to five seconds of removal. Biochemical procedures used to determine ATP (Lamprecht et al., 1974), creatine (Bernt et al., 1974), and phosphocreatine (Lamprecht et al., 1974) concentrations were calculated from the analysis of the first tissue sample (Refer to Appendices J and K). These muscle samples were also used to determine the fluid content of the tissue, as well as protein concentration, using the Lowry's technique (Lowry et al., 1951). Total tissue fluid content was determined by subtracting the wet muscle weight from the dry weight

after freeze drying a portion of the biopsy. The biopsy samples were freeze dried for 16 hours at 10 microns of mercury vacuum and -45°C using a Labconco freeze dryer model 45. Samples were dissected free of visible blood, fat, and connective tissue, freeze dried and weighed again.

The second tissue biopsy sample was oriented cross-sectionally and mounted in OCT solution on a piece of cork and frozen using isopentane cooled in liquid nitrogen. This second sample was used for histochemical analyses. Muscle samples were sectioned at a thickness of eight μm in a –20°C cyrostat. Three pieces of tissue were sectioned in series for each sample, and were placed on glass cover for histochemical analysis. A myofibrillar ATPase assay (Brooke and Kaiser, 1970) was used to determine fiber type (Refer to Appendix L). Pre-incubation solutions were set at a pH of 10.4, 4.3, and 4.45 for type I, type IIa and IIb, and type IIc respectively. Fiber type identification, muscle fiber areas and fiber types percentages were obtained with the assistance of a PSI computer assisted image analysis system (Perceptive Systems Inc., League City, TX). Following the myofibrillar ATPase assay, coverslips were placed into the PSI scanner and images were captured in the computer. Using the images, fiber type was determined by examining the shade of each muscle fiber. Muscle fiber areas were calculated by tracing the border of each muscle fiber with a computer joystick. The PSI system calculated the fiber type percentages based on the fibers circled for fiber area.

Statistical Analysis

Statistical analyses were performed by using a two way analysis of variance (ANOVA) with repeated measures on time. A Newman-Keuls post hoc test was used when significant F ratios were obtained. P values were set at 0.05 level. All values were expressed as means, plus or minus standard deviation. The assistance of the computer software, Statistica, (Oklahoma City) was used.

Chapter 4

Results

Creatine Load

There was a significant increase in cellular [Cr] for all three groups across time (Table 4) with T2 and T3 significantly greater than T1. No significant differences in cellular [PCr] and combined [Cr + PCr] between groups over time were observed. Since there was no significant change in the [Cr], [PCr], or combined [Cr + PCr] in any of the groups, the ALM and AL data were collapsed. The collapsed group (AL + ALM) had significantly higher [PCr] from T1 to T2.

Figure 2 illustrates the mean percent changes in total cellular [Cr], [PCr], and combined [Cr + PCr], in all groups over time. The total free cellular Cr content in the AL and ALM groups increased by 42.6% and 49.1% respectively from T1 to T2, versus 20.5% in the PL group. Although not statistically significant, the combined [Cr + PCr] in the ALM and AL groups also increased from T1 to T2 by 14.4% and 14.3% respectively, while the PL group decreased by 9.6%. There were modest, but not significant increases in [PCr] in the AL and ALM groups following the five day load, 1.0 and 1.5% respectively. Total [PCr] in the PL group declined by 12.8% following the five day loading period, but increased by 12.5% over the initial baseline levels at T3.

Table 4: Total Cellular Creatine and Phosphocreatine Content (mmol/kg)

		T1		T2		T3	
		M	±SD	M	±SD	M	±SD
Cr							
	ALM	33.07	9.43	48.63[a]	8.40	45.67[a]	4.10
	AL	33.89	6.13	48.03[a]	6.45	42.33[a]	9.26
	PL	38.10	7.58	48.03[a]	13.57	47.93[a]	5.87
	ALM+AL	33.48	7.78	48.33	7.43	44.00	6.68
PCr							
	ALM	78.28	3.60	78.14	7.83	80.66	11.34
	AL	79.69	6.41	80.64	7.98	70.65	10.51
	PL	79.01	11.16	65.96	11.81	72.36	9.77
	ALM+AL	78.99	5.01	79.39[b]	7.91	75.66	10.93
Cr+PCr							
	ALM	109.59	8.05	126.31	7.34	110.19	33.60
	AL	113.58	9.69	129.31	12.03	112.98	18.52
	PL	117.12	14.99	113.99	12.95	120.29	8.57
	ALM+AL	111.59	8.87	127.81	9.69	111.59	26.06

[a] T2 and T3 are significantly greater than T1, $P<0.05$
[b] significantly greater PCr from T1 to T2, $P<0.05$

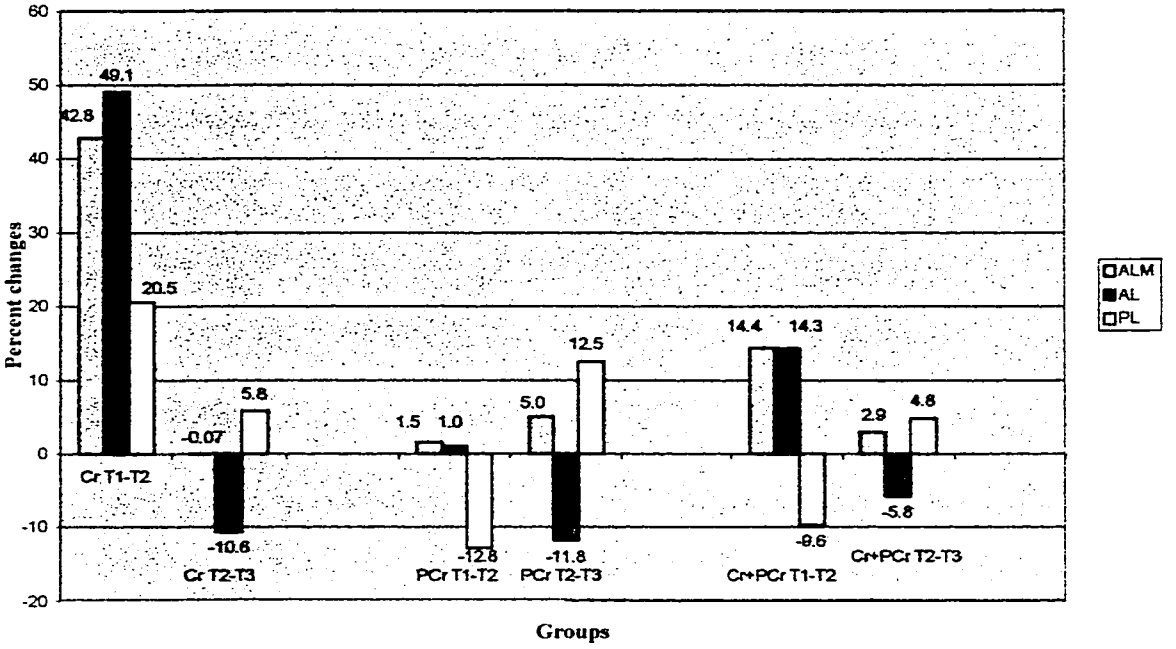

Figure 2: Percent Changes in Total Cellular Creatine, Phosphocreatine and Combined Creatine + Phosphocreatine

Anthropometric Measurements

Absolute body mass changed significantly over time (Table 5). The ALM and AL groups significantly increased mean absolute body mass by 2.0 and 2.2 kg respectively after the five day acute load (T2) compared to pre-load levels. Following the 32 day maintenance load combined with training (T3), the ALM group maintained a significantly higher mean body mass of 2.3 kg compared to pre-load levels (T1). The mean body mass of the AL group, which received a placebo for the duration of the 32 day maintenance phase, was reduced from T2 but remained statistically higher at T3, with a mean increase of 1.25 kg compared to pre-load levels. The mean body mass of the PL group was significantly lower than the ALM and AL groups at T1, T2, and T3. This discrepancy did not statistically change over the course of the study.

All groups decreased in skinfolds from T1 to T2. The PL group significantly changed over time, decreasing at T2 by a mean of 2.74 mm and then increasing at T3 by a mean of 2.30 mm over T1. The AL group had significantly higher skinfold measures than the PL and ALM group throughout the study.

Percent body fat showed a main effect for time in all groups from T1 to T2. All groups had significantly lower body fat at T2 compared to T1 and T3.

