ASPECTS OF CREATINE AND ARGININE SUPPLEMENTATION ON METABOLISM IN HUMANS

By

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SUMMARY

This thesis investigated the effects of dietary supplementation with the guanidino compounds creatine (Cr) and arginine on metabolism in humans. Experiments studied the influence of exercise and Cr + carbohydrate (CHO) ingestion upon muscle glycogen and Cr accumulation, the effects of arginine ingestion upon the fate of ingested CHO, the influence of Cr + CHO supplementation upon muscle glycogen accumulation and endurance exercise performance, and the effects of Cr supplementation upon various indices of health. Biochemical analysis was performed on venous blood samples and on muscle biopsy samples from *vastus lateralis* obtained from subjects during the experiments. Blood samples were analysed for glucose, insulin and lactate concentrations and muscle samples for adenosine triphosphate, Cr, phosphocreatine and glycogen concentrations. Exhaustive exercise, performed prior to Cr + CHO supplementation, augmented muscle Cr accumulation, but Cr accumulation was diminished in non-exercised muscle. This was possibly due to a blunted CHO-stimulated insulin response after exercise, which would diminish insulin-mediated Cr transport. Creatine + CHO supplementation augmented glycogen resynthesis in exercised muscle to a greater extent than CHO supplementation alone. A tendency for greater glycogen synthesis was observed following Cr + CHO supplementation without prior exercise, and was paralleled by a tendency for subsequent submaximal exercise to be prolonged. Ingestion of 10 g arginine with a CHO drink was found not to have any influence upon the fate of the ingested glucose. This was probably due to the resulting plasma arginine concentration being insufficient to influence CHO disposal. Finally, acute (5 day) and prolonged (8 week) Cr supplementation produced no adverse effect upon health, as indicated by markers of haematological, hepatological, muscle and renal function.

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DECLARATION

I hereby declare that the present thesis has been composed by myself and that the work, of which it is a record, has been performed by myself, except where assistance has been acknowledged. No part of this thesis has been submitted in any previous application for a higher degree. All sources of information have been specifically referenced.

Tristan Mark Robinson

Some of the results contained within this thesis have been presented in the following communications:

1. Robinson TM, Sewell DA, Hultman E, Greenhaff PL. (1997). The effect of creatine and carbohydrate ingestion on muscle creatine and glycogen accumulation following glycogen depleting exercise in man. *J Physiol* **499*P***: 74P

2. Sewell DA, Robinson TM, Casey A, Greenhaff PL. (1998). The effect of acute dietary creatine supplementation upon indices of renal, hepatic and haematological function in human subjects. *Proc Nutr Soc* **57**: 17A

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LIST OF ABBREVIATIONS

ADP	:	adenosine diphosphate
ALT	:	alanine aminotransferase
AMP	:	adenosine monophosphate
ANT	:	adenine nucleotide translocase
Arg	:	arginine
ATP	:	adenosine triphosphate
ATPase	:	adenosine triphosphatase
BF	:	blood flow
BMI	:	body mass index
BP	:	blood pressure
Ca^{2+}	:	calcium ion
cAMP	:	cyclic AMP
CHO	:	carbohydrate
CK	:	creatine kinase
CK_C	:	cytosolic creatine kinase
CK_M	:	mitochondrial creatine kinase
Cl^-	:	chloride ion
CPK	:	creatine phosphokinase
Cr	:	creatine
$Cr.H_2O$	:	creatine monohydrate
d.m.	:	dry matter
EDL	:	extensor digitorum longus
G-6-P	:	glucose-6-phosphate
G-6-PDH	:	glucose-6-phosphate dehydrogenase

H^+	:	hydrogen ion
HK	:	hexokinase
HR	:	heart rate
IMP	:	inosine monophosphate
ip	:	intraperitoneal
K^+	:	potassium ion
Km	:	Michaelis constant
LDH	:	lactate dehydrogenase
MAP	:	mean arterial pressure
Na^+	:	sodium ion
NAD^+	:	ß – nicotinamide adenine dinucleotide
NADH	:	ß – nicotinamide adenine dinucleotide, reduced
$NADP^+$	:	ß – nicotinamide adenine dinucleotide phosphate
NADPH	:	ß – nicotinamide adenine dinucleotide phosphate, reduced
NO	:	nitric oxide
PCr	:	phosphocreatine
P_i	:	inorganic phosphate
PK	:	pyruvate kinase
Pla	:	placebo
^{31}P-NMR	:	phosphorous-31 nuclear magnetic resonance
PPO	:	peak power output
RER	:	respiratory exchange ratio
STPD	:	standard temperature and pressure for dry gas
TCA	:	tricarboxylic acid
TCr	:	total creatine

Vmax	:	maximal reaction velocity when substrate concentration is not limiting
VCO_2	:	carbon dioxide production
VO_2	:	oxygen consumption
VO_2max	:	maximum oxygen uptake

Chapter 1

REVIEW OF LITERATURE

1.1 Introduction

In the last decade a substantial amount of research has been conducted upon the effects of the guanidino compound, creatine (Cr), on various aspects of cellular function. The majority of this research has addressed the various roles that Cr and its phosphorylated form, phosphocreatine (PCr) play in muscular function, either in conditions of muscle disease, or in healthy muscle of individuals performing exercise. Much of the recent work has addressed this latter role, and has demonstrated that supplementation of the diet with Cr can increase muscle Cr concentration and lead to subsequent improvements in exercise performance. This research has led to the practice of 'Cr supplementation' becoming very popular worldwide with professional and amateur sportspersons. This review aims to summarise the research that has contributed to our current understanding of muscle Cr metabolism and also to highlight areas for further investigation of the aspects of Cr supplementation and muscle function.

1.2 Historical perspective of creatine research

Creatine was first isolated and identified as a 'new organic constituent of meat' in 1835 by the French scientist, Chevreul (cited by Needham, 1971). In 1847, Liebig observed that Cr was a regular constituent of mammalian muscle, but only present in small amounts in other organs that he investigated (cited by Needham, 1971; Balsom *et al*, 1994). Liebig also noticed that this compound was present in much higher amounts in muscle of wild foxes than in that of captive specimens. From this he was the first to propose that Cr was associated with muscular work. An important discovery relating to the function of Cr in muscle was achieved in 1927 by Fiske and Subbarow (cited in Needham, 1971), who reported a labile

phosphorous in resting muscle of the cat, which they termed 'phosphoryl' Cr. They demonstrated that levels of muscle PCr diminished when the muscle was electrically stimulated, but returned to resting levels in the following recovery period. Eggleton and Eggleton (1928) supported the finding that the labile phosphorous was a compound of Cr. They also reported that the 'combined creatine' (i.e. PCr) was present in a much higher proportion in resting muscle than was present in fatigued or rigor muscle (i.e. myofibril cross bridge detachment is not possible due to a lack of ATP, found following death or poisoning of muscle by metabolic inhibitors). These studies supported earlier findings (Schlossmann, 1924; Tiegs, 1926; both cited in Needham, 1971), which stated that concentration of "diffusible creatine" (i.e. Cr not combined with phosphorous) increased during muscle contraction. These findings suggested that PCr was used in some way during muscular contraction, possibly as an energy source, and the resulting product, Cr was used to resynthesise PCr during recovery from contraction. Work by Lohmann (1928) and Fiske & Subbarow (1929) had identified another compound involved in muscular energy provision (cited in Needham, 1971). This compound, identified as adenosine triphosphate (ATP), was suggested to play a role, in combination with PCr, in muscle energy provision which did not involve production of lactic acid. Subsequent work by Lundsgaard (c. 1930, cited in Needham, 1971) confirmed this relationship, and helped fuel research leading to Cr and PCr being recognised as key intermediates of skeletal muscle metabolism (Bessman & Carpenter, 1985).

Around this time a substance which had been discovered in urine of man by Heintz and Pettenkofer (c.1860, cited in Needham, 1971), was identified by Liebig as creatinine. The excretion of this substance was found to be related to

the amount of active muscle tissue in the body, leading Chanutin (1926, cited in Needham, 1971) to conclude that creatinine was directly derived from the Cr stored in muscle.

These early findings led investigators to determine whether or not Cr could be taken into the body from the diet. The first Cr feeding experiments were conducted in the early part of the 20th century, using Cr extracted from either meat or urine. These experiments showed that levels of Cr and creatinine recovered from urine amounted to less than the amount of Cr given in the feeding. This led to the conclusion that some of the Cr fed to animals or man was retained in the body. This hypothesis was given further support by the findings of Folin and Denis in 1912 and 1914 (cited in Needham, 1971) that cats fed Cr showed increases in muscle Cr content of up to 70%.

In 1939 Ray *et al* (cited in Chaikelis, 1940) discovered that feeding subjects 60 g gelatin (containing 25% glycine) per day, resulted in a delay in fatigue development. At about the same time, Bloch & Schoenheimer (1939) demonstrated that Cr was synthesised from glycine and 2 other amino acids; arginine and methionine. It was subsequently demonstrated that ingestion of 6 g of glycine every day for 10 weeks produced improvements in some measurements of muscle performance (Chaikelis, 1940). It was postulated that glycine ingestion stimulated Cr biosynthesis, thereby increasing muscle Cr concentration sufficiently to improve exercise performance. This suggestion was supported by work of Borsook *et al* (1941), which demonstrated that consuming gelatin or arginine and glycine increased Cr biosynthesis. This and subsequent research led to the elucidation of the Cr biosynthetic pathway involving the 3 amino acids and 2 enzymes (Walker, 1979; Figure 1.1; section 1.3).

Little further research was published concerning Cr supplementation until Sipilä *et al* (1981) released the findings of a year-long Cr treatment of patients with gyrate atrophy of the choroid and retina. This disease is caused by a metabolic disorder resulting in an inability to synthesise Cr. Subjects ingested 1.5 g Cr per day for a year, which reversed the type II muscle fibre atrophy associated with the disease. Over the period of the treatment there was a subjective increase in strength, and anecdotal evidence led the investigators to hypothesise that Cr ingestion may influence exercise performance. Despite this finding, Cr supplementation did not attract further attention until 1992, when Harris *et al* published the results of a number of supplementation trials which had been conducted in their laboratory. The findings of this investigation and the subsequent research which it prompted are discussed in more detail in section 1.7.

1.3 Biosynthesis of Creatine

Creatine is synthesised by the body from three amino acids, glycine, arginine and methionine (Fig. 1.1). In the first reaction, an amidine group is transferred from arginine to glycine, producing ornithine and guanidinoacetate. This reaction is catalysed by L-arginine:glycine amidinotransferase (commonly called transamidinase) and is reversible (Walker, 1960, 1979). The second reaction, catalysed by S-adenosylmethionine:guanidinoacetate N-methyltransferase, involves the irreversible transfer of a methyl group from S-adenosylmethionine to guanidinoacetate, and results in the formation of Cr and S-adenosylhomocysteine (Walker, 1960, 1979). In man, this biosynthesis occurs mainly in the liver and, to a lesser extent, in the pancreas and the initial reaction can also occur in the kidneys (Walker, 1979). In addition to endogenous synthesis, Cr can also be

Figure 1.1 Biosynthesis of creatine. (Walker, 1979)

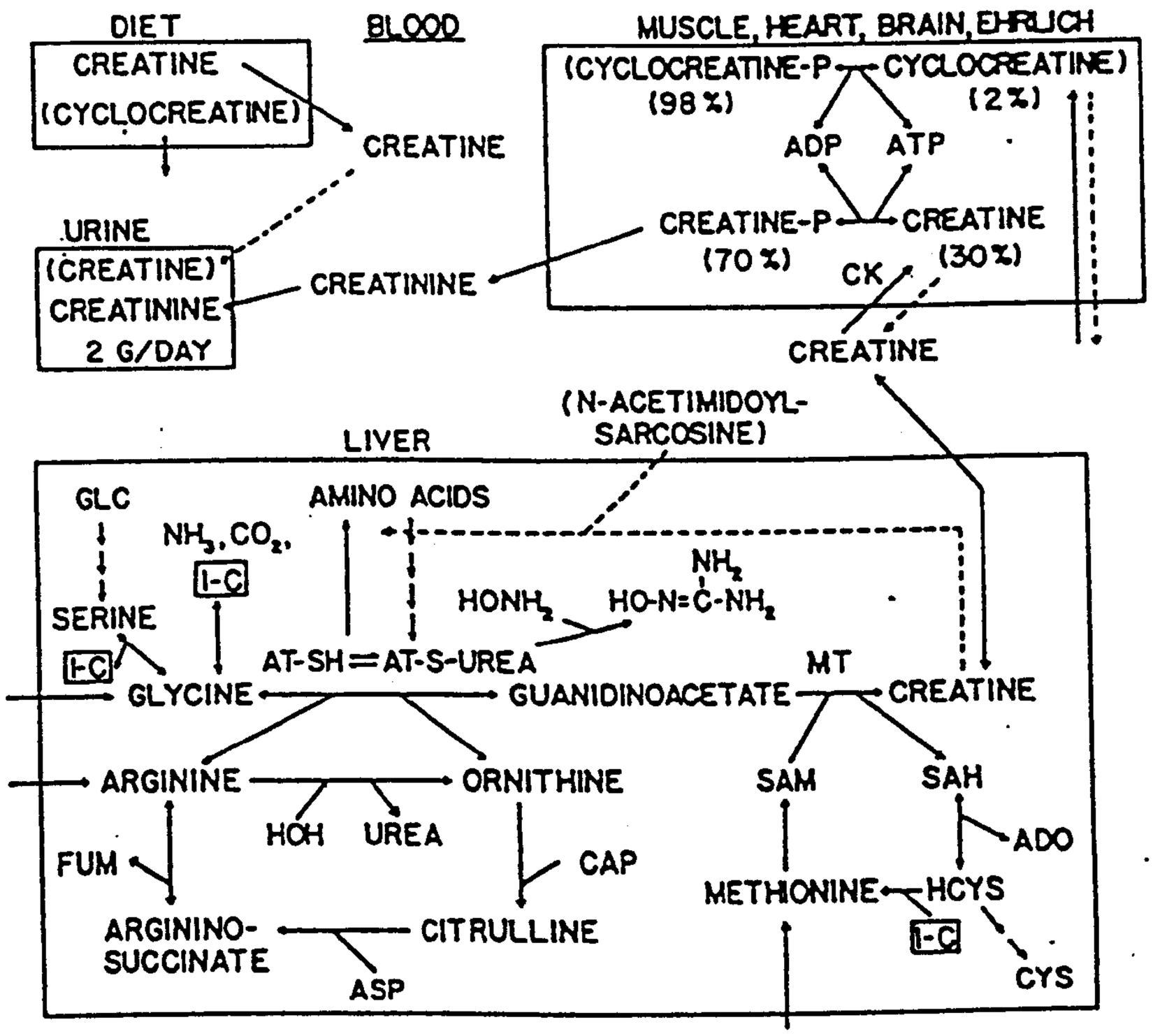


Fig. 1. Reactions involved in the biosynthesis and metabolism of creatine. *De novo* biosynthesis of creatine can occur in liver and pancreas of man and in liver of chicks. In contrast to human liver, human pancreas and chick liver have low arginase levels and cannot synthesize citrulline from ornithine. Controls modulate excessive biosynthesis of creatine during ingestion of creatine-containing diets and periods of inadequate food intake and thus spare amino acid precursors of creatine and the important one-carbon pools. Tissues containing creatine kinase take up and release creatine and its synthetic analog cyclocreatine by unknown mechanisms; percentages phosphorylated in resting muscle are given in parentheses. The creatine–creatine-P pool is continuously depleted throughout life by the formation and excretion of creatinine, about 2 g/day in man; this loss must be replaced from the diet or by biosynthesis. AT—SH, glycine amidinotransferase; MT, guanidinoacetate *N*-methyltransferase; CK, creatine kinase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; CAP, carbamoyl phosphate; 1-C, one-carbon derivative.

obtained in the diet from meat and fish, although biosynthesis alone supplies sufficient Cr to maintain normal muscle function in healthy individuals. This is demonstrated in the case of vegetarians, who receive no Cr from dietary sources, and have serum Cr levels within the normal range, albeit possibly at the lower end of the scale (Delanghe *et al*, 1989). The rate of Cr biosynthesis is controlled by the content of the transamidinase enzyme, which is regulated by feedback inhibition from Cr (Walker, 1979). Creatine feeding has been demonstrated to decrease transamidinase protein content and activity at a pre-translational level, as evidenced by a decrease in transamidinase mRNA (McGuire *et al*, 1984). The major sites of Cr synthesis, the liver, pancreas and kidneys, have relatively low concentrations of Cr, and these sites do not phosphorylate Cr to any significant extent (Walker, 1979), indicating that Cr is transported away from sites of synthesis to sites of storage. This separation of biosynthesis and utilisation provides the opportunity for regulation of uptake and retention of Cr by muscle, and excretion of excess Cr by the kidneys (Walker, 1979).

1.4 Creatine transport

Creatine is transported in blood, with normal resting plasma concentrations in man being 50-100 $\mu mol.l^{-1}$ (Harris *et al*, 1992). The total concentration of Cr and PCr (TCr) in muscle is far greater than in plasma, in the region of 110 - 130 $mmol.kg^{-1}$ (Harris *et al*, 1974), suggesting that Cr enters muscle against a strong concentration gradient. Two mechanisms have been proposed to explain the high Cr concentration in skeletal muscle, a specific transport process for Cr entry into the cell and the ability of the cell to 'trap' Cr within the cell, thereby preventing its loss.

A specific Cr transporter has been identified in brain, heart, kidney and skeletal muscle of rabbit (Guimbal & Kilimann, 1993) and rat (Schloss *et al*, 1994). Investigation of human tissues has shown that mRNA for this Cr transporter are expressed in brain, heart, intestine, kidney, ovaries, skeletal muscle and testes, and that the transporter belongs to a group of transporter proteins involved in neurotransmitter uptake (Nash *et al*, 1994).

The presence of a transport system that mediates Cr entry into cells had, however, been proposed many years prior to the discovery of the transport protein itself. An energy-dependent, saturable entry process was shown to exist for transport of Cr into muscle (Fitch & Shields, 1966; Fitch *et al*, 1968; Daly & Seifter, 1980). Creatine transport into rat extensor digitorum longus (EDL) muscle *in vitro* was inhibited under anaerobic conditions and by the presence of an oxidative phosphorylation uncoupling compound, 2,4-dinitrophenol (Fitch & Shields, 1966). These findings demonstrated that Cr transport was an energy-requiring process. Analysis of the kinetic properties of Cr transport into muscle revealed a saturable process with a Michaelis constant (K_m) for Cr entry of 500 $\mu mol.L^{-1}$ and a maximal reaction velocity (V_{max}) of 600 $\mu mol.L^{-1}.h^{-1}$. Relatively little Cr was lost from the muscle, suggesting that Cr entry and loss were dissociated from one another. It was later demonstrated that the entry process was specific for Cr, or compounds with very similar structure, separating the proposed Cr transporter from other transporters, such as those for transport of amino acids or other small molecules (Fitch *et al*, 1968). The compounds with similar structure to Cr that were transported into the cell were found to inhibit Cr uptake. It was proposed that these compounds might be of use to further investigate the effects of low

cellular Cr concentration upon muscle function, particularly in disease states such as Duchenne muscular dystrophy.

A study of Cr uptake by a variety of cultured cells known to accumulate Cr supported the previous findings that the process was saturable, and inhibited by structural analogues and metabolic inhibitors (Daly & Seifter, 1980). These investigators demonstrated that Cr uptake was a heterogeneous process, with at least two components. One component of Cr transport had a low K_m for Cr (0.02 – 0.04 mM) and contributed to less than 10% of the total Cr transport activity. The other component exhibited a higher K_m (3.1 – 6.4 mM), was dependent upon the presence of Na^+ and Cl^- in the incubation medium and could occur against a strong concentration gradient. It was also shown that Cr uptake by the cultured cells was inhibited in the presence of compounds that inhibited the activity of Na^+/K^+ATPase (Daly & Seifter, 1980). The observation of non-linear Lineweaver-Burke plots of Na^+-dependent Cr uptake prompted the authors to suggest that phosphorylation of Cr taken into the cell stimulated Cr transport to some extent. Subsequent work, however, demonstrated that intracellular phosphorylation of Cr was not necessary for net Cr accumulation by human monocytes and macrophages exhibiting similar uptake (Loike *et al*, 1986).

Cr transport by cultured rat and human skeletal muscle cells was examined by Loike and colleagues (1988). In a series of experiments the authors demonstrated that the concentration of Cr in the culture medium specifically affected the rate of Cr transport. At high extracellular Cr concentrations it appeared that the Na^+-dependent, but not the Na^+-independent, component of transport was down-regulated. This high extracellular Cr concentration did not alter Cr efflux from the cell, nor did it affect the affinity of the transport mechanism for Cr, which

suggested that either the amount of the transport component or its rate of turnover were under regulation. It was shown that Cr had to enter the cell to exert this regulatory effect, but that its phosphorylation was not required. Maximal downregulation of transport activity was between 70 – 80 % and was achieved after 24 h incubation in medium containing a high Cr concentration. The authors demonstrated that the turnover rate of transporter was relatively slow and that protein synthesis was involved in the down-regulation process (Loike *et al*, 1988). The recent identification and cloning of the Cr transporter and its mRNA has enabled investigation of the effects of *in vivo* Cr supplementation on its content in skeletal muscle (Guerro-Ontiveros & Wallimann, 1998). Prolonged (3 - 6 month) Cr feeding in rats was shown to significantly decrease the muscle Cr transporter content. However, the amount of Cr administered to rats in this study was exceptionally high (equivalent to humans ingesting 150 $g.d^{-1}$). The effect of Cr supplementation on Cr transporter content of human muscle is currently unknown. It has been proposed that the high Cr concentration of skeletal muscle is achieved by 'trapping' Cr once it has been transported inside muscle (Fitch & Shields, 1966; Fitch, 1977). Phosphocreatine is a polar molecule, and therefore cannot pass through membranes (Fitch, 1977). As 60% of muscle Cr is in the form of PCr it is thought that this may account for a certain amount of muscular Cr retention, but is not wholly responsible (Fitch, 1977). Other methods of muscle cell Cr entrapment proposed include binding to intracellular components and the existence of membranes which restrict passage of selected compounds (Fitch & Shields, 1966; Fitch, 1977). This latter theory would depend on muscle having a separate method of transporting Cr out of the muscle. This method would act with a lower affinity for Cr than the process allowing Cr to enter the muscle, and

would account for the difference between entry and loss of Cr demonstrated *in vitro* by rat skeletal muscle (Fitch & Shields, 1966). Recently it has been proposed that a fraction of Cr is compartmentalised within the muscle cell and does not contribute to the TCr pool involved in muscle energy metabolism (Hochachka & Mossey, 1998). This was suggested by the finding that, in fish fast-twitch muscle *in vivo*, the enzyme creatine kinase (CK) did not have access to, nor did it interact with, the total intracellular pool of Cr + PCr. The authors proposed that a significant amount of Cr was bound within the cell, making it unavailable for interaction with CK. Some Cr binding sites have been identified (Saks & Ventura-Clapier, 1994), although it has been suggested that further, as yet unidentified, Cr binding sites also exist within muscle (Hochachka & Mossey, 1998).

1.5 Total creatine pool

In a normal 70 kg male the total body Cr pool (TCr) is approximately 120 g (Walker, 1979), the majority of which (>95%) is located in skeletal muscle at a concentration of ~ 125 mmol.kg^{-1} d.m. , where it exists in either its free (40%) or phosphorylated (60%) form, PCr (Harris *et al*, 1974). The remaining body Cr (~5%) is found in small quantities in brain, liver, kidneys and testes (Walker, 1979).

Daily Cr turnover is about 2 g.d^{-1} for a 70 kg male, with Cr being replaced through endogenous synthesis, or dietary Cr intake, or both (Walker, 1979). Since its first discovery in the urine of man, it has been established that creatinine is the sole product of Cr degradation (Needham, 1971). This process is irreversible and occurs non-enzymatically with no further degradation of creatinine. Creatinine

excretion by the kidneys remains relatively constant in an individual, although it does vary between individuals (serum creatinine concentration, 60 - 130 $\mu mol.L^{-1}$; Fitch, 1977; Medicine Publishing Foundation, 1983), and is primarily dependent upon an individual's muscle mass, the major site of creatinine production (Heymsfield et al, 1983). For this reason serum creatinine concentration is commonly used as a marker of skeletal muscle health and kidney function. Excretion of creatinine in the urine increases following a period of Cr supplementation and, upon cessation of supplementation, progressively decreases as muscle Cr concentrations return to pre-supplemented levels (Hultman *et al*, 1996).

1.6 Role of creatine in muscle metabolism

Phosphocreatine is involved in energy transfer reactions for resynthesis of ATP. Catalysed by CK, PCr is broken down to Cr and a phosphoryl group, along with its bond energy, is transferred to adenosine diphosphate (ADP), thereby generating ATP.

$$PCr + ADP + H^+ \xrightleftharpoons{CK} Cr + ATP$$

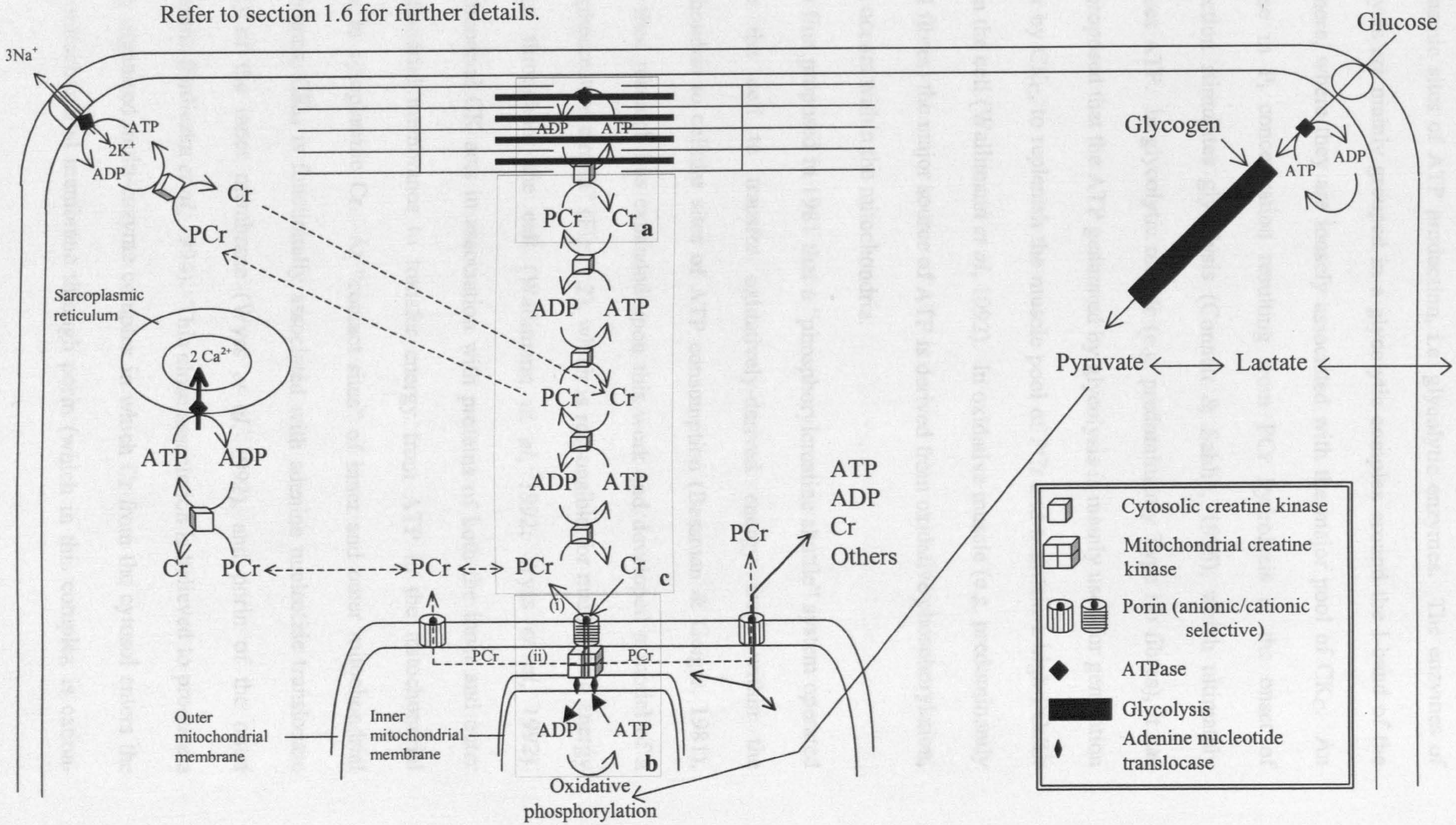
As indicated by the above expression this is an equilibrium reaction, however, as the free energy of PCr breakdown is greater than that of ATP hydrolysis, ATP resynthesis is the more energetically favourable outcome under normal conditions. Creatine kinase is present in the body as a number of different isoenzymes, dependent upon the tissue in which they are found (Burger *et al*, 1964). These isoenzymes have been found in brain, smooth, and striated muscle. Furthermore, it appears that slightly differing CK isoenzymes are compartmentalised within

cells, one type in the cytosol and another within mitochondria (Wallimann *et al*, 1992).

With regard to exercise, the most important CK isoenzymes are those present in skeletal muscle. One type, cytosolic creatine kinase (CK_C), is located in the cytosol mainly in subcellular "compartments", such as sites of energy consumption (e.g. near myosin ATPase in myofibrils, or near ATPases for ion transport), or energy production (e.g. near glycolytic enzymes). The other form of CK, mitochondrial creatine kinase (CK_M), is located between the inner and outer mitochondrial membrane. Much research has been conducted to determine the distribution and function of the CK isoenzymes within the cell, and have been summarised in a number of extensive reviews (Bessman & Carpenter, 1985; Wallimann *et al*, 1992; Wyss *et al*, 1992; Saks *et al*, 1994). These summaries have proposed a number of mechanisms by which CK and its associated substrates contribute to cellular energy homeostasis.

The first main role of PCr is as a 'temporal energy buffer' during the initial onset of muscular activity (Fig. 1.2a). A small proportion (5 – 10 %) of cellular CK_C exists bound to the central portion of myosin filaments (i.e. the 'H-zone' of a sarcomere), in close proximity to sites of ATPase activity (Turner *et al*, 1973; Wallimann *et al*, 1992). Upon contractile activity, the products of ATP hydrolysis are 'pushed' toward the H-zone from either end of the A-band. The close association between CK_C and myosin facilitates the rapid transfer of P_i from PCr to ADP for ATP resynthesis, maintaining a high ATP: ADP ratio, thus enabling contraction to continue. This role of PCr is considered to "buffer" the delay in the initiation of glycolysis that occurs at the onset of muscular contraction. Most of the remaining CK_C of the cell, termed soluble CK_C, is compartmentalised near

Figure 1.2 The "phosphocreatine circuit" theory of cellular energy metabolism.
(adapted from Wyss *et al*, 1992 and Saks *et al*, 1994)
Refer to section 1.6 for further details.



cytoplasmic sites of ATP production, i.e. glycolytic enzymes. The enzymes of glycolysis are mainly grouped in a glycolytic complex around the I-band of the sarcomere, where they are loosely associated with the major pool of CK_C. An increase in P_i concentration resulting from PCr hydrolysis at the onset of contraction stimulates glycolysis (Connett & Sahlin, 1996), which ultimately produces ATP. In glycolytic muscle (e.g. predominantly Type IIb fibres), it has been proposed that the ATP generated by glycolysis is mainly used for generation of PCr by CK_C, to replenish the muscle pool of PCr and maintain a high PCr/Cr ratio in the cell (Wallimann *et al*, 1992). In oxidative muscle (e.g. predominantly Type I fibres) the major source of ATP is derived from oxidative phosphorylation, which occurs within the mitochondria.

It was first proposed in 1981 that a "phosphorylcreatine shuttle" system operated within the cell to transfer oxidatively-derived energy from within the mitochondria to cellular sites of ATP consumption (Bessman & Geiger, 1981). Since then research has expanded upon this work and developed a model of a "phosphocreatine circuit" (Fig. 1.2), which is responsible for maintaining energy balance throughout the cell (Wallimann *et al*, 1992; Wyss *et al*, 1992). Mitochondrial CK acts in association with proteins of both the inner and outer mitochondrial membranes to transfer energy from ATP in the mitochondrial matrix to cytoplasmic Cr. At "contact sites" of inner and outer mitochondrial membranes, CK_M is functionally associated with adenine nucleotide translocase (ANT) of the inner membrane (Wyss *et al*, 1992), and porin of the outer membrane (Brdiczka *et al*, 1994). This close association is believed to produce a highly organised multi-enzyme complex in which Cr from the cytosol enters the outer mitochondrial membrane through porin (which in this complex is cation-

selective) and passes into a channel within CK_M. Adenine nucleotide translocase transfers ATP from the mitochondrial matrix to the CK_M channel, where it is hydrolysed in the presence of Cr, thereby producing ADP and PCr. These products then leave the CK_M channel, ADP returns to the mitochondrial matrix via ANT whilst PCr leaves the complex through porin by electrostatic repulsion due to its negative charge (Fig $1.2b_{(i)}$; Wyss *et al*, 1992). A slightly different model has also been proposed, in which the PCr formed within CK_M enters the intermembrane space of the mitochondrion and leaves through porin in its anion-selective state (Fig $1.2b_{(ii)}$; Wyss *et al*, 1992).

Addition of physiological concentrations (10 – 20 mM) of Cr to a medium containing cardiac muscle cells or fibres (Dzheia *et al*, 1983; Saks *et al*, 1991; Boehm *et al*, 1996) stimulates mitochondrial respiration. Furthermore, the presence of creatine, in a medium containing skinned skeletal muscle fibres, increases the sensitivity of mitochondria to small increases in ADP concentration, thereby promoting mitochondrial respiration (Tonkonogi *et al*, 1998). This Cr-stimulated respiration is achieved through displacement of the CK reaction occurring at the mitochondrial membrane in favour of PCr synthesis, thereby stimulating oxidative phosphorylation. Although it is not possible to demonstrate this effect in whole muscle, it is feasible that changes in muscle Cr concentration will result in a greater capacity of the muscle for mitochondrial respiration.

The term 'shuttle' may be misleading when referring to the transfer of energy from production sites to utilisation sites within the cytosol. It has been proposed that a series of 'microcompartments' exist within the cytoplasmic compartment, in which all components of the CK reaction exist in equilibrium (Saks *et al*, 1994). An increase in cytoplasmic ADP concentration (e.g. near a contracting myofibril)

acts as a 'signal' for ATP resynthesis by the closely associated CK_C, the resultant Cr is then rephosphorylated by CK_C situated slightly further away from the myofibril, producing ADP that, in turn, is itself rephosphorylated. This chain of dephosphorylation/phosphorylation reactions ultimately results in the propagation of a 'metabolic wave' that travels from the site of ATP utilisation, through a series of CK reactions in the cytosol, to the mitochondrial membrane (Fig 1.2c), where Cr is rephosphorylated by mitochondrial ATP, as described above. These 'signals' may arise from any ATP-utilising activity within the cell and if the 'frequency' of the signals arriving at the mitochondrial membrane exceeds a certain threshold, local ADP concentration will rise. Elevated concentrations of both ADP and Cr will stimulate an enhanced rate of oxidative ATP synthesis within the mitochondrion (Saks *et al*, 1994; Wyss *et al*, 1992), thereby increasing the rate of PCr resynthesis by CK_M (i.e. the response to the signal becomes 'amplified').

A high intracellular PCr concentration can also prevent ADP accumulation within the cell (Wallimann *et al*, 1992). Under normal resting conditions PCr, via the CK reaction, maintains ADP at a relatively low concentration at sites throughout the cell. During conditions of metabolic stress, however, such as during maximal exercise or at low glycogen concentration at the end of prolonged exercise, ATP resynthesis will be insufficient to maintain contraction and ADP will accumulate within the cell. Accumulation of ADP can inhibit the activity of ATPases within the cell and result in a net loss of adenine nucleotides from the cell. This latter effect occurs through the activation of adenylate kinase, which catalyses the transphosphorylation of a pair of ADP molecules to ATP, which is utilised by the cell to maintain contraction, and adenosine monophosphate (AMP), which is

converted to inosine monophosphate (IMP) and ammonia. Both IMP and AMP can undergo further degradation to products that, along with ammonia, pass freely through the sarcolemma and out of the muscle cell. It can be seen that accumulation of ADP would be disadvantageous to muscle as it would result in a net loss of adenine nucleotides, ultimately decreasing its ability to sustain contraction. Therefore a high intracellular PCr concentration acts to maintain a high ATP/ADP ratio, thus preventing adenine nucleotide loss.

Type I muscle fibre cells typically have relatively high levels of CK_M and low levels of CK_C (Wallimann *et al*, 1992), whereas in type II muscle fibre cells, the opposite is apparent (Yamashita & Yoshioka, 1991; Wallimann *et al*, 1992). There is also a differential distribution of PCr (and thus Cr) between muscle fibre types, with type II fibres containing more PCr than type I fibres (Söderlund & Hultman, 1991; Söderlund *et al*, 1992, Greenhaff *et al*, 1994; Sahlin *et al*, 1997). These differences reflect the functional characteristics of type I and type II fibres. Type I fibres are slow-contracting, have a low power output, favour aerobic metabolism for resynthesis of ATP during contraction and are relatively resistant to fatigue. These characteristics, coupled with the relatively lower levels of PCr in these cells, suggest that the 'phosphocreatine circuit' is favoured in these muscle types. Type II fibres, however, are fast-contracting, have a high power output, favour ATP resynthesis via anaerobic processes and fatigue rapidly during sustained contraction. Together with the greater proportion of PCr in these fibres, the temporal energy buffering role of PCr is most likely to occur in these muscle types.

1.7 Creatine supplementation in humans

Because of the equilibrium nature of the CK reaction, it has been hypothesised that increasing the concentration of Cr in muscle might increase the availability of PCr for rephosphorylation of ADP, thereby delaying muscle fatigue. Additionally, increasing the availability of Cr in the cytoplasm might have the potential to increase the rate of mitochondrial respiration, and thus oxidative PCr regeneration for supply to sites of energy consumption. As mentioned previously, early Cr feeding studies demonstrated that a fraction of ingested Cr is retained in the body of animals, increasing the body pool of Cr. However, it was not until 1992, when Harris and colleagues reported that high oral doses of Cr produced significant elevation of muscle Cr and PCr concentration in humans, that interest was generated concerning the effects of Cr supplementation upon exercise performance.

