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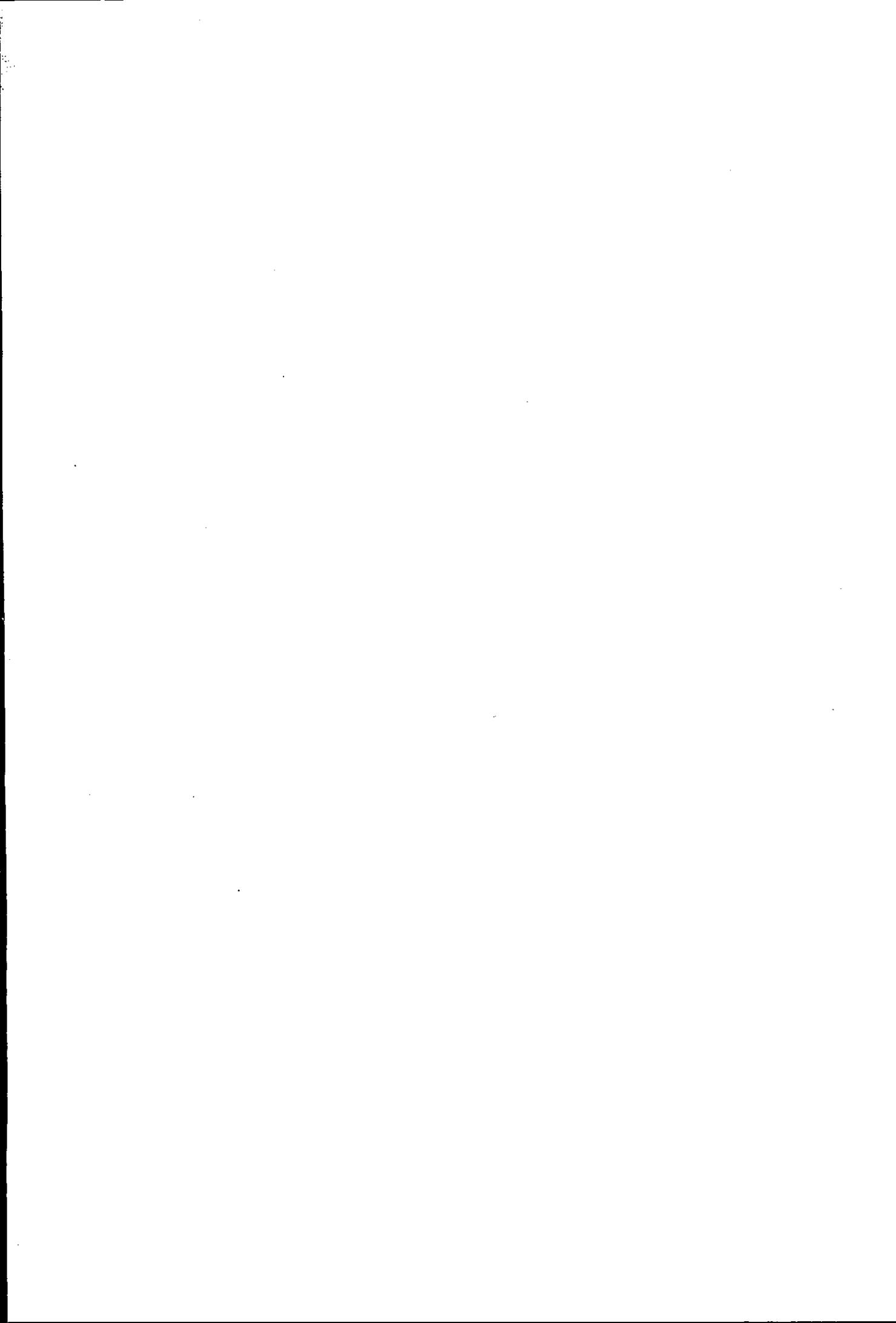
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**AMINO ACID METABOLISM
DURING EXERCISE AND RECOVERY
IN HUMAN SUBJECTS**

by

Mark Bruce

A Doctoral Thesis

Submitted in partial fulfilment of the requirements
for the award of Doctor of Philosophy
of Loughborough University

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ABSTRACT

The depletion of muscle and liver glycogen observed during prolonged submaximal exercise is associated with fatigue. Resynthesis of glycogen stores during the recovery period after exercise is therefore essential for the recovery of endurance exercise capacity. In recent years, attention has focussed on the supplementation of protein in addition to glucose-polymer during recovery from exercise in an attempt to further increase glycogen synthesis. The aims of the first and second studies in this thesis were to investigate the effect of glucose-polymer and amino acid ingestion, and solely amino acid ingestion upon amino acid and carbohydrate metabolism during recovery from glycogen-depleting exercise.

In the first study (Chapter 4), seven well-trained male cyclists completed a bout of glycogen-depleting exercise (~90 min) at 70% $\dot{V}O_{2max}$, then ingested either 1.7 g·glucose·kg body wt⁻¹ glucose-polymer (6.4% w:v) and 0.3 g·kg body wt⁻¹ L-glutamine (GP + GLN), 1.7 g·glucose·kg body wt⁻¹ glucose-polymer (6.4% w:v) and an isoenergetic and isonitrogenous mixture of L-alanine and L-glycine (GP + ALA & GLY), or an equivalent volume of a placebo solution (CON1 and CON2) during a 4 h recovery period. Subjects then cycled to volitional fatigue at 70% $\dot{V}O_{2max}$. Plasma glutamine concentration was increased during the recovery period following the ingestion of glucose-polymer and glutamine ($P < 0.05$). An increase was observed in both plasma glucose concentration and serum insulin concentration ($P < 0.05$) following the ingestion of the solutions containing glucose-polymer, with a tendency for a greater increase in the GP + GLN condition. Furthermore, there was a tendency for the sum of the phenylalanine and tyrosine concentrations to be decreased during the recovery period in the GP + GLN condition. However, cycle time to exhaustion was not statistically different between conditions during the second bout of exercise.

In the second study (Chapter 5), seven well-trained male cyclists completed a bout of glycogen-depleting exercise (~90 min) at 70% $\dot{V}O_{2max}$, then ingested either 0.3 g·kg body wt⁻¹ L-glutamine (GLN) or an isoenergetic and isonitrogenous mixture of L-alanine and L-glycine (ALA & GLY) dissolved in 31.25 ml·kg body wt⁻¹ solution during a 4 h recovery period. Subjects then cycled to volitional fatigue at 70% $\dot{V}O_{2max}$. Plasma glutamine

concentration was increased during the recovery period following the ingestion of glutamine ($P < 0.05$). Furthermore, plasma glucose concentration was increased during the second bout of exercise following the ingestion of glutamine ($P < 0.01$). However, cycle time to exhaustion was not statistically different between conditions during the second bout of exercise.

In the third study (Chapter 6), the hypothesis was tested that oral glutamine or ornithine α -ketoglutarate would enhance TCA cycle intermediate (TCAI) expansion at the start of exercise and hence increase oxidative energy delivery and extend endurance capacity. Following a bout of glycogen-depleting exercise (~90 min) and 18 h of a low-carbohydrate diet, seven well-trained male cyclists ingested either 5 ml·kg body wt⁻¹ placebo solution (CON), 0.125 g·kg body wt⁻¹ L(+) ornithine α -ketoglutarate dissolved in 5 ml·kg body wt⁻¹ placebo solution (OKG), or 0.125 g·kg body wt⁻¹ L-glutamine dissolved in 5 ml·kg body wt⁻¹ placebo solution (GLN), 1 h prior to cycling exercise to exhaustion at 70% $\dot{V}O_{2max}$. Biopsy samples from the vastus lateralis were obtained 1 h post supplement, after 10 min of exercise and at exhaustion. Cycle time to exhaustion was not statistically different between conditions. Glutamine ingestion increased both plasma and muscle glutamine concentration ($P < 0.05$) compared to the other two conditions. Σ TCAI (citrate, malate, fumarate, and succinate, ~85% of total TCAI pool) was not different between conditions 1 h post supplement or at exhaustion, but at 10 min exercise Σ TCAI was greater in the GLN condition (499% increase relative to rest) compared to the CON condition (360%; $P < 0.05$) and the OKG condition (394%). Despite this increase in Σ TCAI at 10 min exercise following glutamine ingestion, no sparing of phosphocreatine utilisation or reduction in muscle lactate accumulation was observed compared to the other conditions at the same time point. The ingestion of glutamine was able to enhance the TCAI pool size at 10 min of exercise, but did not delay the onset of fatigue.

The studies in this thesis have been able to demonstrate that the supplementation of amino acids during recovery from exercise can modulate metabolism during recovery and exercise during a subsequent bout of exercise. However, the supplementation of amino acids to increase the total concentration of the TCA cycle intermediates has demonstrated that this increase may not be as physiologically significant to TCA cycle flux and oxidative energy metabolism during exercise as previously thought.

PUBLICATIONS

Part of the work contained within this thesis has been published in the following publications.

Published paper

Bruce, M., Constantin-Teodosiu, D., Greenhaff, P.L., Boobis, L.H., Williams, C. & Bowtell, J.L. (2001) Glutamine supplementation promotes anaplerosis but not oxidative energy delivery in human skeletal muscle. *American Journal of Physiology Endocrinology and Metabolism*, 280: E669-E675.

Review papers

Rennie, M.J., Bowtell, J.L., Bruce, M., & Khogali, S.E.O. Interaction between glutamine availability and metabolism of glycogen, tricarboxylic acid cycle intermediates and glutathione. *Journal of Nutrition*, 131(9): 2488S-2490S.

Bowtell, J.L. & Bruce, M. Glutamine: An anaplerotic precursor. *Nutrition*. In Press.

Published communications

Bruce, M., Bowtell, J.L & Williams, C. (1999) Effect of glutamine and carbohydrate-electrolyte supplementation on recovery from exhaustive exercise. *Journal of Physiology*, 515.P, 69P.

Bruce, M., Bowtell, J.L & Williams, C. (1999) Effect of oral glutamine and glucose-polymer supplementation on recovery from exercise. *Proceedings of the 4th Annual Congress of the European College of Sports Science, Rome, Italy*.

Bruce, M., Constantin-Teodosiu, D., Greenhaff, P.L., Boobis, L.H., Williams, C. & Bowtell, J.L. (2000) Effect of glutamine and ornithine α -ketoglutarate supplementation on tricarboxylic acid cycle intermediate (TCAI) pool size. *FASEB Journal*, 14(4): A92.

Bruce, M., Constantin-Teodosiu, D., Greenhaff, P.L., Boobis, L.H., Williams, C. & Bowtell, J.L. (2000) Glutamine supplementation promotes anaplerosis but not oxidative energy delivery in contracting human skeletal muscle. *Proceedings of The 4th Oxford Glutamine Workshop, Oxford, UK*.

Bruce, M., Constantin-Teodosiu, D., Greenhaff, P.L., Boobis, L.H., Williams, C. & Bowtell, J.L. (2001) Enhancement of tricarboxylic acid cycle intermediate pool expansion in human skeletal muscle did not extend sub-maximal exercise capacity. *Journal of Physiology*, 531.P, 55P.

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CHAPTER 1

INTRODUCTION

Prolonged submaximal exercise relies heavily upon the body's stores of both carbohydrate and fat to generate energy to fuel muscular work. Moreover, carbohydrate is the primary substrate during prolonged submaximal exercise, with muscle glycogen being quantitatively the most important energy source at intensities greater than 60% $\dot{V}O_{2\max}$ (Romijn et al. 1993). Fatigue during prolonged submaximal exercise is associated with the depletion of both liver and skeletal muscle glycogen stores (Bergström et al. 1967; Hultman and Nilsson, 1971). Under normal conditions, the glycogen stores in man are relatively small (~100 g in liver and ~325 g in skeletal muscle), sufficient for ~120 min of exercise at 75% $\dot{V}O_{2\max}$. Nutritional strategies to increase the glycogen synthesis rate post exercise are essential to achieve complete resynthesis of both skeletal muscle and liver glycogen stores and hence restore exercise capacity.

The ability of carbohydrate supplementation to restore glycogen stores post exercise is well documented in the literature (Ivy, 1991; Ivy et al. 1988a; Ivy et al. 1988b; Reed et al. 1989). More recently however, attention has been directed toward the ingestion of a combination of carbohydrate and protein post exercise to enhance muscle glycogen restoration (Roy and Tarnopolsky, 1998; Zawadzki et al. 1992; Tarnopolsky et al. 1997; Carrithers et al. 2000; van Hall et al. 2000a, Jentjens et al. 2001). The hypothesis behind this nutritional strategy being that the co-ingestion of carbohydrate and protein serves to increase insulin levels to a greater extent than carbohydrate alone, thereby increasing glucose uptake and storage in skeletal muscle (Zawadzki et al. 1992; Tarnopolsky et al. 1997; Jentjens et al. 2001). However, very little research has examined the effect of providing an individual amino acid in combination with carbohydrate, or ingested alone post exercise (Varnier et al. 1995; Wagenmakers et al. 1997; Bowtell et al. 1999, van Hall et al. 2000a).

Glutamine, a 5C non-essential amino acid, is the most abundant amino acid in both plasma and skeletal muscle (with the exception of taurine) (Bergström et al. 1974) and

is synthesised within the tissues of the body, with skeletal muscle (Golden et al. 1982) and liver (Häussinger, 1989) being the most quantitatively important producers. Glutamine is also involved in a variety of metabolic processes in a number of tissues. Glutamine has been shown to increase protein synthesis in skeletal muscle (MacLennan et al. 1987; MacLennan et al. 1988), to increase both liver and skeletal muscle glycogen synthesis (Mouterde et al. 1992; Low et al. 1996; Scislowski et al. 1989; Varnier et al. 1995), to be a major precursor for gluconeogenesis (Nurjhan et al. 1995), to hold a regulatory role in acid-base balance (Golden et al. 1982), and to be an important respiratory fuel for both the gut (Windmueller and Spaeth, 1974) and the immune system (Ardawi and Newsholme, 1983).

The intravenous administration of glutamine alone after exhaustive exercise has been shown to increase muscle glycogen synthesis in human skeletal muscle (Varnier et al. 1995). Furthermore, the provision of glutamine has been shown to increase liver glycogen synthesis in hepatocytes of fasted rats; a situation where it is expected that liver glycogen content will be reduced (Mouterde et al. 1992). Therefore, the provision of glutamine after prolonged exercise, when both muscle and liver glycogen stores are reduced, could conceivably increase both liver and skeletal muscle glycogen synthesis. It was hypothesised that this potential increase in glycogen resynthesis, particularly in the liver, would allow for a better maintenance of blood glucose concentration in a subsequent bout of exercise, thus prolonging endurance capacity, particularly during cycling exercise (Coyle et al. 1983).

The tricarboxylic acid (TCA) cycle provides a link between amino acid metabolism and carbohydrate metabolism, since amino acids are important precursors for TCA cycle intermediates (TCAI). Furthermore, the relative flux of carbon into and out of the TCA cycle will determine the concentration of the TCAI (Lee and Davis, 1979). It is feasible that glutamine may be converted to α -ketoglutarate, one of the TCAI, and therefore hold an important role in anaplerosis [replenishment of the TCAI] (glutaminase (EC 3.5.1.2): glutamine + H₂O \rightarrow glutamate + NH₃; glutamate dehydrogenase (EC 1.4.1.2): glutamate + NAD⁺ \leftrightarrow α -ketoglutarate + NH₃ + NADH). It has been suggested that an increase in the total concentration of the TCAI may be necessary for increased TCA cycle flux and hence oxidative energy production during submaximal exercise

(Wagenmakers, 1998b; Sahlin et al. 1990). Therefore, it was hypothesised that the supplementation of potential anaplerotic precursors, such as glutamine, may modulate oxidative energy delivery and affect the aetiology of fatigue during submaximal exercise.

Therefore, the aims of the studies described in this thesis were to examine the role of amino acid metabolism both during, and in recovery from, prolonged submaximal exercise at whole-body and cellular level. In particular, the aim was to determine whether the supplementation of glutamine and glucose-polymer, or glutamine alone, during recovery from exercise would extend exercise capacity in a subsequent bout of exercise. Secondly, to determine whether the supplementation of glutamine prior to exercise would reduce the delay in the onset of increased oxidative energy production at the start of exercise, and whether this would extend exercise capacity during submaximal exercise.

CHAPTER 2

REVIEW OF LITERATURE

2.1 INTRODUCTION

The main aim of this chapter is to draw together some of the findings from the many physiological and metabolic studies conducted over the last few decades, to present a framework of our knowledge regarding both carbohydrate metabolism and amino acid metabolism during exercise and recovery.

2.2 PROTEIN

The body of a 70 kg man contains about 12 kg of protein (amino acid polymers) and 200-230 g of free amino acids. Skeletal muscle accounts for 40-45% of total body mass and contains approximately 7 kg of protein, primarily in the form of contractile (myofibrillar) proteins. About 120 g of the free amino acids are present intracellularly in skeletal muscle, while only 5 g of free amino acids are present in the circulation. Protein intake typically comprises 10-15% of the total dietary intake in a Western diet and this supply of protein is essential to maintain cellular integrity and function, in addition to health and reproduction. A continual turnover of protein occurs (breakdown + synthesis), with amino acids being constantly removed from the metabolic pool during the synthesis of an array of different functional and structural proteins and reappearing in the pool as the result of protein breakdown.

The contribution of protein to energy expenditure at rest is estimated to be less than 10% of the body's total energy expenditure (Rennie et al. 1981; Brooks, 1987). However, during exercise, the contribution of protein to energy production has been shown to be influenced by carbohydrate availability, with an increase in protein catabolism observed in the glycogen-depleted state (Lemon and Mullin, 1980; Wagenmakers et al. 1991). Amino acids cannot be stored in the body in a manner analogous to carbohydrate and fat, and therefore amino acids in excess of the amount required for protein synthesis are oxidised, thus contributing to energy production.

During endurance exercise there is a net breakdown of body protein, predominantly in the liver and muscle (Rennie et al. 1996a). This is achieved through a decrease in the rate of protein synthesis (Rennie et al. 1981) and an increase in the rate of protein breakdown (Carraro et al. 1990). In particular, exercise causes a decrease in the rate of degradation of contractile proteins and an increase in the rate of degradation of non-contractile proteins (Dohm et al. 1987). Post endurance exercise, whole-body techniques to determine protein balance have indicated that protein synthesis is increased (Rennie et al. 1981; Devlin et al. 1990), which may be linked to an increased blood flow post exercise (Biolo et al. 1997; Tipton and Wolfe, 1998). Moreover, the administration of exogenous amino acids post exercise increases the rate of protein synthesis with a concomitant reduction in the rate of protein breakdown (Biolo et al. 1997; Borel et al. 1997; Rasmussen et al. 2000).

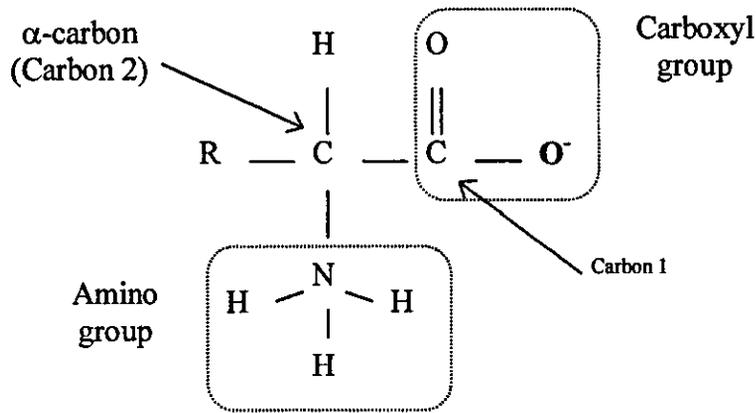
2.3 AMINO ACIDS

Proteins have an important role to play in the formation of body structures and enzymes. Proteins are macromolecules consisting of long chains of amino acid subunits, which are joined together by peptide bonds. Amino acids exist both in the form of proteins and as free amino acids.

Amino acids consist of an amino group, a carboxyl group, a hydrogen atom and a distinctive R group bound to a carbon atom. The basic structure of each of the 20 amino acids is identical, except for the structure of the R group, or side chain, which determines the specific characteristics of each individual amino acid (Stryer, 1995). The exception to this rule is proline that has a secondary amino group and glutamine that has both an amino and amide group.

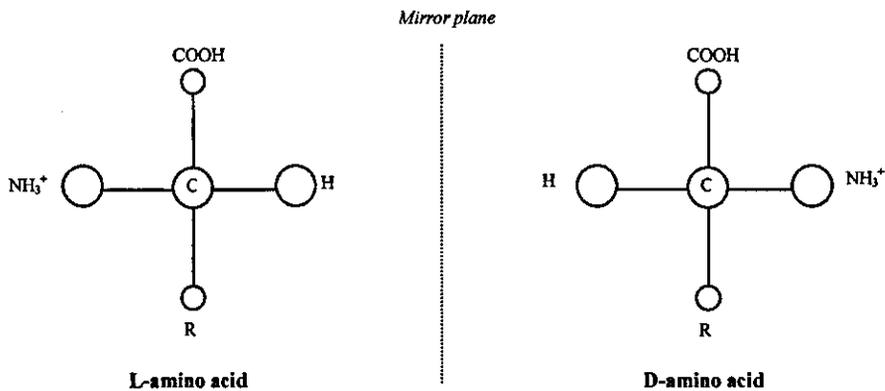
By convention, the carboxyl carbon is deemed carbon 1 in the amino acid structure, with the carbon atom next to carbon 1 being termed the α -carbon or carbon 2 (see Fig. 2.1a). When the α -carbon has an amino group attached (NH_2) it can be classed an amino acid. All of the 20 standard amino acids, except for glycine, have four different groups arranged tetrahedrally around the α -carbon (see Fig. 2.1b) and thus can exist in the D or L configuration. However, D-amino acids rarely occur in nature and are never present in proteins.

Fig. 2.1a General structure of an amino acid (amino and carboxyl groups).



NB: R represents the characteristic side chain.

Fig. 2.1b General structure of an amino acid (stereoisomers L and D).



NB: R represents the characteristic side chain.

Amino acids that can be synthesised internally from existing amino acids and other substances are termed 'non-essential', whereas those amino acids that cannot be synthesised in the body and are required constituents of the diet are termed 'essential'. The free amino acid pool is comprised of those amino acids not incorporated into protein; including plasma amino acids and intracellular amino acids in liver and skeletal muscle. In contrast to the liver, which is able to oxidise most of the twenty amino acids that are present in proteins, human skeletal muscle can oxidise only six amino acids: the branched-chain amino acids (leucine, isoleucine, valine), asparagine, aspartate and glutamate (glutamate can also be derived through the breakdown).

It has been reported that the intracellular concentration of free amino acids is dependent upon muscle fibre population, with slow-twitch fibres demonstrating a higher concentration of most amino acids. The only amino acids demonstrating higher concentrations in fast-twitch fibres were alanine and glycine (Turinsky and Long, 1990). Although these observations were in rat skeletal muscle, it is feasible that a similar pattern should be observed in human skeletal muscle, albeit with a reduction in the absolute differences between fibre types, as amino acid concentrations in rat skeletal muscle have been reported to be higher than in human skeletal muscle (Bergström et al. 1974)

A continuous exchange of amino acids between protein and free amino acid pools exists in humans, as proteins are constantly being synthesised and simultaneously degraded, a process termed 'protein turnover'. Traditionally, the view has been held that the liver is the major site of both the catabolism and synthesis of amino acids. However, it has been demonstrated that the majority of amino acids in the body are in fact incorporated into muscle tissue, and that muscle tissue constitutes the primary site for the degradation of amino acids (Goldberg and Chang, 1978).

2.3.1 AMINO ACID TRANSPORT

The cellular amino acid requirements for both metabolism and protein synthesis must be met by a combination of the biosynthesis of non-essential amino acids and the transport of amino acids into the cell. The transport of amino acids is subject to regulation, and there are a multiplicity of systems with differing capacities and affinities (characterised using the Michaelis-Menton equation; capacity, V_{max} ; affinity, K_m), which complicates the study of amino acid transport and regulation. Those transport systems of substantial importance are Systems-A, -ASC, -L, and N.

Alanine and glycine transport

System-A (A stands for alanine) transports those amino acids with short, polar, or linear side chains, in particular alanine and glycine, although many of the other amino acids are also in part transported via System-A (Collarini and Oxender, 1987). System-A is a high affinity, low-capacity transporter that is sodium-dependent and whose activity is markedly reduced at low extracellular pH. Furthermore, System-A is stimulated by

insulin in an inward direction and is regulated by the availability of amino acids in the extracellular environment, with a substantial up-regulation displayed in the face of low alanine concentrations (Rennie, 1996a). Although amongst the first of the amino acid transporter systems to be kinetically characterised, the importance of System-A in muscle alanine transport may be limited due to its low capacity (Hundal et al. 1989). It is possible that a greater proportion of alanine transport between blood and muscle occurs via Systems-ASC (alanine; serine; cysteine) and System-L (leucine). System-ASC is the major sodium-dependent system and is a medium-affinity, medium-capacity system, whereas System-L is sodium-independent and is a low-affinity, high-capacity system (Collarini and Oxender, 1987). Both systems are unresponsive to insulin in skeletal muscle (Rennie, 1996a).

BCAA and aromatic amino acid transport

System-L appears to be the primary transporter for the branched-chain and aromatic amino acids, but not glutamine to any extent (Collarini and Oxender, 1987). System-L transport activity is regulated in response to the availability of System-L amino acids, principally leucine, isoleucine, valine, lysine, and phenylalanine. System-L activity is unaffected by insulin or any other hormones. None of the amino acids transported by this system are electrically charged at a physiological pH, therefore it effectively acts as a mechanism to equalise concentrations of its substrates across the sarcolemmal membrane.

Glutamate transport

Glutamate exchange between blood and tissues generally occurs at a low rate due to the relatively low activity of the transporter (Rennie, 1996a). Glutamate transport, and also aspartate, most probably occurs via System- X_{AG} (X stands for exchanger) in human skeletal muscle. System- X_{AG} is a high-affinity, low-capacity transport system that is sodium-independent but H^+ dependent (i.e transporter characteristics are altered in the presence of H^+). In addition, System- X_{AG} is hormone insensitive. However, the kinetic characteristics of System- X_{AG} do not appear to adequately explain the >50 fold concentration gradient that is maintained between the muscle and blood (Bergström et al. 1974), nor how muscle is able to extract glutamate from the blood despite this gradient. More work is required in this area before this process is fully understood.

Glutamine transport

In hepatocytes, System-N (nitrogen) is primarily responsible for the uptake of glutamine. System-N may be unique to liver cells, as it has not yet been described in other cell types, and appears to have the greatest physiological activity, presumably because of its medium-range affinity and capacity. System-N is sodium-dependent and is subject to regulation by amino acid availability, similar to System-A. The uptake of glutamine into hepatocytes is inhibited by histidine and asparagine (Fafournoux et al. 1983), in addition, facilitated diffusion enables hepatocytes to release glutamine when the intracellular concentration is elevated (Fafournoux et al. 1983). Until recently it was believed that glutamine metabolising enzymes held an exclusive role in regulating glutamine metabolism in the liver (Fafournoux et al. 1983). However, it has since been shown that glutamine transport across the plasma membrane may also be a potential regulatory site in glutamine metabolism (Häussinger et al. 1985).

System-N^m (m stands for muscle) is the transporter in muscle analogous to System-N in the liver (Hundal et al. 1987). System-N^m is primarily responsible for the transport of glutamine. In addition, System-N^m transports asparagine and histidine in skeletal muscle, but asparagine and histidine are also substrates for Systems-A and ASC and for System-L respectively, and therefore System-N^m has a far lower capacity for these amino acids compared to glutamine. System-N^m is a high-capacity, low-affinity, sodium-dependent transport system, and this combination of affinity and capacity appears to account for the fact that glutamine has the fastest rate of entry into muscle relative to other amino acids (Banos et al. 1973). Furthermore, of all the major amino acid transport systems, System-N^m appears to be the only system that shows acute regulation by insulin (Rennie et al. 1994). Insulin is able to stimulate glutamine transport into skeletal muscle and the absence of insulin causes a reduction in the intramuscular glutamine pool, due to an increased efflux of glutamine from the muscle rather than a decreased influx of glutamine into the muscle (Rennie et al. 1996b).

The activity of System-N^m is elevated under conditions of stress including, acidosis, surgical trauma, sepsis, burns, during corticosteroid treatment, and most probably during exercise. The increase in glutamine efflux from skeletal muscle is generally linked to an increase in transporter system activity (Babij et al. 1986a; Babij et al. 1986b; Rennie et al. 1989), rather than an alteration in enzyme activity i.e. glutamine

synthetase and glutaminase (Babij et al. 1986a; Hundal et al. 1990). The mechanism behind this increase in transporter activity has been linked to the elevation of intracellular sodium which may stimulate transporter activity as opposed to increasing the carrier affinity for glutamine (Rennie et al. 1986). This mechanism would appear to be inherently linked to the observed stoichiometry of glutamine:sodium co-transport which has a coupling ratio of 1:1 (Hundal et al. 1987). Therefore, a decrease in the intramuscular glutamine pool may be mediated through an increase in intracellular sodium, causing an increase in Na^+ -dependent glutamine efflux from the muscle (Hundal et al. 1987). Factors which influence Na^+ transport, mediated either through a change in the activity of the Na^+ - K^+ ATPase or by influencing membrane permeability to Na^+ , could alter the flux of glutamine and in turn the intracellular concentration.

It is conceivable that changes in the plasma concentration of glutamine may modulate the size of the intramuscular pool due to the characteristics of glutamine transport in human skeletal muscle, with an increase in plasma glutamine concentration leading to an increased flux of glutamine into skeletal muscle (Ahmed et al. 1993). Furthermore, it has been suggested that glutamine is likely to be the most rapidly exchanging amino acid across the muscle membrane at physiological glutamine concentrations due to the high capacity of System- N^{m} (Hundal et al. 1987; Ahmed et al. 1993). Indeed, the infusion of glutamine has been observed to increase the intramuscular glutamine pool, highlighting the capacity of System- N^{m} (Varnier et al. 1995).

The modulation of the intramuscular glutamine pool by System- N^{m} is further demonstrated by the observation that System- N^{m} displays adaptive regulation, suggesting that when glutamine requirements are high and the supply limiting, increased synthesis of the transporter protein will occur (Rennie et al. 1994; Tadros et al. 1993). Thus, skeletal muscle has the necessary characteristics to be a net exporter of glutamine (Rennie et al. 1996b).

2.3.2 AMINO ACID METABOLISM

The initial step in the metabolism of amino acids is the removal of the amino group, leaving the carbon skeleton of the amino acid. Chemically, the remaining carbon skeleton is deemed a keto-acid or oxo-acid. The process of removal of the amino group

is termed deamination, resulting in the production of their corresponding keto-acids, and generally ammonia. However, most amino acids actually undergo transamination, rather than deamination. In this process, the amino group is transferred firstly to the catalysing enzyme, leaving the keto-acid, then the enzyme transfers the amino group to an acceptor molecule, which is a different keto-acid, thus forming the corresponding amino acid. The most common amino group acceptor is α -ketoglutarate resulting in the formation of glutamate, thus placing glutamate and α -ketoglutarate in central positions in amino acid metabolism.

The skeletal muscle concentration of the free amino acids aspartate, asparagine and the BCAA, derived either through the net breakdown of muscle proteins or via uptake from the circulation, is lower than their relative occurrence in muscle proteins, implying that they are metabolised in skeletal muscle. Furthermore, some amino acids, such as alanine, glutamine and glutamate are present in the free amino acid pool at concentrations exceeding those in muscle protein and this reflects the fact that they are synthesised in skeletal muscle (Smith and Rennie, 1990). All other amino acids are released in proportion to their occurrence in muscle proteins implying that little or no metabolism occurs. Indeed, during studies involving exercise, both phenylalanine and tyrosine have been used as indicators of protein degradation as they are not subject to any intermediate metabolism (Blomstrand and Saltin, 1999). Similarly, 3-methylhistidine (3-MH) has been used as an indicator of contractile protein degradation, as it is a direct by-product of actin and myosin degradation and cannot be subsequently metabolised (Elia *et al.* 1979). However, it has been demonstrated that 3-MH is also released during gut proteolysis (*i.e.* smooth muscle), which questions the use of 3-MH appearance as an index of contractile protein degradation (McKeran *et al.* 1978; Rennie and Millward, 1983).

The first step in branched-chain amino acid (BCAA) metabolism is the reversible BCAA aminotransferase reaction (BCAT; located predominantly in the mitochondria in humans, Suryawan *et al.* 1998) where leucine, isoleucine and valine are converted to their respective branched-chain α -keto acids (BCKA) and the amino group acceptor α -ketoglutarate is converted to glutamate. To complete the oxidation of the BCAAs, the BCKAs are degraded in an irreversible oxidative-decarboxylation reaction catalysed by

the branched-chain α -keto acid dehydrogenase complex (BCKADH). This reaction is suggested to be rate-limiting in BCAA oxidation. This step commits the BCAA carbon skeleton to the degradative pathway, therefore the activity of the BCKADH is highly regulated through a reversible phosphorylation (inactive)-dephosphorylation (active) mechanism (Harper et al. 1984).

Only 4% of the BCKADH is in the active form in skeletal muscle at rest (Wagenmakers et al. 1989), with human skeletal muscle holding a high capacity for BCAA transamination (Suryawan et al. 1998). The low activation of the BCKADH in human skeletal muscle at rest, suggests that the activity of the BCKADH has the potential to increase many fold. Indeed, skeletal muscle BCKADH activity has been shown to be stimulated by contractile activity, the exogenous provision of BCAA and low muscle glycogen content (Kasperek et al. 1985; Wagenmakers et al. 1989; van Hall et al. 1996b; Jackman et al. 1997; Wagenmakers et al. 1989). Moreover, the activation of BCKADH by exercise and BCAA ingestion have been found to be additive, and the activation of BCKADH by low muscle glycogen content and BCAA ingestion is via different mechanisms (van Hall et al. 1996b). Furthermore, the activation of BCKADH by exercise does not lead to an increase in the total enzyme activity in human muscle, rather the proportion of the enzyme present in the active state (dephosphorylated) increases (Wagenmakers et al. 1989).

The principal transamination reaction in BCAA metabolism, catalysed by BCAT, has been suggested to drain α -ketoglutarate and thus reduce TCA cycle flux which may impede the oxidation of glucose and fatty acids (Wagenmakers et al. 1990). More specifically, in the case of leucine, this reaction will put a net carbon drain on the TCA cycle, as the carbon skeleton of leucine is oxidised to three acetyl-CoA molecules and the aminotransferase step uses α -ketoglutarate as an amino group acceptor. The oxidation of both valine and isoleucine do not lead to the net removal of TCA cycle intermediates, as valine is oxidised to succinyl-CoA and isoleucine to both succinyl-CoA and acetyl-CoA.

The 'draining' effect on α -ketoglutarate by BCAA oxidation is normally counteracted by the regeneration of α -ketoglutarate either through the deamination of glutamate via

the glutamate dehydrogenase reaction or through transamination of glutamate with pyruvate by the alanine aminotransferase reaction (AAT) (Gibala et al. 1997a). However, under conditions of low glycogen or in patients with McArdle's disease (muscle phosphorylase deficiency), such anaplerotic processes are reduced or do not exist (Wagenmakers et al. 1991; Wagenmakers et al. 1990). The reduced availability of pyruvate in these situations has been suggested to decrease the anaplerotic capacity of the AAT reaction, leading to a decrease in the concentration of the TCA cycle intermediates (due to insufficient counterbalance of the carbon-draining effect of the BCAA aminotransferase reaction), and reduction in TCA cycle activity (Wagenmakers, 1998a). Indeed, no increase is observed in arterial or muscle alanine concentration during exercise in McArdle's patients (Wagenmakers et al. 1990; Sahlin et al. 1995), with only a modest decrease in muscle glutamate concentration during incremental exercise (Sahlin et al. 1995) demonstrating a reduced flux through the AAT reaction in these patients.