Table 5: Anthropometric Measures

	T1		T2		T3	
	M	±SD	M	±SD	M	±SD
Weight (kg)						
ALM	78.22	12.80	80.22[b]	13.27	80.55[c]	12.66
AL	79.49	9.60	81.76[b]	9.68	80.74[c]	9.88
PL	72.08[a]	4.19	72.18[a]	4.50	73.00[a]	4.62
Skinfolds (mm)						
ALM	44.31	8.62	42.50	8.35	43.54	8.66
AL	52.47[e]	17.92	50.54[e]	17.55	51.74[e]	18.34
PL	42.62	13.83	39.88[d]	13.03	44.92[d]	16.23
Body fat (%)						
ALM	15.10	1.83	14.86[f]	1.74	15.11	1.52
AL	17.13	4.16	16.76[f]	4.05	17.00	3.95
PL	14.44	4.09	13.74[f]	4.42	14.66	4.48

$P<0.05$

[a] body mass of PL group is significantly different than ALM and AL group at all times

[b] body mass is significantly different, T1 vs T2

[c] body mass is significantly different, T1 vs T3

[d] skinfolds of the PL group significantly decreased at T2, increased at T3

[e] skinfolds of the AL group significantly higher than ALM and PL group at all times

[f] body fat percent had a main effect for time for all groups from T1 to T2

Dietary Results

Table 6 summarizes the three day dietary reports for all groups. Total caloric intake significantly decreased from T2 to T3 in all groups, which can be partially explained by the significant reduction in carbohydrate consumption in all groups from T2 to T3. There was no significant difference in fat or protein, over time. Fluid intake significantly increased by a mean of 1694.9 ml, 996.3 ml, 993.9 ml in the ALM, AL, PL groups, respectively, from T1 to T2. Fluid intake then dropped significantly from T2 to T3 in all groups (ALM=1718.8 ml, AL=1131.1 ml, PL=986.67 ml) but were not significantly different from T1 (Table 6). While significant changes were observed across time, these changes were not different between groups.

Table 7 illustrates protein and fluid intake expressed in relative terms to body weight. No significant differences were reported in either dependent variable.

Urine Output

There was a significant main effect in all groups over time in urine output. T2 was higher than T1 and T3 in all groups (Table 8). There were no group or interaction effects. Table 9 illustrates the fluid intake to urine volume ratio. This ratio indirectly conveys fluid retention. No significant differences were detected.

Table 6: Dietary Results

	T1		T2		T3	
	M	±SD	M	±SD	M	±SD
CHO (g)						
ALM	534.00	139.33	673.33	236.57	514.83[a]	125.97
AL	602.33	304.88	610.83	100.82	461.33[a]	98.37
PL	445.67	110.44	475.00	96.45	406.33[a]	69.71
Protein (g)						
ALM	139.33	24.46	144.67	27.36	148.83	51.73
AL	132.50	53.07	135.33	30.52	129.00	35.74
PL	119.00	41.39	118.67	32.15	88.83	27.48
Fat (g)						
ALM	89.50	25.19	101.50	46.13	100.67	35.27
AL	117.67	32.91	119.00	39.64	96.83	46.28
PL	104.17	29.00	80.17	12.21	65.17	23.96
Fluids (ml)						
ALM	3393.28	1293.81	5088.22[b]	1169.31	3369.33[b]	699.96
AL	3221.55	1343.01	4217.89[b]	716.44	3086.78[b]	901.04
PL	2793.44	760.20	3787.39[b]	709.15	2800.72[b]	688.94
Total Calories (cal)						
ALM	3473.00	610.28	4203.83	923.84	3528.33[c]	567.67
AL	4120.33	1522.01	3996.50	540.25	3274.00[c]	911.39
PL	3215.17	861.42	3253.67	562.34	2658.50[c]	380.37

$P<0.05$

[a] significant differences for all groups from T2 to T3

[b] significantly increased from T1 to T2, significantly decreased from T2 to T3

[c] significant decreases for all groups from T2 to T3

Table 7: Relative Expression of Protein and Fluids

	T1	±SD	T2	±SD	T3	±SD
Protein (g/kg BW)						
ALM	1.86	.45	1.90	.50	1.95	.88
AL	1.67	.63	1.66	.34	1.63	.46
PL	1.64	.55	1.64	.45	1.17	.36
Fluids (ml/kg BW)						
ALM	44.10	15.01	65.69	14.02	42.94	7.97
AL	40.10	14.51	51.73	7.97	40.27	8.76
PL	38.51	10.16	52.16	9.17	38.95	10.75

Table 8: 24 Hour Urine Volume (L)

	T1		T2		T3	
	M	±SD	M	±SD	M	±SD
ALM	2.43	1.23	2.91*	1.51	2.39	1.54
AL	1.42	0.87	2.14*	0.85	1.59	1.02
PL	1.50	0.72	1.68*	0.93	1.53	0.84

*main effect for time, T2 is higher than T1 and T3 for all groups, $P<0.05$

Table 9: Fluid Intake to Urine Volume Ratio

	T1	±SD	T2	±SD	T3	±SD
ALM	1.61	0.64	1.96	0.87	1.84	0.77
AL	2.78	1.50	2.88	1.30	2.92	1.86
PL	2.36	1.34	2.05	0.91	2.25	0.97

Muscle Cross-Sectional Area and Fiber Type Percentage

There was a significant increase in muscle fiber area of type I fibers from T1 to T2 as well as from T1 to T3 in all groups (Table 10). Since there was no significant change in the subgroups of type IIa, IIb, and IIc, the type II fibers for all groups were collapsed into one group for analysis. There was a significant increase in the collapsed type II fibers from T1 to T3 for all groups. No significant changes in the percentage distribution of fiber types were noted over time in any group.

Total Tissue Water Content

Table 11 outlines the total tissue water content expressed as a percentage of the total intracellular and intercellular water content. There was no significant change in total tissue water content over time, between or within groups.

Protein Content

Table 12 contains data illustrating total protein content, as determined by the Lowry's technique. There was a main effect for all groups following the five day acute load with creatine (Table 12). All groups showed a significant difference in protein content over time with T2 measures being lower than T1. The AL group had lower protein content than the PL group at all three times.

Table 10: Fiber Area, Percentage of Fiber Type, and Total Fiber Count

	ALM			AL			PL		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
Fiber Area (μm^2)									
Type I									
M	1083.14	1447.33[a]	1355.28[a]	1387.47	1528.08[a]	1791.13[a]	1498.88	1508.66[a]	1705.62[a]
SD	242.50	441.29	268.22	286.92	510.09	418.06	410.00	435.96	401.80
Type IIa									
M	1856.78	2437.18	2402.98	1951.21	2150.51	2671.46	2091.50	2140.84	2412.62
SD	528.70	969.70	884.00	591.88	754.95	872.40	696.19	549.45	518.26
Type IIb									
M	1541.93	1838.36	2082.19	1624.35	2059.01	1597.33	1575.93	1385.73	1629.98
SD	445.40	1510.98	794.30	384.06	725.42	1022.06	472.83	790.81	884.68
Type IIc									
M	1047.78	1027.70	2008.25	1295.06	1828.03	1841.07	1612.40	1237.68	1814.84
SD	1097.57	1092.38	873.64	1294.01	717.49	1046.76	991.19	1121.23	351.15
Type II									
M	1815.82	1986.70	2314.99[b]	1802.73	2236.80	2220.30[b]	1847.98	1962.62	2079.99[b]
SD	507.93	681.85	604.77	590.03	1096.72	803.57	583.93	599.52	349.43
Fiber percentage (%)									
Type I									
M	48.50	50.90	47.70	45.03	48.27	48.33	43.65	42.10	43.38
SD	14.30	12.93	9.84	11.38	9.18	9.84	14.75	13.45	8.82
Type IIa									
M	38.33	34.49	40.39	42.72	36.70	37.92	38.67	44.90	42.00
SD	15.72	12.05	10.60	15.14	9.18	9.14	12.69	13.65	9.36
Type IIb									
M	9.07	9.64	8.41	8.92	9.75	9.03	14.23	9.17	9.03
SD	2.38	3.91	4.38	6.24	3.01	4.08	6.42	2.96	3.37
Type IIc									
M	4.80	4.16	3.37	3.28	5.18	4.67	3.67	3.78	5.48
SD	3.86	4.78	2.58	3.83	2.86	5.35	2.77	2.84	3.55
Total Fiber Count (#)									
Total		4898			4075			4337	

[a] significant increase from T1 to T2, T1 to T3, $P<0.05$ (main effect)
[b] significant increase from T1 to T3 in all groups, $P<0.05$

Measurements based on a mean of 99 fiber/biopsy across sampling times and groups.