Creatine monohydrate ($Cr.H_2O$) is available as a white crystalline powder which is easily soluble in warm water. Orally ingested Cr is transported in the bloodstream following absorption and is thought to enter skeletal muscle against a concentration gradient in the same way as endogenously synthesised Cr (Harris *et al*, 1992). The $Cr.H_2O$ dosage administered by Harris *et al* (1992) was intended to raise plasma Cr to concentrations above the estimated K_m for Cr entry into muscle (500 μM; Fitch *et al*, 1968). Harris showed that following ingestion of a 5 g dose of $Cr.H_2O$, plasma Cr concentration rose from a baseline of 50-100 $\mu mol.L^{-1}$ to more than 700 $\mu mol.L^{-1}$ one h later. A smaller dose of 1 g was shown to produce only a modest rise (~100 $\mu mol.L^{-1}$) in plasma Cr concentration. Supplementation for a number of days (5 g Cr 4 times per day for 2 or more d) resulted in increases of 20-50% in TCr content of muscle, with concentrations

achieved after supplementation of between 140 - 160 mmol.kg^{-1} d.m. This rise resulted from increases in both PCr (8% increase) and Cr (36% increase) content. The increases in TCr concentration appeared to be less dependent upon the duration of the supplementation and dosage rate than upon the initial TCr content of the individual's skeletal muscle, with subjects with the lowest initial TCr content showing the greatest uptake following supplementation. Creatine supplementation was found to have little effect upon those individuals with initial muscle Cr content at the higher end of the normal range (~ 145 mmol.kg^{-1} d.m.), suggesting an upper limit to muscle Cr concentration.

The retention of Cr by the body was found to be greatest during the first days of supplementation, with a mean of 32% of the administered dose being retained. This was indicated by a comparison of muscle TCr concentration of samples taken on day 2 with those taken on day 4 of supplementation, and estimated by renal Cr excretion of 3 subjects being 40%, 61% and 68% of the ingested dose on days 1, 2 and 3 respectively.

The authors also demonstrated that muscle Cr uptake was augmented when submaximal exercise was conducted during the supplementation period. Five subjects performed 1 h of one-legged exercise on the morning of each day of Cr supplementation, at a moderate intensity that could be maintained for the whole hour. During the supplementation period, three of the five subjects ingested 30 g Cr each day for 4 d, another ingested the same amount for 7 d and a further subject ingested 20 g.d^{-1} for 3.5 d. At the end of the supplementation period the authors reported that muscle TCr concentration was significantly greater (~ 9%) in the exercised leg than in the non-exercised leg. Creatine accumulation by the non-exercised leg was similar to that observed in Cr supplemented subjects not

performing exercise. The authors proposed that the enhanced Cr accumulation was either as a result of increased total blood flow to the exercised muscle, or by a change in the transport kinetics of Cr across the muscle fibre membrane.

This report by Harris and colleagues (1992) suggested an important role of oral Cr supplementation for increasing muscle Cr concentration, and thereby for favourably altering muscle metabolism. The results reported were, however, a collection of preliminary studies using single subjects, or sample populations of relatively small size. Additionally, a wide variation between subjects was seen in muscle TCr accumulation and renal Cr excretion. Furthermore, a variety of supplementation regimens were used, making it uncertain whether muscle Cr accumulation was related to the dosage administered or duration of supplementation. A more recent report described the effects of ingesting different amounts of Cr over varying time periods on muscle Cr accumulation and subsequent degradation in humans (Hultman *et al*, 1996). Creatine supplementation (20 $g.d^{-1}$, 6 d) significantly increased muscle TCr concentration above pre-supplemented levels. Thereafter, muscle TCr concentration gradually decreased with time, and was matched by a parallel increase in urinary creatinine formation and excretion. This suggested to the authors that the rate of creatinine production was directly proportional to the muscle Cr concentration. It was also shown that maintenance of Cr ingestion following supplementation, at a level intended to match the rate of Cr degradation to creatinine (2 $g.d^{-1}$, 28 d; based on Walker, 1979), prevented the decrease in muscle TCr concentration with time (Hultman *et al*, 1996). Creatine supplementation with smaller amounts of Cr over a longer time period (3 $g.d^{-1}$, 28 d) resulted in a slower rate of muscle Cr accumulation compared with that following higher amounts, however, muscle

TCr concentrations were no different at the end of the respective supplementation periods. The authors concluded that muscle Cr concentration could be increased rapidly or at a slower rate by using differing supplementation regimens, and that elevated muscle Cr concentration could be maintained by daily ingestion of amounts of Cr equivalent to daily degradation to creatinine, thereafter.

1.7.1 Effects of creatine supplementation upon exercise performance

Using one supplementation protocol described by Harris *et al* (1992), Greenhaff and colleagues (1993) investigated the effects of Cr supplementation upon skeletal muscle torque production and fatigue. They found that subjects performing repeated bouts of maximal voluntary contractions of the quadriceps muscle group were able to sustain peak isokinetic torque production at a higher level following a regimen of oral Cr supplementation (5 g $Cr.H_2O$, 4 times per day for 5 d). There was also a decreased accumulation of plasma ammonia during exercise following Cr supplementation, compared with that following placebo-supplementation, which suggested that the rate of ADP rephosphorylation was greater during exercise following Cr supplementation.

A number of theories were proposed to explain the improvement in maintenance of force production through an increase in muscle TCr concentration. These included increased ATP availability, increased muscle metabolic buffering capacity, and an increase in the rate of PCr resynthesis from mitochondrial ATP during exercise and/or recovery (Greenhaff *et al*, 1993). The PCr/ADP energy transfer system catalysed by CK_C plays an important role in muscle contraction as it has the ability to maintain a high ratio of ATP to ADP in the cell (Kammermeier, 1987). It was proposed that by increasing muscle Cr

concentration there would be a consequent increase in muscle PCr (as demonstrated by Harris *et al*, 1992), and therefore an increase in anaerobic ATP production by the CK reaction during exercise (Greenhaff *et al*, 1993). The authors calculated that the increases in muscle PCr predicted from Harris' work, using the same supplementation regimen, would be sufficient to increase anaerobic ATP production by approximately 7%, and suggested that this contributed to the 5% increase in peak torque production seen following Cr supplementation in their study.

Another proposed mechanism by which Cr feeding improved exercise performance was that the increases in PCr concentration following Cr ingestion, if similar to those of Harris *et al* (1992), could increase the metabolic buffering capacity of muscle by about 7%. This was based on the predicted contribution of PCr to the acid-base balance of the muscle cell (Sahlin & Hultman, 1980). This effect would be mediated through the increased PCr concentration utilising more of the H^+, generated from glycolysis, in the production of ATP through the CK reaction.

A third proposal for the effect of Cr feeding upon performance of repeated bouts of maximal exercise was that the elevation in muscle TCr content increased the rate of PCr resynthesis from mitochondrial ATP during exercise and/or recovery. It had previously been proposed that mitochondrial ATP synthesis was linked to the phosphorylation of Cr at the mitochondrial membrane (Bessman and Geiger, 1981; Bessman & Carpenter, 1985; Wallimann *et al*, 1992; Wyss *et al*, 1992). The rate of PCr resynthesis from mitochondrial ATP during exercise and/or recovery was proposed to be greater following Cr supplementation (Greenhaff *et al*, 1993).

Similar results were observed by Balsom and colleagues (1993a), in a group of individuals performing repeated bouts of high intensity exercise on a cycle ergometer. Subjects cycled at 140 rpm during 10 six-s bouts of exercise, with each bout separated by 30 s of recovery. A high workload was used so that the subjects, in the control condition, were unable to maintain the required pedalling rate for the whole 6 s beyond bouts 4 to 6. Following Cr supplementation, subjects were able to sustain pedalling rate throughout each bout for a greater number of bouts. The authors proposed reasons similar to Greenhaff *et al* (1993) for these results, namely that there was a greater PCr availability for exercise as a result of elevated pre-exercise PCr and that there was a higher rate of resynthesis during recovery.

Much of the subsequent research into the effects of Cr supplementation upon exercise performance attempted to address one or more of these proposed mechanisms in their experimental design. Subjects supplementing their diet with Cr (30 $g.d^{-1}$, 6 d) showed a significant improvement in final run time over four repeated short (300 m) and longer (1000 m) distances (Harris *et al*, 1993). The total run time of the 1000 m distance was significantly improved following Cr supplementation. After supplementation, the best 300 m and 1000 m run times were also significantly reduced. These data supported the proposal that Cr supplementation improved performance, although could not offer any insight into the mechanisms by which it exerted its action.

Greenhaff *et al* (1994) addressed whether oral Cr ingestion did indeed have an effect on muscle PCr resynthesis after maximal, PCr-depleting exercise. Using human subjects in separate non- and Cr-supplemented conditions (20 $g.d^{-1}$, 5 d), isometric contractions of the vastus lateralis muscle were evoked by electrical

stimulation, causing a substantial depletion of muscle PCr. In the majority of the subjects there was a substantial (25%) increase in muscle TCr concentration following supplementation, however, in three of the subjects there was virtually no effect of Cr ingestion upon muscle TCr content (~ 5% increase). These subjects were termed 'non responders'. It appeared that the subjects who had muscle TCr concentrations close to or less than 120 $mmol.kg^{-1}$ d.m. before supplementation exhibited the greatest increases in muscle TCr following supplementation. This supported similar findings by Harris *et al* (1992). Those subjects with significant increases in muscle TCr concentration demonstrated an accelerated rate of PCr resynthesis after 1 min of recovery from contraction. This study confirmed the previously-raised hypothesis that an increased muscle Cr concentration would facilitate a greater rate of PCr resynthesis by displacing the equilibrium reaction catalysed by CK (Greenhaff *et al*, 1993). It had been estimated, *in vitro*, that the K_m of CK for Cr was 19 $mmol.L^{-1}$ muscle water, whilst for ATP it was 0.6 $mmol.L^{-1}$ (Bergmeyer, 1965). The concentration of Cr in skeletal muscle at rest and following maximal exercise is approximately 13 $mmol.L^{-1}$ muscle water (Harris *et al*, 1992) and 37 $mmol.L^{-1}$ muscle water (Spriet *et al*, 1987), respectively. During the initial stages of recovery from maximal contraction in both Cr- and non-supplemented states, the rate of ATP resynthesis from ADP and PCr would be at its highest, and Cr concentration would be above the K_m of CK_M for Cr. The availability of free Cr would therefore be unlikely to affect the rate of PCr formation by this process. As PCr resynthesis progressed during recovery, however, so would free Cr concentration decline. Once it had decreased to around 19 $mmol.L^{-1}$, free Cr concentration would become a determining factor of the rate of PCr resynthesis. From the study of Greenhaff *et*

al (1994) it would appear that free Cr concentration reached this level in the non-supplemented state after about 60 s of recovery from maximal contraction. From this point, differences in rate of PCr resynthesis were seen between Cr- and non-supplemented states, which produced a 30% higher PCr concentration at the end of recovery in the Cr-supplemented state. It was suggested that this effect was due to free Cr concentration being maintained during the whole recovery period at a level higher than that of the K_m of CK for Cr in the Cr-supplemented state. The data from this investigation provided evidence to suggest that Cr supplementation produced improvements in performance (of repeated bouts of maximal, PCr-depleting exercise) by enhancing the drive for PCr resynthesis by mitochondrially-derived ATP during recovery periods.

Most of the research up until 1994 had investigated the effects of Cr supplementation on maximal exercise of very short duration and short recovery time (Balsom *et al*, 1993a; Greenhaff *et al*, 1993, 1994). Birch and colleagues (1994) examined the effect of Cr supplementation (20 $g.d^{-1}$, 5 d) on performance during three 30 s bouts of maximal isokinetic cycling interspersed with 4 min of recovery. Following supplementation, peak power output (PPO) was increased in bout 1, and mean power output and total work done was increased during bouts 1 and 2. No difference with Cr feeding was observed during bout 3. Peak plasma ammonia 2 min after exercise was lower following Cr supplementation, despite more work being performed during the first 2 exercise bouts. This supported earlier suggestions that Cr supplementation enhanced ATP turnover, as indicated by a decrease in markers of adenine nucleotide degradation (Balsom *et al*, 1993a; Greenhaff *et al*, 1993). The increase in PPO during bout 1 after Cr supplementation was not expected, and could not be explained by the authors.

Reference was made to the finding that chronic 1-year Cr supplementation significantly increased type II muscle fibre cross-sectional area of patients with muscle fibre area (Sipilä *et al*, 1981), and the potential role of Cr in protein synthesis (Ingwall, 1976; Bessman & Savabi, 1988). The authors suggested, however, that the short duration of Cr supplementation employed in their study (5 d) would be unlikely to influence muscle protein synthesis.

Another investigation proposed that if PCr availability and utilisation during exercise was related to force maintenance during maximal exercise (Hultman *et al*, 1991), then elevated PCr concentration following Cr supplementation would attenuate fatigue and increase total work production (Cooke *et al*, 1995). However, peak power, time to peak power, total work and time to fatigue during 15 s of maximal cycling exercise were all unaffected by Cr supplementation (20 $g.d^{-1}$, 5 d). The lack of any improvement of a single bout of exercise complemented previous reports that initial bouts of exercise were unaffected by Cr supplementation (Balsom *et al*, 1993a; Greenhaff *et al*, 1993), but did not concur with improvements in PPO observed by Birch *et al* (1994). The authors suggested that PCr concentration had not altered sufficiently for improvements in exercise performance to be observed (Cooke *et al*, 1995), however, no data was provided to support this suggestion. It is known that Cr accumulation varies between individuals in relation to their muscle Cr concentration before supplementation (Harris *et al*, 1992; Greenhaff *et al*, 1994). The lack of data relating to the success of the Cr supplementation regimen in Cooke and colleagues' study (1995) therefore makes the interpretation of the performance results difficult. Based upon their data, the authors suggested that the proposed ergogenic effect of Cr supplementation was not related to a pre-exercise elevation

of muscle PCr stores, but was due to an increased potential for PCr resynthesis during recovery.

The ability to maintain high power output during maximal exercise following Cr supplementation was again demonstrated by Balsom and colleagues in 1995. Subjects cycled for 5 six-second bouts against a high workload, interspersed with 30 s recovery periods, followed 40 s after the last bout by 10 s cycling against a 5% greater workload. Creatine supplementation (20 $g.d^{-1}$, 6 d) significantly increased muscle TCr concentration, and power output during 10 s of high intensity cycling was better maintained, compared with pre-supplementation results. This improvement was attributed to an increased PCr concentration observed following the preceding exercise bouts, despite identical work to the pre-supplementation trial being performed. Muscle lactate concentration measured 3 min after the exercise was significantly lower in the Cr supplemented condition. Although not observed in this study, blood lactate concentration measured in a previous investigation with a similar exercise protocol (Balsom *et al*, 1993a), reflected this result and suggested lower lactate production following Cr supplementation. The authors proposed that, as the same amount of work was performed before and after supplementation, the contribution to total energy production from PCr hydrolysis was greater as a result of Cr supplementation. In other experiments, however, no effect of Cr supplementation on muscle lactate (Greenhaff *et al*, 1994) or blood lactate (Birch *et al*, 1994; Greenhaff *et al*, 1994) concentration had been observed. It is likely that these contrasting findings were related to the exercise protocols employed in the studies - PCr would be expected to contribute to total ATP resynthesis to a greater extent during 6-s bouts of exercise interspersed with 30-s intervals than it would during 30-s of maximal

exercise. An additional aim of the study (Balsom *et al*, 1995) was to investigate the effect of Cr supplementation on PPO, measured by specific jump tests (Bosco *et al*, 1983). It was proposed that improved PPO might have been achieved through a change in muscle morphology, such as increased type II fibre hypertrophy observed with 1 year Cr supplementation (Sipilä *et al*, 1981), resulting from Cr-stimulated protein synthesis (Bessman & Savabi, 1988). Jump performance did not change following Cr supplementation, suggesting to the authors that no major morphological change had occurred. However, this result might also have been due to a lack of specificity of the test used (Young *et al*, 1997). The authors concluded that exercise performance was improved following Cr supplementation due to a greater utilisation of PCr. This was achieved by a greater rate of PCr resynthesis during recovery after Cr supplementation.

The proposal by Greenhaff *et al* (1994), that Cr supplementation produced improvements in ADP rephosphorylation during exercise, mediated by an increased PCr concentration, was given support by a subsequent report (Casey *et al*, 1996). The authors found that there was a 30% reduction in ATP loss from mixed muscle during maximal isokinetic cycling exercise. Supplementation with Cr also produced improvements in maximal exercise performance, as measured by total amount of work done during two 30 s bouts of cycling at maximal intensity, each separated by 4 min of recovery. A positive correlation was observed between the increase in muscle TCr concentration and the improvement in exercise performance ($r = 0.71$, $p < 0.05$, $n = 8$). This supported the findings of Greenhaff *et al* (1994) that PCr resynthesis during exercise recovery was only improved in subjects that exhibited a marked increase in muscle TCr concentration. In the above study, however, no significant correlation was found

between the change in mixed muscle PCr concentration and the changes in ATP loss or exercise performance (Casey *et al*, 1996). Investigation of different muscle fibre types revealed that a positive correlation existed between the change in PCr concentration following supplementation in type II fibres and the improvement in exercise performance ($r = 0.66$, $p < 0.05$). Additionally, a correlation existed between the change in PCr concentration and the change in PCr degradation (and thus ADP rephosphorylation) in type II fibres during exercise ($r = 0.78$, $p < 0.01$). The finding that no such relationship existed in type I fibres led the authors to suggest that Cr supplementation exerted a greater effect upon type II than type I muscle fibres.

A small number of studies have addressed the question of whether Cr supplementation could influence the performance of prolonged, submaximal exercise (Balsom *et al*, 1993b; Stroud *et al*, 1994; Myburgh *et al*, 1996; Godly & Yates, 1997; Engelhardt *et al*, 1998). Such activity, often termed endurance exercise, predominantly generates energy via oxidative means. At certain stages of endurance exercise, however, anaerobic energy production is also required, such as at the onset of exercise, during short periods when exercise intensity exceeds maximal aerobic capacity (VO_{2max}; e.g. sprint finishes in long distance running or cycling; Godly & Yates, 1997; Engelhardt *et al*, 1998), or when the exercise intensity fluctuates continuously (e.g. terrain running; Balsom *et al*, 1993b). It was proposed by some of these studies that Cr supplementation might improve performance of prolonged exercise that included such energy requirements. The results of these investigations revealed, however, that Cr supplementation did not improve endurance exercise performance (Balsom *et al*, 1993b; Myburgh *et al*, 1996; Godly & Yates, 1997). Creatine supplementation

resulted in a decrease in performance of terrain runners (Balsom *et al*, 1993b), possibly due to an elevation of body mass. Cr supplementation (6 $g.d^{-1}$, 5 d) did improve the performance of short intervals of high intensity cycling during a period of endurance exercise (Engelhardt *et al*, 1998). There was also a tendency for improved endurance performance in some subjects, although no significant improvement was observed. The authors suggested that Cr supplementation might be beneficial to endurance athletes if their exercise activities included short periods of sprinting.

Another mechanism by which Cr supplementation had been proposed to influence prolonged submaximal exercise was by increasing the availability of Cr for transfer of oxidatively-derived energy from mitochondria to sites of utilisation (Stroud *et al*, 1994). It was hypothesised that intracellular Cr availability could influence mitochondrial ATP production to the extent that an increase in Cr concentration might increase oxygen consumption during exercise and recovery. However, whole body respiratory gas exchange (VO_2 and respiratory exchange ratio, RER) and blood lactate concentration of male subjects performing incremental running on a treadmill did not change following Cr supplementation (20 $g.d^{-1}$, 5d). The authors concluded that Cr supplementation did not improve performance of submaximal incremental exercise.

Recent evidence suggests that long-term Cr supplementation may have an anabolic effect on muscle (Vandenberghe *et al*, 1997). If so, this would have implications for treatment of muscle disease and exercise performance. Potential anabolic effects of Cr have previously been proposed, based on *in vitro* evidence that Cr stimulated muscle myofibril protein synthesis in cardiac and skeletal muscle (Ingwall, 1976), however, these results were not supported by a

subsequent investigation (Fry & Morales, 1980). In human patients with gyrate atrophy of the choroid and retina, prolonged supplementation with Cr (1 – 5 years, 1.5 $g.d^{-1}$) was associated with an increase in type II muscle fibre diameter of vastus lateralis muscle (Sipilä *et al*, 1981; Vannas-Sulonen *et al*, 1985). In a recent investigation, Cr supplementation during a 10-week resistance training intervention caused significantly greater improvements in maximal muscle strength and power output during intermittent arm exercise of sedentary female subjects (Vandenberghe *et al*, 1997). Significant increases in muscle PCr concentration (measured by ^{31}P-nuclear magnetic resonance; ^{31}P-NMR) and fat-free mass, above those of subjects receiving placebo supplements, were also observed in Cr supplemented subjects. The authors discussed that this increase might have arisen due to a number of factors, such as increased training intensity in subjects receiving Cr or a delay in the onset of overtraining. However, they also suggested that it was feasible that Cr supplementation facilitated the development of muscle hypertrophy during resistance training. Continuation of Cr supplementation in a subgroup of subjects after the resistance training had finished did not prevent arm power output from diminishing to pre-trained levels. Although no measurements of muscle protein concentration were made in this investigation, the results suggest that prolonged Cr supplementation may augment muscle protein synthesis and have identified a future area for research.

Early studies investigating the influence of Cr supplementation on exercise performance were mainly concerned with the elucidation of the mechanisms by which Cr supplementation exerted its ergogenic effect. A significant proportion of subsequent investigations have applied the use of Cr supplementation to 'field studies' of exercise performance of normal subjects or trained individuals. The

19.9 mmol.kg^{-1} d.m.). This increase is below the suggested 'threshold level' above which significant increases in PCr concentration and PCr resynthesis during recovery are observed (20 mmol.kg^{-1} d.m.; Greenhaff *et al*, 1994; Casey *et al*, 1996). The authors suggested that the relatively small increase in muscle TCr concentration seen in their subjects contributed to the lack of any observed improvement in exercise performance (Snow *et al*, 1998). As had been previously suggested (Casey *et al*, 1996), investigation was required of the mechanisms governing muscle Cr accumulation, with the aim that a method of maximising the response to Cr supplementation could be achieved. If a method could be found to maximise muscle Cr accumulation, it would follow that significant improvements in muscle function and performance, and in recovery from maximal exercise, could be achieved by many individuals, and may reduce the phenomenon of the 'non-responder'.

1.8 Factors affecting muscle creatine accumulation

Before the Cr transporter had been identified, or its transport properties fully described, studies of the effect of various treatments upon muscle Cr uptake had been conducted. Creatinurea, the presence of greater than normal amounts of Cr in urine, induced in rats by exposure to X-rays or by intraperitoneal (ip) injection of Cr could be prevented by administration of insulin to the animals (Koszalka & Andrew, 1968, 1970). Additionally, the elevated blood Cr concentrations observed following ip Cr loading were suppressed when insulin was administered (Koszalka & Andrew, 1970). Subsequent investigation of the effects of insulin on uptake of Cr by muscle of normal and X-irradiated rats demonstrated that insulin enhanced the transfer of Cr from the blood into skeletal muscle (Koszalka &

findings of these investigations have not been conclusive, however, with Cr supplementation producing improvements in exercise performance (Vandenberghe *et al*, 1996, 1997; Volek *et al*, 1997; Aaserud *et al,* 1998; Kelly & Jenkins, 1998; Magnaris & Maughan, 1998; McNaughton *et al*, 1998; Smith *et al*, 1998a; Smith *et al*, 1998b), preventing previously-observed decrement of exercise performance (Ööpik *et al*, 1998), or not producing any measurable improvement in exercise performance (Mujika *et al*, 1996; Redondo *et al*, 1996; Cooke & Barnes, 1997; Javierre *et al*, 1997; Odland *et al*, 1997; Bermon *et al*, 1998; Snow *et al*, 1998). Two recent reviews have also summarised a number of preliminary reports presented as meeting abstracts (Kreider, 1998a,b), demonstrating differing effects of Cr supplementation on exercise performance. The reasons for these differences are most likely multi-factorial, and may depend upon the duration of supplementation (range 3 d – 75 d), the amount of Cr administered (range 10 $g.d^{-1}$ – 30 $g.d^{-1}$), the training status of the subject group (many levels; sedentary, untrained individuals up to elite-trained athletes), the age and gender of the subject group, and the interaction of any other 'treatment' (e.g. strength training). The validity of some of the indicators of performance may also be questioned, as some may not necessarily be measuring factors proposed to be influenced by Cr supplementation.

A key point mentioned in discussion by a number of investigators who examined muscle Cr content following Cr supplementation was the variation in muscle Cr accumulation between subjects (Harris *et al*, 1992; Greenhaff *et al*, 1994, Casey *et al*, 1996). It was noted in one investigation (Snow *et al*, 1998) that Cr supplementation (30 $g.d^{-1}$, 5 d) resulted in a significant, yet small, increase in muscle TCr concentration (mean increase 11.7 ± 2.4 $mmol.kg^{-1}$ d.m., range 2.9 –

Andrew, 1972). It was unknown, however, whether insulin acted directly upon Cr transport, or whether its action was through other effects of the hormone *in vivo*, such as on growth hormone or testosterone. Removing the possibility of these factors by *in vitro* investigation of rat EDL muscle, Haughland and Chang (1975) demonstrated that insulin directly enhanced both the rate of transport and the extent of Cr uptake by rat skeletal muscle. More recent work has shown that net Cr uptake by cultured mouse skeletal myoblast cells is influenced by a number of hormones (Odoom *et al*, 1996). In a number of experiments the authors addressed factors associated with alterations in the rate of Cr transport. The dependence of Cr transport upon the presence of Na^+ was confirmed, with >90 % of uptake occurring via this process. Variation in extracellular Cr concentration upon Cr uptake, such as would occur during Cr supplementation, was studied. An approximately hyperbolic relationship was observed between extracellular and intracellular Cr concentration, with no discernible increase in uptake at extracellular concentrations greater than 500 μM. Of particular interest in this investigation was the K_m of 110 ± 25 μM observed for Cr transport by these skeletal muscle cells. This was substantially lower than that observed by Fitch and colleagues (500μM; Fitch & Shields, 1966; Fitch *et al*, 1968). If a similar K_m exists for human muscle, it is unlikely that plasma availability of Cr will be a limiting factor of transport into muscle, as the majority of Cr supplementation studies use doses of Cr that produce plasma Cr concentrations of approximately 800 $\mu mol.L^{-1}$ (Harris *et al*, 1992).

Inhibition of Na^+/K^+ATPase activity of the cells with ouabain prior to incubation with Cr significantly decreased final Cr content to 72% of control concentration. This supported earlier suggestions that Na^+/K^+ATPase activity was involved in

generating a Na^+ gradient for Cr transport (Loike *et al*, 1986). Selected hormones chosen for their important physiological role or their effects upon Na^+/K^+ATPase were used to investigate their role in net Cr accumulation. Insulin was demonstrated to stimulate Cr accumulation at a supraphysiological concentration (3 nM), but not at physiological concentrations (0.2 - 0.8 nM). Amylin, which is structurally similar to a neuropeptide released to muscle following nerve excitation (calcitonin gene-related peptide, CGRP), more than doubled cellular Cr content at moderate physiological concentrations (60 – 90 nM). At a supraphysiological concentration (40 μM) the thyroid hormone 3,3',5-triiodothyronine (T_3) stimulated Cr uptake from medium containing a high Cr concentration, with a diminishing effect at higher T_3 concentrations (70 – 110 μM). Insulin, amylin (working via the CGRP receptor) and T_3 are all known to stimulate Na^+/K^+ATPase activity of skeletal muscle, which the authors proposed would increase the driving force for Cr accumulation. The authors also demonstrated that the stimulatory effects of insulin and insulin-like growth factor (IGF-1) upon Cr accumulation might involve intracellular signals via phosphorylation of tyrosine residues within the cell.

The potential mechanisms by which exercise might influence muscle Cr transport *in vivo* (Harris *et al*, 1992) were also addressed by *in vitro* investigation (Odoom *et al*, 1996). IGF-1, released *in vivo* in response to elevated circulating concentrations of growth hormone following exercise, was found to stimulate Cr accumulation at physiological concentrations. Concentrations of the catecholamines, noradrenaline and adrenaline also increase during exercise. The authors demonstrated that noradrenaline stimulated Cr uptake in cultured muscle cells. Further investigation using adrenergic agonists and antagonists led the

authors to propose that noradrenaline achieved its effect by activation of β_2 adrenergic receptors. This mechanism was probably mediated through a secondary messenger such as cyclic AMP, and acted through stimulation of the Na^+/K^+ATPase of the muscle cells. Similarly, CGRP released at neuromuscular junctions in response to motoneuron stimulation, might also mediate an exercise-related influence upon muscle Cr transport, as suggested by the effect of amylin. Until recently, the majority of the investigations of factors that influenced muscle Cr uptake had been conducted *in vitro* or *ex vivo*, and *in vivo* investigations had been restricted to studies involving rats. The question of whether any of these proposed mechanisms could improve Cr accumulation during a period of Cr supplementation in humans has recently been addressed (section 1.8.1).

1.8.1 Factors affecting muscle creatine accumulation and its ergogenic effects in humans

The potential role of insulin in stimulating muscle Cr retention in humans was investigated, by stimulation of endogenous insulin release by ingesting a large amount of CHO during a period of Cr supplementation (Green *et al*, 1996c). Subjects consumed a drink containing 93 g CHO when Cr was ingested (4 x 5 $g.d^{-1}$, 5 d), and a high CHO diet was maintained during the supplementation period. Creatine excretion was markedly reduced in subjects receiving Cr + CHO than in subjects supplemented with Cr only and consuming a normal diet. The pattern of plasma Cr and serum insulin appearance and disappearance following oral administration of Cr + CHO were similar, and peak plasma Cr concentration and the area under plasma Cr/time curve were markedly reduced in the Cr + CHO group than in the Cr only group (i.e. Cr disappearance was enhanced). This

pattern of response supported the hypothesis that the insulin response to CHO ingestion could increase skeletal muscle Cr uptake. However, it was possible that a similar series of responses might have been observed if gut absorption of Cr was diminished somehow. This investigation estimated the retention of orally administered Cr by the body, as estimated by urinary Cr content, however, so did not directly measure muscle Cr accumulation. A subsequent investigation provided evidence to support this proposal and showed excellent agreement between whole body Cr retention and muscle Cr accumulation (Green *et al*, 1996a). In this investigation subjects ingested Cr and, in addition to this, one group of subjects consumed a drink containing 93 g CHO 30 min after each Cr dose. At the end of the supplementation period, subjects ingesting Cr + CHO had 60% greater muscle Cr accumulation than subjects ingesting Cr only (33.0 ± 3.4 $mmol.kg^{-1}$ d.m. vs. 20.7 ± 2.4 $mmol.kg^{-1}$ d.m., respectively). Furthermore, it appeared that pre-supplementation TCr concentration did not necessarily influence the extent of Cr accumulation when CHO was ingested during supplementation. Indeed, one individual receiving Cr + CHO with a high initial TCr concentration (152.1 $mmol.kg^{-1}$ d.m.) increased his muscle TCr concentration by more than 40 $mmol.kg^{-1}$ d.m. This would obviously be of benefit to individuals wishing to improve their performance of repetitive, high-intensity exercise, as TCr increases in excess of 20 $mmol.kg^{-1}$ d.m. are required for improvements in postexercise PCr resynthesis (Greenhaff *et al*, 1994) and exercise performance (Casey *et al*, 1996). The authors observed that serum insulin concentration increased 17-fold 20 min after Cr + CHO ingestion and was still significantly greater than basal concentration 60 min after ingestion. This response was similar to that previously observed, where plasma appearance and

disappearance of insulin and Cr were similar (Green *et al*, 1996c). The authors proposed that the combination of CHO ingestion with Cr supplementation augmented muscle Cr accumulation by an insulin-mediated effect.

The magnitude of Cr retention by human muscle in these investigations was greater than previously observed in rat skeletal muscle *in vivo*, when muscle Cr uptake was only slightly increased in the presence of insulin (Koszalka & Andrew, 1972), or not affected at all (Greenhaff *et al*, unpublished data; cited in Green *et al*, 1996a). This prompted questions concerning whether animal models of muscle Cr accumulation were a realistic basis for comparison when results were to be applied to humans (Green *et al*, 1996a), and supported the suggestion that further research of Cr supplementation in humans was required.

The amounts of CHO consumed by subjects that augmented muscle Cr accumulation (Green *et al*, 1996a), elicited insulin responses toward the high end of the physiological range (Marshall, 1993). Investigations demonstrating an effect of insulin upon Cr uptake in cultured mouse skeletal muscle cells have shown that supraphysiological insulin concentrations are required to produce an effect (Odoom *et al*, 1996). It had been proposed that insulin exerted its effect of increasing Na^+-dependent Cr transport by stimulating Na^+/K^+ATPase activity in muscle (Odoom *et al*, 1996). Insulin can also have a number of other effects *in vivo*, however, which would not be apparent *in vitro*. One such effect, which might be expected to influence muscle Cr accumulation, is the stimulation of blood flow to the muscle. This would have the effect of increasing the availability of Cr for uptake by the muscle, an effect that could not be observed by an *in vitro* study. With the aim of further establishing the role of insulin in mediating Cr accumulation by muscle in humans, Steenge *et al* (1998) used an euglycemic

insulin clamp technique at different insulin concentrations. Insulin and glucose were infused intravenously and maintained at a constant concentration throughout a 300 min period on 4 separate occasions. Insulin infusion rate was set to achieve one of 4 steady-state circulating insulin concentrations during each visit, which represented concentrations within the normal human range and a supraphysiological concentration (mean serum insulin concentration; 12, 56, 109 or 199 mIU.L^{-1}). The insulin infusion rate eliciting serum insulin concentration of ~ 109 mIU.L^{-1} was used, as similar concentrations have been achieved following ingestion of a drink containing ~95 g CHO with Cr (Green *et al*, 1996a,c). Creatine was administered as a 5 g oral dose 60 min after equilibration of insulin and glucose concentrations, and was subsequently administered via a nasogastric tube to maintain a plasma Cr concentration of ~ 800 μmol.L^{-1}. Muscle biopsy samples were obtained, and forearm and calf blood flow measurements made, during each experiment. Insulin stimulated muscle Cr accumulation, but only at high or supraphysiological concentrations (100 – 200 mIU.L^{-1}). Total Cr concentration increased by 4.6 mmol.kg^{-1} d.m. and 8.4 mmol.kg^{-1} d.m. during the 225 min period of Cr infusion with steady-state insulin concentrations of 109 and 199 mIU.L^{-1}, respectively. Plasma Cr concentration was lower at several time points during clamps at those insulin concentrations, indicating that muscle Cr transport was increased. Calf and forearm blood flow was increased above basal level during all but one treatment condition (forearm blood flow during lowest insulin concentration), however, the magnitude of the increase was the same, irrespective of the circulating insulin concentration. It was therefore unlikely that increased blood flow contributed to the increase in Cr accumulation seen at higher insulin concentrations. The findings from these investigations suggested that

insulin could augment muscle Cr accumulation in humans, but only at high physiological concentrations. Therefore large amounts of CHO must be ingested with the Cr supplements for any enhancement of Cr accumulation to occur.

It was observed by Harris *et al* (1992) that submaximal exercise performed during Cr supplementation resulted in greater Cr accumulation in the exercised muscle. No similar effect was observed, however, in subjects performing submaximal exercise (cycling at 70% VO_2max) during Cr and CHO supplementation (5 g Cr + 93 g CHO 4 times per day for 5 d; Green *et al*, 1996c). Exercising subjects receiving Cr + CHO showed no different responses of peak plasma Cr concentration, plasma Cr bio-availability, urinary Cr excretion or serum insulin concentration, when compared with subjects ingesting Cr + CHO without prior exercise. It was proposed by the authors that the improvements in muscle Cr accumulation with exercise observed by Harris *et al* (1992) might have resulted through improvements in insulin sensitivity of the exercised muscle, and that any such improvement was overshadowed by the large response to ingested CHO (Green *et al*, 1996c).

Additional factors may also have accounted for these contrasting results. Firstly, in the study of Harris *et al* (1992), small population size, various Cr supplementation regimens and differing times of muscle sampling make interpretation of the results difficult. In addition to this, two subjects were vegetarian. Normal reference values for serum Cr are lower in vegetarians than for individuals who maintain 'normal' diets (Delanghe *et al*, 1989). It has been suggested that vegetarians may have lower than normal muscle Cr concentration and it has been demonstrated that upon Cr supplementation vegetarians exhibit greater Cr retention than non-vegetarians (Green, 1996; Green *et al*, 1997).

Therefore the inclusion of two vegetarian subjects in the exercising group receiving Cr might have skewed accumulation results toward higher values.

Another report proposed that other nutritional factors might affect muscle Cr accumulation in humans (Vandenberghe *et al*, 1996). It was suggested that combining Cr supplementation with ingestion of a compound known to increase Na^+/K^+ pump activity might enhance muscle Cr accumulation and thereby further improve muscle performance. Caffeine was ingested with Cr as it has been proposed to directly stimulate Na^+/K^+ pump activity and also increase plasma adrenaline concentration, which also stimulates Na^+/K^+ pump activity (Clausen, 1986). Examination of muscle PCr concentration using ^{31}P-NMR demonstrated that ingestion of caffeine during Cr supplementation did not increase muscle PCr concentration to any greater extent than supplementation with Cr only. Furthermore, the authors showed that caffeine ingestion during supplementation abolished the ergogenic effect of Cr supplementation on performance of maximal exercise. This result was unexpected and the authors could not provide any explanation for its occurrence based upon their data. The effect did not appear to be related to an acute action of caffeine upon muscle energetics, as plasma caffeine concentration was considered to be minimal at the time of exercise. It was therefore suggested that, to obtain the ergogenic effects of Cr supplementation, beverages containing caffeine (or any other trimethylxanthine compounds) should not be ingested at the same time as Cr.

1.9 Effects of guanidino compounds on CHO disposal

Another unexpected, yet interesting, result was observed by Green *et al* (1996a) in their investigations of the effects of CHO ingestion on Cr accumulation. A

greater increase in body mass was observed of subjects ingesting Cr + CHO than those ingesting Cr only, although this difference was non-significant (Green *et al*, 1996a). This suggested to the investigators that insulin may have contributed to the increased body mass by increasing both Cr and glycogen accumulation by muscle. Subsequent investigation examined the effects of ingesting Cr only, Cr and CHO or CHO only on muscle Cr accumulation and glycogen storage (Green *et al*, 1996b; Green, 1996). Although peak glucose concentration was the same in both groups that received CHO, a greater rate of glucose clearance was observed with Cr + CHO supplementation. This difference appeared to be mediated by a 60% greater peak serum insulin concentration following ingestion (Green, 1996). Muscle glycogen concentration increased significantly following five days of supplementation in the groups that received CHO, but did not change significantly in the Cr only group. The increase in muscle glycogen was twice as great in the Cr + CHO group than in the CHO only group, although this difference was not statistically significant due to a large variation in glycogen concentration in the Cr + CHO group. Furthermore, a positive correlation was observed between individual increases in muscle glycogen and TCr of the Cr + CHO group ($r = 0.75$, $n = 8$, $p < 0.05$). Whilst caution should be taken when interpreting these results (particularly since they have not yet been published in a peer-reviewed journal), they are of interest as an apparent relationship between muscle Cr and glycogen accumulation would have implications for both maximal and submaximal exercise. Submaximal exercise of prolonged duration (i.e. 65% - 80% VO_2max, 60 – 180 min) mainly utilises the body's CHO store for fuel. Pre-exercise muscle glycogen concentration is a major determining factor of the duration of this type of activity (Bergström *et al*, 1967) and a method of

increasing muscle glycogen concentration above normal level might have the potential to improve performance of such exercise.