Under conditions of reduced pyruvate concentration and increased ammonia concentration, a situation which exists during the latter stages of prolonged exercise, the synthesis of glutamine is favoured (Goldberg and Chang, 1978). Indeed, an increase in plasma glutamine concentration has been observed following prolonged submaximal exercise (Sahlin et al. 1990). This increase in the presence of glutamine in the plasma has been attributed to a large portion of the ammonia produced (through the deamination of the BCAA; see section 2.4.3) being bound to glutamate by the action of glutamate synthetase, so that the muscle releases large amounts of glutamine and only small amounts of ammonia (Eriksson et al. 1985).

As the muscle becomes depleted of glycogen, such as during prolonged exercise, BCKADH activation is increased (van Hall et al. 1996b) and BCAA oxidation is accelerated (Rennie et al. 1981), with increasing amounts of the BCAA being extracted from the circulation, contributing to the energy required to sustain exercise (Wagenmakers et al. 1991; van Hall et al. 1996b; van Hall et al. 1995b). Therefore, it has been suggested that the provision of BCAA may serve as an additional, or indeed alternative, fuel source during prolonged exercise.. However, the ingestion of a solution containing BCAA in addition to glucose-polymer failed to affect cycling performance over 100 km in well-trained cyclists, compared to glucose-polymer only (Madsen et al.

1996), with similar findings being reported during cycling to exhaustion at 70% $\dot{V}O_{2max}$ (Galiano et al. 1992), despite elevating plasma BCAA concentrations to $\sim 1000 \mu\text{mol.l}^{-1}$ (normal concentration of plasma BCAA in resting humans is 300-400 $\mu\text{mol.l}^{-1}$).

2.4 AMMONIA METABOLISM

Ammonia is produced by skeletal muscle both at rest and during exercise (Lowenstein, 1972; Mutch and Banister, 1983). The normal resting plasma ammonia concentration is less than 50 μM , but during submaximal exercise to exhaustion the ammonia concentration may increase to 250 μM (Brouns et al. 1990). Ammonia is widely deemed to be a toxic by-product of metabolism, however the ammonium ion may actually serve to regulate glycolysis to a small extent through the activation of phosphofructokinase (PFK) (Lowenstein, 1972; Lowenstein and Goodman, 1978; Spencer and Katz, 1991a; Lowenstein, 1990). In addition, and somewhat paradoxically, ammonia may act as a buffer for lactic acid, thereby helping to maintain pH balance (Mutch and Banister, 1983). During exercise, ammonia production is greatly increased and is related to the intensity and duration of exercise. However, the pathway(s) by which ammonia is produced during exercise has led to much debate in the literature.

During prolonged submaximal exercise ammonia (NH_3)¹ can be produced through two possible avenues in contracting muscle. Firstly, ammonia may be derived from the oxidative deamination of glutamate via a reversible reaction catalysed by glutamate dehydrogenase (EC 1.4.1.2):



This reaction is therefore involved in the coupling of amino acid and ammonia metabolism. The glutamate dehydrogenase reaction activity appears sufficiently high to sustain the ammonia production required for the synthesis of glutamine in resting muscle, a reaction catalysed by glutamine synthetase (EC 6.3.1.2; $\text{glutamate} + \text{ATP} + \text{NH}_3 \rightarrow \text{glutamine} + \text{ADP} + \text{Pi}$) (Wibom and Hultman, 1990; Wibom et al. 1992; Starritt et al. 1999; Wibom et al. 1992). Previously however, it has been suggested

¹ In this thesis NH_3 represents the sum of both ammonia and the ion, ammonium.

that the glutamate dehydrogenase reaction does not contribute significantly to ammonia production in exercising muscle, as its activity in the direction of deamination is below the observed rates of ammonia production (Lowenstein and Goodman, 1978). Furthermore, considering that the reaction is near-equilibrium in nature and that a large decrease is observed in glutamate concentration during the early stages of exercise (Bergström et al. 1985), the ammonia production rate appears to be lower than that expected through this pathway, suggesting that ammonia is produced through additional pathways.

Whether or not the glutamate dehydrogenase reaction is capable of sustaining the rate of ammonia production during exercise is a contentious issue. More recent evidence suggests that the activity of the enzyme is increased with endurance training (Wibom et al. 1992). Endurance trained individuals generally experience a lesser increase in plasma ammonia during prolonged exercise (Graham et al. 1995). Therefore, it is possible in these individuals that the glutamate dehydrogenase reaction may favour glutamate formation. This would increase the availability of glutamate (substrate) for the glutamine synthetase reaction, which could conceivably increase glutamine synthesis and release from muscle (see sections 2.3.2 and 2.6.1 for additional sources of glutamate). Although the response of glutamine synthetase to training has not yet been studied, an increased release of glutamine from endurance trained muscle has been observed suggesting increased activity of this enzyme (Hood and Terjung, 1994). Furthermore, as glutamine synthetase is a mitochondrial enzyme, one may expect an increase in activity in response to training, in addition to other mitochondrial enzymes. The concomitant formation of glutamate through the glutamate dehydrogenase reaction and glutamine through the glutamine synthetase reaction would serve to reduce the amount of free ammonia present, corresponding to the observation in trained muscle. Indeed, if glutamate arises from the amination of α -ketoglutarate via the glutamate dehydrogenase reaction, then the synthesis of glutamine, catalysed by glutamine synthetase, can be seen as a transporter of two molecules of ammonia. The synthesis of both alanine (Graham et al. 1995; MacLean et al. 1994; MacLean et al. 1996; Galassetti et al. 1998) and glutamine (Eriksson et al. 1985; Graham et al. 1995; MacLean et al. 1994; MacLean et al. 1996; Galassetti et al. 1998) after reaction with pyruvate and glutamate respectively, provide a non-toxic mechanism for the removal of

amino groups from the muscle, and as such may act as a buffer for ammonia. The degree to which both glutamine and alanine are synthesised is largely dependent upon the concentration of ammonia within the tissue, with increased levels of ammonia favouring glutamine synthesis rather than alanine synthesis (Goldberg and Chang, 1978). Therefore, when considering muscle ammonia flux it would be imprudent to neglect both alanine and glutamine efflux. However, alanine and glutamine synthesis and subsequent efflux is unable to prevent the gradual rise in plasma ammonia concentration observed during prolonged submaximal exercise. This may be due to the competition between intramuscular enzymes, namely transaminases (i.e. alanine aminotransferase, aspartate aminotransferase) that exists for pyruvate and glutamate during exercise, in addition to the fact that glutamine synthesis is an energy requiring process.

2.4.1 THE PURINE NUCLEOTIDE CYCLE

Evidence for a second source of ammonia production is demonstrated by the observation that extracts of muscle cytosol have been shown to produce ammonia (Lowenstein and Goodman, 1978). This suggests that glutamate dehydrogenase, which occurs almost exclusively in the mitochondrial matrix (Lindena et al. 1996), cannot be solely responsible for ammonia production. This source of ammonia is most likely generated via the breakdown of ATP in the purine nucleotide cycle (PNC), proposed by Lowenstein (Lowenstein, 1972). The PNC generates ammonia through the deamination of AMP to inosine monophosphate (IMP), catalysed by the enzyme AMP deaminase (EC 3.5.4.4), producing ammonia as a by-product (Fig. 2.2). The key positive modulators of AMP deaminase are thought to be H^+ , AMP, free ADP and P_i (Graham et al. 1995).

The AMP deaminase reaction within the PNC is not reversible under normal physiological conditions, therefore a further two reactions exist to convert IMP back to AMP (Fig. 2.2). However, if IMP accumulates and is not reaminated back to AMP, a depletion of the adenine nucleotides (AMP, ADP, ATP) will occur. If IMP is reaminated, the adenine nucleotide pool is restored. The reactions required to reaminate IMP back to AMP are catalysed by adenylosuccinate synthetase and adenylosuccinate lyase. These reactions require the amine group from aspartate to proceed, which is

derived through transamination with glutamate. It is noteworthy that the reactions of the PNC do not act in concert, in other words, AMP deamination does not occur at the same time as IMP reamination. Moreover, AMP deamination occurs during muscle contraction, whilst IMP reamination occurs during muscle recovery (Meyer and Terjung, 1980).

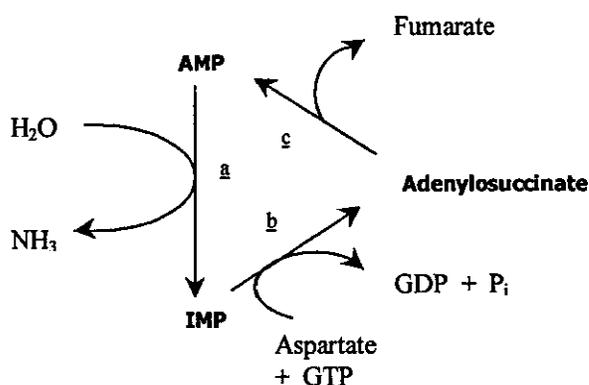


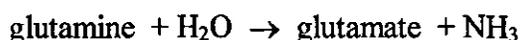
Fig. 2.2 The purine nucleotide cycle. The reactions are catalysed by a) AMP deaminase; b) adenylosuccinate synthetase; c) adenylosuccinate lyase

The net reaction of the PNC includes the deamination of the α -amino group of aspartate. Therefore, theoretically, the six amino acids oxidised in human skeletal muscle (leucine, isoleucine, valine, aspartate, asparagine and glutamate) could also be deaminated via the reactions of the PNC, as the α -amino group of these amino acids is interchangeable by reversible transamination reactions (Wagenmakers, 1998a).

The PNC is also suggested to be involved in nucleotide management. However, if the PNC is actually 'cycling' there is no net formation of IMP or removal of AMP, suggesting that the PNC does not actually contribute to nucleotide management. In this situation, aspartate is deaminated with the formation of ammonia and fumarate by the PNC. Therefore, the PNC may not only be responsible for the production of ammonia, but may be capable of replenishing TCA intermediates by generating fumarate. The generation of fumarate by the PNC in the cytosol may therefore be seen as an 'anaplerotic' reaction, with fumarate being transported into the mitochondrion presumably via a fumarate/malate translocator, which has been shown to exist in rat liver and kidney mitochondria (Atlante et al. 1998). It has been suggested that the increase in fumarate in rat skeletal muscle observed during exercise can be accounted

for by the operation of the PNC (Aragón and Lowenstein, 1980). However, in a more recent study it was reported that in human skeletal muscle a normal exercise-induced increase in fumarate was observed in patients with near total myoadenylate deaminase (AMPD) deficiency (AMPD catalyzes the reaction from adenosine monophosphate (AMP) \rightarrow inosine monophosphate (IMP) + NH₃ within the PNC) (Tarnopolsky et al. 2001). These results are consistent with the concept that the PNC is not quantitatively important in exercise-mediated increase in fumarate. It is however conceivable that species difference may exist in the operation of the PNC.

Whilst not considered as important as the previously discussed pathways for ammonia production, there are a number of other pathways by which ammonia may be produced in skeletal muscle. Glutaminase (EC 3.5.1.2) catalyses the breakdown of glutamine to ammonia:



Although important in ammonia metabolism in the liver, kidney and gut, the activity of this enzyme in skeletal muscle is generally regarded to be low at rest and during exercise, although it has not been measured in human skeletal muscle either at rest or during exercise. Secondly, adenosine deaminase (EC 3.5.4.4) an enzyme important in the control of adenosine concentration may also produce ammonia (adenosine + H₂O \rightarrow inosine + NH₃). However, as the concentration of adenosine in skeletal muscle is very low, suggesting that the quantitative contribution to ammonia production in skeletal muscle is most probably negligible.

Lastly, the deamination of the BCAA also contributes to ammonia production, which is discussed in section 2.4.3.

2.4.2 THE UREA CYCLE

Ammonia is a universal participant in both amino acid synthesis and degradation. However, an accumulation of ammonia above normal physiological concentrations can be toxic. Therefore, cells undergoing active amino acid catabolism must be able to

excrete ammonia at a rate comparable to the rate of ammonia synthesis. Excess ammonia, that is ammonia not involved in the formation of amino acids or the biosynthesis of nitrogen compounds, is converted to urea by the urea cycle (Fig. 2.3) in the liver. It is then secreted into the bloodstream and extracted by the kidneys for excretion in the urine.

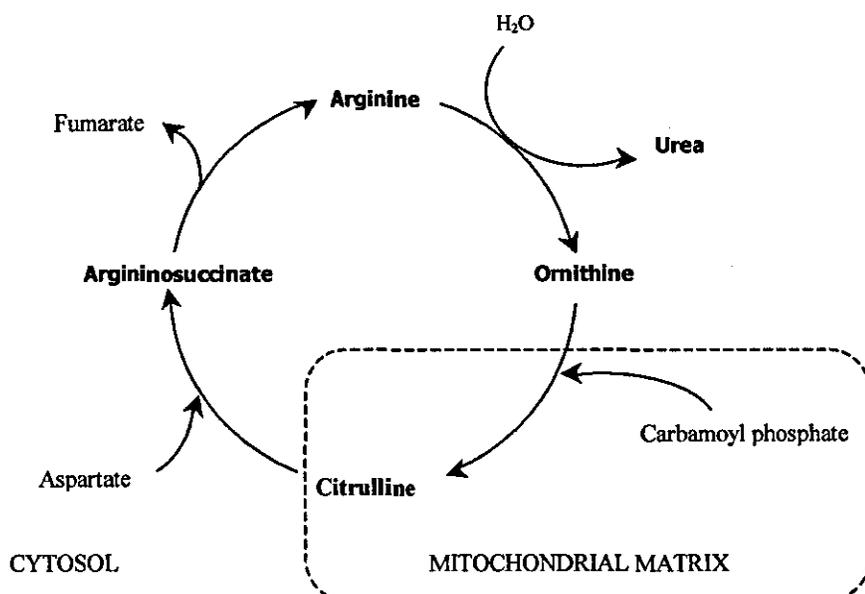
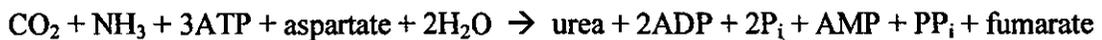


Fig. 2.3 The urea cycle. Urea is formed by the hydrolysis of arginine. The subsequent reactions of the cycle synthesize arginine from ornithine, the other product of the hydrolysis reaction. Firstly, ornithine is carbamoylated to citrulline by carbamoyl phosphate. Citrulline then condenses with aspartate to form argininosuccinate, which is cleaved to arginine and fumarate.

In the urea cycle ammonia combines with CO_2 to form carbamoyl phosphate. A series of reactions then lead to the formation of urea in liver cells (Fig. 2.3). One of the nitrogen atoms of urea is transferred from aspartate, derived from ammonia through the transfer to glutamate via the glutamate dehydrogenase reaction and then through transamination to aspartate. The second nitrogen atom and carbon atom is from carbamoyl phosphate, which is synthesised from CO_2 (Stryer, 1995). Ornithine, an amino acid which is not involved in the synthesis of proteins, acts as the 'carrier' for both the nitrogen and carbon atoms. The compartmentation of the urea cycle is such that ornithine must be transported into the mitochondrion, and citrulline exported into the cytosol for the cycle to proceed.

Carbon balance is maintained during cycling by the conversion of the fumarate to oxaloacetate in the TCA cycle, and then transaminated to aspartate. This series of reactions preserves the carbon skeleton of aspartate in the form of fumarate. The synthesis of fumarate therefore links the urea cycle and the TCA cycle, and is also analogous to the production of fumarate via the PNC. The net reaction for one turn of the urea cycle is:



The fumarate produced is then hydrated to malate, which is in turn oxidised to oxaloacetate. Oxaloacetate then has several possible fates: (1) transamination to aspartate, (2) conversion to glucose through gluconeogenesis, (3) condensation with acetyl CoA to form citrate, or (4) conversion to pyruvate (Stryer, 1995).

2.4.3 AMMONIA METABOLISM DURING EXERCISE

At rest, a small uptake of ammonia is observed in skeletal muscle. During exercise ammonia is released, the magnitude of which is dependent upon the intensity and duration of the exercise. Ammonia production during short, intense exercise is primarily due to the net breakdown of adenine nucleotides to IMP and is also related to fibre type recruitment, with fast-twitch glycolytic fibres being largely responsible for ammonia production due to their significant recruitment during this type of exercise (Meyer et al. 1980; Meyer and Terjung, 1979). However, during the first 30 minutes of prolonged submaximal exercise there is negligible increase in IMP (Sahlin et al. 1990), despite an increase in ammonia concentration (van Hall et al. 1995b).

Therefore, it is questionable whether the PNC contributes significantly to ammonia production during prolonged submaximal exercise (refer to section 2.4.1), and alternative sources such as BCAA oxidation, may contribute to a greater extent during this type of exercise. The fact that ammonia production exceeds the rate of breakdown of adenine nucleotides may indicate ammonia production through the deamination of amino acids via the PNC, at least during this initial period of submaximal exercise (van Hall et al. 1995b). The adequate provision of aspartate through the transamination of glutamate, ensures that the PNC may continue to 'cycle' and reaminate IMP to AMP.

The α -group of aspartate may be derived from any of the six amino acids which can be oxidised within skeletal muscle through reversible transamination reactions involving glutamate. However, as an elevation in skeletal muscle aspartate concentration has been observed during submaximal exercise, rather than decrease if involved in the PNC it has been suggested that PNC cycling may be negligible during exercise of this nature (MacLean et al. 1991). This adds to evidence suggesting that the PNC may not actually 'cycle' under such circumstances (Rennie, 1996a; Tarnopolsky et al. 2001). Indeed, during submaximal cycling to fatigue, a situation whereby ammonia production should be increased (Brouns et al. 1990), no alterations in the key positive modulators of AMP deaminase (H^+ , AMP, free ADP and P_i) have been observed, suggesting a greater contribution from the amino groups of the BCAA to ammonia production (MacLean and Graham, 1993; MacLean et al. 1996; MacLean et al. 1991; van Hall et al. 1995a; MacLean and Graham, 1993; van Hall et al. 1995b; MacLean et al. 1996).

During prolonged submaximal exercise the oxidation of the branched-chain amino acids (BCAA) via transamination and deamination reactions may contribute significantly to ammonia production. The first step in the metabolism of the BCAA is the removal of the NH_3 group by the reversible deamination catalysed by BCAA aminotransferase (BCAT). The NH_3 group is transferred to α -ketoglutarate, forming glutamate, which is then deaminated by glutamate dehydrogenase to produce α -ketoglutarate and NH_3 . Therefore, the transdeamination (transamination and deamination) of the BCAA can contribute to ammonia production during exercise. Muscle ammonia levels have been reported to be significantly greater during exercise when BCAA are ingested prior to exercise. This suggests that the increase in BCAA oxidation results in elevated ammonia production, supporting the important contribution of BCAA transdeamination to ammonia production (MacLean et al. 1994).

It has been suggested that a reduction in muscle glycogen content may increase ammonia production during prolonged submaximal exercise (Broberg and Sahlin, 1989; Broberg and Sahlin, 1988; Wagenmakers et al. 1991). In the glycogen depleted state, IMP formation is increased during exercise (Spencer and Katz, 1991a; Spencer et al. 1992) leading to a stoichiometric depletion of the adenine nucleotides (ATP, ADP, AMP) and a concomitant production of ammonia via the AMP deaminase reaction. In

addition, when muscle glycogen content is reduced during prolonged submaximal exercise, an increased uptake of the BCAA into skeletal muscle has been observed, presumably suggesting an increased oxidation of these amino acids (Lemon and Mullin, 1980; Wagenmakers et al. 1991).

2.4.4 BCAA AND THE CENTRAL FATIGUE HYPOTHESIS

Little is known about the mechanisms of central fatigue (i.e. fatigue resulting from the failure or limitations within the central nervous system). It was suggested in the mid 1980's that changes in plasma amino acid concentrations could play a role in central fatigue by influencing the synthesis and release of neurotransmitters, particularly 5-hydroxytryptamine (5-HT), in the brain (Newsholme, 1986). 5-HT has been linked to a variety of psychological responses including, lethargy, arousal, sleepiness and mood, therefore alterations in the concentration of 5-HT in the brain may modulate muscular fatigue or perceived exertion during exercise. For these reasons, 5-HT has been proposed as a potential mediator of 'central fatigue' (Blomstrand et al. 1988; Blomstrand et al. 1991).

The rate of 5-HT synthesis is dependent on the supply of its precursor, the amino acid tryptophan. The first reaction in the synthesis of 5-HT is catalysed by the enzyme tryptophan mono-oxygenase, which is considered the rate-limiting reaction in 5-HT synthesis (Newsholme and Leech, 1983). Since this enzyme is not saturated with substrate, an increase in the supply of tryptophan could theoretically increase the rate of 5-HT synthesis, implying that the uptake of free tryptophan into the brain is an important step in the regulation of 5-HT synthesis. In addition, the rate of 5-HT synthesis in the brain is also dependent upon the concentration of tryptophan in the blood and also upon the concentration of other large neutral amino acids (LNAA).

The LNAA transporter is a saturable carrier that transports amino acids across the blood-brain barrier, including tryptophan, tyrosine, methionine, phenylalanine and the BCAA (leucine, isoleucine and valine). These amino acids compete with tryptophan for transport into the brain, with the relative rate of transport into the brain being dependent upon the concentration of each amino acid relative to its competitors. Tryptophan is the only amino acid that is transported bound to albumin in the plasma (McMenamy and

Oncley, 1958). In the resting state, ~90% is bound to albumin and ~10% is in the free form. It has been suggested that the free concentration of tryptophan rather than the total concentration governs the rate of entry of tryptophan into the neurons and that free tryptophan therefore competes with other LNAA for transport into the brain (Blomstrand and Newsholme, 1996). The plasma concentration of free tryptophan has been found to increase during prolonged exercise (Blomstrand et al. 1988), which may be related to an increased rate of release of free fatty acids from adipose tissue during exercise, causing an increase in the plasma concentration of free fatty acids. The free fatty acids are transported bound to albumin in the plasma, like tryptophan, and an increase in their concentration decreases the binding of tryptophan to albumin, thereby increasing the plasma free tryptophan concentration (McMenamy and Oncley, 1958).

During the latter stages of prolonged exercise, the mobilisation of free fatty acids is increased which may displace the tryptophan bound to albumin, increasing the free tryptophan concentration. Concomitantly, an increased uptake of the BCAA (the amino acids that compete with tryptophan for transport into the brain) from plasma by skeletal muscle is observed during this period, which may result in an increase in the plasma concentration ratio of free tryptophan:BCAA. As suggested earlier, this may increase the rate of tryptophan entry into the brain, which may increase the brain concentration of 5-HT. In a study in humans, prolonged exercise caused a decrease in the plasma concentration of the BCAA with a concomitant increase in the free tryptophan concentration, akin to the proposed hypothesis (Blomstrand et al. 1988). Therefore, studies have examined the central fatigue hypothesis by manipulating either the plasma BCAA and/or tryptophan concentration. BCAA supplementation has been proposed not only to provide an additional fuel source during prolonged submaximal exercise, but may also be able to reduce the increase in the plasma concentration ratio of free tryptophan to BCAA during prolonged exercise. Through this mechanism, a reduction in the uptake of tryptophan into the brain and subsequent increase in brain concentrations of the neurotransmitter 5-hydroxytryptamine (5-HT) was proposed.

One of the first studies aiming to manipulate the plasma BCAA concentration was conducted by the research group who proposed the original central fatigue hypothesis. Subjects ingested 16 g of a mixture of BCAA in a 5% carbohydrate solution during a field-based marathon (Blomstrand et al. 1991). This dose was able to elevate plasma

BCAA levels and thus decrease the plasma free tryptophan:BCAA ratio. Despite this, marathon performance was not different compared to the placebo group. However, when subjects were divided into fast (≤ 3 h 5 min) and slow runners, a reduction in performance time was observed in the slower runners only. It must be noted that some criticisms concerning the experimental design of this study have arisen and therefore caution should be taken when interpreting the results, particularly considering the results of later studies (van Hall et al. 1995a). Blomstrand *et al.* (1991) also suggested that the slower runners may have depleted their glycogen stores more quickly, therefore becoming more susceptible to BCAA provision compared to the faster runners, as the oxidation of the BCAA during exercise appears to be related to glycogen concentration in skeletal muscle. An inverse relationship has been found between the activity of the branched-chain α -keto acid dehydrogenase complex and skeletal muscle glycogen content, suggesting that BCAA oxidation would be increased in the glycogen-depleted state (Wagenmakers et al. 1991). However, Varnier et al. tested this hypothesis, by infusing BCAA prior to exercise in previously glycogen-depleted subjects, and performance during a graded exercise test was not different when compared to a saline control (Varnier et al. 1994).

In a well controlled laboratory-based study, subjects received a 6% sucrose solution supplemented with either a low (6 g l^{-1}) or high (18 g l^{-1}) concentration of BCAA, or tryptophan (1 g l^{-1}) (van Hall et al. 1995a). Neither the low nor high concentration of BCAA affected cycle time to exhaustion at 70% W_{max} compared with a placebo trial despite an increase in the plasma BCAA concentration. The supplementation of tryptophan would be expected to affect performance in a negative manner according to the central fatigue hypothesis. In this study, tryptophan supplementation did not affect cycle time to exhaustion at 70% W_{max} compared to either BCAA supplementation or control trials. This was despite an estimated 20-fold increase in free-tryptophan influx into the brain. This finding questions the importance of free Tryp:LNAAs for the development of central fatigue.

Therefore, the majority of studies where the plasma concentration ratio of free tryptophan to BCAA was altered through either BCAA or tryptophan supplementation have failed to demonstrate a positive effect on performance. The results from these studies in human subjects would seem to indicate that the manipulation of the plasma

concentration ratio of free tryptophan to BCAA is unable to modulate the central fatigue mechanism that has been previously observed in rats (Chaouloff et al. 1986). Whether this failure in the central fatigue mechanism lies in the transport of amino acids through the blood-brain barrier, the conversion of tryptophan to 5-HT, or the proposed effect of 5-HT on central nervous system function during exercise remains to be elucidated.

More recently, in the rat, it has been demonstrated that brain ammonia levels increase during prolonged exercise, parallel to the increase observed in plasma ammonia (Guezennec et al. 1998). In order to detoxify this ammonia, which in itself may lead to fatigue (Banister and Cameron, 1990), an increase in glutamine synthesis was observed (glutamate + NH₃ → glutamine). It is therefore postulated that an increase in glutamine synthesis will decrease both brain glutamate and gamma-amino butyric acid (GABA) levels, as glutamate is also the precursor for GABA synthesis. GABA, considered as the main inhibitory neurotransmitter, is involved in the regulation of locomotion at spinal level (Cazalets et al. 1994). 5-HT release is inhibited by GABA, mediated via the pre-synaptic 5-HT receptor, therefore a decrease in brain GABA levels may lead to an increase in 5-HT release. Indeed, it has recently been demonstrated that a GABA agonist is able to extend endurance time to exhaustion in the rat (Abdelmalki et al. 1997). The supplementation of glutamine may therefore reduce the requirement for glutamine synthesis, thus sparing glutamate and maintaining GABA levels in the brain. Although an intriguing hypothesis, complimenting the original 'central fatigue' hypothesis, until further research is conducted these mechanisms remain speculative, especially in humans.

2.5 AMINO ACID SUPPLEMENTATION POST EXERCISE

It has been known for more than 30 years that the consumption of carbohydrate during recovery from exercise leads to a greater accumulation of muscle glycogen (Bergström et al. 1967; Hultman, 1967a). More recently however, the consumption of protein in addition to carbohydrate during recovery from exhaustive exercise has been demonstrated to increase muscle glycogen resynthesis to a greater extent than the consumption of carbohydrate alone (Zawadzki et al. 1992). However, the meals administered in this study were not isoenergetic, therefore the observed differences may be due to additional energy intake rather than the specific properties of protein.

In more controlled experiments, the co-ingestion of carbohydrate and protein post-exercise has been shown to stimulate muscle glycogen resynthesis (Tarnopolsky et al. 1997; Jentjens et al. 2001). It is important to note however, that the rate of muscle glycogen synthesis was not greater than the carbohydrate alone condition in these experiments. The increase in muscle glycogen storage post-exercise after the co-ingestion of carbohydrate and protein has been attributed to an increased insulin response promoting storage of carbohydrate (Spiller et al. 1987).

Furthermore, the provision of protein, and more specifically amino acids, in the post-exercise period may enhance whole-body nitrogen balance and preserve whole-body and muscle protein stores (Borel et al. 1997). Therefore, in terms of whole-body recovery, the provision of both carbohydrate and amino acids in the post-exercise period may be more favourable than the ingestion of carbohydrate alone. In particular, the supplementation of a single amino acid has been considered more recently, rather than whole protein. Of particular interest in this context is the amino acid glutamine. A positive relationship has been observed between protein synthesis and the intramuscular glutamine concentration (MacLennan et al. 1987), in addition the intramuscular glutamine concentration falls dramatically as a result of starvation, injury, disease and after prolonged exercise. During these conditions, glutamine synthetase activity has been reported to increase, whereas glutaminase activity was unchanged (Tadros et al. 1993). Furthermore, an increase in the capacity for glutamine uptake via system N^m was observed, suggesting that both system N^m and glutamine synthetase undergo adaptive up-regulation in glutamine-deprived muscle cells. Therefore, the supplementation of glutamine may preserve intracellular stores and exert an anabolic effect.

2.6 GLUTAMINE

Glutamine has an elementary composition of carbon (41.09%), hydrogen (6.9%), oxygen (32.84%) and nitrogen (19.17%) and has a molecular weight of 146.15. Glutamine is a five-carbon amino acid with two amino moieties, an α -amino group, and an easily hydrolysed terminal amide group (C₅H₁₀N₂O₃; see Fig. 2.4). Oxidation of one molecule of glutamine yields 30 molecules of adenosine triphosphate (ATP), an amount

comparable to the 36 molecules of ATP produced from glucose, a six-carbon sugar (Lacy and Wilmore, 1990).

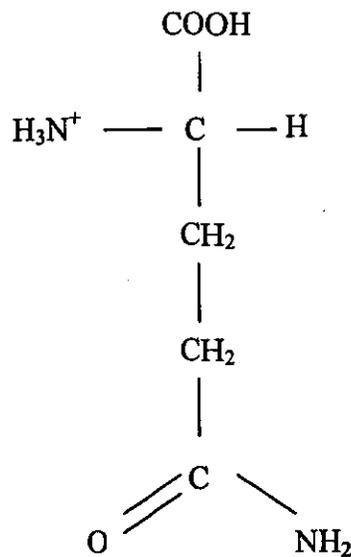


Fig 2.4 Chemical structure of glutamine.

Glutamine, although a non-essential amino acid, has been identified as a conditionally 'essential' amino acid, such that its requirement markedly increases during conditions of metabolic stress (Lacy and Wilmore, 1990). Glutamine has a prominent role in the maintenance of acid-base balance (Golden et al. 1982), and is an important energy source for the cells of the gut (Hartmann and Plauth, 1989; Souba, 1991; Kight and Fleming, 1995; Fleming et al. 1997) and the immune system (Ardawi and Newsholme, 1983; Newsholme et al. 1989). Skeletal muscle and the small intestine appear to be the major sites for glutamine synthesis and uptake, respectively, in humans (Marliss et al. 1971; Windmueller and Spaeth, 1974).

Glutamine is the most abundant free amino acid in plasma and skeletal muscle (with the exception of taurine) (Bergström et al. 1974) and constitutes more than 50% of the free intracellular amino acid pool. The concentration of glutamine in human skeletal muscle is remarkably high, about 20 mmol/kg wet weight which is approximately 30 times the concentration observed in whole blood (0.6 - 0.7 mmol.l⁻¹). Glutamine is present in the free amino acid pool at a concentration exceeding that in muscle protein, as are alanine and glutamate, reflecting the fact that these amino acids are synthesised in muscle. It has been estimated that the synthesis rate of glutamine in human skeletal muscle is

between 20 and 80 g per day and is higher than that of any other amino acid (Darmaun et al. 1986).

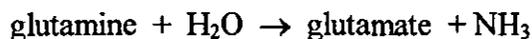
The intramuscular glutamine pool is depleted in response to a variety of situations including glucocorticoid treatment (Muhlbacher et al. 1984), severe illness (Rennie et al. 1986; Biolo et al. 2000), and prolonged exercise (Rennie et al. 1981). This decrease in intramuscular glutamine concentration is also reflected in the plasma glutamine concentration, with both surgery (Parry-Billings and Newsholme, 1992) and prolonged exercise (Parry-Billings et al. 1987) leading to a decrease in plasma glutamine concentration, presumably due to an increased uptake of glutamine by the splanchnic bed.

There is considerable evidence to suggest that a positive correlation exists between positive whole-body protein balance and both the size of the intramuscular glutamine pool (MacLennan et al. 1987; MacLennan et al. 1988; Jepson et al. 1988) and the plasma concentration of glutamine (Watt et al. 1992; Hankard et al. 1998). However, this does not necessarily suggest a cause and effect relationship. The concentration of insulin, a potent anabolic hormone, regulates glutamine transport (see section 2.3.1). A reduction in insulin concentration causes a reduction in the intramuscular glutamine pool, due to an increased efflux of glutamine from the muscle (Rennie et al. 1996b). Therefore, it is feasible that a reduction in intramuscular glutamine concentration and protein synthesis may merely reflect a reduction in insulin concentration. However, the possibility of a relationship between glutamine concentration and protein turnover rates have led to the use of glutamine solutions in both enteral and parental nutrition (Khan et al. 1991; Hardy et al. 1992; Hardy et al. 1992; Smith, 1997; Morlion et al. 1998).

2.6.1 GLUTAMINE METABOLISM

The catabolism of glutamine occurs via one of two isoforms of glutaminase (Curthoys and Watford, 1995), an exclusively mitochondrial enzyme. The liver-type glutaminase is expressed only in periportal hepatocytes of the postnatal liver, coupling ammonia production and urea synthesis. Kidney-type glutaminase is abundant in kidney, brain, intestine and lymphocytes where the resulting ammonia is released without further metabolism.

The reaction catalysed by glutaminase (EC 3.5.1.2), also referred to glutaminase I, gives rise to stoichiometric amounts of glutamate and ammonia. The activity of this enzyme is termed phosphate-dependent.



The activity of glutaminase I in human skeletal muscle is still to be quantified. Previously, the activity of glutaminase I has been reported to be relatively low in both rat skeletal muscle (Ottaway, 1969) and in human skeletal muscle (Swierczynski et al. 1993). Furthermore, the activity of glutaminase I does not appear to be influenced by the concentrations of its substrate or product (Smith et al. 1984), but may be subject to hormonal regulation (Squires et al. 1997). In addition, as glutaminase I is located in mitochondria, the transport of glutamine into the mitochondrion may be a point of regulation, an area requiring further investigation.

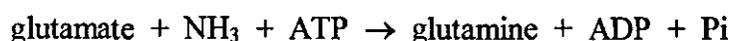
During acidosis, and possibly other stressful circumstances where pH is lowered, such as during exercise, glutaminase I in the kidney demonstrates an increase in activity, whereas the enzyme activity in skeletal muscle does not (Swierczynski et al. 1993). This response would appear to be inherently linked to the restoration of the acid-base balance with the export of glutamine from the muscle, and hence ammonia, leading to an increased catabolism in the kidney. Indeed, an increase in the plasma glutamine concentration should lead to an increase in renal extraction of glutamine with a concomitant increase in flux through the glutaminase I pathway. In the liver, an increase in plasma glutamine concentration above physiological levels leads to an increase in the activity of glutaminase I and subsequent metabolism of glutamine to produce glucose and urea (Curthoys and Watford, 1995). The alterations in hepatic glutaminase I activity appear to be mediated by changes in the rate of transcription, whereas kidney glutaminase I appears to be regulated at the post-transcriptional level (Curthoys and Watford, 1995).