Table 11: Total Tissue Water Content (%)

	T1		T2		T3	
	M	±SD	M	±SD	M	±SD
ALM	67.97	2.55	69.19	2.03	68.89	1.92
AL	67.91	2.42	68.12	1.55	70.44	1.61
PL	67.59	2.23	68.62	2.35	68.68	3.58

Table 12: Total Protein Content of Biopsies (mg/g wet weight)

	T1		T2		T3	
	M	±SD	M	±SD	M	±SD
ALM	76.47	9.77	71.24[b]	6.42	78.55	6.05
AL	75.94[a]	4.26	68.67[ab]	7.75	68.92[a]	7.14
PL	85.84	6.73	72.94[b]	5.19	74.40	10.18

[a] AL group significantly lower than PL group at all times, $P<0.05$
[b] All groups had a significant difference over time, $P<0.05$

Chapter 5

Discussion

Contrary to previous studies that have examined the effects of creatine supplementation on performance, the present study is unique in that it examined the morphological changes responsible for the increase in body mass associated with creatine supplementation with concurrent periodized resistance training. The purpose of the study was to investigate changes in muscle fiber area, total cellular fluid, and cellular protein associated with creatine monohydrate supplementation while undergoing periodized resistance training. In this study, it was hypothesized that the body mass increase was due to an increase in total water content in the muscle cells and therefore, there would be an increase in the muscle fiber area. It was also hypothesized that this increase in cell hydration might stimulate protein synthesis in accordance with the cell swelling theory proposed by Häussinger et al. (1993).

Body Mass Increase

In the present study, absolute body mass significantly changed over time in both the AL and ALM group by 2.2 and 2.0 kg respectively, following the five day acute load. Following the 32 day maintenance load, the ALM group remained 2.3 kg heavier than the pre-load levels. These results are similar to previous studies that have demonstrated an increase in body mass ranging from 0.9 to 2.9 kg following an acute loading period of three to six days (Balsom et al., 1993a; Balsom et al., 1995; Earnest

et al., 1995; Greenhaff et al., 1994; Kelly and Jenkins, 1998; Kreider et al., 1998; Peeters et al., 1999; Söderlund et al., 1994; Vandenberghe et al., 1997; Volek et al., 1997a).

The results of skinfold tests indicated that there was a slight, but not significant decrease from T1 to T2 in the creatine supplementation groups (ALM 44.31 to 42.50 mm, AL 52.47 to 50.54 mm) and then a slight, but not significant increase from T2 to T3 in the same groups (ALM 43.54 mm, AL 51.74 mm). The PL subjects had a significant decrease from T1 to T2 (42.62 to 39.88 mm) and significant increase from T2 to T3 (44.92 mm) These results support the findings of Volek et al. (1997a), who evaluated the effects of creatine supplementation (25 grams per day for seven days) on seven site (triceps, subscapular, mid-axillary, chest, suprailiac, abdomen, and thigh) skinfold measures using 13 healthy resistance trained men as subjects. The results of this study reported a mean gain of 1.3 kilograms in the creatine supplementation group, but no significant changes in the sum of skinfold thicknesses.

In the present study, the calculated percent body fat showed a small but significant decrease from T1 to T2 of less than one percent (ALM 15.10% to 14.86%, AL 17.13% to 16.76%, PL 14.44% to 13.74%). Although the calculated percent body fat was statistically significant, the decrease may not reflect an actual change in body composition. The reason for this is that skinfold measures and subsequent calculation of percent body fat have a standard error of plus or minus three to five percent in males (Durnin and Womersley, 1974). With a less than one percent change in calculated percent body fat over time, it could be concluded that changes detected

in this study are within measurement error and therefore, do not truly reflect changes in the fat component of the body.

Several studies have attempted to explain the mechanism for the changes in body composition following creatine supplementation. Earnest et al. (1995) reported that body mass following a 20 grams per day loading period for 28 days, increased 1.7 kg, of which, 1.5 kg was attributed to an increase in fat free mass, as measured by hydrostatic weighing. Kreider et al. (1998) attempted to measure total body water via bioelectrical impedance following 15.75 grams per day of creatine for a 28 day loading period. No significant change in total body water was noted. The skinfold data from the current study would suggest that the significant increases in body mass by the creatine supplementation groups are not associated with changes in either the fat or fat-free mass.

Cross-Sectional Fiber Area and Fiber Type Percentage

Muscle cross-sectional area analysis indicated a significant increase in fiber area in type I fibers from T1 to T2 and from T1 to T3 in all groups. The percentage increases in cross-sectional area for type I fibers increased from T1 to T2 in the ALM group by 33.6% and from T1 to T3 by 25.1%. The AL group increased from T1 to T2 by 10.1% and from T1 to T3 by 29.1%. By comparison, the PL group's significant changes in type I fiber area were modest, with increases of 0.7% from T1 to T2 and 13.8% from T1 to T3 reported. Since type I fibers have lower resting levels of phosphocreatine compared to type II muscle fibers (Spriet, 1995), the type I muscle

fibers in the creatine supplementation groups may be able to load, or increase their intracellular Cr levels to a greater extent.

Although not statistically significant, type IIa, IIb, and IIc muscle fiber areas in the ALM and AL groups showed a trend towards an increase in fiber area. For instance, type IIa fibers increased by 31.3% and 29.4% for the ALM group from T1 to T2, and T1 to T3, respectively. Similarly, the type IIa fibers in the AL group increased by 10.2% from T1 to T2 and 36.9% from T1 to T3. In contrast, type IIa fibers in the PL group increased by a small 2.4% from T1 to T2 and 15.4% from T1 to T3. These findings are consistent with previous findings by Kraemer et al. (1995), who reported a 24% increase in type IIa fibers following a 12 week resistance training period with no creatine loading.

Type IIb fiber areas increased by 19.2% from T1 to T2 and 35.0% from T1 to T3 in the ALM group. In AL group, type IIb fiber areas increased by 26.8% from T1 to T2 and decreased by 1.7% from T1 to T3. Unlike the ALM and AL groups, type IIb fibers in the PL group decreased by 12.1% from T1 to T2 and increased modestly, by 3.4% from T1 to T3. Type IIc fibers decreased by 1.9% from T1 to T2 in the ALM group and increased by 91.7% from T1 to T3. In comparison, the type IIc fiber areas of the AL group increased by 41.1% and 42.2% from T1 to T2 and from T1 to T3, respectively. Type IIc fibers in the PL group decreased by 23.2% from T1 to T2 and increased by 12.6% from T1 to T3. Although the percentage changes in type IIc fibers may seem large, they make up only one to three percent of the total fibers analysed from the biopsies. Therefore, these changes have little impact on the total

cross-sectional area. The reported lack of overall significance in the cross-sectional area of the type II fibers may be attributed to the small sample size and large variances in measures.

Since there was no significant change in the subgroups of type IIa, IIb, and IIc fibers, the type II fibers were collapsed into one group for analysis. In terms of the combined type II fibers, the creatine loaded groups demonstrated a greater percent increase in muscle fiber size compared to the PL group. The ALM group increased by 9.4% from T1 to T2 and 27.5% from T1 to T3, while the AL group increased by 24.1% from T1 to T2 and 23.2% from T1 to T3 versus an increase of only 6.2% from T1 to T2 and 12.6% from T1 to T3 for the PL group. These results are similar to those reported by Volek et al. (1999), who found an increase in the type II muscle fiber area in the creatine supplemented subjects (29-35%), compared to the placebo counterpart (6-15%). Subjects in that study loaded with 25 grams of creatine per day for one week followed by a maintenance dose of five grams per day for 11 weeks while training.