Of particular interest is the observation that peak insulin concentration was greater when Cr was ingested with CHO, compared with CHO ingestion alone (Green, 1996). A number of investigations have demonstrated that another guanidino compound, arginine (itself a precursor of Cr biosynthesis), can also influence insulin release to affect whole body CHO disposal. For a given individual, plasma insulin concentration increases in response to elevation in blood glucose concentration, to a certain extent, in a dose-dependent manner (Mikines, 1992). The magnitude of the insulin response will influence the rate of muscle glucose transport and glycogen synthase activity. Other factors, including other hormones (e.g. glucagon), and some amino acids (e.g. leucine, arginine) also stimulate insulin secretion from the pancreas (Ganong, 1979). Arginine is a dibasic amino acid that is synthesised by the kidney from citrulline. Physiological roles of arginine include regulation of ammonia excretion through the urea cycle and the biosynthesis of Cr. It is also the unique substrate for production of nitric oxide (NO) used in many tissues as a biological effector molecule (Barbul, 1995). Arginine also enhances the secretion of a variety of hormones, including adrenal catecholamines, glucagon, growth hormone and insulin (Barbul, 1986). Endogenous synthesis of arginine is normally sufficient for daily metabolism, although in times of elevated protein turnover such as growth or trauma arginine has been considered an indispensible amino acid (Nagakawa *et al*, 1963; Seifter *et al*, 1978). Exogenous arginine administered intravenously has been demonstrated to increase glucose turnover in dogs (i.e. increased glucose production and utilisation; Cherrington & Vranic, 1973). This increase is brought about by

stimulation of hepatic glucose production and whole-body glucose utilisation by elevated concentrations of glucagon and insulin, respectively (Cherrington *et al*, 1974). In humans, intravenous administration of arginine, anticipated to elicit plasma concentrations of 4 mM, resulted in a 2 – 3 fold increase in circulating insulin concentration (Efendic *et al*, 1974). This pre-treatment with arginine caused subsequent potentiation (up to 2-fold) of the initial insulin response to infused glucose at a variety of concentrations.

Three mechanisms by which arginine stimulates insulin release have been proposed. Uptake of L-arginine by pancreatic β-cells exposed to D-glucose results in insulin secretion via membrane depolarisation induced by the cationic nature of the amino acid (Blachier *et al*, 1989). Another mechanism proposed is that arginine is metabolised by the urea cycle, producing ornithine, which enters the TCA cycle as substrate for gluconeogenesis, thereby stimulating insulin release through glucose production (Malaisse *et al*, 1989). Finally, arginine-induced plasma elevation of glucose and insulin in rats has been shown to be due, in part, to NO formation (Jun & Wennmalm, 1994), however, differential effects of NO exist upon glucagon and insulin secretion (Panagiotidis *et al*, 1992; Panagiotidis *et al*, 1994; Henningsson & Lundquist, 1998). It has also been demonstrated that L-arginine administration stimulates insulin-mediated glucose uptake through a NO-mediated increase in blood flow (Paolisso *et al*, 1997). Whatever the mechanism by which it acts, it is apparent that arginine influences insulin secretion and, of interest in exercise physiology, augments glucose-stimulated insulin release and whole-body glucose disposal. This phenomenon is of potential value to individuals wishing to improve glycogen repletion following

exercise, as the elevated insulin response may have the effect of improving muscle glucose transport and/or glycogen synthesis.

It is possible that the relationship between muscle Cr and glycogen accumulation following Cr + CHO ingestion (Green *et al*, 1996b) might have been influenced by factors other than an elevated insulin release. Creatine supplementation has been associated with increases in body mass thought to be attributable to body water retention (Hultman *et al*, 1996). It is possible that muscle Cr accumulation would cause muscle cells to retain water to maintain their osmotic potential. This would result in swelling of muscle fibres that had accumulated Cr. Cell volume changes are known to influence a number of cellular functions, including solute transport and intracellular metabolism (Lang *et al*, 1998). A recent investigation using human subjects has shown that hypo-osmolal conditions, which are known to induce cell swelling, exert protein- and glucose-sparing effects on whole-body metabolism (Berneis *et al*, 1999). Glycogen synthesis in the liver is modulated by changes in hepatic cell volume, with an increase observed with cell swelling and a decrease with cell shrinkage (Häussinger, 1996). At present the exact mechanism by which cell volume governs this effect is unclear, however, intracellular signalling involving mitogen activated protein kinase activity has been proposed (Häussinger, 1996). Indirect evidence that swelling of muscle might have a similar effect upon muscle glycogen synthesis (Low *et al*, 1996a; Rennie *et al*, 1996) prompted investigation of glycogen synthesis in cultured muscle cells exposed to solutions of differing osmotic potential (Low *et al*, 1996b). It was demonstrated that in rat muscle myotubes, which have many characteristics of adult muscle (Low *et al*, 1996b), glycogen synthesis was modulated in association with changes in cell volume, and that these changes were not dependent upon

changes in cell glucose uptake. These changes may have been co-ordinated via intracellular signalling processes that acted through tyrosine kinase activity within the cell. Strenuous exercise resulting in muscle lactate accumulation is known to increase muscle cell volume (Sahlin, 1983) and it was proposed that this might assist muscle glycogen resynthesis in the period following exercise (Low *et al*, 1996b). The amino acid glutamine also increases muscle cell volume (Low *et al*, 1996a). Post-exercise glycogen resynthesis was enhanced when glutamine was provided during recovery (Varnier *et al*, 1995) by a magnitude similar to the changes in muscle cell volume. These findings demonstrate that changes in muscle cell volume can influence muscle glycogen synthesis. They also suggest that provision of metabolically-active substrates known to increase cell volume during a post-exercise period might enhance subsequent glycogen synthesis.

1.10 Safety aspects of creatine supplementation

Publication of research findings that Cr supplementation could lead to improvements in exercise performance has caused Cr to become an extremely popular dietary supplement of athletes. Promotion of Cr as an ergogenic aid by a wide range of sport and fitness publications, and anecdotal comments from professional sportspersons on its efficacy has contributed to Cr being one of the highest selling dietary supplements in the USA. During 1997, USA sales of Cr totalled $100 million (£60 million) and forecasts of future sales predicted an annual rise of 20 – 25 % (Rowbottom, 1998). A recent questionnaire survey for a UK newspaper revealed that almost 57% of over 300 elite sporting competitors in the UK had used Cr during their sporting career (Harris & Arthur, 1998). The popularity of Cr supplementation amongst athletes has raised concerns from

sporting and medical bodies, however, concerning whether any side-effects of Cr ingestion exist. Investigations of Cr supplementation to date have only shown one recognisable side effect, that of an increase in body mass following Cr feeding for 5 or more days (e.g. Balsom et al, 1993a,b; Greenhaff *et al*, 1994). Anecdotal suggestions have been made of Cr contributing to liver and kidney damage, causing muscle cramping and strains, and producing dehydration and gastrointestinal discomfort. Very little evidence has been supplied to support these suggestions, yet lay publications and the public media continue to allege that Cr supplementation can be harmful. Recently a case study was reported of renal dysfunction in a football player who had been taking Cr prior to commencement of pre-season training (Pritchard & Kalra, 1998). The patient had pre-existing kidney dysfunction, which responded well to medication. Following Cr supplementation, however, his serum creatinine concentration was elevated above the normal range and creatinine clearance was impaired. One month after cessation of Cr supplementation serum creatinine and creatinine clearance were within normal range. This study did not investigate the effects of Cr supplementation upon kidney function, but merely implied that Cr supplementation caused the impaired function. Another letter to a medical journal has recently described a case of renal insufficiency in a patient four weeks after he had begun ingesting Cr (20 $g.d^{-1}$ $Cr.H_2O$, duration of supplementation not specified; Koshy *et al*, 1999). The patient had experienced 4 days of nausea, vomiting and bilateral flank pain. Upon hospitalisation, the patient was found to have acute focal interstitial nephritis and focal tubular injury and serum creatinine concentration was higher than the normal range. Symptoms disappeared following treatment with intravenous fluid. To date, these two case studies

remain the only scientific evidence to support claims that Cr supplementation may not be appropriate for all individuals. By contrast, oral Cr supplementation has been used for a number of years in the treatment of gyrate atrophy of the choroid and retina (Sipilä *et al*, 1981; Vannas-Sulonen *et al*, 1985) and other muscle diseases without any adverse effects on health being observed. Intravenous administration of PCr has also been demonstrated to improve functional recovery of the heart following myocardial infarction and prevent ischaemic damage during cardiac surgery (Saks *et al*, 1992). Recent animal studies have also demonstrated that Cr has neuroprotective effects, which may be useful in therapy for Huntington's disease and other neurodegenerative diseases (Matthews *et al*, 1998; Klivenyi *et al*, 1999).

Despite the lack of evidence from controlled investigations, anecdotal opinions continue to be reported that Cr supplementation adversely affects health. This has prompted requests for detailed investigation of potential harmful effects of Cr by sporting and medical councils.

1.11 Aims of this thesis

The principal aim of this thesis is to examine some physiological effects of dietary supplementation with the guanidino compounds Cr and arginine in humans. Experiments outlined in this review have suggested that Cr accumulation by human skeletal muscle might be improved by exercise during supplementation (Harris *et al*, 1992), and by CHO ingestion during supplementation (Green *et al*, 1996a). When we consider the possible relationship between muscle glycogen and Cr accumulation (Green *et al*, 1996b) and the influence of exercise upon subsequent glycogen synthesis (Bergström & Hultman, 1966) and possibly Cr

accumulation by muscle (Harris *et al*, 1992), it is of interest whether these responses can be combined. It is hypothesised that the combination of Cr supplementation with a glycogen supercompensation protocol might produce marked increases in muscle glycogen and Cr concentration, above those that have been achieved previously using supercompensation strategies or Cr or Cr + CHO supplementation alone. With the aim of addressing this hypothesis, Chapter 3 describes a study to investigate the effects of a bout of glycogen-depleting exercise upon subsequent muscle glycogen and metabolite concentrations, when subjects' diets were supplemented with CHO or Cr + CHO.

As mentioned in this review, another guanidino compound, arginine, has been shown to have a potent effect upon insulin release into, and glucose clearance from the blood, when administered intravenously. Based upon these findings, and following results obtained in Chapter 3, the effects of oral ingestion of arginine and CHO upon blood glucose and insulin responses were investigated in Chapter 4, to establish whether arginine had an effect on the fate of ingested CHO.

Following from results obtained in Chapter 3, and the previously observed relationship between muscle Cr and glycogen accumulation following Cr + CHO supplementation (Green *et al*, 1996b), it is hypothesised that Cr + CHO supplementation might be beneficial to individuals who perform prolonged exercise. The experiment in Chapter 5 was undertaken to further examine the effects of Cr + CHO supplementation upon muscle Cr and glycogen accumulation and whether these effects (if any) improved subsequent performance of prolonged exercise.

As the popularity of Cr use as a dietary supplement has increased, so has the attention it has received in the media, from both positive and negative viewpoints.

With the aim of addressing concerns recently-raised about potential harmful effects of Cr supplementation on health, Chapter 6 examines the effects of Cr supplementation upon various indices of health, namely haematological, hepatological, muscle and renal function.

In the general discussion, the results of the experiments presented in this thesis are discussed. The practical implications that these results may have are considered and potential interests for future research are also proposed.

Chapter 2

GENERAL METHODOLOGY

2.1 Ethical approval and clinical practice

The University of Nottingham Medical School Research Ethics Committee approved all of the experiments described in this thesis. The experiments were conducted in laboratories of the School of Biomedical Sciences, University of Nottingham Medical School. During all experimental visits a minimum of two investigators were present, at least one of whom was qualified to administer first aid and had experience in the clinical techniques being used. Additionally, a doctor was present within the School to provide medical cover for every experiment. A laboratory record of each experiment was recorded and signed by the investigator.

2.2 Recruitment and screening of subjects

Experimental subjects were recruited by advertisement. Interested volunteers were interviewed for suitability for each experiment and the experimental protocols were explained to them in detail. In addition to the interview, volunteers were provided with an information sheet, which outlined all aspects of the investigation and informed them that they could withdraw from the study at any time without giving prior notice (see Appendix A). A basic medical screening examination was then conducted for all volunteers. Firstly, subjects completed a confidential questionnaire relating to aspects of their health, diet and activity and measurements of height and weight were taken. A 12-lead ECG recording (Cambridge VS-6, Picker International, USA) and measurements of blood pressure (Accutor 1A, Datascope Corp., Paramus, NJ, USA) in supine and standing positions were then performed. With the volunteer's consent, blood samples were obtained by venupuncture of an antecubital vein. These samples

were analysed for full blood count and for indices of renal and hepatological function by the Haematology and Clinical Chemistry departments of the Queen's Medical Centre, Nottingham, respectively. A clinician checked the results of the medical screening and volunteers considered to be in good health were accepted for inclusion in the study. Prior to involvement in each study, subjects signed a consent form, which confirmed that they had read and understood the information sheet concerning the study, further confirmed that their health was normal and stated that their agreement to participate in the study was made of their own free will.

2.3 Measurement of peak oxygen consumption

Equipment and theoretical basis of measurement

In the experiments described in Chapters 4 and 5, tests were conducted on subjects to determine their peak oxygen consumption (VO_2peak). During these tests subjects exercised at incrementally increasing workloads and samples of expired air were monitored throughout. Expired air samples were analysed and recorded at 20-second intervals throughout the exercise period. A headset-mounted mouthpiece connected to a computerised analysis system (Vmax 29, SensorMedics Corporation, Yorba Linda, CA, USA) set to 'mixing chamber' mode was used for analysis and recording. Prior to each test, the analysis system was calibrated for airflow measurement, and accuracy of oxygen and carbon dioxide measurement was determined using certified calibration gases and atmospheric air. The analysis system used measured oxygen concentration using a paramagnetic oxygen analyser, which generated a signal in response to the partial pressure of oxygen in an expired gas sample. Carbon dioxide measurement

was made using a non-dispersive infrared technique, which operates by measuring the amount of infrared energy that is absorbed in a sample of expired gas. Oxygen consumption (VO_2) was automatically calculated by a computer linked to the analysis system, based on the following equation, which is derived as detailed in Appendix B:

O_2 consumption = Volume of oxygen inspired – Volume of oxygen expired

Exercise test for VO_2peak measurement

For assessment of VO_2peak, each subject performed a continuous incremental exercise test on an electrically braked cycle ergometer (Excalibur Sport, Lode N.V. Instrumenten, Groningen, The Netherlands), having not eaten during the preceding 2 h. Each subject was positioned on the ergometer at a suitable saddle height and cycled at a cadence of 70 rpm against increasing workload intensity steps of 50 Watts (W), each step lasting 3 min. The initial exercise intensity was either 100 W or 150 W, depending upon the subject's experience with cycling exercise. As the subject was approaching fatigue, workload increments were increased by only 30 W until the subject could no longer maintain the workload for 3 min. The exercise test was designed to produce exhaustion within 15 - 20 min.

Peak VO_2 was taken to be the average of the last four VO_2 measurements recorded during the final completed stage of exercise. A similar, shorter test was conducted for each subject not less than 3 days later. This involved the subject cycling at a warm-up workload for 3 min, followed by cycling at a workload 30 W below that of the final completed stage of the previous test. The workload was then increased in 30 W increments until the subject could no longer maintain the workload for 3 min. The values of VO_2 measured during both tests were plotted

against workload applied for each stage of the exercise test. From the resultant plots, workloads could be calculated that would elicit 70 % of VO_2peak for use in the test protocols in Chapters 4 and 5.

2.4 Calculation of respiratory exchange ratio and measurement of substrate oxidation

Theoretical basis of measurement

It is possible to estimate the proportion of substrate that is contributing to energy production by assessing the ratio of CO_2 produced to O_2 consumed, in subjects at rest and performing steady-state exercise. This ratio is given the term respiratory exchange ratio (RER) and is based upon the fact that fat, carbohydrate and protein differ in the amount of O_2 used and CO_2 produced when they are utilised as fuel. Carbon dioxide production (VCO_2) can be calculated in a similar manner to O_2 consumption:

CO_2 production = Volume of CO_2 expired – Volume of CO_2 inspired

Given VCO_2, RER can be calculated by the simple equation,

$$RER = \frac{VCO_2\ \%}{VO_2\ \%}$$

In Chapter 4 expired gas samples were collected for estimation of substrate oxidation. In order to calculate the oxidation rates of CHO (VO_{2CHO}) and fat (VO_{2FAT}), the rate of protein oxidation (VO_{2P}) is also required as protein contributes a small proportion to total energy expenditure. In the experiment described, samples were collected at rest and it was assumed that protein oxidation contributed to 12.5 % of total energy expenditure during the collection period (Weir, 1949). Derivation of the equations for VO_{2CHO}, VO_{2FAT} and VO_{2P} calculation are shown in Appendix B.

Equipment for measuring RER

Subjects rested on a bed with their head completely covered by a plastic canopy. The canopy consisted of a domed hood with an inlet for atmospheric air and an outlet from which expired air was collected for analysis by a gas analysis system (GEM, Europa Scientific Ltd., UK). Prior to each test, the analysis system was calibrated for accuracy of oxygen and carbon dioxide measurement using certified calibration gases and atmospheric air. The system generated reports of expired air analysis for every 60 seconds of sampling. From these values, the contributions of different substrates to oxidative metabolism were estimated.

2.5 Forearm blood flow measurement

Theoretical basis of measurement

In Chapter 4, forearm blood flow was measured using venous occlusion plethysmography. This non-invasive technique relies upon the assumption that a limb is a cylinder of fixed length and that any change in limb volume will result in a change in the circumference at all points of that cylinder (Whitney, 1953). The rate of arterial inflow into a limb can be measured by observing changes in limb circumference whilst venous flow out of the limb is halted. A high-pressure arterial occlusion cuff was wrapped around the wrist and a lower pressure venous occlusion cuff was wrapped around the arm above the elbow. To measure changes in limb circumference, a mercury-in-rubber strain gauge was attached around the forearm between the cuffs. For each blood flow measurement the arterial occlusion cuff was inflated to a pressure exceeding arterial systolic pressure to prevent any shift of blood away from the hand into the limb. After one minute of inflation to avoid introduction of artefact to measurements, the low

pressure venous occlusion cuff was inflated above venous pressure, but lower than systolic pressure, to allow arterial in-flow. Arterial flow was monitored for 8 seconds, then the low pressure cuff was deflated to allow venous out-flow of blood. Positioning the forearm above the atria of the heart ensured complete venous drainage in this period. The cuff was inflated and deflated cyclically for approximately 7 measurements.

Equipment for measurement of blood flow

Forearm circumference was measured using a tape measure, and a strain gauge of appropriate size was attached around the forearm. The strain gauge consisted of a compliant silicon rubber tube (bore diameter 0.5 mm, wall thickness 0.25 mm) filled with mercury, closed off at each end with platinum wire (0.9 mm diameter). The platinum wires were attached to a strain gauge holder to complete a Wheatstone Bridge circuit. This circuit enables signals to be generated in proportion to changes in length of the mercury-in-rubber tube. The changes in length are measured by a galvanometer within the gauge, which in turn is attached to a signal amplifier and microcomputer running chart-recording software (Chart V 3.4 for Apple Macintosh). Prior to each experiment, the circuit was balanced using a 10 g weight to maintain tension. The gauge was then calibrated on a wooden forming block. Briefly, the holder was fixed in position whilst the rubber tubing was looped over a mount on the end of the calibration block. A screw of known pitch was used to adjust the mount for five full turns. The chart-recording software recorded the mean size of the resulting signals and subsequent calculations of blood flow were based upon the calibration factor obtained.

Forearm blood flow was calculated using the following equation:

$$\text{Blood flow} = \frac{\text{slope} \bullet \text{pitch calibration screw} \bullet \text{chart speed}}{\text{Calibration factor}} \bullet \frac{200}{\text{limb circumference}}$$

where:

$$\text{Calibration factor} = \frac{\text{sum of distance of each step}}{\text{number of steps}}$$

Pitch of calibration screw = 0.53

Slope is measured in $mV.s^{-1}$, chart speed in $mm.s^{-1}$ and limb circumference in mm.

2.6 Supplementation information

In the experiments in Chapters 3 - 6 subjects consumed a carbohydrate drink containing 18.5 % glucose and simple sugars (Lucozade™, Smithkline Beecham, Coleford, UK). During the 5-day supplementation periods (Chapters 3, 5 & 6) subjects drank a 500 ml serving of Lucozade™ four times each day, evenly distributed throughout the day (e.g. 08:00, 12:00, 16:00, 20:00 hrs). In Chapter 4 subjects were given a single 378 ml serving of Lucozade™ during each experimental visit, and were required to consume the drink within 5 minutes.

In Chapter 4, subjects ingested 10 g doses of the amino acids arginine and glycine (Ajinomoto Co. inc., Kawasaki City, Japan), on separate occasions. The amino acids in powder form were dissolved in 200 ml cold water, to which was added 50 ml of sugar-free fruit cordial. Subjects were required to consume the amino acid drink as quickly as possible. The approximate time for drink consumption was 30 s.

In Chapters 3, 5 & 6, subjects' diets were supplemented with Cr in the form of creatine monohydrate ($Cr.H_2O$; Experimental and Applied Sciences, CO, USA).

Subjects were provided with vials containing $Cr.H_2O$ that they dissolved in a warm non-caffeinated drink and consumed on 4 separate occasions each day, evenly distributed throughout the day (e.g. 08:00, 12:00, 16:00, 20:00 hrs), for 5 days.

2.7 Dietary manipulation

In Chapter 3, all subjects were required to maintain a diet of high CHO content. To achieve this, subjects were provided with appropriate foodstuffs and dietary advice which, if followed, was anticipated to ensure subjects received > 80% of their daily energy intake in the form of CHO (see Appendix A).

In Chapter 4, each subject consumed a standardised meal prior to each experimental visit. An identical meal was consumed before every experimental visit, which commenced no less than 12 h after the meal.

In Chapter 5, subjects were asked to follow and keep a record of their normal diet over the 3 days prior to the first exhaustive exercise visit. They were asked to repeat the recorded diet for the 3 days prior to all subsequent experimental visits, in addition to which they also consumed the experimental supplements.

2.8 Blood sampling procedures and analysis

In Chapters 3 & 4 blood samples were obtained from a vein on the dorsal surface of the hand. Subjects placed their hand in a heated Perspex chamber in which air temperature was maintained at 55 °C in order to arterialise the venous drainage of the hand (Gallen and Macdonald, 1990). After allowing the hand to be warmed for approximately 10 min, local anaesthesia was applied to the skin (1% w/v lignocaine hydrochloride, Antigen Pharmaceuticals Ltd., Roscrea, Ireland) and a

21 gauge venous cannula was inserted retrogradally into a superficial vein on the dorsal surface of the hand. Following cannulation, the subject's hand was returned to the heated chamber and blood samples obtained from the cannula via a 3-way tap system. Cannula patency was maintained throughout the experiment by an isotonic saline drip (0.9% sodium chloride BP, Baxter Healthcare Ltd. Thetford, England, UK).

In Chapter 5, blood samples were obtained from a cannula inserted into a vein of the forearm. Local anaesthesia was applied to the skin (1% w/v lignocaine hydrochloride, Antigen Pharmaceuticals Ltd., Roscrea, Ireland) and an 18 gauge cannula was inserted retrogradally into an antecubital vein. Blood samples were obtained from the cannula via a 3-way tap system and the cannula was kept patent using an isotonic saline drip (0.9% sodium chloride BP, Baxter Healthcare Ltd. Thetford, England, UK). In Chapters 4 & 5, the cannula was secured at the site of insertion by a waterproof adhesive medical covering to prevent its movement whilst the subject exercised.

Throughout experiments in Chapters 3 - 5, a 15 min equilibration period was allowed between insertion of the cannula and collection of the first blood sample. Prior to collection of an experimental blood sample, a 2 ml syringe was used to remove a mixture of saline and blood contained in the dead-space between the tip of the cannula and the 3-way tap. A 10 ml syringe was then used to collect blood for analysis, which was divided equally for serum and plasma analysis. Additionally, 100 μl of the same sample was immediately introduced into an automated analyser (Chapters 3 & 5, YSI 2300 STAT plus, YSI, Yellow Springs, OH, USA; Chapter 4, Hemocue AB, Ångelholm, Sweden) for measurement of glucose and lactate (Chapter 5 only) concentrations. Previous calibration of the

automated equipment by comparison with spectrophotometric glucose and lactate analysis of identical samples has determined a very good degree of accuracy of this equipment (Fox & Greenhaff, personal communication).

In Chapters 3 & 4, blood samples for serum analysis were allowed to clot, centrifuged at 3000 rpm for 10 min and the supernatant was stored at -20 °C. Serum insulin concentration was measured using a commercially available radio-immunoassay diagnostic kit (Diagnostic Products Corporation, Los Angeles, CA, USA) by the Department of Clinical Chemistry, Queen's Medical Centre, Nottingham, UK.

In Chapter 6, blood samples were obtained by venupuncture of an antecubital vein, after subjects had been seated for at least 5 min, and dispensed into separate tubes either containing potassium EDTA or plain tubes containing no anticoagulant. These samples were analysed for indices of haematological function and for indices of hepatological, muscle and renal function by the Departments of Haematology and Clinical Chemistry, Queen's Medical Centre, Nottingham, respectively.

2.9 Muscle sampling procedures and analysis

In Chapters 3 & 5, muscle samples were obtained by a clinician using the percutaneous needle biopsy technique (Bergström, 1962). Prior to sampling, a 10 cm^2 (approx.) area of skin, which covered the area from which the biopsy was to be taken, was shaved and cleaned with an alcoholic iodine solution. Local anaesthetic (1% w/v lignocaine hydrochloride, Antigen Pharmaceuticals Ltd., Roscrea, Ireland) was injected under the skin and into the muscle and a 10 mm incision was made through the skin to allow insertion of the biopsy needle. A

biopsy needle was inserted through the incision into the vastus lateralis muscle from which a sample was cut (~ 50 mg wet weight) and removed from the leg. In Chapter 3, the muscle sample was allowed to stand at room temperature for 1 minute to allow PCr concentration to return to a normal level (Söderlund & Hultman, 1986). Tissue damage due to the muscle biopsy procedure causes Ca^{2+} release in the muscle tissue, which in turn stimulates actomyosin ATPase activity. This will result in a transient decrease in PCr concentration as ATP is consequently resynthesised. An equilibration period of 1 minute at room temperature, prior to muscle tissue freezing, has been demonstrated to more accurately reflect the concentrations of ATP and PCr in resting human muscle (Söderlund & Hultman, 1986). The muscle sample was then frozen in liquid nitrogen and stored at -80 °C for subsequent analysis. In Chapter 5, all muscle samples were frozen in liquid nitrogen immediately following removal so that PCr and ATP concentrations during exercise and immediately following exhaustion could be measured. Once frozen, all muscle samples were freeze-dried and stored at -80 °C.

Prior to analysis, any fat contained in the samples was solubilised by immersion in petroleum ether for 20 minutes and removed by pouring off the solvent. Muscle samples were then dissected free of visible blood and connective tissue and powdered in a mortar and pestle. Aliquots of muscle powder were weighed for subsequent analysis of ATP, PCr, Cr (~ 6.0 – 7.0 mg muscle powder) and glycogen (~ 2.01 – 3.0 mg muscle powder) concentrations.

Muscle samples for measurement of metabolite concentrations were first extracted with 80 $\mu L.mg^{-1}$ d.m. (minimum 500 µL) 0.5 M perchloric acid (PCA) for 10 minutes with intermittent mixing and storage in ice. The extract was then

centrifuged (14000 rpm, 1 min) and 400 μL of the supernatant transferred to an eppendorph tube and neutralised with 100 μL 2.2 M $KHCO_3$. The resultant supernatant was immediately frozen in liquid N_2 and stored at -80°C, prior to analysis. The extraction dilution factor (F) was then calculated for each sample as follows:

$$F = \frac{V_{PCA} \times (V_S + V_{KHCO_3})}{V_S \times Wt_M}$$

where: V_{PCA} = Volume of PCA added (μL)

V_S = Volume of PCA supernatant (μL)

V_{KHCO_3} = Volume of $KHCO_3$ added to the PCA extract (μL)

Wt_M = Weight of muscle powder (mg)

Muscle ATP and PCr concentrations were determined spectrophotometrically in NADPH-linked reactions (Harris *et al*, 1974), as outlined below:

$$\text{Glucose–6–phosphate} + NADP^+ \xrightarrow{\text{G-6-PDH}} \text{P-gluconolactone} + NADPH + H^+$$

$$ATP + \text{Glucose} \xrightarrow{\text{HK}} ADP + \text{G-6-P} + H^+$$

$$PCr + ADP + H^+ \xrightarrow{\text{CPK}} Cr + ATP$$

Briefly, the absorbances at 340 nm of microcuvettes containing all components of the reaction mixture (including sample or water as a blank), other than hexokinase (HK) and creatine phosphokinase (CPK) were measured (A1). Absorbances were also measured following the addition of HK (A2) and CPK (A3) to the reaction mixture. Following addition of each enzyme, cuvettes were incubated at room temperature with constant agitation on a rotating plate until the reaction had reached a plateau. Changes in absorbance measurements of samples were used to

calculate ATP and PCr concentrations of the samples using the following calculations:

$$[\text{ATP}]\ (\text{mmol.kg}^{-1}\ \text{d.m.}) = \frac{V2\ (A2 - Bl2) - V1\ (A1 - Bl1) \bullet F}{6.22 \bullet SV}$$

$$[\text{PCr}]\ (\text{mmol.kg}^{-1}\ \text{d.m.}) = \frac{V3\ (A3 - Bl3) - V2\ (A2 - Bl2) \bullet F}{6.22 \bullet SV}$$

where: V1 = initial reaction volume (μL)

V2 = V1 + volume of HK added (μL)

V3 = V2 + volume of CPK added (μL)

A1 (or Bl1) = Initial absorbance of reagent + sample (or blank)

A2 (or Bl2)= Absorbance following addition of HK to sample (or blank) cuvette

A3 (or Bl3) = Absorbance following addition of CPK to sample (or blank) cuvette

F = Extraction factor

6.22 = millimolar absorption coefficient of NADPH @ 340 nm

SV = Sample volume

By a similar method, muscle Cr concentration was determined spectrophotometrically in NADH-linked reactions (Harris *et al*, 1974), as outlined below:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

$$\text{ADP} + \text{phosphoenolpyruvate} + \text{H}^+ \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP}$$

$$\text{Cr} + \text{ATP} \xrightarrow{\text{CPK}} \text{PCr} + \text{ADP} + \text{H}^+$$

The absorbances at 340 nm of microcuvettes containing all components of the reaction mixture (including sample/blank), other than CPK were measured (A1).

Following addition of CPK, and incubation until the reaction had reached a plateau, absorbances of cuvettes were measured (A2). Changes in absorbance measurements of samples were used to calculate Cr concentrations of the samples using the following calculation:

$$[Cr]\ (mmol.kg^{-1}\ d.m.) = \frac{V2\ (Bl2 - A2) - V1\ (Bl1 - A1) \bullet F}{6.22 \bullet SV}$$

Muscle glycogen concentration was determined by an assay that first involved hydrolysis of glycogen by heating at 80°C for 20 min in 120 μL 0.1 $mol.L^{-1}$ NaOH, followed by neutralisation with 480 μL of buffered HCl (3:1 ratio 0.2 $mol.L^{-1}$ citric acid/0.2 $mol.L^{-1}$ Na_2HPO_4 buffer : 0.1 $mol.L^{-1}$ HCl). The mixture was then incubated at room temperature for 1 h in the presence of 10 μL amyloglucosidase and the supernatant stored at -20°C for subsequent analysis of glucose concentration. The extraction dilution factor (F_G) of the sample was then calculated by the following equation:

$$F_G = \frac{V_{EXT} + Wt_M}{Wt_M}$$

where: V_{EXT} = sum of all extraction reagent volumes

The concentration of glucosyl units in the glycogen digests was determined spectrophotometrically in NADH-linked reactions (Harris *et al*, 1974), as outlined below:

$$ATP + Glucose \xrightarrow{HK} ADP + G\text{-}6\text{-}P + H^+$$

$$Glucose\text{-}6\text{-}phosphate + NAD^+ \xrightarrow{G\text{-}6\text{-}PDH} P\text{-}gluconolactone + NADH + H^+$$

The absorbances at 366 nm of microcuvettes containing all components of the reaction mixture (including sample/blank), other than HK were measured (A1 /

Bl1). Following addition of HK, and incubation until the reaction had reached a plateau, absorbances of cuvettes were measured (A2 / Bl2). Changes in absorbance measurements of samples were used to calculate glucosyl concentrations of the samples using the following calculation:

$$\text{Glucose (mmol.kg}^{-1}\text{ d.m.)} = \frac{\{(A2 \bullet V2) - (A1 \bullet V1)\} - \{(Bl2 \bullet V2) - (Bl1 \bullet V1)\} \bullet F_G}{SV \bullet 3.34}$$

where: 3.34 = millimolar absorption coefficient of NADH @ 366 nm

A small number of muscle samples obtained in Chapter 5 were not of sufficient size (i.e. < 4 mg dry weight) to separate for analysis of metabolite and glycogen concentration. In the case of these samples, the muscle powder was first extracted using 0.5M PCA, using the same method for metabolite extraction. Following metabolite extraction and removal of supernatant for metabolite analysis, the remaining pellet and a separate aliquot of PCA extract were hydrolysed in 100 $\mu L.mg^{-1}$ d.m. 1M HCl for 2 h and the resulting supernatants were stored at -80°C prior to analysis. Glucose concentration of the supernatant of these samples was determined fluorimetrically (λ_{ex} 340 nm, λ_{fl} 460 nm) in NADPH-linked reactions (Lowry & Passoneau, 1973), as outlined below:

$$\text{ATP + Glucose} \xrightarrow{HK} \text{ADP + G-6-P + H}^+$$

$$\text{Glucose–6–phosphate + NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{P-gluconolactone + NADPH + H}^+$$

The glucose concentration of non HCl hydrolysed PCA extract was subtracted from that of the HCl hydrolysed PCA extract, giving the concentration of soluble glycogen in the sample. This value was then added to the insoluble glycogen concentration determined for the HCl hydrolysed muscle sample to give total glycogen concentration of the sample (Jansson, 1981).

To ensure reliability of the assay procedures performed on different occasions,

standard solutions of ATP, PCr, Cr and glucose were also assayed at the same time as muscle extract samples. Very good repeatability was found between measurements of standard solutions for all assays performed on different occasions. Laboratory method sheets for muscle extraction and all analytical assays outlined above can be seen in Appendix B.

2.10 Correction procedures for muscle metabolite and substrate concentrations

Muscle PCr, Cr and glycogen concentrations were corrected to account for variations in non-muscle constituents (e.g. blood, connective tissue) of each sample. An accepted method of correcting for these constituents is to adjust values based on the concentration of ATP measured in each sample (Harris *et al*, 1992), as muscle ATP concentration is considered to not vary with time for any given individual, but will change with exercise. Any non-muscle component of a sample is likely to decrease the ATP concentration measured in that sample. Thus, in a range of muscle samples taken at rest from any given individual, a low ATP concentration of a sample compared with other samples will be indicative of the presence of non-muscle constituents. To correct concentrations to account for contamination, the mean of the two highest ATP concentrations was taken to be representative of the actual concentration for that individual. The resulting value was then divided by the ATP concentration of each sample to produce a 'correction factor' for each sample. The correction factor was then multiplied with the PCr, Cr and glycogen concentrations to give 'ATP-corrected' concentrations. In Chapter 6, muscle samples were obtained at rest, during exercise and immediately following exhaustive exercise. To remove any influence of exercise upon the relative concentration of ATP between resting and

exercised samples, the correction factor was determined for samples obtained within each time point. That is, samples obtained at rest were corrected based upon the mean of the highest 2 ATP concentrations of all of a subject's resting samples, and not of samples from rested and exercised muscle. Correction was similar for samples obtained during exercise and immediately following exhaustion.

2.11 Statistical analysis

The data presented as area under curve (AUC) in Chapter 4 were calculated from the glucose and insulin concentrations of each subject over the measurement period. A curve of best fit was constructed for each series of subject data using a Least Squares method. A third-order polynomial equation was used to predict the curve of best fit, and the area under the curve was found by calculating a definite integral from a lower to an upper value for each consecutive data point and totalling all area values in the measurement period. All AUC analysis was performed using graph analysis software (Kaleidagraph V 3.0.2, Synergy Software, Reading, USA).

In Chapters 3, 4, and 5, results are presented as mean values with standard errors of the mean (S.E.M.). In Chapter 6, results are presented as mean values with standard deviations from the mean (SD) to allow clear interpretation of the clinical data presented therein. In Chapters 3, 4, 5 and 6, comparison of data across experimental groups was assessed for statistical significance using analysis of variance (ANOVA; 2-way ANOVA for treatment and time effects, 1-way ANOVA for treatment or time effects). Significant differences revealed by ANOVA were scrutinised further using, where appropriate, Fisher's Protected

Least Significant Difference t-test and Student's unpaired t-test. Comparison of data within an experimental group was analysed for statistical significance using Student's paired t-test. Where correlations between variables are reported, a Pearson's correlation coefficient was determined and the statistical significance of the relationship determined by linear regression. These statistical tests were conducted using commercially available statistical analysis software (SuperAnova V 1.11, Abacus Concepts Inc., Berkeley, CA, USA; Excel V 5.0, Microsoft Corporation, Redmond, WA, USA).