The action of glutaminase II is actually a combination of a number of isozymes, namely glutamine aminotransferase and ω -amidase. The quantitative importance of these reactions in glutamine metabolism is doubtful due to the relatively low concentration of

the preferred keto acid substrates (phenylpyruvate and the keto analogues of methionine, glycine, serine, cysteine, and asparagine). It has been suggested that glutaminase I may be able to support higher rates of glutamine catabolism than glutamine aminotransferase and ω -amidase in rat skeletal muscle (glutaminase II) (Swierczynski et al. 1993). However, the glutaminase I inhibitor 6-diazo-5-oxo-L-norleucine (DON) does not prevent glutamine catabolism, thereby suggesting that the activity of glutamine aminotransferase and ω -amidase is in fact greater in rat skeletal muscle (Wu et al. 1991). Moreover, it must be recognised that further research is required into glutamine catabolism in skeletal muscle, in particular in human skeletal muscle, as at present the evidence is limited.

The catabolism of glutamine via glutaminase I leads to the direct formation of glutamate, which can in turn be converted to α -ketoglutarate through the glutamate dehydrogenase reaction. The catabolism of glutamine via glutaminase II leads firstly to the formation of α -ketoglutaramide (glutamine transaminase) and then α -ketoglutarate (ω -amidase). Irrespective of the glutamine metabolic pathway, the resultant α -ketoglutarate can conceivably enter the TCA cycle and potentially increase the concentration of the TCA cycle intermediates (see section 2.10).

Glutamine synthetase (EC 6.3.1.2) is responsible for the formation of glutamine from glutamate derived from either the action of glutamate dehydrogenase or through transamination reactions. It has been demonstrated that in postabsorptive humans, BCAA released by proteolysis play a prominent role as a source of α -amino nitrogen for glutamine synthesis (Garber et al. 1976a; Garber et al. 1976b; Chang and Goldberg, 1978; Darmaun and Déchelotte, 1991). The synthesis of glutamine requires glutamate to accept a second ammonia moiety in a reaction catalysed by the enzyme glutamine synthetase.



This enzyme is termed a *synthetase*, rather than a *synthase*, as the reaction links bond formation with the energy released from ATP hydrolysis (Mathews and van Holde, 1996).

Glutamine synthetase is found in most tissues, located in the cytosol, and although skeletal muscle possesses a relatively low activity of this enzyme when expressed per gram of tissue, by virtue of its large mass this tissue is the major site of glutamine synthesis in the body (Smith and Rennie, 1990). Furthermore, glutamine synthetase activity has been reported to be lowest in slow twitch muscle fibres (Falduto et al. 1992). Although glutamine synthetase activity has been detected in rat kidney extracts (Iqbal and Ottaway, 1970), it has not been detected in human kidney (Curthoys and Watford, 1995).

The synthesis of glutamine via the glutamine synthetase reaction is regulated by its product (glutamine) and its substrate (glutamate), in addition to glucocorticoids (Smith et al. 1984; Max, 1990; Feng et al. 1990). The activity of the enzyme in cultured skeletal muscle cells is increased in response to lowered glutamine concentrations and increased glutamate concentrations (Smith et al. 1984). Conversely, the enzyme activity is lowered in the presence of high glutamine concentrations, however glutamine synthetase mRNA levels remain unaltered under such conditions suggesting an independent regulation at a post-transcriptional level (Feng et al. 1990; Opara et al. 1992; Hickson et al. 1996).

Glucocorticoids have been demonstrated to cause an accelerated release of glutamine from skeletal muscle leading to a decrease in intramuscular glutamine levels (Muhlbacher et al. 1984). This response would appear to be largely due to an increased activity of the glutamine transporter system in skeletal muscle (System N^m; see section 2.3.1) (Babij et al. 1986a; Babij et al. 1986b). It has previously been reported that these alterations in intracellular levels of glutamine were not accompanied by any measurable change in the glutamine metabolising enzymes, glutamine synthetase and glutaminase (Muhlbacher et al. 1984). More recently however, dexamethasone, a potent synthetic glucocorticoid, has been shown to increase glutamine synthetase activity, via an increase in glutamine synthetase mRNA level (Max, 1990; Falduto et al. 1992). This would result in an increased production of glutamine in situations where glucocorticoid concentration is increased (Darmaun et al. 1988). Despite glucocorticoids increasing the expression of glutamine synthetase, a decrease is observed in the intramuscular level of glutamine due to increased glutamine efflux from skeletal muscle, as discussed previously.

It has been proposed previously that a 'glutamine substrate cycle', involving glutaminase I and glutamine synthetase, may exist in skeletal muscle (Parry-Billings and Newsholme, 1991). The existence of a 'glutamine substrate cycle' in skeletal muscle could conceivably lead to a futile intracellular cycling between glutamine and glutamate, with skeletal muscle being responsible for both the production and degradation of glutamine. However, this suggestion has been disputed (Rennie et al. 1992) as the enzymes involved, glutaminase and glutamine synthetase, are located in different cellular compartments [glutamine synthetase is cytosolic, whereas glutaminase is mitochondrial] (Kelso et al. 1989). Furthermore, the original proposition by Parry-Billings *et al.* seems to have disregarded the contribution that glutaminase II may make to glutamine degradation in skeletal muscle. Indeed, Wu *et al.* (1991) have demonstrated that glutamine degradation is not inhibited in rat skeletal muscle in the presence of the glutaminase I inhibitor 6-diazo-5-oxo-L-norleucine (DON), suggesting that glutaminase II does contribute to glutamine degradation.

It is feasible however, that a 'glutamine-glutamate cycle' exists between skeletal muscle, the liver and the gut (Marliss et al. 1971). The splanchnic bed takes up most of the glutamine produced and released by skeletal muscle. Glutamine is then converted to glutamate and ammonia by the action of glutaminase I. However, only about 50% of the glutamate produced is available to skeletal muscle, as the splanchnic bed retains ~50% for metabolism in the gut (Souba, 1991; Windmueller and Spaeth, 1974) and liver (Häussinger, 1989) due to the central role that glutamate plays in a variety of transamination and deamination reactions. This may also be complicated by the slow mixing of the intracellular and plasma glutamate pools (Darmaun et al. 1986).

2.6.2 GLUTAMINE SUPPLEMENTATION

The intravenous administration of glutamine is able to increase both the plasma and intramuscular concentrations of glutamine (Watt et al. 1992; Varnier et al. 1995). In the postabsorptive state, 54 ± 4 % of enterally delivered $[2-^{15}\text{N}]$ glutamine tracer is sequestered on the first pass through splanchnic bed (Matthews et al. 1993). More recently, using $[1,2-^{13}\text{C}_2]$ glutamine, it was reported that approximately 58 ± 2 % of an enterally delivered tracer is sequestered on the first pass through splanchnic bed (Haisch

et al. 2000), suggesting that the uptake of the glutamine tracer is not an artefact of the label used. In spite of this substantial extraction and subsequent oxidation by the splanchnic bed, the oral delivery of glutamine has been demonstrated to substantially increase the systemic glutamine concentration (Déchelotte et al. 1991; Hankard et al. 1995; Hankard et al. 1998; Darmaun et al. 1994), thus feasibly providing the extrasplanchnic tissues with an exogenous supply of glutamine.

Although it has been demonstrated that the intravenous infusion of glutamine is able to increase muscle glutamine concentration (Watt et al. 1992; Varnier et al. 1995), and that long-term oral glutamine supplementation is able to increase muscle glutamine concentration in rats (Opara et al. 1992), at present no studies have examined whether an oral glutamine load is able to transiently elevate muscle glutamine concentration in humans. It is feasible however, that an increase in the plasma glutamine concentration will augment the rate of uptake of glutamine uptake by the muscle via the low affinity, high capacity glutamine transporter in skeletal muscle, System N^m (Ahmed et al. 1993) (see section 2.3.1). The Michaelis-Menten constant (K_m ; 0.5-1.5 mM) of glutamine transport in human skeletal muscle of near-plasma glutamine concentration (~0.7 mM) is suggestive that the influx of glutamine into skeletal muscle is modulated by changes in the plasma glutamine concentration (Ahmed et al. 1993). Indeed, skeletal muscle accounts for nearly 50% of glutamine uptake from the plasma (Nurjhan et al. 1995; Stumvoll et al. 1996).

Skeletal muscle is primarily responsible for glutamine synthesis (Marliss et al. 1971), therefore, due to the transport characteristics of glutamine, it is not surprising that the *de novo* synthesis of glutamine is suppressed in response to exogenous glutamine (Hankard et al. 1995), most probably mediated through the modulation of the activity of the enzymes involved in glutamine metabolism (Smith et al. 1984; Feng et al. 1990). Furthermore, the exogenous provision of glutamine is able to increase both splanchnic extraction and oxidation of glutamine indicating a regulatory response to the glutamine load, indeed the splanchnic extraction of glutamine increases in a dose-dependent manner (Hankard et al. 1995).

In fasted dogs, the infusion of glutamine inhibits lipolysis, signified by a decrease in both glycerol and free fatty acid levels, in the absence of any changes in insulin or

glucagon concentration (Cersosimo et al. 1986). Similar findings have been reported in postabsorptive humans (Déchelotte et al. 1991), suggesting that exogenous provision of glutamine exerts an antilipolytic effect.

2.6.3 GLUTAMINE AND EXERCISE

There appears to be a lack of consensus within the literature regarding the effects of exercise upon plasma glutamine concentration. It has been reported that during prolonged submaximal exercise ($\sim 60\text{-}80\%$ $\dot{V}O_{2\max}$) lasting approximately 90 min, the plasma glutamine concentration either remains unchanged or exhibits a moderate increase (Eriksson et al. 1985; van Hall et al. 1995b; van Hall et al. 1999; Sahlin et al. 1990). However, during more prolonged exercise (e.g. marathon), the plasma glutamine concentration has been reported to decline (Parry-Billings et al. 1987; Parry-Billings et al. 1992). In addition, the efflux of glutamine from skeletal muscle is increased during prolonged submaximal exercise (Sahlin et al. 1990; Rennie et al. 1981; van Hall et al. 1999). During recovery from exercise, plasma glutamine concentration has been reported to decline following intermittent high-intensity exercise (Walsh et al. 1998a) and steady-state exercise (Rennie et al. 1981).

Wagenmakers et al. (1991) have suggested that a reduction in muscle glycogen concentration or glucose concentration could be responsible for the fall in plasma glutamine concentration. Glutamate only contributes a small proportion of the carbon required for glutamine synthesis (Wagenmakers et al. 1990), with the production of TCA cycle intermediates from muscle glycogen and glucose via carboxylation of pyruvate conceivably contributing to the carbon skeletons required for glutamine synthesis. Therefore, a reduction in muscle glycogen or glucose concentration may result in a reduction in glutamine synthesis in skeletal muscle. Indeed, the consumption of a low-carbohydrate diet for 3 days led to a greater decline in plasma glutamine concentration during recovery from 60 min of cycling at 70% $\dot{V}O_{2\max}$ compared to the consumption of a normal diet (Gleeson et al. 1998). Despite this apparent requirement for carbohydrate to prevent the decline in plasma glutamine concentration post exercise, the consumption of carbohydrate during exercise has been shown to be unable to

prevent the decline in plasma glutamine concentration post exercise (van Hall et al. 1998).

Exercise may therefore influence the rate of glutamine efflux from skeletal muscle, particularly following very prolonged exercise. Metabolism in those tissues and cells that are influenced by glutamine may be affected in the recovery period after exercise. For example, cells of the immune system require glutamine for both energy provision and nucleotide synthesis; therefore a reduction in plasma glutamine concentration may impair immune function. Thus skeletal muscle may play an important role in immune function (Parry-Billings et al. 1990).

2.6.4 GLUTAMINE AND IMMUNE FUNCTION

It has generally been considered that both lymphocytes and macrophages obtain most of their energy through the oxidation of glucose. More recently it has been established that the rate of glutamine utilisation by these cells is often greater than that of glucose (Ardawi and Newsholme, 1983). Glutamine would appear to be necessary for the optimal function of cells of the immune system *in vitro*, with optimal proliferation of lymphocytes occurring at a glutamine concentration of between 0.3 and 1.0 mM (Ardawi and Newsholme, 1983; Rohde et al. 1995). Indeed, a lowering of the glutamine concentration in the medium has been shown to markedly decrease the rate of proliferation (Newsholme and Parry-Billings, 1990; Calder, 1994). Therefore, the presence of glutamine is required if optimal lymphocyte proliferation is to occur.

However, only ~30% of glutamine carbon is completely oxidised within the cells of the immune system (Ardawi and Newsholme, 1983), with the partial oxidation of glutamine, termed glutaminolysis, contributing to the production of glutamate, aspartate, alanine. These by-products are required for the synthesis of purine and pyrimidine needed for the formation of DNA and RNA (Newsholme et al. 1989). In addition, glutaminolysis also provides precursors for the formation of glucosamine, GTP, and NAD⁺ (Newsholme et al. 1989).

There is now considerable evidence to suggest that prolonged, intense exercise is associated with adverse effects on the cellular immune system (Pederson et al. 1998).

These adverse effects include a lowering of circulating lymphocyte numbers, a reduction in neutrophil function, a decrease in NK cells and a decrease in immunoglobulin levels in blood and saliva. In addition, previous studies have indicated that a single period of prolonged exercise transiently decreases plasma glutamine concentration (Parry-Billings et al. 1987; Parry-Billings et al. 1992). Furthermore, a transient decrease in plasma glutamine concentration concomitant with a suppression of peripheral lymphocyte proliferation has been observed in chronically exercised rats (Koyama et al. 1998). In humans, a reduction in both plasma glutamine concentration and neutrophil function has been observed following very prolonged (~164 min) exercise at 55% $\dot{V}O_{2max}$ (Robson et al. 1999). These findings would seem to imply a causal relationship between exercise-induced immunosuppression and transient hypoglutaminaemia post exercise. This relationship may be partly due to the fact that exercise places increased demands on muscle and other organs, both in terms of provision and requirement, for glutamine post exercise. This may force the immune system into 'glutamine debt', thus impairing immune function (Pederson et al. 1998).

For these reasons, the plasma glutamine concentration has been proposed to be a useful tool in monitoring overtrained athletes, in whom depressed plasma glutamine levels are often observed (Rowbottom et al. 1997; Rowbottom et al. 1996, Kingsbury et al. 1998). However, the use of plasma glutamine as an indicator of overtraining status has recently been deemed insensitive (Bagger et al. 1997). Plasma glutamine concentration is influenced by short-term exercise, nutritional status, diet, infection and physical trauma, it is important that these factors are considered when using plasma glutamine concentration as a marker of overtraining (Walsh et al. 1998b). Nonetheless, it would appear that plasma glutamine level or the plasma glutamine to glutamate ratio may provide useful markers, and may be more effective when combined with other known markers of overtraining.

Therefore it is feasible that, the provision of glutamine post exercise may aid immune function. Based on subjective evidence, the supplementation of glutamine post exercise has led to fewer reported incidences of infections in the week following the bout of exercise (Castell et al. 1996). Under more controlled conditions, recovery from experimentally-induced overtraining may be more rapid with the provision of

glutamine, however symptoms of overtraining could not be prevented with glutamine supplementation (Bagger et al. 1997). The provision of glutamine has also been shown to alter lymphokine-activated killer (LAK) cell activity, but not natural killer (NK) cell activity (Rohde et al. 1995), in addition to suppressing tumour growth in tumour-bearing rats (Shewchuk et al. 1997b).

It would appear that a degree of circumstantial evidence exists in support of the supplementation of glutamine to enhance immune function. However, the purported beneficial effects of glutamine supplementation post exercise have not been observed in all research studies.

For example, the supplementation of glutamine post exercise has been shown to have no effect upon lymphocyte distribution and was unable to prevent the post exercise decline in plasma glutamine concentration (Castell et al. 1997). Lymphocyte metabolism and function was not enhanced after the provision of a high-protein diet (2% w/w glutamine) to exercise-trained rats for 7 days (Shewchuk et al. 1997a). However, this may have been due to the fact that the protocol employed did not induce a transient decrease in plasma glutamine concentration post exercise and did not alter plasma glutamine concentration following consumption of the high-protein diet. Moreover, despite abolishing the post exercise decrease in plasma glutamine concentration, the supplementation of glutamine *in vivo* has been demonstrated to have no effect on the post exercise changes in immune function (Rohde et al. 1998). Furthermore, it has been suggested that the fall in plasma glutamine concentration does not account for the decrease in neutrophil function following prolonged exercise, suggesting there is no direct relationship between plasma glutamine concentration and immune function (Walsh et al. 2000).

Therefore, changes in immune function post exercise may not to be related to a decrease in plasma glutamine concentration and may be more related to the level of the circulating stress hormones, cortisol and adrenaline, or the cytokines (soluble glycoproteins that are produced and mediate communication between and within immune and non-immune cells). Indeed, strenuous exercise has been shown to increase circulating levels of the cytokine interleukin (IL)-6 (Rohde et al. 1997), suggesting that

exercise induces a strong systemic anti-inflammatory response similar to the response seen in trauma (Pederson et al. 1999).

2.7 ORNITHINE α -KETOGLUTARATE (OKG)

OKG is a salt formed of two molecules of ornithine and one molecule of α -ketoglutarate. The pKs of ornithine (10.8) and α -ketoglutarate (1.9 & 1.4) give a pH of ~ 7 in aqueous solution over a wide range of OKG concentrations (up to 5 mM) (Cynober, 1995), making it possible to provide OKG either enterally or parenterally.

In recent years, attention has focused on the qualitative improvement of nutritional support required in response to catabolic situations, in particular the use of specific substrates that play key roles in the control of protein metabolism. The role of the amino acid glutamine has already been discussed in this context (see section 2.6), additionally, the substrate ornithine α -ketoglutarate (OKG) has been shown to improve the nutritional status of hypercatabolic patients (Vaubourdolle et al. 1987; Le Boucher and Cynober, 1997). OKG has received particular attention over recent years due to its efficiency in various malnutrition states and its ability to generate anabolic signals, both hormonal and metabolic.

In the extreme catabolic state, for example in burn injury or sepsis, OKG has been shown to improve nitrogen balance and decrease hyperphenalaninemia (Cynober et al. 1987). OKG has also been shown to reduce urinary 3-methylhistidine excretion (Le Boucher et al. 1997), demonstrating that OKG can inhibit myofibrillar degradation, and counteract the trauma-induced decrease in the intramuscular glutamine pool (Cynober, 1995; Le Boucher et al. 1997). Furthermore, OKG has been shown to stimulate the secretion of both insulin and human growth hormone (hGH), however the effect appears to be highly dependent on nutritional and metabolic status (Cynober et al. 1990; Cynober et al. 1984; Vaubourdolle et al. 1987).

OKG has been shown to generate glutamine in muscle (Vaubourdolle et al. 1991) and the jejunum (Le Boucher et al. 1997). However, the mechanism by which OKG increases the muscle glutamine pool is controversial. In particular, the suggestion that the α -ketoglutarate moiety is solely responsible for the improvement in glutamine status

has been questioned (Hammarqvist et al. 1990; Wernerman et al. 1990), with more recent research suggesting an interaction between the ornithine and α -ketoglutarate pathways when OKG is provided (Cynober, 1999).

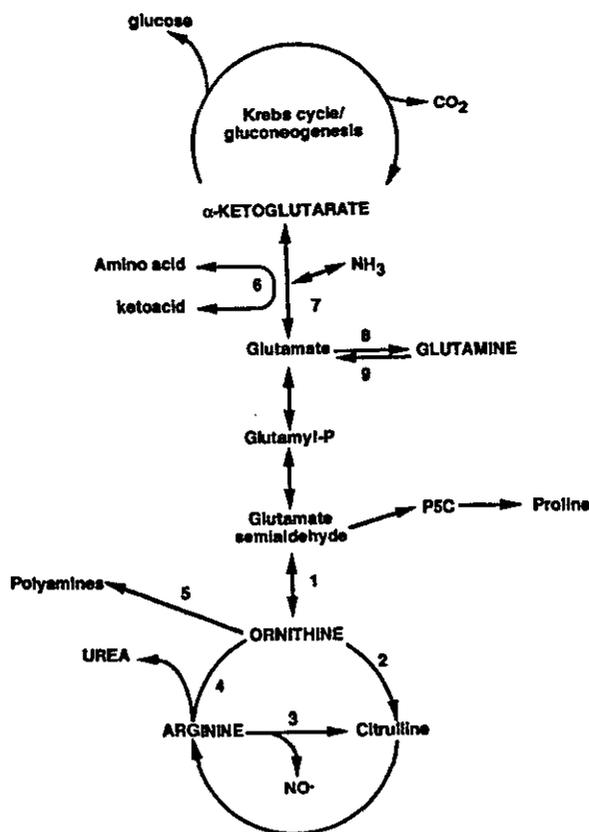


Fig 2.5 Ornithine and α -ketoglutarate metabolic pathways. Key enzymes are 1) ornithine aminotransferase; 2) ornithine carbamoyltransferase; 3) Nitric oxide synthase; 4) arginase; 5) ornithine decarboxylase; 6) transaminases; 7) glutamate dehydrogenase; 8) glutamine synthetase; 9) glutaminase.

Indeed, firm evidence of an interaction between ornithine and α -ketoglutarate was provided when a group of healthy individuals received either 10 g OKG, 6.4 g ornithine or 3.6 g α -ketoglutarate. Only the administration of OKG was able to significantly increase arginine and proline plasma levels and glutamine production by the kidney (Cynober et al. 1990). The biochemical explanation of the metabolic interaction between ornithine and α -ketoglutarate is that these two compounds share a common metabolic pathway through which the TCA cycle and the urea cycle are linked (α -ketoglutarate \leftrightarrow glutamate \leftrightarrow glutamyl-phosphate \leftrightarrow glutamate semialdehyde \leftrightarrow ornithine; see Fig. 2.4). *In vivo*, the main role of ornithine is transamination to glutamate. However, as the reactions involved are almost in equilibrium, the

administration of both ornithine and α -ketoglutarate as OKG diverts these molecules and their direct metabolites toward other pathways generating glutamine, arginine, proline, and polyamines, all potent modulators of protein metabolism.

OKG, through its α -ketoglutarate moiety, has an inherent link with the TCA cycle (see Fig 2.5). The positioning of α -ketoglutarate in the TCA cycle, at the beginning of the second span of the cycle (see section 2.10), is a point at which carbon could feasibly enter the cycle. It has been demonstrated that α -ketoglutarate infusion is able to increase the intramuscular α -ketoglutarate content in anaesthetised dogs (Roth et al. 1991), therefore it is feasible that after being transported into skeletal muscle the α -ketoglutarate moiety of OKG could enter the mitochondria and the TCA cycle (refer to section 2.12). In skeletal muscle, given the significance of the TCA cycle, this may have implications for oxidative energy production, particularly during exercise.

2.8 GLYCOGEN METABOLISM

It was demonstrated in a series of experiments in the late 1960's, early seventies (Bergström et al. 1967; Hermansen et al. 1967; Hultman, 1967a, Hultman and Nilsson, 1971) that both skeletal muscle glycogen content and liver glycogen content is reduced following prolonged exercise. Furthermore, this depletion in glycogen stores was associated with fatigue during submaximal exercise (Bergström et al. 1967). Therefore, the restoration of both muscle and liver glycogen during the recovery period after a bout of prolonged exercise is important to the recovery of endurance exercise capacity.

2.8.1 LIVER GLYCOGEN METABOLISM

The liver is able to release glucose into the circulation, unlike skeletal muscle, due to the presence of the enzyme glucose-6-phosphatase, which converts glucose-6-phosphate (G-6-P) to glucose. Glucose derived from both gluconeogenesis and glycogenolysis in the liver is released into the circulation to preserve glucose homeostasis, therefore the synthesis and storage of glycogen in the liver is vital.

Glucose is extracted from the blood via the hepatic portal vein and is transported into liver cells via the facilitative glucose transporter 2 (GLUT 2). GLUT2 is a low-affinity, high capacity transport system, allowing the uninhibited (non-rate limiting) flux of glucose into or out of liver cells due to its high K_m (~17mM) (Mueckler, 1994; Thorens, 1996). Therefore, the rate of glucose uptake into the liver via GLUT2 increases in parallel with any rise in blood glucose, such as after a meal or when a glucose load is delivered.

The transport of glucose into the liver cell is near-equilibrium, and distinct from other tissues, the phosphorylation of glucose to form G-6-P in the liver is catalysed by glucokinase which has a very high K_m for glucose, between 8 and 12mM (Newsholme and Leech, 1983; Wehmeyer et al. 1994). G-6-P has four main fates: glycogen synthesis; fatty acid synthesis; the pentose phosphate pathway; or hydrolysis to form glucose once more (Newsholme and Leech, 1983). Therefore, if a sufficient increase in blood glucose concentration occurs, there will be a concomitant rise in the concentration of G-6-P in liver cells, which will reduce glycogenolysis and increase glycogenesis. Thus, the synthesis of liver glycogen is dependent on the production of G-6-P, the formation of which is proportional to the blood glucose concentration.

Much debate has occurred over recent years as to whether hepatic glycogen synthesis occurs through an 'indirect' pathway, via gluconeogenic processes, or through a 'direct' pathway (glucose \rightarrow G-6-P \rightarrow G-1-P \rightarrow UDP glucose \rightarrow glycogen) (Magnusson and Shulman, 1991). It is known however, that following a glucose load, hepatic glucose production is suppressed and hepatic glycogen synthesis occurs. In postabsorptive humans, it has been demonstrated using ^{13}C nuclear magnetic resonance spectroscopy (NMR), a validated procedure (Gruetter et al. 1994), that ~50% of liver glycogen is synthesised through the 'direct' pathway following the administration of a glucose load and this increases to ~70% 4 h after the ingestion of a mixed meal (Magnusson and Shulman, 1991; Taylor et al. 1996a; Taylor et al. 1996b). The K_m of glucokinase is in the order of 8 mM and blood glucose concentration is ~6 mM following a glucose load (dependent upon the size of glucose load), it is likely therefore that the direct pathway contributes to a significant portion of liver glycogen synthesis. In addition, using ^{13}C NMR, it has been demonstrated that in the fasted state, both hepatic glycogen deposition

and glycogen breakdown may occur simultaneously (Magnusson et al. 1994) indicating the versatility of liver glycogen metabolism.

Following prolonged submaximal exercise, it has previously been observed that a greater proportion of an oral glucose load escapes hepatic retention, thereby allowing the repletion of muscle glycogen to take precedence over hepatic glycogen repletion (Mæhlum et al. 1978). However, more recent evidence using ¹³C magnetic resonance spectroscopy (MRS), has suggested that the role of liver glycogen homeostasis has previously been underestimated (Casey et al. 2000). When a relatively small amount of glucose was provided post-exercise (1 g/kg body mass), differences were observed between conditions in liver glycogen content, but not muscle glycogen content. These findings would appear to suggest that the supply of glucose to the liver has priority over skeletal muscle when glucose availability is limited.

2.8.2 SKELETAL MUSCLE GLYCOGEN METABOLISM

Following prolonged exercise, resulting in muscle glycogen depletion, the degree of muscle glycogen resynthesis is largely determined by the availability of glucose to the skeletal muscle cell. The transport of glucose into the skeletal muscle cell occurs via facilitated diffusion and the glucose transporter in skeletal muscle is expressed in four isoforms, glucose transporter 1 (GLUT-1), glucose transporter 3 (GLUT-3) (Stuart et al. 2000), glucose transporter 4 (GLUT-4) and glucose transporter 5 (GLUT-5). GLUT-1 is located predominantly on the plasma membrane and appears to be expressed to a lesser extent and involved in basal, not insulin or exercise stimulated, glucose transport (Douen et al. 1990). GLUT-4, on the other hand is located largely intracellularly in the unstimulated state, and its translocation to the plasma membrane from intracellular stores is stimulated by insulin and contractile activity (Douen et al. 1990; Cushman et al. 1998; Ploug et al. 1987; Ploug and Ralston, 1998). Furthermore, muscle glycogen concentration appears to modify GLUT-4 translocation in response to muscle contractions, with glycogen depletion causing an increase in GLUT-4 translocation at the cell surface (Derave et al. 1999).

When the concentration of glucose in the systemic circulation is high, and thus readily available to skeletal muscle, upon entry into the cell glucose is phosphorylated to G-6-P

by the enzyme hexokinase (the first step in the glycolysis or glycogen synthesis pathway). The G-6-P has two possible fates in the skeletal muscle cell, either entry into the glycolytic pathway, or use for the synthesis of glycogen, the rate-limiting reaction catalysed by glycogen synthase.

Glycogen synthase exists in two forms, an active I-form and an inactive D-form. The conversion of glycogen synthase from one form to another is mediated through phosphorylation and dephosphorylation reactions, alternating between the D-form and the I-form respectively. Phosphorylation, resulting in a decrease in glycogen synthase activity, is catalysed by specific kinases; and dephosphorylation, which results in an increase in glycogen synthase activity, is catalysed by specific phosphatases (Dent et al. 1990). Prolonged submaximal exercise which results in a marked degradation of muscle glycogen, leads to an increased availability of phosphorylated sites on the glycogen synthase enzyme which become available to glycogen synthase phosphatase and lead to an increase in glycogen synthase activity (Yan et al. 1992).

Glycogen synthesis is catalysed by a self-glucosylating protein primer, glycogenin (Shearer et al. 2000). Glycogenin autocatalytically generates an oligosaccharide primer of 7-11 glucosyl units, which serves as a substrate for glycogen synthase, as glycogen synthase can only extend an existing glycogen chain. Glycogen synthase and branching enzyme then act to catalyse the formation of two physiologically distinct pools of glycogen, pro- (PG) and macroglycogen (MG) (Adamo and Graham, 1998a; Adamo et al. 1998b). Each glycogen molecule, PG or MG, contains one molecule of glycogenin, therefore it has been suggested that glycogenin may be a potential regulator of glycogen synthesis in human skeletal muscle (Shearer et al. 2000). Following exhaustive submaximal exercise, MG stores are reported to show a greater relative depletion, PG being more sensitive to dietary carbohydrate post exercise demonstrating a more rapid resynthesis following depletion. However, the supercompensation associated with the consumption of high-carbohydrate diet following glycogen-depleting exercise is due to greater synthesis of the MG pool (Adamo et al. 1998b).

It is evident that the control of glycogen synthesis in skeletal muscle post exercise may not rest solely upon the activation of the enzyme glycogen synthase. Consideration must also be given to factors including glucose transport/phosphorylation which, using

¹³C NMR, have been shown to control insulin-stimulated glucose disposal (Price et al. 1994; Jucker et al. 1999; Price et al. 2000).

Effect of insulin

The presence of insulin stimulates glycogenesis in skeletal muscle through the dephosphorylation and activation of glycogen synthase, the rate-limiting enzyme (Yan et al. 1993) (Lawrence and Roach, 1997). An insulin-stimulated protein kinase (GSK3) has been shown *in vitro* to phosphorylate and activate PP1 (protein phosphatase-1), more specifically PP1G, the glycogen-bound form of PP1 (Dent et al. 1990). PP1G has been shown to be the principal enzyme involved in the regulation of glycogen metabolism compared to other forms of PP1 (Dent et al. 1990). However, the precise molecular mechanism by which insulin activates glycogen synthase is still to be elucidated.

The integral role of insulin in glycogen synthesis is demonstrated by the insulin-mediated activation of both glycogen synthase and GLUT-4 translocation. It is also conceivable that glycogen synthase activity and glucose transport may interact to control the rate of glycogen resynthesis after glycogen-depleting exercise (Ivy, 1991). Indeed, it has recently been suggested that the control of glycogen synthesis by insulin in skeletal muscle is distributed between glucose transport and glycogen synthase (Azpiazu et al. 2000).

Effect of exercise

The restoration of muscle glycogen after exercise has been demonstrated to occur in a bi-phasic pattern (Price et al. 1994). Using ¹³C NMR measurements, when muscle glycogen content was reduced to <30 mmol/kg wet weight at the end of exercise, a rapid phase of glycogen resynthesis was identified during the first 30-60 min of recovery. This initial rapid phase of glycogen resynthesis was insulin-independent and was followed by an insulin-dependent and G-6-P dependent slower second phase. It appears that the initial phase of glycogen repletion is more dependent upon the glycogen concentration within skeletal muscle. Indeed, it has been demonstrated that glycogen-depleting exercise itself can activate glycogen synthase independent of any activation by insulin (Wallberg-Henriksson et al. 1988; Price et al. 1994). Furthermore, it has been demonstrated that the rate of glycogen resynthesis is dependent upon the absolute

glycogen content at the cessation of exercise (Ivy, 1991; Zachwieja et al. 1991) (Price et al. 2000), with glycogen synthase activity being inversely proportional to the glycogen concentration (Yan et al. 1992). Thus, in the glycogen-depleted, post exercise state, both skeletal muscle (Yan et al. 1993) and most likely hepatic glycogen synthase activity are elevated (Niewoehner and Nuttall, 1995).

2.9 GLUCONEOGENESIS

Gluconeogenesis is defined as the metabolic process by which glucose is formed from non-carbohydrate precursors, which include lactate, pyruvate, tricarboxylic acid cycle intermediates, the carbon skeletons of most amino acids and glycerol (Newsholme and Leech, 1983). In the postabsorptive state, gluconeogenesis plays an integral role in the maintenance of blood glucose homeostasis, contributing about one-third of all glucose produced by the liver. Gluconeogenesis occurs primarily in the liver (90%), with the kidney contributing to a smaller extent (10%). Gluconeogenesis becomes important whenever dietary carbohydrate is insufficient to meet demands, for example, during fasting or starvation, low-carbohydrate diets, or prolonged exercise. During these situations lipolysis is accelerated, and glycerol, lactate and amino acids become quantitatively more responsible for contributing carbon to the glucose pool through gluconeogenesis.

Gluconeogenesis is regulated by the mobilisation and delivery of gluconeogenic precursors from peripheral sites, and also hormonally by insulin (inhibition) and glucagon (stimulation) (Consoli et al. 1990; Wasserman et al. 1992). In addition, the release of cortisol, during prolonged exercise or other conditions of low body carbohydrate stores, decreases protein synthesis and increases protein breakdown, thereby increasing the availability of amino acids for gluconeogenesis.

