^{1}H-MRS Evaluation of the Phosphocreatine-Creatine (PCr/Cr) Pool in Human Muscle

by

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ABSTRACT

Phosphocreatine (PCr) has been shown to effectively buffer ATP levels at high work rates in skeletal muscles. Our main goal was to assess whether or not the pool of PCr and Cr (Cr_{tot}) is the same in different metabolic states. Twelve healthy power trained (PWR) athletes (VO_2max 48 ± 1.9 ml/kg/min) and 12 healthy endurance trained (END) athletes (VO_2max 69.9 ± 1.5 ml/kg/min) completed a plantar flexion of the right foot against an increasing load until volitional fatigue. This was performed while lying supine in a 3 Tesla superconducting magnet with the gastrocnemius medialis (m. gastrocnemius). centered in a circumscribing coil. Total work production was calculated for the entire activity. Immediately following exhaustion a pressure cuff was inflated for 5 min (>350 mmHg superior to the knee) to allow collection of spectra prior to PCr resynthesis. A PRESS (Point Resolved Spectroscopy) sequence was used to resolve the ^{1}H-visible Cr/PCr peak (3.02 ppm) during rest and ischemic fatigue. Standardized echo time (TE) of 100ms for 164 averages was used in collecting data from a 4.5 cm^3 volume of interest (VOI) in the m. gastrocnemius. Upon removal of the cuff, recovery of Cr_{tot} was assessed for 10 min. Comparisons of rest vs. ischemic fatigue states in both groups indicated at least a 140% increase in the ^{1}H-MRS visible pool of Cr_{tot}. After 10 min of recovery the Cr_{tot} pool returned to within 27% of its resting state. These results suggest that there is a separate pool of Cr in the muscle which may be unavailable to creatine kinase until the onset of intense exercise. It is interesting to note that while both the transverse relaxation time (T_2) for Cr_{tot} and the choline/carnitine/taurine peak (3.1 ppm) decreased by 65% and 26% respectively, the T_2 of water was unchanged.

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ABBREVIATIONS USED

ADP.....adenosine diphosphate

AGAT.....L-Arginine: glycine amidinotranserase

AMP.....adenosine monophosphate

ARG.....arginine

ATP.....adenosine triphosphate

CHO.....the ^{1}H-MRS peak visible at 3.1 ppm and contains the resonances of choline, taurine and carnititne

CK.....creatine kinase

Cl^-.....chloride ion

CO_2.....carbon dioxide

Cr.....creatine

Cr_{TOT}.....the total phosphocreatine and creatine pool

CUFF.....the spectral acquisition period directly after exercise, when the pneumatic pressure cuff was inflated placing the lower extremity was in a state of ischemic fatigue

GAMT.....S-adenosylmethionine: guanidinoacetate methyltransferase

GLY.....glycine

H_2O .....water

IMP.....inosine monophosphate

K^+.....potassium ion

La.....lactate

LB.....the proposed "loosely bound" pool of phosphocreatine/creatine

MRS.....magnetic resonance spectroscopy

m. gastrocnemius.....gastrocnemius medialis

MET.....methionine

Na^+.....sodium ion

NH_3^+ .....ammonia

NMR.....nuclear magnetic resonance

Pi .....inorganic phosphate

PCA .....perchloric acid

PCr.....phosphocreatine

PFK.....phosphofructokinase

REST.....the spectral acquisition period prior to exercise

S96.....an additional study which determined the transverse relaxation time for the peaks at 3.02, 3.1and 4.7 ppm both during REST during CUFF

TB..... the proposed "tightly bound" pool of phosphocreatine/creatine

UB..... the proposed "unbound bound" pool of phosphocreatine/creatine

VO_2max.....maximum amount of oxygen consumed per minute

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INTRODUCTION

Basics Of The Phosphagen System

Phosphocreatine (PCr) has been of interest to physiologists since the 1930's. While creatine (Cr) was first identified in meat extract in 1835 by Chevreul, it was not until the early 1900's that researchers started considering Cr as an important factor in muscle contraction. Thunberg, reported in 1911 that Cr stimulated oxygen consumption and carbon-dioxide production in muscle. Shortly thereafter, Fiske identified PCr and showed that it decreased during contraction and was restored during recovery. Lundsgaard later showed that iodoacetate-treated muscle could contract normally in the absence of lactate formation and was accompanied by the breakdown of PCr, which led him to conclude that PCr was directly supplying the energy for contraction. In 1934, Lohmann linked ATP and PCr by demonstrating enzyme-catalyzed phosphoryl group transfer between these two compounds - now recognized as the creatine kinase reaction (see Needham, 1971).

The creatine kinase reaction (CK, EC 2.7.3.2) catalyzes the following reaction:

$$Mg.ADP + PCr + H^+ \text{ <----> } Mg.ATP + Cr \quad \text{(equation 1)}$$

where ADP is adenosine diphosphate and ATP is adenosine triphosphate. This is a near equilibrium reaction, with K_{eq} of 185 (pH 7, free $[Mg^{2+}]$ = 1.0 mM, I = 0.25 M, temperature = 38^{o}C) (Teague et al., 1992). For such *in-vivo* near equilibrium reactions, large amounts of enzyme are required for generating a change in the net forward flux of the reaction, because the driving force is small. This enzyme occurs mainly in muscle and nervous tissues in the human body as well as in many species in the animal kingdom. Its primary and largely undisputed contribution to cellular metabolism is the "buffering" of ATP concentrations during changes in

metabolic rate. This is possible, since CK has a low K_m for ADP (~20-30 μM) and the ADP concentration at rest is ~5-40 μM. This means that CK is highly sensitive to changes in ADP and is therefore central to the maintenance of energetic homeostasis during exercise. Since PCr is not saturating to the reaction, CK can also respond to changes in PCr. Thus with burst activity an increase in ADP will drive the CK reaction to the right (equation 1), but this will slow down as the availability of PCr declines.

Maintenance of a constant 5'-adensosine triphosphate (ATP) pool is necessary for normal cellular function. There are two major pathways within the cell that produce ATP as a utilizable energy source; anaerobic and aerobic pathways. While the latter are more efficient and produce significantly more (for each cycle through the pathway 36 mol ATP/mol glucosyl unit are produced) usable energy than the former; it is the anaerobic energy production (3 mol ATP/mol glucosyl unit) which is crucial to the maintenance of high-intensity exercise as discussed below (Newsholme et al., 1986). *In-vivo,* the two systems operate in tandem. For example, the CK reaction and glycolysis can function anaerobically to supply ATP and support cell function during the onset of burst activity, while oxidative phosphorylation requires approximately 15 s to become active and about a minute to start making a significant contribution. Combined these two systems provide cells with their maximum metabolic rates. These pathways are very finely tuned in muscle tissue and are geared to promote the highest level of activation possible. It is this tight coupling which is of interest to this paper, and thus skeletal muscle will be the tissue of central focus.

At the beginning of high intensity work rates, anaerobically derived ATP provides up to 90% of the total ATP required since oxygen is in short supply. So it may be considered that the cells anaerobic pathways act as an "energy buffer" minimizing any decreases in the ATP pool until the aerobic pathways can be initiated. In humans, for example, the approximate contributions of anaerobic and aerobic sources to total ATP production during high-intensity exercise lasting ~3 min. are 80%/20% in the initial 30 s, 45%/55% from 60-90 s and 30%-70% from 120-180 s (Saltin et al., 1990). Other functions for PCr are presently the center of a great deal of controversy

which will be considered in a later section. First, it is pertinent to consider how the CK reaction is linked to the energy supply of the cell.

It has long been known that the activity of CK is closely linked to the hydrolysis of ATP by ATPases:

$$H_2O + ATP <----> ADP + Pi + H^+ \quad \text{(equation 2)}$$

where H_2O represents water and Pi is inorganic phosphate. An increase in the activity of myofibrillar ATPase causes a small decrease in the steady state concentration of ATP and increases those of ADP, Pi and H^+. All of these metabolites have been shown to act as significant regulators in muscle metabolism (see Newsholme et al., 1986 for review). If considering high-energy phosphate metabolism in the cell, the direct regulation of ATP production by ADP availability makes sense, yet the capacity for ADP to regulate is still actively debated. In order to tightly couple the critical balance between energy supply (metabolism) and energy demand (work), muscle respiration would best be controlled by a signal indicative of the magnitude of cell labor. However, it has been proposed that Pi may play even a greater role than ADP in regulation of energy metabolism (Schnyder et al., 1988). To complicate the question of regulation further it is noticed that with continued activity the changes in the concentrations of the above regulators will result in larger changes in the concentrations of other regulators, such as adenosine monophosphate (AMP), PCr, and ammonia (NH_3), as shown in the following reactions.

The consequent increase in ADP will stimulate the adenylate kinase reaction:

$$ADP + ADP <----> ATP + AMP \quad \text{(equation 3)}$$

The relationship between the concentrations of the three adenine nucleotides in muscle is therefore determined by the equilibrium constant for this reaction ([ATP][AMP]/2[ADP]) and the overall

[ATP]/[ADP] ratio. The AMP concentration in muscle is maintained very low and consequently small changes in the [ATP]/[ADP] ratio cause much larger relative changes in [AMP]. For example, an exercise-induced fivefold increase in [ADP] corresponds to a 27-fold increase in [AMP].

The increase in [AMP] and the decrease in [ATP] results in the increase in the activity of AMP deaminase, which catalyzes;

$$AMP + H_2O \text{ ----> } IMP + NH_3^+ \quad \text{(equation 4)}$$

This results in an increase in the concentration of NH_3^+. The greater significance of this reaction is the reduction (albeit small) of the total concentration of adenine nucleotides to inosine monophosphate (IMP), thus removing them from the pool supporting activity.

Another consequence of the reduction in the [ATP]/[ADP] ratio is to favor PCr breakdown which leads to an increase in the concentration of Pi. This is clearly evident, when the above equations (1) and (2) are summed to create the following equation:

$$PCr \text{ ----> } Cr + Pi \quad \text{(equation 5)}$$

The changes in the concentrations of these regulators ([ATP]/[ADP], Pi, AMP, PCr and NH_3^+) have far reaching effects on cellular metabolism. For example, AMP is a potent activator of glycogen phosphorylase and phosphofructokinase (PFK). The increase in the activity of the former will lead to an increase in the activity of PFK and result in the stimulation of glycolysis and eventually glucose transport into the cell. Some of these metabolites (ADP, Pi, ATP) have also been closely linked to the regulation of oxidative phosphorylation (Balaban, 1990; Meyer et. al. 1986; Veech et al. 1979; Foley et al., 1991).

Origin Of Phosphocreatine - How Is It Made And How Is It Taken Up By The Muscle Cell ?

Cr consists of the three amino acids: arginine (ARG), glycine (GLY), and methionine (MET). L-Arginine: glycine amidinotranserase (AGAT) catalyzes what is recognized as the rate limiting step in Cr synthesis, which is the transfer of the amidino group of Arg to Gly to yield L-ornithine and guanidionoacetate. The latter compound is then methylated at the amidino group by S-adenosylmethionine: guanidinoacetate methyltransferase (GAMT) to produce Cr (Bloch et al., 1940). This appears a simple reaction; however, in the body Cr metabolism is complicated by the fact that these reactions occur mainly in the kidney and liver, thus necessitating a transport of Cr to tissues which require it (the three muscle types of skeletal, smooth, and cardiac muscle, as well as nervous tissue).

AGAT and GAMT appear to have evolved with the appearance of the lampreys (Van Pilsum et al., 1972). While virtually all vertebrates examined contain these enzymes in some of their tissues, they were also found in some invertebrate species (annelids, echinoderms, and urochordates etc.) signifying that they either accumulate Cr from their environment or from the diet. Many of the lower vertebrates express both AGAT and GAMT in their livers and kidneys. Mammalian livers tend to contain high levels of GAMT, but only low levels of Cr, and without CK activity, therefore contain no PCr. These higher vertebrates do however contain relatively high amounts of AGAT in the pancreas and kidneys, which subsequently contain low activities of GAMT. Based largely on these findings and the fact that nephrectomized animals show drastic reductions in Cr synthesis (Horner et al., 1959), it has been postulated that the main route of Cr biosynthesis in mammals begins with the formation of guanidinoacetate in the kidney; this is transported through the blood to the liver where it is methylated to Cr. This final product is then exported to the various Cr-requiring tissues. However, this scenario may not hold for skeletal muscle tissue, since the GAMT activity in skeletal muscle has been shown to exhibit the potential to synthesize all the Cr needed by this tissue (Daly et al., 1985).

While the contribution of various tissues to the formation and transport of Cr is still debated, so is its uptake. In skeletal, cardiac, and smooth muscle cells, as well as astroglia cells and erythrocytes, a Na^+/Cl^- transporter has been observed to co-transport Cr into the cell (Daly et al., 1985; Ku et al., 1980; Möller et al., 1989; Guimbal et al., 1993). Since the concentration of Cr in blood amounts to between 300-400 μM, the accumulation of Cr in most tissues is against a large concentration gradient from the blood. It has been proposed that this is driven by the electrochemical potential difference of extracellular versus intracellular $[Na^+]$ (Wyss et al., 1994). Cloning of the Cr transporter cDNA, followed by northern blot analysis, revealed the largest amounts of this Cr transporter mRNA in kidney, heart and skeletal muscle, but none was found in the liver, spleen or small intestine. This discovery is augmented by the finding that ingestion of Cr-monohydrate supplements appears to be able to bolster the Cr pool in skeletal muscle. The uptake of plasma Cr by muscle has been suggested in animal studies to be enhanced by insulin (Walker et al., 1979; Haughland et al., 1975). This was given convincing support in a recent human study comparing biopsy, urine and plasma analyses before and after six days of Cr supplementation (Green et al., 1996). These researchers were able to show a 60% greater increase in the PCr/Cr pool (Cr_{TOT}) of skeletal muscle when a 0.5 L solution of ~18.5% wt/vol. glucose and simple sugars was consumed after each 5 g. Cr dose. The fact that Cr is transported in the plasma, and that intramuscular total Cr stores increase with oral ingestion of Cr monohydrate has led some researchers to conclude that Cr can enter the bloodstream directly and be taken up by skeletal muscle (Hoberman, et al. 1947; Harris et al., 1992i; Greenhaff et al., 1994). If this elevation in Cr could result in an increase in PCr, then the energetic buffering capacity of this anaerobic energy store would be expanded.

Human Research In High Intensity Exercise Shows Phosphocreatine To Be The Major ATP Buffer In The Short Term

CK is an enzyme which has attracted a great deal of attention over the last 30 years due to

its importance maintaining energetic homeostasis within the cell during short term burst type activity. This may be considered its "anaerobic role" and is best exhibited in the electric organ of the electric fish, *Narcine brasiliensis,* where CK is observed to support the enormous energy demand placed on the cell when activation occurs and there is a 2,000-fold increase in Na^+/K^+ ATPase activity (Blum et al., 1990).

In humans, PCr has been observed to effectively buffer the ATP pool during the onset of intense activity. After repeated short bouts of sprint type activity (such as a 30s sprint) it is observed to contribute up to 30% of the energy required to maintain the work done (Hultman et al., 1983; Spriet et al., 1989). During such intense ballistic type activity the flux rates through the myosin, Ca^{2+}, and Na^+/K^+ ATPases are increased several hundred fold, which results in the hydrolysis of ATP at extremely high rates. This allows the human to attain a power output of 2.5 - 3 times greater than that required to attain VO_{2max}, but this can only be maintained for short periods of time. The loss of ATP must be replenished from either anaerobic or aerobic energy stores. It was observed that the total aerobic energy contribution for such a sprint is only 20 percent. Therefore, anaerobic metabolism must be providing the remaining 80 percent of the ATP required (Saltin et al., 1990; Trump et al., 1996; Margaria et al., 1969). In the 1960's researchers began looking in earnest at defining the contribution of these energy components to exercise. In 1964, Margaria proposed the "Serial Mobilization Theory", which suggested that PCr is the first and only substrate for ATP resynthesis during the first ten seconds of intense activity, then anaerobic glycogenolysis and then aerobic metabolism are called upon to support the muscle's activity (Margaria et al., 1964 and 1969).

With the advent of muscle biopsy analysis in the early 1960's it became easier to discriminate the different contributions of the various pathways to energy provision by analyzing muscle over different time courses of an exercise bout. This method allows the extraction of a piece of skeletal muscle via a needle which is then flash frozen in liquid nitrogen, freeze dried, allowing the metabolites to later be extracted in perchloric acid (PCA). From these extracts PCr,

ATP, ADP, lactate as well as many other metabolites are determined through enzyme analysis. The equation generally used to calculate total ATP provision is:

$$\text{Total ATP provision} = \Delta[PCr] + 1.5[La] + 2\,(\Delta\,[ATP] - \Delta\,[ADP]) \quad \text{(equation 6)}$$

However, when analyzing biopsies taken after high intensity, short duration exercise some assumptions were required: (1) that the uptake of extracellular glucose and other fuels is minimal; (2) that there is minimal oxidation of lactate within the cell; (3) and, there is no significant accumulation of glycolytic intermediates. It is usually not possible to fully account for any lactate which may escape into the blood, and therefore the amount of lactate calculated will always be underestimated.

Under specific conditions, most of these assumptions have been validated (Cheetham, et al., 1986; Spriet et al., 1983), yet they express an obvious limitation in the *in-vitro* analysis of muscle biochemistry. Nevertheless, with this technique in hand the Serial Mobilization Theory was scrutinized. It was found that in the first 10 s of a 30 s wingate trial, PCr was indeed contributing close to half of the high energy phosphate required (PCr 44%; glycolysis 53%; aerobic 3 %) (Saltin et al., 1971). Later biopsy analysis was used in conjunction with electrical stimulation, and it was found that PCr decreased within 2 s of maximal stimulation, but lactate was also formed, challenging the thought that PCr is the sole contributor to the energy requirements in the first seconds of exercise (Hultman et al., 1983). During repeated 30 s bouts with intermittent periods of rest the contribution of PCr is observed to change as expressed in the following table (combined results from Trump et al., 1996, Spriet et al., 1989):

	BOUT 1	BOUT 2	BOUT 3
PCr	25%	31%	15%
glycolysis	55%	44%	15%
Aerobic	20%	25%	70%

It was observed that the resynthesis of ATP from gylcogenolysis decreases significantly, yet there was only a 40% decrease in the power output over the three bouts. While the absolute contribution of PCr to the energy requirement also decreases, the relative contribution increases in the second bout and then decreases, suggesting that by the third bout, most of the ATP is being provided by aerobic metabolism. Studies such as these concluded that PCr, while likely not the sole contributor, does indeed act as the major energy "buffer" in the cell until aerobic stores are enabled.

Other Possible Roles For The Phosphocreatine/Creatine Pool And Our Present Understanding Of This Pool From Magnetic Resonance Spectroscopy

Models of respiratory control in skeletal muscle emphasize either kinetic or thermodynamic features of regulation. The classic kinetic model proposed by Chance and Williams in 1956 still provides the conceptual basis for many modern studies of human muscle metabolism. This model proposes that oxidative phosphorylation is limited primarily by ADP availability, and therefore there is a Michaelis-Menten type dependence of respiratory rate on cytoplasmic [ADP]. In this model Pi is generally assumed not to play a role because the K_m of the mitochondrial Pi transport mechanism (~0.25 mM) is several fold lower than the lowest Pi content observed in resting muscle, and thus must always be nearly saturated (Jacobus et al., 1985; McFarland et al., 1994). An alternative kinetic model is the "creatine shuttle", as proposed by Bessman and Saks (Bessman, 1954; Bessman et al., 1981; Saks et al., 1994). This model is conceptually similar to the classic model, but considers the ADP supply limited at the miochondrial inner membrane by diffusion of Cr to the mitochondrial CK, rather than by cytoplasmic [ADP] limiting the activity of cytoplasmic CK.

In contrast to these models, thermodynamic models of respiratory control suppose that many reactions of oxidative phosphorylation are near equilibrium when oxygen is not limiting. According to this view, the net cytoplasmic ATPase rate ultimately determines respiration rate. It

also suggests that the metabolic changes which occur due to a change in this ATPase rate result from the relaxation of all the reactions in this energy supply network to a new steady state (Kushmerick et al., 1983). Compared with the kinetic models, these thermodynamic models lead to two main predictions. First, respiration rate should depend on the cytoplasmic free energy of ATP hydrolysis, and should therefore be sensitive to changes in cytoplasmic Pi as well as to adenine nucleotide levels. Second, the relationship between cytoplasmic phosphates and oxygen consumption should not be fixed by kinetic properties of mitochondrial enzymes but would more likely depend on intramitochondrial NADH/NAD ratio.