2.12 Coefficient of variance for analytical measurements

The coefficients of variance (CV %) for measurements of metabolite and substrate concentrations of blood and muscle samples are presented in Table 2.1. The CV % was determined by repeated measurements (n = 10) of the same sample and was calculated as follows:

$$CV\ \% = \frac{\text{standard deviation of repeated measurement}}{\text{mean of repeated measurement}} \bullet 100$$

Table 2.1 Coefficient of variance for analytical measurements

Measurement	n	CV %
Blood		
Glucose (Hemocue AB)	10	2.1
Glucose (YSI 2300)	10	1.1
Lactate	10	0.9
Muscle		
ATP	10	0.6
Cr	10	1.7
PCr	10	0.9
Glycogen	10	1.1
(spectrophotometric method)		
Blood flow	7	13.7

Chapter 3

EFFECTS OF EXHAUSTIVE EXERCISE AND CREATINE AND CARBOHYDRATE SUPPLEMENTATION UPON SKELETAL MUSCLE ACCUMULATION OF CREATINE AND GLYCOGEN

3.1 INTRODUCTION

In their paper describing the effects of Cr supplementation upon muscle Cr accumulation, Harris *et al* (1992) reported that performance of exercise during a period of Cr supplementation increased the magnitude of accumulation. Submaximal exercise performed by 5 subjects immediately prior to Cr ingestion on each day of supplementation resulted in 9% greater TCr concentration in exercised muscle than in non-exercised muscle. It was postulated that this effect may have been brought about by an exercise-induced increase in limb blood flow to the exercised muscle and thereby Cr delivery, or by a change in the kinetics of muscle Cr transport (Harris *et al*, 1992). It should be noted, however, that the differences observed between limbs were relatively small and highly variable between individuals. Furthermore, the greatest exercise induced increase in muscle Cr accumulation was observed in two vegetarian subjects. Preliminary evidence indicates that the magnitude of muscle Cr retention during Cr supplementation is, at least initially, markedly greater in vegetarians compared with non-vegetarians (Green *et al*, 1997). It would appear, therefore, that the small number of subjects, the variation in the supplementation protocol and the inclusion of vegetarians in the study of Harris *et al* (1992) has not provided clear insight into the effect of exercise on muscle Cr accumulation. Subsequent investigation of the effect of 1 h submaximal exercise performed during a period of Cr + CHO supplementation showed no improvement in whole body Cr retention (Green *et al*, 1996c). However, the authors proposed that any stimulatory effect of exercise might have been overshadowed by the magnitude of the response to ingesting Cr with CHO. Creatine supplementation for 10 days, combined with exercise training, has been reported to produce improvements in

high intensity running performance in rats (Brannon *et al*, 1997). Although no difference in muscle Cr concentration was observed between training + Cr-supplemented and non-training + Cr-supplemented rats, it was suggested that the improvements in performance in the former group were attributable to changes in muscle Cr + PCr stores and an increase in muscle citrate synthase activity. During the initial 10-day Cr loading period of this study, exercise was restricted to submaximal treadmill running for only 7 $min.d^{-1}$, for familiarisation purposes. This relatively short exercise duration may not have been sufficient to study a true influence of exercise upon Cr accumulation. Presently there is inconclusive evidence of any effect of exercise on muscle Cr accumulation. It is pertinent, therefore, to examine more closely the influence of exercise upon muscle Cr accumulation in humans.

Whole-body Cr retention (Green *et al*, 1996c) and muscle TCr concentration (Green *et al*, 1996a) increases when large amounts of simple CHO are ingested in conjunction with Cr. An observation of the former study was that consumption of CHO with Cr resulted in a greater increase in body mass than when individuals consumed Cr alone. Further investigation of this supplementation regimen (Green *et al*, 1996b, Green, 1996) also revealed a tendency for greater glycogen accumulation in muscle when Cr + CHO was ingested, compared with Cr or CHO only. Furthermore, a significant correlation between the extent of muscle glycogen and TCr accumulation following Cr + CHO supplementation was observed ($r = 0.75$, $n = 8$, $p < 0.05$). This correlation was not evident when Cr or CHO alone were ingested. This finding is of interest, particularly as pre-exercise availability of muscle glycogen is a principal determinant of endurance exercise performance (Bergström *et al*, 1967).

The classically accepted method of increasing muscle glycogen stores is by 'glycogen loading', which involves depletion of muscle glycogen, usually by exercise, followed by consumption of a high CHO diet for several days (e.g. Bergström & Hultman, 1966; Sherman & Costill, 1984). Methods such as these can increase muscle glycogen concentrations to between 150% and 200% of normal resting levels. This response has been attributed to an increase in glycogen synthase activity in the post-exercise period and an exercise-induced increase in insulin sensitivity of the glycogen depleted muscle (Bergström & Hultman, 1966). Based on the proposed relationships between exercise and Cr accumulation (Harris *et al*, 1992), between muscle Cr and glycogen accumulation during Cr + CHO supplementation (Green *et al*, 1996b) and between glycogen-depleting exercise and subsequent glycogen resynthesis (Bergström & Hultman, 1966), it is of interest whether glycogen-depleting exercise could influence subsequent muscle Cr accumulation and whether the glycogen supercompensation response could be augmented by Cr ingestion.

Aims

The principal aims of this study were to examine a number of factors that have been proposed to increase muscle Cr accumulation in humans. Firstly, to determine whether exercise enhanced muscle Cr accumulation, a 1-legged cycling model of exercise was used to allow comparison of Cr accumulation between exercised and non-exercised muscle. In an attempt to stimulate insulin-mediated Cr transport, CHO was administered with Cr, which provided further opportunity to examine the relationship between muscle Cr and glycogen accumulation observed during Cr + CHO supplementation.

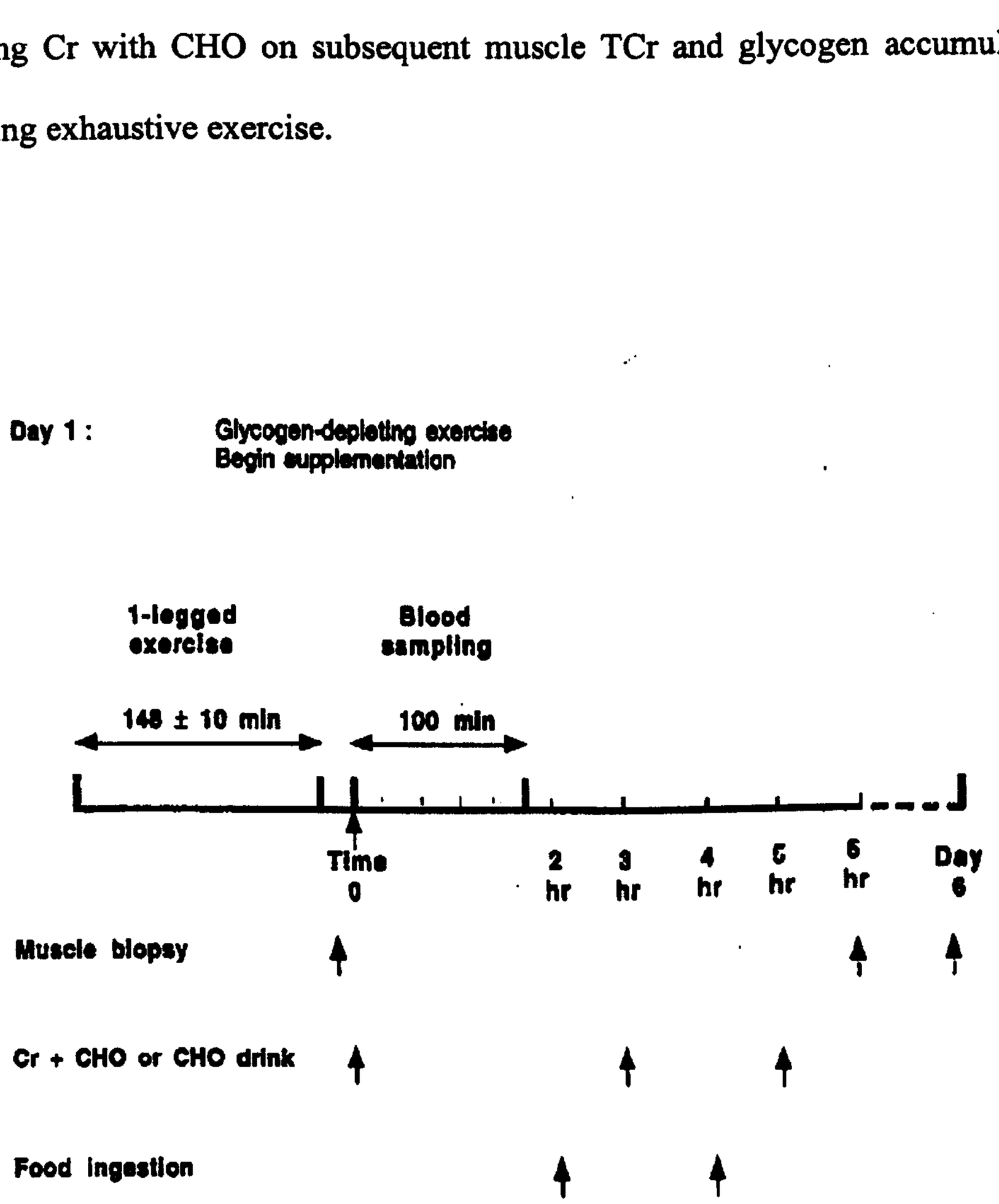
3.2 METHODS

Fourteen healthy males (Means (± S.E.M.); age 23 (1) yr., BMI 22 (0.5) $kg.m^{-2}$) participated in the study. All subjects were moderately active and none were highly trained. Subjects reported to the laboratory on the morning of the study following an overnight fast, having abstained from alcohol consumption and strenuous exercise for 48 h.

Protocol and treatment groups

An outline of the experimental protocol is shown in Fig. 3.1. Subjects, in pairs, performed one-legged cycling exercise on a cycle ergometer, one subject on either side of the ergometer, supporting themselves with their contralateral leg (Bergström & Hultman, 1966). A cycling cadence of 70 rpm was maintained and subjects worked against a load predetermined to raise heart rate to between 160 and 170 bpm. Consumption of water was allowed *ad libitum* throughout the exercise. Subjects continued cycling until near fatigue, when they were allowed to rest for a maximum of 5 min. This was followed by short periods of cycling and rest until the required cadence could not be maintained for longer than 2 min, or until a subject chose not to continue. If one subject exercised longer than his partner did, an investigator continued cycling in the partner's position. Upon exhaustion, subjects rested supine on a recovery bed and a muscle biopsy sample (Post-ex) was obtained from the vastus lateralis of the exhausted (EX) and non-exhausted (NEX) limbs, using the needle biopsy technique (Bergström, 1962). Further muscle samples were also obtained from each of the subjects' limbs six hours post-exhaustion (6 h) and 5 days later (5 day). Following the first muscle

Figure 3.1 Outline of an experimental protocol to determine the effect of ingesting Cr with CHO on subsequent muscle TCr and glycogen accumulation following exhaustive exercise.



biopsy, a cannula was inserted into an antecubital vein of the subject's arm and 8 ml blood samples were obtained at 20 min intervals for a period of 100 min. The cannula was kept patent during the sampling period using an isotonic saline drip. Following exercise, subjects were randomly allocated into two dietary treatment groups:

CHO group consisted of 7 subjects (age 24 (2) yr., BMI 21.3 (0.3) $kg.m^{-2}$). During the initial 6 hours of recovery from exercise each subject ingested a warm sugar-free drink, followed immediately by 500 ml of a drink containing approximately 93 g CHO (Lucozade™) on 3 separate occasions, as described below.

Cr + CHO group consisted of 7 subjects (age 23 (2) yr., BMI 22.5 (0.8) $kg.m^{-2}$). During the initial 6 hours of recovery from exercise each subject consumed 5 g creatine monohydrate powder (Experimental and Applied Sciences, Golden, Colorado, USA) dissolved in a warm sugar-free drink, followed immediately by 500 ml Lucozade™ on 3 separate occasions, as described below.

The first supplement was ingested immediately following collection of a post-exercise blood sample (0 min), which occurred within 30 min of the end of the exercise and Post-ex biopsy sample. In the 6 hour period of recovery from exercise, prior to further muscle sampling, subjects ingested two additional doses of their respective supplements at 2 h intervals (i.e. 3 h and 5 h after the initial supplement). In addition to this, all subjects consumed a small meal, containing approximately 70 g CHO per meal, 1 h prior to supplement dose 2 and 3 (i.e. 2 h and 4 h after the initial supplement).

Subjects repeated ingestion of the supplements four times each day (see Chapter 2), for a further four days. In addition to the supplements, all subjects were

provided with high CHO foodstuffs (i.e. potatoes, rice and cereal) and dietary advice to maintain a diet of high CHO content (CHO contributing > 80% daily energy intake). During this period all subjects followed their normal daily activities, but did not perform any strenuous exercise.

Muscle sample treatment and analysis.

Muscle biopsy samples obtained were treated and analysed for Cr, PCr and glycogen concentrations, as described in Chapter 2.

Blood sample treatment and analysis.

It has previously been observed that exhaustive exercise, such as that employed during 'glycogen loading', can result in a diminished insulin response to ingested glucose (Bloom *et al*, 1976; Galbo, 1985; Ivy *et al*, 1985). In order to ascertain whether this occurred in the present investigation, the blood sampling protocol outlined above was repeated during a separate visit to assess subjects' non-exercised responses to the supplements ingested. Analysis of blood samples for glucose and insulin concentrations was performed as described in Chapter 2.

3.3 RESULTS

Subjects reported compliance with all aspects of the protocol and did not report any ill effects. There was no significant body mass change of the CHO group following 5 days of supplementation, however, body mass of the Cr + CHO group increased significantly (pre-supplementation 72.8 (4.6) kg, post-supplementation 73.8 (4.6) kg; $P < 0.05$).

Muscle metabolite and substrate changes.

No change in muscle ATP concentration was observed in limbs of either group throughout the study (Table 3.1).

No significant changes in muscle TCr concentration were observed in the EX and NEX limbs of either group 6 h following one legged exercise (Fig. 3.2) or in the limbs of the CHO group after 5 days (Fig. 3.2a). There was a significant increase in muscle TCr concentration in the EX (23% increase; $P < 0.001$) and NEX (14% increase; $P < 0.05$) limbs of the Cr + CHO group following 5 days of supplementation, with the EX limb having a 68% greater accumulation ($P < 0.01$; Fig. 3.2b).

In accordance with the changes in muscle TCr concentration, no changes in PCr or Cr concentration were observed in limbs of the CHO group at any time. A significant increase in PCr concentration was observed in the NEX limb of the Cr + CHO group following 5 days of supplementation (Table 3.1) and there was also a significant increase in Cr concentration in the EX limb of this group (all $P < 0.05$).

The exercise protocol almost completely depleted glycogen in the EX limbs of the CHO group and Cr + CHO group (to 7% and 5% of the concentration measured in the NEX limb post-exercise, respectively; $P < 0.001$; Fig. 3.3). Muscle glycogen concentration of the NEX limb after the exercise period was used as a measure of the initial glycogen concentration of the EX limb, as it has been previously shown that this one-legged cycling protocol does not affect muscle glycogen concentration in the supporting leg (Casey *et al*, 1995).

Table 3.1 Muscle metabolite concentrations (mmol.kg^{-1} d.m.) of the exhausted (EX) and non-exhausted (NEX) limbs of CHO and Cr + CHO treated groups immediately after one-legged exercise (Post-ex) and 6 h and 5 days after supplementation. Values are mean concentrations (± S.E.M.).

		EX			NEX		
		Post-ex	6 h	5 day	Post-ex	6 h	5 day
CHO	ATP	24.3 (0.6)	23.9 (0.3)	24.3 (0.4)	24.9 (0.4)	24.3 (0.4)	24.6 (0.5)
group	PCr	89.9 (4.6)	89.1 (1.9)	83.4 (2.1)	84.6 (1.7)	87.2 (1.4)	83.2 (2.5)
	Cr	41.3 (1.6)	44.5 (2.2)	42.1 (2.2)	42.4 (1.5)	41.8 (1.4)	42.6 (1.8)
Cr +	ATP	23.9 (0.9)	24.2 (0.5)	22.4 (0.7)	24.5 (0.5)	23.6 (1.0)	24.0 (1.0)
CHO	PCr	84.4 (4.5)	90.8 (2.6)	97.6 (2.5)	78.8 (4.1)	81.6 (4.2)	89.9 (1.6)*
group	Cr	41.7 (4.6)	43.4 (5.1)	57.1 (3.1)*	42.9 (4.6)	39.1 (3.2)	48.8 (2.7)

* $P < 0.05$, significant differences from post-exercise concentrations.

Figure 3.2 Muscle total creatine (TCr):

a) from the exhausted (EX) and non-exhausted (NEX) limbs of the CHO group post one-legged exercise and 6 h and 5 days after CHO supplementation;

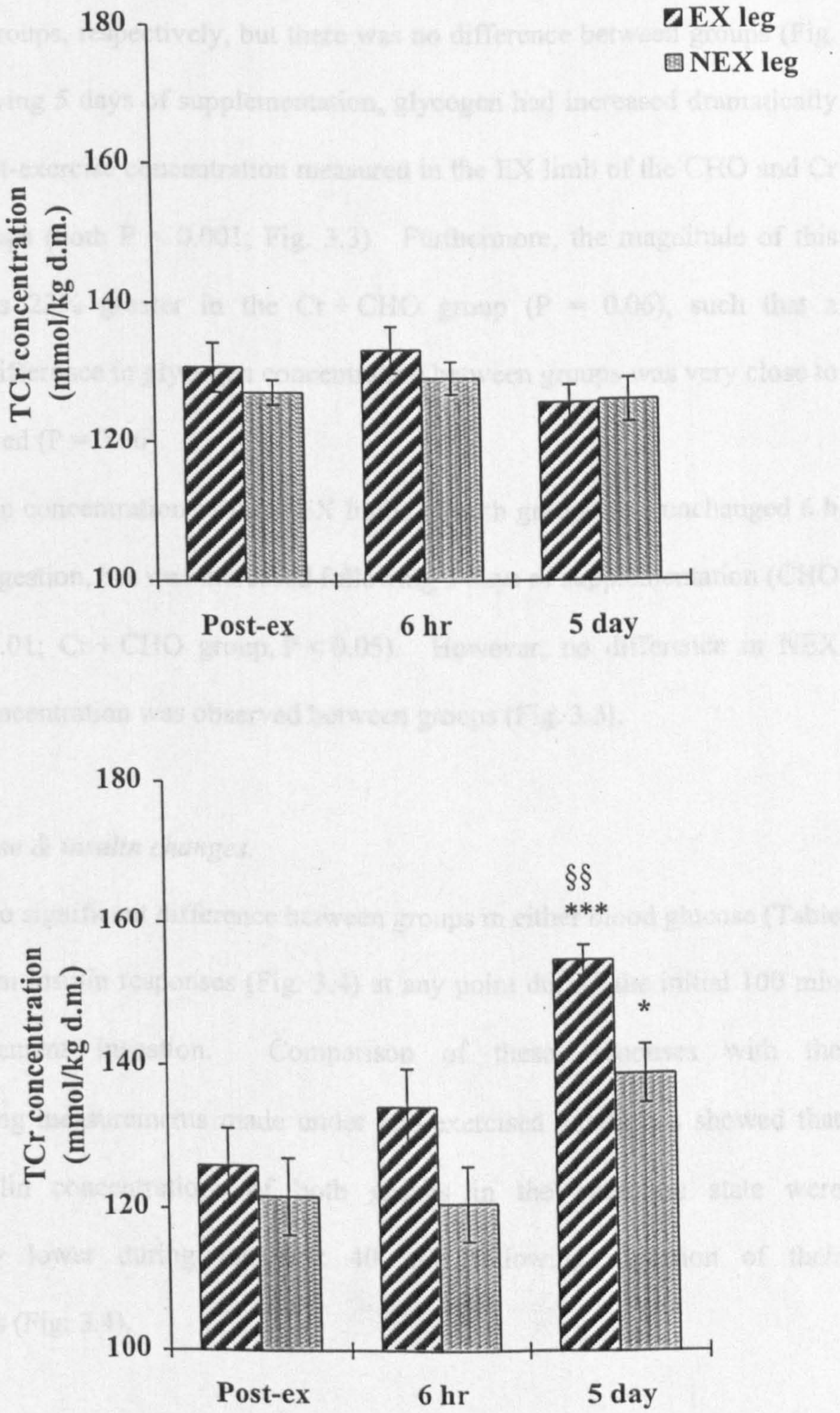
b) from the EX and NEX limbs of the Cr + CHO group post one-legged exercise and 6 h and 5 days after Cr + CHO supplementation.

Values represent means ± S.E.M.

* $P < 0.05$ and *** $P < 0.001$, significant differences from post-ex concentration;

§§ $P < 0.01$, significant difference between EX and NEX limbs after 5 days of supplementation.

Fig. 3.2


a
180
160
140
120
100
TCr concentration (mmol/kg d.m.)
EX leg
NEX leg
Post-ex
6 hr
5 day
b
180
160
140
120
100
TCr concentration (mmol/kg d.m.)
§§
***
*
Post-ex
6 hr
5 day
Time sample taken

Six hours following ingestion of the first supplement, glycogen had been restored in the EX limb to 68% and 72% of non-exhausted concentration in the CHO and Cr + CHO groups, respectively, but there was no difference between groups (Fig. 3.3). Following 5 days of supplementation, glycogen had increased dramatically from the post-exercise concentration measured in the EX limb of the CHO and Cr + CHO groups (both $P < 0.001$; Fig. 3.3). Furthermore, the magnitude of this increase was 23% greater in the Cr + CHO group ($P = 0.06$), such that a significant difference in glycogen concentration between groups was very close to being achieved ($P = 0.06$).

The glycogen concentration of the NEX limbs of both groups was unchanged 6 h following ingestion, but was increased following 5 days of supplementation (CHO group, $P < 0.01$; Cr + CHO group, $P < 0.05$). However, no difference in NEX glycogen concentration was observed between groups (Fig. 3.3).

Blood glucose & insulin changes.

There was no significant difference between groups in either blood glucose (Table 3.2) or serum insulin responses (Fig. 3.4) at any point during the initial 100 min post supplement ingestion. Comparison of these responses with the corresponding measurements made under non-exercised conditions showed that serum insulin concentrations of both groups in the exercised state were significantly lower during the first 40 min following ingestion of their supplements (Fig. 3.4).

Figure 3.3 Muscle glycogen concentrations in the EX and NEX limbs post one-legged exercise and 6 h and 5 days after carbohydrate (CHO group) or creatine and carbohydrate (Cr + CHO group) supplementation.

Values represent means ± S.E.M.

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, significant differences from post-ex concentration of the same leg.

Fig. 3.3

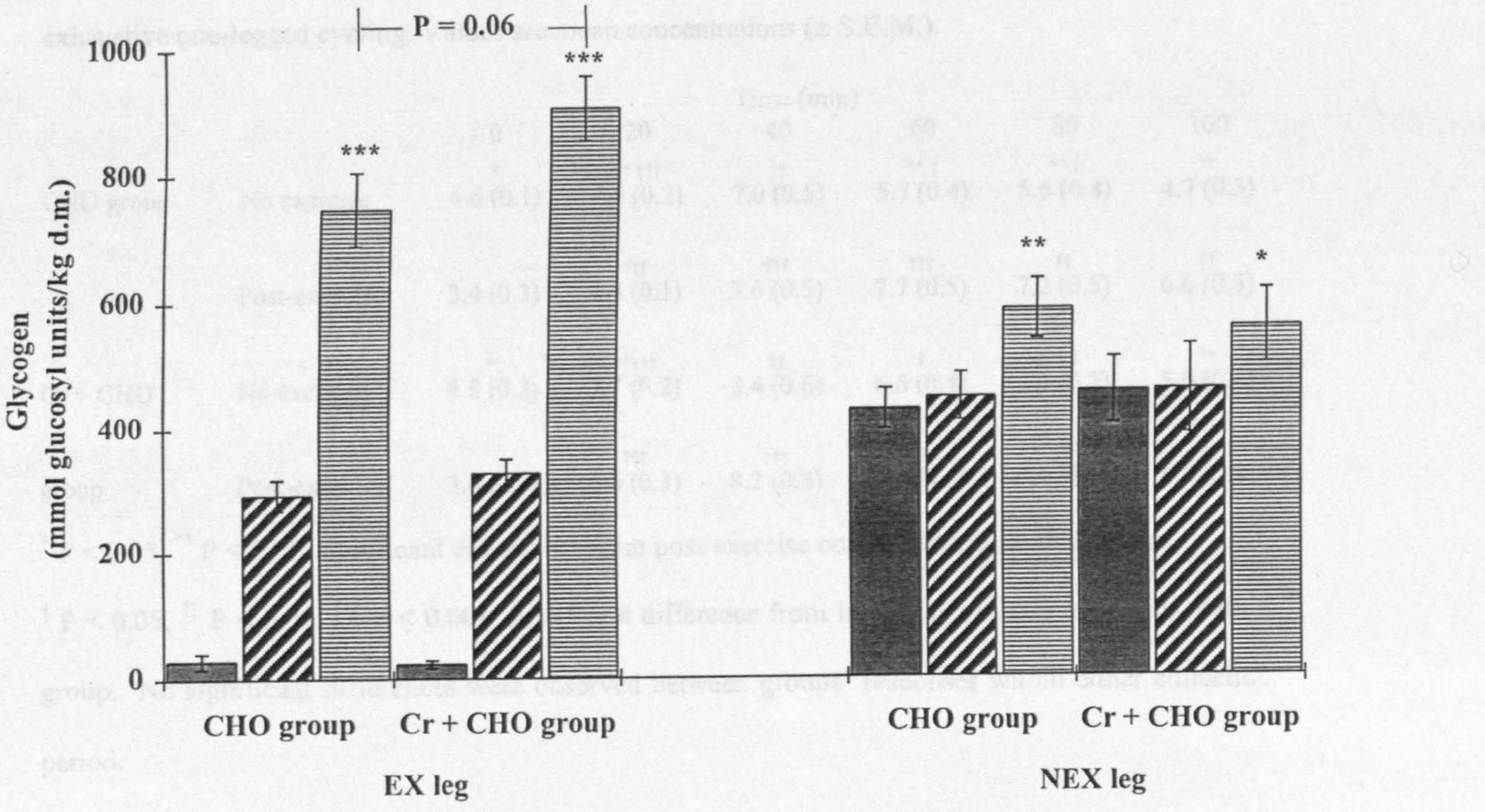

Post-ex
6 hr
5 day
P = 0.06
***
***
**
*
1000
800
600
400
200
0
Glycogen
(mmol glucosyl units/kg d.m.)
CHO group
Cr + CHO group
CHO group
Cr + CHO group
EX leg
NEX leg

Table 3.2 Whole blood glucose concentrations ($mmol.L^{-1}$) of CHO, and Cr + CHO groups for 100 min following ingestion of 500 ml CHO solution, under non-exercised conditions, and following exhaustive one-legged cycling. Values are mean concentrations (± S.E.M.).

		Time (min)					
		0	20	40	60	80	100
CHO group	No exercise	* 4.6 (0.1)	** ††† 7.6 (0.2)	†† 7.0 (0.5)	** † 5.7 (0.4)	** † 5.6 (0.4)	** 4.7 (0.3)
	Post-exercise	3.4 (0.3)	††† 6.4 (0.1)	††† 7.6 (0.5)	††† 7.7 (0.5)	†† 7.2 (0.5)	†† 6.6 (0.5)
Cr + CHO	No exercise	** 4.8 (0.2)	**††† 7.7 (0.2)	†† 8.4 (0.6)	† 6.6 (0.4)	** † 5.5 (0.2)	** 5.0 (0.4)
group	Post-exercise	3.6 (0.2)	††† 6.6 (0.3)	††† 8.2 (0.3)	††† 8.0 (0.5)	††† 7.1 (0.3)	††† 6.8 (0.4)

* $P < 0.05$, ** $P < 0.01$, significant differences from post-exercise concentrations, within group.

† $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$, significant difference from initial concentration (0 min), within group. No significant differences were observed between groups' responses within either collection period.

Figure 3.4 Serum insulin concentrations:

a) of CHO group subjects at rest and after exhaustive exercise in response to ingestion of 500 ml CHO solution (~ 90 g CHO). * $P < 0.05$, significantly different from post-exercise concentration.

b) of Cr + CHO group subjects at rest and after exhaustive exercise in response to ingestion of 5 g creatine monohydrate and 500 ml CHO solution.

Values represent means ± S.E.M.

* $P < 0.05$ and ** $P < 0.01$, significant differences from post-exercise concentration.

3.4 DISCUSSION

A major finding of the present study was the difference in TCr concentration between the EX and NEX limbs of the Cr + CHO group after 5 days of supplementation. This demonstrates that a single bout of exhaustive exercise prior to Cr supplementation can markedly augment skeletal muscle Cr accumulation and that this response is restricted to the exercised muscle, which may be of practical importance. The present observation supports the indication of Harris *et al* (1992) that exercise can augment muscle Cr accumulation. In their investigation, the authors proposed that exercise increased muscle Cr uptake by increasing blood flow to the exercised muscle during the post-exercise period, or by increasing the kinetics of muscle Cr transport. The authors implied that increasing blood flow to the exercised muscle would maximise the exposure of that muscle to Cr concentrations above the Michaelis constant (K_M) for its transport. Although muscle blood flow was not measured in the present investigation, these results suggest that an increased blood flow following exercise was unlikely to have been responsible for the greater Cr content of the EX limb in the present study. This contention is supported firstly by the lack of any difference in TCr concentration between limbs after 6 hours of supplementation, when differences in limb blood flow would be expected to be at their greatest. Secondly, evidence demonstrating that the K_M of muscle Cr transport is substantially lower (20 - 110 $\mu mol.L^{-1}$; Loike *et al*, 1986; Nash *et al*, 1994; Odoom *et al*, 1996) than the value of 500 $\mu mol.L^{-1}$ proposed by Fitch & Shields (1966), and cited by Harris *et al* (1992), makes it unlikely that Cr availability will limit transport using conventional regimens of Cr supplementation.

Creatine transport into muscle cells is dependent upon the presence of extracellular Na^+ (Daly & Seifter, 1980). A specific Cr transporter has been identified and cloned (Guimbal & Kilimann, 1993; Mayser *et al*, 1992; Nash *et al*, 1994), which is highly expressed in skeletal muscle. After 5 days of Cr + CHO supplementation, there was an 11% difference in the TCr concentration between the EX and NEX limbs in the current study, which is in agreement with a 9% difference between limbs previously observed (Harris *et al*, 1992). Given that both limbs were exposed to the same plasma Cr concentration over the 5 days of supplementation, it is feasible that exercise may have enhanced Cr accumulation by increasing the maximal rate (V_{max}) of Cr transport in the exercised limb. Indeed, exercise has been demonstrated to enhance the transport of other amino acids (Biolo *et al*, 1995), which has been suggested to be only partly dependent upon increased muscle blood flow (Tipton & Wolfe, 1998). An exercise induced augmentation of muscle Cr transport may have occurred by a number of as yet unknown mechanisms. For example, Odoom *et al* (1996) have suggested that an increase in the V_{max} of Cr transport may occur as a result of an allosteric effect on the transporter itself, by recruitment or synthesis of new transporters, or by producing changes in the forces driving Cr transport. In support of the latter suggestion, it has been proposed that the majority of Cr transport is achieved by a Na^+ - Cr co-transport system (Loike *et al*, 1986; Odoom *et al*, 1996), which makes use of the sarcolemmal Na^+/ K^+ pumps. It has been suggested that sarcolemmal Na^+/ K^+ pump total activity can be increased through either an increase in intrinsic pump activity, or by a net gain in the number of pumps (Odoom *et al*, 1996). Exercise training has been demonstrated to produce increases in the number of muscle Na^+/ K^+ pumps, which attenuates the loss of K^+ from muscle during

subsequent exercise (McKenna *et al*, 1996, 1997). In humans, Green and colleagues (1993) have shown that exercise training of sufficient duration and intensity (2 $h.d.^{-1}$, 65% VO_{2max}, 6 d) can upregulate Na^+/ K^+ pump concentration within the first week of training. Additionally, it has been demonstrated in rats that a single acute bout of running exercise can increase muscle concentration of some Na^+/ K^+ pump subunits in the sarcolemma and also elevate mRNA levels for additional Na^+/ K^+ pump subunits (Tsakiridis *et al*, 1996). It is therefore feasible that in the present study the single bout of exhaustive exercise produced an upregulation of net muscle Na^+/ K^+ pump activity in the EX limb which facilitated muscle Cr transport. In support of this conclusion is the finding that pharmacological activation and inhibition of Na^+/ K^+ pump activity in mouse myoblast cells was paralleled by up- and down-regulation of cellular Cr accumulation *in vivo* (Odoom *et al*, 1996).

The Na^+/ K^+ ATPase pump activity in skeletal muscle is also influenced by insulin (Hundal *et al*, 1992; Marette *et al*, 1993). The abundance of some pump subunits in the muscle membrane has been shown to increase with acute exposure to insulin, most likely by recruitment from intracellular compartments. It is likely that this change contributes to the enhancement of muscle Cr accumulation that is observed when CHO is ingested with Cr (Green *et al*, 1996a). It remains to be seen whether any interaction exists between exercise- and insulin- induced increases in total pump activity.

Total creatine accumulation in the EX muscle in the present study (28.6 ± 4.8 $mmol.kg^{-1}$ d.m.), was no greater when compared with the magnitude of accumulation previously observed in non-exercised subjects ingesting Cr + CHO (33 ± 3.4 $mmol.kg^{-1}$ d.m.; Green *et al*, 1996a). Furthermore, the magnitude

of TCr accumulation in the NEX muscle of the Cr + CHO group (17.0 ± 4.7 mmol.kg^{-1} d.m.), although significantly elevated above the initial concentration, was comparable with that achieved previously in non-exercised individuals supplemented with Cr only (20.7 ± 2.4 mmol.kg^{-1} d.m.; Green *et al*, 1996a). Given the diminished insulin response to oral CHO observed following exhaustive exercise in the present study, these findings also indirectly support a role for insulin in promoting Cr transport. Specifically, the exercise-induced blunting of CHO-mediated insulin release observed in the present study (Fig. 3.4) would be expected to negatively affect insulin-mediated stimulation of muscle Cr transport, particularly since recent work has demonstrated that high physiological concentrations of insulin are required to stimulate muscle Cr accumulation in humans (Steenge *et al*, 1998). This phenomenon is of practical importance in terms of optimising muscle TCr accumulation during supplementation i.e. individuals wishing to maximise muscle Cr transport by consuming CHO might refrain from performing exhaustive or prolonged exercise during the supplementation period.

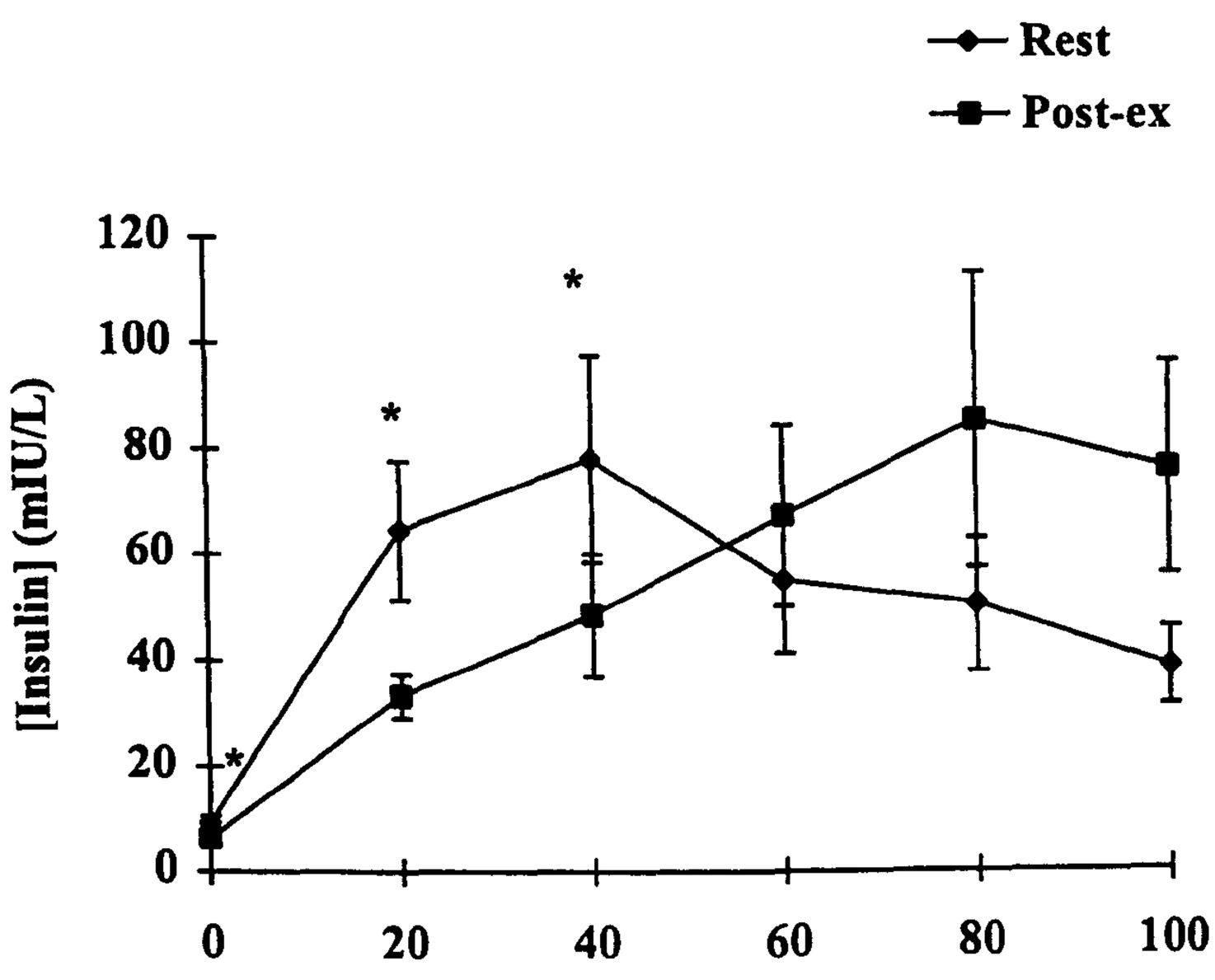
The present study found that combining Cr and CHO ingestion with prior glycogen-depleting exercise augmented glycogen resynthesis during recovery and, similar to muscle Cr accumulation, this response was restricted to the exercised muscle. This is the first documentation of this response in humans. Furthermore, the increase in glycogen concentration was of a magnitude that one would consider sufficient to produce a significant improvement in endurance exercise performance. Green *et al* (1996c) reported a significantly greater peak insulin concentration following the ingestion of Cr + CHO, compared with the ingestion of CHO alone and also showed a significant relationship between muscle Cr and

glycogen accumulation ($r = 0.75$, $n = 8$, $P < 0.05$) following 5 days of supplementation in non-exercised individuals. In the present study there was no significant difference in the glucose stimulated insulin response between groups following exercise. However, the extent of glycogen depletion produced by the present study would be expected to stimulate insulin-independent glycogen resynthesis for a major part of the initial recovery period (Price *et al*, 1994). Therefore the effect of diminished circulating insulin concentration observed following exercise might have, at least in terms of glycogen synthesis, been negligible during the early stage of resynthesis. However, the clear lack of an effect of Cr + CHO on glycogen accumulation in the NEX limb after 6 h and 5 days of recovery in the present study goes against an increase in insulin availability being responsible for the augmentation of glycogen resynthesis. Presumably one could have expected to see an increase in glycogen concentration in both limbs following Cr + CHO ingestion, albeit to a lesser extent in the NEX limb, had this mechanism been in operation. This is supported further by the lack of any difference in insulin responses between groups at rest following ingestion of their respective supplements (Fig. 3.4).