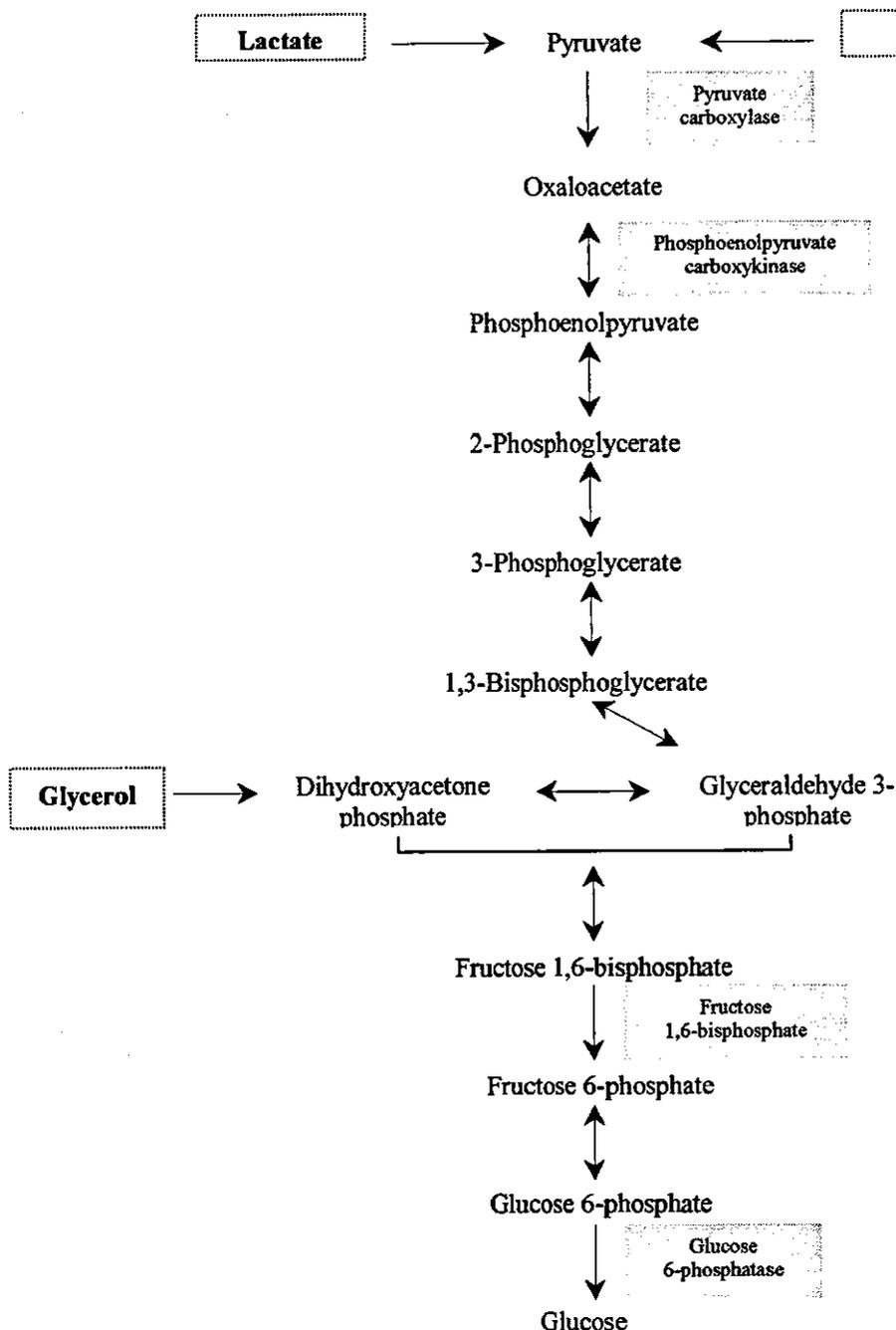


Fig. 2.6 Pathway of gluconeogenesis. The distinctive reactions of the pathway are indicated by the shaded enzymes. The other reactions are common to glycolysis. The entry points for lactate, amino acids and glycerol are indicated.

The production of glucose through gluconeogenesis is via the reversal of the glycolytic pathway (Fig. 2.6). Most of the glycolytic reactions are reversible, however, three of the reactions are irreversible; the pyruvate kinase reaction, the phosphofructokinase (PFK) reaction and the glucokinase reaction. However, to overcome these irreversible reactions, additional by-passing reactions exist. A two-stage conversion of pyruvate to phosphoenolpyruvate bypasses the pyruvate kinase reaction. This involves the reactions

with enzymes pyruvate carboxylase, yielding oxaloacetate, a tricarboxylic acid cycle intermediate, and phosphoenolpyruvate carboxylase, yielding phosphoenolpyruvate. Fructose 1,6-bisphosphatase converts fructose 1,6-bisphosphate to produce fructose 6-P, by-passing the irreversible PFK reaction. Lastly, the enzyme glucose 6-phosphatase, not present in muscle cells (see section 2.8.1), converts the glucose 6-phosphate to glucose, overcoming the irreversible hexokinase/glucokinase reaction. Within liver cells, the first enzyme of gluconeogenesis, pyruvate carboxylase, is located in the mitochondrial matrix. The last enzyme, glucose-6-phosphatase is bound to the smooth endoplasmic reticulum. The other enzymes of the pathway are located in the cytosol.

2.9.1 AMINO ACIDS AS GLUCONEOGENIC PRECURSORS

Previously, the view was held that the most important amino acid gluconeogenic precursor was alanine, which led Felig et al. (1970) to introduce the concept of 'glucose-alanine' cycle. Indeed, alanine has been suggested to be the major gluconeogenic substrate during recovery from exhaustive exercise in fasting conditions (Favier et al. 1987). More recently however, the incorporation of labelled carbon into plasma glucose during the infusion ^{14}C glutamine in postabsorptive subjects has demonstrated that glutamine may also be an important gluconeogenic precursor (Nurjhan et al. 1995). Although the overall carbon transfer to glucose from glutamine and alanine is comparable (Nurjhan et al. 1995; Stumvoll et al. 1996), glutamine is quantitatively more important than alanine in transporting protein-derived carbons through plasma and supplying the glucose pool (Nurjhan et al. 1995). Indeed, the existence of a 'glucose-glutamine' cycle analogous to the 'glucose-alanine' or Cori cycles has been proposed (Perriello et al. 1995). However, in contrast to the 'glucose-alanine' and Cori cycles, the recently proposed 'glucose-glutamine' cycle is able to provide a positive transfer of protein-derived new carbon to the glucose pool.

In postabsorptive humans, 45% of plasma glutamine originates from its direct release from tissue protein (Matthews and Campbell, 1992; Nurjhan et al. 1995), 13% originates from plasma glucose (Perriello et al. 1995), with a further 5% coming from plasma glutamate (Darmaun et al. 1994; Matthews and Campbell, 1992). The remaining glutamine entering the circulation is most probably derived from conversion of other amino acids to glutamine in skeletal muscle (Garber et al. 1976c; Chang and Goldberg, 1978a; Chang and Goldberg, 1978b; Goldberg and Chang, 1978).

The infusion of glutamine in postabsorptive subjects, which increased the plasma concentration threefold, led to a seven fold increase in the conversion of glutamine into plasma glucose (Perriello et al. 1997). The disparity between the increase in the plasma concentration of glutamine and the incorporation of glutamine into glucose suggests that glutamine may exert a stimulatory and regulatory effect upon gluconeogenesis. This stimulatory effect is demonstrated by the fact that physiological increases in plasma glutamine have the ability to accelerate both glutamine and alanine conversion to glucose (Perriello et al. 1997). In addition, it has previously been shown that an excess

of one gluconeogenic substrate may inhibit gluconeogenesis from other gluconeogenic precursors, however the role of glutamine as a gluconeogenic precursor was not considered (Jahoor et al. 1990).

Glutamine has been shown to be of greater importance as a gluconeogenic precursor in the kidney, with gluconeogenesis from alanine essentially limited to the liver (Stumvoll et al. 1998). The difference between the two gluconeogenic organs in substrate selection appears to be largely due to differences in enzyme regulation and substrate supply.

2.10 THE TRICARBOXYLIC ACID CYCLE (TCA CYCLE)

In 1937 Sir Hans Krebs proposed the notion that organic fuels are oxidised through a cyclic pathway. This cyclic pathway, the Krebs cycle, was named after its discoverer, but subsequently has adopted the titles of the Citric acid cycle, and the Tricarboxylic acid (TCA) cycle.

The primary function of the TCA cycle is to completely oxidise the acetyl group of acetyl-CoA producing CO₂. This occurs with the concomitant reduction of the electron carriers, NADH and FADH₂, whose subsequent oxidation in the electron transport chain is accompanied by the formation of ATP by oxidative phosphorylation. This accounts for approximately two thirds of the ATP formation and oxygen consumption in mammals, with the remaining third attributed to the β-oxidation of fats. The pivotal step of the TCA cycle involves the condensation of acetyl-CoA (2C) with oxaloacetate (4C), to yield citrate (6C) (Figure 2.7). Citrate is then converted to aconitate (6C), and then isocitrate (6C), losing one carbon skeleton as CO₂, forming α-ketoglutarate² (5C). A second carbon skeleton is lost as CO₂ in the formation of succinyl-CoA (4C). The four carbon skeleton forms succinate, fumarate, malate and then oxaloacetate in one complete turn of the cycle, where oxaloacetate can again condense with acetyl-CoA to begin another turn of the cycle.

² α-ketoglutarate is more commonly referred to as 2-oxoglutarate. However, in this thesis, for continuity α-ketoglutarate will be used.

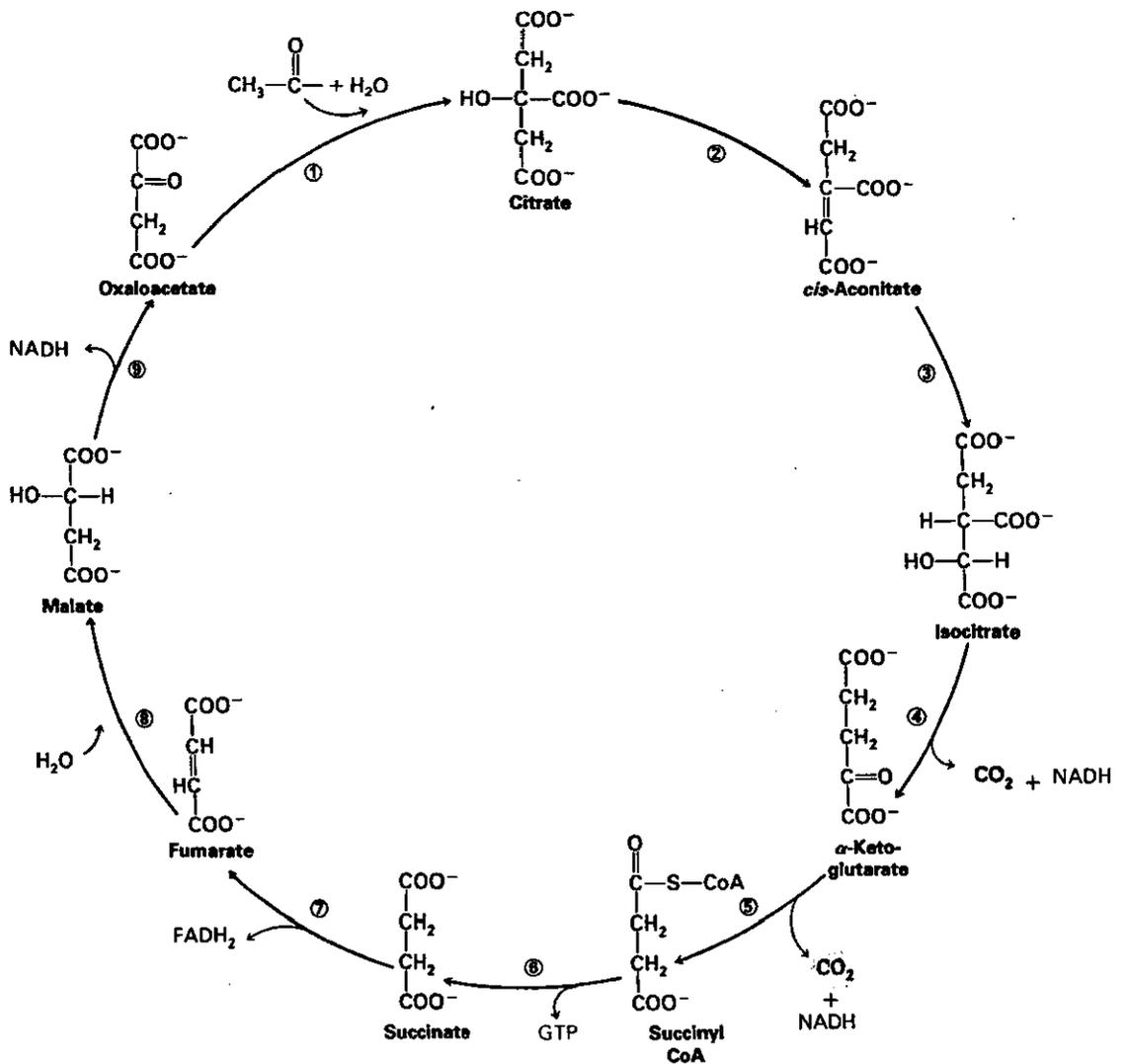


Fig. 2.7 The tricarboxylic acid (TCA) cycle. This series of reactions is catalysed by the following enzymes, as numbered in the diagram (parentheses indicates type of reaction): ① Citrate synthase (condensation); ② Aconitase (dehydration); ③ Aconitase (hydration); ④ Isocitrate dehydrogenase (oxidative carboxylation); ⑤ α -ketoglutarate dehydrogenase complex (oxidative decarboxylation); ⑥ Succinyl CoA synthetase (substrate-level phosphorylation); ⑦ Succinate dehydrogenase (oxidation); ⑧ Fumarase (hydration); ⑨ Malate dehydrogenase (oxidation). N.B Acetyl-CoA is shown entering the TCA cycle at reaction ①

The TCA cycle is located within the mitochondrion, however, at least six of the nine TCA cycle intermediates (TCAI) are found in both the cytosol and mitochondrion (Newsholme and Leech, 1983). Present techniques for the rapid freezing of the muscle sample (i.e. immediately plunging in liquid nitrogen) causes the cell structure to be destroyed and thus do not allow resolution of the subcellular compartmentalisation of the TCAI. Indeed, at present, it is impossible to attribute the measured concentration of

a given TCAI solely to the TCA cycle. The determination of the subcellular compartmentalisation of the TCAI is important to further our understanding of TCA cycle function. In addition, the concentration of the intermediates *cis*-Aconitate and succinyl-CoA is too low to measure using currently available techniques, and thus no values for these intermediates have been quoted in the literature.

It is noteworthy that the TCA cycle can only oxidise 2C acetyl units. The entry into the TCA cycle of carbon skeletons, other than acetyl-CoA, at various points in the cycle will merely increase the concentration of the intermediates, unless they are subsequently removed from the cycle.

The oxidation of acetyl groups in the TCA cycle and the oxidation of reduced coenzymes in the electron transfer chain are tightly coupled to the phosphorylation of ADP to form ATP. Therefore, regulation of the TCA cycle and the electron transfer chain is closely linked to the rate of ATP utilisation. In addition the rate of the TCA cycle is also controlled by three enzymes catalysing irreversible steps, citrate synthase, isocitrate dehydrogenase and α -ketoglutarate dehydrogenase (Figure 2.6), as well as the rate at which the reduced coenzyme products of the cycle, NADH and FADH₂, are oxidised in the respiratory chain.

The most important factor controlling TCA cycle activity is the mitochondrial ratio of NADH to NAD⁺ (mitochondrial redox state; [NADH]/[NAD⁺]). This ratio is linked to the mitochondrial ATP/ADP ratio and ultimately to the cytosolic phosphorylation potential. Indeed, the concentration of NADH determines the activity of the three non-equilibrium reactions that regulate the activity of the TCA cycle (citrate synthase, isocitrate dehydrogenase and α -ketoglutarate dehydrogenase). An increase in the NADH concentration, or an increase in the [NADH]/[NAD⁺] ratio³, inhibits these enzymes through negative allosteric inhibition. Furthermore, citrate synthase activity is regulated by citrate concentration, and both isocitrate dehydrogenase and α -ketoglutarate dehydrogenase are activated by the intramitochondrial Ca²⁺.

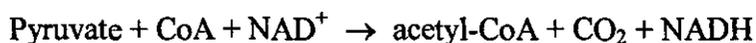
³ In the case of the two dehydrogenase reactions that regulate TCA cycle activity (isocitrate dehydrogenase and α -ketoglutarate dehydrogenase), an increase in the [NADH]/[NAD⁺] ratio would mean a decrease in the concentration of NAD⁺, because the total concentration of NADH would be constant.

In the rested state, the mitochondrial redox state is highly reduced, such that the NADH concentration is high, in addition the rate of ADP entry into the mitochondrion will be low. In exercising muscle, the rate of ADP entry into the mitochondrial matrix increases and stimulates the rate of electron transfer from NADH to oxygen. This increases the rate of oxygen utilisation and the formation of NAD^+ , thereby increasing the mitochondrial redox state, and concomitantly, the activity of the TCA cycle as NAD^+ is a substrate for three of the cycle reactions (isocitrate \rightarrow α -ketoglutarate; α -ketoglutarate \rightarrow succinyl CoA; malate \rightarrow oxaloacetate).

The TCA cycle has been considered as two physiological pathways: the span from acetyl-CoA to α -ketoglutarate and the span from α -ketoglutarate to oxaloacetate (Newsholme and Leech, 1983). In skeletal muscle, during sustained exercise, this division is only academic, since flux through the two pathways must be identical and regulated in a concerted manner. Nonetheless, in the context of this thesis this division may explain how it is possible for carbon skeletons to feed into the cycle at the level of α -ketoglutarate.

2.11 PYRUVATE DEHYDROGENASE COMPLEX (PDC)

The link between pyruvate formed through glycolysis (cytosol) and the TCA cycle (mitochondrion) is via the pyruvate dehydrogenase enzyme complex (PDC). PDC is a multiple enzyme complex composed of three tightly bound enzyme components (pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase) and is situated in the inner mitochondrial membrane and catalyses the irreversible reaction whereby pyruvate undergoes oxidative decarboxylation to acetyl-CoA (requiring NAD^+ and CoA). Thus PDC controls the flux of pyruvate derived acetyl-CoA into the TCA cycle. The net reaction catalysed is:



Once acetyl-CoA is formed, the acetyl units have two principal fates in skeletal muscle; oxidation to CO_2 in the TCA cycle, or incorporation into acetylcarnitine. A high rate of acetyl group formation by the PDC results in the transfer of the acetyl group from CoA to carnitine via the carnitine acetyltransferase reaction forming acetylcarnitine, thus

maintaining the availability of CoA for TCA cycle function. Therefore, carnitine acts as a buffer for the excess acetyl-CoA produced by the PDC when the rate of acetyl-CoA formation is greater than its rate of entry into the TCA cycle, demonstrated by the large increase observed in intramuscular concentration of acetylcarnitine at the onset of exercise (Putman et al. 1993; Constantin-Teodosiu et al. 1993; Constantin-Teodosiu et al. 1992).

The phosphorylation state and hence the activity of PDC is largely regulated by the concerted action of PDC kinase and PDC phosphatase. Dephosphorylation of PDC by PDC phosphatase produces the active form (PDC_a) and this transformation is dependent on the simultaneous reduction in activity of PDC kinase (Figure 2.8). The *in vivo* regulation of skeletal muscle PDC_a is believed to be important to the integration of carbohydrate and fat oxidation within this tissue (Randle et al. 1963). During starvation or carbohydrate deprivation, PDC_a is lowered when the ratio of acetyl-CoA to CoASH increases due to an increased availability and oxidation of fat fuels. This reduction in PDC_a conserves intramuscular and whole-body carbohydrate stores in the face of reduced carbohydrate availability (Putman et al. 1993).

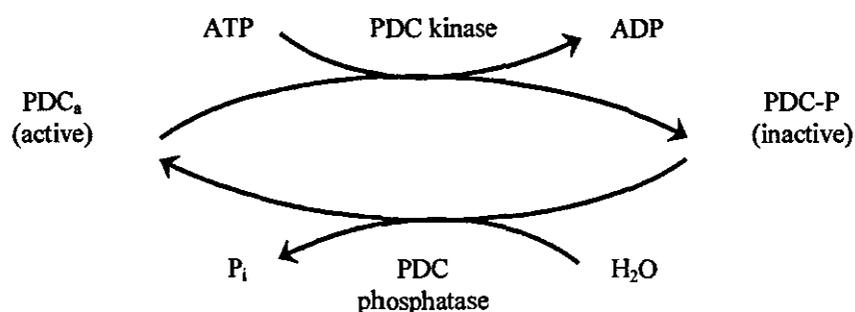


Fig. 2.8 The pyruvate dehydrogenase (PDC) complex. This reaction is regulated by phosphorylation and dephosphorylation. In the dephosphorylated state, PDC is active. However, when a phosphate group is transferred from ATP to PDC, the PDC becomes inactive. Phosphorylation is catalysed by PDC kinase, whereas the phosphate group is removed by PDC phosphatase.

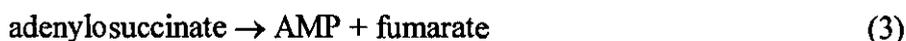
At the onset of exercise, a near-complete transformation (~5 fold increase) of PDC to PDC_a is observed as acetyl units are delivered to the TCA cycle (Putman et al. 1993; Constantin-Teodosiu et al. 1992). This is primarily due to an activation of PDC phosphatase by a high intramitochondrial concentration of Ca²⁺ during exercise, whilst

PDC kinase activity is inhibited by a high mitochondrial concentration of ADP. Conversely, high intramitochondrial [acetyl-CoA]/[CoA], [ATP]/[ADP] and [NADH]/[NAD⁺] ratios result in PDC kinase activation. The increased ratios reflect an abundance of substrate for both the TCA cycle and electron transport chain, respectively, thus further decarboxylation of pyruvate by the PDC would be superfluous.

2.12 ANAPLEROTIC PROCESSES IN SKELETAL MUSCLE

The term “anaplerosis” is derived from the Greek word meaning “to fill up”. In the physiological setting and in the context of this thesis, anaplerosis refers to metabolic pathways that allow entry of carbon into the TCA cycle, by routes other than the entry of carbon as acetyl-CoA via the citrate synthase reaction (Kornberg, 1966). The entry of carbon into the TCA cycle increases the total concentration of the TCA cycle intermediates (TCAI), and thus expands or “fills up” the TCAI.

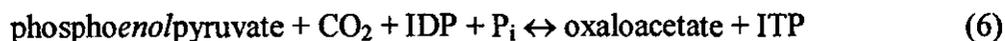
A number of processes could feasibly contribute to anaplerosis during exercise, but their physiological significance has been the subject of much debate (Aragón and Lowenstein, 1980; Spydevold et al. 1976; Sahlin et al. 1990). The proposed processes include the purine nucleotide cycle (PNC), three reactions catalysed by AMP deaminase (Eqn 1), adenylosuccinate synthase (Eqn 2) and adenylosuccinate lyase (Eqn 3), respectively (see section 2.4.2):



One complete turn of the PNC results in the deamination of aspartate, the consumption of GTP and the production of NH₃ and fumarate. The fumarate produced may feasibly enter into the TCA cycle.

Other potential anaplerotic reactions are catalysed by glutamate dehydrogenase (GDH; Eqn 4), pyruvate carboxylase (PC; Eqn 5), phosphoenolpyruvate carboxykinase

(PEPCK, Eqn 6), alanine aminotransferase (AAT; Eqn 7) and malic enzyme (ME; Eqn 8):



The central role of pyruvate in many of the anaplerotic reactions is highlighted in Figure 2.9. Indeed, it has been reported that the AAT reaction is the principal mechanism for TCAI expansion during the initial period of exercise (Sahlin et al. 1990; Gibala et al. 1997a; Spencer et al. 1991b) Furthermore, the AAT reaction is dependent upon the availability of both pyruvate and glutamate to proceed. However, if the AAT were the principal mechanism for TCAI expansion, one might expect to observe an increase in α -ketoglutarate, one of the products of the AAT reaction. No such increase has been reported during the initial period of exercise when it is purported the AAT reaction is most active. On the other hand, an increase is observed in intramuscular alanine concentration, the second product of the AAT reaction, together with a decrease in glutamate concentration (Gibala et al. 1997a; Sahlin et al. 1990).

Taken together, these findings suggest that the AAT reaction may indeed contribute most significantly to anaplerosis during the initial period of exercise. However, the anaplerotic carbon derived from the AAT reaction does not appear to accumulate as α -ketoglutarate. There is a disproportionate increase in the TCAI succinate, malate and fumarate during the initial period of exercise (Gibala et al. 1998). As α -ketoglutarate is in equilibrium with glutamate via the glutamate dehydrogenase reaction, there is most probably a rapid conversion of α -ketoglutarate to these intermediates due to the tight regulation of the α -ketoglutarate dehydrogenase complex. Indeed, an increase in α -ketoglutarate content causes a rapid activation of the α -ketoglutarate dehydrogenase complex (Bunik et al. 1991) suggesting a rapid conversion of α -ketoglutarate to other TCAI. Furthermore, any delay in the freezing of the muscle biopsy sample would favour the accumulation of the TCAI in the second span of the TCA cycle.

Interestingly, the changes observed in muscle TCAI pool size observed following pharmacological activation of the PDC with dichloroacetate (DCA) or during exercise, have been largely mediated by changes in the intermediates located in the second span of the TCA cycle, namely succinate, malate and fumarate and oxaloacetate (Constantin-Teodosiu et al. 1999; Gibala and Saltin, 1999a; Gibala et al. 1998). Moreover, malate appears to undergo the largest increase of any of the measured TCAI during exercise (Sahlin et al. 1990; Sahlin et al. 1995; Gibala et al. 1997a; Gibala et al. 1998; Gibala et al. 1997b; Gibala and Saltin, 1999a; Aragón and Lowenstein, 1980).

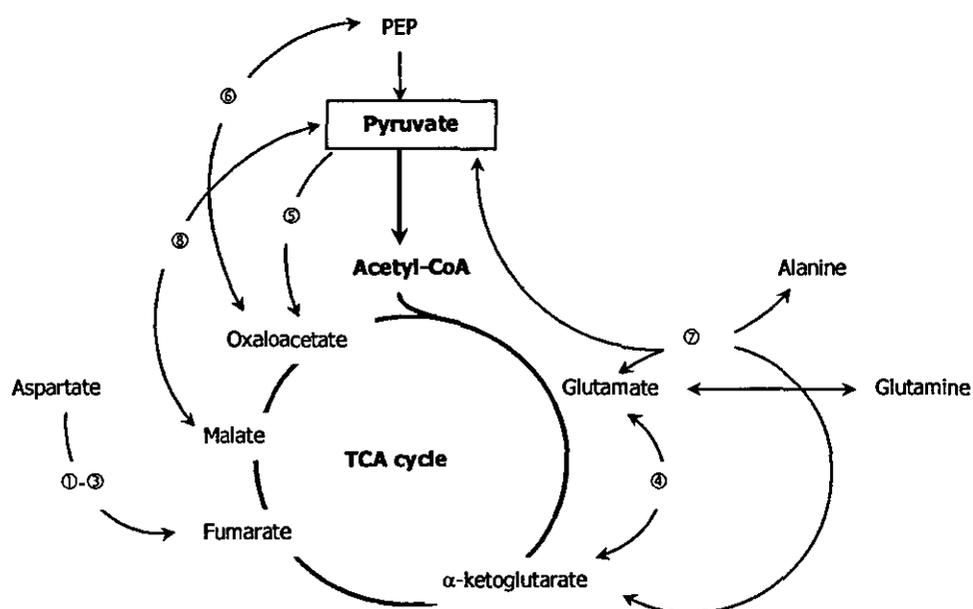


Fig. 2.9 Anaplerotic reactions in human skeletal muscle. Reactions shown catalysed by: ①-③ net reaction of the PNC; ④ glutamate dehydrogenase (GDH); ⑤ pyruvate carboxylase (PC); ⑥ phosphoenolpyruvate carboxykinase (PEPCK); ⑦ alanine aminotransferase (AAT); ⑧ and malic enzyme (ME); PEP (phosphoenolpyruvate) reversible reaction to pyruvate catalysed by pyruvate kinase (PK).

2.13 GLUTAMINE AND GLYCOGEN RESYNTHESIS IN THE LIVER AND SKELETAL MUSCLE

Alterations in the concentration of amino acids in portal blood appear to activate cell volume regulatory mechanisms in the liver. The uptake of amino acids in the absorptive

state may act as a signal governing the metabolic state of the liver (Häussinger et al. 1990). In addition, it has been suggested that a similar mechanism involving cell volume changes may exist in skeletal muscle *in vitro* (Parry-Billings et al. 1991). Furthermore, a cell volume change, more specifically cell swelling, has been shown to be a prerequisite for the amino acid-dependent activation of the hepatic System N transporter (Bode and Kilberg, 1991).

Cell swelling may be regarded as an initial primary signal by which glycogen synthesis is activated both in the liver and in skeletal muscle. Glycogen synthesis may be modulated by cellular hydration state, both in the liver and skeletal muscle (Lang et al. 1998). Cell swelling would appear to favour the synthesis of glycogen (anabolic signal), whereas cell shrinkage appears to act in the opposite manner (catabolic signal). The precise mechanism by which cell volume changes may stimulate glycogen synthesis in both liver and skeletal muscle cells remains unclear. However, it has been suggested that the cytoskeleton architecture, in particular transmembrane integrins may be responsible for transducing the mechanical stimuli (i.e. cell swelling) into chemical signals which evoke metabolic responses (i.e. glycogen synthesis) (Ingber, 1997; Low and Taylor, 1998).

In hepatocytes, it is likely that cell swelling activates glycogen synthase phosphatase and acetyl-CoA carboxylase (Baquet et al. 1990; Baquet et al. 1991b). More specifically, swelling appears to activate PI3-kinase (phosphatidylinositol 3-kinase) which mediates the swelling induced activation of both glycogen synthase and acetyl-CoA carboxylase (Low and Taylor, 1995; Krause et al. 1996a; Krause et al. 1996b). Furthermore, due to the inherent link between cell swelling and cell pH via Na^+/H^+ exchange, which is activated by insulin, glycogen synthesis in hepatocytes may be stimulated by acidification, with a concomitant inhibition of glycolysis (Peak et al. 1992). A further potential mechanism by which glycogen synthesis is mediated is by the influx of amino acids causing a decrease in intracellular Cl^- concentration (Meijer et al. 1992a). This would allow glycogen synthesis to proceed, as a high concentration of Cl^- ions inhibit glycogen synthase phosphatase, thus impairing glycogen synthesis.

Indeed, it has been demonstrated that cell swelling *per se* is able to stimulate glycogen synthesis both in isolated rat hepatocytes (Baquet et al. 1990; Mouterde et al. 1992)

and in rat skeletal muscle cells (Scislowski et al. 1989; Low et al. 1996). The provision of glutamine to the perfused rat liver is able to induce liver cell swelling (Häussinger et al. 1990). Upon entry into the *in vitro* liver cell, glutamine is able to stimulate hepatic glycogen synthesis and lipogenesis from glucose and inhibit ketogenesis (Katz et al. 1976; Baquet et al. 1990; Baquet et al. 1991a; Meijer et al. 1992; Peak et al. 1992; Lavoinne et al. 1987; Lavoinne et al. 1996). Furthermore, the ability of glutamine to modulate liver glycogen synthesis has also been demonstrated *in vivo*. Niewoehner & Nuttall (1996) demonstrated using a rat model, that the oral provision of glutamine is able to stimulate liver glycogen synthesis through an activation of glycogen synthase, probably mediated via the cell-swelling mechanisms discussed previously.

In humans, the intravenous infusion of glutamine has been shown to promote skeletal muscle glycogen resynthesis during recovery from exhaustive exercise compared to the infusion of an isonitrogenous and isoenergetic mixture of alanine and glycine (Varnier et al. 1995). The proposed mechanism for the increase in muscle glycogen is that glutamine may act as a glycogenic precursor or through the activation of skeletal muscle glycogen synthase via the mechanisms discussed previously.

However, more recent work suggests that the supplementation of glutamine in combination with glucose post exercise is unable to increase muscle glycogen resynthesis to a greater extent than glucose alone (van Hall et al. 2000b). Furthermore, glycogen synthase activity was similar in both conditions (Wagenmakers et al. 1997; van Hall et al. 2000b). These results are complimentary when compared to those of Bowtell *et al.* (1999) who found that a combination of glutamine and glucose was unable to further stimulate post exercise glycogen resynthesis compared to glucose alone (Bowtell et al. 1999). Therefore, there appears to be no additive effect on muscle glycogen resynthesis when both glutamine and glucose are provided post exercise. It is conceivable that the supplementation of a sufficient glucose load (~1.5 g/kg body wt; Ivy et al. 1988b) might cause maximal stimulation of glycogen synthase through an elevation in insulin concentration, with no further stimulation possible through the addition of glutamine.

2.14 GLUCOSE TOXICITY AND GLUCOSAMINE— A ROLE FOR GLUTAMINE?

Previous studies have demonstrated a role for the hexosamine biosynthetic pathway in glucose regulation of insulin-stimulated glucose transport in adipocytes (Marshall et al. 1991b). Over-activity of the hexosamine biosynthetic pathway has recently been suggested to be one of the possible mechanisms mediating glucose-induced insulin resistance or glucose toxicity (McClain and Crook, 1996; Virkamäki et al. 1997). Skeletal muscle accounts for the major portion (~75%) of insulin-mediated glucose disposal, thus it is important to outline mechanisms that may affect insulin action in this tissue.

Glucose flux through the hexosamine biosynthetic pathway is controlled by its first and rate-limiting enzyme, glutamine:fructose-6-phosphate amidotransferase (GFAT) (Marshall et al. 1991b; Marshall et al. 1991c). GFAT activity has been shown to be present and measurable in human skeletal muscle, but its activity has been found to be 5-18 fold greater in the liver (Yki-Järvinen et al. 1996; Nerlich et al. 1998).

GFAT transfers an amino group from glutamine to fructose-6-phosphate (F-6-P) to form glucosamine-6-phosphate (GlcN-6-P) and glutamate (McKnight et al. 1992). The final products of the hexosamine biosynthetic pathway are uridine diphosphate-N-acetyl-glucosamine (UDP-GlcNAc) and other nucleotide hexosamines (McClain and Crook, 1996). The routing of glucose through the hexosamine biosynthetic pathway and the formation of hexosamine products appear to regulate GFAT activity (Traxinger and Marshall, 1991). In addition, the products of the hexosamine biosynthetic pathway appear to act as negative feedback regulators limiting excessive muscle glycogen deposition post exercise by gradually decelerating skeletal muscle glucose uptake (McClain and Crook, 1996; Nelson et al. 1997). Indeed, GFAT activity is subject to feedback inhibition by the end product of the hexosamine biosynthetic pathway, UDP-GlcNAc in human skeletal muscle (Yki-Järvinen et al. 1996).

Increasing the available fructose-6-phosphate (F-6-P), whilst maintaining the available glutamine, has been shown to increase GFAT activity (Nelson et al. 1997). Concomitantly, GFAT activity in rats after an overnight fast, as measured by an

accumulation of GlcN-6-P, is decreased by ~30% compared to *ad libitum* fed rats (Buse et al. 1996; Nelson et al. 1997). The apparent K_m of GFAT for glutamine in several mammalian tissues has been reported to be between 0.4 and 1.6 mmol.l⁻¹ (Kornfeld, 1967; Traxinger and Marshall, 1991; Robinson et al. 1995). The apparent K_m of GFAT for F-6-P in muscle being ~2.4 mmol.l⁻¹ (Robinson et al. 1995), glucose flux through the hexosamine biosynthetic pathway *in vivo* is therefore highly dependent upon the concentration of F-6-P.

It is important to recognise that the hexosamine biosynthetic pathway metabolises only 2-3% of glucose in adipocytes under normal circumstances (Marshall et al. 1991b). The proportion of glucose metabolised via the hexosamine biosynthetic pathway appears to be largely determined by GFAT, as this is the rate-limiting enzyme (Marshall et al. 1991a) and accumulation of the hexosamine products seems to parallel GFAT activity (Hebert and McClain, 1995). Therefore, it is conceivable that an increase in GFAT activity, mediated through a concomitant increase in glucose, glutamine and insulin, may increase the flux of glucose through the hexosamine biosynthetic pathway. Indeed, absolute flux via the hexosamine biosynthetic pathway was increased in muscle *in vivo* during hyperglycaemia and hyperinsulinemia (Robinson et al. 1995), as it has been observed that both glucose and insulin serve to regulate GFAT activity (Daniels et al. 1996).