In an attempt to better understand these models it is relevant to consider some of these other possible roles for the Cr_{TOT} pool: the shuttling of high energy Pi between the sites of ADP formation and consumption (Rojo et al., 1991; Bessman et al., 1981); the preferential channeling of ATP to the sarcoplasmic reticulum (SR) bound Ca^{2+} and Na^{+}/K^{+} ATPases (Rossi et al., 1990); and the facilitated diffusion of ATP in the cytoplasm as driven by CK. All of these processes are critical to cell function and they are suggested to be tightly linked to the CK reaction. Therefore, to understand the process which determines the availability of this PCr, it is inherently important to understanding the energetic balance within the cell.

Various isozymes of CK have been observed which lend a great deal of support to the suggested roles above. The cytosolic CK is composed of the monomeric units M (muscle) and B (brain) and is generally found as a dimer. The MM-CK is bound to the myosin-ATPases and to the sarcolemma with access to both the Ca^{2+} and Na^{+}/K^{+} - ATPases. It is therefore recognized to be involved in maintaining the contractile process. The BB-CK isozyme is found exclusively in nervous tissue and plays a similar role to that of MM-CK in muscle. The MB-CK isozyme is found in such low concentrations in muscle and nervous tissue that it is often disregarded as a key player in metabolism. The final major isozyme of CK is the mitochondrial (mi) CK which is reversibly bound to the outer surface of the inner mitochondrial membrane. It is functionally coupled to oxidative phosphorylation and since its discovery has caused much debate over the functions of the

PCr/Cr pool (Jacobs et al., 1964; Meyer et al., 1985; Jacobus et al .,1985).

In 1955, it was suggested that the kinetics of mitochondrial oxidative phosphorylation could be described by the availability of ADP, an idea that predated our knowledge of the adenine nucleotide translocase (Chance et al., 1956). While it was first postulated that the extramitochondrial phosphorylation potential ([ATP]/[ADP]/[Pi]) was the parameter that determined the immediate rates of oxygen consumption, it was later proposed that mitochondrial respiratory control was simply a function of the [ATP]/[ADP] ratio, independent of the tissue content of Pi. These two separate veins of thought have polarized the debate on cellular control of oxidative phosphorylation. Control by the cytoplasmic phosphorylation potential is based on near-equilibrium thermodynamic considerations, whereas the [ATP]/[ADP] ratio is founded on the kinetics of the adenine nucleotide translocase. While these theories share some characteristics they are still greatly different and have been the subject of controversy which has led to a great deal of research on CK. These theories arose from the biochemical observation that the tissue content of [ADP] appeared to be considerably higher than the estimated K_m of the adenine nucleotide translocase for ADP. For example the acid-extracted content of ADP in the heart is almost 1.0 mM, whereas the translocase K_m is approximately 20-30 μM (Jacobus et al., 1985). Under these conditions the translocase would clearly be saturated with ADP. Therefore, other control theories were thought to be required; yet, some still adhered to traditional thought since they have found free ADP in tissues which would fall within the ADP kinetic control range (Ackerman et al., 1980).

The discovery of the specific isozymes of CK led researchers to predict a compartmentation of adenylates within the cell for both practical and energetic efficiency reasons. The idea that a large fraction of cytoplasmic ATP is not available for free diffusion to the myofibrils first evolved from their observation that ischemic cardiac muscle stops contracting when PCr is depleted but that ATP levels remain at 80% or more of controls (Gudbjarnsaon et al., 1970). If the activity of the mi-CK at the inner mitochondrial membrane can maintain near equilibrium at the surface, then any

diffusion gradient for ATP and ADP necessarily results in greater gradients of PCr and Cr (Meyer et al., 1984). Therefore, under the appropriate experimental conditions (i.e.: high ATP/ADP ratio and high total Cr/total adenylate ratios) the flux of high-energy phosphate out of mitochondria would be predominantly in the form of PCr, and the flux of acceptor to the mitochondria would be in the form of Cr.

Bessman and co-workers, about 35 years ago, postulated a direct coupling between the adenylate translocator and hexokinase (Gots et al. 1972 and 1974). It was subsequently shown that HK specifically binds to the outer membrane pore (Fiek et al., 1982). In support of this, various laboratories have observed HK to have a significantly lower K_m for ADP when the enzyme was utilizing mitochondrial ATP as compared to cytosolic ATP. In addition, the V_{max} with mitochondrial ATP was about half of that with cytosolic ATP (Gots et al., 1972 and 1974; Wicker et al., 1993). In sum, it would seem that the functional advantage of these complexes (presumably composed of the kinases, porin, and adenylate translocator) would be molecular channeling of ADP into the inner compartment in order to keep a high phosphorylation potential in the cytosol while ATP turnover is high. In this way, the mitochondrial energy metabolism could be activated (without general [ADP] increase) during rapid ATP turnover in muscle and brain by an interplay between MM and mi-CK supplying the intramitochondrial ADP and by bound HK providing mitochondrial substrates via glycolysis.

This has led to the belief that there is compartmentation of metabolites and isozymes in the cell as a basis for the important role of the CK system (Ishida et al., 1994). This was further supported in later studies which have shown that the outer mitochondrial membrane appears to have a relatively low permeability for adenine nucleotides (Lawson et al., 1979). It has been proposed that, due to the specific localization and interaction of Cr_{TOT} with other cellular systems by a mechanism of direct substrate channeling in multienzyme complexes, these CK isozymes integrate energy metabolism by forming an efficient "phosphocreatine shuttle" for intracellular energy transport. This suggests that the CK system minimizes the dissipation of ATP free energy

by minimizing adenylate diffusion gradients between mitochondria and sites of ATP utilization. Inherent to this scheme is the assumption that not all of the available Cr_{TOT} is in contact with CK at all times. Yet all the present phosphorous magnetic resonance spectroscopy (^{31}P-MRS) studies to date have assumed that CK is in equilibrium and that all the Cr_{TOT} is available to CK at all times (Meyer et al., 1985; Wiseman et al., 1995).

If the "shuttle" theory is correct then large fluctuations in the ATP/ADP pool, during the rapid onset of energy consuming processes would require PCr as an energy buffer as well as energy transferring system. In this manner, the high ATP turnover during excitation would increase ADP levels only locally but the Cr levels globally. This increase in ADP locally at the mitochondria has been hypothesized to act as a signal to activate mitochondrial metabolism (Jacobus et al., 1985). It has been further suggested that during rest, metabolites other than free ADP or Pi also regulate the mitochondrial activity, such as the Ca^{2+} modulation of NADH dependent dehydrogenases (Pfaff et al., 1968). When oxidative phosphorylation generates the energy needed for building up glycogen and protein stores, mitochondrial metabolism may be activated by metabolites which are utilized in these pathways, such as glucose. The activation is purported to be performed by miCK. The capacity of this octamer to connect two artificial membranes (Rojo et al., 1991) supports this idea and lends credence to the belief that peripheral kinases (such as hexokinase) might interact with the adenylate translocator via the pore protein (located at the outer mitochondrial membrane), while kinases in the inner membrane space such as miCK might functionally couple porin and the outer membrane with the adenylate translocator and the inner membrane (Kottke et al., 1991; Schnyder et al., 1988; Pfaff et al., 1968).

These results obtained from biochemical approaches are consistent with a CK system that is highly compartmentalized, with complex energy buffering distribution and regulatory networks and distinct localization of CK isozymes at subcellular sites of energy production and utilization, strongly suggesting the existence of a "shuttle"(Wallimann et al., 1989; Saks et al., 1994). This theory was scrutinized by MRS.

The introduction of MRS to the field of muscle biochemistry allowed these questions to be addressed in a completely different manner - *in-vivo*. At first ^{31}P-MRS was employed to view PCr and its changes from rest to exercise. For all these experiments it was and still is assumed that all the ATP and PCr are detectable by ^{31}P-MRS (i.e. are MRS "visible"). One of the initial observations was that the PCr concentration at rest is considerably greater than when measured from biopsy (Chance et al., 1982), a curious finding indeed, when one considers that PCA extracted muscle should liberate all PCr from the tissue sample. Some possible explanations for this are that there is a large release of Ca^{2+} during the biopsy procedure or that freezing may cause some PCr degradation. If this is correct, however, then the estimate of ATP from biopsy analysis of PCr would be underestimated (equation 6). These studies have developed quite a controversy since it has been reported that the resting PCr concentration in skeletal muscle from MRS studies is 170 mmol/kg/dm, which is significantly greater than the Cr_{TOT} determined biochemically to be 120 mmol/kg/dm (Spriet et al., 1989; Chance et al., 1982). This has led some researchers to suggest that there may be some binding of Cr or PCr in the muscle cell during rest (Wallimann et al., 1992; Savabi, 1988).

Therefore, the aspect of PCr/Cr binding was addressed using *in-vivo* ^{31}P-MRS saturation transfer measurements in muscle (Wiseman et al., 1995; McFarland et al., 1994). These saturation transfer studies measured the CK forward flux in resting and stimulated isolated and perfused rat soleus muscle. It was observed that the flux through the CK reaction did not increase during steady state stimulation over a ten-fold range of ATPase rates. This contrasts with the "shuttle" model which would predict the flux through CK to match that of the ATPases (Wallimann et al., 1992). An interesting finding in the comparison of these studies is that the *in-vivo* reaction rates can differ significantly from the rates measured *in-vitro* . The CK activity measured *in-vivo* was approximately 3.0 $mM \cdot s^{-1}$ which is merely a tenth of that measured through enzymatic analysis (28 $mM \cdot s^{-1}$). In addition, the reaction flux at rest does not appear to increase with increasing work load.

These and other MRS studies have made conclusions on the regulation of cell metabolism based on their calculations of the [ADP] from the observed PCr/Pi peaks. A problem arises because this key metabolite can not be directly measured. A large portion of the cellular ADP is bound to actin and other proteins and therefore not free in the cytoplasm and hence MRS invisible. In addition, even in rigorously stimulated muscle, the true free level of ADP is less than 250 μM which is too low to be detected by ^{31}P-MRS (Meyer et al., 1982). Therefore the common solution to this problem is to calculate free cytoplasmic ADP from the CK reaction. The equation used to derive ADP from the Cr_{TOT} equilibrium is:

$$[ADP] = ([ATP]/[PCr])/[Cr]/(K_{CK}[H^+]) \quad \text{(equation 7)}$$

Assuming that the Cr and Pi contents in skeletal muscle are nearly equal, that the PCr+Cr sum remains constant, and that K_{CK} is 1.66×10^9 M^{-1}, then the following equation has been used, developing a direct proportion between Pi/PCr and free ADP:

$$[Pi]/[PCr] = [ADP] * K_{CK}[H^+]/[ATP] \quad \text{(equation 8)}$$

Therefore, if ATP, pH, and Cr_{TOT} do not significantly change, then there should be a Michaelis-Menten relationship between Pi/PCr and respiration rate or workload in skeletal muscle, which would agree with the traditional dogma of respiratory control in the cell (Chance et al., 1956; Wiseman et al., 1995). If this were not the case then ^{31}P-MRS would not be a viable tool to address the fluctuations in the ATP/ADP pool as these are based on the visible PCr pool.

Because of these extensive assumptions, some researchers have questioned the MRS data, suspecting that the experimentally reported near-equilibrium situation for the entire CK system, as observed by *in-vivo* ^{31}P-MRS measurements, may be a consequence of certain limitations of the MRS method and/or may be due to a peculiar unexpected *in-vivo* behavior, in MRS terms, of the

CK system itself. Recently, support for a structured CK system has been found in ^{31}P-MRS studies on transgenic mice which no longer express MM-CK. Surprisingly these mice are able to use PCr as an energy source, but no CK flux was observed. In the wild type mice, which expressed 50% or more of the normal activity level of MM-CK the measured CK flux increased to 100% of normal. Thus it would appear that a large portion of CK in the intact tissue is not available to contribute to the CK flux measurements by current *in-vivo* ^{31}P-MRS (Van Deursen et al., 1993; Walliman, 1996). In fact, it has been suggested that nearly 50% of the total cellular CK is bound to subcellular structures and is therefore "invisible" *in-vivo*. If this is the case then these bound CK's may behave quite differently than the proposed equilibrium nature of the enzyme, in that the CK fluxes through these bound isoforms may increase with higher metabolic rates and remain MRS invisible (Walliman, 1996).

In an attempt to clarify the discrepancies between the MRS and biochemical data a recent study compared results from both methods and showed good reproducibility between MRS and biopsy data for sub-maximal work loads. This study considered the PCr pools in human muscle during incremental work loads (up to 90% of maximal voluntary contraction (MVC)) separated by periods of rest and found that both the ratio between muscle PCr and ATP at rest and the changes in PCr during muscle contractions were similar (except at 90% of MVC) when determined by biochemical and MRS analyses (Bangsbo et al., 1993). The muscle [ADP] determined by biochemical analysis were not changed at the low work loads (28% and 64% MVC) and were only slightly elevated at the end of 90% MVC, whereas there was a 2-12 fold increase in the free [ADP] as observed by MRS. This is a very significant detail since, as stated, the calculation of the free ADP concentration is fully dependent on the assumption that the CK equilibrium is maintained in contracting muscles.

In order to test this assumption various researchers have used [1-^{14}C]-Cr in various tissue preparations (Savabi, 1988; Mossey, 1995; Lee et al., 1961). Savabi observed rat atria after incorporation of labeled Cr during periods of rest, anoxia, and post anoxic recovery. The tissue

content and specific activity of Cr (SA_{Cr}) and PCr (SA_{PCr}) were determined after each change in metabolic state. She surprisingly found a higher specific activity for tissue PCr (1.87 times) than Cr, independent of the percentage of total intracellular Cr that was present as free Cr. This provided very good evidence for the existence of two separate pools of free Cr. She suggested that in the normal oxygenated state ~9% of the total intracellular Cr is free to participate in energy transport, ~36% is either bound or somehow unavailable to the CK reaction, and the rest exists as PCr. She also found that the newly synthesized PCr (in this experiment the labeled PCr) is not readily mixed with the rest of the PCr since the SA_{PCr}/SA_{Cr} increased during anoxia. This endorses the concept of a compartmentalized intracellular environment. Support for this research goes back to 1961 when Lee and Visscher carried out similar experiments on rabbit heart and obtained similar results (SA_{PCr}/SA_{Cr} = 1.61) (Lee et al., 1961). Along this line of thought, evidence for an MRS undetectable ATP pool has also been observed (Zahler et al., 1987) - why not then an undetectable PCr/Cr pool? In a more recent study analyzing trout white muscle in four different metabolic states (Mossey, 1995), found SA_{PCr}/SA_{Cr} to always be much higher than one (values ranging from 3.1 at rest to 5.45).

If these data are correct, they raise serious concerns for much of the ^{31}P-MRS studies which have reported ADP values during changing tissue metabolic states. In contrast to traditional views, these data suggest a structured environment in the cell and one in which CK would likely not have access to Cr_{TOT} at all times. Additional support is found in the evidence that sarcomeric muscle is a very highly organized tissue showing paracrystaline subcellular structures where many enzymes are localized exclusively at distinct subsarcomeric regions to fulfill a regionally specific metabolic function (Kraft et al., 1996). Depending on the muscle fiber type, significant amounts of the CK isozymes are more or less firmly bound to subcellular structures. In the case of mi-CK, the enzyme is situated exclusively within the mitochondria, where it has been proposed to be involved in metabolic channeling of high energy phosphates (Saks et al., 1994; Wallimann et al., 1992). Recently proton nuclear magnetic resonance (^{1}H-MRS) has become refined enough to approach the question of the status of Cr_{TOT} in the muscle cell. This method is able to resolve a

CH_3 (3.02 ppm) and a CH_2 (3.93 ppm) peak, both of which are considered to represent all of the MRS visible PCr and Cr. ^{1}H-MRS studies of the methyl group of Cr (which represents Cr_{TOT}) has led to the hypothesis that the Cr pool has liquid-crystal-like behavior, likely due to the highly organized structure of the tissue (Kreis et al., 1994 and 1997).

Phosphocreatine/Creatine Pool Varies Between Muscle Fiber Types

Two types of fibers are found in skeletal muscle, fast (FT) and slow (ST) twitch, also known as Type II or white and Type I or red, respectively. This classification is based on the respective physiological properties and oxidative capacities of these fibers; the FT category is composed of fast twitch oxidative (Type IIa) and fast twitch glycolytic (Type IIb) fibers. While many different enzymes have been used to establish this classification, it is the differential sensitivity of the cell's myosin ATPase isozyme to extremes of pH which is the most widely used method, and it is on this basis that human muscle is classified into at least these 3 types of fiber. Type I fibers have high activities of the enzymes of the Tricarboxylic Acid Cycle (TCA) cycle, of fatty acid oxidation and of the electron transfer chain in addition to a high concentration of triacylglycerol; thus they characteristically have a relatively high oxidative capacity. Type IIb fibers have a low oxidative and high glycolytic capacity and a low content of triacylglycerol. Type IIa display a high oxidative and high glycolytic capacity and an intermediate content of triacylglycerol.

The biochemical and anatomical changes in the fibers as one moves from I to IIa to IIb are generally as follows (Saltin et al., 1988): The content of glycolytic enzymes increase four to five fold. The amount of sarcoplasmic reticulum (SR) increases two times. There is a significant increase in diameter of the fibers. There is an increase in the capacity for taking up Cr. There is an increase in the expression of the GLUT 4 receptor but a decrease in expression of hexokinase; the former of which is involved in the transport of glucose into the cell, while the latter is the rate limiting enzyme for glycolysis for incoming glucose. In type IIa fibers, 90 percent of the CK is in

the form of MM-CK, which is double that which is found in type I fibers. This increase in MM-CK is matched to an increase in myosin ATPase and therefore an overall increase in ATP turnover capacity. On the other hand there is 12 times the amount of mi-CK found in Type I compared to Type IIa fibers .

The average amount of Cr in a muscle cell is considered to be approximately 30 mmol/kg dm. Upon closer inspection differences between fibers become evident. Type IIa have closer to 35 mmol/kg dm which is matched by a higher expression of CK (0.2 mM). The Cr concentration in Type I fibers is closer to 20 mmol/kg dm. The PCr concentrations are also significantly different with 75 and 85 mmol/kg found in Types I and II respectively. Upon closer examination it is observed that the amount of PCr in a tissue is linearly related to its CK activity (Iyengar M., 1984). The glycogen content also varies such that Type I has 400 mmol/kg dm and Type IIa significantly greater amount at 470 mmol/kg dm. Not surprisingly, however, the ATP level in the two fiber types is held fairly constant at 25 mmol/kg dm. In sum, it is observed that type II fibers are more independent of external aerobic stores and are therefore likely to be the primary muscle fibers working at the onset of activity. Experimentally this has been shown in electrical stimulation studies in which the quadriceps fermoris was stimulated for between 10 - 30 s. These studies showed a faster and greater degradation of PCr as well as a greater decrease in ATP in type II fibers. Therefore, the interaction of CK and PCr can be most convincingly observed Type II fibers.

Significant Aspects Of Oral Creatine Supplementation

It is obvious from the previous discussion that the energy stored as PCr is crucial to the maintenance of ATP provision during high intensity exercise. The ability to replenish the PCr store during recovery periods between repeated bouts of such ballistic activity is also important to continued performance. This is made very evident by the fact that it becomes increasingly difficult to reactivate the glycolytic pathway during such repeated bouts. This has intrigued the scientific

and sports communities for over 15 years; if it were feasible to bolster this pool, could performance be enhanced?

The total PCr/Cr content in human skeletal muscle is generally between 115-140 mmol/kg dm, where ~60-65% (70-90 mmol/kg dm) is found in the form of PCr (Harris et al., 1947). Approximately two grams (or 1.7%) of Cr is turned over in the body per day and excreted as creatinine. This lost Cr is replaced through various foods in our diet, with red meat being a particularly good source, or through de-novo biosynthesis (Crim et al., 1976). It has been known for some time that Cr ingestion will contribute to the total body pool (Chanutin, 1926). Early studies which looked at urinary concentrations of Cr and creatinine following Cr ingestion or isotopically labeled (^{15}N) Cr were unable to account for the amount of Cr ingested in the daily excretions of the subjects (Hoberman, et al. 1947; Bloch et al., 1939; Chanutin, 1926). It has now been confirmed that a significant amount of this Cr is taken up by skeletal muscle. When recreationally active subjects were asked to ingest 20-30 g Cr/day for two to seven days, their Cr_{TOT} increased from 127 to 147 mmol/kg dm while the PCr also increased from 84 to 90 mmol/kg dm (Harris et al., 1992).