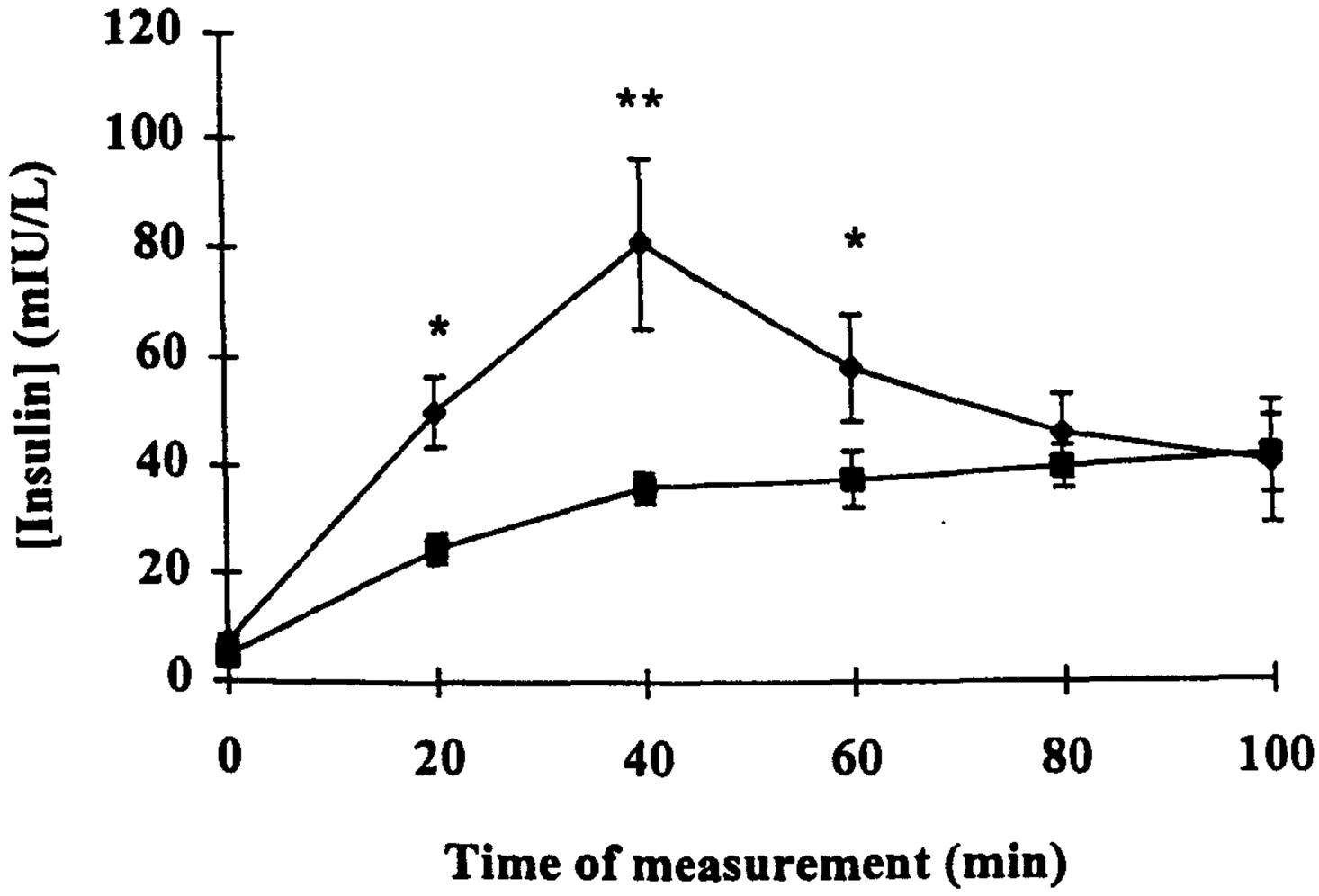
It has recently been reported that muscle glycogen synthesis is modulated by muscle cell volume changes (Low *et al*, 1996a). Osmotically-induced swelling of primary rat myotubes increased glycogen synthesis independently of changes in glucose uptake. Creatine supplementation in man has been shown to increase body mass (e.g. Greenhaff *et al*, 1994), in particular fat-free mass (Dentowski *et al*, 1997; Kreider *et al*, 1998), and has also been shown to increase body water content over the supplementation period (Dentowski *et al*, 1997). It has been suggested that Cr accumulation results in water retention by the muscle (Hultman

Fig. 3.4

a


Rest
Post-ex
[Insulin] (mIU/L)
120
100
80
60
40
20
0
*
*
*
0
20
40
60
80
100

b


[Insulin] (mIU/L)
120
100
80
60
40
20
0
*
**
*
0
20
40
60
80
100
Time of measurement (min)

et al, 1996), which would be expected to cause muscle swelling. Indeed, it has recently been reported that 3 days of Cr ingestion (~ 21 $g.d^{-1}$) significantly increased total body water and intracellular volume by 2% and 3.1%, respectively, as measured by multifrequency bioimpedence analysis (Zeigenfuss *et al*, 1998). Whilst this method of measurement is not the most sensitive for measuring fluid changes (c.f. magnetic resonance spectroscopy), these results suggest that Cr supplementation can increase muscle volume. If this is the case, it is possible that the greater accumulation of Cr in the EX limb of the Cr + CHO group produced a greater muscle cell volume change than in the corresponding limb of the CHO group, thereby contributing to the higher glycogen concentration in the Cr + CHO group following 5 days of supplementation. Such a response would also, at least partly, explain the lack of an effect of Cr + CHO on glycogen synthesis in the NEX limb, where Cr accumulation was markedly lower than previously observed following Cr + CHO ingestion (Green *et al*, 1996a).

In conclusion, this study demonstrated that exhaustive exercise could enhance creatine accumulation in exercised muscle through an as yet undetermined mechanism. This effect was restricted to the exercised muscle itself and did not appear to be due to an increase in muscle blood flow. Exhaustive exercise diminished glucose-stimulated insulin release, however, thereby attenuating any enhancement of Cr accumulation due to CHO ingestion. Finally, Cr + CHO supplementation following glycogen-depleting exercise augmented glycogen resynthesis in the exercised muscle which might be considered sufficient to significantly improve endurance exercise performance.

Chapter 4

EFFECTS OF ARGININE INGESTION UPON THE FATE OF INGESTED GLUCOSE IN RESTED AND EXERCISED HUMANS

4.1 INTRODUCTION

Ingestion of Cr with a drink containing a large amount of CHO has been shown to result in an enhanced insulin release compared with ingestion of CHO only (Green, 1996). Whilst a similar response was not observed in Chapter 3, in subjects at rest or following glycogen-depleting exercise, glycogen concentration was markedly greater in supercompensated muscle after 5 days of Cr + CHO supplementation than in comparative muscle supplemented with CHO only. When CHO is consumed after exercise, the rate of glycogen resynthesis is directly related to the insulin response to the ingested supplement (Zawadzki *et al*, 1992). Insulin promotes glucose transport into cells for glycogen synthesis, possibly by increasing GLUT4 protein content in the muscle membrane (Klip & Marette, 1992; Rodnick *et al*, 1992). Secondly, insulin activates glycogen synthase (Ivy & Holloszy, 1981), presumably via an intracellular signal for the enzyme's dephosphorylation (Connett & Sahlin, 1996). It follows that any mechanism that can enhance insulin release when a CHO beverage is ingested after exercise might improve glycogen resynthesis. The amino acid, arginine, is another guanidine-based compound which, when administered intravenously to humans, has been shown to stimulate insulin release (Floyd *et al*, 1966) and augment glucose-stimulated insulin release (Efendic *et al*, 1974). It can also induce peripheral vasodilation (Giugliano *et al*, 1997), thereby increasing muscle blood flow. As a consequence of these latter two effects, arginine (specifically L-arginine) significantly increases insulin-mediated glucose uptake (Paolisso *et al*, 1997). Dietary supplementation with relatively large amounts (~25 g) of arginine, in humans, has been found to result in higher plasma insulin concentrations after a meal than in subjects following a normal diet (Beaumier *et al*, 1995).

Different types of exercise have different effects upon muscle glycogen storage. Prolonged exercise that depletes muscle glycogen concentration results in a high rate of subsequent glycogen storage if adequate CHO is supplied (Bergström & Hultman, 1966). Exercise that has a significant eccentric component, however, can result in impairment of glycogen resynthesis (Costill *et al*, 1990; Pascoe & Gladden, 1996). There is considerable interest in the optimisation of muscle glucose uptake during exercise recovery, as evidenced by the many different CHO-containing beverages marketed for post-exercise 'energy replenishment'. Prolonged consumption (7 d) of a high-arginine diet, however, was shown to cause excessive loss of sodium in the urine, and an associated loss of free water. This water loss consequently led to a weight loss in subjects (Beaumier *et al*, 1995). High doses of arginine are also unpalatable (personal observation) and might also cause gastrointestinal discomfort in some individuals (Gater *et al*, 1992). These side-effects of high-dose arginine consumption would negatively affect individuals who ingested them in a beverage designed for post-exercise repletion of CHO stores. Based upon the observations that Cr + CHO supplementation increased muscle glycogen storage (Chapter 3) it is of interest to study whether a palatable quantity of arginine could also influence the fate of ingested glucose, in rested individuals and following different forms of exercise.

Aim

The aim of the present study was to examine the effects of ingesting a palatable dose of arginine upon the fate of glucose ingested at the same time. These effects were investigated in rested subjects and following two different types of exercise.

4.2 METHODS

Six healthy males participated in the study (mean (± S.E.M.), age 25 (2) years; BMI 25.9 (1.8) $kg.m^{-2}$). All subjects reported that they were moderately active, but were not involved in any exercise training.

Experimental protocol

On six separate occasions each subject reported to the laboratory following an overnight fast. The meal prior to each visit was identical for each subject. Upon arrival, subjects lay supine for 30 min whilst resting measurements of heart rate (HR), blood pressure (BP) and forearm blood flow (BF) were recorded and an arterialised venous blood sample was collected (see Chapter 2) for glucose and insulin determination. Immediately following this baseline measurement period, subjects were required to perform one of three 'activities' in a randomised manner:

a) 45 min laying semi-upright on an experimental bed (Rest).

b) 1 h of repeated bouts of squatting exercise, carrying a backpack containing weight equivalent to 25% of their body weight (Resistance). Each squat required the subject to bend his leg at the knee and lower the weight until the axis of the hips was parallel with the axis of the knees, followed by a return to the standing position. Subjects performed 20 repetitions of the squatting exercise in a 1-min period followed by 3 min of rest in a standing position, whereupon they repeated the squatting exercise. All subjects completed 15 bouts of this work/rest cycle in the 1 h period. Heart rate was monitored throughout the exercise period using a telemetric heart rate monitor (Polar PE3000, Polar, Kempele, Finland).

c) 1 hr cycling exercise (Cycling). Each subject cycled at a cadence of 70 rpm against a workload predetermined to elicit an oxygen consumption approximately 70% of his maximum aerobic capacity. During exercise, subjects ingested 200 ml of water every 15 min to maintain euhydration. Heart rate was monitored by a 3-lead ECG throughout the exercise period.

Each activity was aimed at modifying glucose disposal differently; resting responses were considered as a control treatment, cycling exercise was anticipated to increase muscle glucose storage and resistance exercise was expected to result in a relative decrease in the amount of glucose stored. Visits were separated by at least 1 week.

Following each activity, subjects rested supine for 30 min whilst measurements of HR, BP and BF were recorded, and samples of blood and expired air were collected. Subjects then ingested 250 ml of a sugar-free fruit drink containing, in a randomised manner, either 10 g glycine (placebo) or 10 g L-arginine. This was immediately followed by ingestion of 378 ml of a CHO solution containing 70 g simple sugars (Lucozade™). At the time of the investigation, subjects and investigators were unaware of the identity of the amino acid drink. During the subsequent 3 h post-ingestion period, blood samples were collected every 20 min, expired air samples were collected during the last 15 min of each 30 min period and HR, BP and BF were recorded during the last 8 min of each 30 min period.

4.3 RESULTS

Blood glucose

Blood glucose concentration decreased significantly following resistance exercise prior to arginine ingestion (Fig. 4.1b; $P < 0.05$), and following cycling exercise (Fig. 4.1c; $P < 0.001$) prior to both treatments. Following CHO ingestion, blood glucose concentration increased significantly in rested subjects and following resistance and cycling exercise (all $P < 0.001$). Glucose concentration peaked 40 min after CHO ingestion following all activities (Table 4.1) and gradually declined toward resting concentration. Glucose concentration was no longer significantly different from resting concentration 140 min, 160 min and 140 min following CHO ingestion in subjects when rested, following resistance exercise or cycling exercise, respectively. This response following arginine ingestion was no different from when placebo was ingested (Fig. 4.1a,b,c). The area under blood glucose concentration / time curve (Glucose AUC), following any activity, was no different following arginine ingestion than when placebo was ingested (Table 4.1).

Serum insulin

One subject involved in the investigation demonstrated a hyperinsulinaemic response to the ingested CHO on all occasions. The pattern of insulin response to ingested CHO was similar to the other subjects, however, insulin concentration was 3 times greater than the mean of the rest of the group. To avoid misinterpretation of the results, data for that subject's insulin response were not included in the analysis or reports of the group's response to the treatments.[1]

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[1] Refer to note 4.1 at end of Chapter.

Figure 4.1 Whole blood glucose concentration prior to activity and during a 3 h period following ingestion of 70 g CHO and either 10 g placebo (filled symbols) or arginine (open symbols) in subjects (n = 6):

a) without prior exercise (Rest)

b) following resistance exercise (Resistance)

c) following cycling exercise (Cycling)

Values represent mean ± S.E.M.

Arrows indicate time of drink ingestion.

* $P < 0.05$, significant difference from pre-activity concentration, prior to arginine ingestion. *** $P < 0.001$ significant difference from pre-activity concentration, prior to both treatments. ††† $P < 0.001$, significant increase from post-activity, pre-ingestion concentration, both treatments.

Fig. 4.1

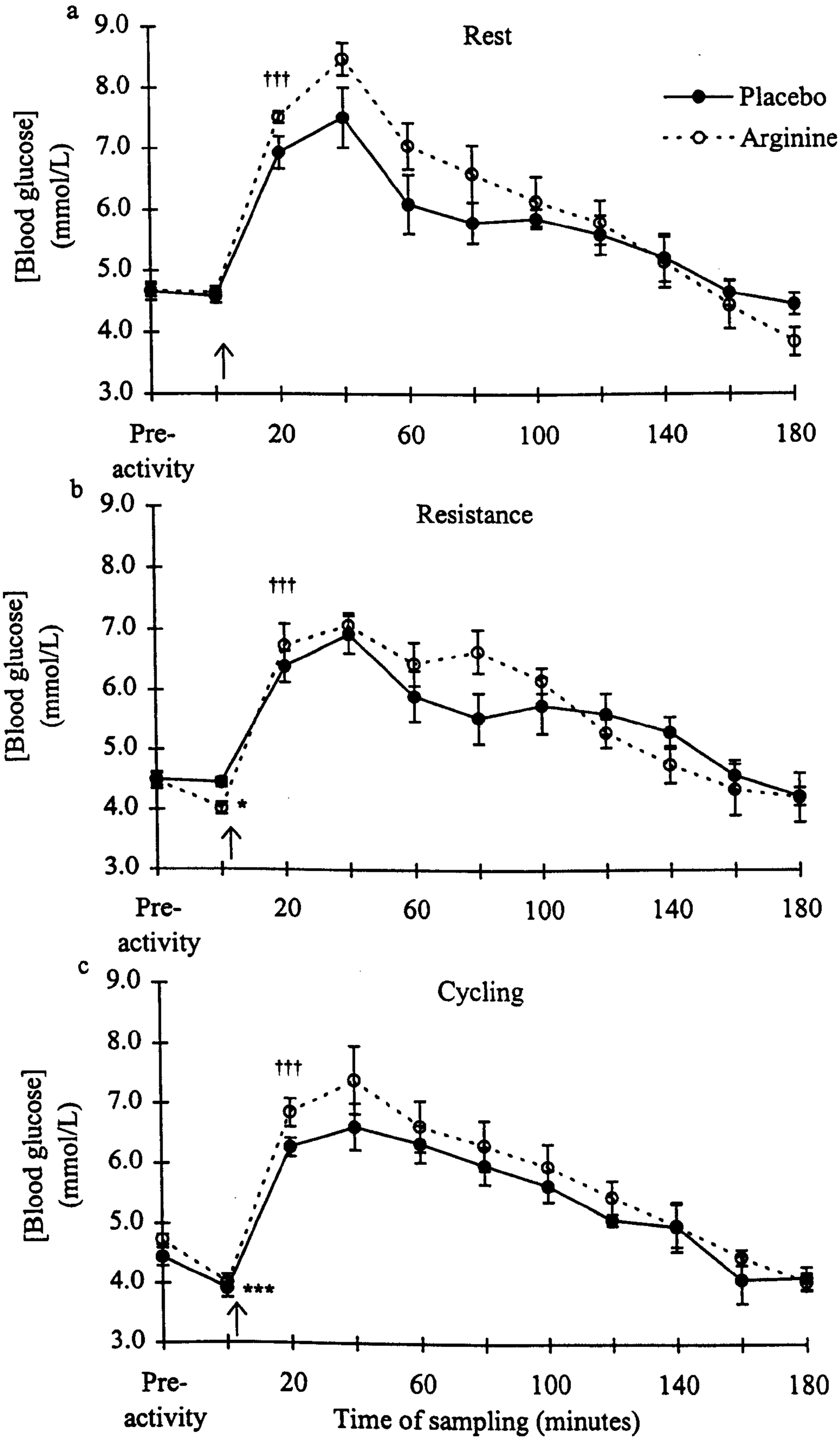

a
Rest
[Blood glucose] (mmol/L)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
†††
Placebo
Arginine
Pre-activity
20
60
100
140
180
b
Resistance
[Blood glucose] (mmol/L)
†††
*
c
Cycling
[Blood glucose] (mmol/L)
†††
***
Time of sampling (minutes)

Table 4.1 Blood glucose and insulin responses (area under curve, AUC) and peak glucose and insulin concentrations during 3 h of exercise recovery following CHO and either placebo (Pla) or arginine (Arg) ingestion.

Values are mean (± S.E.M.), n = 6 (* n = 5).

	Rest		Resistance		Cycling	
	Pla	Arg	Pla	Arg	Pla	Arg
Glucose AUC	1037	1100	999	1028	968	1031
(mmol/L/3h)	(35)	(23)	(44)	(28)	(34)	(31)
Insulin AUC*	5608	5464	4881	5028	4459	4274
(mIU/L/3h)	(565)	(886)	(410)	(598)	(359)	(251)
Peak glucose	7.5	8.48	6.9	7.1	6.62	7.4
(mmol/L)	(0.5)	(0.3)	(0.3)	(0.2)	(0.4)	(0.6)
Peak insulin*	53	58	49	59	42	43
(mIU/L)	(9)	(17)	(5)	(13)	(4)	(13)

Serum insulin significantly increased in response to CHO ingestion after all activities (all $P < 0.001$; Fig. 4.2), peaked after 40 min and had returned to resting concentrations by the end of the measurement period ($P > 0.05$ at 180 min, all conditions). No difference in peak insulin concentration was observed between activities, nor were there any differences in peak insulin concentration within any activity when arginine was ingested. No significant difference was observed, between insulin concentrations following arginine or placebo ingestion, at any time point during the measurement period. The area under the insulin concentration / time curve (Insulin AUC), over the post-ingestion period following each activity was no different following arginine ingestion than when placebo was ingested (Table 4.1)

Carbohydrate oxidation

Total CHO oxidation over the 3 h post-ingestion period (Fig. 4.3) was significantly lower following resistance ($P < 0.01$) and cycling exercise ($P < 0.001$), compared with CHO oxidation at rest. Within any activity, there was no significant difference in CHO oxidation when arginine was consumed, compared with placebo ingestion, although a consistent tendency for less CHO oxidation was apparent (Fig. 4.3).

Forearm blood flow

Forearm blood flow did not change from resting, pre-ingestion levels following ingestion of CHO and either supplement when subjects were rested or after they had performed resistance exercise (Fig. 4.4a,b). Forearm blood flow increased significantly above pre-exercise levels after cycling ($P < 0.01$). The rate of

Figure 4.2 Serum insulin concentration prior to activity and during a 3 h period following ingestion of 70 g CHO and either 10 g placebo (filled symbols) or arginine (open symbols) in subjects (n = 5):

a) without prior exercise (Rest)

b) following resistance exercise (Resistance)

c) following cycling exercise (Cycling)

Values represent mean ± S.E.M.

Arrows indicate time of drink ingestion.

††† $P < 0.001$, significant increase from post-activity, pre-ingestion concentration, both treatments.

Fig. 4.2

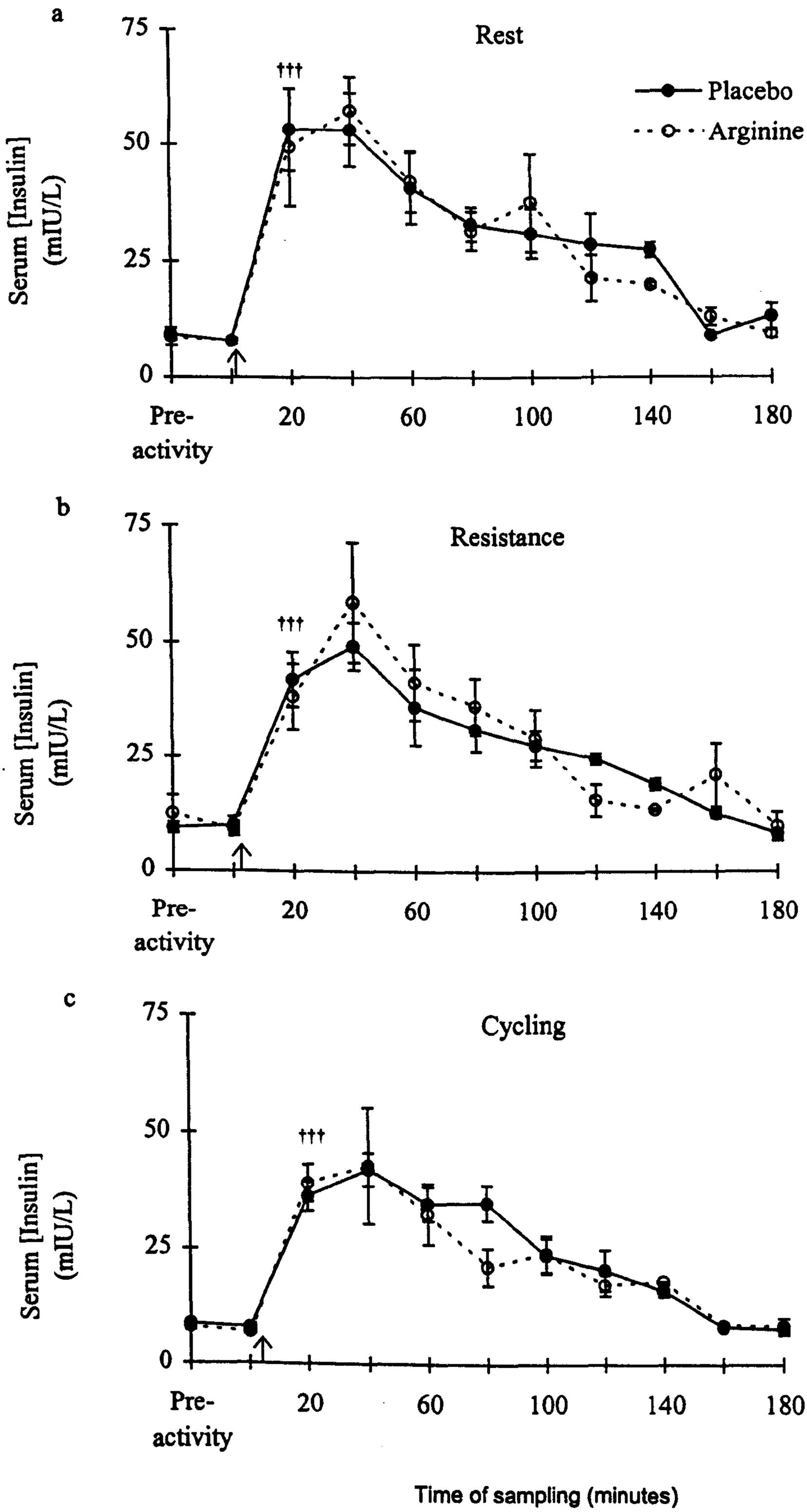

a
Rest
Placebo
Arginine
Serum [Insulin] (mIU/L)
75
50
25
0
†††
Pre-activity
20
60
100
140
180
b
Resistance
c
Cycling
Time of sampling (minutes)

Figure 4.3 Total CHO oxidation during a 3 h period following ingestion of 70 g CHO and either 10 g placebo (solid bars) or arginine (hatched bars) in subjects (n = 6) without prior exercise (Rest), following resistance exercise (Resistance), and following cycling exercise (Cycling).

Values represent mean ± S.E.M.

** $P < 0.01$, *** $P < 0.001$, significantly different from rested values, both treatments

Fig. 4.3

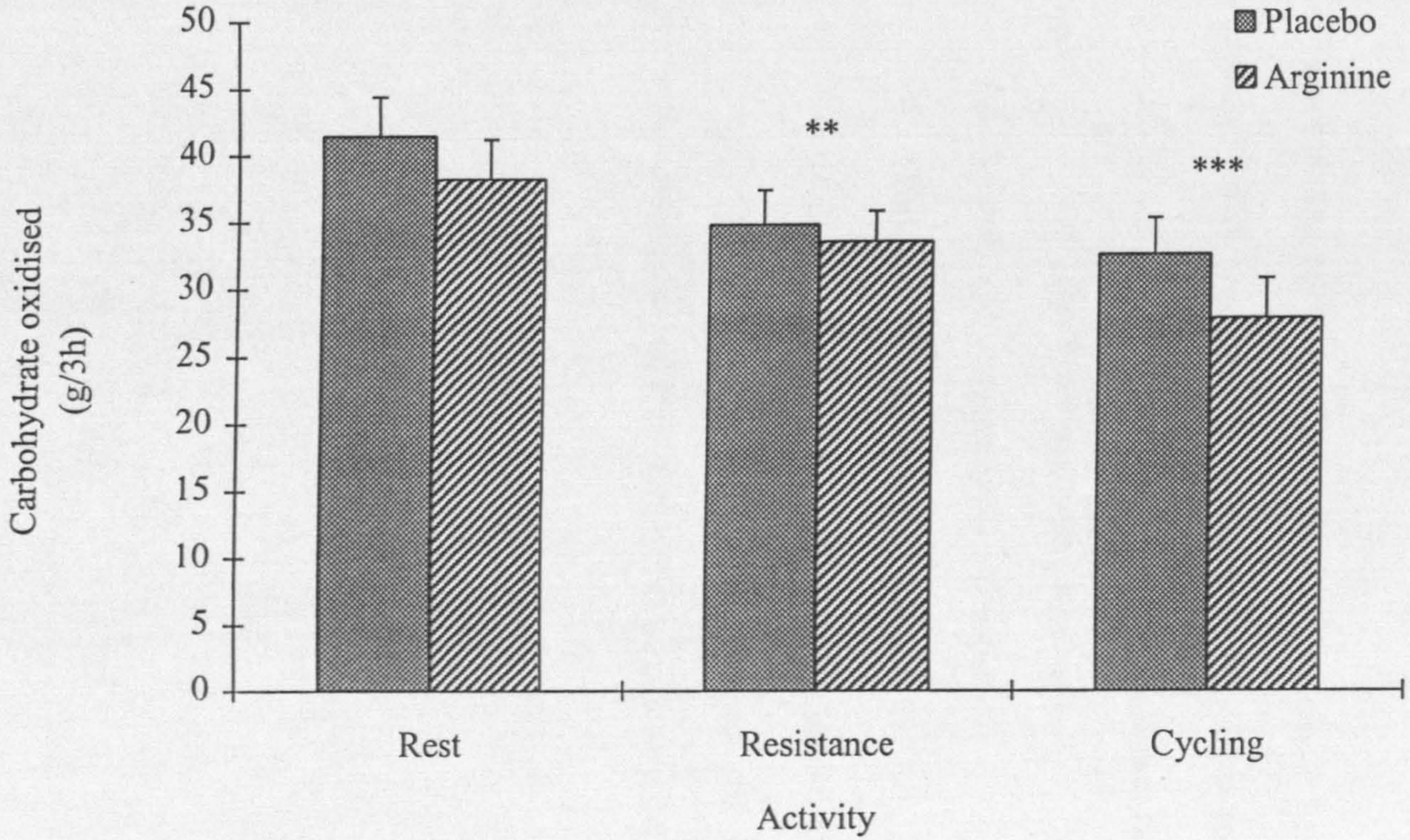

Placebo
Arginine
Carbohydrate oxidised (g/3h)
50
45
40
35
30
25
20
15
10
5
0
**
***
Rest
Resistance
Cycling
Activity

Figure 4.4 Forearm blood flow prior to activity and during a 3 h period following ingestion of 70 g CHO and either 10 g placebo (filled symbols) or arginine (open symbols) in subjects (n = 6):

a) without prior exercise (Rest)

b) following resistance exercise (Resistance)

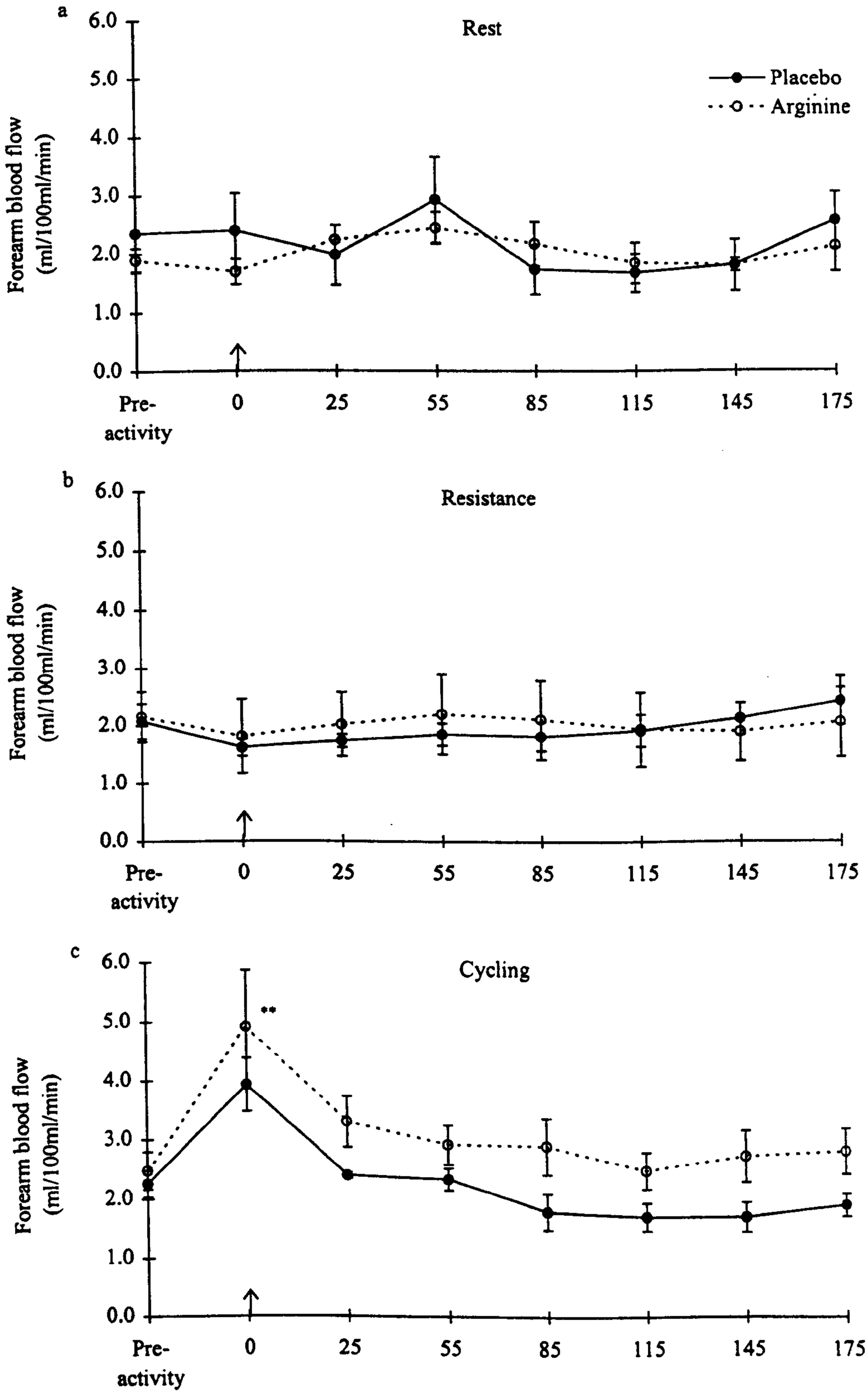
c) following cycling exercise (Cycling)

Values represent mean ± S.E.M.

Arrows indicate time of drink ingestion.

** $P < 0.01$ significant difference from pre-activity measurement, both treatments.

Fig 4.4


a
Rest
Placebo
Arginine
Forearm blood flow (ml/100ml/min)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Pre-activity
0
25
55
85
115
145
175
b
Resistance
Forearm blood flow (ml/100ml/min)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Pre-activity
0
25
55
85
115
145
175
c
Cycling
**
Forearm blood flow (ml/100ml/min)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Pre-activity
0
25
55
85
115
145
175
Time of measurement (minutes)

decline in forearm blood flow following exercise was no different following arginine ingestion than when placebo was ingested (Fig. 4.4c).

Blood pressure and heart rate

Mean arterial pressure decreased significantly following resistance ($P < 0.01$) and cycling ($P < 0.05$) exercise, but remained constant during the remainder of the 3 h measurement period (Fig. 4.5). Mean arterial pressure was no different following arginine ingestion compared with after placebo ingestion after any activity. No effect of arginine treatment upon HR was observed after any activity.

Palatability and tolerance of amino acid drinks

All subjects reported that one drink (subsequently known to contain arginine) had an unpleasant flavour, some reported it leaving a 'plastic' aftertaste. One subject reported gastrointestinal discomfort and diarrhoea during the day following two of the three occasions that arginine was ingested. No subjects reported any problems associated with ingestion of the placebo drink.

Figure 4.5 Systolic (square symbol), diastolic (circular symbol) and mean arterial (no symbol) blood pressures prior to activity and during a 3 h period following ingestion of 70 g CHO and either 10 g placebo (filled symbols) or arginine (open symbols) in subjects (n = 6):

a) without prior exercise (Rest)

b) following resistance exercise (Resistance)

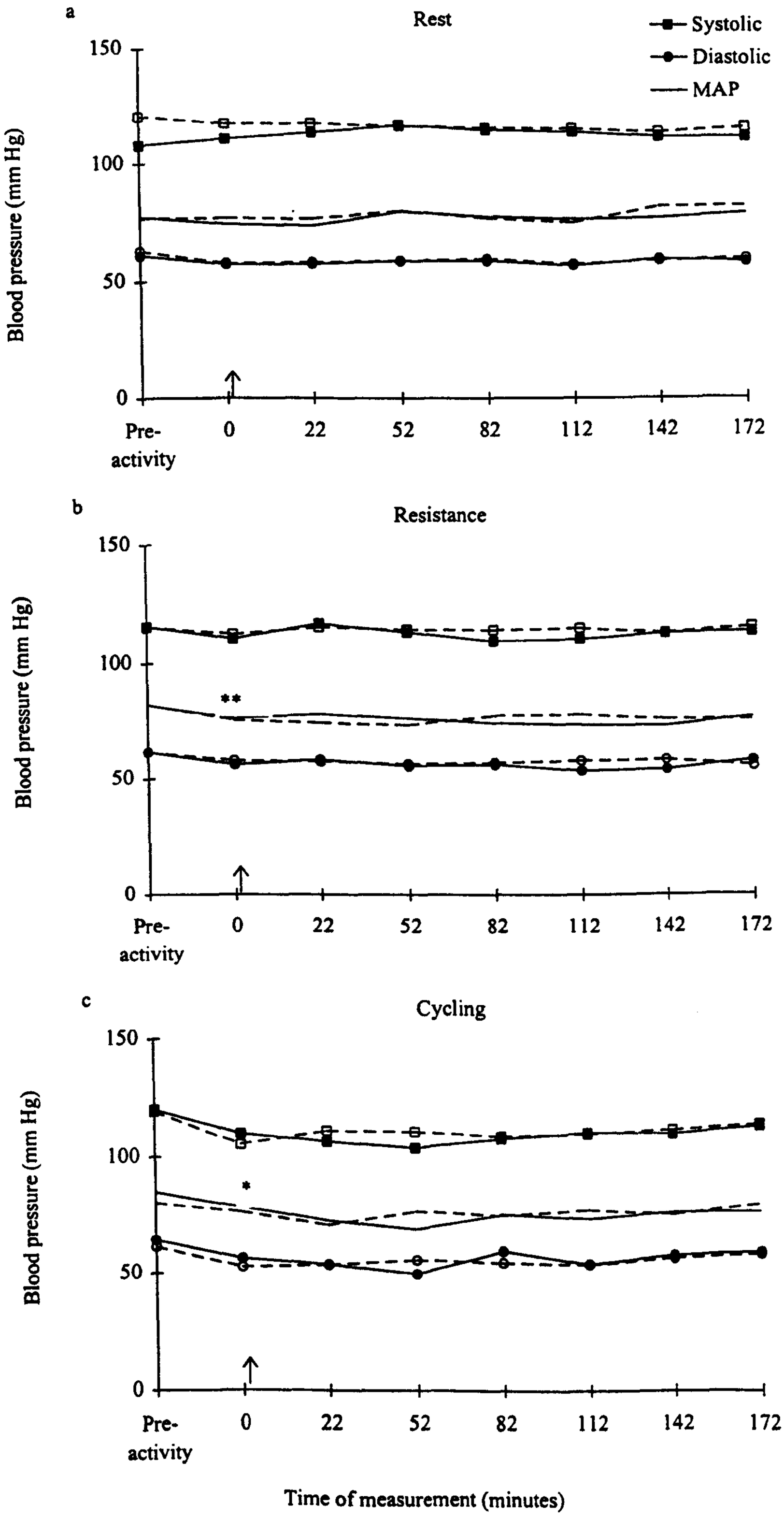
c) following cycling exercise (Cycling)

Values represent mean values for treatments. Standard error bars are not shown for purpose of clarity.

Arrows indicate time of drink ingestion.

* $P < 0.05$, ** $P < 0.01$, significant differences from pre-activity measurements, prior to both treatments.