The activity of GFAT has been reported to be greater in slow twitch muscle fibres (Yki-Järvinen et al. 1997), suggesting that GFAT activity may be greater in endurance trained athletes, as they generally have a higher percentage of slow twitch muscle fibres. In spite of this finding, GFAT activity has been demonstrated to be unaffected by a prolonged bout of exercise, although it may be subject to nutritional regulation post exercise (Nelson et al. 1997). Therefore it is feasible that the post exercise supplementation of glucose and glutamine, substrates of the GFAT reaction, may be expected to increase the activity of GFAT thus increasing the product of the reaction, namely glucosamine.

Pre-exposure to glucosamine is able to induce insulin resistance in skeletal muscle through a reduction in the ability of insulin to increase glucose transport (Robinson et al. 1993). Upon entry into the cell via facilitated diffusion, using the same transport

system as glucose, glucosamine is phosphorylated to glucosamine-6-phosphate (GlcN-6-P) and enters the hexosamine biosynthetic pathway distal to GFAT (Marshall et al. 1991b; Robinson et al. 1995).

Glucosamine-induced insulin resistance of glucose transport appears to be restricted to those cells expressing the glucose transporter GLUT-4 (i.e. skeletal muscle and adipocytes). Glucosamine appears to act by impairing the insulin-induced translocation of GLUT-4 from intracellular stores to the plasma membrane. *In vitro*, the pre-exposure or infusion of glucosamine has been shown to cause insulin resistance by impairing insulin-induced GLUT-4 translocation in rat skeletal muscle (Robinson et al. 1993; Baron et al. 1995). However, glucosamine does not alter basal glucose transport or insulin stimulation of GLUT-1 translocation and mitogen-activated protein kinase in adipocytes (Heart et al. 2000).

As GLUT-4 is integral in insulin-stimulated glucose transport, any defect in the translocation of this glucose transporter will ultimately reduce skeletal muscle glycogen synthesis due to a decrease in available G-6-P. The production of glucosamine, via GFAT and the hexosamine biosynthetic pathway, has been shown to impair GLUT-4 translocation from its insulin-sensitive intracellular pool. This decreases glucose uptake in to the muscle cell and thus limits skeletal muscle glycogen synthesis. A ~60% reduction in muscle G-6-P has been observed in glucosamine infused rats, suggesting impaired glucose transport (Giaccaari et al. 1995). Indeed, the activity of GFAT, indicative of flux through the hexosamine biosynthetic pathway, has been shown to be inversely related to whole-body glucose disposal rates (Daniels et al. 1996). Furthermore the oral provision of glutamine, in addition to ¹⁴C labelled glucose, has been shown to induce a ~40% decrease in skeletal muscle glucose uptake, possibly due to increased flux through the hexosamine biosynthetic pathway and increased glucosamine production (Sawada et al. 1998). It has been reported that the effects of glucosamine are evident ~30 min after infusion in rats (Baron et al. 1995), with the intrinsic activity of GLUT-4 being reduced within 2 h of glucosamine infusion (Hawkins et al. 1999).

As the hexosamine biosynthetic pathway is able to regulate insulin-stimulated glucose transport, one may expect that hexosamine metabolism is also able to regulate glycogen

synthesis (Giaccari et al. 1995). Indeed, both the over-expression of GFAT or pre-exposure to glucosamine, have been shown to reduce the ability of insulin to stimulate glycogen synthase, mediated via a down-regulation in basal PP1 activity, in addition to reducing the ability of insulin to stimulate PP1 (Crook and McClain, 1996; Robinson et al. 1993).

Hepatic glucose transport is mediated through the insulin-independent GLUT2 (Mueckler, 1994; Thorens, 1996), and is therefore not subject to modulation by glucosamine production. It has been postulated that the production of glucosamine, which may impede glucose uptake in skeletal muscle through the provision of glucose and glutamine, may force a compensatory increase in hepatic glucose uptake, leading to an increase in liver glycogen synthesis (Bowtell et al. 1999). Indeed, the provision of glutamine in addition to glucose-polymer post exercise has been shown to increase whole body non-oxidative glucose disposal, in other words whole body carbohydrate storage, compared to glucose-polymer alone (Bowtell et al. 1999), with the most likely site of increased carbohydrate storage outside of skeletal muscle being the liver. When compared with recent findings demonstrating that skeletal muscle glycogen synthase failed to be activated to a greater extent when combination of glucose and glutamine was provided post exercise compared to glucose alone (Wagenmakers et al. 1997), it would certainly appear feasible that an increase in liver glycogen may occur in this situation.

CHAPTER 3

GENERAL METHODS

3.1 INTRODUCTION

The research proposals and all procedures described in this thesis were approved by the Ethical Advisory Committee of Loughborough University and were carried out in accordance with the 'Code of Practice for Workers having Contact with Body Fluids'. Prior to giving written consent to participate in the study, subjects were fully informed of the demands that would be placed upon them and also the possible risks and discomforts. Subjects were given the opportunity to ask for further information and clarification of the tests to be undertaken, in addition subjects were advised that they may cease participation in the study at any point, without need for explanation. Subjects were also required to complete a medical questionnaire prior to participation in the study and provide details of their training background. Subjects with a medical condition potentiating an undue personal risk were excluded from the study. In addition, as prolonged cycling is extremely demanding, subjects who were familiar with endurance exercise were accepted as volunteers. All testing was carried out in the Department of Physical Education, Sports Science and Recreation Management laboratory at Loughborough University, Loughborough, UK.

3.2 DATA COLLECTION

In all studies, a friction-braked cycle ergometer was used (Monark, Model 824E). Resistance was applied by the addition of pre-determined weights to a hanging cradle. In order that subjects could assume a position on the ergometer similar to their normal cycling position, a seat post that provided both vertical and horizontal adjustment (Cranlea & Co, Birmingham, UK) and handlebar stem that allowed multiple adjustment (Look Ergostem, Look, France) were fitted. In addition, a racing saddle and racing handlebars were fitted to the ergometer. Power output was calculated via an interface with a BBC microcomputer through high frequency logging of the flywheel angular velocity. Through prior calibration of the cycle ergometer, changes in kinetic energy of the flywheel were incorporated into the power output calculations, which were recorded

every 30 seconds. In addition, flywheel revolutions were recorded through an analogue counter (calibrated prior to the commencement of each ride) to enable power output to be calculated. The use of a BBC microcomputer enabled a visual display of both power output and pedal frequency (revolutions per minute). Furthermore, subjects were provided with continual visual feedback of their current pedal frequency via a digital display on the ergometer. However, during the cycle to exhaustion rides, subjects were unable to see the time elapsed and were never given any indication of this.

In all studies, heart rates were recorded using a short range radio telemeter (Vantage NV, Polar Electro, Finland). Subjects weight was measured using a balance scale (Model 3306ABV, Avery Ltd., Birmingham, UK) and their height measured using a static measurement scale.

Expired gas was collected using the Douglas bag technique. During expired air collection, subjects wore a nose clip and breathed through a low resistance one-way respiratory valve connected to a 200 litre capacity Douglas bag by lightweight smooth bore tubing (Falconia Ltd., Manchester, UK). This enabled a closed circuit to be formed for the collection of expired air over a given time period.

The expired gas collections during recovery were for 6 min and those taken during exercise were 1 min collections. During recovery, expired air was always collected with the subject assuming a seated position and with the subject having rested for ~10 minutes to ensure consistency. In addition, the subject was given the mouthpiece and nose-clip ~45 seconds prior to expired gas collection to allow the subject to become accustomed to the breathing apparatus. This avoided the possibility of hyperventilation that may alter the CO₂ content of expired gas, which in turn could give rise to a spurious $\dot{V}CO_2$ value.

The percentage of oxygen (O₂) and carbon dioxide (CO₂) in the dry expired air (silica gel was used as a drying agent) was measured using a paramagnetic O₂ and infra-red CO₂ analyser (Servomex Model 1400, Crowborough, UK). The O₂ analyser operates on the basis of the susceptibility of O₂ to a paramagnetic gas and provides a digital readout to one decimal point. The CO₂ analyser provides a digital readout to two

decimal points. Both analysers were calibrated against nitrogen, a certified reference gas (Cryoserve Ltd., Worcester, UK), and atmospheric air prior to each series of expired air analyses. The calibration of the analysers was checked at regular time intervals and were re-calibrated if necessary.

After the O₂ and the CO₂ concentrations of the expired gas had been measured, the volume of expired gas was measured using a Harvard digital dry gas meter (Harvard Apparatus, Edenbridge, UK). The temperature of the expired gas was measured using a thermister (Edale Instruments Ltd., Type 2984 Model C) fitted to the inner surface of the gas outlet pipe of the dry gas meter. The analogue readout of the thermister was calibrated prior to each set of analyses.

The measured gas concentrations and volume were standardised for temperature, atmospheric pressure and water vapour content (STPD). Using the Haldane transformation formula, oxygen uptake ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), minute ventilation ($\dot{V}E$) and the respiratory exchange ratio (RER) were then calculated. The use of open circuit spirometry allowed for energy expenditure under steady state conditions to be estimated using indirect calorimetry. The proportion of energy derived from carbohydrate and fat were estimated using the non-protein RER, with the carbohydrate and fat oxidation rates determined from measurements of $\dot{V}O_2$ and $\dot{V}CO_2$ (Frayn, 1983).

All experimental tests administered in this thesis were conducted in the laboratory under neutral environmental conditions (18-22°C). Ambient conditions in the laboratory were carefully monitored throughout the experimental period during all studies, and controlled where possible. Wet and dry bulb temperatures were measured using a whirling hydrometer (Brannan Thermometers Ltd., Cumberland, UK), operated in close proximity to the subject during rest and were subsequently used to calculate relative humidity (%) using an appropriate conversion scale.

3.3 PRELIMINARY MEASUREMENTS

Familiarisation

All subjects who volunteered to take part in the studies described in the thesis were familiarised with the laboratory environment and experimental procedures. In all studies a series of preliminary tests were conducted prior to the main experimental trial to determine the oxygen cost of submaximal cycling (cycling economy) and maximal oxygen uptake ($\dot{V}O_{2\max}$). In addition, a 60 minute familiarisation ride was performed prior to the main experimental trial in studies 1 and 2. During all measurements using the cycle ergometer, subjects were required to pedal at 75 revolutions per minute, as this was considered to be reflective of the subject groups normal cadence using the ergometer.

Determination of cycling economy

This test determined the oxygen cost of cycling over a range of submaximal work-rates ($\dot{V}O_2$ -work rate). The work rates were selected to each subject's cycling ability and corresponded to work rates between 60 – 90% of the individual's $\dot{V}O_{2\max}$. This test was continuous and involved subjects performing four minute stages at four different work rates with the load being increased every stage. Expired air collections were made during the last minute of each four minute period and were analysed for oxygen and carbon dioxide, to determine $\dot{V}O_2$ and $\dot{V}CO_2$ as described previously. In addition, heart rate and flywheel revolutions were recorded during the last minute of each stage. By applying linear regression to the four values of $\dot{V}O_2$ obtained and their corresponding work rate values, individual relationships between $\dot{V}O_2$ and work rate were established for each subject.

Determination of maximum oxygen uptake

Maximum oxygen uptake ($\dot{V}O_{2\max}$) was determined using a continuous, incremental test to volitional exhaustion. Subjects performed three minute stages with the load being increased 0.5 kg at the end of each stage. The starting work rate was based upon the

subjects cycling ability and also to ensure a test duration of approximately 10 minutes. Expired gas collections were made during minutes 1 min 45 s to 2 min 45 s of each stage. Continuous verbal encouragement was given to each subject during the test. A final collection was taken after subjects had indicated that they could only continue for one more minute. From this collection the subjects $\dot{V}O_{2max}$ was determined. Criterion for establishing a valid $\dot{V}O_{2max}$ test were, a heart rate approximating an age-predicted maximal value and an RER value greater than 1.0.

Using the data obtained from both the $\dot{V}O_2$ -workrate test and the $\dot{V}O_{2max}$ test, the $\dot{V}O_2$ equating to both 60 and 70% of the subjects $\dot{V}O_{2max}$ was determined, which in turn was equated to a work rate and subsequently a load corresponding to 60 and 70% $\dot{V}O_{2max}$.

Familiarisation ride

To ensure that the calculated loads for both 60 and 70% $\dot{V}O_{2max}$ were correct and that subjects were fully familiarised with prolonged cycling and the procedures that were to be used, subjects performed a 60 minute familiarisation prior to the main experimental trial in studies 1 and 2. Subjects performed 10 min at 60% $\dot{V}O_{2max}$ and the remaining 50 min at 70% $\dot{V}O_{2max}$ with expired air collections being made every 10 min. If necessary, the load was adjusted accordingly to ensure that the subject was cycling at 70% of their $\dot{V}O_{2max}$.

Glycogen depletion protocol

In all studies, subjects performed a bout of glycogen depleting exercise. The protocol implemented was identical in all studies. Subjects initially performed a 5 min warm-up at ~60% $\dot{V}O_{2max}$, and then undertook a bout of exercise of varying intensity (Fig 3.1), at a pedal rate equivalent to ~75 rpm. After 30 min of exercise at 70% $\dot{V}O_{2max}$, subjects rested passively for 2 min. The workload was then doubled and they completed 3 x 50 s

bursts of activity separated by 2 min rest. This burst of high-intensity activity was designed to substantially reduced type II fibres of glycogen.

Finally, they then cycled for a further 45 min at $\sim 70\% \dot{V}O_{2\max}$ to ensure both a further depletion of glycogen in type I fibres and to ensure that blood lactate concentration at the end of exercise was low to minimise glycogen resynthesis from lactate during recovery. The glycogen depleting exercise was designed to deplete both type I and type II muscle fibres of glycogen as validated by Vøllestad et al. (Vøllestad et al. 1992). Subjects were offered 2 ml water/kg body mass every 15 min during exercise to ensure euhydration during the bout of glycogen-depleting exercise.

3.4 DIETARY ANALYSIS

Subjects were required to complete a weighed food intake diary in the 48 hours preceding each main trial in all studies. Furthermore, before each of the main trials subjects were asked to replicate the food consumed prior to the first main trial. Subjects were asked to refrain from consumption of food and beverages from 10 o'clock the night prior to the main trial to ensure a 12 h overnight fast. Subjects were offered water upon arrival at the laboratory; the volume consumed was monitored and in all cases was negligible and insufficient to affect plasma volume and hydration status. Subject's food record diaries were analysed for nutritional content, in particular the percentage of carbohydrate, fat and protein consumed and their daily energy intake (Comp-Eat 5.0, Lifeline Nutritional Services, London).

In study 3, a low-carbohydrate diet (~ 35 % carbohydrate; ~55 % fat; ~10 % protein; ~5.9 MJ) was prescribed to subjects, after the glycogen-depleting exercise and before the main trial. Each subject had their prescribed diet devised individually using computer software (Comp-Eat 5.0, Lifeline Nutritional Services, London). The subject's normal diet was recorded for two days and the low-carbohydrate diet was based upon this, providing approximately 50 % of their average daily energy intake (food was not provided for subjects in this instance).

3.5 SOLUTIONS

In all studies in this thesis, a solution was provided either during recovery from exercise (studies 1 & 2) or prior to exercise (study 3). The solution contained the assigned amino acid(s) or ornithine α -ketoglutarate (study 3) to allow oral consumption. In study 1, a glucose-polymer solution was provided, in addition to a placebo solution. In studies 2 & 3 only a placebo solution was provided. In study 3, the ornithine α -ketoglutarate provided to the subjects was supplied by Laboratoires Jacques Logeais, Paris, France. All solutions provided were artificially sweetened, palatable and indistinguishable from the other solutions provided during each study. The composition of the solutions used is detailed in Table 3.1

Table 3.1 Composition of solutions provided during studies

	Glucose-polymer ¹	Placebo ²	Placebo ³	Placebo ⁴
Carbohydrate (g·100 ml ⁻¹)	6.4	—	—	—
pH	3.40	3.31	3.42	3.42
Osmolality (mOsm·kg)	296	75	67	67
Sodium (mg·100 ml ⁻¹)	48	51	52	52

¹Brand name: Lucozade Sport (SmithKline Beecham Consumer Healthcare, UK); ²Solution used in study 1; ³Solution used in study 2; ⁴Solution used in study 3.

3.6 ESTIMATION OF WHOLE BODY REHYDRATION

Whole body rehydration during the first and second studies of this thesis was estimated according to the method of Gonzalez-Alonso *et al.* (1992). The percentage gain in body weight during the recovery period relative to the weight loss during the previous bout of exercise provided a suitable index of rehydration. The amount of ingested fluid retained within the body after the recovery period equated to the percent rehydration.

$$\% \text{ rehydration} = \frac{[(BW_{\text{pre}} - BW_{\text{post}}) - (BW_{\text{pre}} - BW_{\text{rec}})]}{\text{Fluid intake (kg)}} \times 100$$

BW_{pre} = pre exercise body weight (kg)

BW_{post} = post exercise body weight (kg)

BW_{rec} = post recovery exercise body weight (kg)

3.7 COLLECTION, TREATMENT, STORAGE AND ANALYSIS OF BLOOD SAMPLES

Sample collection

In all studies, venous blood samples were obtained from an antecubital vein using an indwelling cannula (Venflon 2, 18 G, Ohmeda, Sweden) attached to a 10 cm extension tube (Connecta, Ohmeda, Sweden) for sampling, which was inserted under local anaesthetic (0.5 ml of 1% w:v lignocaine, Antigen Pharmaceuticals, Ireland). The cannula was kept patent by periodic flushing with sterile saline (sodium chloride injection, 0.9 % w/v, Steri-Amp, Steripak Ltd., Cheshire, UK). Blood samples were taken with the subject adopting a seated position, akin to the position assumed whilst sat

on the cycle ergometer. This consistency was also due to the fact that differences have been observed in plasma volume depending upon the position of the individual at the time of collection and in the preceding time (Hagan et al. 1978; Shirreffs and Maughan, 1994). In all cases, basal blood samples were obtained after 15 min of passive rest on the examination couch. A 12 ml blood sample was taken both at rest and during exercise in all studies. During exercise, venous blood samples were taken at regular intervals without any interruption to cycling. The exhaustion blood sample was obtained during the last minute of exercise.

Treatment, storage and analysis of blood samples

In all studies, venous blood was drawn off in untreated plastic syringes (Plastipak, Becton Dickinson, Sweden) and dispensed into either lithium-heparinised tubes, EDTA tubes or serum (containing no anticoagulant) tubes (Sarstedt, Leicester, UK). The venous blood in serum tubes was left to clot for 1 h on ice to obtain serum.

Plasma NH₃ (all studies) and glutamine (study 1)

1 ml of the venous blood was dispensed into calcium-heparinised flip-top Eppendorf tubes (50 units per tube) and centrifuged for 3 min at 12000 rev.min⁻¹ (Eppendorf, Model 5412). The plasma obtained was snap-frozen in liquid nitrogen before being analysed within 48 h for ammonia concentration using a commercially available kit (Sigma Diagnostics, Procedure No. 171-UV, see Appendix C). 2 x 1 ml aliquots were treated in a similar fashion and the plasma was later stored at -70°C, before being analysed at a later date for glutamine concentration (see comparison of methods of glutamine measurement, within this chapter).

Plasma non-esterified fatty acids (NEFA) and glucose⁴ (studies 1 & 2)

An aliquot (~3 ml) of the whole venous blood was dispensed into EDTA tubes and centrifuged for 15 min at 6000 rev.min⁻¹ at approximately 4°C, and the resulting plasma was stored at -20°C before being analysed at a later date. This plasma was analysed for NEFA; (Wako Chemicals, Osaka, Japan, see Appendix C) and glucose using a commercially available kit (Boehringer Mannheim, GmbH) and an automated analyser (Cobas Mira Plus, Roche Diagnostic Systems, Basel, Switzerland; see Appendix A).

Blood lactate⁴ (studies 1 & 2)

Two 20 μ l aliquots of venous blood were drawn from the tube and immediately deproteinised in an Eppendorf containing 200 μ l perchloric acid (2.5% w:v) prior to centrifugation (Eppendorf, Model 5412). The supernatant from these samples was subsequently analysed for blood lactate via fluorometric analysis (Maughan, 1982).

Plasma and blood lactate and glucose⁴ (studies 2 & 3)

1 ml of venous blood was presented at an automated glucose and lactate analyser (Yellow Springs Instruments (YSI), 2300 STATPLUS, Ohio, USA) for the determination, in duplicate, of blood lactate and glucose concentration. After analysis, this aliquot of venous blood was then immediately centrifuged for 3 min at 12000 rev.min⁻¹. The plasma obtained was presented at the YSI automated analyser for the determination, in duplicate, of plasma lactate and glucose concentration.

Serum insulin (all studies) and NEFA (study 3)

An aliquot (~4 ml) of the venous blood was dispensed into serum tubes and after 1 h on ice, serum was obtained by centrifugation for 15 min at 6000 rev.min⁻¹. The serum was stored at -70°C and analysed at a later date for insulin (Radio-immunoassay kit, Diagnostic Products Corporation, California, USA; see Appendix E). The serum obtained was also analysed for NEFA (study 3; Wako Chemicals, Osaka, Japan, see Appendix C).

Haemoglobin and haematocrit (all studies)

Two 20 μ l aliquots of venous blood were drawn from the tube using calibrated micro pipettes (Acupette Pipettes, Scientific Industries Ltd.) and mixed with 5.0 ml of Drabkins Reagent (Boehringer Mannheim, GmbH). Haemoglobin concentration was photometrically determined using the cyanmethaemoglobin method (Cecil Instruments 393 Series 2 Digital Grating spectrophotometer). Triplicate 50 μ l samples of blood were drawn from the same sample using heparinised capillary tubes. These samples were later micro-centrifuged for 15 minutes at 11,000 rev.min⁻¹ (Hawksley Ltd) and

⁴ A comparison in the methods of analysis between plasma and blood glucose and lactate was made for the samples obtained in study 2. Please refer to Appendix N for the full unpublished paper on this comparison.

packed cell volume was determined using a sliding haematocrit reader (Hawksley Ltd). Percent changes in plasma volume from rest were estimated using the method described by Dill & Costill (1974) based upon changes in haemoglobin concentration and packed cell volume.

Plasma amino acids (all studies)

The remaining whole blood in the tube was immediately centrifuged for 15 minutes at 3°C at 6,000 rev.min⁻¹ (Burkard μ P Koolspin). Plasma obtained was stored at -20°C and later analysed for amino acids using high performance liquid chromatography (HPLC) using the method of Ou et al. (1996) in the Department of Anatomy and Physiology, University of Dundee.

Comparison of methods of glutamine measurement

Plasma samples obtained in the first study were analysed for glutamine using an enzymatic spectrophotometric procedure (Lund, 1985). Plasma glutamine concentration was determined by measuring the glutamate concentration in plasma before and after treatment with glutaminase (EC 3.5.1.2), the enzyme responsible for degrading glutamine to glutamate. The plasma glutamine concentration was calculated by subtracting the untreated glutamate concentration from the glutamate concentration in the sample treated with glutaminase. Plasma glutamine concentration was also determined using high performance liquid chromatography (Ou et al. 1996). A comparison of the two methods revealed that the enzymatic procedure resulted in plasma glutamine values ~24% higher than the HPLC method. This disparity between the two methods may be due to the instability of glutamine in the acidic or alkaline media used in HPLC analysis. The difference between the two methods was similar both at low and high glutamine concentrations and therefore the different methods will provide qualitatively similar, if quantitatively different values.

Coefficients of variation for blood and plasma assays

The coefficient of variation (CV) for all blood and plasma assays is shown in Table 3.2 and was calculated from repeated measurements on the same sample.

Table 3.2 Coefficient of variation of blood and plasma assays (n=10)

Assay	Coefficient of variation (%)
Blood lactate (manual ¹)	2.2 %
Blood glucose (automated ²)	1.3 %
Blood lactate (automated ²)	2.2 %
Plasma glucose (automated ³)	2.8 %
Plasma ammonia	6.7 %
Plasma NEFA ³	2.1 %
Serum NEFA ⁴	2.0 %
Serum insulin	6.9 %
Plasma glutamine ⁵	4.3 %

¹Method used in study 1; ²Method used in studies 2 & 3 (YSI 2300 STATPLUS analyser); ³Method used in studies 1 & 2 (Cobas Mira analyser); ⁴Method used in study 3 (Cobas Mira analyser); NEFA = non-esterified fatty acids; ⁵Enzymatic method used for comparative purposes in study 1 only (GP + GLN vs GP + ALA & GLY).

3.8 COLLECTION, TREATMENT, STORAGE AND ANALYSIS OF MUSCLE SAMPLES

Collection of muscle samples

Muscle samples were obtained from the vastus lateralis by a percutaneous needle biopsy technique (Bergström, 1975) with suction applied. The skin and fascia over the anterior aspect of one thigh was anaesthetised (2-3 ml of 1% w:v lignocaine, Antigen Pharmaceuticals, Ireland) and small incisions (~1 cm) were made in skin and subcutaneous layer of fascia to allow extraction of muscle samples using a biopsy needle.

Human vastus lateralis is comprised of 52% (range 36-71%) type I, 36% (range 23-57%) type IIa, and 10% (range 0-35%) type IIb fibres (Schantz et al. 1983), and therefore a biopsy sample represents a mixture of these fibre types. Furthermore, vastus lateralis has been shown to be actively involved during cycling exercise (Essén, 1978), making this muscle an appropriate site for sampling.

Treatment and storage of muscle samples

In order to measure the concentration of metabolic intermediates in a tissue at the time point of sampling, the metabolic reactions must be inhibited very rapidly. Therefore,

the biopsy needle containing the muscle sample was immersed into liquid nitrogen within 1-2 s after removal from the leg. The samples were then quickly removed from the biopsy needle, placed into screw-top Eppendorfs (with pierced tops), and kept in liquid nitrogen for 24-48 h until they were freeze-dried.

Each sample was freeze-dried for 12-18 h using a freeze-drier (Edwards Modulyo, Crawley, UK) and stored at -70°C in an anhydrous state. This procedure allows long-term storage of the samples with reactions inhibited. Furthermore, in this state it is easier to remove fat, blood contaminants and connective tissue from the muscle sample.

Fat was removed from the freeze-dried muscle by washing the sample twice with 1 ml petroleum ether ($30-40^{\circ}\text{C}$). Each time the sample was mixed and centrifuged before removing the ether. Finally, the tube was left open in a fume cupboard to allow evaporation of ether until the sample was completely dry. At a later date, the freeze-dried muscle was dissected free of visible connective tissue and blood, and then powdered using an agate pestle and mortar at $18-23^{\circ}\text{C}$. The powdered muscle sample was then weighed using an electrical balance accurate to five decimal places (Mettler-Toledo AG245, Switzerland), placed in a screw-top Eppendorf, centrifuged ($22,000 \times g$, 1 min) to ensure all the fibres collected at the apex of the Eppendorf and stored at -70°C until later analysis.

Extraction procedure for measurement of mixed muscle metabolites

Samples were extracted according to the method of Harris *et al.* (1974). 0.5 M ice-cold perchloric acid (HClO_4 ; PCA) containing 1mM ethylenediamine tetra-acetic sodium salt (EDTA), was added to each sample for 10 min [volume added (μl) = weight of muscle powder / 12.5; round up volume to nearest 50 μl]. This procedure rapidly inactivates and precipitates protein. Each sample was gently vortexed at 30 s intervals, and kept on ice in the time intervals, for a total of 10 min (<10 min results in a significantly lower release of ATP compared to 10 and 20 min periods; Harris *et al.*, 1974). The vortexing was vigorous enough to free the fibres from the apex of the Eppendorf. Each sample was then centrifuged ($22,000 \times g$, 1 min; $3-4^{\circ}\text{C}$) to ensure the sample collected at the apex of the Eppendorf. A known volume of the resulting supernatant (i.e. extract) was removed, recorded, and stored in flip-top Eppendorfs. The supernatant was neutralised

with 2.2 M potassium bicarbonate (KHCO_3) liberating CO_2 and producing a white salt [volume added in μl = volume of extract in μl /4]. The samples were then centrifuged ($22,000 \times g$, 1 min; $3-4^\circ\text{C}$), the supernatant was removed, and placed in screw-top Eppendorfs and kept on ice. To ensure that the neutralisation step was complete, the pH of the extract was checked using Litmus paper to ensure a pH measurement of 7.0. The samples were then stored at -80°C until later analysis.

Measurement of muscle metabolites

Prior to each assay the muscle extracts were taken from storage at -80°C and defrosted rapidly by immersing the samples in hot water, then vortexed to ensure homogeneity of the extract. The muscle extracts were assayed for the different metabolites using modifications of the methods described by Harris *et al.* (1974), Lowry & Passonneau (1972) and Lund (1985). Adenosine triphosphate (ATP), phosphocreatine (PCr) and creatine (Cr) were assayed spectrophotometrically (Beckman DU 650). Lactate, pyruvate, citrate, succinate, malate, fumarate, glutamate and glutamine were assayed flurometrically (Hitachi F2000). All assays were based on enzyme catalysed reactions. Detailed methods for all muscle assays performed are presented in the Appendix (F-M).

Buffers, co-factors (Grade-I) and enzymes were obtained as standard commercial items from either Boehringer Mannheim or Sigma Chemicals. All reagents were prepared with double-distilled water and kept at 4°C . Standards were prepared using Grade-I chemicals on the day of analysis or were from pre-prepared frozen stock (-20°C).

Calculations for muscle parameters

ATP, phosphocreatine (PCr) and creatine (Cr) concentrations were determined spectrophotometrically using the method of Harris *et al.* (1974 & 1983). The contents of these metabolites were measured indirectly from changes in the absorbance of NADH and NADPH. Both the oxidised and reduced forms of these nucleotides absorb visible light at 260 nm, but only the reduced form absorbs visible light at 340 nm. The formation of ATP, PCr and Cr do not directly involve this interconversion (i.e. there is no spectral change), but may be assayed using the concept of 'coupled reactions'. In such reactions, the complete utilisation of the metabolite is coupled to a reaction utilising the NAD^+/NADH or $\text{NADP}^+/\text{NADPH}$ systems by means of common

intermediates (see individual assays). Metabolite concentration can then be calculated from the recorded changes in absorbance according to the Beer- Lambert law:

$$C = \Delta A / (\epsilon \times d)$$

A = absorbance

c = concentration (of metabolite, mmol kg⁻¹ dm)

ϵ = molar extinction coefficient (1M NADH $\epsilon_{340\text{nm}} = 6.22 \text{ cm}^2 \cdot \mu\text{mol}^{-1}$)

d = light path (cms)

The calculation of metabolite concentration for assays using fluorometric methods, was based upon the inter-conversion of the coenzymes NAD⁺/NADH or NADP⁺/NADPH respectively. The calculation was dependent upon the generation of a standard curve, which was always linear ($r = 0.98$ to $r = 1.00$), and metabolite concentrations were calculated using the resulting standard regression equation (see individual assays).

Blanks (double-distilled water), standards, dilution factors and extraction factors were taken into consideration where necessary. In addition, changes in muscle metabolites were normalised to the mean total creatine (TCr) content for each subject (sum of phosphocreatine concentration ([PCr]) and creatine concentration ([Cr]) (118.0 ± 1.9 mmol/g dry wt)) to correct for variability in solid non-muscle constituents between biopsies (with the exception of lactate). The TCr content did not change significantly between conditions or over time ($P > 0.05$) in study 3.

Coefficients of variation for assays

The coefficients of variation (CV) for all muscle assays are shown in Table 3.3 and was calculated from repeated measurements on the same extract ($\text{CV} = (\text{standard deviation} / \text{mean}) \times 100$). All muscle metabolite concentrations were determined in dry weight to avoid changes in concentration due to water shift during exercise.

Table 3.3 Coefficient of variation of muscle metabolite assays (n=10)

Assay	Coefficient of variation (%)
ATP	1.5 %
PCr	2.3 %
Cr	2.9 %
Pyruvate	9.6 %
Lactate	3.6 %
Citrate	3.2 %
Malate	3.1 %
Fumarate	8.7 %
Succinate	6.0 %
Glutamine	5.3 %
Glutamate	4.9 %

ATP = adenosine triphosphate; PCr = phosphocreatine; Cr = creatine

CHAPTER 4

EFFECT OF GLUCOSE-POLYMER AND GLUTAMINE SUPPLEMENTATION DURING RECOVERY ON EXERCISE METABOLISM IN A SUBSEQUENT BOUT OF EXERCISE

4.1 INTRODUCTION

Glycogen is a branched-chain polysaccharide containing glucose residues and is stored primarily in the liver and skeletal muscle. Glycogen stored in the liver is used primarily to maintain blood glucose levels. In contrast, the role of glycogen stored in skeletal muscle is to provide a source of energy during muscular contraction, in particular during moderate to intense exercise. Fatigue during prolonged submaximal exercise is associated with the depletion of muscle glycogen stores (Bergström et al. 1967). Furthermore, a reduction in liver glycogen concentration (Hultman and Nilsson, 1971) with a concomitant reduction in blood glucose concentration has been suggested as a possible cause of fatigue (Coyle et al. 1983). Therefore, the resynthesis of glycogen, both liver and skeletal muscle, during the recovery period after exercise is important to the recovery of endurance exercise capacity (Casey et al. 2000).

It is known that the consumption of carbohydrate during recovery from exercise leads to a greater accumulation of both muscle and liver glycogen (Hultman, 1967a; Casey et al. 2000). More recently however, research has focussed upon the supplementation of both carbohydrate and protein during recovery from exercise (Zawadzki et al. 1992; Roy and Tarnopolsky, 1998; Carrithers et al. 2000; Jentjens et al. 2001). It has been demonstrated that a combination of carbohydrate and protein can stimulate muscle glycogen resynthesis to a greater extent than placebo (Tarnopolsky et al. 1997; van Hall et al. 2000a). Furthermore, it has been demonstrated consistently that the addition of protein to a carbohydrate solution consumed during recovery from exercise is able to increase insulin concentration to a greater extent than when carbohydrate alone is consumed (Spiller et al. 1987; Tarnopolsky et al. 1997; van Hall et al. 2000; Jentjens et al. 2001). It has been clearly demonstrated that insulin modulates glycogen metabolism by activating glycogen synthase, the enzyme involved in glycogen synthesis, and also by increasing the skeletal muscle cell permeability to glucose,

thereby increasing muscle glucose uptake (Ivy and Kuo, 1998). However, the co-ingestion of carbohydrate and protein post exercise appears to be unable to increase muscle glycogen synthesis to a greater extent than carbohydrate alone, when sufficient carbohydrate is provided (Tarnopolsky et al. 1997; van Hall et al. 2000a; Jentjens et al. 2001).

During exercise a net loss of body protein is observed due to an increase in whole-body protein breakdown and a decrease in whole-body protein synthesis (Rennie et al. 1981). However, during recovery from exercise an anabolic response is elicited due to an increase in whole-body protein synthesis (Rennie et al. 1981). Essential amino acids appear to be the primary stimulators of muscle protein synthesis (Rasmussen et al. 2000; Svanberg et al. 1996), rather than non-essential amino acids (Smith et al. 1998), with insulin playing a less significant role in muscle protein synthesis than previously thought (Gautsch et al. 1998; Long et al. 2000). Therefore, the ingestion of essential amino acids with carbohydrate postexercise may prove the optimal combination to promote skeletal muscle anabolism post exercise.