When the fiber areas of all fiber type sub-groups (type I and type II) were combined, the results showed a mean increase of 9.4 to 33.6% in the creatine loaded groups versus 0.7 to 13.8% in the placebo group. Kraemar et al. (1995) suggested that type II muscle fibers may respond more favorably to resistance training compared with the smaller type I muscle fibers when no creatine supplementation is used. From the present study, it would appear that both types of fibers showed increased fiber area of similar magnitude. This finding conflicts with Sipilä et al. (1981), where gyrate

patients suffering from atrophied type II fibers were able to increase the diameter of type II fibers with no reported change in type I fiber area. The results of the present study may be a response of the combined effect of creatine supplementation and resistance training versus resistance training alone or creatine supplementation alone.

In the current study, fiber percentage did not significantly change over time. The lack of change in the percent fiber type might be expected as the duration and intensity stimulus from training was insufficient, or less than optimal to produce major shifts in fiber type percentages that could be identified with the ATPase assay used in this study.

Cellular Water and Protein Content in the Muscle

The results of intracellular and intercellular water content measured as a percentage of total tissue mass indicated no significant change over time in any group. The total tissue protein content decreased in all groups from T1 to T2, as measured by the Lowry's technique. The type I and type II fiber areas of the creatine groups increased at a greater extent than the PL group after the five day acute load and again at the end of the study at T3. Based on these results, the increase in muscle cross-sectional area may be attributed to a shift in intracellular water from intercellular compartments.

In contrast, since there was a 2.2 and 2.0 kg increase in body mass over a five day loading period in both creatine groups, and no statistical difference in muscle fiber cross-sectional area, intracellular water increases may be ruled out as a possible

explanation for the increase in body weight. It can be speculated that intercellular water may be a factor. Within the limitations of this study, intracellular and intercellular water were not distinguished, and as a result, it was difficult to determine the proportional changes within these compartments.

The current study was unable to support or refute Häussinger's cell swelling theory. According to Häussinger et al. (1993), cellular hydration state is related to protein synthesis. Cell swelling, or an increase in intracellular fluid, may act as an anabolic proliferative signal. From Häussinger's theory, it can be speculated that if there was an increase in cell size due to water, there might be the possibility of protein synthesis over the long term.

Results of total protein content demonstrated a significant decrease from T1 to T2 in all groups (ALM by 6.8%, AL by 9.6%, PL by 15.0%). However, total protein content of the ALM group increased 2.1% following the maintenance phase, whereas the AL and PL groups remained lower than the initial content (AL decreased by 9.2%, PL decreased by 13.3%). If cell swelling, or hyper-hydration due to an increase in intracellular water occurred in the ALM group, it may be speculated that the 32 day maintenance period of the experiment may have been the initial anabolic proliferation signal for protein synthesis suggested by Häussinger et al. (1993). However, the duration of this phase (32 days), may have been insufficient length to note any significant changes in protein content that could be detected by the Lowry's technique.

A possible explanation for the decrease in the total protein content may have been due to the sampling time of the tissue. Muscle biopsies were performed

approximately 24-36 hours post workout and hence, may have reflected an acute catabolic phase associated with the immediate post-exercise period.

Dietary Fluid Intake and Urine Volume

In an attempt to assess water retention by the body, 24 hour urine volume outputs and daily fluid intakes were examined. The results of the 24 hour urine collection indicated a significant increase in absolute volume in all groups during the acute load phase from T1 to T2 (ALM 0.48 L, AL 0.72 L, PL 0.18 L). From T1 to T3, the ALM group had a slight decrease of 0.04 L, while the AL and PL groups increased 0.17 L and 0.03 L, respectively. The increase in urine volume output at T2 was mirrored by an increase in daily dietary fluid consumption from T1 to T2 for all groups, ranging from 1.0 L to 1.7 L. These reported increases in absolute urine volume and fluid intake are contrary to that of Hultman et al. (1996), who reported that creatine ingestion markedly reduced urinary volume by 0.6 L during the initial days of supplementation. These researchers suggest the increased body mass be likely attributed to body water retention reflected by these two measures. Ziegenfuss et al (1997), using magnetic resonance imaging (MRI) supported this conclusion. They reported a significant 6.6% increase in thigh intracellular fluid volumes and a two to three percent increase in total body and intracellular volumes, as measured by multi-frequency BIA, after short term creatine supplementation. Subsequent work by Ziegenfuss in 1998, reported a two percent increase in total body water and a three percent increase in intracellular water, with no change in extracellular water after three

days of supplementation (0.35 g/kg FFM per day). The combined reported findings by Ziegenfuss seem to indicate that weight gain associated with short term creatine supplementation is primarily water retention, with the majority in the intracellular compartment.

To further investigate the possibility of water retention, the ratio of fluid intake versus urine output was calculated. An increase in the ratio would be an indirect measure of fluid retention within the body. Both creatine treatment groups, showed a slight, but not significant increase in this ratio over time (Table 9). The ALM group ratio increased during the acute five day load (T1 to T2) by 0.35. Following the completion of the 32 day maintenance phase, this ratio was still higher than the pre-load levels of 0.23. The AL group displayed a similar, but a slightly lower trend, with values increasing from T1 to T2 and T2 to T3 by 0.10 and 0.14, respectively. By contrast, the ratios for the PL group were lower at both T2 (0.31) and T3 (0.11) when compared to the start of the study. Although not statistically significant, these trends may suggest that the experimental groups using oral Cr supplementation while training, may have retained more fluid than the placebo group.

Total Creatine Load

In this study, total free cellular [creatine] significantly increased for all groups from T1 to T2, and remained significantly higher at T3. The ALM group changed from a mean of 33.07 to 48.63 to 45.67 mmol/kg while the AL group increased from a mean of 33.89 to 48.03 and decreased to 42.33 mmol/kg for T1, T2, and T3,

respectively. The PL group had a mean of 38.10 mmol/kg of cellular [creatine] at T1, increased to 48.03 mmol/kg at T2, and remained constant at T3 with 47.93 mmol/kg. The combined [Cr + PCr] had no significant change in all groups, although the creatine supplementation groups increased 14.4% and 14.3% for the AL and ALM groups respectively, after the five day acute load. The reported percentage and absolute increases in creatine in the present study, following a five day acute load, are at the lower end of creatine values from the majority of studies that have either used muscle biopsies or NMR to analyze muscle creatine content. In the current study, the reported 14.4% and 14.3% increase in the AL and ALM groups respectively, are slightly below the average of 12 previous studies that reported a mean increase of 18.5%, with a range of 15-22% (Williams et al., 1999).

In absolute terms, the current study's [Cr+PCr] rose by 14.4 and 15.5 mmol/kg dm for the AL and ALM groups, respectively. Again, this increase in absolute [Cr+PCr] is considerably below the mean reported increases of 22 mmol/kg dm, with a range of 20-27 mmol/kg dm (Williams et al., 1999). The minimal or no change associated with the phosphorylated creatine after five days of supplementation is surprising since the mean increase in absolute PCr reported in previous works are 14.3 mmol/kg dm, with a range of 3.4-26 mmol/kg dm (Williams et al., 1999). The collapsed group (AL + ALM) had significantly higher [PCr] from T1 to T2. The pre-load values of [Cr + PCr] are within the normal ranges of 115-140 mmol/kg dm as noted by Balsom et al. (1994) and Greenhaff (1995). In addition, the present study

supports that approximately 70-90 mmol/kg dm of the total cellular creatine or approximately 60-65%, is found in the form of phosphocreatine.

As described by Harris et al. (1992), there are great variances within individuals as to the rate of creatine uptake through oral supplementation. They suggested that the pre-supplementation total muscle [creatine] levels seem to be an important factor in determining the readiness for creatine uptake during any supplementation period. Greenhaff et al. (1996) indicated that 20-30% of individuals do not respond to creatine loading. In one of Greenhaff's studies (1994), a standard creatine loading protocol (20 grams per day for five days) substantially increased muscle [Cr] by a mean of 29 mmol/kg dm, or 25% in five of eight subjects. However, in the remaining three subjects, creatine supplementation had little or no effect on muscle [Cr], producing increases of eight to nine mmol/kg dm, or about a five to seven percent increase.