The amount of Cr uptake by muscle may vary considerably from individual to individual, and it has been suggested that there is an upper limit to the amount of Cr a muscle cell is able to contain. Greenhaf et al. in 1994, showed a measurable effect from Cr ingestion on PCr resynthesis during recovery from maximal-intensity exercise was only observed in individuals who demonstrated more than a 20 mmol/kg dry matter increase in muscle Cr_{TOT} concentration after Cr ingestion. Thus, the data suggest a beneficial effect of Cr ingestion on metabolism and performance during both exercise and recovery, but, this may depend on the magnitude of the increase in muscle Cr_{TOT} concentration during supplementation.

It has been suggested and some experimental evidence obtained showing that this increase in Cr_{TOT} enhances performance. After ingesting 20g Cr/day for 5 days a mean increase of 23.1

mmol/kg dm in Cr_{TOT} was observed in nine subjects, which correlated with a 4% increase in both peak and total work production over two bouts of 30 s isokinetic cycling (Casey et. al., 1996). This is likely due to an increased buffering capacity of the ATP pool, since this pool was only reduced to within 69% of that prior to Cr loading - despite the increase in work production. These researchers also showed an increase in PCr in both type I and II fibers; however, the change in PCr in Type II, but not Type I were positively correlated with changes in PCr degradation during exercise and changes in total work production for exercise bout 1 and 2.

The evidence suggesting that oral Cr supplementation augments the muscle Cr_{TOT} is significant, although the best regime for consumption is yet to be found. However, once the supplemental Cr is taken up by the muscle fibers it would be very interesting to know if it becomes phosphorylated or if it remains as Cr; or, if a more structured environment is considered to be present in the muscle, then which pool does this exogenous Cr join. A recent study, which analyzed the plasma, urine and skeletal muscle of 24 subjects who loaded Cr, found that supplementation resulted in an increase in both muscle PCr and Cr concentrations; however, the overall PCr/Cr ratio decreased (Green et al., 1996), a curious finding which suggests that the free Cr concentration is increased to a greater extent than PCr. It was also observed that the PCr/Cr ratio was the same regardless of the amount of muscle Cr accumulation after loading (i.e.: despite the high variability in the amount of Cr taken up by the individuals, they all attained similar Cr_{TOT}'s). This suggests that the magnitude of muscle Cr accumulation dictates the amount of PCr formed; therefore, providing more evidence that Cr may play a key role in the regulation of oxidative ATP production. Since it is recognized that the synthesis of PCr is mediated by mi-CK and is linked to both the oxidative ATP production and cytoplasmic PCr resynthesis, then if Cr regulates the amount of available PCr it would indirectly impact on ATP production. Furthermore, since a constant ATP concentration is assumed (samples were taken at rest), the PCr/Cr ratio could be expected to dictate the free ADP concentration and therefore the stimulus to mitochondrial ATP respiration. This study supports this notion by finding a significant increase in muscle free ADP after supplementation - evidence that Cr may play a more central role in cellular energetics.

Rationale For Thesis Research

In summary, over the last 25 years or so, many studies have been conducted on the nature and functional properties of the phosphagen system in mammalian muscles. The only universal agreement generated by this research is that at high power outputs PCr can effectively buffer ATP concentrations until glycolytic and aerobic pathways are brought into play. Otherwise, the field has gradually become polarized into two camps or into two views of CK-phosphagen function. For convenience these two conceptually very different views of phosphagen function can be termed model 1 and model 2.

Model 1, the traditional view, assumes (i) that the total acid-extractable pool of Cr + PCr occurs in aqueous solution and is fully accessible to CK, (ii) that solution chemistry 'rules' apply globally in muscle cells in vivo, (iii) that CK as a result maintains the entire pool of PCr + Cr essentially at equilibrium under all metabolic states, and (iv) that the main CK-phosphagen function is to 'buffer' ATP concentrations during large scale changes in muscle work and in ATP turnover rates.

Although this view dominates the current literature and extends to other phosphagen systems in invertebrate muscles, several alternative hypotheses differ strikingly from the above. As these share many features in common (their differences usually arise from studying different tissues or from using different techniques), they can be lumped together as model 2. The main conceptual features of this model consider (i) that the structural organization of phosphagen containing cells physically constrains Cr_{TOT} (these constraints in brain and in skeletal, cardiac, and smooth muscle may differ), (ii) that solution chemistry rules may apply *in vivo* to mostly localized PCr/Cr pools, again with possibilities for tissue specificity, (iii) that intracellularly localized CK isoforms *in-vivo* create complex pathways of PCr and Cr metabolism, as discussed in detail above, and (iv) as a result, the CK-phosphagen system serves in intracellular directional transfer functions

as well as in buffering of ATP concentrations.

As there were no signs of a synthesis coming together in the field in early planning stages of this study, what seemed to be needed next were new approaches that could further differentiate between these two competing views of CK-phosphagen function. One possible approach was to use isotopically labeled Cr to probe the functional properties of this system in muscles in different stages - this had already been done by Savabi (1988) with a mammalian heart preparation and by Mossey (1995) using a fish muscle preparation. While the data from both studies seemed incompatible with predictions stemming from model 1 above, these approaches have their own limitations and possible artifacts. The main problem is that all these kinds of isotope data are interpretable if - and only if - the assumption of complete equilibration of ^{14}C-Cr with unlabelled Cr is realized. The main cause for any such lack of equilibration would have to be poorly perfused or even ischemic muscle regions; upon acid extraction, unlabelled Cr from these regions would dilute the ^{14}C-Cr and thus could artifactually lead to lower specific activities than predicted by model 1. While this is not considered probable in the *in vivo* experiments by Mossey (1995), no proof was supplied rejecting the error possibility. In Savabi's experiments this potential for error is exacerbated by the *in vitro* nature (atrial muscle strips) of her preparation.

To avoid these kinds of complications in experimental design, we searched for a different means of assessing and evaluating the phosphagen system in mammalian muscles. Of several possibilities, we settled upon ^{1}H-MRS. Using a 3T, 1 m bore whole body nuclear magnetic resonance (NMR) spectrometer, we reasoned that it should be possible to monitor the functional behavior of the phosphagen system *in-vivo*, non-invasively, and essentially in real time. We took advantage of the fact that MRS cannot discriminate between the methyl ^{1}H resonances of PCr and of Cr at 3.02 ppm. Hence, in one set of measurements it would be possible to obtain information (i) on the relative concentration (from the 3.02 ppm resonance intensity) of CR_{TOT} and (ii) on the Cr microenvironment (from the relative translational mobility of Cr_{TOT}). We took advantage of having access to human volunteer subjects for the study, since they are much easier to work with

than are animal models. Finally, we focused on the gastrocnemius muscle, taking advantage of the established fact that it is composed predominantly of fast twitch type IIa and IIb fibers, both with elevated Cr_{TOT} compared to slow twitch fibers or to cardiac muscle (Johnson et al., 1973).

Since it appeared at the outset that the *in-vivo* behavior of the total PCr and Cr expected by models 1 and 2 should be very different, this study designed predictions which could be tested to measurably discriminate between the two models. In particular, model 1 predicts (i) constant pool size of Cr_{TOT} in different and presumably all metabolic states, and thus (ii) complete CK access to the total pool of PCr + Cr, so that solution thermodynamics applied globally to the tissue. Model 2 in contrast would clearly predict (i) that the NMR visible pool of PCr + Cr could vary in different metabolic states, and (ii) for this and other (structural) reasons that CK need not have access to Cr_{TOT} under all conditions (i.e. that solution chemistry rules need not apply globally to the tissue). Additionally, model 1 predicts that any exogenous Cr incorporation (from dietary supplementation) would be the same in all metabolic states; while model 2 might predict, and in any event would certainly be able to tolerate, Cr incorporation that would be most NMR visible under specific metabolic states.

Accordingly, using ^{1}H-MRS, this study set out to test the above predictions by evaluating the status of Cr_{TOT} in the gastrocnemius muscle of human volunteer subjects under two reproducible and definable metabolic states (rest and ischemic fatigue) with and without supplementary Cr in the diet.

METHODS

Experimental Subjects:

Thirteen healthy power trained athletes (PWR) and twelve healthy endurance trained athletes (END) volunteered to participate in this study. PWR were actively involved in recreational power weight lifting (approx. 10 hrs/week). END were all recreationally involved in some form of endurance activity (approx. 14 hrs/week) and 8 were training for competitions (approx. 18 hrs/week).

Maximal oxygen consumption (VO_2max) was assessed on each subject using a cycle ergometer and measuring expired carbon dioxide (CO_2) until fatigue was reached. Informed written consent was obtained from all subjects after a verbal explanation of the study design and possible risks. The study was approved by the Human Ethics Committees of the Universities of British Columbia and Alberta.

Ergometer Design, Leg Preparation And Exercise Protocol:

Within a week of the initial experimental trial each subject performed an incremental cycle ergometer test to fatigue to measure VO_2max. The subjects were allowed to pedal freely and warm up for three minutes, after which the subject cycled at 80 revolutions/minute against a resistance of 2.0 kg. While continuously sampling gas exchange through a mouthpiece, the resistance was incremented by 0.5 kg every two minutes until the oxygen consumption/minute was stable and continuous, which was operationally defined as VO_2max.

From at least 24 hours before but no more than seven days after their VO_2max test, subjects visited the MRS facility to rehearse the experimental protocol. The subject lied supine on the magnet table with a polyurethane foot pedal strapped to the right foot and the pneumatic cuff

placed just above the right knee (Figure 1 and 2). The right foot was strapped into the foot pedal device so that the axis of rotation of the foot pedal occurred in the same plane as the anatomic axis of plantar flexion of the ankle. Within the bore of the magnet a non ferromagnetic ergometer was positioned around the RF coil. On one end of the ergometer, the pedal attached to the subject's right foot fit into a hinge which allowed plantar flexion of the ankle from neutral (0^{o}) to $+15^{o}$.

The end of a kevlar/nylon rope was attached to the base of the foot pedal and the other passed through the rear bore of the magnet and over a pulley to connect to a plastic container into which an initial load of 10 kg. was placed. One kg. of weight was incrementally added to the container every minute. The plastic container and its load was stabilized by a triangular wooded frame (Figure 3). The exercise protocol was designed as a graduated test to fatigue. The subject performed a plantar flexion movement of the right foot once per second (60 contractions/min.) against the increasing load until he reached exhaustion. Cadence was maintained by the use of an audible beep. The subject was instructed to relax after pedal depression, and not provide any resistance, thus allowing the weight attached to the base of the foot pedal to return it immediately to the neutral position and therefore minimize any eccentric work. During this initial familiarization trial, the subjects successfully learned to relax after pedal depression and the author is confident that any eccentric contribution to the work done was minimal.

Significant verbal encouragement was provided during the exercise to assure exhaustion. Fatigue was taken as the inability to maintain rhythm of displacement. Immediately upon fatigue, the pressure cuff was inflated and held at a pressure of >300 mmHg for 5 min. This condition of ischemic fatigue will be referred to as CUFF for convenience. No MRS measurements were taken during the pre-experimental trials, as this was merely an opportunity for the subject to become acquainted with the exercise and pressure cuff.

Figure 1. Representation of the lower right extremity prepared for the experimental protocol. The leg is resting on a foam pad which sits in a polyurethane cast. The pressure cuff, atapulgite bag and foot pedal are all clearly visible.

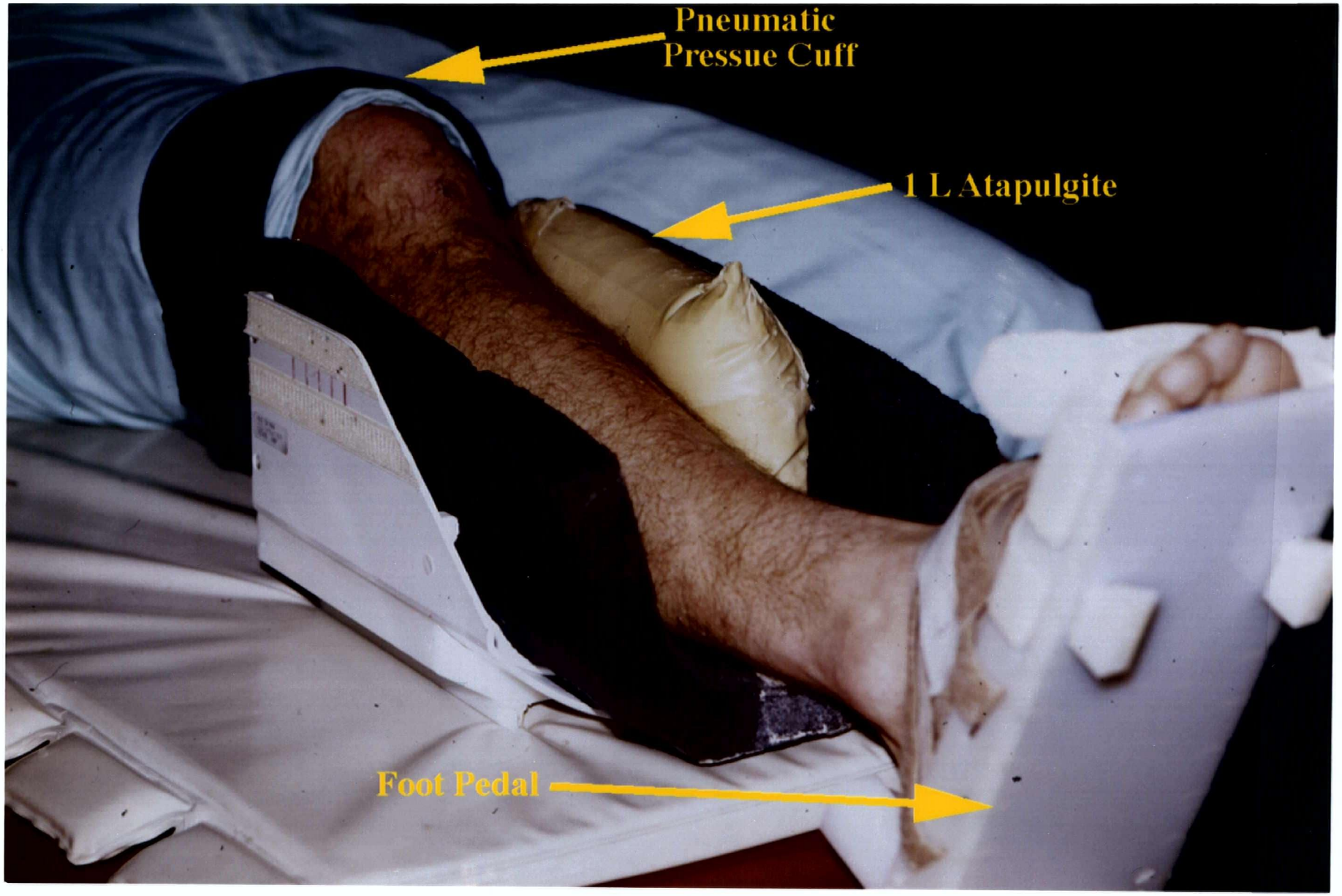

Pneumatic
Pressue Cuff
1 L Atapulgite
Foot Pedal

Figure 2. Example of the preparation for the experimental protocol of the lower right extremity for a given subject. The individual is resting supine on the magnet table with his feet in the bore of the Magnex 3 Tesla magnet. The leg is resting on a foam pad which sits in a polyurethane cast. The pressure cuff, atapulgite bag and foot pedal are all clearly visible.

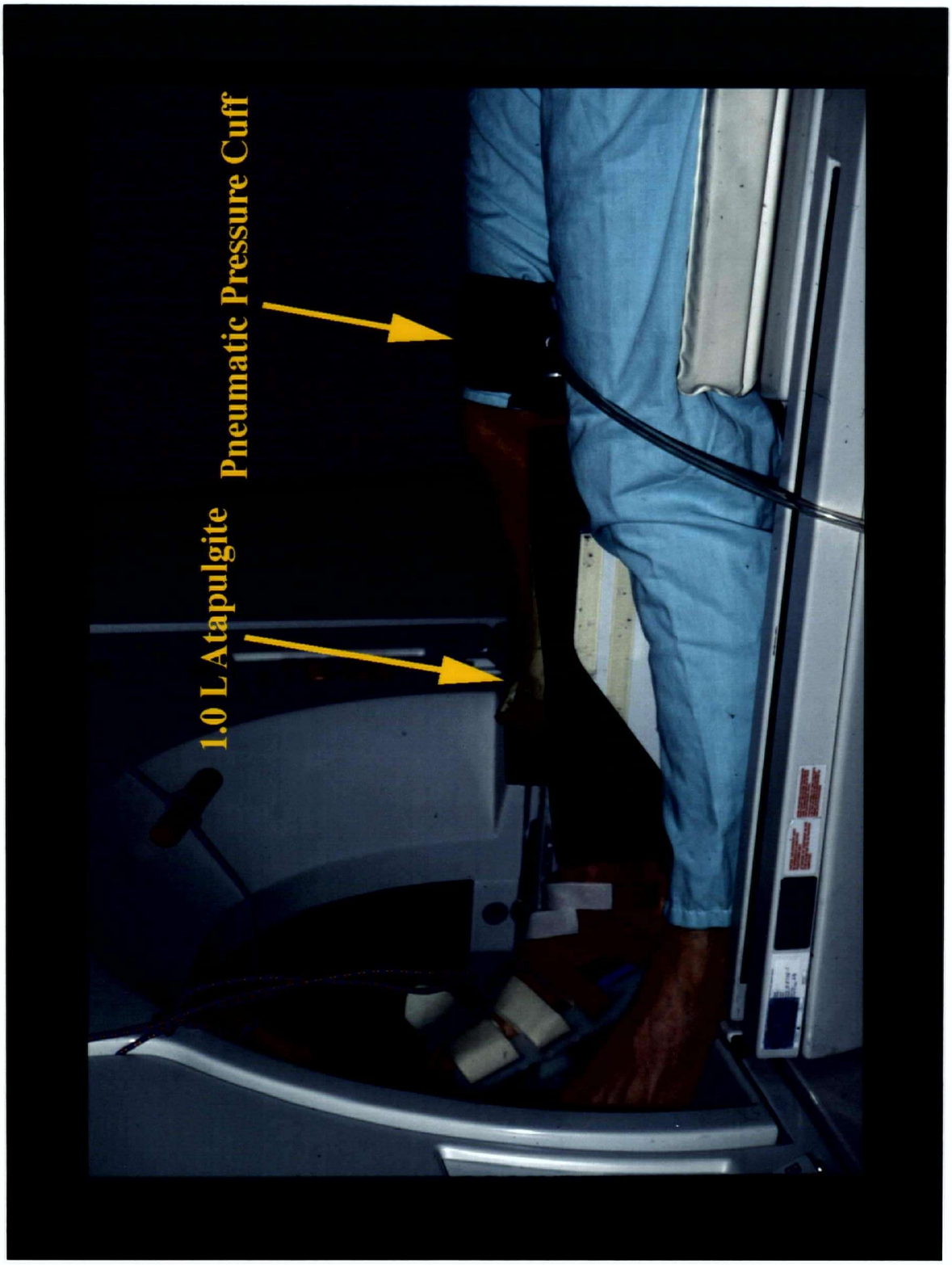
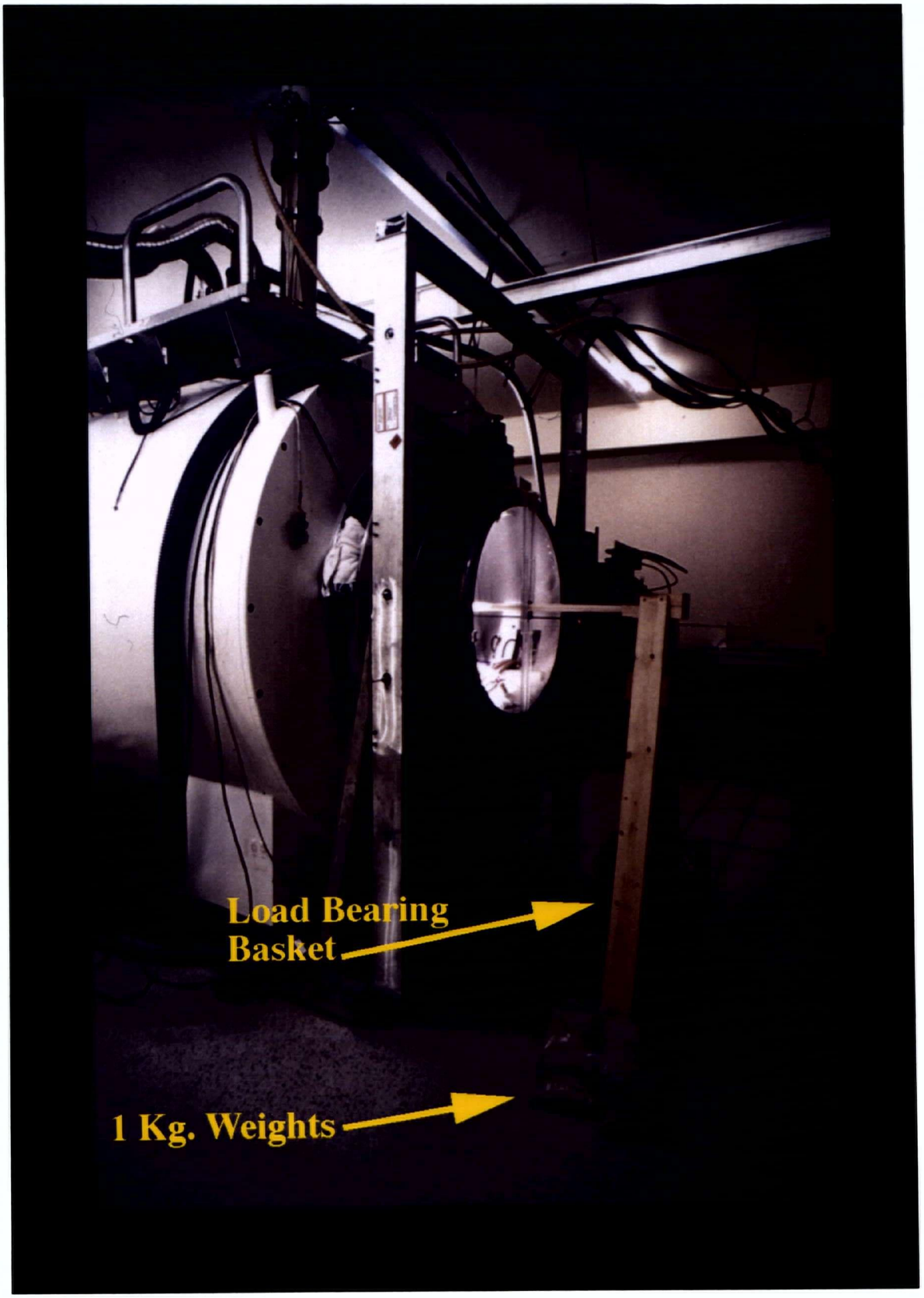

1.0 L Atapulgite
Pneumatic Pressure Cuff

Figure 3. Photograph of the Magnex 3 Tesla magnet exhibiting the weight bearing end of the foot pedal ergometer. Examples of the one kg weight bags are shown.