Fig. 4.5


a
Rest
Systolic
Diastolic
MAP
150
100
50
0
Blood pressure (mm Hg)
Pre-activity
0
22
52
82
112
142
172
b
Resistance
**
c
Cycling
*
Time of measurement (minutes)

4.4 DISCUSSION

The aim of this investigation was to determine whether an amount of arginine, when administered orally, could influence the fate of glucose ingested at the same time. Previous investigations have demonstrated that intravenous administration of arginine augments glucose-stimulated insulin release (Efendic *et al*, 1974) and insulin-mediated whole body glucose disposal (Paolisso *et al*, 1997). Normal daily uptake of arginine from the diet is approximately 5 $g.d^{-1}$ (Visek, 1986). Consumption of a meal containing approximately 25 g arginine causes higher plasma insulin concentrations than a meal containing a normal arginine intake (Beaumier *et al*, 1995). However, such a high amount of arginine can be unpalatable (personal observation) and might cause gastrointestinal distress (Gater *et al*, 1992). Therefore, an amount of arginine lower than this, but above the normal daily intake was used in the current investigation. The results of the investigation suggest that, at the dosage administered, oral arginine ingestion does not influence CHO disposal, either when given to rested subjects or after different types of exercise. Glucose concentrations following CHO ingestion with arginine were no different from placebo conditions, after different activities. These results are reflected by the observation that serum insulin responses (peak insulin and insulin AUC) to CHO ingestion were no different from placebo conditions when arginine was consumed. This suggests that plasma arginine concentration did not reach a sufficiently high level to exert any influence upon insulin-stimulated glucose disposal.

Recent preliminary evidence (Gannon *et al*, 1998) has reported glucose and insulin responses of healthy subjects receiving arginine (1 $mmol.kg^{-1}$ lean body mass), glucose (25 g) or arginine + glucose. The amount of arginine administered

was approximately the same as the 10 g doses used in the current investigation. Blood samples collected over 2 h post-ingestion revealed that arginine alone stimulated a modest rise in glucose concentration, but did not stimulate insulin release. When arginine was provided with glucose it decreased peak glucose concentration, compared with glucose ingestion alone, but prolonged the rise in blood glucose over the post-ingestion period. From their evidence the authors proposed that arginine delayed glucose absorption and/or affected endogenous glucose production or clearance from the blood. Whilst the current investigation has no comparative data for glucose only ingestion, it might be suggested that no impairment of glucose absorption was apparent. Comparison with the response to the ingestion of glycine (which is not known to have any effect upon glucose or insulin metabolism) with glucose, peak glucose concentration after arginine ingestion was not significantly different following any activity. However, it cannot be discounted that both arginine and glycine might have similar effects upon CHO absorption.

Arginine is also known to stimulate glucagon release (Barbul, 1986), which in turn stimulates glycogenolysis in the liver (Ganong, 1979). It is possible that the stimulation of glucagon release by arginine elevated endogenous glucose production, contributing to the slight increase in peak glucose concentration seen in subjects when rested and following cycling exercise. The glucagon response to arginine may only have been small in the present study, however, as it is diminished relative to increases in circulating glucose concentration (Palmer *et al*, 1975).

The information provided by total CHO oxidation gives an indication of the fate of the ingested CHO, which can be utilised for energy immediately or stored in

muscle or liver as glycogen (Richter, 1996). Following exercise, glucose storage is greater in muscle than in the liver (Maehlum *et al*, 1978). The degree of CHO oxidation is less when the drive for CHO storage is high. This is demonstrated in the present study by the lesser CHO oxidation following both forms of exercise, compared with that in rested subjects (Fig. 4.3). There was no significant difference in CHO oxidation between treatments after any activity, although a consistent tendency for less CHO oxidation (and presumably greater CHO storage) following arginine treatment was apparent. The amount of CHO storage suggested by this small difference is unlikely, however, to represent any significant improvement in post-exercise substrate replenishment.

The results show that there was no effect of the arginine treatment upon forearm blood flow in rested subjects or after resistance exercise. Blood flow appeared to be higher when arginine was consumed following cycling exercise (Fig 4.4c). This apparent effect was, however, most likely as a consequence of the post-exercise blood flow being slightly higher prior to CHO ingestion. Examination of the decrease in blood flow from pre-ingestion levels through to the end of the measurement period revealed no effect of arginine ingestion upon blood flow, compared with the response to placebo. The vasodilatory effect of arginine is partly mediated by endogenous insulin release (Giugliano *et al*, 1997), which in the present study was no different between arginine and placebo treatments. Nitric oxide is also involved in vasodilation, but despite arginine being the unique precursor to NO, no evidence exists that arginine availability is limiting to NO production, or that increasing its availability produces a consequent increase in NO formation (Kurz & Harrison, 1997). Therefore it can be suggested that the

lack of an effect of arginine ingestion upon forearm blood flow was most probably because of the lack of any stimulatory effect on insulin release.

The majority of investigations that have administered arginine intravenously to humans have used doses that produce plasma arginine concentrations in the millimolar range (Palmer *et al*, 1975; Roti *et al*, 1986; Giugliano *et al*, 1997). Plasma arginine concentration following ingestion of food containing approximately 25 g arginine elevated plasma arginine concentration by less than 400 $\mu mol.L^{-1}$ to a concentration of approximately 600 $\mu mol.L^{-1}$ (Beaumier *et al*, 1995). It has recently been demonstrated that only 70% of an oral dose of arginine is available for use by the body (Bode-Böger *et al*, 1998). These authors also demonstrated that a 6 g oral dose of arginine was insufficient to influence BP or peripheral blood flow. It is feasible, therefore, to suggest that the dosage of arginine given in the present study did not raise plasma arginine concentration sufficiently to produce a glycogenic effect. It would be of interest to determine the minimum amount of ingested arginine necessary to produce these desired effects. The amount of arginine ingested in the present study was near the limit of palatability and one subject experienced gastrointestinal intolerance during two of the days that arginine was ingested. These findings, coupled with the loss of water and sodium in the urine following prolonged arginine feeding previously observed (Beaumier *et al*, 1995) all suggest that including high amounts of arginine in a CHO beverage would not be a practical method of improving post-exercise CHO replenishment.

In summary, a 10 g oral dose of arginine was found to not have any effect upon blood glucose disposal of human subjects following oral CHO ingestion, either when rested, or following different types of exercise known to differentially affect

glucose disposal. These results suggest that addition of arginine to a CHO beverage would not provide any additional benefit to post-exercise CHO replenishment.

Note 4.1 The author accepts that if an individual's data for a particular measurement is to be removed from those of an experimental group, then no data from that individual should be reported. It would be considered more appropriate to either remove that subject's data from the investigation, or to increase the size of the experimental group sufficiently to dilute any effect of large variations between subjects.

Chapter 5

EFFECTS OF CREATINE AND CARBOHYDRATE SUPPLEMENTATION UPON MUSCLE GLYCOGEN AND CREATINE ACCUMULATION AND SUBSEQUENT ENDURANCE EXERCISE PERFORMANCE

5.1 INTRODUCTION

Studies investigating the effect of Cr supplementation on muscle TCr accumulation have noted a wide variability in accumulation between subjects (Harris *et al*, 1992; Greenhaff *et al*, 1994; Casey *et al*, 1996). Generally, accumulation was greatest in individuals with low initial TCr concentrations. This variability was reduced when large amounts of CHO were ingested during Cr supplementation, due to improvements in Cr accumulation of all individuals (Green *et al*, 1996a). Supplementation with Cr + CHO also produced a tendency for greater muscle glycogen storage than was achievable by supplementation with CHO alone, although this tendency was not significant (Green *et al*, 1996b; Green, 1996). Chapter 3 demonstrated similar results in previously exercised muscle of subjects following 5 d ingestion of Cr + CHO. Further analysis of the changes arising from Cr + CHO supplementation in non-exercised subjects (Green *et al*, 1996b) revealed a significant positive correlation between the changes in muscle concentration of TCr and glycogen ($r = 0.75$, $n = 8$, $p < 0.05$), which was not evident when Cr or CHO alone were ingested. These findings are of interest as elevation of muscle glycogen concentration could have important implications for treatment of disease states such as diabetes mellitus, and pre-exercise availability of muscle glycogen is a principal determinant of endurance exercise performance (Bergström *et al*, 1967).

It is accepted that muscle CHO stores are influenced by dietary CHO intake, however increasing dietary CHO intake from around 55% of energy intake to 80-90% will only increase muscle glycogen concentration by approximately 50 $mmol.kg^{-1}$ d.m. (Maughan *et al*, 1997). It can be calculated that this increase in muscle glycogen would be sufficient to fuel exercise at 70% VO_2max for

approximately 15 minutes (Sahlin, 1986). In the investigation by Green (1996) the magnitude of the difference in glycogen concentration between individuals receiving Cr + CHO and CHO only was approximately 75 mmol.kg^{-1} d.m. This difference in concentration could be sufficient to extend exercise at 70% VO_2max by approximately 20 min above that achieved by CHO supplementation only (based upon Sahlin, 1986). By the same calculation, the difference between glycogen accumulation by exercised muscle of the Cr + CHO and CHO only groups in Chapter 3 could supply energy for an additional 40 min of exercise. Therefore it would appear that combination of Cr and CHO ingestion could not only be of benefit to individuals performing high-intensity exercise, but also be used by individuals to enhance endurance performance.

Aims

The aims of the present investigation were to examine whether muscle glycogen concentration could be increased by ingesting Cr with CHO, and if so, whether endurance exercise performance could be improved through this supplementation regimen.

5.2 METHODS

Thirteen healthy male subjects (Means (± S.E.M.); age 30 (2) yr; BMI 24.8 (0.7) kg.m^{-2}) participated in this study. All subjects regularly performed strenuous exercise for 1 h or more on at least 3 occasions per week. Mean VO_2peak of the subjects was 51.6 (1.4) ml.kg^{-1}.min^{-1} (range 45 – 59 ml.kg^{-1}.min^{-1}).

Experimental design

After initial health screening and exercise testing (see Chapter 2), suitable subjects reported to the laboratory on 4 separate occasions and performed cycling exercise to the point of exhaustion. Details of experimental conditions and protocols are outlined below.

Visit 1: Familiarisation with exhaustive exercise

To familiarise subjects with the exercise protocol, all subjects visited the laboratory in the morning, following an overnight fast, and performed cycling exercise to exhaustion, as outlined below. This visit was included to allow subjects to familiarise themselves with the experience of exercising at relatively high workloads to the point of exhaustion. It was anticipated that the familiarisation session would make subjects more likely to stop exercise during subsequent test visits due to substrate depletion rather than for any other reason.

Visit 2: Exhaustive exercise following a normal diet (EX1)

At least one week following the familiarisation visit, subjects reported to the laboratory in the morning, following an overnight fast, for the first test visit (EX1). During the 3 days prior to EX1, each subject consumed his normal diet, which was recorded in a food diary. During this 3-day period, subjects refrained from strenuous exercise and consumption of alcohol. Subjects' nude weights were recorded upon arrival at the laboratory for the EX1 visit. Subjects then rested supine in preparation for the experimental protocol outlined below.

Visit 3: Exhaustive exercise following CHO supplementation (EX2)

Two weeks following the EX1 visit, subjects reported to the laboratory in the morning, following an overnight fast, for the second test visit (EX2). For 5 days prior to the EX2 visit, subjects had consumed four 500 ml servings of a CHO drink (Lucozade™) per day (see Chapter 2). During the 5-day CHO supplementation period subjects consumed their normal diets, repeating their dietary intake, as previously recorded, over the 3 days prior to the EX2 visit. Subjects did not perform any strenuous exercise during the CHO supplementation period. Subjects' nude weights were recorded on the day prior to ingestion of the first CHO supplement and upon arrival at the laboratory for the EX2 visit. Subjects then rested supine in preparation for the experimental protocol outlined below.

Visit 4: Exhaustive exercise following Cr only or Cr + CHO supplementation (EX3)

Two weeks following the EX2 visit, subjects reported to the laboratory in the morning, following an overnight fast, for the third test visit (EX3). For this visit, subjects were randomly allocated into 2 supplementation groups. One group ingested 5 g $Cr.H_2O$ (Cr only group, $n = 6$) and the other group ingested 5 g $Cr.H_2O$ and 500 ml of a CHO drink (Cr + CHO group, $n = 7$). For 5 days prior to the visit, subjects ingested their respective supplements four times per day (see Chapter 2). During the 5-day supplementation period subjects consumed their normal diet, repeating their dietary intake, as previously recorded, over the 3 days prior to the EX3 visit. Subjects did not perform any strenuous exercise during the supplementation period. Subjects' nude weights were recorded on the day prior to

ingestion of the first supplement and upon arrival at the laboratory on the morning of the EX3 visit. Subjects then rested supine in preparation for the experimental protocol outlined below.

Experimental protocol

Whilst the subject rested supine on a bed, his left arm was cannulated and a resting blood sample was obtained (see Chapter 2). The subject's leg was then prepared for muscle biopsy sampling. Three incisions (two in the case of EX1) were made through the skin of the central area of the ventral side of the thigh. Each incision was approximately 5 mm long and spaced approximately 3 cm apart from each other (Harris *et al*, 1974). A resting muscle sample was obtained (Rest), using the percutaneous needle biopsy technique (see Chapter 2), by insertion through the uppermost of the incisions, whereupon it was immediately frozen in liquid nitrogen. A sterile dressing was applied over the incisions, which was held in place using an adhesive elasticated bandage. Following 15 min recovery from the muscle sampling procedure, the subject performed cycling exercise to exhaustion.

Subjects performed 3 min of warm-up exercise on an electrically braked ergometer (Excalibur Sport, Lode N.V. Instrumenten, Groningen, The Netherlands) at a constant cadence of 70 rpm against a workload of 100 W. The electrical resistance of the cycle was then adjusted to produce a workload designed to elicit an oxygen consumption of 70% VO_2peak. Every 15 min during exercise, expired air samples were collected for measurement of VO_2 and RER (see Chapter 2), and heart rate was recorded. During the three test visits (EX1, EX2, EX3), a blood sample was also obtained every 15 min. To maintain

euhydration subjects ingested 200 ml water at ambient temperature every 15 min throughout the exercise period. Subjects were verbally encouraged to continue cycling for as long as possible to the point of volitional exhaustion, or until they could no longer maintain the required cadence.

Immediately at the point of exhaustion of visit EX1, the subject was supported in the saddle by an investigator and a muscle sample was obtained from the leg through the previously-prepared incision. The muscle sample obtained at that time was termed 'Time of exhaustion visit 1 following normal dietary conditions' ($TEX1_{Normal}$). During visits EX2 and EX3, each subject stopped at the same time as his exhaustion time during visit EX1, and another muscle sample was immediately taken. Muscle samples obtained at these times are termed 'Time of exhaustion visit 1 following CHO ($TEX1_{CHO}$), Cr ($TEX1_{Cr}$) or Cr + CHO ($TEX1_{Cr+CHO}$), depending upon the dietary condition preceding the exercise visit. Following the removal of a TEX1 muscle sample, a new sterile dressing was applied to the biopsy area and the subject continued cycling to the point of exhaustion. Muscle samples obtained at exhaustion during test visits EX2 and EX3 are termed TEX2 and $TEX3_{Cr}$ (or $TEX3_{Cr+CHO}$), respectively. All muscle samples were frozen in liquid nitrogen immediately following removal from the leg (time from stopping exercise to freezing was 12 (2) s). An additional blood sample was obtained five minutes after the time of exhaustion, whilst the subject recovered on a bed.

Analyses of muscle samples for ATP, PCr, Cr and glycogen concentration, and blood samples for glucose (all subjects) and lactate (8 subjects only) concentration were performed as outlined in Chapter 2.

5.3 RESULTS

Body mass

A significant increase in body mass occurred following CHO supplementation (1.0 (0.3) kg, n = 13, $P < 0.01$) and Cr + CHO supplementation (1.7 (0.3) kg, n = 7, $P < 0.001$). No significant change in body mass was seen following Cr supplementation (-0.2 (0.3) kg, n = 6, $P > 0.05$).

Muscle glycogen concentration and utilisation during exercise

Resting glycogen concentrations were not significantly elevated above those of the normal dietary condition following CHO supplementation, Cr supplementation or Cr + CHO supplementation (Fig. 5.1). Muscle glycogen concentrations at $TEX1_{CHO}$ and $TEX1_{Cr}$ were not significantly different from glycogen concentration at $TEX1_{Normal}$ (Fig. 5.2). Muscle glycogen concentration at $TEX1_{Cr+CHO}$ (214 (69) $mmol.kg^{-1}$ d.m., n = 5) was significantly greater than at $TEX1_{Normal}$ (100 (20) $mmol.kg^{-1}$ d.m., n = 13; $P < 0.05$; Fig. 5.2). Muscle glycogen concentration was reduced to 21%, 13% and 16% of the resting pre-exercise concentration by exercise, during EX1, EX2 and EX3 visits, respectively. Glycogen concentrations at TEX2, $TEX3_{Cr}$ and $TEX3_{Cr+CHO}$ were not different from those at $TEX1_{Normal}$ (Fig. 5.2).

There was a trend for greater glycogen utilisation, from the time of exercise commencing to TEX1, after CHO or Cr + CHO supplementation, although utilisation did not differ significantly from that of the whole exercise period after normal dietary conditions (Table 5.1).

Figure 5.1 Resting muscle glycogen concentrations after normal dietary conditions and following CHO, Cr or Cr + CHO supplementation.

Values are mean (± S.E.M.)

Numbers in parentheses refer to the number of samples contributing to each mean value.

Fig. 5.1

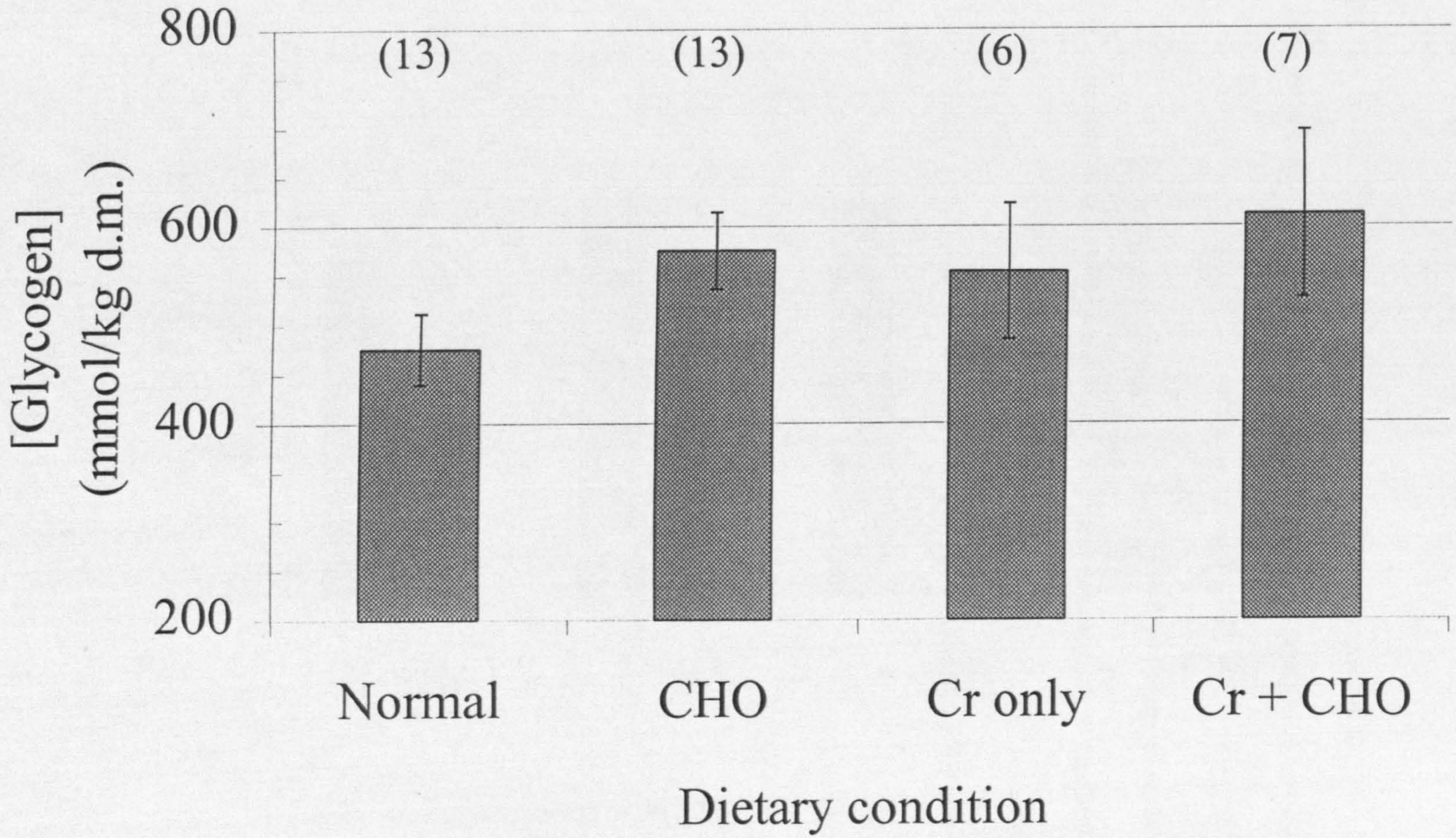

800
600
400
200
(13)
(13)
(6)
(7)
[Glycogen]
(mmol/kg d.m.)
Normal
CHO
Cr only
Cr + CHO
Dietary condition

Figure 5.2 Muscle glycogen concentrations at rest, and during and immediately following exhaustive exercise, after normal dietary conditions and following CHO, Cr or Cr + CHO supplementation.

Values are mean (± S.E.M.)

Numbers in parentheses refer to the number of samples contributing to each mean value.

* $P < 0.05$, significant difference from $TEX1_{Normal}$.

Fig. 5.2

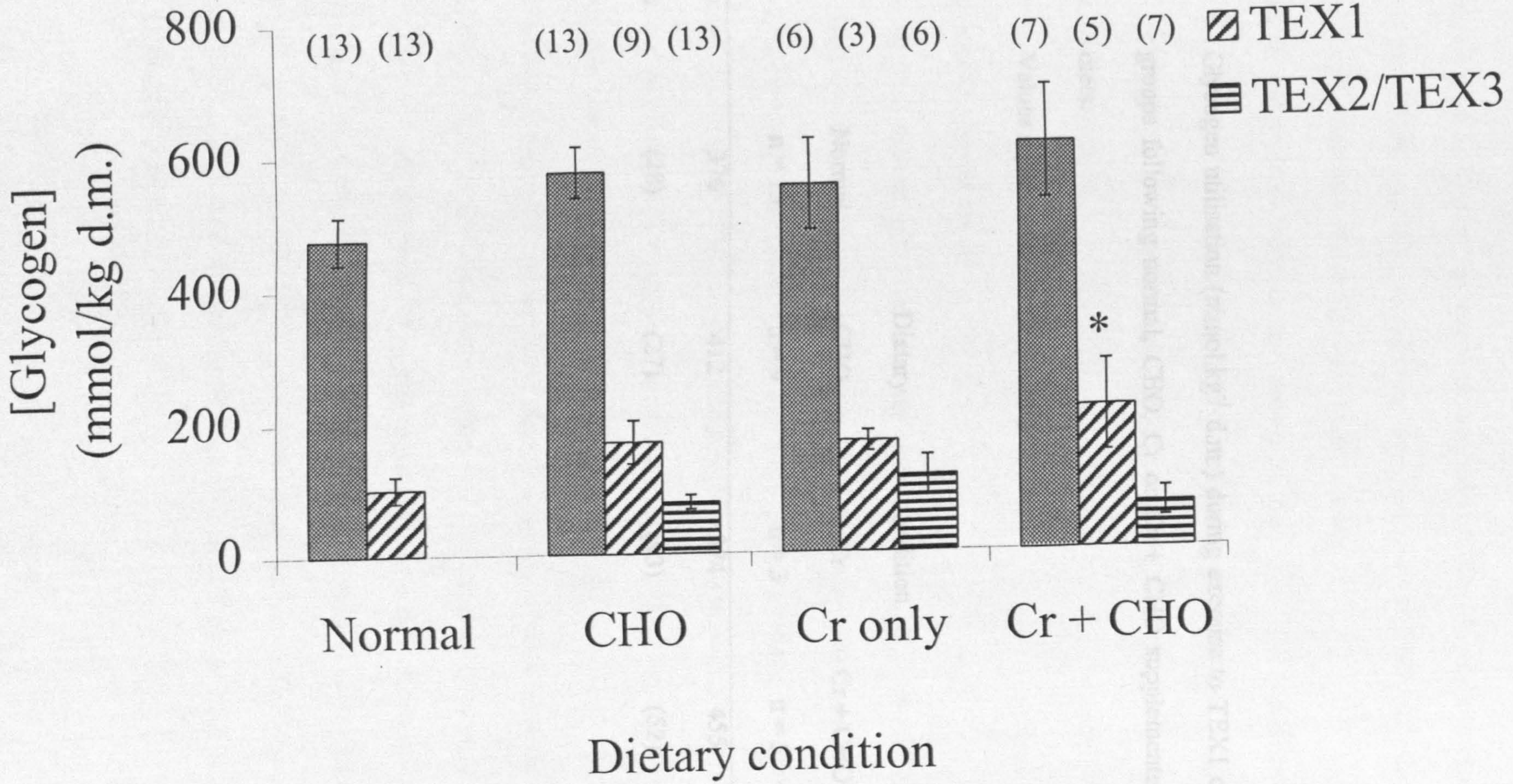

Rest
TEX1
TEX2/TEX3
800
600
400
200
0
[Glycogen]
(mmol/kg d.m.)
(13) (13)
(13) (9) (13)
(6) (3) (6)
(7) (5) (7)
*
Normal
CHO
Cr only
Cr + CHO
Dietary condition

Table 5.1 Glycogen utilisation (mmol.kg^{-1} d.m.) during exercise to TEX1 of groups following normal, CHO, Cr or Cr + CHO supplemented diets.

Values represent mean (± S.E.M.)

	Dietary condition			
	Normal	CHO	Cr	Cr + CHO
	n = 13	n = 9	n = 3	n = 5
Glycogen utilisation	376 (28)	412 (27)	374 (93)	455 (52)

Exercise time to exhaustion

On one occasion, an EX2 exercise test was stopped at TEX1 due to a subject experiencing pain in the muscle sampling area. For this reason that subject's data was not included for analysis of exercise time to exhaustion.

Mean exercise times to exhaustion are shown in Fig. 5.3. No significant difference in exercise time to exhaustion was observed between dietary conditions, although there was a tendency for longer exercise duration following Cr + CHO supplementation.

Muscle metabolite concentrations

Mean resting TCr concentration of subjects was similar to that previously observed (Harris *et al*, 1992). Resting muscle TCr concentrations increased significantly following Cr supplementation (13.8 (5) $mmol.kg^{-1}$ d.m., $P < 0.01$; Fig. 5.4) and following Cr + CHO supplementation (25.2 (7.0) $mmol.kg^{-1}$ d.m., $P < 0.001$; Fig. 5.4). Muscle TCr concentrations of Cr and Cr + CHO groups were not significantly different following supplementation ($P = 0.19$).

Resting muscle PCr concentration increased significantly from pre-supplemented conditions following Cr + CHO supplementation (6.7 (2.7) $mmol.kg^{-1}$ d.m., $P < 0.05$, Table 5.2), but not following Cr supplementation. No difference in resting PCr concentration was observed between the Cr -supplemented and Cr + CHO – supplemented groups. Muscle PCr concentrations at $TEX1_{Cr}$, $TEX1_{Cr+CHO}$, $TEX3_{Cr}$ or $TEX3_{Cr+CHO}$ were not significantly different from those at $TEX1_{Normal}$ (43.4 (3.3) $mmol.kg^{-1}$ d.m.).

Figure 5.3 Exercise times to exhaustion after normal dietary conditions and following CHO, Cr or Cr + CHO supplementation.

Values are mean (± S.E.M.)

Numbers in parentheses refer to the number of samples contributing to each mean value.

Fig. 5.3

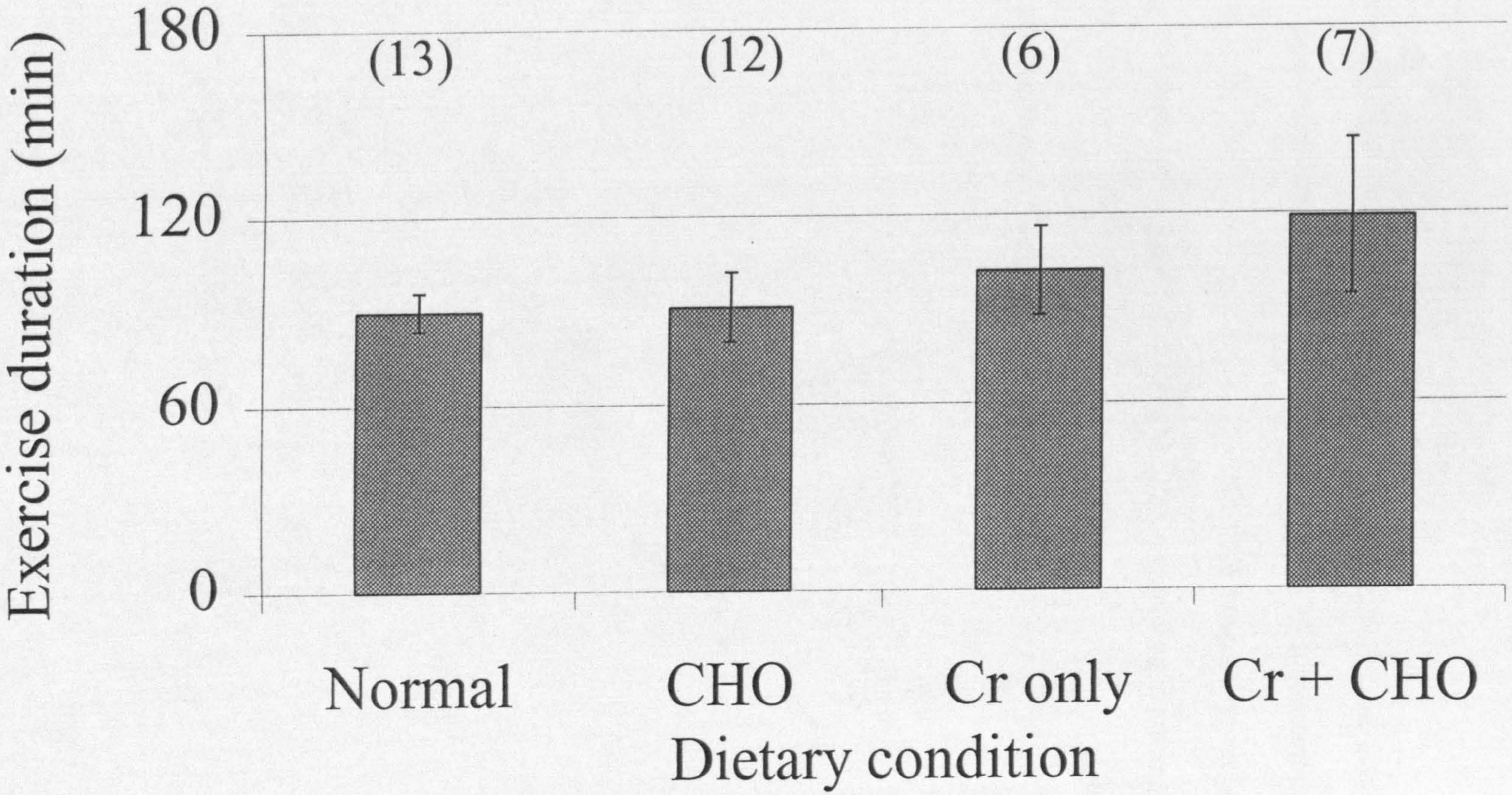

180
120
60
0
Exercise duration (min)
(13)
(12)
(6)
(7)
Normal
CHO
Cr only
Cr + CHO
Dietary condition

Figure 5.4 Resting muscle total creatine concentrations after normal dietary conditions and following CHO, Cr or Cr + CHO supplementation.

Values are mean (± S.E.M.)

Numbers in parentheses refer to the number of samples contributing to each mean value.

** $P < 0.01$, *** $P < 0.001$, significant difference from Normal concentration.

Fig. 5.4

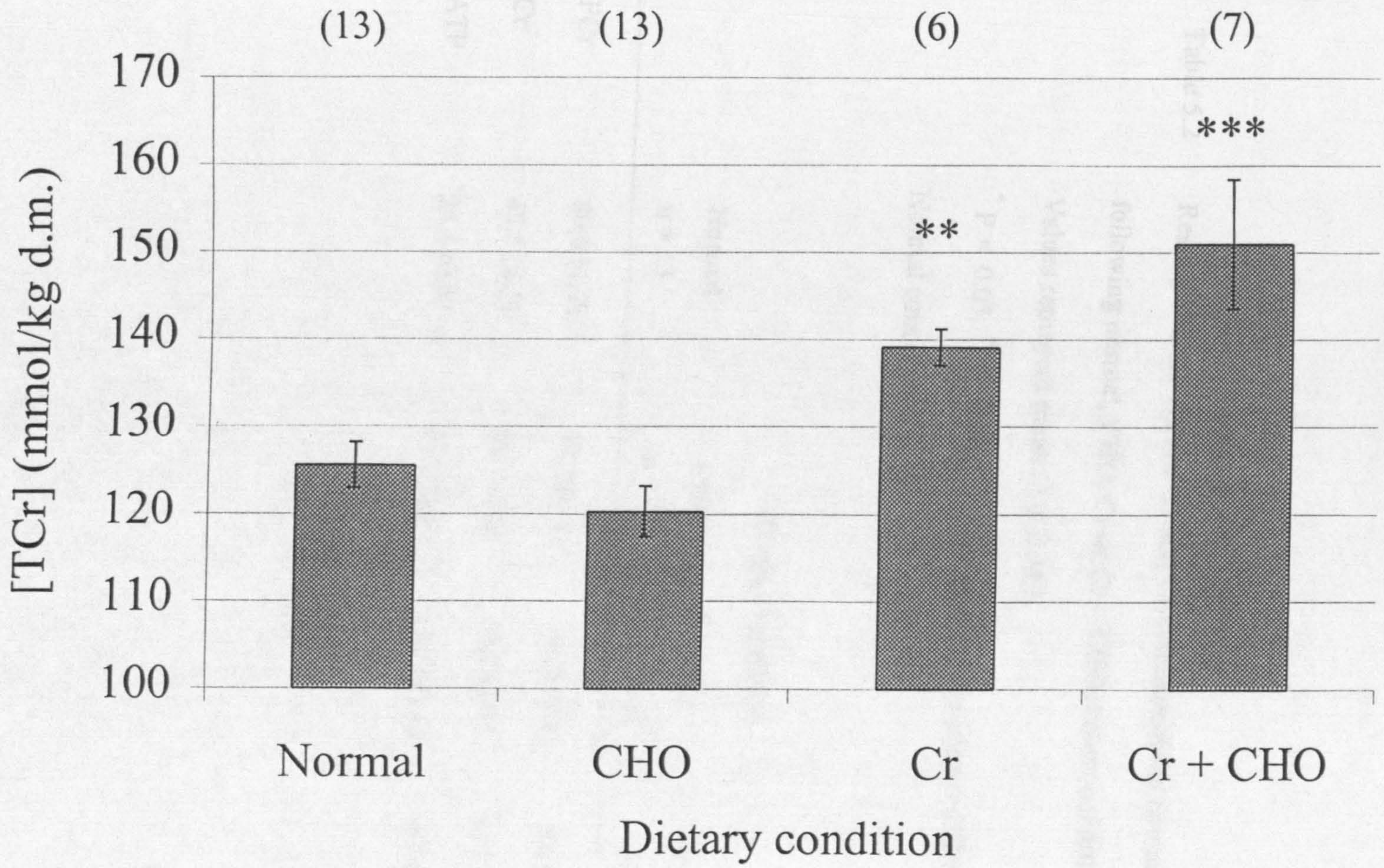

(13)
(13)
(6)
(7)
170
160
150
140
130
120
110
100
[TCr] (mmol/kg d.m.)
**
***
Normal
CHO
Cr
Cr + CHO
Dietary condition

Table 5.2 Resting muscle ATP, PCr and Cr concentrations (mmol.kg^{-1} d.m.) following normal, CHO, Cr or Cr + CHO dietary conditions.

Values represent mean (± S.E.M.)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant difference from Normal concentrations.

	Dietary condition			
	Normal	CHO	Cr	Cr + CHO
	n = 13	n = 13	n = 6	n = 7
PCr	80.0 (1.7)	77.1 (1.8)	81.8 (2.5)	88.9 (3.5)*
Cr	45.5 (2.5)	43.3 (1.9)	57.2 (2.2)**	62.1 (3.5)***
ATP	24.4 (0.6)	24.2 (0.9)	24.0 (1.4)	22.6 (1.8)

Resting muscle Cr concentration increased significantly from pre-supplemented conditions following Cr supplementation (9.4 (3.4) mmol.kg^{-1} d.m., $P < 0.01$) and following Cr + CHO supplementation (18.5 (4.9) mmol.kg^{-1} d.m., $P < 0.001$; Table 5.2). Resting Cr concentration was no different between Cr and Cr + CHO groups following supplementation.

Resting muscle ATP concentrations of both groups were similar on all occasions and did not change following dietary intervention (Table 5.2). ATP concentration immediately following exhaustion was not significantly different from that at rest during any experimental visit.

Blood glucose and lactate during exercise

Concentrations of blood glucose and lactate were compared within and between treatments for exercise time points up to 60 min. After this time point the number of samples contributing to each group's mean concentration decreased due to some subjects fatiguing earlier than others.

Blood glucose concentration did not change significantly from resting concentrations during the first 60 min of exercise following any dietary condition. No significant differences in blood glucose concentrations were observed at any time point (Rest – 60 min) between dietary conditions (Table 5.3). Blood glucose concentrations 5 min post-exhaustion were not significantly different from normal conditions, following any dietary condition (Normal, 4.71 (0.26); CHO, 5.04 (0.45); Cr, 4.72 (0.2); Cr + CHO, 4.93 (0.28) mmol.L^{-1}; $P > 0.05$).

Table 5.3 Whole blood glucose concentrations ($mmol.L^{-1}$) of experimental groups at rest and during the first 60 min of exercise, following normal, CHO, Cr or Cr + CHO dietary supplementation.

Values represent mean (± S.E.M.)