Since the pre-supplementation levels of Cr and PCr are at or slightly below normal ranges in this study, it is surprising that a greater boost did not occur following the five day loading period. It should be noted, however, that the greatest increases occurred in subjects with the lowest initial creatine content as suggested by Harris (1992). As an example, a subject from the ALM group, with an initial total creatine content of 108.30 mmol/kg dm, increased to 136.66 mmol/kg dm or 25% after the acute five day loading period. In contrast, a subject from the AL group, with relatively high pre-experimental level of cellular Cr of 120.79 mmol/kg dm, had only a slight increase to 120.89 mmol/kg dm after the same five day loading phase. Such variances

reflect the individual dose response cited by Harris et al (1992), and make it difficult to generalize conclusions from mean data. The ability for an individual to increase their cellular Cr stores through oral supplementation may be an individual response that group data simply does not mirror.

Another possible explanation for the lower response to Cr loading in the present study may be a result of the high exogenous levels of Cr consumed in the daily diet of subjects. Higher than normal consumption of red meat and fish, which are sources of dietary Cr, may blunt the response to additional oral supplementation. In the present study, three day dietary records revealed protein intakes which ranged from 139.33 to 148.83 grams per day, 129.00 to 135.33 grams per day, and 88.83 to 119.00 grams per day, for the ALM, AL, and PL groups, respectively. As a group, these subjects had high protein diets. The subjects protein intake, expressed in relative terms ranged from 1.22-1.85 g/kg of body weight, is at the upper end of the recommended daily relative intake of protein for weight training males, which is 0.82 to 1.94 g/kg of body weight (Williams, 1995). Due to the small sample size of this study, the suggestion that dietary protein has an effect on creatine uptake can only be speculated and should be investigated further. These results may simply reflect a percentage of non-responders, as suggested by Greenhaff et al. (1996).

Although the present study did not show a large boost in total creatine following supplementation, this result may in part explain why greater changes in cell morphology in the creatine monohydrate groups were not observed over the duration of the study.

Chapter 6

Conclusions

Within the limits of this study, the following conclusions have been made:

1. Cross-sectional area of type I muscle fibers increased in all groups over time.
2. No significant changes in cross-sectional area were noted for any type IIa, IIb, or IIc subgroups.
3. Collapsed type II fiber subgroups increased in fiber area from T1 to T3 in all groups.
4. No significant percentage change in total fluid content was observed. However, it was speculated that the increase in muscle fiber cross-sectional area was due to an increase in intracellular fluid.
5. Total protein content decreased at T2 compared to T1 and T3.
6. There was a body mass increase associated with creatine supplementation and concurrent resistance training when administered at an acute dose of 0.3 g/kg per day for five days and a further maintenance dose of 0.03 g/kg per day for 32 days.
7. No significant body composition changes were observed over time with creatine supplementation.
8. No significant changes in fiber type percentage occurred over time.
9. There were individual differences in the cellular uptake of creatine by the subjects participating in this study.

Implications and Recommendations for Future Research

Related to the present study, further investigation surrounding the fluid and protein content should be investigated (ie. intercellular fluid versus intracellular fluid and protein synthesis initiated by cell swelling). Future research should focus on evaluating the safety of administering creatine to athletes, in particular, in combination with other proposed performance enhancing drugs (ie. drug interaction). Finally, long term affects of creatine supplementation have not been published in detail. More research in this area is recommended to examine possible negative side effects and unknown adaptations from long term creatine supplementation.

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APPENDIX A

Ethics Review Approval

The Ethics Committee of the Faculty of Physical Education and Recreation (University of Alberta):

Name	Position
Dr. Jane Watkinson	Professor and Associate Dean
Dr. Stu Petersen	Professor
Dr. Dick Jones	Professor, Pulmonary Medicine

have reviewed the proposal entitled:

Creatine monohydrate supplementation: An examination of dose response and muscle cell adaptation during concurrent periodised resistance training

submitted by

Dan Syrotuik / Rob Calvert / Lorraine Sim / Gord Bell / Rob Burnham / Ian Maclean

_____	finds it within acceptable standards for human experimentation
__X__	finds it within acceptable standards subject to the following revisions
_____	finds it unacceptable in its present form

Revisions Required:
Since the urine collection / biopsy procedures are inconvenient / invasive, it would be appropriate to inform the subjects WHY they are required.

Please provide this information on both the proposal and the informed consent form. Resubmit one revised copy to the Ethics Committee.

Jane Watkinson — April 7, 1997

Dr. Jane Watkinson, Committee Chair — Date
Faculty of Physical Education and Recreation

APPENDIX B

FACULTY OF PHYSICAL EDUCATION AND RECREATION
UNIVERSITY OF ALBERTA
EDMONTON, ALBERTA
T6G 2H9

PARTICIPANT INFORMATION PACKAGE

CREATINE MONOHYDRATE SUPPLEMENTATION: AN EXAMINATION OF DOSE RESPONSE AND MUSCLE CELL ADAPTATION

Investigator:

Rob Calvert, B.P.E. 433-9838	Gordon Bell, Ph.D.
Lorraine Sim, B.P.E. 434-0466	Robert Burnham, M.D.
Dan Syrotuik, Ph.D. 492-1018	Ian MacLean, M.Sc.

Introduction/Background

Creatine monohydrate has become a popular nutritional supplement and appears to increase the muscle's ability to maintain power output during strenuous high intensity exercise and increase body mass. Research supports the finding that creatine supplementation can enhance intramuscular storage of both creatine and phosphocreatine resulting in increased performance and recovery during repeated bouts of high intensity anaerobic exercise. It remains to be determined what actually accounts for the increase in body mass. In addition, the dose of creatine monohydrate necessary to achieve and maintain optimal cellular levels of creatine while concurrently performing a periodized resistance training program requires further clarification.

The Study

We are interested in examining a creatine supplementation protocol for resistance training athletes, as well as the cellular adaptations that occur within. The study will require a 9 week commitment. Following a 4 week training phase to familiarize all participants with the resistance training protocol, subjects will be randomly assigned to one of three experimental groups: 1) 5 day creatine load / 4 week maintenance, 2) 5 day creatine load / 4 week placebo maintenance, 3) 5 day placebo load / 4 week placebo maintenance. All 45 subjects (15 per group) will train four times per week utilizing a personalized, resistance training program. In order to maintain control over the workload, this will be the only formal training allowed for the duration of the study. The investigators must clear any other physical activity.

All subjects will participate in the following: 1 repetition maximum (RM) test, 8 RM test, work capacity test, anthropometric test, muscle biopsy, 3 day dietary record, and a 24 hour urine collection.

Physiological Testing: Subjects will be required to be available for approximately 3 hours for each testing weekend: before week 1, and after weeks 4, 5, and 9

1. 1 RM strength tests:
To assess strength levels throughout the course of the study, 1 RM tests will be performed prior to the beginning of week 1, and repeated at the end of weeks 4, 5, and 9. Done on both the bench press and inclined leg press, the 1 RM test will be used to determine the maximal amount of weight that each participant can lift once with proper technique.

2. 8 RM strength tests:
8 RM tests will be performed before the start of week 1, and at the end of week 4 in order to establish each individual's optimal training program. The 8 RM test is designed to determine the maximum amount of weight each participant can lift for 6-12 repetitions utilizing proper technique. The exercises for the 8 RM test will be leg extension, leg curl, calf raise, inclined bench press, shoulder press, lat. pull, upright row, dumbbell fly, seated row, bicep curl, tricep pressdown, and lateral raise.

3. Work capacity tests:
Relative muscular endurance will be assessed by lifting 80% of your 1 RM until you are unable to complete a repetition using proper lifting mechanics. The tests will include leg press and bench press. These tests will be conducted prior to week 1, and after weeks 4, 5, and 9.

4. Anthropometry tests:
Height, weight, sum of skinfolds, thigh and arm girth measures will be conducted before week 1, and at the end of weeks 4, 5, and 9.

5. Muscle biopsy:
At the end of weeks 4, 5, and 9, muscle biopsies will be taken from the vastus lateralis (outer thigh muscle) by a physician. This procedure involves the sterile preparation of the mid-thigh area, a small injection of 1-2 ml of local anaesthetic, and a small incision (<1 cm) through the skin and fascia on the lateral portion of the thigh. A biopsy needle is then inserted and a small sample of muscle tissue (~50 mg – about half the size of a tic tac) is obtained using suction. There is usually some discomfort associated with this procedure. After being frozen, subjects usually report feeling only 'pressure' during the removal of the biopsy. No sutures are required; instead, a steri-strip band-aid will be used to close the incision. One to two days after the procedure, the biopsy site will usually feel like a slight 'charley horse' with some local bruising. Muscle biopsies are an optimal way to measure creatine/phosphocreatine, cross-sectional area, water content, and to determine fiber type of a skeletal muscle fiber, in one procedure.