Load Bearing
Basket
1 Kg. Weights

Experimental Protocol

Within a week of the initial familiarization trial each subject returned to perform their first of two experimental trials. The preparation for these trials was exactly the same as the pre-experimental ones except that extreme care was taken to prevent and account for all extraneous movements. A velcro strap was placed across the hips to prevent horizontal movement of the subject in B_0. The right knee was supported at approximately a 20^o angle so that the gastrocnemius medialis (m. gastrocnemius) would be in the center of the RF coil. The lower right extremity from the knee to ankle was placed in a rubberized foam support which was attached to a polyurethane housing secured to the magnet table. A rubber bag filled with 1L of atapulgite (Kaopectate) was placed around the m. gastrocnemius to improve the magnetic field homogeneity and optimize the fat suppression (Figure 1 and 2). On the 'non-leg' side of the atapulgite bag a standard Cr phantom of 20mMol in 0.083 ml was placed to allow for correction of spectra for day to day or subject to subject fluctuations in the magnetic environment (Figure 4). A rubber tube with approximately 4 ml of water was placed laterally across the anterior portion of the calf to enable assessment of any movement in the B_0 plane. The calf was then strapped firmly with velcro into the housing to prevent any unwanted movement during the exercise. The right foot was placed in the pedal and strapped in with velcro straps. The pneumatic pressure cuff was placed superior to the knee in an uninflated state. The magnet table was then moved into the bore of the magnet and the foot pedal hooked into the pedal ergometer.

MRS data were collected during this initial period of rest (REST) which took approximately 30 min. First a series of images were attained from the leg and the volume of interest (VOI) chosen, from which the spectra would be derived (Figure 4). After resting spectra were collected the subject exercised via plantar flexion of the right foot to exhaustion as described above. Immediately upon attaining fatigue, the pressure cuff was inflated (>300 mmHg) and the subject ceased exercising. This was maintained for 5 min. while the CUFF MRS data were collected. Figure 5 represents this diagramatically.

Figure 4. Representative nuclear magnetic resonance image of the right lower extremity with the VOI (4.5 cm^3) illustrated in red and external Cr standard (20 mM) located outside the lower left quadrant of the leg.

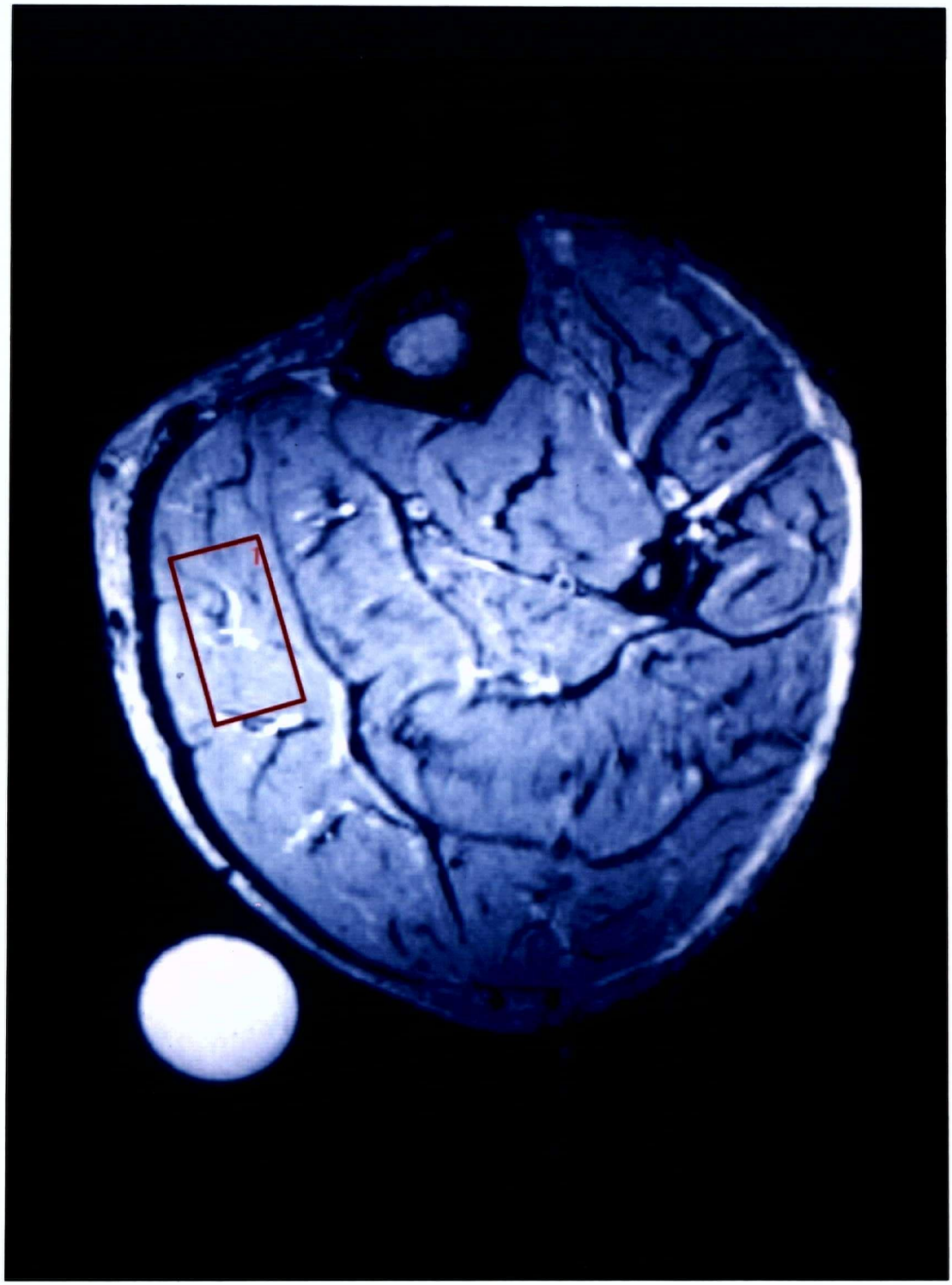
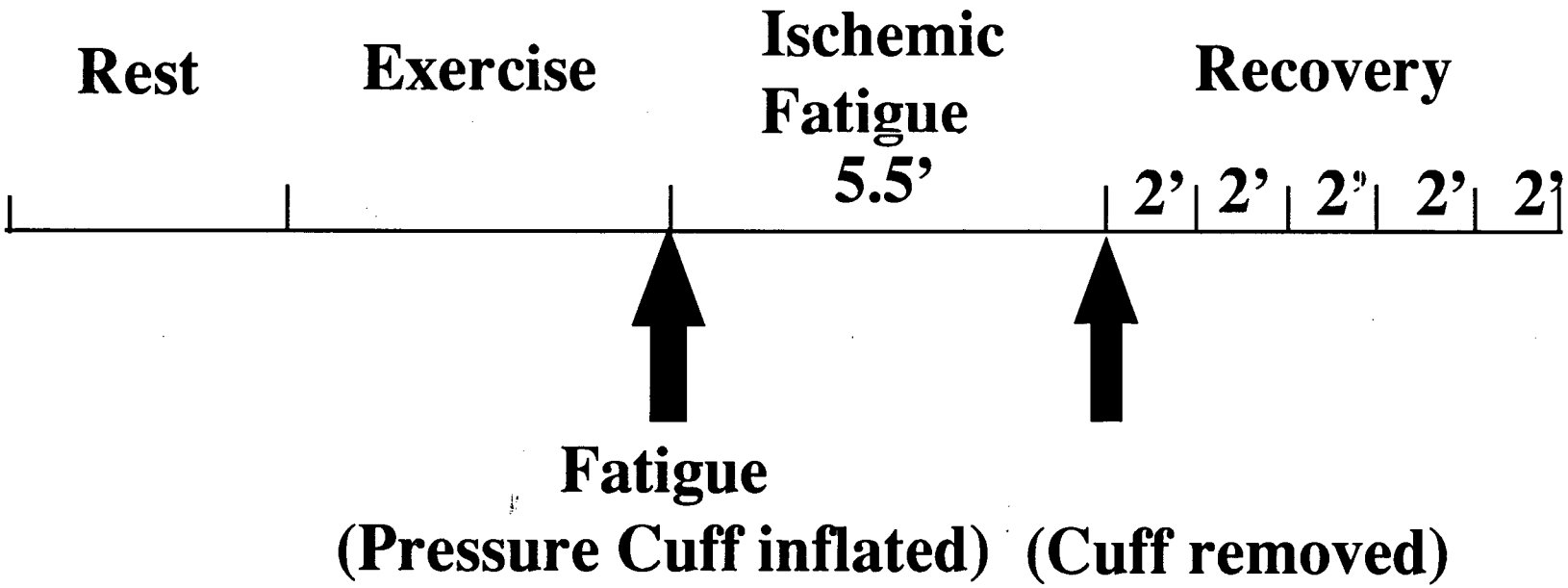


Figure 5. Diagram of the experimental protocol

Figure 5 **Experimental Protocol**



Work Assessment:

Total work was calculated directly from the mass being lifted. A copper cable was attached to the heel of the foot pedal. This cable was attached to a displacement transducer which relayed the change in displacement every 0.107 s. These data were compiled and any eccentric work was removed. The resultant horizontal displacement values were converted to vertical displacement from which the distance the load was moved could be determined.

Creatine Loading Regime:

Prior to leaving the MRS facility after the first experimental trial, the subject received a bag of gelatin capsules filled with either 5g of sucrose or 5 g of Cr-monohydrate. Six subjects were randomly chosen from each of the two subject pools (END and PWR) to load Cr. The Cr-monohydrate was made by Experimental and Applied Sciences (EAS). The subjects were instructed to take 1 capsule 4 times a day for a total of 20 g. Each dose was to be consumed with approximately 0.75 L of some juice with a high glycemic index (ex: orange, grape or apple juice). The reason for consuming the juice was to increase the insulin levels in the individual and assist uptake of Cr by the muscle tissue, as discussed in the introduction. This loading regime was maintained for seven days. On the seventh day the subject returned to the MRS facility for his second and final experimental exercise trial.

Magnetic Resonance Spectroscopy Data Acquisition:

Data were acquired using a Magnex 3 Tesla magnet with actively shielded gradients, and quadrature birdcage resonator. System hardware was controlled using the Surrey Medical Imaging Systems (SMIS) operating system. A series of gradient echo images (echo time (TE) = 22 ms, repetition time (TR) = 500 ms, field of view (FOV) = 20 cm, slice thickness = 5 mm, digital resolution = 512x512) were used to allow volume of interest (VOI) placement. Following

localized shimming (~0.1 ppm), water suppressed inversion null PRESS (Jung et al., 1990) localized spectra (TE = 100 ms, TR = 2 s, inversion recovery time (TIR) = ~600 ms for REST and ~650 ms for the CUFF spectra) were obtained from both a 1.45 x 1.45 x 2.9 cm volume located in the m. gastrocnemius (164 averages), and a 1.45 x 1.45 x 2.9 cm volume located in the Cr reference vial (164 averages). The radio frequency (RF) power was set such that the specific absorption rate (SAR) was as close as possible to the limit of 0.4 W/kg, and the gradient strength then maximized to 0.3 ppm/mm (~0.1 G/cm). At this low gradient strength coupled with the increased spectral dispersion experienced at 3T, a spatial shift of 5.7 mm between the water and Cr peaks resulted along each of the three gradient axes. Therefore, the 3 localization slices were frequency shifted by -216 Hz to that of the Cr resonance in the water suppressed measurements.

Following the acquisition of a baseline Cr spectrum at REST, the subject exercised to volitional exhaustion, at which point a pressure cuff was applied. During CUFF a second Cr spectrum was obtained. On completion of this second acquisition, the cuff was released, and a series of 5 Cr spectra (64 averages, TE = 100 ms) were acquired during the recovery period.

In a follow up experiment six (referred to as (S96))of the subjects (4 END, 2 PWR) returned to complete another trial which focused primarily on the determination of the transverse relaxation times (T_2) for the peaks with resonance 3.02 ppm (Cr), 3.1 ppm (choline/taurine/carnititne) and 4.7 ppm (water) both during REST CUFF. Data were obtained by repeating acquisitions with the PRESS sequence and where the TE time was incremented through 40, 60, 80, 100, 130, 160 ms at REST and 40, 60, 80 ms during CUFF. Fewer TE data points were acquired during the cuff period due to safety considerations and also the limitation of pain tolerance by the subjects during this procedure.

Data Analyses:

The raw MRS free induction decay (FID) spectra was placed through fourier

transformation using the SPECANA software from SMIS U.K. All spectra were given an exponential filter of one, heights were standardized to 4000 units and both zero and first order phasing was utilized to attain an acceptable baseline. Data in this form were then imported into the PERCH spectrum analysis program (distributed by PERCH project, Department of Chemistry, University of Kupio, Finland) for peak analysis. This allowed precise analysis of the peaks of interest. All peaks were fit to a gaussian curve. The T_2 relaxation rates for Cr were estimated by plotting the natural log of the peak area versus the TE times and applying a linear fit, and as such, the slope of the line was equivalent to the T_2.

Statistics:

Data are means ± standard error, unless otherwise noted. The peak intensity data were compared by using a paired t-test. The work data were compared using a Student's t-test. Statistical significance was accepted at $P<0.05$.

RESULTS

Experimental Subject s

Thirteen power trained athletes (PWR) and twelve endurance trained athletes (END) volunteered to participate in this study. The mean age, weight, and height for PWR were respectively 24.5 ± 2 years, 81.5 ± 3.8 kg, 176 ± 1 cm. END had a mean age, weight, and height of 26.0 ± 1 years, 73.8 ± 2 kg, 180.0 ± 2 cm respectively. Maximal oxygen consumption (VO_{2max}) was assessed on each subject using a cycle ergometer and measuring gas exchange until fatigue was reached. The mean VO_{2max} for PWR and END were significantly different ($p<0.01$) at 48±1.9 ml/kg/min and 69.9 ± 1.5 ml/kg/min. respectively.

Magnetic Resonance Spectra:

Well defined ^{1}H-MRS spectra of the Cr peak located at 3.02 ppm at both REST and CUFF were acquired (Figure 6). Excellent resolution was attained for the Cr peak (3.02 ppm) from the choline, taurine, carnitine (CHO) peak. Note the near 40% decrease in Cr intensity as the gastroc. changes from REST to CUFF (intensity values in Table 1).

This resolution was maintained through the recovery collection periods. The REST, CUFF and recovery spectra are compared in Figure 7 (pre Cr loading) and Figure 8 (post Cr loading). Both of these spectra are from the same individual. It is clear that there is a significant decrease in Cr peak intensity (>40%) with a subsequent increase (<50%) in CHO as the leg changes metabolic states. The Cr signal returns with recovery, but after 10 min. is still only 80% recovered. In other words, after 10 min. Cr_{TOT} has not returned to it's resting state.

Spectra were also attained from the external (20 mM) Cr standard both pre and post exercise. This was unchanged between the two states but did fluctuate between individual subjects. An example is provided (Figure 9).

Figure 6. Representative *in-vivo* magnetic resonance spectra for both resting (left) and ischemic fatigue (right) states recorded from the medial gastrocnemius. Both spectra are from the same subject and the same experimental trial prior to Cr loading Water was suppressed to allow visibility of the metabolites of interest. In both states the Cr_{TOT} is clearly resolved from the choline/taurine/carnitine intensities.

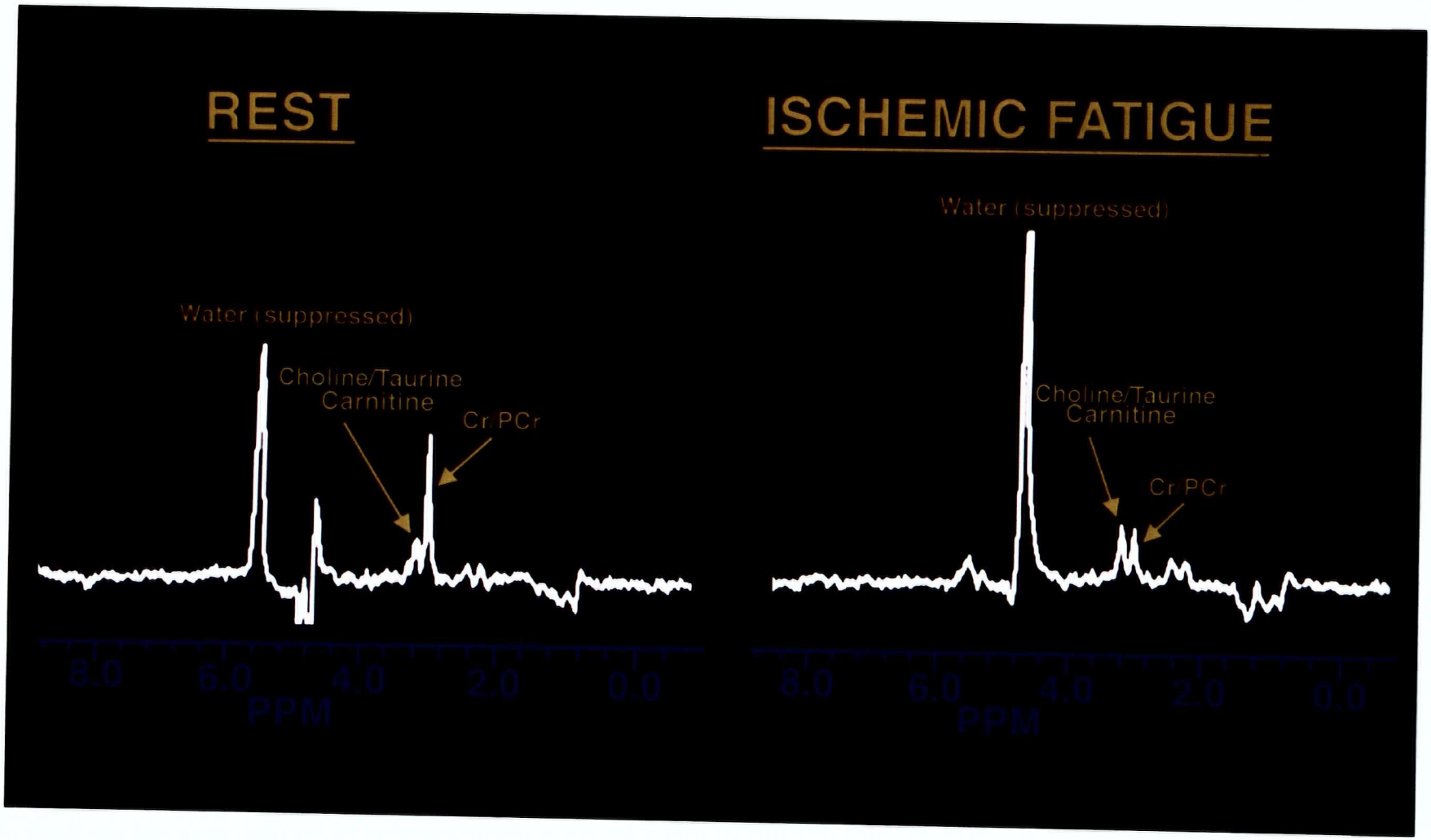

REST
Water (suppressed)
Choline/Taurine
Carnitine
Cr PCr
8.0
6.0
4.0
2.0
0.0
PPM
ISCHEMIC FATIGUE
Water (suppressed)
Choline/Taurine
Carnitine
Cr PCr
8.0
6.0
4.0
2.0
0.0
PPM

Table 1: Change in peak intensities and T2, for various metabolites in the medial gastrocnemius prior to creatine (Cr) and sucrose loading at rest and ischemic fatigue for both the observed spectra (TE = 100 ms) and the calculated values (TE = 0 ms).