The provision of the non-essential amino acid glutamine, which is considered 'conditionally essential' (Lacy and Wilmore, 1990), has been shown to stimulate glycogen synthesis both in skeletal muscle myotubes (Low et al. 1996) and in rat liver cells (Lavoine et al. 1987; Mouterde et al. 1992). The mechanism has been attributed to the activation of glycogen synthase via cell swelling induced by increased uptake of this amino acid (Baquet et al. 1990; Low et al. 1996). An increase in the plasma concentration of glutamine, either through oral provision or intravenous administration, will lead to an increase in intramuscular glutamine concentration (Varnier et al. 1995) due to the rapid uptake of glutamine into skeletal muscle via System N^m (Ahmed et al. 1993). Indeed, it has been demonstrated that the intravenous infusion of glutamine after exhaustive exercise promoted a greater degree of muscle glycogen resynthesis than the infusion of an isoenergetic and isonitrogenous mixture of alanine and glycine (Varnier et al. 1995). In addition, the oral provision of glutamine after exhaustive exercise is able to increase muscle glycogen resynthesis, however a combination of glutamine and glucose-polymer was unable to increase muscle glycogen resynthesis to a greater extent than glucose-polymer alone (Bowtell et al. 1999). Van Hall et al. (2000b) recently confirmed this finding and demonstrated that the co-ingestion of glucose-polymer and

glutamine after exhaustive exercise does not further increase the activation of glycogen synthase, and thus the rate of muscle glycogen resynthesis, compared to glucose-polymer alone.

However, the ingestion of glutamine and glucose-polymer after exhaustive exercise appears to promote whole-body carbohydrate storage to a greater extent than glucose-polymer alone. The most likely site of increased carbohydrate storage being the liver (Bowtell et al. 1999). This response has been attributed to an increase in flux through the hexosamine pathway, due to an increase in the intracellular concentration of both glucose and glutamine, resulting in the formation of glucosamine (Crook et al. 1995). Glucosamine induces insulin resistance by causing an impairment of GLUT-4 translocation from an insulin-sensitive intracellular pool to the sarcolemmal membrane (Baron et al. 1995), in addition to decreasing the insulin sensitivity of glycogen synthase (Crook et al. 1995). Therefore, glucose uptake into skeletal muscle is reduced, reducing skeletal muscle glycogen synthesis through a lesser activation of glycogen synthase and reduced availability of glucose to form glycogen. However, the uptake of glucose in the liver is via GLUT-2, so hepatic glucose uptake is not impaired.

It was hypothesised that the supplementation of both glucose-polymer and glutamine post exercise, would promote hepatic glycogen resynthesis, but that muscle glycogen resynthesis would not be different compared to the supplementation of glucose-polymer alone. However, the addition of glutamine may promote increased skeletal muscle and whole body anabolism during recovery from exercise.

Fatigue during prolonged running is not associated with a decline in blood glucose concentration (Tsintzas et al. 1995; Williams et al. 1990). However, fatigue during prolonged cycling appears to be associated to a decline in blood glucose concentration (Coyle et al. 1983; Derman et al. 1996), possibly linked to the increased glucose uptake by skeletal muscle observed during cycling (Richter et al. 1988; Richter, 1996). Therefore, if liver glycogen content can be increased to a greater extent during recovery from exercise after the consumption of glucose-polymer and glutamine, blood glucose concentration may be better maintained in a subsequent bout of exercise, which could conceivably delay the onset of fatigue.

The aims of this study were to investigate the effect of oral glutamine and glucose-polymer supplementation compared to alanine and glycine plus glucose-polymer supplementation during recovery from exhaustive exercise and to determine the effect upon endurance capacity in a subsequent bout of exercise.

4.2 METHODS

Subjects. Seven well-trained, healthy male subjects participated in this study. Their mean (\pm SEM) age, height, body mass and $\dot{V}O_{2\max}$ were 24.0 ± 1.6 y, 180.4 ± 1.8 cm, 78.7 ± 1.6 kg and 4.83 ± 0.13 l.min⁻¹ (61.5 ± 2.0 ml.kg⁻¹.min⁻¹), respectively.

Preliminary tests. Subjects reported to the laboratory approximately 1 week before the experiment and undertook two preliminary tests in order to determine: (i) the oxygen cost of submaximal cycling, and (ii) maximal oxygen uptake ($\dot{V}O_{2\max}$). The protocols of these tests have been described elsewhere (see Chapter 3). Subjects were fully familiarised with the experimental procedures to be used during the experimental trials.

Protocol of the study. Subjects were instructed to consume their habitual diet and refrain from exercise or strenuous physical activity for 48 h prior to each experiment. On arrival at the laboratory on the morning of the experiment, the overnight fasted subject rested in a supine position and a cannula was inserted into an antecubital vein and a resting blood sample was obtained. The experimental procedure utilised in this study was a standard 'recovery protocol' similar to a protocol adopted previously in the literature (Fallowfield and Williams, 1997), consisting of two bouts of exercise separated by a 4 h passive recovery period.

Subjects performed a bout of glycogen depleting exercise (EX1), as described previously (Chapter 3). EX1 was immediately followed by a 4 h passive recovery period, during which they consumed one of the assigned solutions. They then immediately cycled to exhaustion at $\sim 70\%$ $\dot{V}O_{2\max}$ (EX2) (Fig. 4.1) during which subjects were offered no fluid.

The two bouts of exercise (EX1 and EX2) were separated by a 4 h controlled recovery during which the subjects remained within the laboratory and their activity levels were minimal. After completion of EX1 subjects were rested for ~10 min before their post exercise nude bodyweight was measured. Any excess sweat on the subject's skin was towelled off prior to their weight being recorded.

Subjects undertook four trials during which they received one of three different drinks during the 4 h recovery period. On two occasions subjects consumed 31.25 ml·kg⁻¹ body wt of an artificially sweetened placebo (CON1) and (CON2) (SmithKline Beecham Consumer Healthcare, Gloucestershire, UK), during the experimental trials subjects consumed either 1.7 g glucose·kg body wt⁻¹ (6.4 % w:v glucose-polymer) and 0.3 g·kg body wt⁻¹ L-glutamine (Sigma-Aldrich Chemicals, Dorset, UK) (GP + GLN) (Smithkline Beecham Consumer Healthcare, Gloucestershire, UK), or 1.7 g glucose·kg body wt⁻¹ (6.4 % w:v glucose-polymer) and an isoenergetic and isonitrogenous mixture of L-alanine (0.1829 g·kg body wt⁻¹) and L-glycine (0.1543 g·kg body wt⁻¹) (GP + ALA & GLY) (Sigma-Aldrich Chemicals, Dorset, UK).

Subjects received 780 ml of solution in the first drink with the remaining solution being divided into 5 equal aliquots. In the glucose-polymer conditions (GP + ALA & GLY and GP + GLN), the first drink was formulated to supply 50 g of carbohydrate. Subjects consumed the first of the six drinks after 30 min of recovery, after the first expired air collection, and then every 30 min thereafter. The last drink was administered at the 3 h point during recovery (1 h before exercise) to minimise the possibility of gastro-intestinal stress in the forthcoming exercise bout. Urine voided during the recovery period was collected and a cumulative volume recorded.

Prior to EX2, after voiding the bladder of urine, subject's post-recovery nude body weight was recorded in order to calculate rehydration status. Subjects then completed a 5 minute warm up at 60% $\dot{V}O_{2max}$, prior to commencing EX2. During EX2, subjects were instructed to cycle to volitional exhaustion at a workrate equivalent to ~70% $\dot{V}O_{2max}$, in order to determine their endurance capacity in terms of cycle time to exhaustion. Subjects were given no indication of the time completed during EX2. In order to ensure the reproducibility of cycle time to exhaustion, the point of exhaustion

was defined as the inability to maintain the required power output, despite strong verbal encouragement. Subjects indicated when they could only continue for one more minute, at which point the final expired air collection and blood sample was taken.

Expired air collections were taken prior to the commencement of EX1, then at 30 min, 60 min and 80 min during EX1. During recovery, collections were taken at 30 min intervals, with a collection being taken every 10 min and at exhaustion during EX2 (Fig. 4.1). Venous blood samples were taken prior to EX1 and after 30 min, 60 min and at cessation of EX1. During recovery, samples were taken at 60, 75, 90, 105, 120, 180 and 240 min, with a subsequent sample being taken at exhaustion during EX2 (Fig. 4.1).

Dry bulb temperatures within the laboratory during the main experimental trial were 19.9 ± 0.3 °C, 20.5 ± 0.4 °C, 20.7 ± 0.5 °C and 20.0 ± 0.2 °C for CON1, CON2, GP + GLN and GP + ALA & GLY conditions respectively. Relative humidity within the laboratory during the main experimental trial was 52.7 ± 1.9 %, 49.6 ± 4.0 %, 49.6 ± 2.2 % and 49.9 ± 2.3 % for CON1, CON2, GP + GLN and GP + ALA & GLY conditions respectively. No statistical difference was observed in the relative humidity measurements between trials.

Analysis. Expired air samples were collected and analysed as previously described (Chapter 3). Venous blood samples were analysed for blood lactate and plasma glucose concentration, plasma ammonia, NEFA and amino acid concentration, serum insulin concentration, and haemoglobin concentration and haematocrit values (Chapter 3). Percentage changes in plasma volume were calculated as previously described (see Chapter 3).

Pre-exercise energy intake. The energy intake of the subject's habitual diet during the 48 h prior to the main trial are presented in Table 4.1. No differences were observed between conditions in the pre-exercise diet.

Table 4.1 Energy content and percentage of the primary dietary nutrients of the pre-exercise diet for all conditions (mean \pm SEM). Data presented is mean of 2 days diet

	CON1	CON2	GP + ALA & GLY	GP + GLN
Energy (MJ/day)	12.9 \pm 0.6	13.6 \pm 0.9	13.2 \pm 0.7	13.1 \pm 0.9
Carbohydrate (%)	57.8 \pm 3.0	59.7 \pm 2.5	58.5 \pm 3.9	59.0 \pm 3.1
Fat (%)	28.9 \pm 3.2	26.6 \pm 3.7	28.5 \pm 4.2	27.1 \pm 3.4
Protein (%)	13.7 \pm 1.3	13.6 \pm 0.9	13.1 \pm 1.1	13.9 \pm 1.5

4.3 REPRODUCIBILITY OF PROTOCOL

The reproducibility of endurance capacity has previously been reported to be poor, with cycle time to exhaustion varying by as much as 26.6 % over five trials (Jeukendrup et al. 1996). During this study, subjects undertook four main experimental trials that were separated by at least ten days.

In order to assess the reproducibility of the protocol and the physiological measurements, subjects undertook two placebo trials. This also allowed the efficacy and sensitivity of using cycle time to exhaustion as tool for measuring any improvement in endurance capacity to be assessed. The first trial that subjects completed was always a placebo trial, and the order in which subjects completed the remaining three conditions (CON, GP + GLN, GP + ALA & GLY) was allocated by systematic rotation using a double-blind design.

In summary, the control trials not only provided a benchmark with which to compare the experimental trials, but also acted as a means of assessing the reproducibility of the protocol. It would have been desirable to include more than two trials to gain a better assessment of reproducibility. However, it was felt that the demands of completing more than four trials in total would have been too great and may have reduced subject compliance.

Subjects were asked to cycle to volitional exhaustion, with the cessation of exercise determined by the subject. However, if the subject failed to maintain the required

power output despite strong verbal encouragement on more than five occasions, then the test was terminated.

Statistical analysis. The data were analysed by two-way analysis of variance (ANOVA) for repeated measures (time X condition). Cycle time to exhaustion data were analysed by one-way ANOVA. When the ANOVA resulted in a significant *F* ratio, Fisher's post hoc test was used to locate differences between means. Statistical significance was accepted at the 5% level ($P < 0.05$). Results are presented as means \pm SEM.

A "Limits of Agreement" analysis was used to assess the reproducibility of cycle time to exhaustion, illustrated using a Bland and Altman plot (Bland and Altman, 1986; see Fig 4.2)

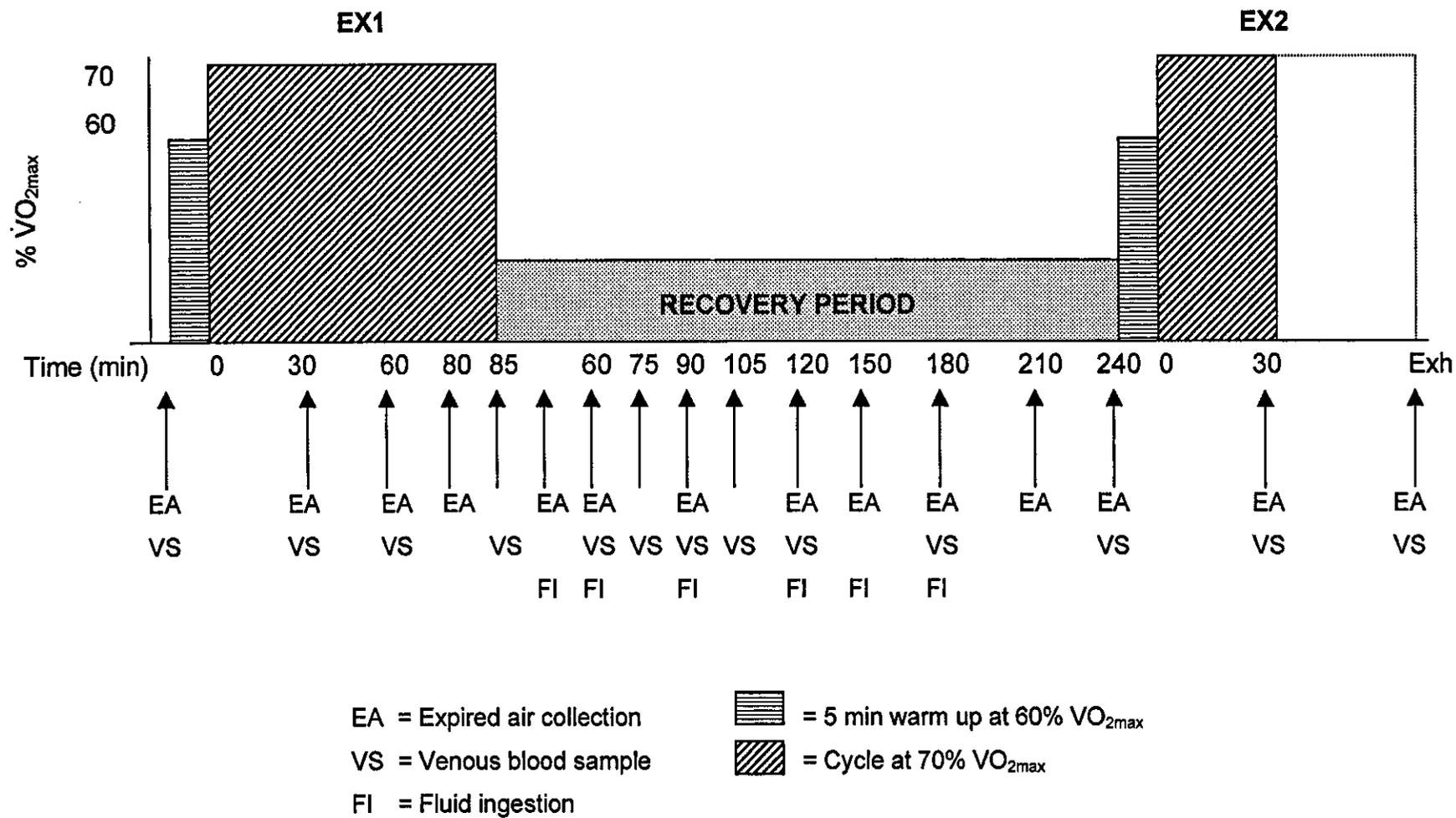


Fig. 4.1 Schematic illustration of the experimental protocol

4.4 RESULTS

Cardiorespiratory data. Pulmonary oxygen uptake ($\dot{V}O_2$), expired minute ventilation (data not shown) and $\% \dot{V}O_{2\max}$ were all increased during exercise ($P < 0.05$), however there were no differences between conditions (Table 4.2). Power output was not different between conditions in either EX1 (CON1: 238 ± 4 ; CON2: 239 ± 5 ; GP + ALA & GLY: 236 ± 4 ; GP + GLN: 234 ± 2 , watts) or EX2 (CON1: 236 ± 4 ; CON2: 228 ± 7 ; GP + ALA & GLY: 220 ± 10 ; GP + GLN: 227 ± 11 , watts).

Heart rate increased during both bouts of exercise (Table 4.2). The average heart rate during EX1 was 147 ± 3 b.min⁻¹, 152 ± 4 b.min⁻¹, 147 ± 3 b.min⁻¹ and 149 ± 3 b.min⁻¹ in the CON1, CON2, GP + ALA & GLY and GP + GLN conditions respectively, however there were no differences between conditions. The average heart rate during exercise EX2 was 169 ± 2 b.min⁻¹, 167 ± 1 b.min⁻¹, 154 ± 1 b.min⁻¹ and 161 ± 1 b.min⁻¹ in the CON1, CON2, GP + ALA & GLY and GP + GLN conditions respectively. Heart rate was significantly higher ($P < 0.05$) in the control conditions compared to the glucose-polymer conditions during EX2.

Endurance capacity. Cycle time to exhaustion tended to be longer during the glucose-polymer conditions relative to the control conditions (CON1: 37.3 ± 2.0 ; CON2: 33.8 ± 1.8 ; GP + ALA & GLY: 40.6 ± 5.8 ; GP + GLN: 41.8 ± 6.2 , min), but the differences did not attain statistical significance. The percentage difference in cycle time to exhaustion between conditions is presented in Table 4.3.

No difference was observed between the two placebo conditions both in terms of physiological responses throughout the experimental period and endurance capacity (CON1: 37.3 ± 2.0 ; CON2: 33.8 ± 1.8 min). In addition, based on individual times, a ~21% variation (range: 2.3% - 72.1%) was observed between the two conditions in cycle time to exhaustion.

In the present study, the reproducibility of endurance capacity, in terms of the calculated 95% limits of agreement are 3.5 ± 17.0 min (Fig 4.2). Therefore, a given subject's

endurance capacity in a second cycle to exhaustion should lie between -13.5 to $+20.5$ min of their first cycle to exhaustion. It is evident in Fig 4.2 that the data is skewed by two subjects (signified by triangular data points). Considering the small number of subjects in the present study, the effect of individual data has a greater bearing on the group data. If the same calculations are applied to 5 subjects (signified by square data points), the calculated 95% limits of agreement, expressed with bias become -1.3 ± 5.4 min, equating to a second cycle to exhaustion of between -6.7 to $+4.1$ min relative to the first cycle to exhaustion (see later in chapter for full discussion).

Plasma glucose and blood lactate concentration. Plasma glucose concentration was not different between conditions in EX1 (Fig. 4.3). During recovery, the plasma glucose concentration increased ($P < 0.05$) in both conditions where glucose-polymer was ingested (GP + ALA & GLY and GP + GLN), whilst the concentration in the control trials remained at a value similar to that at the end of EX1. Plasma glucose concentration increased more rapidly in the GP + GLN condition and was higher ($P < 0.05$) than the GP + ALA & GLY condition at 60 and 75 min of recovery. Plasma glucose concentration in the GP + GLN condition tended to be elevated above the GP + ALA & GLY condition for the first three hours of the recovery period. The peak in plasma glucose concentration was observed at 60 min of recovery in both glucose-polymer conditions, 30 min after the ingestion of the first drink. The area under the plasma glucose curve (AUC) during the recovery period had a tendency to be greater in the GP + GLN condition during the second hour of the recovery period (CON1: 5.2 ± 0.2 ; CON2: 5.2 ± 0.2 ; GP + ALA & GLY: 6.3 ± 0.6 ; GP + GLN: 7.0 ± 0.4 , all $\text{mmol}\cdot\text{h}^{-1}$). Plasma glucose concentration was significantly higher ($P < 0.05$) in the GP + GLN condition than the control conditions until 180 min of recovery. In the GP + ALA & GLY condition, the concentration was significantly higher ($P < 0.05$) than the control conditions at 60, 75 and 90 min of recovery. At the end of the recovery period, plasma glucose concentration was not different between conditions. During EX2, plasma glucose concentration increased from the value at the end of the recovery period in the GP + GLN condition, but decreased in all other conditions (CON1: -3.8 ± 9.7 ; CON2: -5.7 ± 5.4 ; GP + ALA & GLY: -8.8 ± 7.8 ; GP + GLN: $+12.3 \pm 12.4$, all %). The percentage change in the GP + GLN condition was significantly different ($P < 0.05$) to

the GP + ALA & GLY condition. However, no difference between conditions was observed in plasma glucose concentration at exhaustion.

Blood lactate concentration increased ($P < 0.05$) to a similar extent during EX1 and EX2 in all conditions (Fig 4.4). During recovery, the area under the blood lactate concentration curve (AUC) was higher ($P < 0.01$) in the GP + GLN condition compared to all other conditions. In addition, the AUC during recovery in the GP + ALA & GLY condition was higher ($P < 0.05$) compared to the control conditions (AUC: CON1: 4.9 ± 0.5 ; CON2: 5.6 ± 0.7 ; GP + ALA & GLY: 6.6 ± 0.8 ; GP + GLN: 8.0 ± 0.7 , mmol.l⁻¹.4 h).

Carbohydrate and fat oxidation rates. During recovery, the respiratory exchange ratio was higher ($P < 0.05$) in the glucose-polymer conditions compared to the control condition (Table 4.3), however no differences were observed between conditions in either bout of exercise, although an increase was observed relative to baseline in all conditions ($P < 0.05$). The rate of carbohydrate oxidation and fat oxidation increased during exercise ($P < 0.05$). During EX2, total fat oxidation had a tendency to be lower in the glucose-polymer conditions compared to control conditions (CON1: 26.0 ± 3.4 ; CON2: 22.3 ± 2.3 ; GP + ALA & GLY: 21.5 ± 3.0 ; GP + GLN: 21.8 ± 4.4 , g), and total carbohydrate oxidation had a tendency to be higher (CON1: 90.5 ± 9.4 ; CON2: 83.2 ± 9.7 ; GP + ALA & GLY: 102.8 ± 16.2 ; GP + GLN: 111.3 ± 15.4 , g) in the glucose-polymer conditions compared to the control conditions.

Carbohydrate and fat oxidation rates were not different between conditions during EX1. Throughout the recovery period, although no statistically significant differences were noted between conditions, fat oxidation was higher during the control conditions (CON1: 0.084 ± 0.004 ; CON2: 0.085 ± 0.005 ; GP + ALA & GLY: 0.056 ± 0.009 ; GP + GLN: 0.047 ± 0.008 g.min⁻¹) and carbohydrate oxidation was lower (CON1: 0.129 ± 0.012 ; CON2: 0.140 ± 0.014 ; GP + ALA & GLY: 0.201 ± 0.019 ; GP + GLN: 0.218 ± 0.022 g.min⁻¹) compared to the glucose-polymer conditions.

During EX2, carbohydrate oxidation rate was greater ($P < 0.05$) in the GP + GLN condition after 10 min of exercise compared to the control conditions (CON1: $2.31 \pm$

0.14; CON2: 2.31 ± 0.16 ; GP + ALA & GLY: 2.63 ± 0.15 ; GP + GLN: 3.02 ± 0.21 g.min⁻¹), an observation not evident in the GP + ALA & GLY condition. At exhaustion, the carbohydrate oxidation rates were similar between conditions. Fat oxidation rates were greater ($P < 0.05$) at 10 and 20 min and at exhaustion during EX2 in the control conditions compared to the glucose-polymer conditions.

Total grams of carbohydrate oxidised during the recovery period and EX2 was significantly greater ($P < 0.05$) in the GP + GLN condition compared to the control conditions (CON1: 125 ± 9 ; CON2: 123 ± 13 ; GP + ALA & GLY: 153 ± 25 ; GP + GLN: 165 ± 25 g), an observation not apparent in the GP + ALA & GLY condition.

Serum insulin and plasma NEFA concentration. Serum insulin concentration was not different between conditions during EX1 (Fig. 4.5). During recovery, serum insulin concentration increased in both glucose-polymer conditions and reached a peak concentration after 60 min of recovery (30 min after ingestion of the first drink). Serum insulin concentration remained elevated in the glucose-polymer conditions compared to the control conditions until 180 min of recovery ($P < 0.01$). There was a tendency for the serum insulin concentration to be higher in the GP + GLN condition (57.5 ± 6.1 $\mu\text{IU}\cdot\text{ml}^{-1}$) compared to the GP + ALA & GLY condition (50.2 ± 7.4 $\mu\text{IU}\cdot\text{ml}^{-1}$) in the first hour after ingestion of the drink, however this difference was not statistically significant. Serum insulin concentration in the control conditions remained at a level similar to basal throughout recovery. During EX2, serum insulin concentration decreased in all conditions, with no difference observed between conditions.

Plasma NEFA concentration was not different between conditions during EX1 (Fig. 4.6). During recovery, plasma NEFA concentration increased in the control conditions and was higher compared to the glucose-polymer conditions ($P < 0.05$). Plasma NEFA concentration decreased in the glucose-polymer conditions, but not to a value significantly lower than basal. There was a tendency for the plasma NEFA concentration to be lower in the GP + GLN condition compared to the GP + ALA & GLY condition during the recovery period. During EX2, plasma NEFA concentration increased in all conditions with a greater increase ($P < 0.05$) in the glucose-polymer conditions (CON1: $+167 \pm 28$; CON2: $+147 \pm 14$; GP + ALA & GLY: $+852 \pm 154$; GP

+ GLN: 828 ± 283 , all %). There was no difference in the plasma NEFA concentration between conditions during EX2.

Plasma ammonia and amino acid concentration. Plasma ammonia concentration increased relative to basal value ($P < 0.05$) to a similar extent during EX1 in all conditions (CON1: $+647 \pm 85$; CON2: $+658 \pm 89$; GP + ALA & GLY: $+619 \pm 85$; GP + GLN: 619 ± 83 , all %), reaching a peak concentration at the 60 min time point in all conditions (Fig. 4.7). No difference was observed between conditions during EX1. During recovery, there was a tendency for plasma ammonia concentration to be elevated above basal levels in all conditions, with no differences observed between conditions. During EX2, the increase in plasma ammonia concentration was greater in the control conditions (CON1: $+709 \pm 227$; CON2: $+755 \pm 208$; GP + ALA & GLY: $+353 \pm 112$; GP + GLN: 336 ± 71 , all %), however no difference was observed between conditions.

During the recovery period, after glucose-polymer and glutamine ingestion, plasma glutamine concentration was significantly greater ($P < 0.05$) than the other three conditions for the first 90 min of recovery, and remained elevated above the control conditions until 180 min of recovery (Fig. 4.8). During the recovery period, after glucose-polymer and alanine and glycine ingestion, plasma alanine and glycine concentrations were significantly elevated ($P < 0.01$) compared to the other three conditions (Table 4.5). In addition, plasma serine concentration was significantly elevated ($P < 0.01$) during the recovery period after glucose-polymer and alanine and glycine ingestion (Table 4.5).

Total plasma BCAA concentration decreased by 8 % during EX1 in all conditions, parallel to the ~6-fold increase in plasma ammonia concentration (Table 4.5). After consumption of the drink, plasma BCAA concentration was lower in the glucose-polymer conditions relative to the control conditions during the recovery period (4-h time point: CON1: 385.3 ± 19.0 ; CON2: 383.7 ± 30.6 ; GP + ALA & GLY: 198.6 ± 14.2 ; GP + GLN: 172.7 ± 18.0 μM , $P < 0.05$), with a tendency for the plasma BCAA concentration to be lower in the GP & GLN condition compared to the GP + ALA & GLY condition ($P = 0.08$). At exhaustion, plasma BCAA concentration decreased in the control conditions relative to the concentration at the end of the recovery period

compared to the two glucose-polymer conditions (CON1: -6.3 ± 7.8 ; CON2: -8.0 ± 2.0 ; GP + ALA & GLY: $+31.8 \pm 8.7$; GP + GLN: $+67.1 \pm 21.4$, all %; $P < 0.001$).

There was a tendency for both plasma phenylalanine (area under the curve (AUC): CON1: 64.7 ± 3.4 ; CON2: 65.5 ± 3.1 ; GP + ALA & GLY: 59.6 ± 6.8 ; GP + GLN: 47.5 ± 3.8 , all $\text{mM}\cdot\text{h}^{-1}$) and tyrosine (AUC: CON1: 70.3 ± 4.1 ; CON2: 72.2 ± 2.8 ; GP + ALA & GLY: 64.1 ± 6.8 ; GP + GLN: 58.0 ± 6.8 , all $\text{mM}\cdot\text{h}^{-1}$) concentration to be lower during the recovery period in the GP & GLN condition compared to the other three conditions. Indeed, the sum of plasma phenylalanine and tyrosine concentrations (Fig. 4.9) was significantly lower in the GP + GLN condition from 90 min recovery until exhaustion (compared to CON1), from 60 min recovery until exhaustion (CON2) and at 60, 90 and 180 min of recovery in the GP + ALA & GLY condition (all $P < 0.05$). However, the sum of plasma phenylalanine and tyrosine concentrations was significantly lower ($P < 0.05$) in the GP + ALA & GLY condition than the control conditions at 240 min of recovery and at exhaustion only.

The sum of the essential amino acids (His, Iso, Leu, Lys, Met, Phe, Thr, Try & Val) was significantly lower ($P < 0.05$) during the recovery period in the GP + GLN condition compared to the other three conditions (Fig. 4.10). The sum of the non-essential amino acids (Ala, Arg, Asp, Asn, Glu, Gln, Gly, Ser & Tyr), sum of the non-metabolisable amino acids (Thr, Met, Phe, Lys, Gly & Tyr) and the sum of all amino acids were significantly higher ($P < 0.01$) during the recovery period in the GP + ALA & GLY condition compared to the other three conditions.

From 75 min of recovery, the free tryptophan:BCAA ratio was significantly higher in the GP + GLN condition ($P < 0.05$) compared to the other three conditions (Table 4.5). At exhaustion, the free tryptophan:BCAA ratio was significantly higher ($P < 0.005$) compared to the other three conditions in the GP + GLN condition (CON1: 0.13 ± 0.02 ; CON2: 0.12 ± 0.01 ; GP + ALA & GLY: 0.09 ± 0.01 ; GP + GLN: 0.17 ± 0.01)

Rehydration, urine output data and plasma volume. The extent of rehydration during the recovery period was not different between conditions (CON1: 105.4 ± 7.6 ; CON2: -94.6 ± 12.3 ; GP + ALA & GLY: 99.7 ± 7.9 ; GP + GLN: 104.2 ± 7.3 , all %; see Chapter

3 for calculation). Total urine output during the recovery period was not different between conditions (CON1: 2274 ± 262 ; CON2: 2170 ± 191 ; GP + ALA & GLY: 1997 ± 209 ; GP + GLN: 2225 ± 246 , all ml). Plasma volume changes were not different between conditions during EX1. During recovery there was a tendency for plasma volume to be greater in the glucose-polymer conditions. No difference was observed in plasma volume at exhaustion between conditions (data not shown).

Table 4.2 Oxygen uptake ($\dot{V}O_2$), $\% \dot{V}O_{2max}$, heart rate (HR), respiratory exchange ratio (RER), carbohydrate oxidation rate (CHO Ox) and fat oxidation rate (Fat Ox) at rest and during first bout of exercise period in the control, GP + ALA & GLY and GP + GLN conditions.

	Condition	Basal	EX1		
			30 min	60 min	85 min
$\dot{V}O_2$ (l.min ⁻¹)	CON1	0.22 ± 0.03	3.39 ± 0.10*	3.23 ± 0.14*	3.35 ± 0.15*
	CON2	0.23 ± 0.02	3.30 ± 0.08*	3.19 ± 0.10*	3.24 ± 0.14*
	GP + ALA & GLY	0.22 ± 0.01	3.27 ± 0.14*	3.15 ± 0.17*	3.18 ± 0.18*
	GP + GLN	0.21 ± 0.01	3.25 ± 0.12*	3.08 ± 0.11*	3.18 ± 0.13*
$\% \dot{V}O_{2max}$	CON1	4.6 ± 0.6	70.1 ± 0.9*	66.8 ± 1.8*	69.2 ± 1.6*
	CON2	4.7 ± 0.5	68.4 ± 1.1*	66.0 ± 1.5*	66.9 ± 1.6*
	GP + ALA & GLY	4.7 ± 0.3	67.6 ± 2.5*	65.2 ± 2.9*	65.8 ± 3.4*
	GP + GLN	4.4 ± 0.2	67.3 ± 1.6*	63.8 ± 1.2*	65.7 ± 1.6*
HR (b.min ⁻¹)	CON1	—	138 ± 5	156 ± 5 [#]	156 ± 6 [#]
	CON2	—	144 ± 7	160 ± 4 [#]	160 ± 5 [#]
	GP + ALA & GLY	—	141 ± 5	155 ± 6 [#]	158 ± 6 [#]
	GP + GLN	—	142 ± 5	154 ± 7 [#]	157 ± 7 [#]
RER	CON1	0.87 ± 0.03	0.92 ± 0.01*	0.90 ± 0.01*	0.91 ± 0.01*
	CON2	0.92 ± 0.02	0.92 ± 0.01	0.90 ± 0.01	0.91 ± 0.01
	GP + ALA & GLY	0.86 ± 0.02	0.92 ± 0.01*	0.88 ± 0.01	0.89 ± 0.01
	GP + GLN	0.91 ± 0.02	0.92 ± 0.01	0.89 ± 0.01	0.89 ± 0.01
CHO Ox (g.min ⁻¹)	CON1	0.16 ± 0.04	2.91 ± 0.09*	2.53 ± 0.16*	2.79 ± 0.27*
	CON2	0.20 ± 0.03	2.91 ± 0.18*	2.54 ± 0.13*	2.66 ± 0.21*
	GP + ALA & GLY	0.14 ± 0.02	2.84 ± 0.27*	2.22 ± 0.21*	2.44 ± 0.26*
	GP + GLN	0.17 ± 0.02	2.80 ± 0.18*	2.31 ± 0.16*	2.40 ± 0.17*
Fat Ox (g.min ⁻¹)	CON1	0.05 ± 0.01	0.49 ± 0.02*	0.57 ± 0.05*	0.52 ± 0.05*
	CON2	0.03 ± 0.01	0.45 ± 0.08*	0.55 ± 0.05*	0.52 ± 0.05*
	GP + ALA & GLY	0.05 ± 0.01	0.46 ± 0.06*	0.66 ± 0.04*	0.58 ± 0.04*
	GP + GLN	0.03 ± 0.01	0.47 ± 0.04*	0.59 ± 0.02*	0.60 ± 0.05*

Values are means ± SEM for 7 subjects. * $P < 0.05$ vs. basal value, # $P < 0.05$ vs. 30 min (EX1). N.B. Heart rate was not measured at rest.

Table 4.2 (cont'd) Oxygen uptake ($\dot{V}O_2$), $\% \dot{V}O_{2max}$, heart rate (HR), respiratory exchange ratio (RER), carbohydrate oxidation rate (CHO Ox) and fat oxidation rate (Fat Ox) during the recovery period in the control, GP + ALA & GLY and GP + GLN conditions.