6. 24 Hour urine sample:
Subjects will be required to collect their urine for a period of 24 hours at the end of weeks 4, 5, and 9. For each of the three 24 hour periods, a sterile plastic bottle will be supplied to each subject to take home for collection. The urine collection will be used to measure creatinine levels, which are an indicator of creatine turnover.

7. Recording of diet:
Subjects will be required to record their food and fluid intake on a 3 day dietary intake form (as instructed by the investigators) during the last 3 days of weeks 1, 4, 5, and 9 of the study.

Physiological training

FOR THE DURATION OF THE STUDY, ALL TRAINING/ACTIVITY NOT INCLUDED IN THE TRAINING PROGRAM PROVIDED MUST BE CLEARED BY ONE OF THE INVESTIGATORS!!

Participants will be required to complete a 4 week personalized strength training program prior to being randomly assigned to 1 of the 3 treatment groups. Following the assignment to a treatment group, all subjects will continue to train for another 5 weeks.

The strength training program will require approximately 60-90 minutes, 4 times per week (4-6 hours/week). The 4 days stipulated for workouts are Mondays, Tuesdays, Thursdays, and Fridays. Two of the workouts will target the chest, back, and shoulders using 8 exercises (bench press, incline bench press, shoulder press, lateral raises, upright row, lat. pulldown, seated row, dumbbell flys). The remaining 2 workouts will target the arms and legs (incline leg press, leg extension, hamstring curl, calf raises, bicep curls, tricep pressdown). The volume and intensity will be determined by initial strength tests and reassessed at the end of weeks 4, and 5. <u>For the purpose of this study, it is vital that the training program be strictly adhered to.</u> You will be asked to refrain from performing any exercises, sets, or reps other than those specified. Proper warm up, cool down, stretching, and abdominal work are recommended to all participants. Guidelines will be available from the investigators upon request.

Risks

The effort required for strength testing and training will be maximal but should not differ greatly from your previous participation in sporting activities. Creatine monohydrate supplementation dosages used in this study have not been shown to have any negative side effects. The muscle biopsy, as with any invasive procedure, will cause discomfort and has a small risk of infection associated with it. Subjects are asked to report any abnormal effects (numbness, excessive pain, or swelling) immediately.

Commitment

Participants are expected/required to:

1. commit for the duration of the study (approximately 9 weeks)
2. follow their prescribed training program and limit any other training
3. train and test with maximal effort
4. be adequately rested, hydrated, and prepared for testing and training
5. provide 3 muscle biopsy samples
6. be available on campus once per day to receive their supplementation
7. direct any questions or concerns to one of the investigators

Consent

I acknowledge that I have read this form and fully understand the procedures and risks associated with this study. I consent to participate in this study with the understanding that I may withdraw at any time without repercussion. I understand that my personal results will be available for me after the completion of the study. I also understand that the data obtained will be used for research and publication and that all data will be secured with the investigators to ensure confidentiality. I will receive a copy of the consent upon request. I consent to participate in this research project.

Name: _____ (print) Signature: _____ (sign)

Address: _____ Date: _____

_____ Phone: _____

Postal Code: _____

Witness: _____ (sign) Investigator: _____ (sign)

SUBJECT INFORMATION SHEET

NAME: _____
(Last) (First) (Middle)

ADDRESS: _____

_____

PHONE: _____ E-MAIL: _____

AGE: ____ UNIVERSITY PROGRAM (YEAR): _____

HEIGHT: _____ WEIGHT: _____ (lbs or kg)

PREVIOUS WEIGHT TRAINING EXPERIENCE (# OF YEARS): _____

AVERAGE # OF TIMES / WEEK: _____

OF MONTHS OUT OF THE LAST 12: _____

PREVIOUS CREATINE SUPPLEMENTATION EXPERIENCE (IF ANY, DATE LAST USED):_____

****

1. Have you ever had heart trouble?

   YES NO If yes, explain: _____

2. Do you have a bone or joint problem that could be aggravated by exercise?

   YES NO If yes, explain: _____

3. Do you suffer from deseases of the lungs, kidneys, and/or liver?

   YES NO If yes, explain: _____

4. Have you ever or are you presently using any anabolic substances?

   YES NO If yes, explain: _____

5. Do you know of any other reason why you should not do physical activity?

   YES NO If yes, explain: _____

To the best of my knowledge, I declare the above information to be true.

Name: _____ Signature: _____

Date: _____ Witness: _____

APRIL, 1997

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
		1	2	3	4	5
6	7	8	9	10	11	12
13	14 Meeting	15 1RM, 8RM, 80% test	16 1RM, 8RM, 80% test	17	18 workout 3-day diet pick-up programs	19 workout
20	21 workout	22 workout	23	24 workout	25 workout	26
27 workout	28 workout	29	30 workout			

MAY, 1997

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
				1	2 8 RM Test 3-day diet ← bottle pick-up	3 8 RM Test →
4 24 hour urine	5 1RM, 80% test	6 load biopsy	7 load workout	8 load workout ←	9 load workout 3-day diet → bottle pick-up	10 load workout
11 24 hour urine	12 1RM, 80% test	13 biopsy maintenance- (until June 14)	14 workout	15 workout	16 workout	17
18 workout	19	20 workout	21	22 workout	23 workout	24
25	26 workout	27 workout	28	29 workout	30 workout	31

JUNE, 1997

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
1	2 workout	3 workout	4	5 workout	6 workout	7
8	9 workout	10 workout	11	12 workout	13 workout 3-day diet →	14 end maintenance
15 24 hour urine	16 1RM, 80% test	17 biopsy	18	19	20	21
22	23	24	25	26	27	28
29	30					

APPENDIX C

DOSAGE INSTRUCTIONS

(Take as directed daily)

Phase #1 – Loading Phase:

1. Since you are only receiving a half dose on Day 1, consume the solution in two equal parts, separated by a minimum of 3 hours. Try to avoid consumption during meal times (ie. empty stomach)

2. On the remaining 4 'loading' days, consume the 1 litre of solution in four equals parts (approx. 250 ml or 1 cup) over the course of a day with a minimum of 3 hours between drinks if possible

3. Try to schedule your consumption no later than ½ hour prior to a meal or at least 1 hour after a meal (ie. empty stomach)

Phase #2 – Maintenance Phase:

1. Follow guideline #3, but only consume 250 ml or 1 cup per day for the entire 32 day load period

APPENDIX D

EIGHT REPETITION MAXIMUM (8 RM) SELF TEST

NAME: _____ DATE: _____

INSTRUCTIONS:

A 8 RM strength test is a measure of the maximal amount of weight you can lift eight times using **proper technique** without the aid of a partner (spotter). Make sure you have a partner to spot for safety and allow a minimum of three minutes between attempts (sets). A partner should make sure that **proper technique** is followed. For example, if you complete 8 reps using proper technique and attempt another rep and complete it but **without** proper technique, then the test would be complete. It is very unlikely that you will be able to find the weight you can only lift eight times before failure, so the acceptable range is 7 to 11 reps. IF you are not within this range, please repeat step #4 below. The test procedure is outlined below:

1. Complete warm-up and static stretching (avoid a prolonged warm-up).
2. Complete an easy 10 rep set of the exercise to be tested.
3. Complete a second set of 8 reps that would be described as difficult.
4. Add a small amount of weight based on #3 above in an attempt to determine the weight you can lift between 6 and 12 times before failure. Always attempt another rep to ensure you have reached a point of failure to continue the exercise
5. Repeat for all the exercises in the order listed below.
6. Make sure you record the units of the weight lifted and include the weight of the barbell.