Metabolite	T2 (ms)		Peak Area at TE = 100 ms		Peak Area extrapolated to TE = 0ms	
	Rest	Ischemic Fatigue	Rest	Ischemic Fatigue	Rest	Ischemic Fatigue
Cr (3.02 ppm)	117 ±4.10	40 ±6.50	0.69 ±0.04º,** (n=24)	0.34 ±0.02** (n=24)	1.61 ±0.09 º (n=24)	3.99 ±0.28 (n=24)
Choline/Carnitine Taurine (3.30 ppm)	61 ±4.3	45 ±2.4	0.585 ±0.027º,*	1.1 ±0.038*	1.62 ±0.183 º	4.28 ±0.38
Water (4.70 ppm)	30.3 ±1.80 (n = 3)	32 ±1.00 (n = 3)	14.5 ±1.60** (n = 3)	18.35 ±1.90** (n = 3)	382.4 ±50.40 (n = 3)	418.4 ±43.50 (n = 3)

*** Observed at TE = 60 ms**
**** Observed at TE = 100 ms**
Values are expressed as mean ± SEM, n=6 in each case, unless otherwise noted.
Significance was assessed using paired Student's t-test
º: significantly different from ischemic fatigue (P<0.01)

(all Cr values corrected to individual Cr standards)

Figure 7: Representative magnetic resonance spectra for the external creatine standard. Spectra were not different between exercise states.

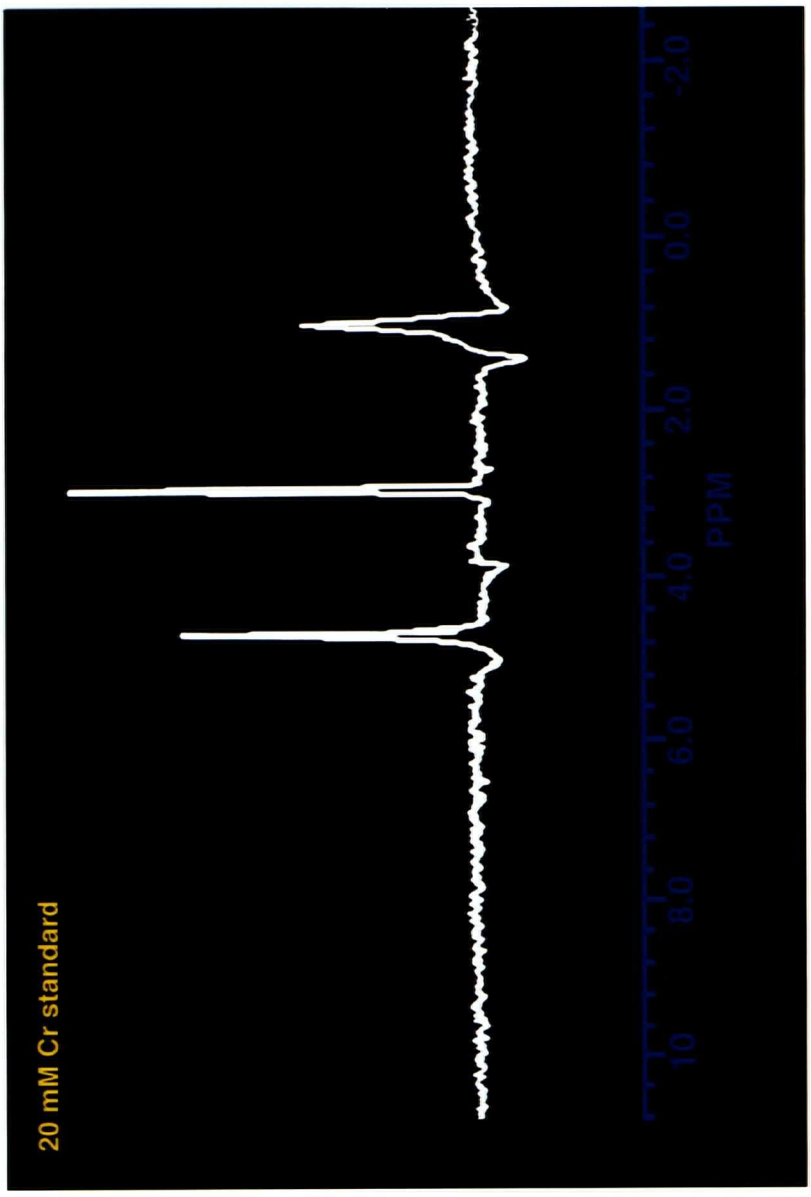

20 mM Cr standard
10
8.0
6.0
4.0
2.0
0.0
-2.0
PPM

Figure 8: Representative *in-vivo* magnetic resonance spectra for resting, ischemic fatigue, and sequential recovery states recorded from the medial gastrocnemius. All spectra are from the same individual on one experimental trial prior to Cr loading. Water was suppressed to allow visibility of the metabolites of interest. In all states the Cr_{TOT} is clearly resolved from the choline/taurine/carnititne intensities.

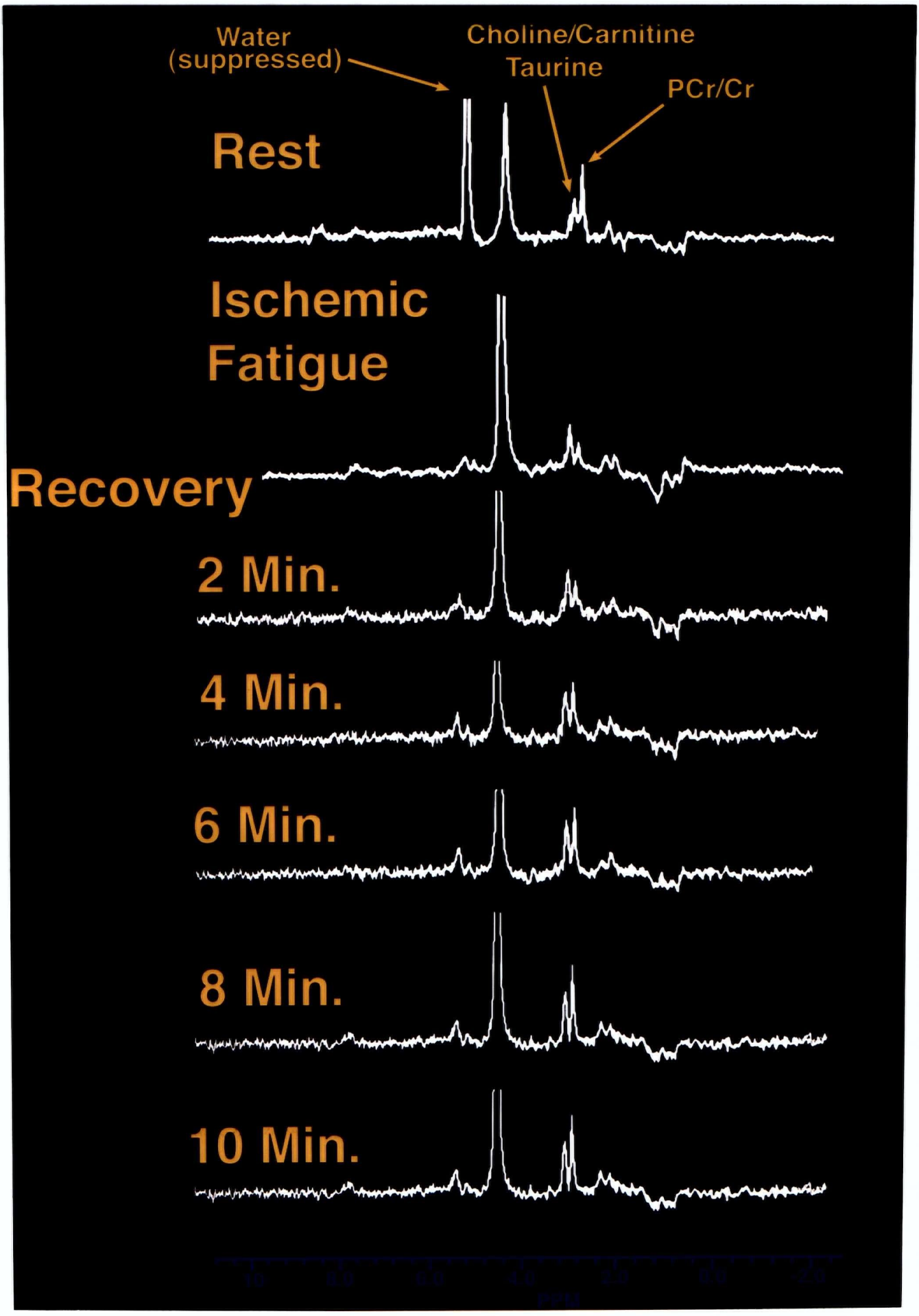
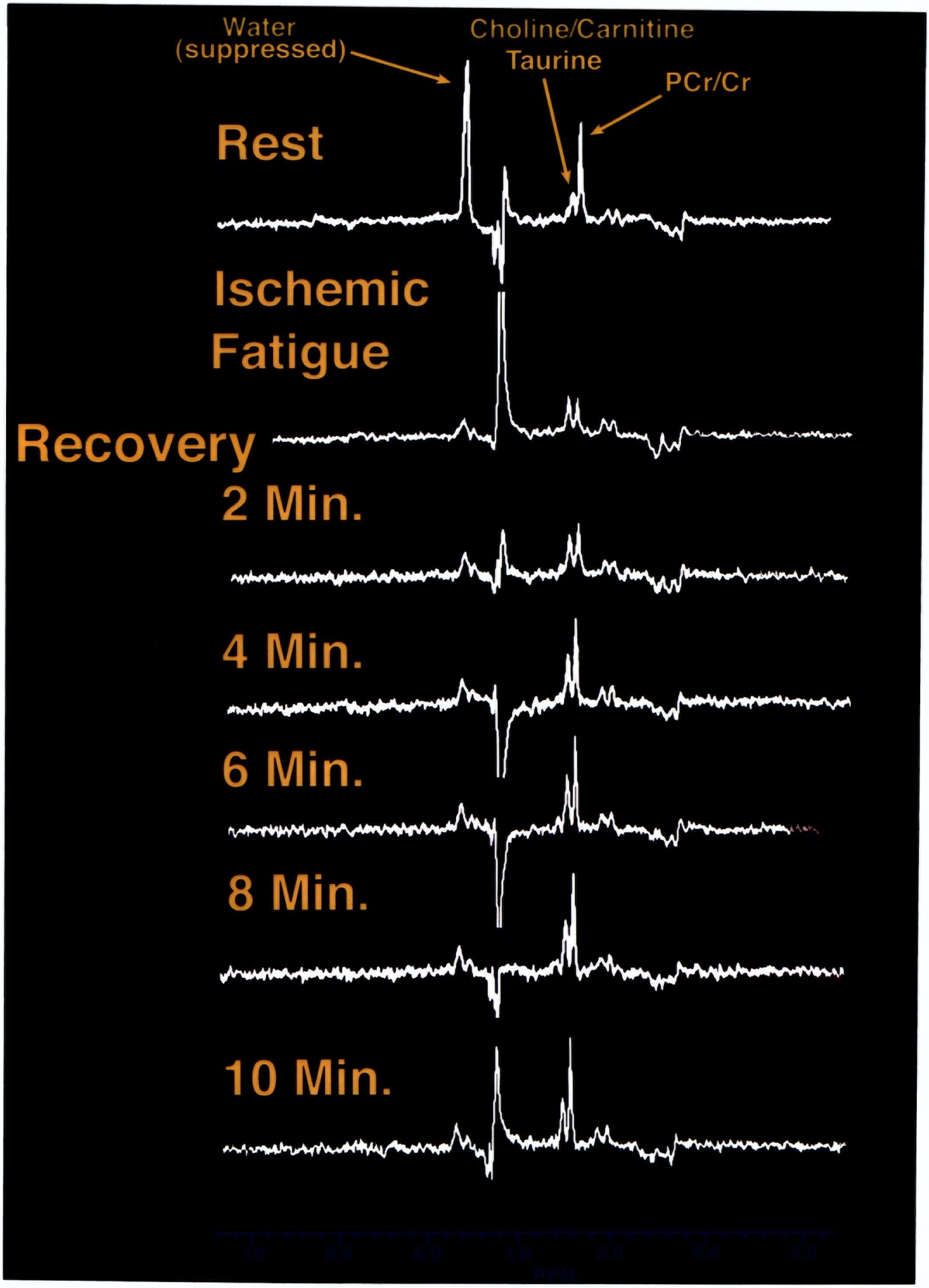

Water
(suppressed)
Choline/Carnitine
Taurine
PCr/Cr
Rest
Ischemic
Fatigue
Recovery
2 Min.
4 Min.
6 Min.
8 Min.
10 Min.
PPM

Figure 9: Representative *in-vivo* magnetic resonance spectra for resting, ischemic fatigue, and sequential recovery states recorded from the medial gastrocnemius. All spectra are from the same individual on one experimental trial post 7 days of Cr loading. Water was suppressed to allow visibility of the metabolites of interest. In all states the Cr_{TOT} is clearly resolved from the choline/taurine/carnititne intensities.


Water
(suppressed)
Choline/Carnitine
Taurine
PCr/Cr
Rest
Ischemic
Fatigue
Recovery
2 Min.
4 Min.
6 Min.
8 Min.
10 Min.

Metabolite Peak Intensities and Transverse Relaxation Times:

The Cr metabolite data assessed from PWR and END were taken at TE = 100 ms. The data collected from S96 were assessed at TE = 60 ms since prior to these trials the pre-emphasis of the gradients were adjusted so that now the signal to noise ratio was significantly better at this TE. Therefore, the optimal peak analysis was at this TE. All peak intensities, once assessed, were corrected to the external standard and calculated to TE = 0 using the formula:

$$A_{TE} = A_0 e^{-TE/T_2}$$

In order to utilize this equation the T_2 for Cr needed to be calculated. The S96 data were collected for this purpose and are displayed in Figure 12. From these spectra it is clear that there is the T_2 effect for Cr as the leg changes from REST to CUFF. The line width broadening of Cr_{TOT} as well as the rapid decrease in signal are trademarks of a shortened T_2.

The change in T_2 is expressed in Figure 11a (REST) and 11b (CUFF). Each of the individual 6 subjects from S96 is shown. From these data a line may be fitted to the points and the slope of that line will be the T_2. This was assessed for Cr, CHO, and water from the S96 data. The T_2 for Cr decreased 3 fold, whereas for CHO, it decreased approximately 60%. The T_2 for Cr decreased significantly from 117 ± 4 ms at REST to 40 ± 7 ms during CUFF (Table 1). The T_2 for CHO also had a significant decrease from 61 ± 4 ms during REST to 45.2 ± 2 during CUFF. The T_2 of water was virtually unchanged between REST and CUFF as it increased from 30 ± 2 ms to 32 ± 1.0 ms. The effect on the observed data was unexpectedly large (Table 1). The 50% intensity decrease from REST to CUFF in Cr observed at TE =100 ms, becomes a 3 fold increase calculated at TE = 0. The mean CHO peak intensity was significantly different between REST (1.58 ± 0.1) and CUFF (4.16 ± 0.1). Water did not show a significant change in T_2 between REST and CUFF nor was there a noticeable difference in peak intensity. The mean water peak intensity was 89.66 ± 9.8 at REST and 92.42 ± 9.6 during CUFF.

The T_2 value from S96 allowed the Cr intensity data from the PWR and END athletes to be extrapolated back to TE = 0 ms (Table 2). While there is a significant difference between REST and CUFF for all PWR and END athletes there was no significant difference between the PWR and END groups either before or after Cr loading.

This is again clearly expressed in Table 3 and 4 which show the recovery of Cr intensity 10 min after the release of the CUFF. Since the T_2 for Cr during recovery was not calculated, it was not possible to calculate the Cr peak intensity at TE = 0 . It is, however, possible to compare the relative intensities of these peaks. There was neither a significant difference in the recovery on a 2 min. to 2 min. basis nor was there a difference in the overall rate of recovery before Cr loading. Hence the populations were pooled for the for Cr intensity which were taken at two minute intervals: 0.42 ± 0.03, 0.49 ± 0.02, 0.53 ± 0.03, 0.54 ± 0.05, 0.6 ± 0.04. The mean REST and CUFF for these populations are represented in Figure 10, to show the trend for Cr_{TOT} recovery.

Work Done::

The differences in work done between the populations both before and after Cr loading are expressed in Table 5. END tended to be able to continue the exercise bout for a longer duration and subsequently completed more work. However, this was only evident in the Cr loaders before loading began. It should be noted that the amount of work data collected from PWR is low (due to displacement cable problems) and precludes any powerful statistical conclusion from these data.

Body Mass Changes:

All groups increased body mass during the one week loading period (whether they were taking sucrose or Cr); however only the END Cr loaders gained a significant amount of weight (Table 6).

Figure 10: Line graph representation for the mean changes observed in the Cr_{TOT} peak intensity at TE = 100 ms for rest, ischemic fatigue, and during ten minutes of recovery in the Endurance (END) and Power (PWR) trained athletes prior to one week of sucrose (SRC) or creatine (Cr) loading. Cr intensity is represented on the ordinate axis. Time during the trial is represented on the abscissa; -10 min. refers to spectra accumulated at REST; -5 to 0 min. refers to CUFF; and 0 to 10 min. refers to spectra acquired during recovery.