		RECOVERY							
Condition		30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
$\dot{V}O_2$ (l.min ⁻¹)	CON1	0.30 ± 0.03	0.29 ± 0.02	0.28 ± 0.03	0.26 ± 0.02	0.29 ± 0.03	0.26 ± 0.03	0.26 ± 0.02	0.25 ± 0.03
	CON2	0.27 ± 0.02	0.30 ± 0.03	0.29 ± 0.03	0.30 ± 0.04	0.28 ± 0.03	0.29 ± 0.03	0.28 ± 0.03	0.27 ± 0.03
	GP + ALA & GLY	0.28 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.25 ± 0.04	0.29 ± 0.03	0.27 ± 0.02	0.28 ± 0.01	0.27 ± 0.02
	GP + GLN	0.24 ± 0.02	0.31 ± 0.03	0.28 ± 0.02	0.27 ± 0.03	0.29 ± 0.03	0.27 ± 0.03	0.28 ± 0.03	0.26 ± 0.03
$\% \dot{V}O_{2max}$	CON1	6.1 ± 0.6	6.0 ± 0.4	5.8 ± 0.5	5.4 ± 0.4	5.9 ± 0.6	5.5 ± 0.5	5.4 ± 0.4	5.2 ± 0.6
	CON2	5.6 ± 0.5	6.2 ± 0.6	6.0 ± 0.7	6.2 ± 0.8	5.9 ± 0.6	5.9 ± 0.6	5.8 ± 0.6	5.5 ± 0.6
	GP + ALA & GLY	5.8 ± 0.4	6.1 ± 0.5	6.1 ± 0.4	5.1 ± 0.8	6.2 ± 0.5	5.5 ± 0.4	5.7 ± 0.3	5.5 ± 0.4
	GP + GLN	5.0 ± 0.4	6.4 ± 0.5	5.8 ± 0.5	5.6 ± 0.5	6.0 ± 0.6	5.5 ± 0.5	5.7 ± 0.5	5.3 ± 0.5
HR (b.min ⁻¹)	CON1	—	—	—	—	—	—	—	—
	CON2	—	—	—	—	—	—	—	—
	GP + ALA & GLY	—	—	—	—	—	—	—	—
	GP + GLN	—	—	—	—	—	—	—	—
RER	CON1	0.80 ± 0.03	0.87 ± 0.02	0.81 ± 0.02	0.82 ± 0.01	0.84 ± 0.01	0.84 ± 0.01	0.85 ± 0.02	0.79 ± 0.03
	CON2	0.79 ± 0.02*	0.87 ± 0.02	0.84 ± 0.01*	0.82 ± 0.01*	0.83 ± 0.02*	0.87 ± 0.01	0.85 ± 0.02*	0.82 ± 0.01*
	GP + ALA & GLY	0.79 ± 0.03*	0.84 ± 0.02	0.91 ± 0.02 ^{a,b}	0.89 ± 0.02 ^{ab}	0.90 ± 0.01 ^{ab}	0.92 ± 0.01 ^a	0.92 ± 0.01 ^{a,b}	0.90 ± 0.01 ^{a,b}
	GP + GLN	0.79 ± 0.01*	0.87 ± 0.02	0.92 ± 0.02 ^{ab}	0.93 ± 0.02 ^{ab}	0.93 ± 0.01 ^{ab}	0.94 ± 0.02 ^{ab}	0.94 ± 0.02 ^{ab}	0.92 ± 0.04 ^{ab}
CHO Ox (g.min ⁻¹)	CON1	0.12 ± 0.04	0.19 ± 0.03	0.09 ± 0.03	0.12 ± 0.02	0.15 ± 0.03	0.14 ± 0.01	0.14 ± 0.02	0.08 ± 0.03
	CON2	0.08 ± 0.02	0.19 ± 0.02	0.15 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	0.19 ± 0.03	0.15 ± 0.02	0.12 ± 0.02
	GP + ALA & GLY	0.09 ± 0.04	0.15 ± 0.03	0.24 ± 0.03	0.19 ± 0.04	0.23 ± 0.02	0.23 ± 0.02	0.25 ± 0.03	0.22 ± 0.02
	GP + GLN	0.08 ± 0.01	0.20 ± 0.03	0.23 ± 0.03	0.24 ± 0.03	0.26 ± 0.03	0.26 ± 0.03	0.26 ± 0.02	0.21 ± 0.02
Fat Ox (g.min ⁻¹)	CON1	0.10 ± 0.02	0.07 ± 0.01	0.10 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.02
	CON2	0.10 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	0.10 ± 0.02	0.09 ± 0.02	0.06 ± 0.01	0.08 ± 0.02	0.08 ± 0.02
	GP + ALA & GLY	0.10 ± 0.02	0.08 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	GP + GLN	0.09 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.02

Values are means ± SEM for 7 subjects. *different to basal value ($P < 0.05$); ^adifferent to CON1 ($P < 0.05$); ^bdifferent to CON2 ($P < 0.05$). N.B. Heart rate was not measured at rest.

Table 4.2 (cont'd) Oxygen uptake ($\dot{V}O_2$), $\% \dot{V}O_{2max}$, heart rate (HR), respiratory exchange ratio (RER), carbohydrate oxidation rate (CHO Ox) and fat oxidation rate (Fat Ox) during the second bout of exercise in the control, GP + ALA & GLY and GP + GLN conditions.

	Condition	EX2		
		10 min	20 min	Exh
$\dot{V}O_2$ (l.min ⁻¹)	CON1	3.54 ± 0.17*	3.60 ± 0.08*	3.31 ± 0.09*
	CON2	3.47 ± 0.17*	3.20 ± 0.04*	3.14 ± 0.19*
	GP + ALA & GLY	3.11 ± 0.14*	3.16 ± 0.20*	2.98 ± 0.23*
	GP + GLN	3.19 ± 0.11*	3.19 ± 0.19*	2.99 ± 0.18*
$\% \dot{V}O_{2max}$	CON1	71.9 ± 0.1*	69.7 ± 2.5*	66.5 ± 1.4*
	CON2	68.2 ± 1.5*	66.3 ± 1.6*	64.8 ± 2.6*
	GP + ALA & GLY	64.4 ± 2.3*	65.3 ± 3.6*	61.4 ± 3.8*
	GP + GLN	66.0 ± 1.1*	66.3 ± 1.6*	61.7 ± 2.6*
HR (b.min ⁻¹)	CON1	165 ± 5	170 ± 5	172 ± 5
	CON2	165 ± 5	169 ± 4	169 ± 5
	GP + ALA & GLY	153 ± 5 ^{ab}	155 ± 6 ^{ab}	154 ± 7 ^{ab}
	GP + GLN	159 ± 5 ^{ab}	163 ± 6 ^{ab}	162 ± 7 ^{ab}
RER	CON1	0.87 ± 0.01	0.88 ± 0.01	0.87 ± 0.02
	CON2	0.88 ± 0.01	0.87 ± 0.02	0.88 ± 0.02
	GP + ALA & GLY	0.91 ± 0.01*	0.91 ± 0.01*	0.88 ± 0.02
	GP + GLN	0.94 ± 0.01 ^{ab}	0.92 ± 0.01	0.89 ± 0.01
CHO Ox (g.min ⁻¹)	CON1	2.31 ± 0.14*	2.54 ± 0.14*	2.39 ± 0.38*
	CON2	2.31 ± 0.16*	2.09 ± 0.23*	2.37 ± 0.48*
	GP + ALA & GLY	2.63 ± 0.15*	2.66 ± 0.26*	2.20 ± 0.41*
	GP + GLN	3.02 ± 0.21*	2.74 ± 0.27*	2.22 ± 0.31*
Fat Ox (g.min ⁻¹)	CON1	0.78 ± 0.06*	0.75 ± 0.03*	0.75 ± 0.10*
	CON2	0.69 ± 0.06*	0.74 ± 0.08*	0.67 ± 0.09*
	GP + ALA & GLY	0.47 ± 0.02 ^{ab}	0.48 ± 0.02 ^{ab}	0.58 ± 0.08 ^{ab}
	GP + GLN	0.35 ± 0.07 ^{ab}	0.47 ± 0.07 ^{ab}	0.58 ± 0.07 ^{ab}

Values are means ± SEM for 7 subjects. *different to basal value ($P < 0.05$); ^adifferent to CON1 ($P < 0.05$); ^bdifferent to CON2 ($P < 0.05$).

Table 4.3 Percentage difference in endurance capacity between conditions (mean ± SEM).

	CON1	CON2	GP + ALA & GLY	GP + GLN
CON1		-7.1 ± 8.0	9.6 ± 16.4	12.8 ± 18.1
CON2			22.3 ± 18.0	23.6 ± 16.3
GP + ALA & GLY				5.4 ± 10.4
GP + GLN				

Table 4.4 Plasma amino acid concentrations at rest and during the first bout of exercise during the control, GP + ALA & GLY and GP + GLN conditions

	Condition	Basal	EX1		
			30 min	60 min	85 min
Alanine	CON1	375 ± 32	483 ± 47*	465 ± 30*	395 ± 36
	CON2	367 ± 52	458 ± 19*	489 ± 73**	439 ± 67
	GP + ALA & GLY	345 ± 25	506 ± 34**	511 ± 59**	460 ± 34**
	GP + GLN	368 ± 45	567 ± 68**b	602 ± 74**abc	564 ± 77**abc
Glutamate	CON1	37 ± 5	33 ± 3	34 ± 4	41 ± 6
	CON2	41 ± 7	31 ± 2	36 ± 4	43 ± 6
	GP + ALA & GLY	37 ± 3	36 ± 1	38 ± 4	45 ± 3
	GP + GLN	41 ± 2	35 ± 3	31 ± 5	40 ± 4
Valine	CON1	187 ± 9	190 ± 15	177 ± 5	168 ± 9
	CON2	172 ± 4	185 ± 14	173 ± 4	167 ± 10
	GP + ALA & GLY	178 ± 8	177 ± 9	165 ± 17	175 ± 8
	GP + GLN	172 ± 9	181 ± 13	164 ± 6	167 ± 9
Isoleucine	CON1	61 ± 5	62 ± 5	55 ± 5	54 ± 6
	CON2	58 ± 2	61 ± 3	55 ± 1	52 ± 3
	GP + ALA & GLY	60 ± 3	60 ± 4	53 ± 3	51 ± 2
	GP + GLN	52 ± 2	55 ± 3	49 ± 2	48 ± 1
Leucine	CON1	107 ± 6	109 ± 8	93 ± 8	88 ± 7*
	CON2	100 ± 2	111 ± 10	97 ± 2	89 ± 4
	GP + ALA & GLY	103 ± 4	93 ± 6 ^{ab}	83 ± 7 ^f	85 ± 3*
	GP + GLN	90 ± 3 ^a	96 ± 5	86 ± 2	85 ± 3
Tyrosine	CON1	55 ± 5	65 ± 5*	67 ± 5*	65 ± 6*
	CON2	55 ± 7	63 ± 5	65 ± 3*	60 ± 4
	GP + ALA & GLY	48 ± 4	54 ± 7 ^{ab}	63 ± 6*	59 ± 8*
	GP + GLN	48 ± 5	54 ± 8 ^{ab}	54 ± 6 ^{ab,c}	57 ± 6*
Phenylalanine	CON1	43 ± 2	47 ± 4	46 ± 3	46 ± 4
	CON2	44 ± 5	47 ± 3	48 ± 2	47 ± 4
	GP + ALA & GLY	42 ± 3	44 ± 3	48 ± 7	50 ± 6*
	GP + GLN	36 ± 3 ^{ac}	41 ± 4	40 ± 3 ^{ac}	42 ± 3 ^c
Tryptophan	CON1	45 ± 2	46 ± 5	44 ± 4	43 ± 6
	CON2	39 ± 3	45 ± 5	44 ± 5	44 ± 4
	GP + ALA & GLY	36 ± 4 ^a	35 ± 4 ^{ab,d}	38 ± 5	41 ± 6
	GP + GLN	43 ± 3	45 ± 4	43 ± 3	44 ± 4

Values are means ± SEM for 7 subjects, expressed in $\mu\text{mol/l}$. NH_3 , ammonia. *different to basal value ($P < 0.05$), **different to basal value ($P < 0.01$), ^adifferent to CON1 ($P < 0.05$), ^bdifferent to CON2 ($P < 0.05$), ^cdifferent to GP + ALA & GLY ($P < 0.05$), ^ddifferent to GP + GLN ($P < 0.05$)

Table 4.4 (cont'd) Plasma amino acid concentrations during recovery and the second bout of exercise during the control, GP + ALA & GLY and GP + GLN conditions

	Condition	RECOVERY							EX2
		60 min	75 min	90 min	105 min	120 min	180 min	240 min	Exh
Alanine	CON1	260 ± 37**	304 ± 35	271 ± 33*	269 ± 30*	234 ± 29**	223 ± 25**	320 ± 27	374 ± 41
	CON2	312 ± 38	301 ± 22	312 ± 37	265 ± 18*	260 ± 34**	204 ± 19*	267 ± 25**	339 ± 51
	GP + ALA & GLY	602 ± 38** ^{a,b,d}	627 ± 61** ^{a,b,d}	611 ± 49** ^{a,b,d}	621 ± 56** ^{a,b,d}	647 ± 57** ^{a,b,d}	684 ± 42** ^{a,b,c}	541 ± 66** ^{a,b,d}	381 ± 35
	GP + GLN	394 ± 42 ^a	396 ± 45 ^{ab}	378 ± 44 ^a	341 ± 41	352 ± 37 ^{ab}	315 ± 37 ^{ab}	275 ± 35*	376 ± 43
Glutamate	CON1	27 ± 5	27 ± 3	29 ± 4	25 ± 3*	24 ± 4*	27 ± 4	35 ± 3	48 ± 8*
	CON2	31 ± 4	30 ± 2	30 ± 4*	26 ± 3**	32 ± 5	30 ± 5	42 ± 10	52 ± 12*
	GP + ALA & GLY	39 ± 4 ^a	43 ± 9 ^{ab}	34 ± 3	34 ± 2*	35 ± 2 ^a	39 ± 5 ^a	38 ± 4	39 ± 5 ^b
	GP + GLN	42 ± 4 ^a	40 ± 2 ^a	41 ± 3 ^{ab}	29 ± 3	34 ± 3	35 ± 4	31 ± 3 ^b	39 ± 4 ^b
Valine	CON1	149 ± 13**	161 ± 11*	156 ± 14**	158 ± 12*	155 ± 12**	163 ± 14*	192 ± 9	183 ± 14
	CON2	168 ± 15	163 ± 9	175 ± 17	150 ± 7	170 ± 17	158 ± 18	194 ± 12	176 ± 12
	GP + ALA & GLY	162 ± 8	166 ± 18	147 ± 8** ^b	145 ± 6**	136 ± 11** ^b	120 ± 10** ^{a,b}	116 ± 10** ^{a,b}	137 ± 13** ^{a,b}
	GP + GLN	145 ± 7 ^b	141 ± 4** ^{b,c}	127 ± 7** ^{a,b}	118 ± 9** ^{a,b,c}	113 ± 6** ^{a,b,c}	102 ± 11** ^{a,b}	101 ± 10** ^{a,b}	143 ± 7** ^{a,b}
Isoleucine	CON1	45 ± 3**	47 ± 3**	47 ± 3**	43 ± 4**	50 ± 3*	57 ± 4	73 ± 4**	68 ± 8
	CON2	52 ± 5	53 ± 6	52 ± 7	48 ± 4*	56 ± 6	60 ± 11	74 ± 9**	68 ± 8**
	GP + ALA & GLY	48 ± 2*	43 ± 2** ^b	43 ± 2**	39 ± 2**	38 ± 2** ^{a,b}	31 ± 2** ^{a,b}	33 ± 2** ^{a,b}	47 ± 3** ^{a,b}
	GP + GLN	40 ± 3 ^b	38 ± 2** ^b	33 ± 3** ^{a,b,c}	30 ± 3** ^{a,b}	28 ± 3** ^{a,b,c}	24 ± 3** ^{a,b}	27 ± 3** ^{a,b}	46 ± 1 ^{a,b}
Leucine	CON1	72 ± 7**	79 ± 6**	78 ± 8**	73 ± 7**	86 ± 7**	100 ± 9	120 ± 8	107 ± 10
	CON2	91 ± 8	87 ± 7	84 ± 9*	81 ± 6*	97 ± 10	95 ± 14	116 ± 11*	107 ± 8
	GP + ALA & GLY	78 ± 3**	69 ± 2** ^b	63 ± 3** ^{a,b}	59 ± 4**	55 ± 5** ^{a,b}	45 ± 5** ^{a,b}	50 ± 5** ^{a,b}	78 ± 10** ^{a,b}
	GP + GLN	73 ± 4 ^b	66 ± 4** ^b	57 ± 5** ^{a,b}	52 ± 5** ^{a,b}	47 ± 4** ^{a,b}	39 ± 5** ^{a,b}	44 ± 5** ^{a,b}	80 ± 5 ^{a,b}
Tyrosine	CON1	47 ± 5	53 ± 3	48 ± 3	52 ± 2	48 ± 4	41 ± 5**	55 ± 7	67 ± 6*
	CON2	57 ± 3 ^a	52 ± 2	51 ± 3	48 ± 1	52 ± 3	43 ± 4*	52 ± 4	56 ± 5 ^a
	GP + ALA & GLY	53 ± 7	45 ± 4	51 ± 5	48 ± 5	44 ± 6	37 ± 5*	33 ± 4** ^{a,b}	46 ± 4 ^{a,b}
	GP + GLN	47 ± 3 ^b	48 ± 4	44 ± 7	43 ± 5 ^a	37 ± 4 ^{a,b}	31 ± 5** ^{a,b}	32 ± 5** ^{a,b}	42 ± 4 ^{a,b}
Phenylalanine	CON1	41 ± 2	44 ± 3	44 ± 2	46 ± 3	42 ± 3	46 ± 4	55 ± 5**	61 ± 7**
	CON2	45 ± 2	49 ± 4	46 ± 4	43 ± 2	43 ± 2	44 ± 5	51 ± 4	54 ± 5**
	GP + ALA & GLY	46 ± 6	43 ± 5	42 ± 4	41 ± 4 ^a	40 ± 4 ^a	38 ± 6	38 ± 6 ^{a,b}	41 ± 4 ^b
	GP + GLN	35 ± 2 ^{b,c}	36 ± 2 ^{a,b}	33 ± 4 ^{a,b,c}	33 ± 3 ^{a,b,c}	32 ± 2 ^{a,b,c}	30 ± 3 ^{a,b,c}	32 ± 4 ^{a,b}	41 ± 4 ^{a,b}
Tryptophan	CON1	34 ± 6**	33 ± 4**	34 ± 5**	33 ± 4**	33 ± 4**	35 ± 4**	39 ± 3	44 ± 5
	CON2	40 ± 4	36 ± 3	37 ± 4	34 ± 2	36 ± 2	31 ± 4*	40 ± 3	40 ± 4
	GP + ALA & GLY	41 ± 6	40 ± 5	35 ± 5	36 ± 5	33 ± 5	31 ± 5	25 ± 4 ^{a,b,d}	24 ± 4 ^{a,b,d}
	GP + GLN	38 ± 3	40 ± 2	36 ± 4	37 ± 3	34 ± 3**	29 ± 3**	32 ± 4** ^{a,b}	45 ± 1

Values are means ± SEM for 7 subjects, expressed in μmol/l. NH₃, ammonia. *different to basal value ($P < 0.05$), **different to basal value ($P < 0.01$), ^adifferent to CON1 ($P < 0.05$), ^bdifferent to CON2 ($P < 0.05$), ^cdifferent to GP + ALA & GLY ($P < 0.05$), ^ddifferent to GP + GLN ($P < 0.05$)

Table 4.4 (cont'd) Plasma amino acid concentrations at rest and during the first bout of exercise during the control, GP + ALA & GLY and GP + GLN conditions

	Condition	Basal	EX1		
			30 min	60 min	85 min
Lysine	CON1	109 ± 9	142 ± 12*	126 ± 16	132 ± 16
	CON2	108 ± 10	139 ± 13	122 ± 14	122 ± 13
	GP + ALA & GLY	89 ± 13	111 ± 22	115 ± 27	101 ± 17
	GP + GLN	106 ± 10	126 ± 17	113 ± 14	113 ± 14
Ornithine	CON1	43 ± 2	49 ± 3	40 ± 4	38 ± 3
	CON2	46 ± 4	44 ± 3	39 ± 3	43 ± 4
	GP + ALA & GLY	49 ± 7	43 ± 6	43 ± 9	48 ± 7 ^c
	GP + GLN	49 ± 4	43 ± 5	45 ± 4	42 ± 3
Methionine	CON1	23 ± 2	25 ± 3	24 ± 3	24 ± 2
	CON2	23 ± 1	25 ± 2	25 ± 2	24 ± 3
	GP + ALA & GLY	23 ± 2	24 ± 2	25 ± 2	23 ± 2
	GP + GLN	25 ± 1	28 ± 2	28 ± 2 ^a	29 ± 2 ^{abc}
Threonine	CON1	119 ± 8	109 ± 7	112 ± 9	102 ± 10
	CON2	115 ± 9	113 ± 9	114 ± 12	106 ± 12
	GP + ALA & GLY	114 ± 10	120 ± 10	110 ± 10	115 ± 10
	GP + GLN	102 ± 8	111 ± 9	105 ± 7	106 ± 10
Glycine	CON1	244 ± 32	230 ± 17	199 ± 12	205 ± 28
	CON2	206 ± 14	223 ± 14	230 ± 24	202 ± 13
	GP + ALA & GLY	242 ± 33	256 ± 37	230 ± 27	233 ± 36
	GP + GLN	249 ± 43	268 ± 40	245 ± 41	251 ± 45
Histidine	CON1	45 ± 7	46 ± 5	42 ± 5	41 ± 4
	CON2	47 ± 8	41 ± 4	51 ± 8	49 ± 9
	GP + ALA & GLY	48 ± 9	44 ± 9	48 ± 8	45 ± 6
	GP + GLN	39 ± 4	44 ± 5	48 ± 2	49 ± 5
Aspartate	CON1	10 ± 1	8 ± 1	9 ± 1	9 ± 1
	CON2	9 ± 1	9 ± 1	10 ± 1	10 ± 2
	GP + ALA & GLY	11 ± 1	11 ± 1 ^a	12 ± 2 ^a	12 ± 1 ^a
	GP + GLN	11 ± 1	11 ± 1 ^a	12 ± 1 ^a	12 ± 1 ^{ab}
Asparagine	CON1	43 ± 5	37 ± 2	33 ± 1	33 ± 3 [†]
	CON2	37 ± 4	38 ± 3	37 ± 3	35 ± 3
	GP + ALA & GLY	36 ± 3	38 ± 3	33 ± 3	35 ± 4
	GP + GLN	34 ± 2	37 ± 2	34 ± 2	35 ± 3
Serine	CON1	123 ± 5	124 ± 9	127 ± 10	118 ± 9
	CON2	115 ± 6	112 ± 15	122 ± 12	116 ± 10
	GP + ALA & GLY	118 ± 6	122 ± 8	112 ± 9	124 ± 8
	GP + GLN	119 ± 5	128 ± 8	114 ± 6	117 ± 8
Arginine	CON1	90 ± 4	91 ± 7	89 ± 6	81 ± 7
	CON2	83 ± 5	95 ± 8 [†]	91 ± 7	84 ± 7
	GP + ALA & GLY	79 ± 7 ^a	86 ± 8	81 ± 10	86 ± 10
	GP + GLN	72 ± 4 ^{ab}	84 ± 6 ^{†a}	77 ± 6 ^{ab}	82 ± 8

Values are means ± SEM for 7 subjects, expressed in μmol/l. NH₃, ammonia. *different to basal value ($P < 0.05$), **different to basal value ($P < 0.01$), ^adifferent to CON1 ($P < 0.05$), ^bdifferent to CON2 ($P < 0.05$), ^cdifferent to GP + ALA & GLY ($P < 0.05$), ^ddifferent to GP + GLN ($P < 0.05$)

Table 4.4 (cont'd) Plasma amino acid concentrations during recovery and the second bout of exercise during the control, GP + ALA & GLY and GP + GLN conditions

	Condition	RECOVERY							EX2
		60 min	75 min	90 min	105 min	120 min	180 min	240 min	Exh
Lysine	CON1	112 ± 21	102 ± 13	103 ± 12	107 ± 11	113 ± 16	114 ± 12	118 ± 19	136 ± 18
	CON2	114 ± 12	101 ± 16	98 ± 17	102 ± 12	107 ± 15	98 ± 19	117 ± 18	115 ± 20
	GP + ALA & GLY	94 ± 12	86 ± 10	85 ± 9	76 ± 7	76 ± 10 ^a	65 ± 10 ^{ab}	56 ± 7 ^{ab}	58 ± 2 ^{ab}
	GP + GLN	79 ± 3 ^{ab}	78 ± 6	70 ± 9 [*]	71 ± 5 ^{*a}	66 ± 7 ^{*ab}	61 ± 8 ^{**ab}	65 ± 10 ^{*ab}	74 ± 8 ^{ab}
Ornithine	CON1	36 ± 4	36 ± 2 [*]	35 ± 3 [*]	35 ± 2 [*]	37 ± 5	39 ± 3	39 ± 2	37 ± 3
	CON2	41 ± 3	34 ± 1 ^{**}	35 ± 3 [†]	35 ± 2 [†]	38 ± 3 [*]	35 ± 1 ^{**}	37 ± 3 [*]	31 ± 3 ^{**}
	GP + ALA & GLY	49 ± 7 ^{ab}	50 ± 9 ^{abd}	46 ± 7 ^{ab}	41 ± 7 [*]	43 ± 8	39 ± 8 [*]	34 ± 4 ^{**}	39 ± 9 ^{**b}
	GP + GLN	42 ± 4 [*]	42 ± 4 ^{ab}	40 ± 5 ^{ab}	43 ± 4	40 ± 4 ^{**}	41 ± 3 [*]	40 ± 5 [*]	38 ± 3 ^{**}
Methionine	CON1	19 ± 3 [*]	20 ± 1	19 ± 1 [*]	18 ± 1 [*]	18 ± 1 ^{**}	19 ± 2 [*]	22 ± 3	24 ± 3
	CON2	20 ± 3	20 ± 2	19 ± 2	17 ± 1 [†]	18 ± 2 [*]	17 ± 2 ^{**}	21 ± 2	22 ± 2
	GP + ALA & GLY	21 ± 1	19 ± 1 [*]	19 ± 1 [*]	20 ± 1	21 ± 3	21 ± 3 ^b	19 ± 4 [*]	22 ± 4
	GP + GLN	23 ± 2 ^a	22 ± 2	20 ± 2 [*]	20 ± 2 [†]	18 ± 1 ^{**}	16 ± 1 ^{**c}	15 ± 1 ^{**abc}	20 ± 1 [*]
Threonine	CON1	86 ± 7 [†]	88 ± 5 ^{**}	87 ± 8 [†]	85 ± 6 [†]	82 ± 7 ^{**}	87 ± 7 [†]	110 ± 8	103 ± 9
	CON2	97 ± 7	97 ± 8	96 ± 8	85 ± 6 [†]	92 ± 6 [*]	91 ± 7 [*]	101 ± 10	100 ± 10
	GP + ALA & GLY	136 ± 15 ^{*abd}	158 ± 20 ^{**abd}	133 ± 1 ^{abd}	136 ± 17 ^{*abd}	134 ± 16 ^{abd}	129 ± 15 ^{abd}	106 ± 13 ^d	114 ± 27 ^d
	GP + GLN	89 ± 6	90 ± 6	84 ± 7	74 ± 5 ^{**}	75 ± 7 [*]	72 ± 7 ^{**}	64 ± 7 ^{**ab}	74 ± 5 ^{**ab}
Glycine	CON1	163 ± 20 ^{**}	165 ± 16 ^{**}	167 ± 21 ^{**}	156 ± 14 ^{**}	156 ± 17 ^{**}	162 ± 16 ^{**}	191 ± 21	199 ± 21
	CON2	174 ± 11	181 ± 17	181 ± 18	155 ± 10	163 ± 11	154 ± 9	182 ± 24	190 ± 26
	GP + ALA & GLY	554 ± 57 ^{*abd}	659 ± 78 ^{*abd}	670 ± 66 ^{*abd}	677 ± 48 ^{*abd}	757 ± 77 ^{*abd}	894 ± 69 ^{*abd}	714 ± 101 ^{*abd}	423 ± 45 ^{*abd}
	GP + GLN	186 ± 24 [*]	183 ± 26 [*]	170 ± 25 ^{**}	159 ± 17 ^{**}	157 ± 16 ^{**}	151 ± 14 ^{**}	149 ± 13 ^{**}	177 ± 28 ^{**}
Histidine	CON1	31 ± 6 [*]	41 ± 3	34 ± 4	40 ± 3	36 ± 5	37 ± 5	54 ± 11	57 ± 8
	CON2	37 ± 5	36 ± 4	37 ± 2	37 ± 5	37 ± 5	37 ± 6	52 ± 9	47 ± 8
	GP + ALA & GLY	41 ± 8	43 ± 8	42 ± 8	38 ± 8	35 ± 7	32 ± 6 [*]	42 ± 8	34 ± 5 ^a
	GP + GLN	36 ± 3	36 ± 2	34 ± 5	38 ± 2	27 ± 2	24 ± 4 [*]	27 ± 4 ^{abc}	36 ± 4 ^a
Aspartate	CON1	7 ± 1	8 ± 1	7 ± 1 [*]	8 ± 1	6 ± 1 [*]	7 ± 1 [*]	9 ± 1	11 ± 2
	CON2	9 ± 1	10 ± 1	9 ± 1	8 ± 1	9 ± 1 ^a	9 ± 1	11 ± 1	12 ± 1 [*]
	GP + ALA & GLY	11 ± 1 ^a	11 ± 1 ^a	14 ± 2 ^{**ab}	13 ± 2 ^{ab}	12 ± 2 ^{ab}	12 ± 1 ^{ab}	12 ± 1	10 ± 2
	GP + GLN	13 ± 1 ^{ab}	14 ± 1 ^{ab}	14 ± 2 ^{ab}	11 ± 1 ^{ab}	12 ± 2 ^{ab}	13 ± 2 ^{ab}	10 ± 1	12 ± 1
Asparagine	CON1	27 ± 3 ^{**}	29 ± 2 ^{**}	28 ± 3 ^{**}	28 ± 2 ^{**}	27 ± 2 ^{**}	30 ± 2 ^{**}	35 ± 3	35 ± 3
	CON2	32 ± 3 ^b	33 ± 3	32 ± 3 [*]	28 ± 1 ^{**}	32 ± 3 [*]	32 ± 3 [*]	37 ± 3	37 ± 3
	GP + ALA & GLY	40 ± 2 ^{abd}	41 ± 3 ^{*abd}	38 ± 2 ^{abd}	39 ± 2 ^{abd}	39 ± 3 ^{abd}	39 ± 2 ^{abd}	34 ± 2 ^d	27 ± 3 ^{**ab}
	GP + GLN	29 ± 2 [*]	29 ± 2 [*]	28 ± 2 ^{**}	25 ± 2 ^{**}	26 ± 2 ^{**b}	26 ± 2 ^{**b}	24 ± 2 ^{**ab}	27 ± 2 ^{**ab}
Serine	CON1	96 ± 7 ^{**}	92 ± 6 ^{**}	94 ± 8 ^{**}	88 ± 5 ^{**}	87 ± 8 ^{**}	97 ± 8 ^{**}	118 ± 10	114 ± 7
	CON2	99 ± 9	97 ± 7	92 ± 8 [*]	81 ± 5 ^{**}	93 ± 8 [*]	91 ± 8 [*]	104 ± 9	111 ± 9
	GP + ALA & GLY	126 ± 9 ^{abd}	155 ± 23 ^{*abd}	131 ± 8 ^{abd}	128 ± 14 ^{abd}	143 ± 10 ^{**abd}	161 ± 12 ^{**abd}	158 ± 14 ^{†abd}	131 ± 13 ^{bd}
	GP + GLN	95 ± 5 [*]	93 ± 6 ^{**}	87 ± 6 ^{**}	76 ± 5 ^{**}	78 ± 5 ^{**}	80 ± 6 ^{**}	77 ± 7 ^{fab}	91 ± 4 ^{**ab}
Arginine	CON1	69 ± 8 ^{**}	75 ± 6 ^{**}	68 ± 7 ^{**}	71 ± 6 ^{**}	67 ± 5 ^{**}	70 ± 5 ^{**}	82 ± 12	79 ± 7
	CON2	79 ± 5	80 ± 5	75 ± 5	68 ± 5 ^{**}	68 ± 5 ^{**}	67 ± 6 ^{**}	74 ± 8	76 ± 7
	GP + ALA & GLY	76 ± 9	75 ± 9	69 ± 8	71 ± 9	67 ± 6 [*]	66 ± 9 [*]	64 ± 7 ^{**a}	64 ± 9 ^{**ab}
	GP + GLN	68 ± 4 ^b	70 ± 6	66 ± 7	59 ± 3 ^{ad}	60 ± 6 [*]	61 ± 7	55 ± 6 ^{**ab}	61 ± 5 ^{ab}

Values are means ± SEM for 7 subjects, expressed in μmol/l. NH₃, ammonia. ^{*}different to basal value ($P < 0.05$), ^{**}different to basal value ($P < 0.01$), [†]different to CON1 ($P < 0.05$), ^bdifferent to CON2 ($P < 0.05$), ^cdifferent to GP + ALA & GLY ($P < 0.05$), ^ddifferent to GP + GLN ($P < 0.05$)

Table 4.5 Summed plasma amino acid concentrations at rest and during the first bout of exercise during the control, GP + ALA & GLY and GP + GLN conditions

	Condition	Basal	EX1		
			30 min	60 min	85 min
Tryp:BCAA	CON1	0.13 ± 0.01	0.13 ± 0.02	0.14 ± 0.01	0.14 ± 0.02
	CON2	0.12 ± 0.01	0.13 ± 0.01	0.14 ± 0.02	0.14 ± 0.01*
	GP + ALA & GLY	0.11 ± 0.02	0.11 ± 0.01 ^a	0.13 ± 0.01	0.13 ± 0.02*
	GP + GLN	0.14 ± 0.01 ^c	0.14 ± 0.01 ^c	0.14 ± 0.01	0.15 ± 0.01
n-m AA	CON1	593 ± 43	618 ± 17	574 ± 34	574 ± 51
	CON2	550 ± 32	611 ± 13	604 ± 29	560 ± 28
	GP + ALA & GLY	559 ± 40	609 ± 53	591 ± 51	581 ± 46
	GP + GLN	565 ± 49	627 ± 33	585 ± 40	598 ± 58
BCAA	CON1	355 ± 18	361 ± 28	325 ± 17	311 ± 21*
	CON2	330 ± 7	357 ± 27	324 ± 6	307 ± 16
	GP + ALA & GLY	341 ± 9	330 ± 16	302 ± 26	311 ± 9
	GP + GLN	314 ± 13	332 ± 20	299 ± 7	300 ± 10
AAA	CON1	144 ± 7	158 ± 10	157 ± 12	154 ± 13
	CON2	138 ± 14	155 ± 10	157 ± 7*	151 ± 10
	GP + ALA & GLY	126 ± 8	133 ± 13 ^{ab}	149 ± 15*	150 ± 15*
	GP + GLN	126 ± 8	140 ± 14	137 ± 9 ^{ab}	143 ± 12
EAA	CON1	738 ± 36	776 ± 42	720 ± 45	700 ± 47
	CON2	706 ± 28	768 ± 41	728 ± 22	699 ± 33
	GP + ALA & GLY	664 ± 28	709 ± 36	685 ± 67	686 ± 21
	GP + GLN	781 ± 40	726 ± 32	676 ± 8	683 ± 33
NEAA	CON1	1500 ± 68	1590 ± 77	1559 ± 51	1463 ± 110
	CON2	1396 ± 77	1574 ± 56	1630 ± 121*	1520 ± 120
	GP + ALA & GLY	1398 ± 72	1644 ± 99*	1580 ± 125	1565 ± 120
	GP + GLN	1425 ± 91	1727 ± 133 ^{ab}	1702 ± 132 ^{ab}	1705 ± 160 ^{ab}
Tot. AA	CON1	2281 ± 97	2415 ± 104	2318 ± 86	2200 ± 149
	CON2	2147 ± 104	2387 ± 88	2396 ± 139	2262 ± 152
	GP + ALA & GLY	2140 ± 76	2395 ± 128	2309 ± 197	2299 ± 134
	GP + GLN	2138 ± 110	2496 ± 151*	2423 ± 189*	2431 ± 189*

Values are means ± SEM for 7 subjects, expressed in µmol/l. n-m AA, sum of non-metabolised amino acids (Thr, Met, Phe, Lys, Gly & Tyr); BCAA, sum of branched-chain amino acids (Leu, Iso, Val); AAA, sum of aromatic amino acids (Try, Tyr & Phe); EAA, sum of essential amino acids (His, Iso, Leu, Lys, Met, Phe, Thr, Try, & Val); NEAA, sum of non-essential amino acids (Ala, Arg, Asp, Asn, Glu, Gln, Gly, Ser, & Tyr); Tot. AA, sum of all amino acids.