EXERCISE	LOAD AND UNIT OF MEASURE (EG. LB OR # PLATES)	NUMBER OF REPS
inclined bench press		
shoulder press		
lat pulls		
upright rows		
dumbbell fly		
seated row		
lateral raise		

EIGHT REPETITION MAXIMUM (8 RM) SELF TEST

NAME: _____ DATE: _____

INSTRUCTIONS:

A 8 RM strength test is a measure of the maximal amount of weight you can lift eight times using **proper technique** without the aid of a partner (spotter). Make sure you have a partner to spot for safety and allow a minimum of three minutes between attempts (sets). A partner should make sure that **proper technique** is followed. For example, if you complete 8 reps using proper technique and attempt another rep and complete it but **without** proper technique, then the test would be complete. It is very unlikely that you will be able to find the weight you can only lift eight times before failure, so the acceptable range is 7 to 11 reps. IF you are not within this range, please repeat step #4 below. The test procedure is outlined below:

7. Complete warm-up and static stretching (avoid a prolonged warm-up).
8. Complete an easy 10 rep set of the exercise to be tested.
9. Complete a second set of 8 reps that would be described as difficult.
10. Add a small amount of weight based on #3 above in an attempt to determine the weight you can lift between 6 and 12 times before failure. Always attempt another rep to ensure you have reached a point of failure to continue the exercise
11. Repeat for all the exercises in the order listed below.
12. Make sure you record the units of the weight lifted and include the weight of the barbell.

EXERCISE	LOAD AND UNIT OF MEASURE (EG. LB OR # PLATES)	NUMBER OF REPS
leg curl		
leg extension		
calf raises		
barbell bicep curl		
tricep pressdown		

APPENDIX E

Table 13: Strength Results

	T1		T2		T3	
	M	±SE	M	±SE	M	±SE
1RM bench press						
ALM	79.2	8.5	80.3	8.2	86.0[b]	9.3
AL	90.6	7.1	93.2	7.7	99.1[b]	9.4
PL	87.5	7.1	89.4	6.9	93.2[b]	7.0
1RM incline leg press						
ALM	298.6	32.9	309.1	33.0	347.7[b]	39.5
AL	308.1	37.4	321.7	34.8	361.0[b]	33.1
PL	298.6	33.5	298.5	37.0	321.4[b]	37.7
Total lifting volume, bench press						
ALM	497.4	73.5	465.0	91.0	711.0[b]	65.2
AL	610.1	83.2	712.7[a]	95.3	709.4[b]	82.8
PL	662.0	52.4	661.2	93.4	689.0[b]	93.7
Total lifting volume, incline leg press						
ALM	2791.6	507.1	2842.2	595.1	3306.5[b]	499.6
AL	3384.8	607.1	3470.5	346.1	4426.6[b]	849.1
PL	3563.2	899.7	3418.2	921.2	4136.7[b]	919.2
Strength/mass ratio, bench press						
ALM	1.0	0.1	1.0	0.1	1.1[b]	0.1
AL	1.1	0.1	1.1	0.1	1.2[b]	0.1
PL	1.2	0.1	1.2	0.1	1.3[b]	0.1
Strength/mass ratio, incline leg press						
ALM	3.8	0.2	3.8	0.2	4.3[b]	0.3
AL	3.8	0.4	3.9	0.3	4.4[b]	0.3
PL	4.1	0.4	4.1	0.4	4.4[b]	0.4

[a] T1 vs. T2 significant difference, $P<0.05$
[b] T1 vs. T3 significant difference, $P<0.05$

APPENDIX F

WHAT DO YOU EAT??

You will be recording your daily intake of food and fluids for 3 consecutive days. They must be a Thursday/Friday/Saturday or a Sunday/Monday/Tuesday combination. Your dietary intake will be analyzed.

It is imperative that you record EVERYTHING that you eat and drink (water as well!). In addition, you must be as ACCURATE as possible when determining the amount (volume or weight) of the food and drink you are recording. It may be difficult for those in residence or for those who are not in complete control of your food intake (preparation, amount, etc.).

HINTS FOR RECORDING DIETARY INTAKE

ACCURACY

1. Accurate measurement Read the weights or volumes of foods or drinks from packages. Example: milk carton, juice box, chocolate bar, potato chips. A 'fistful' of meat = 100 gm, 'fistful veggies = 1 cup, 1 cheese single = 1 oz.

2. Method of cooking Indicate how your food was cooked. Example: fried, steamed, baked, broiled, etc.

3. 'Extras' Don't forget the EXTRAS. Example: ketchup, mustard, mayonnaise, gravy, or butter

4. Food types Be specific about TYPES of food/drink. Example: cheddar cheese, 2% milk, margarine or butter. Whenever possible, identify brand names of the foods

5. Cooked or dry measurement Indicate whether the food measurement is of 'cooked' or 'dry'. Example: chicken weight before or after cooking

6. Specific parts Indicate the exact part of the food you ate or what was removed before eating. Example: chicken (white or dark, bone in or out, skin or skinless), baked potato (skin or skinless), ground beef (lean, extra lean, regular)

7. Labels Whenever possible, attach the nutritional information label from the container (box/can/bag). This will help identify specific brand food nutrients. If you can't remove the label, copy the information onto a piece of paper

BEVERAGES

8. **TEA AND COFFEE** should be included, along with cream, milk, and sugar

9. Don't forget **WATER**

10. Yes, you do have to record **BEER** and other **ALCOHOL**!

PREPARED OR RESTAURANT MEALS

11. Use **PORTION PAKS** whenever possible. Example: salad dressing, butter, jams, peanut butter, cheese. It is easier to quantify the volume of these foods... 1 portion pak = 1 tablespoon

12. Fast foods Include **FAST FOOD** items by name. Example: McDonald's, Pizza Hut, Wendy's.

13. Recipes Record the **amount/volume** of ingredients, the number of servings the entire recipe makes and how many servings you ate

14. Restaurant meals When you eat at a restaurant (other than a fast food place, eg. Earl's), record the name of the meal you ate, list the different ingredients on your plate and the quantities of each

TAKE THE RECORD BOOK WITH YOU AT ALL TIMES...IT'S EASIER TO RECORD WHAT YOU ARE EATING.

APPENDIX G

MUSCLE BIOPSY: WHAT IS IT AND HOW TO CARE FOR IT AFTER IT HAS BEEN PERFORMED

WHAT IS A MUSCLE BIOPSY?

As part of the research project you are participating in, a small piece of muscle will be taken from the outside of your thigh. This is called a muscle biopsy. This will be performed by a qualified physician who is experienced in the procedure. It is performed using a needle approximately the size of a pencil. The skin will be cleansed and then a small area will be frozen with local anesthetic (similar to the freezing you get when you go to the dentist). Then, using a scalpel blade, a 5 millimetre skin incision will be made. This should be painless. The muscle biopsy needle is then inserted through the incision into the outside thigh muscle and the sample is obtained. The needle is in the leg for approximately 10 to 15 seconds. Most individuals describe a mild to moderately uncomfortable 'pressure' feeling when the needle is in the muscle. Following the biopsy, firm pressure will be applied over the incision for approximately 10 minutes to ensure that the bleeding has stopped. Skin tape will be applied over the incision to close it, followed by the application of a pressure dressing.

WHO SHOULD NOT HAVE A MUSCLE BIOPSY?

Individuals with bleeding disorders (ie. low platelet counts, hemophilia), allergies to local anaesthetics (ie. dental freezing), or infection of the skin overlying the biopsy site or generally (ie. fever) should not undergo a muscle biopsy. Please inform the physician who is going to be doing your biopsy if you have any of these disorders.

WHAT ARE THE POTENTIAL COMPLICAITONS OF HAVING A MUSCLE BIOPSY?

2/3 of individuals experience a charley horse sensation of their thigh for a couple of days after the biopsy. Otherwise, complications are infrequent and usually mild. Occasionally individuals feel light headed after the biopsy, and it has been reported that approximately 1 out of 100 will actually faint. This is a temporary condition and results in no long term ill effects. Bleeding into the muscle or persistent bleeding of the skin occurs 1 out of every 100 to 250 biopsies. Rare, but more severe complications reported have included puncture of one of the main arteries or nerves of the thigh. These may require surgical repair. These complications have not been seen by the physician performing your biopsy, despite having considerable experience with the technique.

HOW SHOULD YOU CARE FOR YOUR WOUND FOLLOWING THE BIOPSY?

It is advisable to keep the pressure dressing on for at least 24 hours after the biopsy and the skin tape on for 3 to 4 days. If it is necessary to bathe or shower, saran wrap or a plastic bag can be taped over the biopsy site to protect it from the water. You can continue with your normal everyday activities following the biopsy, but it is generally advisable to avoid heavy, strenuous leg activity until the charley horse sensation has subsided.

Please contact your physician if the following are observed: severe pain or progressive swelling of the thigh; fever; chills; pus draining from the incision site; or pain, swelling/coldness of the ankle or foot.