Dietary Condition		Time of measurement (min)				
		Rest	15	30	45	60
Normal	(n = 13)	4.73	4.24	4.34	4.33	4.30
		(0.17)	(0.10)	(0.15)	(0.16)	(0.2)
CHO	(n = 13)	4.59	4.39	4.69	4.51	4.34
		(0.10)	(0.10)	(0.17)	(0.11)	(0.07)
Cr only	(n = 6)	4.54	4.41	4.57	4.71	4.72
		(0.28)	(0.15)	(0.21)	(0.31)	(0.3)
Cr + CHO	(n = 7)	4.45	4.33	4.5	4.54	4.24
		(0.11)	(0.14)	(0.29)	(0.37)	(0.1)

Blood lactate was increased above resting concentrations during exercise following all dietary conditions (all $P < 0.05$). Blood lactate concentrations during the first 45 min of exercise were significantly higher than normal conditions following CHO supplementation ($P < 0.05$; Table 5.4), but not following Cr or Cr + CHO supplementation. Blood lactate concentrations 5 min post-exhaustion were not significantly different from normal conditions, following any dietary condition (Normal, 2.58 (0.31); CHO, 3.90 (0.74); Cr, 3.10 (0.47); Cr + CHO, 2.79 (0.23) $mmol.L^{-1}$; $P > 0.05$).

VO_2 and RER during exercise

The workloads used in the exercise protocols produced VO_2 measurements of approximately 73% VO_2peak. A tendency for RER values greater than those following normal dietary conditions was observed during exercise following CHO and Cr + CHO supplementation, although no significant difference was observed at any time point (Fig. 5.5).

Table 5.4 Whole blood lactate concentrations ($mmol.L^{-1}$) of experimental groups at rest and during the first 60 min of exercise, following normal, CHO, Cr or Cr + CHO dietary supplementation..

Values represent mean (± S.E.M.)

* $P < 0.05$, significant difference from corresponding time point under normal dietary conditions.

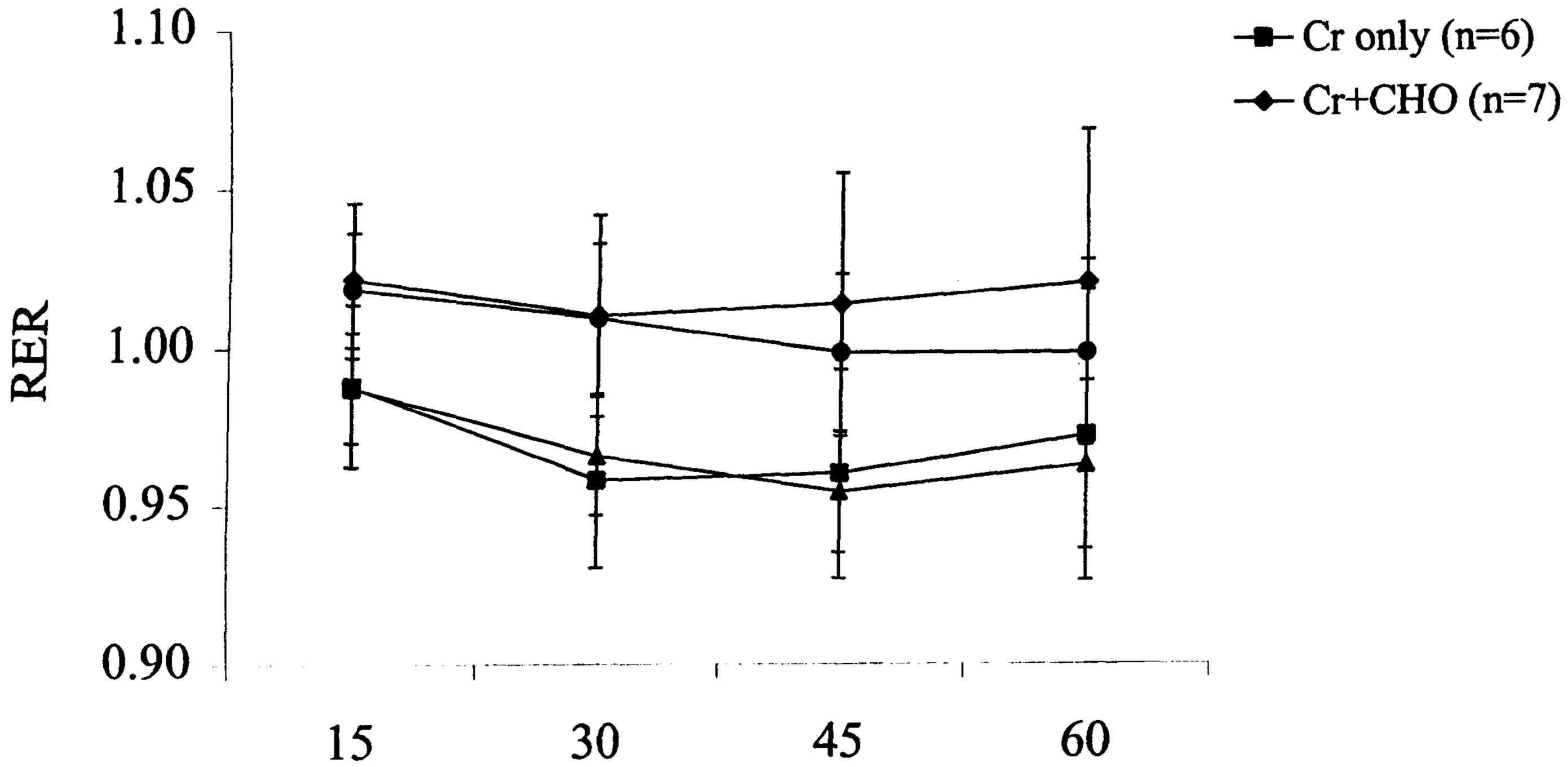
Dietary Condition		Time of measurement (min)				
		Rest	15	30	45	60
Normal	(n = 8)	0.68	2.79	2.72	2.56	2.70
		(0.09)	(0.28)	(0.31)	(0.25)	(0.26)
CHO	(n = 8)	0.90	3.94*	4.16*	3.80*	3.55
		(0.08)	(0.33)	(0.44)	(0.37)	(0.46)
Cr only	(n = 3)	0.66	3.27	3.31	3.16	3.15
		(0.12)	(0.73)	(0.58)	(0.53)	(0.56)
Cr + CHO	(n = 5)	0.78	3.46	3.54	3.51	3.65
		(0.14)	(0.34)	(0.51)	(0.53)	(0.47)

Figure 5.5 Respiratory exchange ratio measurements taken during the first 60 min of exhaustive exercise after normal dietary conditions and following CHO, Cr or Cr + CHO supplementation.

Values are mean (± S.E.M.)

Numbers in parentheses refer to the number of samples contributing to each mean value.

Fig. 5.5


Normal (n=13)
CHO (n=13)
Cr only (n=6)
Cr+CHO (n=7)
1.10
1.05
1.00
0.95
0.90
RER
15
30
45
60
Time of measurement (min)

5.4 DISCUSSION

The primary aim of this study was to investigate the effect of Cr + CHO supplementation upon muscle glycogen accumulation. Previous investigation of non-exercised subjects (Green *et al*, 1996a) and results in Chapter 3 had shown that there was a strong tendency for greater muscle glycogen storage with Cr + CHO supplementation than with CHO supplementation alone. In the present study, however, although muscle glycogen concentration tended to be higher than normal after CHO and Cr + CHO supplementation, these differences were not significant. Furthermore, there was no difference in post-supplementation muscle glycogen concentration between CHO alone and Cr + CHO treatments. The observation that glycogen concentration at $TEX1_{Cr+CHO}$ was two-fold greater than at $TEX1_{Normal}$ suggests, however, that Cr + CHO supplementation can be of benefit to endurance exercise performance. Indeed, there was a tendency for improved exercise duration in those subjects following Cr + CHO supplementation (26 (20) min longer than normal), compared with their exercise times after CHO supplementation (15 (8) min longer than normal). Examination of the glycogen concentrations of those subjects only, after each dietary condition, suggests that there was a tendency for greater glycogen storage following Cr + CHO supplementation. Carbohydrate supplementation increased glycogen concentration by only 61 (26) $mmol.kg^{-1}$ d.m. ($P > 0.05$), whereas after Cr + CHO supplementation, glycogen concentration was 172 (56) $mmol.kg^{-1}$ d.m. higher than under normal dietary conditions ($P < 0.05$). Glycogen concentrations of those subjects after CHO and Cr + CHO supplementation were not significantly different, however.

The lack of any difference in glycogen accumulation between CHO and Cr + CHO treatments of the whole experimental population is similar to results seen in Chapter 3. In that investigation, there was no difference in resting glycogen concentration of non-exercised muscle, of groups ingesting Cr + CHO or CHO alone for 5 days. However, in previously exercised muscle, glycogen concentration was greater following Cr + CHO supplementation than after supplementation with CHO alone. This suggests, therefore, that to achieve the maximum effect of Cr + CHO supplementation upon muscle glycogen accumulation, glycogen-depleting exercise must be performed prior to Cr + CHO supplementation.

Performance of prolonged exercise was not significantly improved above normal conditions by any of the dietary conditions employed in the present study, although there was a tendency for increased exercise duration following Cr + CHO supplementation. It is possible that this was due to there being no significant effect of these treatments upon muscle glycogen concentration. One might have expected, however, that the significantly greater glycogen concentration at $TEX1_{Cr+CHO}$ would have improved exercise performance above normal conditions. Scrutiny of individuals' results reveal that one subject in this group stopped exercise 60 min prior to his $TEX1_{Normal}$ time following Cr + CHO supplementation, despite a glycogen concentration near 200 $mmol.kg^{-1}$ d.m. It is likely that this was for a reason other than substrate depletion, particularly as the remainder of the subjects in that group had exhaustion glycogen concentrations ≤ 71 $mmol.kg^{-1}$ d.m. under the same conditions. Removal of this subject's exercise time reveals that exercise duration following Cr + CHO supplementation was extended by 40 (15) min above normal conditions. This highlights a potential

problem associated with using small numbers of subjects in experimental groups, especially when a large variation in concentration exists between subjects. If one measurement for a particular subject is substantially higher or lower than the remainder of the groups' measurements, the mean result will be skewed toward that particular direction. When experimental populations are small, such measurements can potentially overshadow results that may otherwise be considered significant, and ultimately lead to misinterpretation of the data. In the present study, comparison of TEX1 measurements following Cr or Cr + CHO supplementation could be particularly affected by this. This is because the sample size was reduced furthermore by some subjects stopping exercise before TEX1 during EX2 and EX3 visits.

An elevation of resting glycogen concentration prior to exercise would be expected to result in greater rates of glycogen utilisation during exercise (Gollnick *et al*, 1972; Sherman *et al*, 1981; Hargreaves *et al*, 1995). In the present study, glycogen utilisation appeared to parallel the tendency for higher glycogen concentration after CHO and Cr + CHO supplementation, although utilisation did not differ significantly from normal dietary conditions. This may have resulted in there being no observable improvement in exercise performance, despite a relative increase in glycogen concentration following CHO and Cr + CHO supplementation.

Other factors may have contributed to the apparent lack of effect of Cr + CHO supplementation upon muscle glycogen accumulation and subsequent exercise performance. It was suggested in Chapter 3 that Cr accumulation by muscle may indirectly increase muscle glycogen concentration by increasing cell volume. The relatively smaller Cr accumulation of the Cr + CHO group in the present study,

compared with that previously observed (Green *et al*, 1996b; Green, 1996) might be expected to not influence cell volume to as great an extent. This may possibly account for the relatively small increase in glycogen accumulation of the Cr + CHO group seen in the present study. It is of interest that subjects showing the smallest increases in TCr concentration (11.3 – 13.1 $mmol.kg^{-1}$ d.m.) following Cr + CHO supplementation also displayed the smallest increases in glycogen concentration (42 – 91 $mmol.kg^{-1}$ d.m.). However, no significant correlation was observed between these changes in the whole group. This does not support the previously observed relationship between TCr and glycogen accumulation following Cr + CHO supplementation (Green *et al*, 1996b). It is possible that other factors known to influence glycogen synthesis may have affected glycogen accumulation in the present study. The greatest individual increase in glycogen concentration after Cr + CHO supplementation was observed in a subject with the highest VO_2peak. Endurance trained individuals have enhanced sensitivity to insulin, which is thought to be mediated by a greater concentration of glucose transport protein in their muscle (Andersen *et al*, 1993). The enhanced capacity for muscle glucose transport of these individuals may influence glycogen accumulation to a greater extent than a change in cell volume. This is supported by the observation that a significant correlation existed between VO_2peak and glycogen accumulation following Cr + CHO supplementation ($r = 0.87$, $n = 7$, $P < 0.05$).

It is possible that the exercise to exhaustion protocol employed in the present study was not reproducible enough to provide a reliable measurement of exercise performance (Krebs & Powers, 1989). A similar exercise protocol, in which well trained subjects cycled continuously at 75% of their maximal power output until

exhaustion, was demonstrated to have a coefficient of variance of almost 27% (Jeukendrup *et al*, 1996). Two different time-trial cycling tests using 10 subjects per test were more reproducible over 5 separate visits (coefficient of variation both ~ 3.5%). The reproducibility of these tests in groups of untrained subjects is currently unknown, and may be no more reliable than the test used in the present study. Nevertheless, it may be prudent to consider using such tests in future investigations that measure endurance performance.

The protocol used in this study also allowed investigation of the effects of Cr + CHO supplementation upon muscle Cr accumulation. Although Cr supplementation significantly increased muscle TCr concentration of both groups in the present study, unlike a previous investigation (Green *et al*, 1996a), there was no greater increase when CHO was ingested during Cr supplementation. Furthermore, comparison of the present results with those using an identical supplementation protocol (Green *et al*, 1996a) suggest that Cr accumulation was blunted in both groups supplemented with Cr. Although not the central issue of this study, these results are interesting, particularly as the study aimed to investigate a possible relationship between muscle Cr and glycogen accumulation. Examination of individuals' results showed that 2 subjects in the Cr group appeared to be 'non-responders' (Greenhaff *et al*, 1994; Casey *et al*, 1996), demonstrating increases in TCr concentration of less than 10 $mmol.kg^{-1}$ d.m. Removal of those subjects' contributions to the group's mean revealed Cr accumulation values similar to previous investigations (21.1 (3.0) $mmol.kg^{-1}$ d.m.). Previously, CHO ingestion during Cr supplementation had been shown to substantially increase muscle TCr concentration of many subjects and appeared to abolish the phenomenon of the 'non-responder' (Green *et al*, 1996a). However, in

the present study, although a tendency for increased Cr accumulation existed, subjects ingesting Cr + CHO did not have significantly greater TCr concentration than those supplemented with Cr alone. Again, examination of individuals' results showed that 3 subjects demonstrated only modest increases in TCr concentration (11.3 – 13.1 mmol.kg^{-1} d.m.). Removal of these subjects' values caused the group mean to resemble that observed in a previous investigation (34.8 (7.6) mmol.kg^{-1} d.m.; similar to Green *et al*, 1996a). As mentioned previously, it is possible that the population sizes of the Cr (n = 6) and Cr + CHO (n = 7) groups were not large enough to show significant differences in concentrations, particularly when a large variability in measurements between individuals existed. It is suggested that increasing the size of the experimental groups in the present investigation would allow clearer interpretation of results. Nevertheless, the observation that some subjects showed only modest increases in TCr concentration suggests that ingestion of CHO during Cr supplementation may not augment Cr accumulation in all individuals. The reasons for these results cannot presently be adequately explained. As with glycogen accumulation, changes in TCr concentration may possibly be influenced by an individual's training status. Training can influence factors which may affect Cr accumulation, such as muscle fibre composition (Komi & Karlsson, 1978), muscle Na^+/K^+ pump concentration (Klitgaard & Clausen, 1989; Green *et al*, 1993; McKenna *et al*, 1993; Madsen *et al*, 1994) and glucose-stimulated insulin release (Heath *et al*, 1983; King *et al*, 1987; Rodnick *et al*, 1987; Engdahl *et al*, 1995; Hickner *et al*, 1997). The training status of the subjects used in the present study, as indicated by VO_2peak, covered a range from untrained (~45 mL O_2.kg^{-1}.min^{-1}) up to moderately trained (50 – 59

mL O_2.kg^{-1}.min^{-1}). However, no correlation was observed between VO_2peak and Cr accumulation.

In conclusion, Cr ingestion did not significantly improve glycogen accumulation during a period of CHO supplementation, and did not improve subsequent performance of prolonged exercise. Furthermore, Cr + CHO supplementation did not produce any significant improvement in muscle Cr accumulation above that achieved by Cr supplementation alone. It is suggested that low population sizes and large variations of the experimental groups used may have confounded the significance of the results of the present study. Further investigation is required of the effects of Cr + CHO supplementation upon muscle glycogen and TCr accumulation and subsequent exercise performance.

Chapter 6

EFFECTS OF CREATINE SUPPLEMENTATION UPON INDICES OF HAEMATOLOGICAL, HEPATOLOGICAL, MUSCLE AND RENAL FUNCTION

6.1 INTRODUCTION

Much of the research concerning Cr supplementation has demonstrated improvements in exercise performance, particularly upon repeated bouts of short-lasting maximal exercise. Other effects of Cr supplementation include an increase in body mass (Balsom *et al*, 1993a; Greenhaff *et al*, 1994; Stroud *et al*, 1994), which has been attributed to increased fat-free mass (Earnest *et al*, 1995). It has been proposed that this increase is due to enhanced water retention by the body (Hultman *et al*, 1996), possibly resulting from changes in muscle osmolarity as Cr is accumulated. A consequence of increased water uptake by muscles is a tendency for swelling, or increased definition of muscles, a phenomena which has not passed unnoticed by bodybuilders. As a result of research findings being cited in popular fitness and sporting media, Cr has become a very popular dietary supplement used by amateur and professional athletes.

Recently, however, anecdotal opinions have been expressed about the potential effects of ingesting Cr on aspects of health, such as muscle cramping, muscle-tendon injury and renal dysfunction (Anon, 1998a,b; Josefson, 1998). These concerns have prompted comments from officials of sporting and health bodies that there is insufficient information concerning the safety of Cr ingestion over a prolonged period. Opinions such as these, however, have not been substantiated with scientific evidence, other than two recent case reports of renal dysfunction accompanying oral creatine supplementation (Pritchard & Kalra, 1998; Koshy *et al*, 1999), albeit one in a patient with pre-existing kidney disease (Pritchard & Kalra, 1998). Despite its apparent widespread use amongst athletes (Harris & Arthur, 1998), there is little published evidence of the effects of Cr

supplementation on indices of health, such as haematological, hepatic, and renal function or muscle damage.

A problem with interpreting anecdotal 'side-effects' of Cr supplementation is the variation in dosage that individuals supplement with. In the majority of controlled studies, the dosing regimen established by Harris and colleagues (1992) of 20 g Cr on a daily basis for 5 d has been used to load muscles with Cr. The elevated muscle TCr concentrations achieved by this regimen can be maintained by continuing Cr ingestion at a "maintenance dose" of approximately 2 g every day (Hultman *et al*, 1996), which represents the rate of daily Cr degradation to creatinine (Walker, 1979). However, some individuals may consume much higher doses during a loading phase and subsequent maintenance (Anon, 1998a).

Aim

The aim of the present study was to obtain information relating to indices of haematological, hepatic and renal function and muscle damage in young healthy adult subjects, before and after a regimen of "Cr loading", and also after Cr loading followed by an 8-week "maintenance dose".

6.2 METHODS

Subjects and experimental groups

Forty-eight healthy subjects volunteered to take part in the present series of experiments. None reported any history of kidney or liver related illness. All subjects were moderately active, but none were highly trained. The subjects were divided randomly into 7 experimental groups to examine two different aspects of Cr supplementation.

Effects of a Cr loading regimen

Group 'Cr_{LOAD}' consisted of 7 male subjects (mean (± SD) age 23 (4) years; BMI 22.4 (2.1) $kg.m^{-2}$) who ingested 5 g of Cr dissolved in a warm drink followed by 500 ml of a CHO containing solution 4 times daily for 5 d. This regimen was used because it has previously been shown to augment muscle Cr accumulation by ~ 60% compared with accumulation when Cr alone was ingested (Green *et al*, 1996a). Blood samples for analysis were obtained before supplement ingestion on the first day and on the day after the ingestion regimen had been completed.

Group 'P_{LOAD}' (a placebo group) consisted of 7 male subjects (age 24 (5) years; BMI 21.3 (0.9) $kg.m^{-2}$) who ingested 500 ml of a CHO containing solution 4 times daily for 5 d. Blood samples for analysis were obtained as for Cr_{LOAD} group.

Group 'Cr_{LOAD+6}' consisted of 6 male subjects (age 24 (3) years; BMI 25.6 (6.3) $kg.m^{-2}$) who ingested 5 g of Cr plus 1g glucose dissolved in a warm drink 4 times daily for 5 d. Blood samples for analysis were obtained prior to the start of supplementation and six weeks after the ingestion regimen had been completed.

Group 'P_{LOAD+6}' (a placebo group) consisted of 6 subjects (age 22 (2) years; BMI 21.7 (2.0) $kg.m^{-2}$; 3 males), who ingested 6 g of glucose dissolved in a warm drink 4 times daily for 5 d. Blood samples for analysis were obtained as for Cr_{LOAD+6} group.

Effects of Cr loading and an 8-week maintenance regimen

Group 'Cr_{MAINT}' consisted of 7 female subjects[1] (age 26 (8) years; BMI 23.3 (1.9) $kg.m^{-2}$) who ingested a loading dose of 5 g Cr dissolved in a warm drink 4 times daily for 5 d, and who then maintained an intake of 3 g Cr in a warm drink, once

[1] Refer to Note 6.1 at end of Chapter

daily for 8 weeks. Blood samples for analysis were obtained before supplement ingestion on the first day of the loading dose and on the day after the maintenance regimen had been completed.

Group '$Cr_{MAINT+EX}$' consisted of 9 female subjects (age 27 (6) years; BMI 23.4 (3.2) $kg.m^{-2}$) who ingested a loading dose of 5 g Cr dissolved in a warm drink 4 times daily for 5 d, and who then maintained an intake of 3 g Cr in a warm drink, once daily for 8 weeks. During the 8 week maintenance period subjects engaged in a resistance training program, involving 3 one-hour supervised sessions on resistance equipment each week. This exercise program was used to create similar conditions to those of individuals who ingest Cr during periods of training to improve athletic performance. Blood samples for analysis were obtained as for the Cr_{MAINT} group.

Group '$P_{MAINT+EX}$' (a placebo group) consisted of 6 female subjects (age 28 (5) years; BMI 24.5 (3.6) $kg.m^{-2}$) who ingested a "loading dose" of 5 g of a glucose polymer (dextrose) in a warm drink 4 times daily for 5 d, and who then maintained an intake of 3 g of dextrose in a warm drink, once daily for 8 weeks. During the 8 week supplementation period subjects engaged in a resistance training program identical to subjects in the $Cr_{MAINT+EX}$ group. Blood samples for analysis were obtained as for the Cr_{MAINT} group.

Supplement formulations

Subjects in the Cr_{LOAD+6}, P_{LOAD+6}, Cr_{MAINT}, $Cr_{MAINT+EX}$ and $P_{MAINT+EX}$ groups were provided with supplements in a double-blind manner. During the 5 d "loading dose" period, subjects in all groups ingested their supplements as described in Chapter 2. During the 8-week "maintenance dose" subjects ingested their daily supplement dose at the same time each day.

Blood sampling protocol

Subjects reported to the laboratory on the morning of the experiments following an overnight fast, having abstained from alcohol consumption and strenuous exercise during the previous 48 h . Height and body mass in light clothing were recorded. Blood samples were obtained and treated as described in Chapter 2. Samples were subsequently analysed for indices of renal function (serum sodium, potassium, urea and creatinine concentrations), hepatic function (serum gamma glutamyl transferase, alkaline phosphatase, alanine aminotransferase activity, and albumin and total bilirubin concentrations) and indices of haematological function (haemoglobin concentration was measured and white blood cells and platelets counted). Additionally, an index of muscle damage (serum creatine kinase activity) was measured in the groups assessed six weeks following acute supplementation and following the more chronic maintenance regimen.

6.3 RESULTS

All subjects reported adherence to the experimental protocol and complete ingestion of the supplements. None of the subjects reported any adverse effects as a result of ingesting the supplements.

Effects of a Cr loading dose regimen

Group mean data of the indices measured were within the normal range prior to, the day following and 6 weeks following the loading regimen (Table 6.1). On the day following Cr loading there was no significant difference in any of the indices measured prior to Cr loading, however, moderate decreases were observed in serum urea and bilirubin concentrations of the P_{LOAD} group. Although there were

no differences in absolute concentrations, there was a significant between-group difference in the change in serum creatinine concentration over time (Cr_{LOAD}, 24 (10) μmol/L; P_{LOAD}, -3 (2) μmol/L; $P < 0.05$). This difference was not evident six weeks following the completion of the loading dose (Cr_{LOAD+6}, 2 (2) $\mu mol.L^{-1}$; P_{LOAD+6}, 3 (2) $\mu mol.L^{-1}$; $P > 0.05$). Serum sodium and urea concentrations had increased in the Cr_{LOAD+6} group six weeks after completion of Cr loading (Table 6.1). The change in serum urea concentration with time was significantly different in the Cr_{LOAD+6} group compared with its placebo group (Cr_{LOAD+6}, 1.2 (0.3) mmol/L; P_{LOAD+6}, -0.4 (0.2) mmol/L; $P < 0.001$). A moderate decrease in serum albumin concentration was observed in the P_{LOAD+6} group.

Effects of a Cr maintenance dose regimen

Group mean data of the indices measured were within the normal range prior to the Cr loading regimen, and on the day after the maintenance dose was completed (Table 6.2). Moderate increases in serum potassium, creatinine and albumin concentrations were observed in both groups receiving Cr. Additionally there was a small decrease in alanine aminotransferase activity in the Cr_{MAINT} group and an increase in alkaline phosphatase activity in the $Cr_{MAINT+EX}$ group. There was a significant decrease in serum urea concentration in the $P_{MAINT+EX}$ group. The changes in serum creatinine concentration over time in the groups receiving Cr were significantly different from the placebo group, (Cr_{MAINT}, 26 (6) μmol/L; $Cr_{MAINT+EX}$, 17 (4) μmol/L; $P_{MAINT+EX}$, -6 (3) μmol/L; $P < 0.001$, $P < 0.01$, respectively).

Table 6.1

†Measurement units for indices of renal, hepatological, muscle and haematological function; sodium (mmol/L), potassium (mmol/L), urea (mmol/L), creatinine (μmol/L), gamma glutamyl transferase (Gamma GT; U/L), alkaline phosphatase (alk. phos; U/L), alanine aminotransferase (ALT; U/L), albumin (g/L), total bilirubin (μmol/L), creatine kinase (CK; U/L), haemoglobin (Hb; g/100ml), white blood cells (WBC; x 10^9/L), platelets (x 10^9/L).

* P <0.05, ** P < 0.01, and *** P < 0.001 indicate significant differences between pre- and post- supplementation values, within each group.

Table 6.1 Normal range and mean values (SD) of indices of renal, hepatic, muscle and haematological function pre (all groups), immediately after (Cr_{LOAD}, P_{LOAD} groups) and 6 weeks after (Cr_{LOAD+6}, P_{LOAD+6} groups) 5 days of creatine (Cr) or placebo (P) ingestion.

Clinical index†	Normal range	'Cr_{LOAD}' group		'P_{LOAD}' group		'Cr_{LOAD+6}' group		'P_{LOAD+6}' group	
		Pre	Post	Pre	Post	Pre	6week	Pre	6week
Sodium	135 - 145	141 (2)	141 (1)	141 (2)	142 (1)	139 (1)	141 (1)*	138 (1)	140 (2)
Potassium	3.5 - 5.0	4.0 (0.4)	3.9 (0.3)	4.0 (0.3)	4.3 (0.4)	4.0 (0.2)	4.0 (0.3)	4.1 (0.2)	4.0 (0.2)
Urea	2.5 - 7.0	4.4 (1.1)	3.8 (1.3)	4.9 (1.0)	3.5 (1.1)***	4.1 (0.4)	5.3 (0.6)**	4.7 (0.7)	4.2 (0.7)
Creatinine	60 - 130	88 (15)	112 (30)	96 (14)	92 (18)	103 (10)	105 (10)	82 (8)	85 (12)
Gamma GT	0 - 65	20 (5)	19 (4)	24 (7)	23 (8)	27 (7)	27 (9)	21 (7)	22 (6)
Alk. phos.	30 - 110	67 (17)	72 (17)	71 (16)	72 (18)	72 (9)	72 (13)	64 (15)	61 (15)
ALT	5 - 40	25 (7)	24 (7)	22 (3)	25 (4)	27 (8)	30 (7)	19 (3)	21 (3)
Albumin	35 - 50	44 (3)	43 (2)	44 (2)	44 (2)	47 (4)	45 (1)	47 (2)	45 (2)***
Bilirubin	5 - 17	14 (5)	12 (6)	16 (3)	12 (3)**	12 (2)	10 (2)	12 (4)	10 (3)
CK	up to 200	not measured		not measured		152 (85)	127 (34)	92 (42)	123 (108)
Hb	11.5 - 18.0	14.0 (0.8)	14.0 (0.9)	14.6 (0.3)	14.4 (0.6)	14.9 (0.8)	14.7 (0.6)	13.9 (1.3)	13.4 (1.1)
WBC	4.0 - 11.0	5.0 (1.5)	5.5 (0.9)	5.7 (1.4)	7.0 (1.4)	6.2 (1.0)	6.7 (1.3)	5.3 (1.4)	5.3 (1.2)
Platelets	150 - 400	197 (47)	212 (39)	233 (63)	243 (60)	263 (48)	236 (40)	223 (62)	214 (43)

Table 6.2

†Measurement units for indices of renal, hepatological, muscle and haematological function; sodium (mmol/L), potassium (mmol/L), urea (mmol/L), creatinine (μmol/L), gamma glutamyl transferase (Gamma GT; U/L), alkaline phosphatase (alk. phos; U/L), alanine aminotransferase (ALT; U/L), albumin (g/L), total bilirubin (μmol/L), creatine kinase (CK; U/L), haemoglobin (Hb; g/100ml), white blood cells (WBC; x 10^9/L), platelets (x 10^9/L).

** $P < 0.01$, and *** $P < 0.001$ indicate significant differences between pre- and post- supplementation values, within each group.

Table 6.2 Normal range and mean values (SD) of indices of renal, hepatic, muscle and haematological function pre- and immediately after 5 days of creatine (Cr) or placebo (P) ingestion followed by an 8-week Cr or P maintenance regimen in the presence and absence of exercise training.

Clinical index†	Normal range	'Cr$_{MAINT+EX}$' group		'Cr$_{MAINT}$' group		'P$_{MAINT+EX}$' group	
		Pre	Post	Pre	Post	Pre	Post
Sodium	135 - 145	140 (2)	139 (2)	140 (2)	140 (1)	141 (2)	140 (1)
Potassium	3.5 - 5.0	3.9 (0.2)	4.1 (0.2)**	3.9 (0.2)	4.1 (0.3)**	3.9 (0.3)	4.2 (0.2)
Urea	2.5 - 7.0	3.6 (0.7)	3.6 (0.8)	4.2 (0.7)	3.8 (0.4)	4.3 (0.6)	3.6 (0.8)**
Creatinine	54 - 117	69 (6)	86 (15)***	68 (4)	95 (17)***	78 (9)	72 (5)
Gamma GT	0 - 40	19 (5)	21 (4)	19 (5)	20 (4)	41 (54)	31 (28)
Alk. phos.	30 - 110	52 (6)	60 (12)**	53 (8)	55 (9)	67 (18)	64 (11)
ALT	5 - 40	27 (4)	22 (3)	26 (4)	21 (4)**	30 (5)	28 (9)
Albumin	35 - 50	40 (2)	43 (2)**	40 (3)	44 (3)**	41 (3)	43 (2)
Bilirubin	5 - 17	10 (4)	10 (2)	9 (2)	10 (2)	12 (6)	12 (2)
CK	up to 200	142 (149)	142 (48)	91 (59)	91 (34)	186 (194)	198 (145)
Hb	11.5 – 16.5	12.2 (1.0)	12.2 (0.8)	12.3 (1.1)	12.8 (0.8)	13.3 (1.0)	13.2 (1.1)
WBC	4.0 - 11.0	5.9 (0.6)	6.2 (1.2)	6.5 (1.3)	6.8 (1.5)	6.7 (1.4)	6.0 (0.6)
Platelets	150 - 400	244 (59)	249 (58)	268 (21)	292 (59)	274 (43)	267 (70)

6.4 DISCUSSION

This study has reported the effects of short-term (20 $g.d^{-1}$ for 5 d) and chronic (3 $g.d^{-1}$ for 56 d) Cr supplementation on a range of indices of haematological, hepatic, renal and muscle function in young healthy adults. Data from blood samples taken prior to supplementation, on the day following and six weeks after an established Cr loading dose regimen (Hultman *et al*, 1996), and following a subsequent 8-week maintenance dose of 3 $g.d^{-1}$ have been examined in the present study. Mean concentrations of all indices were within the normal range (Medicine Publishing Foundation, 1983) at all times. Information examining some of these indices in older adults (~51 years) during 8 week Cr supplementation has been published in abstract form (Almada *et al*, 1996; Earnest *et al*, 1996), whilst more recent work has reported some clinical chemistry indices in male athletes ingesting Cr for 28 d (Kreider *et al*, 1998) and renal responses in healthy males after short-term supplementation (Poortmans *et al*, 1997). In none of these reports were adverse responses reported.

Creatinine has been established as the sole end product of Cr degradation, being formed non-enzymatically in an irreversible reaction (Fitch & Sinton, 1964; Fitch *et al*, 1968). As skeletal muscle is the major store of the body Cr pool (Walker, 1979), it is therefore the main site of creatinine production. In normal healthy individuals plasma creatinine concentration is dependent upon total muscle mass (Heymsfield *et al*, 1983), thus daily renal creatinine excretion is relatively constant in a given individual, but can vary between individuals (Fitch, 1977). In the present study, serum creatinine concentration on the day following Cr loading was within the normal range and was not significantly greater than pre-supplementation concentration. These results compare well with those of

Poortmans and colleagues (1997), who found no difference in subjects' plasma creatinine concentrations between Cr supplemented (20 $g.d^{-1}$, 5 d) and placebo conditions. The relatively greater change in serum creatinine concentration over time compared with that of the placebo group in the present study merely reflects the contribution of the increased muscle Cr concentration to the rate of muscle Cr degradation to creatinine. This has been reported previously, together with a parallel increase in urinary creatinine excretion (Hultman *et al*, 1996). This change in creatinine production, coupled with the fact that creatinine concentration remained within the normal range, suggests that kidney function was unaffected by Cr loading. Six weeks after discontinuation of Cr loading there was no indication of an elevated serum creatinine concentration. Muscle TCr will have returned close to pre-supplementation levels by this time (Hultman *et al*, 1996), as will have renal creatinine excretion. Maintaining elevated muscle Cr concentration by ingesting 3 $g.d^{-1}$ Cr after Cr loading increased serum creatinine above pre-supplementation concentrations. However, these concentrations were again within the normal range and again, were likely due to the increase in muscle Cr concentration. Similar results have been observed following 28 d Cr supplementation (15 $g.d^{-1}$) and training in young athletes (Kreider *et al*, 1998), however, serum creatinine concentration did not change in older adults following 8 week Cr supplementation (20 $g.d^{-1}$, 5 d followed by 10$g.d^{-1}$ for 51 d; Earnest *et al*, 1996).

No difference in serum urea concentration was observed on the day following Cr supplementation. This is in contrast to studies in rats, which showed elevated plasma urea concentrations on the day following a 7 d period of Cr administration (Ööpik *et al*, 1996). The authors attributed this result to an increased availability

of arginine for urea synthesis, resulting from a decrease in its use in endogenous Cr biosynthesis. The increase in serum urea concentration six weeks following the Cr loading regimen is likely to be of little clinical significance. The serum concentration of urea represents primarily a balance between urea formation from protein catabolism and its excretion in urine. As urea is formed almost solely in the liver from the catabolism of amino acids it is unlikely that any changes in serum urea at this time would be seen as a direct result of Cr supplementation. The breakdown pathway for Cr is via transformation to creatinine and is not a reversal of its synthesis from its amino acid precursors. This contention is supported by the lack of any difference in serum urea concentration on the day following a Cr loading regimen. It has been proposed that Cr supplementation may promote lean tissue synthesis, at least during an exercise training period (Vandenberghe *et al*, 1997; Kreider *et al*, 1998). If this was the case in the present study, it is possible that the elevated urea concentration resulted from an increased lean tissue mass following Cr supplementation contributing to a greater degree of net protein catabolism during the six week post-supplementation period. This explanation seems unlikely in this case, however, given that Cr was ingested for only 5 d without any concurrent training, and is refuted further by the finding that serum urea was unchanged after more chronic supplementation with training ($Cr_{MAINT+EX}$ group).

Serum sodium concentration was increased six weeks after Cr loading. However, the absence of any similar change on the day after completing supplementation suggests that this was unlikely to be due to supplementation, and the magnitude of the change would be expected to be of little clinical significance. Similarly the

increase in serum potassium concentration following chronic Cr supplementation was small and again unlikely to be of clinical significance.

All hepatic function indices were within the normal limits before and after Cr loading. The changes observed in serum alkaline phosphatase ($Cr_{MAINT+EX}$) and alanine aminotransferase (ALT) activity (Cr_{MAINT}), and albumin (P_{LOAD+6}, $Cr_{MAINT+EX}$, Cr_{MAINT}) and bilirubin (P_{LOAD}) concentrations with time were all marginal and occurred in both directions. Elevated ALT activity is a commonly used indicator of liver cell damage, however, in the present study its activity decreased over time. A small increase in ALT has been observed following 28 d Cr supplementation (Kreider *et al*, 1998), however, no similar changes in hepatic function indices have been observed by other investigators (Almada *et al*, 1996; Earnest *et al*, 1996; Kreider *et al*, 1998; Poortmans *et al*, 1997). It is suggested that the changes in the present study are unlikely to be of clinical significance and probably unrelated to the experimental protocol.

Haematological indices were unchanged by the supplementation protocols. Similar observations have been made by Kreider *et al* (1998). In an investigation of athletes ingesting Cr whilst engaged in body mass reduction for weight-classed competition, haemoglobin concentration was slightly increased post-supplementation (Ööpik *et al*, 1998). However, this small increase was likely to have been mediated by a 3.8% decrease in plasma volume observed at the same time, and not as a result of Cr supplementation.

Serum CK activity, an accepted marker of muscle damage, was unchanged six weeks following acute Cr supplementation, nor was it changed following more chronic supplementation in the presence and absence of resistance training. This latter finding is of particular interest, as anecdotal comments have linked Cr

supplementation to the development of muscle cramps and muscle-tendon injury during or following exercise (Anon, 1998a; Anon 1998b; Josefson, 1998). In the present study there were no reports of muscle cramping or injury. An increase in CK activity has been observed in males, but not females, following 8 weeks Cr supplementation (Almada *et al*, 1996).