(• : represents the standard mean of the Cr intensity, assessed from 24 subjects prior to the loading regime)

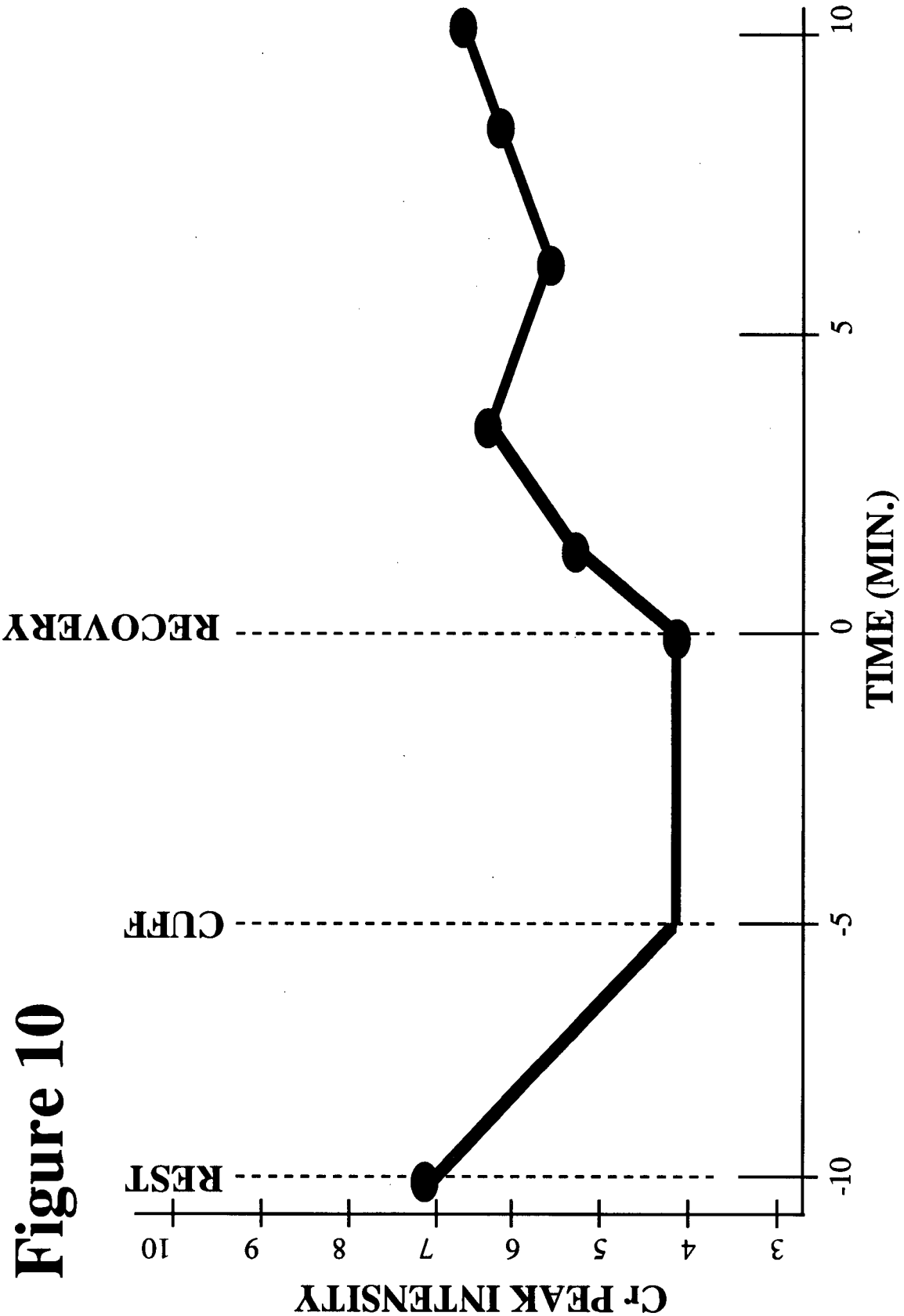

Cr PEAK INTENSITY
3
4
5
6
7
8
9
10
TIME (MIN.)
-10
-5
0
5
10
REST
CUFF
RECOVERY

Figure 10

Figure 11a: The natural log of individual creatine peak intensities taken at six increasing echo times (TE) during rest for six subjects. This expresses the variability between the subject data which was used to determine T_2.

Figure 11a:

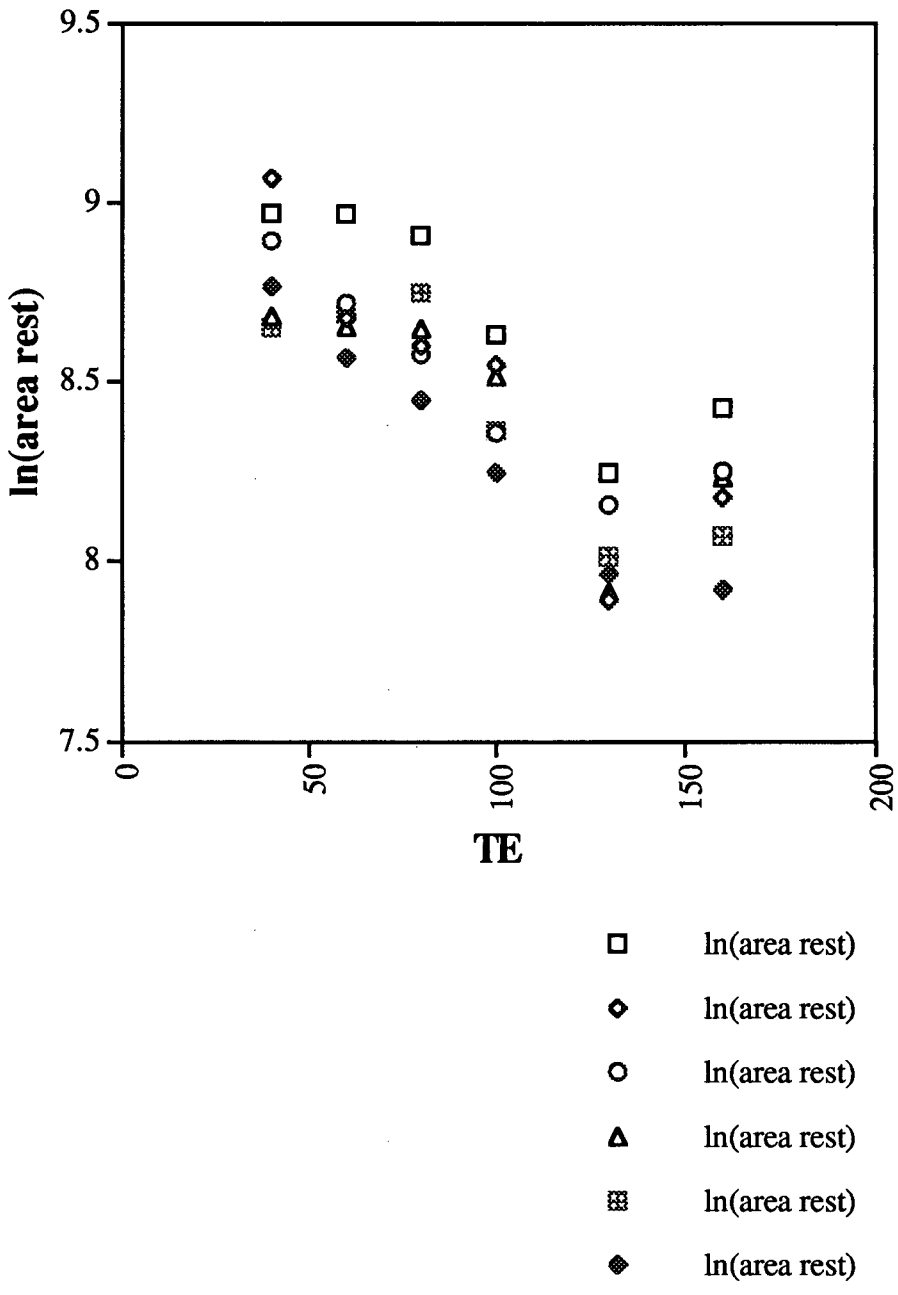

9.5
9
8.5
8
7.5
ln(area rest)
0
50
100
150
200
TE
ln(area rest)
ln(area rest)
ln(area rest)
ln(area rest)
ln(area rest)
ln(area rest)

Figure 11b: The natural log of individual creatine peak intensities taken at three increasing echo times (TE) during rest for six subjects. This expresses the variability between the subject data which was used to determine T_2.

Figure 11b:

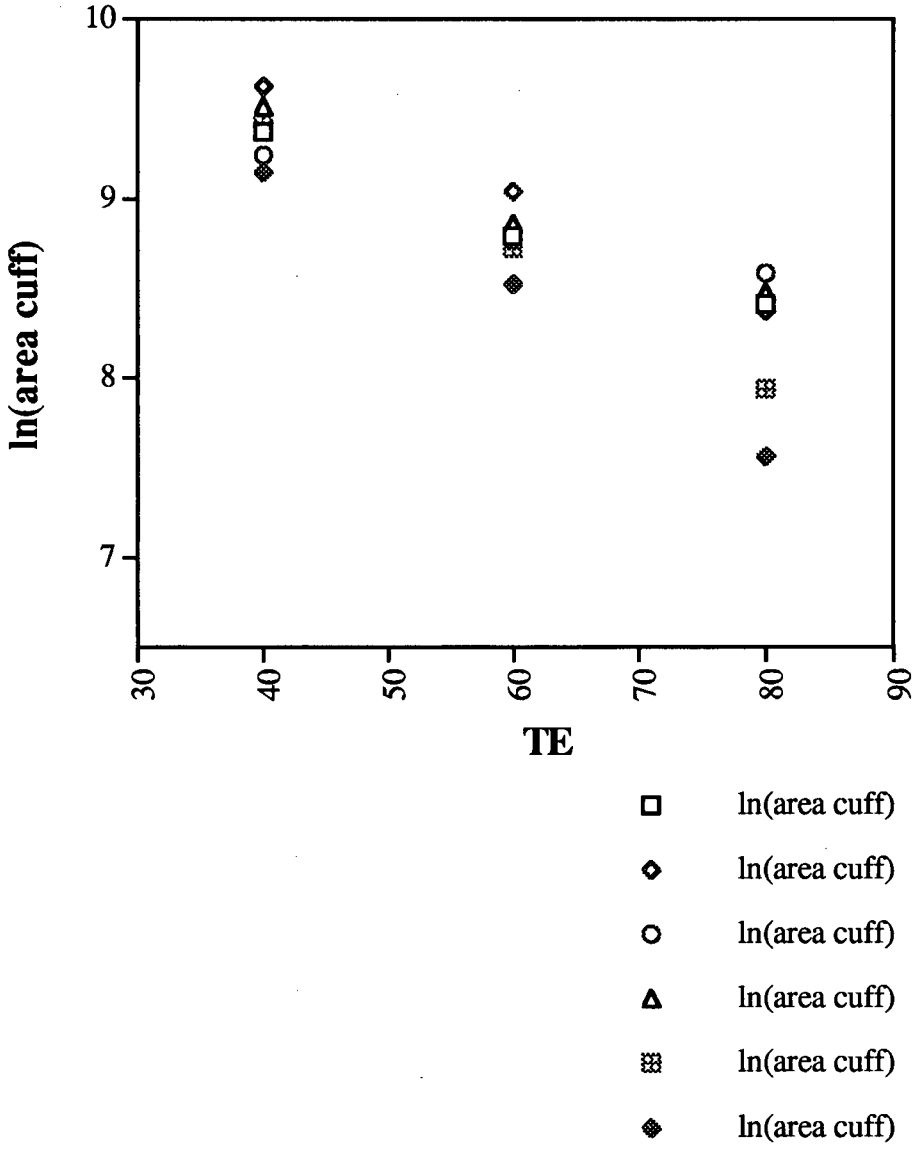

10
9
8
7
ln(area cuff)
30
40
50
60
70
80
90
TE
ln(area cuff)
ln(area cuff)
ln(area cuff)
ln(area cuff)
ln(area cuff)
ln(area cuff)

Figure 12: The change in peak intensity for metabolites at 3.1 ppm (trimethylamines; TMA), 3.02 ppm (creatine; Cr_{TOT}) and the unknown peak at 2.9 ppm (unlabeled) for both rest and ischemic fatigue. Note the more rapid decrease in signal with increased line broadening during ischemic fatigue which is characteristic of shorter transverse relaxation times. Echo time (TE) is expressed in ms along the abscissa.

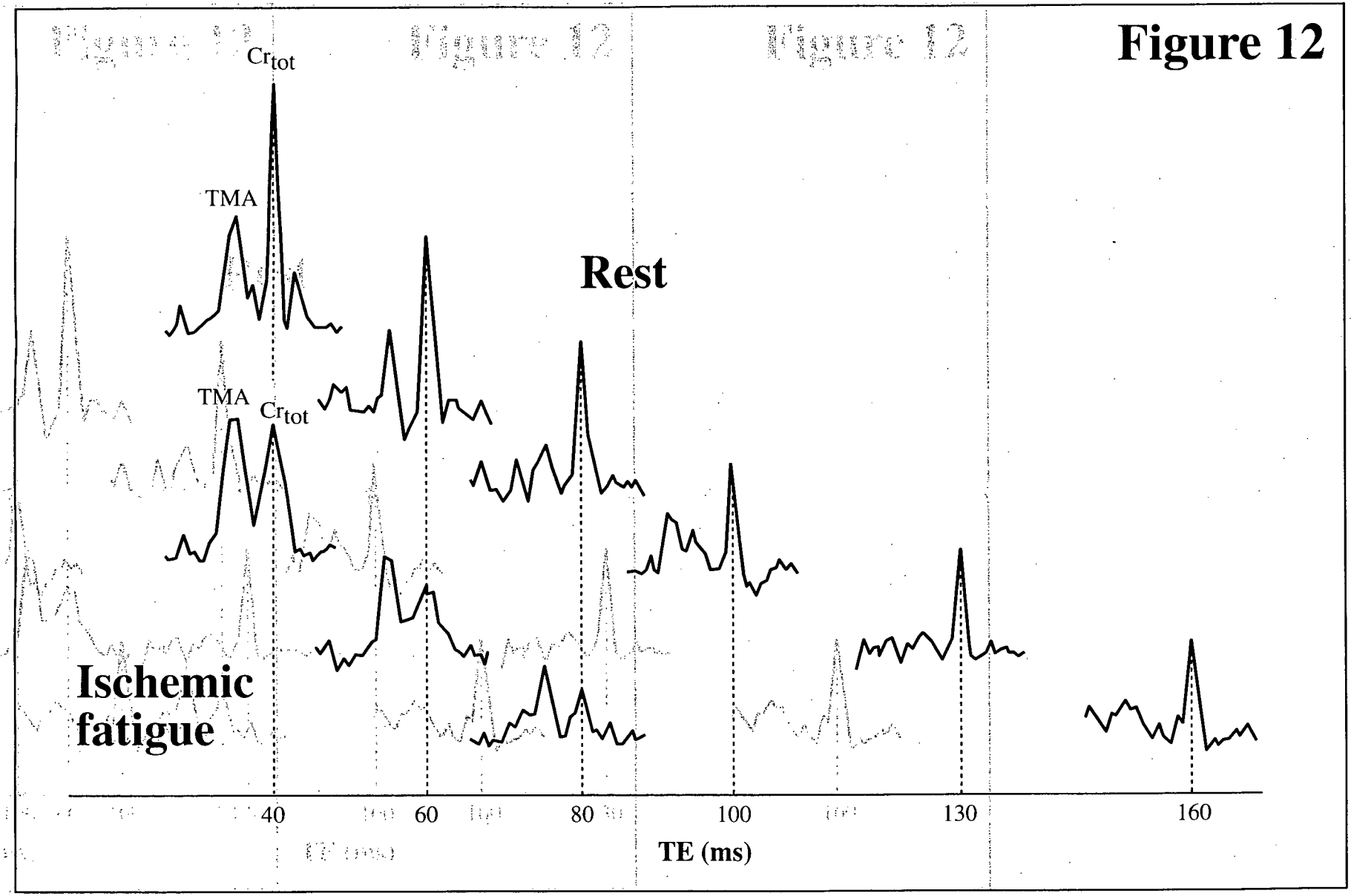


Figure 12
Cr$_{tot}$
TMA
Rest
TMA
Cr$_{tot}$
Ischemic fatigue
40
60
80
100
130
160
TE (ms)

TABLE 2: Changes in Cr peak intensity in the medial gastrocnemius calculated at TE = 0 ms for rest and ischemic fatigue in both Power (PWR) and Endurance (END) trained subjects for both pre and post sucrose (SRC) and (Cr) loading. Spectra were acquired at TE = 100 ms for 164 averages.

		END				PWR		
		REST	CUFF	REST/ CUFF		REST	CUFF	REST/ CUFF
SRC	PRE LOAD	1.57 ± 0.15	4.67 ± 0.6	0.36 ± 0.06	SRC	1.78 ± 0.15	4.2 ±0.51	0.45 ± 0.04
	POST LOAD	1.72 ± 0.16	5.28 ± 0.63	0.36 ± 0.03		1.51 ± 0.15	4.7 ± 0.55	0.33 ±0.03*
Cr	PRE LOAD	1.43 ± 0.08	3.70 ± 0.65	0.43 ± 0.06	Cr	1.3 ± 0.08	3.53 ± 0.67	0.42 ± 0.08
	POST LOAD	1.58 ± 0.15	4.95 ± 0.46*	0.33 ± 0.03*		2.34 ± 0.23*	5.92 ± 0.58*	0.40 ±0.01

Values are expressed as mean ± SEM, n=6 in each case. Significance was assessed using paired Student's t-test

*: significantly different from PRE LOAD ($P<0.05$)

(all values corrected to individual Cr standards)

TABLE 3: Observed changes in the CrTOT peak intensity at TE = 100 ms for rest, ischemic fatigue, and during ten minutes of recovery (REC) in the Power trained athletes both before and after one week of sucrose (SRC) or creatine (Cr) loading

		Rest 5.5 min.	Ischemic Fatigue 5.5 min.	REC 1 2 min.	REC 2 2 min.	REC 3 2 min.	REC 4 2 min.	REC 5 2 min.
SRC	PRE LOAD	0.76 ± 0.06***	0.34 ± 0.04	0.44 ± 0.07 (n=5)	0.47 ± 0.05 (n=5)	0.55 ± 0.07 (n=5)	0.60 ± 0.11 (n=5)	0.57 ± 0.09 (n=5)
	POST LOAD	0.65 ± 0.06***	0.40 ± 0.05	0.53 ± 0.05	0.57 ± 0.05	0.54 ± 0.06	0.59 ± 0.05**	0.59 ± 0.53**
Cr	PRE LOAD	0.55 ± 0.03***	0.30 ± 0.06	0.39 ± 0.01* (n=4)	0.51 ± 0.03* (n=4)	0.53 ± 0.02* (n=4)	0.53 ± 0.02* (n=4)	0.58 ± 0.04* (n=4)
	POST LOAD	1.00 ± 0.09***	0.50 ± 0.05	0.59 ± 0.05	0.71 ± 0.05	0.69 ± 0.05	0.77 ± 0.05	0.74 ± 0.03

Values are expressed as mean ± SEM, n=6 in each case, unless otherwise noted.
Significance was assessed using paired/unpaired Student's t-test

*: significantly different from POST LOAD ($P<0.05$)
**: significantly different from Cr LOAD ($P<0.05$)
***: significantly different from ishemic fatigue ($P<0.01$)

(all values corrected to individual Cr standards)

TABLE 4: Observed changes in the CrTOT peak intensity at TE = 100 ms for rest, ischemic fatigue, and during ten minutes of recovery (REC) in the Endurance trained athletes both before and after one week of sucrose (SRC) or creatine (Cr) loading

		Rest 5.5 min.	Ischemic Fatigue 5.5 min.	REC 1 2 min.	REC 2 2 min.	REC 3 2 min.	REC 4 2 min.	REC 5 2 min.
SRC	PRE LOAD	0.67 ± 0.06***	0.38 ± 0.05	0.45 (n=1)	0.57 (n=1)	0.58 (n=1)	0.48 (n=1)	0.52 (n=1)
	POST LOAD	0.73 ± 0.07***	0.43 ± 0.05	0.55 ± 0.01 (n=3)	0.66 ± 0.03 (n=3)	0.55 ± 0.08 (n=3)	0.60 ± 0.09 (n=3)	0.54 ± 0.13 (n=3)
Cr	PRE LOAD	0.64 ± 0.06***	0.30 ± 0.05	0.41 ± 0.04 (n=2)	0.5 ± 0.07 (n=2)	0.5 ± 0.08 (n=2)	0.44 ± 0.02 (n=2)	0.50 ± 0.02 (n=2)
	POST LOAD	0.67 ± 0.06***	0.41 ± 0.04	0.51 ± 0.05 (n=5)	0.55 ± 0.07 (n=5)	0.59 ± 0.04 (n=5)	0.6 ± 0.06 (n=5)	0.56 ± 0.07 (n=5)

Values are expressed as mean ± SEM, n=6 in each case, unless otherwise noted.
Significance was assessed between the Rest and Ischemic Fatiuge states using paired Student's t-test
No comparative statistics were performed on the recovery data, as the sample sizes were too small to attain any power

***: significantly different from ishemic fatigue (P<0.01)

(all values corrected to individual Cr standards)

Table 5: Differences in work done between power (PWR) and Endurance (END) trained subjects after seven days of loading either creatine (Cr) or sucrose (SRC)

		END	PWR
SRC	PRE LOAD	11.4 ± 1.6	9.2 ± 2.9 (n = 5)
	POSTLOAD	10.9 ± 1.0	8.2 ± 1.5 (n = 4)
Cr	PRE LOAD	12.4 ± 1.4*	7.8 ± 2 º
	POSTLOAD	11.3 ± 1.8	10.1 ± 0.1** (n = 2)

Values are in kilojoules, expressed as mean ± SEM, n=6 in each case, unless otherwise noted.

Significance was assessed using paired and unpaired Student's t-test

*: significantly different from PWR GROUP ($P<0.05$)

**: significantly different from SRC LOADER ($P<0.05$)

º: significantly different from POST LOAD ($P<0.05$)

Table 6: Differences in body mass between power (PWR) and Endurance (END) trained subjects after seven days of either creatine (Cr) or sucrose (SRC) loading.