APPENDIX H

Photograph of the Muscle Biopsy Procedure

APPENDIX I

ACID EXTRACTION FOR METABOLITE ASSAYS

The same acid extract can be used to measure glycogen, Cr, ATP, and PCr for each muscle.

1. Weigh out a piece of frozen muscle, and place it in a glass tissue homogenizer on ice. You will need at least 10 mg of tissue.

2. Multiply the weight (in mg) by 6 and add that volume (in μl) of 0.6 N perchloric acid (PCA).

3. Quickly grind the tissue until homogenous, then using a manual pipet with a small yellow tip (cut off the narrow end of the tip), stir up the suspension and withdraw 2 aliquots of 25 μl for subsequent glycogen analysis. Transfer to a labeled microcentrifuge tube and freeze.

4. Centrifuge the remaining homogenate for 5 min. at 12, 000 rpm in the table top Biofuge A.

5. Using a manual pipet, withdraw a know volume of supernatant and transfer to another tube, record volume.

6. Multiply this volume by 0.22, this is the volume of saturated Trizma base you need to add to neutralize the acid extract. Add this volume now.

7. Freeze the neutralized extracts or analyze forthwith.

APPENDIX J

ATP/PCr ASSAY
(Lamprecht et al., 1974)

Reaction Scheme

$$PCr + ADP \rightarrow \text{creatine} + ATP$$
(creatine kinase)
$$\downarrow$$
$$ATP + \text{glucose} \rightarrow \text{glucose-6-phosphate} + ADP$$
(hexokinase)
$$\downarrow$$
$$\text{glucose-6-phosphate} + NADP + H_2O \rightarrow \text{6-phophogluconic acid} + NADPH$$
(glucose-6-phosphate dehydrogenase)

Reagents

1. Triethanolamine buffer (50 mM, pH 7.6): Dissolve 0.85 ml triethanolamine in about 80 ml dH_2O. Adjust pH to 7.6 with HCl, then dilute to 100 ml with dH_2O. This is stable at 4°C.

2. Reaction medium (soup) (5 mM $MgCl_2$, 0.2 mM ADPm 10 mM glucose, 0.3 mM NADP, 1 U / ml G-6-PDH): Dissolve 0.010 g $MgCl_2$, 1.2 mg Na_2NADP and 10 U g-6-PDH in 10 ml TRA buffer. This should be made fresh daily.

3. HD (20 U /100 µl): Dilute 5 µl HK from vial in 100 µl TRA buffer, fresh daily

4. CK (25 U /100 µl): Dissolve 1.0 mg powder in 1 ml TRA buffer, fresh daily

Procedure

Pipet successively into cuvets:

15 µl sample PCA extract

680 µl soup

Mix and wait until OD reading is stable. Record this value as OD1.

5 5 µl HK HK

Mix and wait until OD reading is stable. Record this value as OD2.

5 µl CK

Mix and wait until OD reading is stable. Record this value as OD3.

Do duplicates for each sample. Run a blank using 50 5 µl 0.6 N PCA neutralized with Trizma

Calculations

The muscle concentrations of ATP and PCr, in µmoles/g or mM are calculated as follows:

PCr or ATP = (OD2-OD1) sample – (OD2-OD1) blank / 6.22 x dilution

The dilution for ATP = 700/15 x 7/1 x 1.22/1
cuvet homogenate neutralization
= 398.5

The dilution for PCr = 705/15 x 7/1 x1.22/1
cuvet homogenate neutralization
= 401.4

APPENDIX K

CREATINE ASSAY
(Bernt et al., 1974)

Reaction Scheme

ATP + creatine → phosphocreatine + ADP
(creatine kinase)

ADP + phosphoenolpyruvate → ATP + pyruvate
(pyruvate kinase)

NADH + pyruvate → lactate + NAD
(lactate dehydrogenase)

Soup (100 ml)

100 mM Trizma base	1.211 g	
0.2 mM NADH	0.014 g	
0.1 mM PEP	0.0023 g	
10 mM $MgSO_4$	0.120 g	
100 mM KCL	0.746 g	
>3 U/ml LDH + >5 U/ml PK	0.3 ml	* mixed together already
0.2 mM ATP	0.011 g	
		pH to 9.1

Procedure

Add 10 µl of neutralized PCA muscle extract to cuvet, then add 750 µl of soup. Wait until reading stabilizes, then read OD1. Next add 10 U of CK to each cuvet, and allow the reaction to run to completion. Read OD2 and calculate.

- Since the old Boehringer CK is about 20 U/mg, if we add 5 mg CK to 100 µl of soup, we would have 100 U in 100 µl, and could then add 5 µl to each cuvet

Calculation

$$[mM] = \frac{\Delta OD}{6.22} \times \frac{770\ (CK-5)}{10} \times 7 \times 1.22$$

APPENDIX L

ADENOSINE TRIPHOSPHATASE STAIN
(Brooke and Kaiser, 1970)

Materials:

1. 0.1 M Sodium Barbital
   5.15 g powder plus deionized water → 250 ml store at room temperature (RT)
2. 0.18 M Calcium Chloride
   2.65 g CaCl2.2H20 plus deionized water → 100 ml store at RT
3. 1% Calcium Chloride
   5.0 g CaCl2.2H20 plus deionized water → 500 ml store at RT
4. 2% Colbalt Chloride
   6.0 g CoCl2.6H20 plus deionized water → 300 ml store at RT
5. Barbital Acetate Solution
   1.47 g sodium barbital powder
   8.82 g sodium acetate crystal (CH3C00Na-3H20 FW=136.08)
   deionized water →300 ml (3.51 g anhydrous) store at RT
6. 0.1 N Hydrochloric Acid
   2.505 ml conc. HCl plus deionized H20 → 300 ml
7. Adenosine Triphosphate, disodium salt
   Powder, store desiccated at –20 C
8. Ammonium Sulfide (light solution, original stock is 21%)

Pre-Incubation Solutions:

10.4 ATP (type II fibers stain darkest, type I lightest)

2.0 ml 0.1 M Sodium Barbital
2.0 ml 0.18 M Calcium Chloride
6.0 ml deionized water

Adjust pH to 10.4 just prior to use (original pH @ 9.4) with 3-4 drops of 0.1 N NaOH
Place coverslips in solution for EXACTLY 15 minutes at room temperature
Place into prepared ATP solution for 15 minutes

4.3 ATP (type I dark, type IIb intermediate, type IIa lightest)
4.45 ATP (type I dark, type II light, type IIc intermediate)

2.5 ml Barbital Acetate Solution
5.0 ml 0.1 N HCl
4.0 ml deionized water

pH to 4.3 or 4.45 just prior to use (original pH @ 4.7-4.9) with 0.1 N HCl (1-2 drops)
Place coverslips in solution for EXACTLY 5 minutes at room temperature
Quickly rinse in deionized water or 9.4 pre-inc. solution to avoid pH shock
Place coverslips into prepared ATP solution for 35 minutes

ATP Solution

2.0 ml 0.1 M Sodium Barbital
1.0 ml 0.18 M Calcium Chloride
7.0 ml deionized water
17 mg ATP powder (15-20 mg) disodium slat

add in order listed to prevent ATP from precipitating
Prepare just prior to use and adjust pH to 9.35
The pH after the ATP addition will be approximately 8.2

Method:

1. Pre-incubate at the necessary pH and time
2. Incubate in the ATP solution for the appropriate time
3. Wash in 3 changes of 1% Calcium Chloride for a total of 10 minutes
4. Place in 2% Cobalt Chloride for 5 minutes
5. Wash in 3-5 changes of an approximately 0.005 M Sodium Barbital solution (eg. Approximately 2.5 ml 0.1 M Barbital plus deionized water → approx. 50 ml)
6. Wash in about 20 changes of tap or deionized water
7. In a FUME HOOD: prepare a 2% ammonium sulfide solution from the stock for the 10.4 ATP (0.2 ml stock NH_4SO_2 + 9.8 ml deionized H_2O) and a 5% ammonium sulfide solution for the 4.3 and 4.45 ATP (0.5 ml stock NH_4SO_2 + 9.5 ml deionized H_2O). Place the coverslips in the proper solution for 25 seconds
8. Rinse well with tap water (several changes in hood)
9. Mount with Canada Balsam