In conclusion, results from this study provide evidence that there are no obvious adverse clinical effects of acute or more chronic Cr supplementation on indices of haematological, hepatic, muscle and renal function. Additionally, all subjects reported no adverse effects of Cr supplementation and no incidences of muscle injury occurred in individuals ingesting Cr during a resistance training program. It can therefore be suggested that there is no obvious health risk associated with Cr supplementation to healthy individuals when ingested in the recommended quantities that have been scientifically proven to have an ergogenic effect.

Note 6.1 It is accepted that variations in hormone concentrations may influence the indices measured in the present investigation, particularly in females. In an attempt to control for such influences, blood samples obtained from each female subject pre- and post-supplementation were taken at approximately the same stage of her menstrual cycle. All females were pre-menopausal and were taking oral contraceptive medication at the time of the investigation.

Chapter 7

GENERAL DISCUSSION

The experiments described in this thesis were conducted to examine various effects upon human metabolism of oral supplementation with the guanidino compounds creatine and arginine. Creatine supplementation has received a lot of attention since it was first shown that muscle TCr concentration could be increased by oral Cr ingestion (Harris *et al*, 1992). The first demonstrations that Cr supplementation could improve exercise performance of repeated bouts of high-intensity exercise (Greenhaff *et al*, 1993; Balsom *et al*, 1993a) stimulated a great amount of subsequent investigation. This included studies of the effects of Cr ingestion upon performance of different activities and also of the mechanisms by which Cr supplementation produced these effects. It was noted in some investigations that some individuals demonstrated little or no change in muscle TCr concentration following Cr supplementation, and that a large variation in TCr accumulation existed between individuals. Methods proposed to improve muscle TCr accumulation included the performance of submaximal exercise (Harris *et al*, 1992) and ingestion of CHO (Green *et al*, 1996a) during the period of Cr supplementation.

Chapter 3 of this thesis examined the effects of combining exhaustive exercise with Cr + CHO supplementation upon subsequent accumulation of TCr and glycogen in skeletal muscle. Using a 1-legged model of exercise, it was demonstrated that exhaustive exercise performed prior to Cr + CHO supplementation resulted in greater Cr accumulation in exercised than in non-exercised muscle. No increase in TCr concentration of the exercised muscle was apparent after 6 h of Cr supplementation immediately following exhaustive exercise, which suggested that an exercise-induced increase in blood flow to the muscle was not the cause of the increased accumulation. Therefore other factors,

probably localised to the exercised muscle itself, may be responsible for this effect. Further investigation is required, therefore, into mechanisms by which exercise augments Cr accumulation. The combined exercise / supplementation protocol used in the study did not result in muscle TCr concentrations above those achieved by Cr + CHO supplementation without exercise (Green *et al*, 1996a), however, and appeared to diminish Cr accumulation in non-exercised muscle. It was demonstrated that the exercise protocol resulted in diminished glucose-stimulated responses of subjects compared with their responses when in a rested state. This provides further support to the proposed importance of insulin in muscle Cr transport (Steenge *et al*, 1998). It is intriguing, however, that such a difference in Cr accumulation existed between limbs. Although the post-exercise blunting of the CHO-stimulated insulin response is likely to have affected Cr transport in the early period of supplementation, it is unlikely to have lasted beyond the first day of supplementation (LeBlanc *et al*, 1981). It is possible that by this time the process of Cr transporter down-regulation had begun, diminishing net Cr uptake despite a return to a normal insulin response to glucose. This proposal is given support by the observation that less Cr is retained by the body during days 2 and 3 of supplementation than during the first day of supplementation (Harris *et al*, 1992).

Another interesting finding of Chapter 3 was that the glycogen supercompensation response, seen following glycogen-depleting exercise and a high CHO diet, was augmented when Cr was also ingested. This suggested that muscle Cr accumulation could influence muscle glycogen synthesis by some mechanism. One explanation proposed for this effect was that muscle cell volume increased due to Cr accumulation, which stimulated glycogen synthesis. This proposal has

been given support by findings, recently published, that acute Cr supplementation increases intracellular volume in man (Zeigenfuss *et al*, 1998). The augmentation of glucose-stimulated insulin release by ingestion of Cr with CHO (Green *et al*, 1996c) could be another mechanism by which Cr ingestion might improve muscle glycogen storage. However, in Chapter 3 no difference in insulin response to CHO was observed between subjects receiving CHO or Cr + CHO under rested conditions or following exercise. Furthermore, there was no difference, between groups, in glycogen concentration of the non-exercised muscle. These results suggest that further investigation of the effects of Cr + CHO ingestion upon insulin release are warranted.

The finding, in Chapter 3, that Cr + CHO supplementation resulted in greater glycogen storage in exercised muscle, led to the investigation undertaken in Chapter 4. In this experiment, the effects of another guanidino compound, arginine, upon physiological factors known to influence CHO storage were studied. It was proposed that oral ingestion of arginine might increase the muscle storage of an ingested quantity of CHO, by enhancing insulin release and increasing blood flow to muscles. This investigation (performed using subjects under rested conditions and following types of exercise known to differentially affect glucose storage) aimed to assess whether arginine ingestion could be a beneficial aid to post-exercise glycogen replenishment. There was no indication, however, that arginine ingestion improved the storage of ingested CHO following any type of activity. Although not measured in the study, it was likely that the quantity of arginine ingested was insufficient to increase plasma arginine concentrations to levels that would influence insulin release or stimulate peripheral vasodilatation. A question for future research would be to determine

the oral arginine dose necessary, to reproduce the effects observed when arginine is administered intravenously. However, from a practical point of view, it is unlikely that such information would be of use to individuals wishing to improve muscle CHO storage after exercise. Dietary intake of ~30 $g.d^{-1}$ arginine leads to sodium and water loss in urine (Beaumier *et al*, 1995). Furthermore, the observation that some individuals experience gastrointestinal disturbances following ingestion of 6 – 10 g doses of arginine (present investigation; Gater *et al*, 1992) suggests that higher oral doses of arginine may not be well tolerated. One way in which this problem may be overcome would be to combine the ingestion of two or more insulinotropic amino acids.

Results of Chapter 3 supported a previous observation that a tendency existed for greater muscle glycogen accumulation when Cr was ingested with CHO (Green *et al*, 1996b; Green, 1996). The amounts of additional glycogen stored, compared with those stored following CHO supplementation, were proposed to be sufficient to prolong the duration of subsequent exhaustive submaximal exercise. In Chapter 5, the effects of CHO, Cr + CHO and Cr supplementation regimens upon muscle glycogen and Cr accumulation were examined, along with their effects upon subsequent endurance exercise performance. It was shown that Cr ingestion during CHO supplementation did not significantly improve muscle glycogen accumulation compared with CHO supplementation alone. As a consequence of this, performance of endurance exercise was not significantly improved, although a tendency for prolonged exercise duration was apparent following Cr + CHO supplementation. For practical reasons, the experimental group was split into 2 sub-groups, which effectively decreased the statistical power of the tests used to analyse the data. It was suggested that the results were inconclusive due to low

sample numbers in each of the Cr treatment groups and that more conclusive information would be obtained if additional subjects were included in the investigation.

Chapter 5 also showed that, although muscle Cr concentration tended to be higher following Cr + CHO supplementation, TCr concentrations were not significantly different from those of individuals supplemented with Cr alone. This may also have been due to the small numbers of subjects in the experimental groups and a wide individual variation in their TCr concentrations. Examination of the muscle TCr accumulation of subjects following Cr and Cr + CHO supplementation revealed that this was less than had been previously observed in subjects receiving identical supplements. This may partly have been due to some individuals demonstrating little or no change in muscle TCr concentration (non-responders). Indeed, some subjects receiving Cr alone and some in the Cr + CHO group could be considered as 'non-responders'. Removal of these subjects' values from their respective group mean TCr concentrations suggested that the remainder of the subjects in each group had responses similar to those previously observed in subjects receiving the same treatments (e.g. Green *et al*, 1996a). It was suggested that further insight into the effects of Cr + CHO supplementation could be achieved, by increasing the number of subjects in each experimental group. Although the results of Chapter 5 suggested that Cr + CHO supplementation may not improve endurance performance, the results of Chapter 3 suggest that performing glycogen-depleting exercise prior to Cr + CHO supplementation may achieve this goal. The changes in muscle glycogen concentration produced by the protocol in Chapter 3 were far greater than by Cr + CHO supplementation in rested individuals. Therefore, the next direction of research in this area should

examine endurance performance of individuals before and after a supplementation protocol that involves glycogen-depleting exercise followed by Cr + CHO ingestion. Careful consideration should be made of the method for measuring endurance performance and of the training status of experimental subjects, to minimise within- and between- subject differences that may overshadow the statistical power of the results.

Another direction for future research is to investigate the influence that an individual's training status has upon muscle Cr accumulation. To date, there has been no investigation that has compared muscle Cr accumulation of trained and untrained subjects. In a critical review of the ergogenic effects of Cr on exercise performance in competition-like tasks, it was proposed that highly trained athletes did not benefit from Cr supplementation as much as untrained subjects (Mujika & Padilla, 1997). The training status of an individual can be suggested to influence a number of factors that may affect Cr accumulation.

An individual's training status is known to influence muscle Na^+/K^+ pump concentration, a factor that provides a driving force for muscle Cr transport. Muscle total Na^+/K^+ pump activity has been proposed to influence Cr accumulation by its ability to generate a Na^+ gradient across muscle cell membranes. An increase in net Na^+/K^+ pump activity (by increases in the total number of pumps or in pump activity, or both) would increase the drive for Na^+-dependent Cr transport (Odoom *et al*, 1996). Exercise training results in increases in muscle total Na^+/K^+ pump concentration (Klitgaard & Clausen, 1989; Green *et al*, 1993; McKenna *et al*, 1993; Madsen *et al*, 1994), with strength training having a greater effect than endurance training (Clausen, 1998). It can be proposed, therefore, that trained subjects might achieve a greater degree of Cr accumulation

than untrained individuals, due to a higher concentration of muscle Na^+/K^+ pumps. Net activity of Na^+/K^+ pumps also depends upon the activity of the individual pumps themselves. Insulin has a potent stimulatory effect upon Na^+/K^+ pump activity and is believed to act by increasing the affinity of the pumps for intracellular Na^+, thereby promoting Na^+ transport from the cell (Clausen, 1998). An increase in serum insulin concentration, such as that following ingestion of CHO, would be expected to increase Na^+/K^+ pump activity. It is this phenomenon that has been suggested to account for the greater Cr accumulation seen with Cr + CHO supplementation (Greenhaff, 1997). Trained individuals exhibit a diminished insulin response to a given amount of CHO than untrained individuals (Heath *et al*, 1983; King *et al,* 1987; Rodnick *et al*, 1987; Engdahl *et al*, 1995; Hickner *et al*, 1997). It is known that high physiological concentrations of insulin are necessary to stimulate Cr transport into human muscle *in vivo* (Steenge *et al*, 1998). Such high concentrations can usually be achieved by ingesting large amounts of CHO. As glucose-stimulated insulin release is diminished in trained subjects, it is possible that they may not benefit from CHO ingestion during Cr supplementation. Some of the diminished insulin response seen in trained subjects is attributable to the effect of the preceding bout of exercise. This was shown in trained individuals, who had an increase in glucose-stimulated insulin response above their normal response, after 10 days of detraining (Heath *et al*, 1983). An interesting direction for future research would, therefore, be to examine Cr accumulation of trained individuals. Experimental interventions might include Cr supplementation following various periods of detraining, or comparison of muscle Cr accumulation in trained and untrained subjects under

conditions where circulating insulin concentrations are identical (e.g. by use of an insulin clamp technique and naso-gastric Cr feeding).

The type of training that individuals are involved in may also influence their capacity for Cr accumulation. Although mainly genetically-determined, muscle fibre composition can be influenced, to a limited extent, according to the type of activity that an individual is regularly engaged in (Komi & Karlsson, 1978). PCr concentration varies between fibre types (Soderlund *et al*, 1992; Greenhaff *et al*, 1994; Sahlin *et al*, 1997), with type II fibres having approximately 20% more PCr than type I fibres. Although not demonstrated, it is possible that this is achieved by type II fibres having a greater number of Cr transporters than type I fibres. It could be proposed, therefore, that individuals with a greater proportion of type II fibres (e.g. sprinters, weightlifters) would have greater muscle TCr concentrations following supplementation than individuals with a more even distribution of type I and II fibres. Examination of PCr distribution across fibre types before and after supplementation has shown, however, that type II fibres have the same change in PCr concentration as type I fibres (Casey *et al*, 1996b). Although no information is available for single fibre concentration of Cr, it is reasonable to assume that similar changes in muscle Cr concentration between fibre types occur with Cr supplementation. This would suggest that any differences in muscle fibre composition of subjects would be unlikely to influence the extent of muscle Cr accumulation.

The rise in the popularity of Cr as a dietary supplement has ultimately led to an increase in the attention it has received from the public media. An unfortunate accompaniment to this publicity is the opportunity for anecdotal opinions to be expressed, which subsequently become regarded as factual information. In the

case of Cr supplementation, such anecdotal claims have implicated Cr supplementation with causing muscle injury, cramping, dehydration, and gastrointestinal disturbance and, in one extreme case, the deaths of three American college wrestlers. However, these claims have not been substantiated with any scientific evidence, indeed scrutiny of the circumstances associated with the wrestlers' deaths led the U.S. Department of Health and Human Services to conclude that Cr was not associated with the deaths (Centers for Disease Control and Prevention, 1998). Two recent case reports remain the only scientific evidence linking Cr supplementation to impaired health (Pritchard & Kalra, 1998; Koshy *et al*, 1999). Both of these reports linked Cr supplementation to kidney dysfunction, although neither could definitively conclude that the supplementation caused the conditions. A small number of investigations have examined the effects of Cr supplementation on various aspects of health, such as kidney function (Earnest *et al*, 1996; Kreider *et al*, 1998; Poortmans *et al*, 1997), liver function (Almada *et al*, 1996; Earnest *et al*, 1996; Kreider *et al*, 1998; Poortmans *et al*, 1997) and muscle damage (Almada *et al*, 1996). Chapter 6 investigated the effects of Cr supplementation on markers of haematological, hepatological, muscle and renal function in healthy individuals. No obvious indication of impaired function was observed for any of the aspects measured. The majority of concerned comments expressed in the media from individuals or governing bodies have been related to the effects of Cr supplementation on the kidneys. It is likely that these concerns stem from the use of serum creatinine concentration as a marker of glomerular function in the kidney. Creatinine concentration above the normal range in the blood traditionally has indicated impairment of renal function, and a change in concentration over time is usually as a result of a change in

glomerular filtration rate (Marshall, 1993). Diagnosis of impaired renal function arising from serum creatinine measurement makes the assumption that creatinine production is constant in the body. This is not the case following Cr supplementation, however, as creatinine production rises with an increased muscle Cr store (Hultman *et al*, 1996). Furthermore, creatinine excretion in the urine rises during Cr supplementation and is maintained at elevated levels for a week or more following ingestion of the last supplement (Hultman *et al*, 1996). Under circumstances where renal dysfunction is indicated in an individual who has supplemented with Cr, it might be appropriate to assess the clearance rate from the circulation of intravenously infused inulin. Inulin clearance is accepted as being equal to the glomerular filtration rate, as it is completely filtered from the blood by the glomeruli and is not secreted, reabsorbed or metabolised by renal tubules (Marshall, 1993). It may be prudent to incorporate this method into any future laboratory-based investigations of the effects of Cr supplementation upon kidney function.

The general findings from Chapter 6 suggest that there are no obvious adverse effects to an individual's health from short-term Cr supplementation (i.e. 20 $g.d^{-1}$ for 5 days) or prolonged Cr ingestion (e.g. 3 $g.d^{-1}$ for 8 weeks). The effects of Cr supplementation for longer periods (> 8 weeks) upon health are currently unknown. It would be of value to obtain clinical information, similar to that measured in Chapter 6, from individuals ingesting Cr over longer periods such as during a season of athletic training. Information relating to the incidence of injury or other side effects attributed to Cr ingestion should also be recorded. This data, when compared with closely-matched control individuals undergoing identical training regimens, could allow assessment of whether Cr supplementation *per se*

contributes to these side effects, or whether they arise as a result of increased workloads attempted during training. At present there is no information concerning the effects of ingesting amounts of Cr higher than those proven to have an ergogenic effect. Anecdotal comments have claimed that some athletes use 10 – 30 times the recommended doses (Anon, 1998a). As there appears to be no additional benefit of ingesting very large amounts of Cr for long periods (the majority of the daily 20 g administered is excreted in urine after the initial days of supplementation, Harris *et al*, 1992), this practice should be discouraged. Perhaps some responsibility should fall upon researchers to promote sensible use of Cr, by reinforcing this message in scientific publications.

In summary, the experiments reported in this thesis have demonstrated that Cr accumulation by muscle can be enhanced by prior exhaustive exercise (Chapter 3), but that the stimulatory effect of CHO ingestion upon Cr accumulation is abolished under these conditions, possibly due to diminished glucose-stimulated insulin release following exercise. It was also shown that CHO ingestion during Cr supplementation might not augment Cr accumulation in all individuals (Chapter 5), suggesting that further research is required into this method of Cr supplementation. Ingestion of Cr + CHO enhanced glycogen storage in exercised muscle, to a greater extent than CHO ingestion alone (Chapter 3). Supplementation with Cr + CHO without prior exercise did not significantly improve glycogen accumulation, however, and did not significantly improve subsequent exercise performance. This suggests that it may be necessary to perform glycogen-depleting exercise prior to Cr + CHO supplementation to achieve glycogen concentrations that could improve endurance performance. Arginine, which is involved in Cr biosynthesis and has also been associated with

improvements in CHO disposal, was found to be ineffective in promoting CHO storage in rested or exercised subjects when given orally (Chapter 4). It is likely that this was due to an inability to achieve a plasma arginine concentration sufficient to stimulate mechanisms that would promote CHO storage. Finally, Chapter 6 showed that there was no obvious impairment to health of acute (5 d) or more chronic (8 week) periods of Cr supplementation at doses shown to significantly increase muscle TCr concentration.

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Anon (1996). *Guide to examinations for higher degrees by research*. The University of Warwick, Graduate School.

APPENDICES

APPENDIX A

Subjects' information sheets

Effects of exhaustive exercise and creatine and carbohydrate supplementation upon muscle creatine and glycogen accumulation

Mr Tristan M. Robinson, Dr Paul L. Greenhaff, Dr Dean A. Sewell
Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham. NG7 2UH.

Subject information sheet

Within the Department of Physiology and Pharmacology we are interested in the effect of muscle glycogen depletion upon subsequent glycogen resynthesis and creatine uptake into skeletal muscle.

Creatine is important to energy transduction during normal muscle function. Almost all of the human pool of creatine is contained in skeletal muscle. Humans have the ability to synthesise creatine, but can also receive it in their diet from meat and fish.

In this present study we will be asking volunteers to consume creatine and/or glucose following 1-legged exhaustive exercise on a bicycle ergometer. To measure muscle creatine and glycogen concentration, a small sample of muscle (a biopsy) will be taken from each leg following exercise, 6 hours following exercise and after 5 days of supplementation with creatine and/or glucose. The creatine dose to be administered will be 5g each time, this amount has not been shown to produce any adverse effects in human individuals.

You are free to withdraw from this study at any stage, if you so wish.

Response to a creatine and/or glucose dose at rest

On the day prior to the visit, please refrain from:

a) heavy exercise
b) consumption of alcohol
c) consumption of food from 9 p.m. onwards. You are allowed to drink water.

On the day of the visit:
Please report to room B8a, medical school building at 11 a.m.
You will be required to lie on a bed, and rest for approximately two hours. A cannula will be inserted into a vein in your arm and a blood sample will be taken. You will then be given a warm fruit drink to consume, possibly containing creatine, followed by 500 ml of Lucozade. Blood samples will then be taken every 20 minutes for the following 100 minutes. After this you are free to leave.

High carbohydrate food list

Please try to eat only foods listed below:

Provided:

- Potatoes (boiled or baked)
- Rice
- Cereal (e,g, Kellogg's Cornflakes, Rice Crispies)
- Pasta (e.g. spaghetti)

Other suggested items:

- Bread
- Jam, Honey, Golden syrup, Marmalade
- Baked beans
- Fruit, esp. Bananas, Apples
- Dolly mixtures, Liquorice allsorts, Bioled sweets, fruit gums etc.
- Ginger snap biscuits & Fig rolls
- Vegetables (except for nuts)
- Any soft drinks
- Chutney, pickles etc.
- Dried fruit (e.g. Figs)

Low carbohydrate content, please eat as little as possible.

- Semi-skimmed milk (we'd prefer you to use skimmed milk)
- Margarine/butter
- Meat (including sausages, hamburgers etc.)
- Chocolate & cakes
- Fish
- Cream
- Cheese

Effects of arginine ingestion on blood response to an oral glucose load at rest and following exercise

Mr Tristan M. Robinson, Dr Paul L. Greenhaff, Dr Dean A. Sewell
Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham. NG7 2UH.

Subject information sheet

Within the Department of Physiology and Pharmacology we are interested in the supply of glucose to skeletal muscle for energy storage. Uptake of glucose by muscle is mediated by insulin which is secreted following ingestion of a meal. The naturally occuring amino acid, arginine, can be obtained from the diet and, although not an essential amino acid, is necessary for cell growth and normal tissue repair following injury. Additionally, arginine is known to stimulate insulin secretion.

The aim of this study is to look at the effect of drinking an arginine solution on blood glucose and insulin concentration following oral glucose ingestion. In addition, we wish to investigate the effect of two different forms of exercise upon this response, as exercise is also known to influence insulin release and blood glucose disappearance.

Following routine medical screening, some preliminary exercise tests will be used to determine the intensity of exercise at which you will perform during the study. There will then be six further visits to the laboratory over the following seven weeks, each separated by at least one week. At each visit, following a 48 hour period refraining from alcohol consumption and strenuous activity, and an overnight fast, you will be required to undertake one of the following three regimens;

i) Consume a test drink and rest on a bed for 3 hours,
ii) Exercise on a cycle ergometer for an hour at an intensity which will raise heart rate to approximately 170 bpm. You will then consume a test drink and rest on a bed for 3 hours,
iii) Perform repeated bouts of 'squat' weightlifting exercise, lifting a weight determined in the preliminary test. You will then consume a test drink and rest on a bed for 3 hours.

Each activity will be performed twice, on separate occasions, during the study period.

During each visit a small plastic needle (a cannula) will be inserted into a vein of your arm, this will be used to obtain small blood samples in the recovery period following consumption of the test drink. Measurements of expired gases, heart rate, blood pressure and blood flow will also be taken in this period.

You are free to withdraw from this study at any time that you wish.

An inconvenience allowance of £ 100 has been allocated for your compliance with and completion of this study.

UNIVERSITY OF NOTTINGHAM MEDICAL SCHOOL

Healthy Volunteer's Information Sheet

The effect of creatine and carbohydrate ingestion on muscle glycogen and phosphocreatine metabolism during prolonged exhaustive cycling exercise in man

Dr Dean A. Sewell, Dr Paul L. Greenhaff, Mr Tristan M. Robinson*, Cardiovascular, Metabolism and Nutrition Section, School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH.

*Person to whom correspondence should be sent

Subject Information Sheet

Within the School of Biomedical Science we are interested in muscle *function and fatigue* during exercise. We are presently investigating the intake and metabolic consequences of the *consumption of creatine*, a compound involved in energy production in muscle. Creatine is synthesised in the body, but can also be obtained from the diet by eating meat.

In this present study we will be asking volunteers to consume powdered creatine and glucose. We will measure the amount of creatine and other energy stores in muscle samples.

After routine medical screening and the completion of a health questionnaire, we will ask you to perform a short exercise test to exhaustion on a cycle ergometer to measure maximal oxygen uptake (VO_{2max}). The test will last for approximately 12 minutes and involves breathing through a mouthpiece so that we can sample the air breathed out. A few days later you will return to repeat this test, to confirm your VO_{2max} value. After this visit you will be required to make 4 visits to the laboratory in the following six weeks. We ask that you do not exercise or drink any alcohol for 48hrs before each visit.

On the next visit (the first test session), following an overnight fast (from 9 p.m. the previous evening) you will be asked to cycle to exhaustion at an intensity of 70% VO_{2max}. It is expected that this will take approximately two hours. A small sample of muscle (a biopsy) will be taken from your leg prior to exercise and immediately following the end of exercise. Muscle samples are taken from a small incision (approximately 4 mm) through the skin, local anaesthetic is injected into the area to prevent discomfort during the procedure. Some subjects report slight soreness but no complications are anticipated. During each test visit a small plastic needle (a cannula) will be inserted into a vein of your arm, this will be used to obtain small blood samples during the exercise.

Following a week of normal diet and activity you will be given supplements of a glucose drink, to take over a period of 5 days, in addition to your usual diet. **During this supplementation period we require you not to perform any exercise.** The day following the last day of supplementation you are required to return to the laboratory to repeat the exercise/sampling test session, during

which an additional muscle sample will be taken (3 samples in total during this visit).

A week following this exercise session you will be given supplements of either creatine (to be dissolved in a warm drink) and glucose drink, or creatine only, to take over a period of 5 days, in addition to your usual diet. Again, we require you not to perform any exercise during this 5-day period. On the day following this supplementation period you will be required to return to the laboratory to repeat the test session outlined above, including muscle sampling.

An inconvenience allowance of £150 is available for completion of the study.

You are free to withdraw from this study at any stage, if you so wish.

Guidelines for diet & activity during the study.

On the day prior to **any** visit to the laboratory:

- Do not consume any food or drink, other than water, after 9 p.m.
- Do not drink any alcohol

During the three days prior to the Familiarisation visit:

- Follow your normal diet and record everything you eat in the food diary provided. You will be required to keep to this diet prior to future visits.
- Do not engage in any strenuous activity (e.g. training, anything other than 'normal daily activity')

During the 5-day carbohydrate (CHO) supplementation period (prior to Test Session 2):

- You are asked to drink a 500 ml 'serving' of Lucozade, at four equally-spaced times each day (e.g. 8 am, 12 pm., 4 pm., 8 pm.)
- Do not engage in any strenuous activity
- Keep to your 'normal diet', as recorded in your food diary, during the 3 days prior to the next visit.

EITHER

During the 5-day creatine and carbohydrate (Cr + CHO) supplementation period (prior to test session 3):

- You are asked to drink a warm-to-hot drink containing one vial of creatine powder, at four equally-spaced times each day, followed immediately by 500 ml of Lucozade (see note below concerning creatine drink preparation)
- Do not engage in any strenuous activity
- Keep to your 'normal diet', as recorded in your food diary, during the 3 days prior to the next visit.

OR

During the 5-day creatine (Cr) supplementation period (prior to test session 3):

- You are asked to drink a warm-to-hot drink containing one vial of creatine powder, at four equally-spaced times each day, (see note below concerning creatine drink preparation)
- Do not engage in any strenuous activity
- Keep to your 'normal diet', as recorded in your food diary, during the 3 days prior to the next visit.

NB. You may partake in physical activity/training in the period between a visit to the laboratory and the first day of any supplementation.

<u>Creatine drink preparation</u>

You are provided with vials containing 5 g creatine monohydrate. To prepare a drink, creatine must be dissolved in a warm-to-hot, <u>but not boiling</u>, drink. Ensure that all of the powder is dissolved in the drink (e.g. by stirring). We ask that you do not dissolve the creatine in coffee.

<u>Care of biopsied area</u>

You are advised to keep the area covered with the transparent sheet applied after the biopsies are obtained. This will prevent the area from becoming wet when washing, and also maintain sterility of the area. DO NOT TAKE ASPIRIN OR ANY ASPIRIN-CONTAINING PRODUCT in the days following biopsy sampling.

APPENDIX B

Derivation of equations for calculations used in this thesis

Oxygen consumption

The volume of oxygen inspired is calculated by multiplying the volume of air inspired per minute (V_I; corrected to standard temperature and pressure for dry gas, STPD) with the proportion of inspired air that is made up of oxygen (F_IO_2). The volume of expired oxygen is calculated in a similar manner, with the expired volume of air (STPD corrected) and proportion of air that is oxygen given the terms V_E and F_EO_2, respectively.

Therefore VO_2 can now be expressed as:

$$VO_2 = (V_I \bullet F_IO_2) - (V_E \bullet F_EO_2)$$

Carbon dioxide production

$$VCO_2 = (V_E \bullet F_ECO_2) - (V_I \bullet F_ICO_2)$$

where F_ECO_2 and F_ICO_2 are the proportions of carbon dioxide in expired and inspired air, respectively.

Calculation of substrate oxidation from RER

Protein oxidation (VO_{2P}) can be estimated by the equation

$$VO_{2P} = VO_2 \bullet 0.125$$

Where 0.125 is the proportional contribution of protein to the total VO_2 under thermoneutral conditions at rest.

Given VO_{2P}, we can calculate the non-protein component of VO_2 (VO_{2NP}):

$$VO_{2NP} = VO_2 - VO_{2P}$$

By similar methods we can calculate VCO_{2P} and VCO_{2NP}:

$$VCO_{2P} = VO_{2P} \bullet 0.81$$

$$VCO_{2NP} = VCO_2 - VCO_{2P},$$

where 0.81 is the RER value for protein oxidation.

Consequently, the non-protein component of RER (RER_{NP}) can be calculated:

$$RER_{NP} = \frac{VCO_{2NP}}{VO_{2NP}}$$

The percentage contribution of CHO and fat to RER_{NP} can be calculated:

$$\%CHO_{NP} = \frac{RER_{NP} - 0.707}{1 - 0.707} \bullet 100$$

$$\%FAT_{NP} = (100 - \%CHO_{NP})$$

where 0.707 is the RER value for fat oxidation and 1 is the RER value for CHO oxidation.

From the resultant values, the rate of oxidation of CHO (CHO_{OX}) or fat (FAT_{OX}) can be calculated:

$$VO_{2CHO} = \frac{\%CHO_{NP} \bullet VO_{2NP}}{100}$$

$$CHO_{OX} = \frac{VO_{2CHO}}{0.788} \quad (g.min^{-1})$$

$$VO_{2FAT} = \frac{\%Fat_{NP} \bullet VO_{2NP}}{100}$$

$$FAT_{OX} = \frac{VO_{2FAT}}{2.019} \quad (g.min^{-1})$$

where 0.788 is the amount of oxygen consumed per gram of CHO oxidised (mean of glycogen and glucose oxidation) and 2.019 is the amount of oxygen consumed per gram of fat oxidised. From these oxidation rates, the total amount of substrate utilised over any given period can be calculated.

Analytical Assay Procedures

Method for ATP and PCr determination

The 3 reactions involved

1 Glu 6-P + NADP ----G6PDH---> P-gluconolactone +NADPH

2 ATP + Glu -----HK-------> ADP + Glu 6-P

3 PCr + ADP ----CPK------> Cr + ATP

No	Reagents	Final conc. (mM)	conc. stock (mM)	mass/vol	vol/375µl
D1	TEA pH 7.5-7.6	100	1000	18.6mg/100ml	37.5
	$Mg(Ac)_2.4H_2O$	10	100	2.14mg/100ml	
	$EDTA.Na.2H_2O$ (+4•C)	1	10	0.372mg/100ml	
F1	DTT (+4°C)	1	50	7.8mg/ml	7.5
F2	$NADP.Na_2.4H_2O$ (+4°C)	1	25	21.48mg/ml	15
F3	$ADP.Na.2H_2O$ (-20°C)	0.04	10	5.01mg/ml	1.5
F4	glucose	5	20	22.5mg/ml	15
**	G-6-PDH	Dilute1p in 2p H_2O		1.1mg/ml	1.125
	H_2O				297.4
	HK	Dilute 1p in 1p H_2O		2.0mg/ml	3µl
	CPK	Dilute in $NaCO_3$/BSA		15mg/ml	3µl

Note:

G-6-PDH	order Boehringer (cat. no. 127035) 1.1mg/ml Dilute 1 part in 2 with water.
HK	order Boehringer ammonium sulfate suspension (cat no. 127175) 2mg/ml. Dilute 1 part in 1.
CPK	order Sigma lyophilised enzyme (cat no. C-3755). Dissolve 15mg/ml in 0.5% $NaHCO_3$ + 0.05% BSA (D5).

Procedure

• Always remember that reagents and samples are to be kept on ice when ever possible, but also allowed to come to room temperature.

• Before starting remove reagents f1,f2,f3 & f4 from fridge/freezer for weighing, keeping in the bag with dessicant for 15 min.

• Prepare reagents f1-f4 and enzymes, calculating the exact proportions required for the number of samples and blanks that are being done.

• Take an aliquot of D1 sufficient for the number of samples to be done, and if not doing reaction 1 ensure G-6-PDH is added to the mixture.

• Mix together all reagents thoroughly in proportions described above.

• Never do more than 20 samples in one run, placing a blank sample every 6-10 samples.

• To the cuvettes 50µl of sample is added and 450µl of mixed reagents is added

• The absorbance is then measured and at 20 s intervals, aliquots of 3µl of the first enzyme are added.

• Twenty minutes from the addition of the first enzyme absorbance. is measured again, and at 20 s intervals the next enzyme is added

• after 45min total the final absorbance measurement is made at 20 s intervals.

X

Glycogen method

Extraction

Solutions needed

1. G5 0.1 M NaOH stored at 4°C
2. G6 0.1M HCl stored at 4°C
3. G7 0.2 M citric acid, 0.2M Na_2HPO_4, pH =5, stored at 4°C
4. AGS Amyloglucosidase (Boehringer, from *Aspergilus niger*) 200mg/ml, made on the day.

G7 must then be mixed with the G6 at a ratio of 3:1.
NOTE this ratio may need to be slightly adjusted if G5 (which is added to the sample) prior to the addition of G6/G7 mix, is not sufficient to neutralise all G6/G7 mix. Therefore, before mixing the G7 and G6 the proportion of G5 required to fully neutralise G6 needs to be checked. Ideally it would be close to a 1:1 ratio, but this is not always the case. Do this by adding equal proportions of G5 and G6 and check the pH is neutral, and if this is not the case repeat it again with increasing proportions of G5 or G6 (as appropriate). Remember this will affect the relative proportions of G6/G7, i.e. if more G5 is required to neutralise G6, then less G6 is added to the G6/G7 mix proportionally.

Remember to have heating block turned on and set to 80°C also before commencing.

Method
Bring freeze-dried weighed samples to room temperature in dessicant, and store on ice.

To each vial add the appropriate amount of NaOH according to the table below (limit samples done at any one time to no more than ten). After adding NaOH to each sample, vortex vigorously until all the sample is dissolved and the solution has a distinct green tinge. Place samples into heating block set at 80°C to allow full solubilisation and reaction with the NAOH for 10 minutes.

Dilution Table

Powder (mg)	0.1M NaOH (µl)	Buffer G6+G7 (µl)	AGS (µl)
0.0-1.0	75	300	15
1.0-2.0	100	400	15
2.01-3.0	120	480	15
3.01-4.0	160	640	20
4.01-5.0	200	800	25
5.01-6.0	240	960	30

After 10 minutes remove samples and neutralise by the addition of G6/G7 mix to each vial, followed by the addition of AGS.

Samples must then be left at room temperature with caps off, for 1 hour.
The samples must then be spun for one minute at 14,000rpm and the supernatant taken.

Extraction factor calculation

$$\text{Ex. Factor} = \frac{(\mu l)\ G5 + (\mu l)\ G6/G7 + (\mu l)\ AGS}{(mg)\ \text{of weighed sample}}$$

Glucose assay

$$\text{Glu-6-P} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{P-gluconolactone} + \text{NADH}$$

$$\text{Glu} + \text{ATP} \xrightarrow{\text{HK}} \text{Glu-6-P} + \text{ADP}$$

RV =450 SV= 40

Reagents

	Reagents Mass/volume	Final conc. (mM)	Vol 450 (µl)	*20 (µl)	conc. of stock (mM)
G1	Triethanolamine 7.00g/100ml	100	120	2400	375
	KOH 0.8g/100ml	40		150	
	$Mg(AC)_2.4H_2O$ 2.4g/100ml	30		112.5	
	$EDTA.Na.2H_2O$ 0.14g/100ml Adjust to pH 8.2 With KOH	1		3.75	
H1	ATP 27.72mg/ml	0.75	5	150	45
H4	DTT 9.36mg/ml	1	5	150	60
F8	NAD 19.92	1	10	300	30
	H_2O		225	4500	

G6PDH order Sigma G-5885 (sulphate free!) dilute vial in 200µl H_2O only lasts a matter of a week once dissolved

Hexokinase order Sigma H-4502 (sulphate free!) 200 units dilute 200µl H_2O

Enzymes should be mixed in a 1:1 ratio sufficient for 3µl to be added per cuvette.

Set spectrophotometer to 366nm. Read each samples abs then add 3µl of enzyme mix every sample, and let them incubate for no more than 10 minutes and read the abs again.

Measurement of Creatine

Principle

The 3 reactions involved:

1 Pyruvate + NADH ----LDH---> Lactate +NAD

2 ADP + PEP -----PK-------> ATP + Pyruvate

3 Cr + ATP ----CPK------> PCr + ADP

Method

1) Make up the following reagent mixture with these amounts per cuvette:

Reagent Mass/volume	Volume per cuvette (µl)
Glycine buffer	150
KCl 15g/100ml	7.5
ATP $Na_2.5H_2O$	30
PEP	22.5
NADH	7.5
LDH	0.75
PK	0.75
H_2O	232.5

Vortex and keep on ice.

---

2) Prepare the enzyme:

CPK Dilute in 0.5% $NaCO_3$/ 0.05% BSA 15mg/ml 6µl per cuvette

Vortex and keep on ice.

---

3) Set spectrophotometer to read at 340 nm for 1200 s using "METABOLI" program.

---

4) Pipette 450µl of the reagent mix into each cuvette.

---

5) Defrost the samples quickly in hot water and vortex.

---

6) Pipette 30µl of water / sample into each cuvette. Mix and start reading.

---

7) After 4 min add 6µl of CPK to each cuvette. Agitate to mix. Continue reading for 20 min where a plateau marks the end point of the reaction.

Note:

CPK order Sigma lyophilised enzyme (cat no. C-3755).
Dissolve 15mg/ml in 0.5% $NaHCO_3$ + 0.05% BSA (D5).

Procedure

• Always remember that reagents and samples are to be kept on ice when ever possible, but also allowed to come to room temperature.

• Before starting remove reagents from fridge/freezer for weighing, keeping in the bag with dessicant for 15 min.

• Prepare all reagents and enzymes, calculating the exact proportions required for the number of samples and blanks that are being done.

• Mix together all reagents thoroughly in proportions described above.

• Never do more than 20 samples in one run, placing a blank sample every 6-10 samples.

• To the cuvettes 30µl of sample is added and 450µl of mixed reagents is added

• The absorbance is then measured and at 20 s intervals, aliquots of 6µl of the first enzyme are added.

• Thirty min from the addition of CPK absorbance. is measured again.

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