		END	PWR
SRC	PRE LOAD	79.6 ± 2	78.9 ± 4
	POSTLOAD	81 ± 3	79.3 ± 4
Cr	PRE LOAD	69.5 ± 1 *	84.4 ± 7
	POSTLOAD	70.4 ± 4	85.2 ± 7

Values are in kilograms, expressed as mean ± SEM, n=6 in each case.

Significance was assessed using paired Student's t-test

* : significantly different from POST LOAD ($P<0.05$)

Effects Of Cr Loading

After one week of Cr loading the END Cr loaders did not express a larger Cr_{TOT} pool during REST, but did show a significant increase during CUFF. The PWR athletes exhibited increased Cr_{TOT} during both REST and CUFF after one week of Cr loading. Loading sucrose had no effect on the Cr intensity (Table 2). Cr loading did not increase the rate or amount of recovery of Cr_{TOT} in END (Table 4). PWR showed a consistently greater Cr_{TOT} over the 10 min. recovery period. Cr loading increased the amount of work done in the PWR yet showed no difference in END (Table 5). However, END did significantly increase their body mass after one week of Cr loading, whereas PWR showed no increase (Table 6).

DISCUSSION

Empirical vs. Calculated Results

This study has been able to show acceptable ^{1}H-MRS spectra derived from well defined regions within the human m. gastrocnemius. This has been elucidated through the use of a 3T magnet, a circumscribing coil and a PRESS sequence (which increases the signal received from metabolite methyl singlets in the muscle). The combined use of these techniques provided better resolution of the Cr_{TOT} (3.02 ppm) peak from the CHO (3.1 ppm) peak, than was previously recorded *in-vivo* (Schick et al., 1993; Unitt et al., 1992).

The empirical finding from the observed data demonstrates that during the transition from exercise to fatigue a decrease in Cr_{TOT} intensity and in its T_2 relaxation time takes place. The observed decrease in Cr_{TOT} intensity was about 40%; however, when corrected for the change in T_2, this decrease transformed into more than a two-fold increase in estimated Cr_{TOT} intensity at TE = 0 ms (Table 1).

That the Cr_{TOT} of human skeletal muscle changed its MRS visible concentration between REST and CUFF is the most significant finding from this work. This was not expected and suggests that some of the Cr_{TOT} may not be available to CK during all metabolic states. This is in direct contrast to the accepted traditional thought of CK-phosphagen function (model 1) in the cell, which maintains that CK is freely moving in the cytoplasm and the inner-mitochondrial membrane and that all Cr/PCr are free to react with it at all times (Kushmerick et al., 1983).

The calculated increase in Cr_{TOT} is very dependent on the T_2 determined for the Cr_{TOT} peak at 3.02 ppm. When the observed (TE =100 ms) data were extrapolated back to TE = 0 ms using the standard conversion ($A_{TE} = A_0\, e^{-TE/T_2}$) it is plainly evident that any small shift in T_2 will have a large impact on the area calculated at TE = 0 (Table 1). The sensitivity of the results to T_2 can be softened by using a shorter TE; however, in this study acceptable resolution of the 3.02

peak was not attained for CUFF and REST (see Figure 12) until TE = 100 ms. The possibility of a shift in T_2 for Cr_{TOT} during exercise has very recently become a topic of debate among MRS spectroscopists, and although our data are consistent with other recent work done on the leg (Styles et al., 1995), the main focus of this discussion will be to try to persuade the reader that the T_2 measurements presented here are sound and represent the transverse relaxation times of the 3.02 ppm peak at REST and CUFF. These T_2 measurements are the foundation of support for the finding that Cr_{TOT} increases with exercise. It is therefore important to understand what may be affecting the T_2 of Cr_{TOT} and other metabolites so that a clearer interpretation of the environment CK is operating in may be generated. These factors will now be considered.

Possible Explanations For Changes In The Transverse Relaxation Time:

During the exercise bout the temperature in the leg would be expected to increase a nominal amount (at most a few °C) and would likely remain elevated during the cuffing. This would invariably increase the ability of water to tumble freely in B_O. Since tissue relaxation depends on the dynamics of water molecules, anything which affects the state of water should impinge on the T_2 of the other metabolites in the cell (Bronskill et al., 1993). This coupled to the expected tissue edema should cause the T_2 of Cr_{TOT} to increase (seemingly opposite to the observed result). A possible explanation for the lack of temperature effect finding is that something could be decreasing the T_2 and countering the effect of temperature; or, it may also be argued that temperature would not have a noticeable effect on the T_2 of Cr. Since this variable was not measured, it is only possible to postulate. It is expected that if the temperature of the cellular environment did increase, it was not sufficient enough to have a significant impact on the T_2. Evidence for this may be found in a study which observed human muscle at rest (without an increase in muscle temperature) and discovered two different T_2's for the Cr_{TOT} (Styles et al., 1995). Cr loading may cause muscular edema which would tend to increase the T_2 or the visible Cr by approximately 5% (Kreis et al., 1997i). However, when individual images were checked pre and post Cr loading, no edema was witnessed in this or other studies (Kreis et al., 1995).

Deoxymyoglobin (dMb) has been observed to decrease the T_2 of cellular metabolites through its paramagnetic nature in the cell (Shen et al., 1996). This finding holds great significance to this study since, during CUFF, virtually all myoglobin (Mb) in the intracellular compartment of skeletal muscle and the hemoglobin (Hb) in the microvasculature of the VOI would be deoxygenated and thus in their respective paramagnetic states. During REST, the Mb and Hb are mostly saturated with oxygen and therefore diamagnetic. If dMb and deoxygenated hemoglobin (dHb) were the cause of the reduced T_2, then other metabolites in the cell should have been affected. A relatively slight decrease is evident in the CHO peak at 3.1 ppm. Water, however, does not show a T_2 change with changing states of the muscle, which suggests that some other factor is causing the reduction in T_2 for Cr and possibly CHO. Evidence to the contrary is found in animal studies. In various T_2 weighted diffusion studies on the brain, dHb was observed to decrease the T_2 for water a few percent (Hanstock et al., 1994; Dreher et al., 1994). However, since it is highly unlikely that the Hb in the brain deoxygenated to the state of dHb or dMb in fatigued ischemic muscle, it would be expected that this large reduction in both dHb and dMb would decrease the T_2 of water in muscle a great deal. This is not observed.

Let us consider that dMb and dHb did reduce the T_2 of water and that this was countered by an increase in intracellular water. Exercise physiologists expect that following such an exhaustive exercise bout there is a modest increase in intracellular water of 5-15% (M. Lindinger, personal communication) which would tend to increase the T_2 of water and likely other metabolites, by providing more room for the molecules to spin in (Bronskill et al., 1993). Imagine this as a fine balancing act between the increase in paramagnetic centers (which lower T_2) with the localized edema of exercise. It seems unlikely, however, that this additional water would correct the T_2 back to within precisely 1.7 ms of the resting value. Therefore, although not tested, it is strongly suggested that dMb is not acting as a paramagnetic center, causing a decrease in T_2 for water, Cr or any other metabolite observed.

The recovery data support this conclusion (Tables 5 and 6). All subjects were able to recover their Cr_{TOT} to within 20% of resting values once the pressure cuff was removed. Immediately upon removal of the pressure cuff, the m. gastrocnemius would experience a reactive hyperemia due to the 5.5 min. of ischemia (Anderson et al., 1985). This flood of oxygenated Hb would be able to rapidly reoxygenate the dMb (easily within one to two minutes) yet it takes more than 10 min. for the Cr_{TOT} to recover, suggesting that dMb is not the cause of the shift in T_2. Therefore, something besides the increase in temperature or the paramagnetic nature of dMb or dHb is altering the T_2 of the Cr peak at 3.02 ppm.

Other studies have also found more than one T_2 for Cr, suggesting 2 different states or pools of Cr/PCr. A recent study found ^{1}H-MRS concentrations of Cr/PCr to be significantly greater than those determined by biochemical assay, which suggests some loss of Cr_{TOT} during biopsy (Styles et al., 1995; Bottomley et al., 1997). They also reported T_2's similar to those we found in the present study for water (31.8 ms), but those for CHO (113 ms) were quite different. In regards to Cr, they suggest that Cr_{TOT} is found as an non-homogeneous pool at rest which exhibits two very different T_2's (23.4 and 212 ms). Although these vary from those found in this study it should be noted that the standard deviations for these values were high (7 and 59 respectively for n=6). It is possible that the relatively large VOI ($9cm^3$) used by these researchers resulted in spectra derived from a mixture of muscles as well as fat. This non-homogeneous sample could represent various environments for Cr and therefore various T_2's. It is also likely that with such a large VOI and with TE's up to 528 ms, they were able to discriminate between carnitine, carnosine, taurine and choline (no spectra was shown!), whereas our peak is likely a mixture of these peaks. Although different, their report of two T_2's, is in agreement with the findings of this study and supports the concept of at least two pools of Cr/PCr in the cell - one which is freely tumbling (T_2 = 212 ms) and one which has its movement restricted (T_2 = 23.4 ms).

Schick et al. (1993) also found two T_2's for Cr/PCr at 110 and 140ms which were determined from a relatively small VOI ($2cm^3$) in the soleus muscle. They assigned the former T_2

to the Cr/PCr -CH_2 peak at 3.9 ppm and the latter to the Cr/PCr -CH_3 peak at 3.02 ppm, again suggesting Cr_{TOT} exists in at least two states in the cell; and in this case at rest! It would appear, therefore, that there are indeed two spin-spin relaxation constants for Cr_{TOT} which need to be addressed and given a firm value. One possibility is that the lower T_2 (40 ms) is representative of the Cr pool which is actively involved in buffering the ATP pool and the longer T_2 (117 ms) of resting muscle is representative of the Cr pool which maintains basal cellular function. This suggests a structured environment for Cr_{TOT} and would support model 2.

Model 1 predicts that no change would be observed in the peak intensity of Cr as the muscle cell changes its metabolic state. It proposes that Cr_{TOT} is free in solution throughout the cell and available to CK at all times. Therefore, this model suggests that as the muscle begins to exercise, the flux through CK will increase, and PCr will tend to be converted to Cr. Both should remain MRS visible and thus the peak intensity should not change. Even the empirical observation of a reduced peak intensity at 3.02 ppm refutes this. The observation of a calculated increase in Cr peak intensity supports model 2 in that it suggests a structured environment for CK where only a portion of Cr_{TOT} is available to CK during rest, with the majority of Cr_{TOT} becoming available during exercise. How the cell is structured and where this "hidden" portion of Cr_{TOT} is situated in the cell are questions that need to be addressed and at present can merely be theorized about.

A Proposal For The State of The Phosphocreatine/Creatine Pool in Muscle :

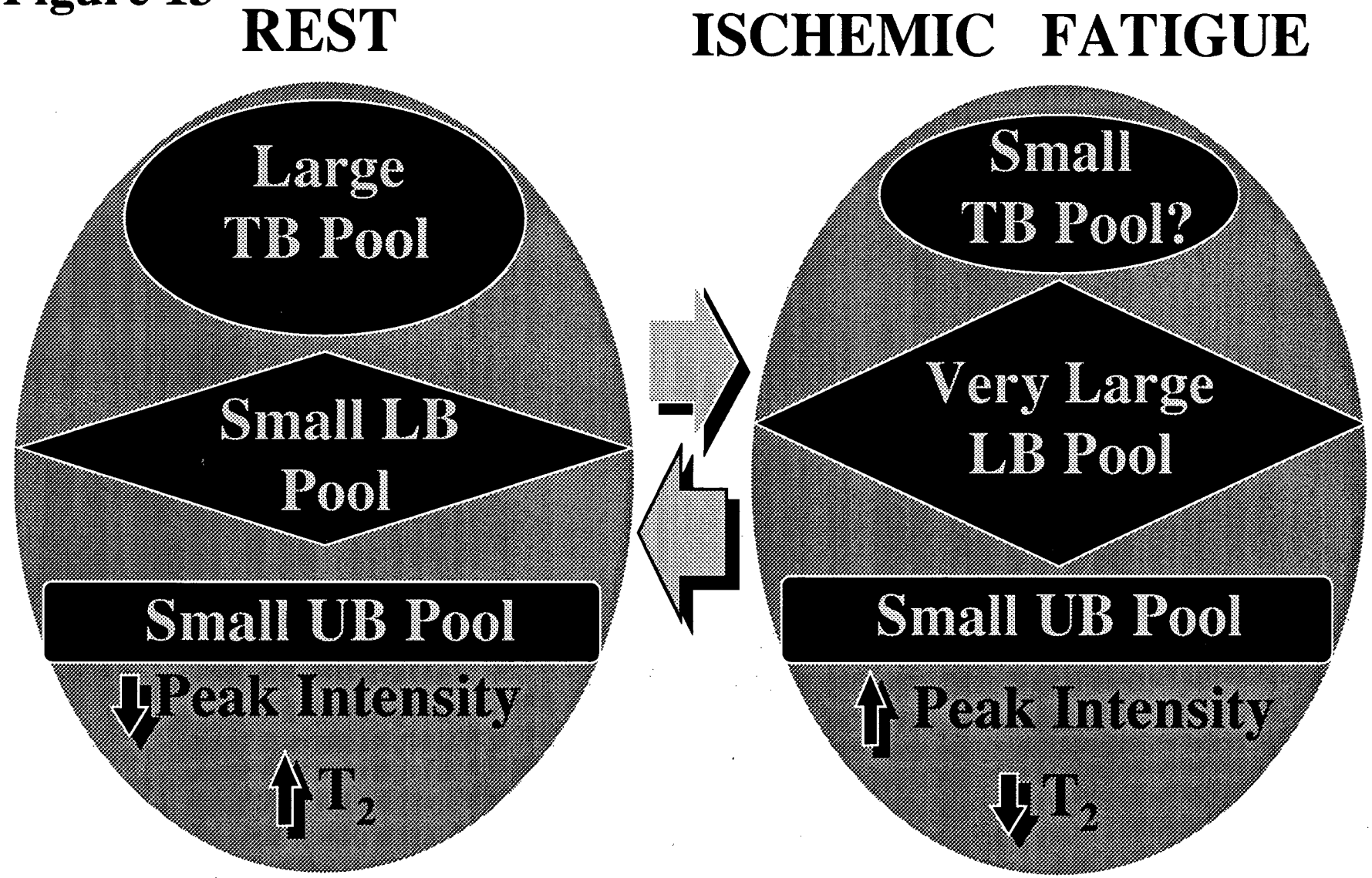
Without any other possible explanation available in the literature for possible changes in Cr_{TOT} pool size or T_2, it becomes necessary to postulate on changes in the intracellular environment, such as changes in viscosity or in binding states, either of which could alter both T_2 and peak intensity. At this time, there is no information available as to the nature of any such changes, with much work needed to be done in this field.

With this in mind, the following speculative model is tentatively suggested: The shorter T_2 (that found in CUFF) may be considered to represent a pool of Cr which is loosely bound (LB) in the cell and is visible at both rest and CUFF; however, at rest it represents merely a small portion of the MRS visible pool and thus its T_2 is overshadowed by the longer T_2 observed at rest. LB is likely to consist of Cr/PCr, which is associated with MM/mi-CK and which are actively buffering the ATP pool. The longer T_2 (that prevalent at rest) represents the majority of Cr_{TOT} at rest - that pool has the greatest amount of freedom and is therefore unbound (UB). It is reasonable to believe that this pool is mostly PCr and largely free within the cytoplasm actively supporting basal metabolism. This study also supports the belief of a large tightly bound pool of Cr/PCr at rest (TB). The majority of this pool is likely Cr which is tightly bound either with the mitochondrial membrane or at the myofibril. During CUFF most of the cell's Cr_{TOT} will be found as Cr. From the observed increase in Cr peak intensity it would appear that the TB pool at rest becomes available during exercise and supports the buffering of the ATP pool in the cell. During CUFF, TB is then observed as part of the LB pool, conceivably loosely bound to CK and still MRS visible.

Thus, three pools of Cr/PCr are predicted to make up Cr_{TOT}. At rest there is a low Cr peak intensity which has a relatively high degree of mobility (T_2) represented as UB. The LB pool is a very small component of this low peak intensity but should be MRS visible. The TB pool is large and invisible at rest. As the metabolic rate of the cell increases, it is predicted that TB becomes available to the CK reaction to assist in the defense of any increase in ADP. When the muscle is observed in a state of ischemic fatigue the peak intensity is much higher, but the pool is less mobile, suggesting that TB has entered into the LB pool. While the UB pool is likely still present, it is small in size, and its T_2 is indecipherable from the T_2 of LB, which is much more visible (Figure 13).

Figure 13. Diagram representing the proposed explanation for the increase in creatine intensity and decrease in T_2. ↑ represents an increase, ↓ represents a decrease.

Figure 13


REST
ISCHEMIC FATIGUE
Large TB Pool
Small LB Pool
Small UB Pool
Peak Intensity
T_2
Small TB Pool?
Very Large LB Pool
Small UB Pool
Peak Intensity
T_2

Other Possibilities For The State Of The Phosphocreatine/Creatine Pool In Muscle

Other theories predicting the state of Cr_{TOT} have been postulated and deserve mention. It has been suggested that Cr/PCr may be in a liquid crystal like lattice, which would support a theory of more than one physical state for Cr_{TOT} (Kreis et. al., 1994). These researchers have now completed a number of studies on human muscle using ^{1}H-MRS and have introduced the concept that dipolar coupling may be part of the reason for the observation of two states of Cr_{TOT} (Boesch et al., 1996; Kreis et al., 1994 and 1997). They suggest that their findings indicate two pools of Cr/PCr and have recently suggested that one might be Cr and the other PCr (Kreis, personal communication and Kreis et al., 1997ii). The dipolar coupling behavior of molecules implies partial restriction of the motion or rotation of the molecule - which would explain the T_2 differences in this thesis, yet Kreis et. al. have not documented a shift in T_2 with exercise. While their human work has been done on a magnet of considerably lower field strength (1.5 T) (Kreis et al., 1994; Kreis et al., 1997i; Kreis et al., 1997ii), similar findings have been shown in rat muscle at 4.7 T (Ntziachristos et al., 1996) suggesting that this phenomenon is independent of magnetic field. Direct comparison, however, is still considered difficult, since the smallest VOI utilized by this group was always greater than 7 cm^3, which is larger than that used in this thesis (6.1 cm^3).

However, in most of their work a PRESS sequence was utilized similar to that used in this thesis. When they extended their localization sequence into a two-dimensional J-resolved spectroscopy sequence, a spectrum clearly showing homonuclear coupling was observed. Such splitting may be due to electron-mediated J-coupling, which is usually observed in isotropic liquids, but normally not varying in size or direct dipole-dipole interaction. It is more likely, however, that changes in chemical shift or J-coupling constants are due to chemical exchange between different molecular entities (possibly Cr/PCr and CK). But, artifacts such as anisotropic susceptibility differences between muscle, fat and air play a role in causing this (recall that such artifacts were avoided in this thesis by using atapulgite).

The initial interest of Kreis's group was with a peak at 3.9 ppm which they occasionally observed as a split peak (3.9 and 4.0 ppm). They found they could alter this splitting by moving the leg in relation to B_0. By expressing the frequency difference between two peaks as a function of knee bending within the magnet, (i.e.: the angle between the external magnetic field (B_0) and the leg) they were able to show an orientation dependence of this spectral splitting which strongly suggested a direct dipolar interaction (Kreis et al., 1994; Ntziachristos et al., 1996). This is very intriguing since the N-CH_2 protons of Cr have a resonance at 3.93 ppm, and one would predict that if coupling were to occur here, it would also be prevalent at the 3.02 ppm peak.

The 3.93 ppm peak was then tested in rat skeletal muscle both *in-vivo* and post mortem. As the legs of the rats were turned from 8º to 55º relative to B_0, the N-CH_2 peak of Cr went from a doublet to a singlet with an overall larger line width (Ntziachristos et al., 1996). This coupling appears only *in-vivo* , and it disappears after death with a time constant similar to the post-mortem decay rate of PCr in muscle, which suggests that PCr is the main contributor to these peaks at 3.93 ppm. This phenomenon was tested on the CH_3-Cr peak at 3.02 by analyzing a VOI of mixed human muscle. It was found that when the leg was held in an angle of 54.7º the peaks at 2.9, 3.02, and 3.1 ppm all convalesce into one, thus this orientation is referred to as the "magic angle" (Kreis et al., 1995). Therefore, if the peak observed at 3.02 ppm is part of a doublet or triplet, then once it is subtracted from the spectra there should be a peak left which is or partially represents Cr_{TOT}. Recent quantitative difference spectroscopy revealed that most of the resonances at 3.02 ppm affected by dipolar coupling can be assigned to Cr and/or PCr (Kreis et al., 1997i). In rat muscle at 4.7 T, two peaks are located directly upfield from Cr/PCr when the leg is held at 8º to B_0. The first of these is assigned to the trimethylamine (TMA) compounds while that further upfield is considered to represent carnitine and taurine (the combination of all 3 are referred to as CHO in this thesis). When TE is kept constant and the leg is moved to the rat's "magic angle" (or 55º) these peaks combine to one, suggesting dipolar couplings of these three peaks. These observed triplet-like peaks are in general agreement with a dipolar coupled system of three

chemically equivalent spins. The outer lines of the triplet overlap with the peak at 3.1 ppm and the unidentified peak at 2.9 ppm. Therefore it would appear that both aspects of the Cr_{TOT} spectrum (that at 3.93 and 3.02) are affected by dipolar coupling at REST. The main conclusion from this work is that in one and two-dimensional spectra of human muscle, the orientation of muscle fibers within the magnet will alter the spectra (split or combine peaks) and that this is caused by direct dipole-dipole interaction in a molecule (possibly Cr or PCr) within the oriented phase (Kreis et al., 1994). If this is correct, then the spectra observed in this thesis represent the triplet of Cr at REST which goes more to a singlet during CUFF. The most serious consequence of this finding is the effect on the T_2 data reported here. If correct, then T_2 can not be measured from the existing data since there are at least two components at 3.02 ppm. The first is the Cr singlet which changes with the T_2 and decays normally and the second peak is part of a triplet which is coupled in a ratio of 1:2:1 at 2.9:3.0:3.1 ppm and is expected to decay in relation to a coupling constant - different from the normal decay. The problem which arises is that components of triplets change with TE and therefore can only be found at a given TE. In other words, if the configuration of the peak at 3.02 has multiple T_2's and the amplitude of the peak is affected by the TE, then the T_2 was measured incorrectly. However, the T_2's reported here may be reasonable, since the standard errors for the values reported here are low and it is unlikely that all the legs from all the volunteers would have identical fibers.

With this in mind the REST and CUFF spectra taken at various TE's were reconsidered. It appears that at TE = 40 ms the full Cr signal is present (Fig. 12) during CUFF. At 3.02 ppm there is a single prominent positive peak (the coupled component). If there was coupling then the same peak would be expected at 80 ms, but in the opposite direction. This is confirmed when viewing the uncorrected fourier transformed data taken at TE = 80 ms. These spectra exhibit a prominent negative peak at 3.02 ppm. Therefore, the T_2 reported for the CUFF should be acceptable since it follows normal coupling behavior and its signal decreases with T_2 and as a function of TE.

The signal measured to calculate the T_2's reported decreased as the TE increased (Figure

12), a finding which is normal and expected. This decrease also depended on the T_2 relaxation rate. If this signal was coupled (doublet, triplet etc..), then it may oscillate in phase (+ or -, as observed in the CUFF signal at 3.02 ppm) as the TE is varied, with the maximum signal being recovered at TE = 1/(some coupling constant). Bearing this in mind, when viewing the REST spectra acquired at various TE's, there is a peak at TE = 40 ms with a mean separation from the main Cr peak of 13.8 Hz (Figure 12). If this results from a triplet (as suggested by Kreis et al. 1997), then a maximum recovery of the 2.9 ppm component should appear at TE ~ 76 ms. Therefore, at 40 ms no coupling should be visible. However, if the component is from a doublet (one component at 2.9 ppm and the second at 3.1 ppm), then the coupling is 2 x 13.8 Hz, and the maximum signal would be recovered at 38 ms (which is very close to the 40 ms used in the experimental design). This question needs to be addressed.

In the most recent work from Kreis et al. (1997ii), they observed ^{1}H-spectra from human tibialis anterior during 3 min. of isometric work and during recovery. During exercise, they observe the disappearance of the 2.9 ppm and 3.93 ppm peak, with a subsequent line broadening of the 3.02 ppm peak suggesting that part of the signal observed at this linewidth is a triplet. Yet when observing the changes between rest and exercise, they report no T_2 difference. However, upon personal communication, they do admit that there may be some T_2 decrease and possibly T_1 increase in the 3.02 ppm peak during exercise (R. Kries and R. Balaban, personal communication). Outside of this discussion, the author was unable to find any reported T_2's during fatigue or ischemic fatigue.

If dipolar coupling were the cause of the T_2 findings in this study, then what could be causing the coupling effect. Some possible explanations are that : 1) PCr/Cr may be hindered from isotropic tumbling by being constrained in the small spaces between actin/myosin chains, or 2) PCr/Cr might be temporarily bound to macromolecules which themselves are strictly ordered within the muscle cells, or 3) the dipolar coupled peaks might originate from large molecules which are permanently bound to ordered structures in muscle, but have enough side chain mobility to be

observable and partially average dipolar couplings. This last reason is certainly not the case for Cr or PCr (since they are relatively small molecules) and may therefore be discarded. Either of the first two reasons are certainly feasible when considering Cr_{TOT}. For example, it may be postulated that CK could be the macromolecule which could transfer it is ordering within the cell to PCr/Cr. The former reason is one of the most likely and would support the idea of the "shuttle" (model 2), but it will also be one of the more difficult to prove.

The Effects Of Loading Creatine Monohydrate On Performance And The Magnetic Resonance Spectra Gathered From Muscle

With the above discussion in mind it may be difficult to consider any argument regarding the benefits or concessions of Cr loading. This stems largely from the fact that if the T_2's determined in this study are incorrect, then the CUFF Cr_{TOT} intensities are overestimations. It is important to note however, that all the Cr_{TOT} intensities were corrected to separately assessed Cr standards and to the same T_2's. Therefore, all these values are relative and thus comparable. In addition, there are other effects which have been linked to Cr loading (performance enhancement during exercise and weight gain) which were monitored and can be used to elucidate the possibility that Cr was taken up by the muscle.

The Cr loading regime used in this study has proven effective in previous in-vitro analysis of increasing the total Cr concentration in muscle ~20%, of which approximately 30% is in the form of PCr (Harris et al., 1974). Recent in-vitro work has shown that a more rapid way to increase the muscle Cr store is to ingest a dose of 0.3 g/kg body mass for six days (Hultman et al., 1996). However, both PWR and END athletes were observed to take up Cr into the gastroc. after one week of loading. Upon closer observation some constraints appear to be placed on this loading. The END athletes began the loading regime with a larger Cr_{TOT}, and since they are not expected to have the metabolic machinery to load as effectively as PWR, they increased their pool size slightly. The power trained athletes are expected to have the metabolic machinery and they

started with a lower mean Cr_{TOT}. From numerous fiber-type analyses of human muscle, it is observed that the END athletes would be expected to have a greater concentration of Type I fibers, while the PWR trained a larger percentage of Type IIb's (see Saltin et al., 1988 for review). Thus, it is expected that the PWR trained would be able to take up more and maintain more of the Cr, as it is the Type IIb's which are recognized as the more anaerobically driven of the three fiber types. This hypothesis has found credence in the data from this study. The PWR trained athletes increased their Cr peak intensity by 44% during REST and 40% during the CUFF, while the END athletes were only able to increase Cr_{TOT} by 10% and 25% during REST and CUFF respectively. Both populations support the theory that orally consumed supplements of Cr-monohydrate will be taken up by the muscle and add to the Cr_{TOT} pool. Yet it appears that the fiber types constituting the muscle could have an effect on the amount an individual is able to load. This ability of the PWR athletes to load more may have its origins at the cellular level. It is possible that Cr loading promotes an increase in Cr transporter production to allow this extra exogenous Cr into the cell (Walker et al., 1979). This is inferred when the difference for Cr intensities are considered between END and PWR being 0.76 and 0.97 for REST and CUFF respectively.

No one was able to exhibit more than a 44% increase in Cr peak intensity. It is therefore suggested that there may be some set limit of Cr that the muscle cell (either IIa,IIb, or I) can hold. Harris et. al. (1992) has shown that increases in Cr_{TOT} were indeed related to the starting content. All but 2 of 18 subjects demonstrated an increase in Cr_{TOT}, and these "non-responders" already had Cr_{TOT}'s above 145 mmol/kg dm prior to supplementation. All subjects appeared reach an upper Cr_{TOT} of ~155 mmol/kg dm following supplementation which supports the belief that there is an upper limit for Cr in muscle (Harris et al., 1992). It has also been suggested that there must be an upper limit to the amount of Cr the muscle cell can take up and that Cr entry into the cell is initially dependent on the extracellular Cr concentration but is subsequently downregulated in the presence of elevated extracellular and intracellular Cr (Hultman et al., 1996, Loike et al., 1986)

The loading does not appear to be uniform between the subject groups, which indirectly

supports the theory of more than one pool of Cr_{TOT}. It is clear that Cr intensity goes up in all individuals during CUFF; whether PWR or END, Cr or sucrose loader. Both END and PWR were able to increase Cr_{TOT} during CUFF after one week of loading, yet only PWR increased the Cr_{TOT} during REST. END did not express any increase in Cr_{TOT} during REST after loading, which leads to the question: Where does this "extra" Cr suddenly come from during CUFF? It is the belief of the author that in an individual with a large percentage of Type I fibers (END) any supplemental Cr taken up by the muscle cell will be stored in the TB pool and only brought into use during increased metabolic rate. The END subjects train for more aerobic type activity and can be thought to use this extra Cr in it's energy transport capacity rather than as an energy buffer. PWR athletes on the other hand commonly train in an anaerobic capacity which requires the use of PCr more as an energy buffer and would therefore be predicted to load Cr into the UB pool.

Confirmation for the MRS findings that showed an increase in the Cr pool may be found in the fact that there was a significant body mass gain in the END Cr loaders, and while there was a trend for a weight gain in the PWR Cr loaders, it was not significant. A 1-2% weight gain is typical in Cr loading trials of seven days or more (Harris et al., 1992ii; Greenhaff, 1994; Kreis et al., 1996).

The performance data is a little harder to interpret since the data sets for the PWR athletes are incomplete. The main reason for this was a malfunction in the cable for measuring displacement. With that in mind, it would appear that Cr loading did enhance the performance of the PWR trained athletes. However, the MRS data would suggest that both groups were able to bolster their Cr_{TOT} pools. Why then would the performance of the END athletes decrease? With the present data it is difficult to draw any conclusions regarding performance benefits. Each subject was asked to rank the benefit they felt their supplement (sucrose or Cr) provided them, with 1 = none to 5 = most. As an anecdote it may then be added that out of 24 subjects only three found a benefit greater than "3" and of those one was a sucrose loader. At present there is a great deal of research being performed on Cr supplementation and its apparent benefit to exercise performance

(Harris et al., 1992ii; Greenhaff, 1994; Kreis et al., 1996; Moreno et al., 1996). It is the belief of this author that although oral Cr supplementation may bolster the PCr/Cr pool, such loading does not necessarily provide a benefit and if it did it is likely to only support ballistic type activity (30 s sprint) and not a prolonged exhaustive exercise such as the one in this study

Quantification of the Cr loading in this study may be questioned if the argument for dipolar coupling is accepted. If this were the case and there was a "magic angle", then any change which may be affected by oral Cr supplementation may be overshadowed by the peaks at 2.9 and 3.1 ppm which have been considered to hold resonance for Cr_{TOT} (Kreis et al., 1995). Indeed, in a recent MRS study involving 5 days of Cr loading, subjects whose spectra were observed at the "magic angle" appeared to show a modest increase in the 3.02 ppm peak at REST (Kreis et al., 1997i). Yet, when these researchers took the difference of the pre-load from the post load spectra, the line width appeared much narrower, suggesting that something as of yet unaccounted for is still underneath the 3.02 ppm peak which may need to be added to the Cr intensity already calculated. Along this vein of thought, they admit to having great difficulty in trying to quantify the increase in Cr_{TOT}, with interindividual differences causing most of the problem (i.e.: placebo loaders showed significant increases in Cr over 5 day period) (Kreis et al., 1997i).

Where do we go from here?

With the assumption that the anisotropic susceptibility differences are only relevant to spectra derived from muscle close to the outside of the leg, pilot trials have been performed on the human soleus (Chris Hanstock, personal communication). By using this muscle, atapulgite was not necessary. Lactate was observed in this preparation and exhibited predictable dipolar coupling. The 3.02 ppm Cr peak at TE = 40 ms was observed as one defined peak at REST. Various TE's were considered without any coupling evidence. This questions the coupling found in TA and m. gastrocnemius, since both of these must contend with susceptibility effects. This possible artifact must be considered. A narrower water suppression pulse was also applied in these pilot trials,

which allowed clear resolution of the 3.93 ppm Cr peak. This was observed as a doublet and exhibited coupling behavior, therefore suggesting that coupling may still be a factor in this muscle.

When the resting data from this thesis were analyzed, only the main Cr peak (3.02 ppm) was considered. Therefore if coupling is a factor, then it is likely that the values reported here for rest are underestimations. To correct for this problem it will be necessary to determine the T_2 of both the coupled and uncoupled Cr (if coupling exists). With this in hand it will be possible to extrapolate both peaks back to TE = 0 ms and attain the true value for Cr peak intensity at rest. In the original data analysis the narrow 3.02 ppm peak (REST) was compared with the broader 3.02 ppm peak (CUFF). If there is a dipolar coupled element present in the rest spectrum it was not fully taken into account in the peak estimation (only the central peak of the coupled triplet would have been included). On the other hand, if the CUFF Cr peak area contains contaminants (i.e.: the shoulders observed after resolution enhancement are from a metabolite other than Cr), then that Cr peak area is overestimated. The heart of the matter then is: Can Cr be assigned to these so-called "dipolar coupled" Cr peaks? Extract evidence does not indicate any other metabolite at 3.0 ppm other than Cr; therefore, it would seem logical that these extra Cr peaks need to be taken into account when making peak area estimates of the total Cr pool. If this is the case, then the rest data need to be modified to include the extra Cr peak area.

In order to estimate the effects of T_2 on peak area, it is necessary to have an understanding of: (a)the dipolar J-modulation effects of the coupling at different TE's; and (b)the effects of the local environment in which the coupled and non-coupled Cr are present (i.e.: do both forms of Cr have the same or different T_2 when contributing to the spectrum). If it is not possible to determine these T_2's (due to limitations of the cuffing procedure), then it will be necessary to infer the coupled T_2. During REST the 2.9 peak vanishes by TE = 80 ms and does not reappear; nor is it evident during CUFF at TE = 40 ms (Figure 12). This suggests that it either has a very short T_2 or that it does not oscillate as a normal coupled peak. In a recent animal study this peak did exhibit normal coupling behavior - but the T_2 was not stated (Ntziachristos et al., 1996). If this peak is

assumed to hold normal coupling behavior with Cr, then the CH_3-Cr peak at CUFF can be considered the coupled form of Cr and a T_2 of 40 ms measured in this study can be assumed for this peak since all the coupled components are expressed under one peak. A bolder assumption must then follow; namely, that the environment for coupled Cr at REST is the same as at CUFF. This is somewhat complicated by the observation that showed the dipolar coupling of the 3.02 and 2.9 peak during REST is 13-14 Hz whereas it is at 4-5 Hz during CUFF (Fig. 12). The peak is much broader during the latter state which could mean that the coupling changed or that the nuclei changed their orientation. In other words, the nuclei rotated 30-40º from B_0 in the CUFF state whereas at REST the nuclei were aligned along B_0. Considering the lengths taken to secure the volunteers legs, it is not likely that such a large rotation would have occurred during the exercise. Therefore the question which must be asked is: Why does the coupling decrease during CUFF?

A possible explanation could be in a microstructural change which is occurring as the muscle fibers shift from the REST to CUFF state. At rest the myosin heads of the muscle filaments are unattached to the actin group. If at fatigue more actin-myosin cross-links were present (due to the reduction in the availability of ATP), then the orientation of the myosin head would be altered with respect to B_0 and therefore change the local environment in which Cr moves. With recovery this peak at 2.9 ppm should re-appear, which does not appear to be the case in this study. Kreis et al. have shown that after 5s of recovery from exhaustive exercise the Cr peak at 3.02 ppm gets significantly broader and a shoulder appears on its right hand side. By 228 s into recovery the 2.9 peak has reappeared (Kreis et al., 1997ii). If this is coupled to the 3.1 ppm peak, as a doublet, then it too would need to be isolated. This is hard to assess from these data since at 1.5 T the dipolar coupled peak (3.1 ppm) is at a chemical shift very similar to the carnitine measured with a 3 T magnet (Chung et al., 1997, Briere et al., 1995).

It is suggested that the peak at 3.02 ppm changes shape with both changes in TE and due to T_2 decay (Styles et al., 1995). At REST this peak may be considered a triplet, with one component at 2.9 ppm, an equivalent component at 3.1 ppm, and a large component at 3.02 ppm. The 2.9

peak needs to be isolated. This area can then be doubled and subtracted from the Cr peak (3.02 ppm) to illustrate the "uncoupled" Cr component. In other words, it is still debated as to whether there are two components of Cr under the peak, one of which is coupled (viewed during REST) and the other uncoupled (viewed at CUFF). If it is possible to tease these apart and assuming that the uncoupled Cr peak is unaffected by the triplet peak, then a fascinating hypothesis comes to light. It is possible that the coupled 3.02 peak is PCr and the uncoupled is Cr, since their respective areas are approximately 1:4, which is very similar to the accepted proportion of PCr/Cr. If these peaks can be isolated at REST it may then be possible to observe changes in both PCr and Cr during changing metabolic states using only ^{1}-H-MRS (J. Bell and R. Kreis, personal communication).

There is no reason to believe that the T_2 of the coupled and non-coupled should be the same and in fact the observation of the 2.9 ppm component in the TE = 40 ms REST spectra suggest that its T_2 may be somewhat shorter than the 117 ms estimated for the main 3.02 ppm Cr peak. If it is assumed that the T_2 of the main Cr peak (3.02 ppm) is ~117 ms as calculated during REST, and the 2.9 component is 40 ms (as assessed during CUFF, which would also force the assumption that it is in a constrained environment), then the data acquired at 40 ms during rest (Figure 12) may be taken and used to individually adjust the peak areas for the 3.02 ppm peak (T_2 = 117 ms) and 2.9ppm peak (T_2 = 40 ms) to estimate their areas at TE = 0 ms. The combined Cr area at rest and at TE = 0 ms would then become 2.90 vs. 3.99 during the CUFF, a mere 27% increase as opposed to the 3 fold increase reported (see table below)!

If it is assumed that the T_2 of the coupled Cr is similar to that estimated during the fatigue state, then the following table indicates how this would affect our peak area estimates reported in Table 1. In the following table TE = 0 ms (same T_2) refers to the assumption that the T_2 of the main Cr and coupled Cr peaks at rest are the same (i.e.: 117 ms), whereas TE = 0ms (different T_2) refers to the assumption that the T_2 of the main Cr (117 ms) and the coupled Cr (40 ms) peaks at rest are different.

Peak Area

	Observed TE = 100 ms	Calculated TE = 0 ms	Calculated TE = 0 ms (same T_2)	Calculated TE = O ms (different T_2)
Rest	0.69	1.61	2.30	2.92
Fatigue	0.34	3.99	3.99	3.99

CONCLUSIONS

The field of phosphagen research has become polarized over the last 25 years or so into two views or models for PCr and Cr function *in-vivo*. In this study, an in vivo, noninvasive, ^{1}H-MRS approach was used to test predictions that discriminate between these two models of phosphagen function. In one set of experiments, the human m. gastrocnemius of volunteer subjects (power and endurance trained) was interrogated under two conditions: rest and ischemic fatigue. Both the 3.02 ppm ^{1}H resonance intensity and the apparent transverse relaxation time (T_2) for Cr_{TOT} were found to change. At fatigue, the 3.02 ppm intensity increased significantly, implying that the pool size of PCr + Cr increased; at the same time, the T_2 decreased, implying a decreased mobility. Since in fatigue, Cr makes up the bulk of Cr_{TOT}, these effects are most probably caused by changes in the concentration of MRS visible Cr and by changes in its freedom to tumble. In another set of experiments, Cr supplementation was found to increase the 3.02 ppm intensity more in the ischemic fatigue state than at rest, suggesting that exogenous Cr uptake is most clearly expressed in the former metabolic condition. None of these three major findings are compatible with the model 1 or traditional view of phosphagen function in muscle - but all of them can be accommodated by model 2.

In the process of this study, and in parallel studies by Kreis et al (1997i, ii), the problem of dipolar coupling became evident to us and has raised interpretative difficulties with these measurements. As these are resolved, they may lead to some tempering of the above conclusions, but the qualitative agreement of these data with model 2, rather than with model 1, concepts of phosphagen function will most assuredly remain.

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