*different to basal value ($P < 0.05$), **different to basal value ($P < 0.01$), ^adifferent to CON1 ($P < 0.05$), ^bdifferent to CON2 ($P < 0.05$), ^cdifferent to GP + ALA & GLY ($P < 0.05$), ^ddifferent to GP + GLN ($P < 0.05$)

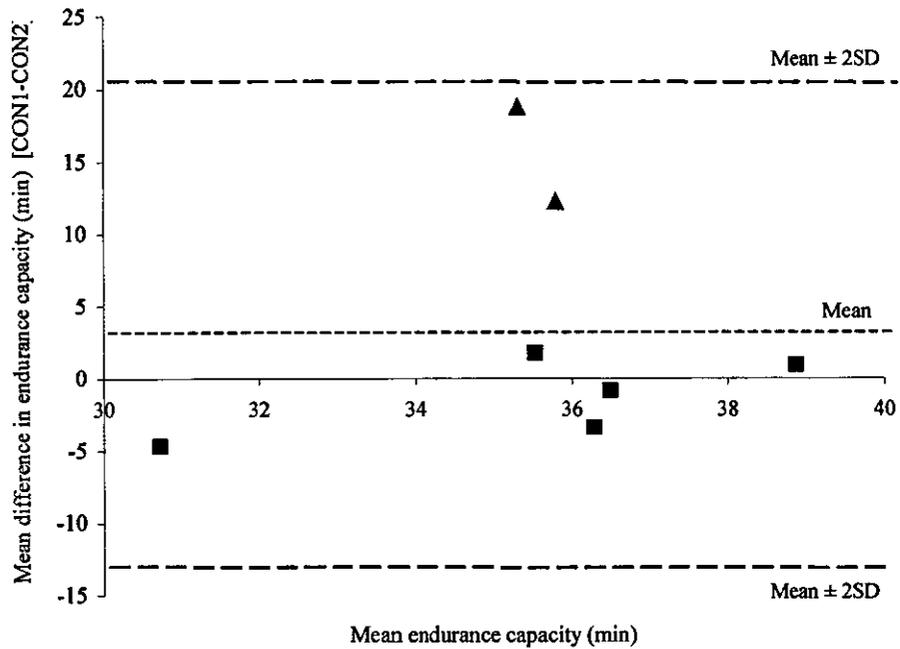
Table 4.5 (cont'd) Summed plasma amino acid concentrations during recovery and the second bout of exercise during the control, GP + ALA & GLY and GP + GLN conditions

	Condition	RECOVERY							EX2
		60 min	75 min	90 min	105 min	120 min	180 min	240 min	Exh
Tryp:BCAA	CON1	0.13 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	0.11 ± 0.01	0.10 ± 0.01*	0.13 ± 0.02
	CON2	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.01
	GP + ALA & GLY	0.14 ± 0.02**	0.14 ± 0.02** ^a	0.14 ± 0.02**	0.15 ± 0.02** ^{a,b}	0.15 ± 0.02** ^{a,b}	0.16 ± 0.02** ^{a,b}	0.12 ± 0.02	0.09 ± 0.01 ^{a,b}
	GP + GLN	0.15 ± 0.01	0.16 ± 0.01** ^{a,b}	0.16 ± 0.01** ^{a,b,c}	0.19 ± 0.01** ^{a,b,c}	0.18 ± 0.01** ^{a,b,c}	0.18 ± 0.01** ^{a,b}	0.18 ± 0.01** ^{a,b,c}	0.17 ± 0.01** ^{a,b,c}
n-m AA	CON1	467 ± 46**	471 ± 29**	467 ± 36**	463 ± 26**	460 ± 40**	470 ± 31**	552 ± 49	590 ± 54
	CON2	507 ± 19	500 ± 16	493 ± 26	450 ± 13*	476 ± 19	446 ± 31*	524 ± 54	536 ± 64
	GP + ALA & GLY	903 ± 66** ^{a,b,d}	1010 ± 93** ^{a,b,d}	1000 ± 76** ^{a,b,d}	997 ± 64** ^{a,b,d}	1073 ± 92** ^{a,b,d}	1186 ± 84** ^{a,b,d}	965 ± 113** ^{a,b,d}	701 ± 56** ^{a,b,d}
	GP + GLN	459 ± 29*	457 ± 28*	421 ± 35**	400 ± 23**	385 ± 23** ^b	359 ± 21** ^a	356 ± 32** ^{a,b}	427 ± 41** ^{a,b}
BCAA	CON1	266 ± 23**	287 ± 20**	282 ± 24**	274 ± 16**	291 ± 20**	320 ± 25	385 ± 19	357 ± 29
	CON2	311 ± 27*	303 ± 20	311 ± 30	280 ± 15*	322 ± 32	313 ± 41	384 ± 31*	351 ± 25
	GP + ALA & GLY	288 ± 10*	278 ± 16**	253 ± 11**	242 ± 10**	229 ± 17** ^{a,b}	195 ± 16** ^{a,b}	199 ± 14** ^{a,b}	262 ± 25** ^{a,b}
	GP + GLN	258 ± 11** ^b	245 ± 9** ^{a,b}	217 ± 14** ^{a,b}	201 ± 17** ^{a,b}	189 ± 12** ^{a,b}	166 ± 18** ^{a,b}	173 ± 18** ^{a,b}	269 ± 12** ^{a,b}
AAA	CON1	122 ± 12*	130 ± 8	125 ± 7	131 ± 5	123 ± 9*	122 ± 10*	149 ± 12**	172 ± 13
	CON2	142 ± 8*	137 ± 6	135 ± 10	125 ± 3	131 ± 7	119 ± 12	143 ± 9**	150 ± 13*
	GP + ALA & GLY	139 ± 13	128 ± 10	128 ± 11	125 ± 11	117 ± 12*	109 ± 12**	96 ± 10** ^{a,b}	111 ± 6 ^{a,b}
	GP + GLN	120 ± 7 ^b	123 ± 8	113 ± 15 ^b	113 ± 10	103 ± 8 ^{a,b}	89 ± 10 ^{a,b}	95 ± 12 ^{a,b}	123 ± 11 ^{a,b}
EAA	CON1	589 ± 50**	615 ± 38**	602 ± 42**	604 ± 30**	614 ± 40**	657 ± 44	784 ± 47	782 ± 57
	CON2	663 ± 37*	642 ± 17	645 ± 39*	598 ± 14*	656 ± 36	631 ± 62	764 ± 61	728 ± 63
	GP + ALA & GLY	667 ± 30	665 ± 48	609 ± 29	588 ± 37*	568 ± 46**	514 ± 47** ^{a,b}	484 ± 38** ^{a,b}	551 ± 30** ^{a,b}
	GP + GLN	559 ± 21** ^{b,c}	546 ± 18** ^{b,c}	494 ± 36** ^{a,b,c}	475 ± 31** ^{a,b,c}	440 ± 25** ^{a,b,c}	397 ± 35** ^{a,b,c}	406 ± 43** ^{a,b}	559 ± 29** ^{a,b}
NEAA	CON1	1171 ± 95**	1237 ± 79**	1186 ± 90**	1162 ± 72**	1107 ± 76**	1149 ± 54**	1373 ± 117	1393 ± 77
	CON2	1314 ± 84	1314 ± 62	1311 ± 83	1157 ± 39*	1216 ± 82	1159 ± 60*	1322 ± 97	1402 ± 129
	GP + ALA & GLY	2053 ± 134** ^{a,b,d}	2209 ± 191** ^{a,b,d}	2143 ± 136** ^{a,b,d}	2167 ± 135** ^{a,b,d}	2291 ± 167** ^{a,b,d}	2495 ± 136** ^{a,b,d}	2108 ± 204** ^{a,b,d}	1580 ± 116 ^d
	GP + GLN	1505 ± 96 ^{a,b}	1509 ± 104 ^{a,b}	1443 ± 100*	1302 ± 85	1327 ± 74*	1303 ± 85	1147 ± 81** ^a	1324 ± 87
Tot. AA	CON1	1795 ± 141**	1888 ± 113**	1823 ± 132**	1801 ± 98**	1758 ± 114**	1844 ± 91**	2196 ± 154	2212 ± 128
	CON2	2019 ± 119	1989 ± 75	1991 ± 119	1790 ± 46**	1910 ± 115	1825 ± 121*	2123 ± 149	2162 ± 192
	GP + ALA & GLY	2769 ± 147** ^{a,b,d}	2924 ± 215** ^{a,b,d}	2798 ± 149** ^{a,b,d}	2796 ± 164** ^{a,b,d}	2903 ± 202** ^{a,b,d}	3049 ± 174** ^{a,b,d}	2627 ± 234** ^{a,b,d}	2170 ± 128
	GP + GLN	2106 ± 106*	2098 ± 109	1977 ± 109	1819 ± 101*	1807 ± 88*	1741 ± 103**	1593 ± 128** ^{a,b}	1921 ± 115 ^a

Values are means ± SEM for 7 subjects, expressed in μmol/l. n-m AA, sum of non-metabolised amino acids (Thr, Met, Phe, Lys, Gly & Tyr); BCAA, sum of branched-chain amino acids (Leu, Iso, Val); AAA, sum of aromatic amino acids (Try, Tyr & Phe); EAA, sum of essential amino acids (His, Iso, Leu, Lys, Met, Phe, Thr, Try, & Val); NEAA, sum of non-essential amino acids (Ala, Arg, Asp, Asn, Glu, Gln, Gly, Ser, & Tyr); Tot. AA, sum of all amino acids.

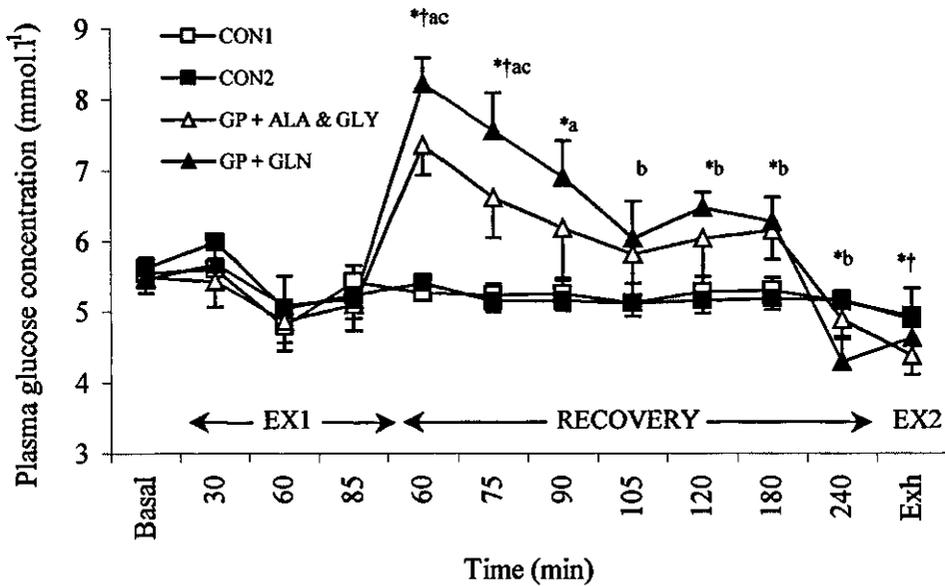
*different to basal value ($P < 0.05$), **different to basal value ($P < 0.01$), ^adifferent to CON1 ($P < 0.05$), ^bdifferent to CON2 ($P < 0.05$), ^cdifferent to GP + ALA & GLY ($P < 0.05$), ^ddifferent to GP + GLN ($P < 0.05$)

Fig. 4.2 Bland-Altman plot showing mean difference in endurance capacity between CON1 and CON2 conditions.



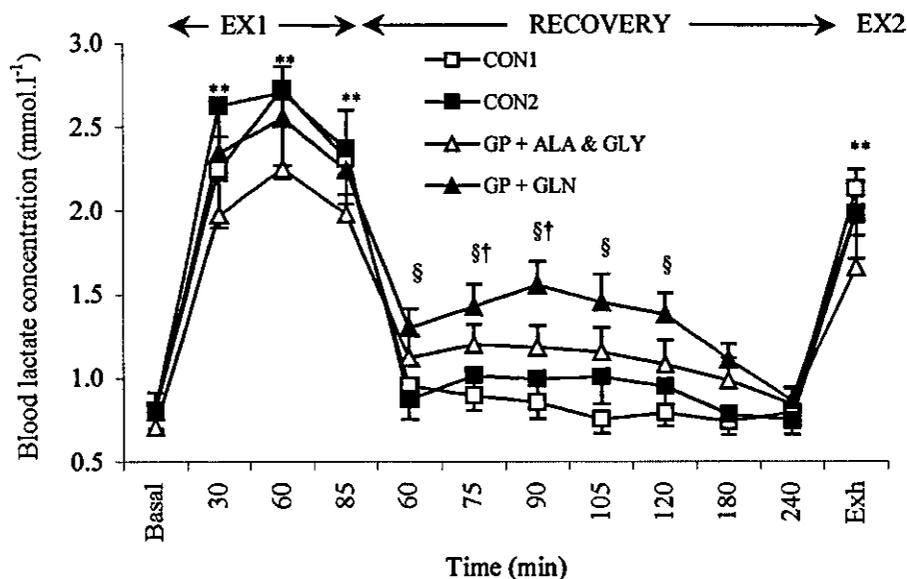
(see section entitled "Endurance capacity" for description of square and triangular symbols).

Fig. 4.3 Plasma glucose concentration during the experimental period in the control, GP + ALA & GLY and GP + GLN conditions. Values are means \pm SEM for 7 subjects.



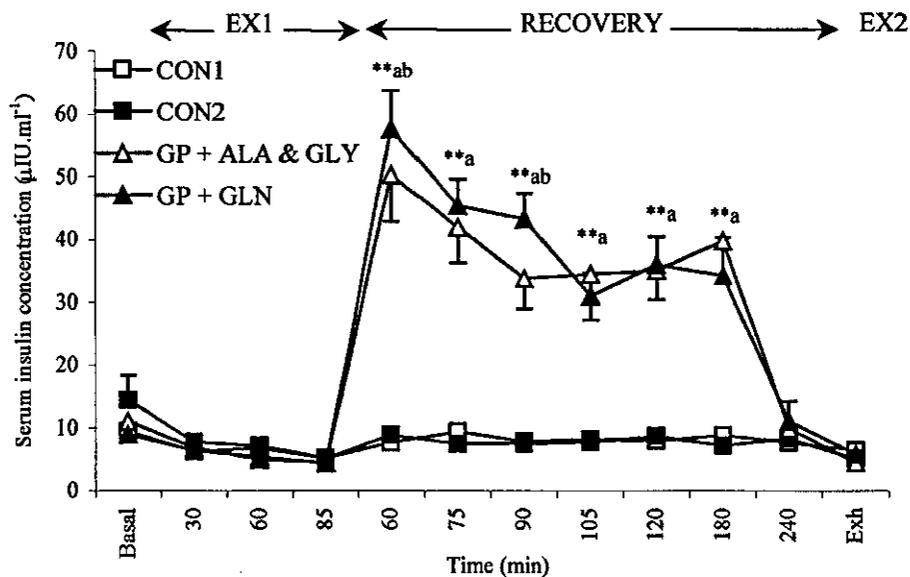
- * = significantly different to basal in GP + GLN condition ($P < 0.05$)
- † = significantly different to basal in GP + ALA & GLY condition ($P < 0.05$)
- a = significantly different to control in glucose-polymer conditions ($P < 0.05$)
- b = significantly different to control in GP + GLN condition ($P < 0.05$)
- c = significantly different to GP + ALA & GLY condition in GP + GLN condition ($P < 0.05$)

Fig. 4.4 Blood lactate concentration during the experimental period in the control, GP + ALA & GLY and GP + GLN conditions. Values are means \pm SEM for 7 subjects.



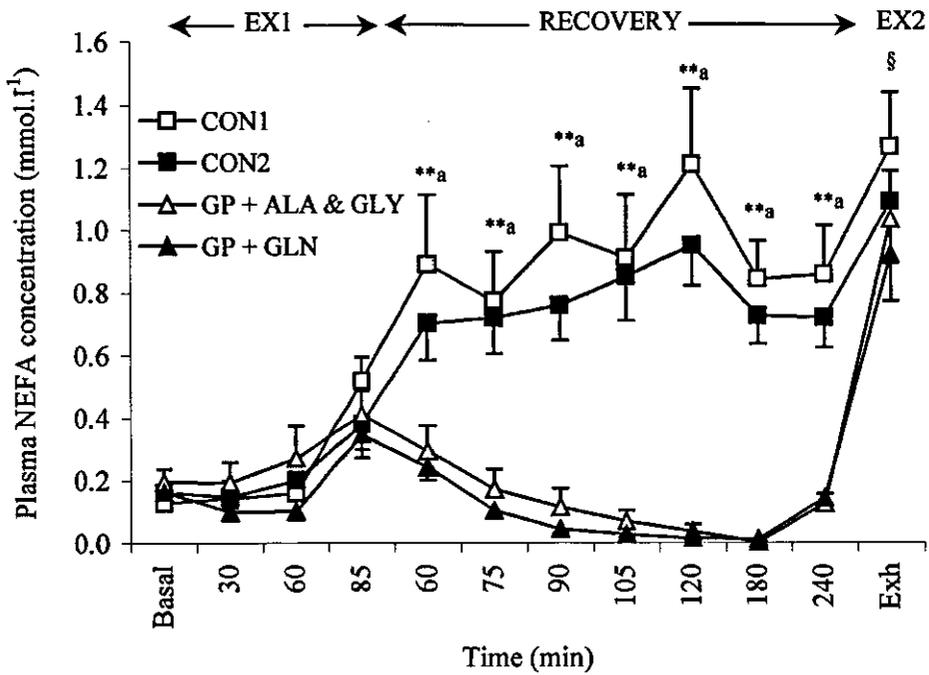
** = significantly different to basal in all conditions ($P < 0.01$)
 \S = significantly different to basal in GP + GLN condition ($P < 0.05$)
 \dagger = significantly different to basal in GP + ALA & GLY condition ($P < 0.05$)

Fig. 4.5 Serum insulin concentration during the experimental period in the control, GP + ALA & GLY and GP + GLN conditions. Values are means \pm SEM for 7 subjects.



** = significantly different to basal in GP + ALA & GLY and GP + GLN conditions ($P < 0.01$)
 a = significantly different to control conditions in glucose-polymer conditions ($P < 0.01$)
 b = significantly different to GP + ALA & GLY condition in GP + GLN condition ($P < 0.05$)

Fig. 4.6 Plasma NEFA concentration during the experimental period in the control, GP + ALA & GLY and GP + GLN conditions. Values are means \pm SEM for 7 subjects.

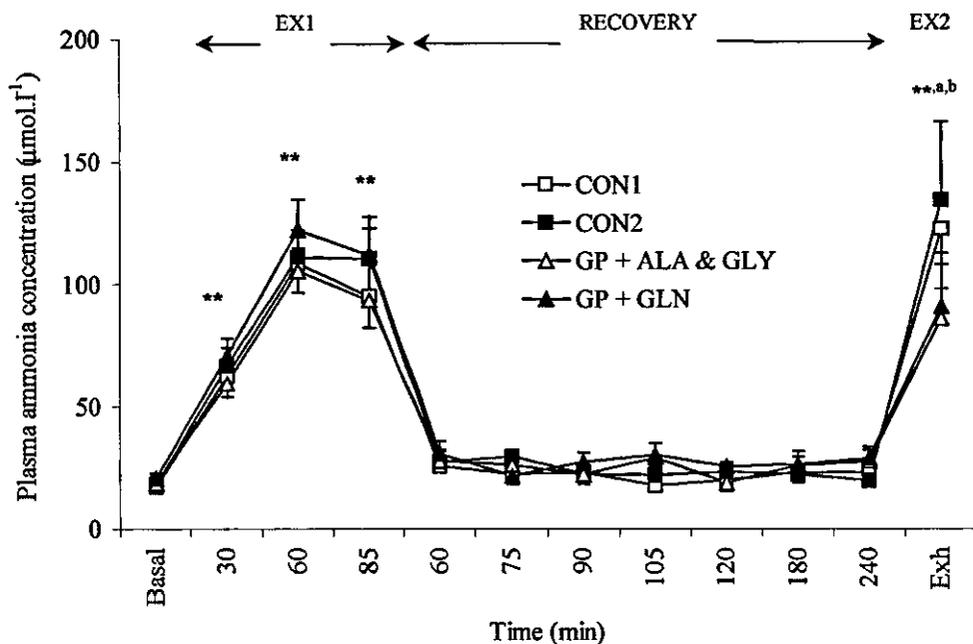


** = significantly different to basal in control conditions ($P < 0.01$)

§ = significantly different to basal in all conditions ($P < 0.01$)

a = significantly different to glucose-polymer conditions in control conditions ($P < 0.01$)

Fig. 4.7 Plasma ammonia concentration during the experimental period in the control, GP + ALA & GLY and GP + GLN conditions. Values are means \pm SEM for 7 subjects.

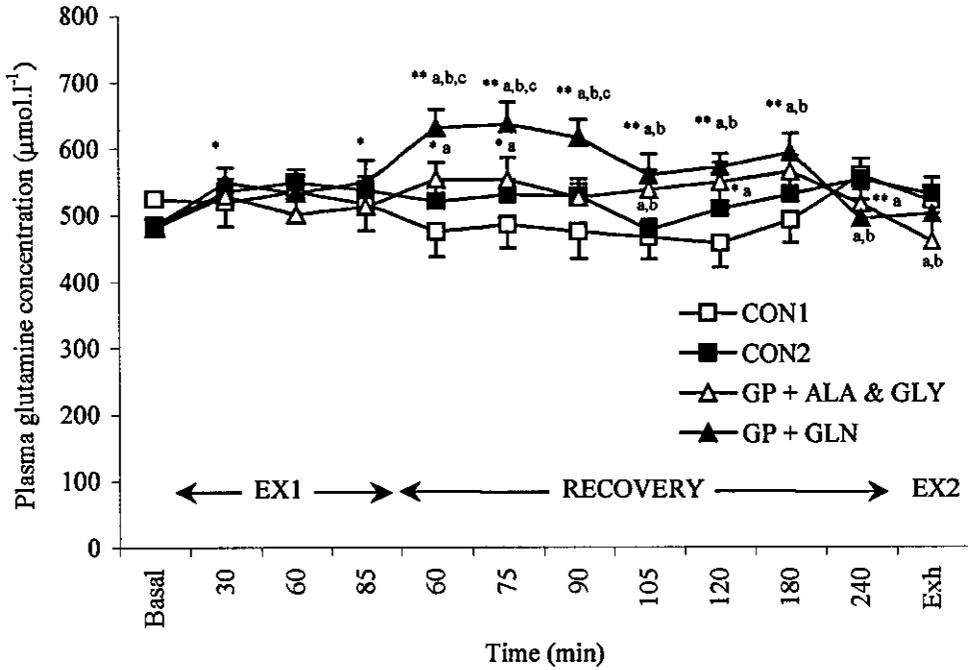


** = significantly different to basal in all conditions ($P < 0.01$)

a = glucose-polymer conditions significantly different to CON1 ($P < 0.05$)

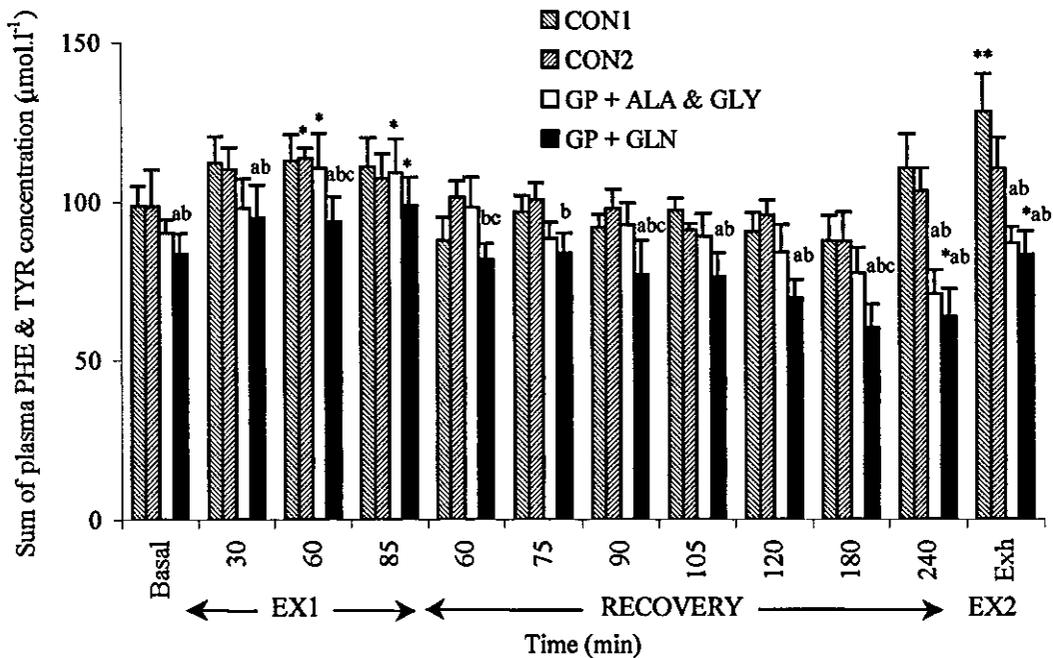
b = glucose-polymer conditions significantly different to CON2 ($P < 0.05$)

Fig. 4.8 Plasma glutamine concentration during the experimental period in the control, GP + ALA & GLY and GP + GLN conditions. Values are means \pm SEM for 7 subjects.



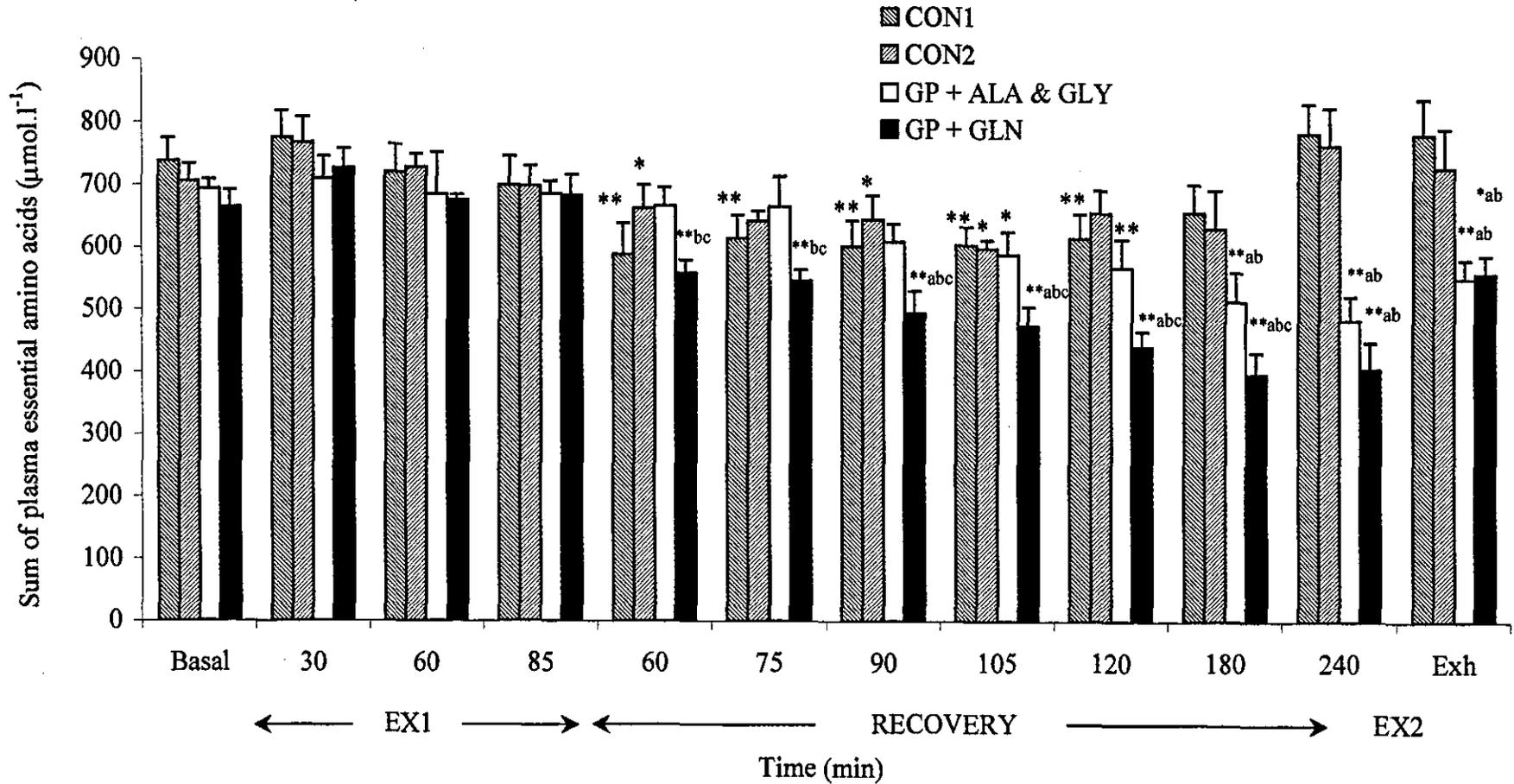
* = significantly different to basal ($P < 0.05$) ** = significantly different to basal ($P < 0.01$)
 a = significantly different to CON1 ($P < 0.05$) b = significantly different to CON2 ($P < 0.05$)
 c = significantly different to GP + ALA & GLY ($P < 0.05$)

Fig. 4.9 Sum of plasma phenylalanine and tyrosine concentration during the experimental period in the control, GP + ALA & GLY and GP + GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal ($P < 0.05$) ** = significantly different to basal ($P < 0.01$)
 a = significantly different to CON1 ($P < 0.05$) b = significantly different to CON2 ($P < 0.05$)
 c = significantly different to GP + ALA & GLY ($P < 0.05$)

Fig. 4.10 Sum of plasma essential amino acid concentration during the experimental period in the control, GP + ALA & GLY and GP + GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal ($P < 0.05$) ** = significantly different to basal ($P < 0.01$) a = significantly different to CON1 ($P < 0.05$)
 b = significantly different to CON2 ($P < 0.05$) c = significantly different to GP + ALA & GLY ($P < 0.05$)

4.5 DISCUSSION

The main finding of this study was that the supplementation of glucose-polymer and glutamine during recovery from exhaustive exercise did not extend endurance capacity in a subsequent bout of exercise compared to placebo and glucose-polymer and alanine & glycine.

Glutamine is transported across the intestinal brush border by both sodium-dependent and -independent systems (Taylor et al. 1989). Due to this co-transport, and previous work that has demonstrated that the addition of glutamine to oral rehydration solutions increases sodium absorption and therefore bulk water flow (Rhoads et al. 1991; van Loon et al. 1996), it has been suggested that the ingestion of glutamine may promote a more rapid intestinal absorption in the gut. However, despite implementing a more frequent sampling period in the second hour of recovery, the peak in plasma glucose concentration occurred 30 min after consumption of both of the glucose-polymer containing drinks. However, both the peak plasma glucose concentration and the area under the plasma glucose curve had a tendency to be greater during the second hour of recovery in the GP + GLN condition compared to the GP + ALA & GLY condition, despite receiving the same glucose-polymer dose. Indeed, the proportion of the area under the plasma glucose curve during the recovery period was greater ($P < 0.05$) in the GP + GLN condition during the second hour of recovery (CON1: 33%; CON2: 33%; GP + ALA & GLY: 35%; GP + GLN: 37%). Therefore, the time course for the appearance of glucose in the systemic circulation appears to have been greater in the GP + GLN condition, suggestive that the addition of glutamine may have increased the rate of intestinal absorption. However, it must be recognised that the plasma concentration merely reflects the balance between entry and removal of glucose into or out of the systemic circulation.

The ingestion of glutamine with the glucose-polymer solution was able to elevate the plasma glutamine concentration by ~30 % (relative to the baseline concentration). Therefore, in spite of the reported ~50 % extraction and substantial oxidation of enterally delivered glutamine by the splanchnic bed, in addition to a reduction in glutamine *de novo* synthesis (Matthews et al. 1993; Hankard et al. 1995; Haisch et al. 2000), the oral glutamine load used in the present study was sufficient to increase

glutamine concentration in the systemic circulation. Furthermore, it has been reported that the plasma concentration of glutamine decreases by up to 25% following prolonged submaximal exercise (van Hall et al. 1998), therefore it appears that the glutamine dose provided in the present study was able to counteract any post exercise reduction in plasma glutamine concentration. However, a reduction in plasma glutamine concentration was still evident in the control conditions, where neither glutamine nor glucose-polymer was provided. Furthermore, although the supplementation of glucose-polymer during exercise has been shown to be able to counteract the post exercise decrease in plasma glutamine concentration (van Hall et al. 1998), it is feasible that the supplementation of glucose-polymer post exercise may also serve to maintain plasma glutamine concentration by providing carbon skeletons for glutamine synthesis via the carboxylation of pyruvate (Wagenmakers et al. 1991).

It has been reported that the intravenous administration of glutamine is able to increase plasma glutamine concentration by ~70 %, which caused intramuscular glutamine concentration to increase by ~16 % (Varnier et al. 1995). In the present study, the oral provision of glutamine and glucose-polymer was able to increase plasma glutamine concentration by ~34 %, therefore theoretically increasing intramuscular glutamine concentration by ~8 %. Furthermore, hepatic glycogen synthase activation and glycogen synthesis is increased in rats orally fed glutamine (Niewoehner and Nuttall, 1996). Therefore, an increase in plasma glutamine of the order observed in the present study should be sufficient to increase both muscle and liver glutamine concentration, and therefore modulate metabolism in these tissues.

The non-essential amino acids alanine and glycine were supplemented to provide an isoenergetic and isonitrogenous comparative condition. It has been demonstrated that alanine is able to mimic some of the properties of glutamine, for example, alanine is both synthesised and exported by skeletal muscle (Chang and Goldberg, 1978c) and is an important gluconeogenic precursor, predominantly in the liver (Stumvoll et al. 1998). Glycine, on the other hand, is deemed metabolically inert within skeletal muscle, however this amino acid is able to interact with metabolism in other tissues, in particular the liver.

During prolonged submaximal exercise with normal glycogen levels, both plasma alanine and glutamine concentration appear to increase during the first 30 min and then gradually return to resting values when exercise is continued for 90 min (van Hall et al. 1995b). Concomitant to this observation, a gradual reduction in the release of both alanine and glutamine from the muscle during prolonged submaximal exercise has been observed (van Hall et al. 1999). Indeed, a similar pattern was observed in the present study with an initial increase observed in both plasma alanine and glutamine concentration during the first bout of exercise (with normal glycogen levels), which then decreased towards the end of exercise (~90 min).

During the second bout of exercise, plasma alanine concentration again increased in all conditions, except when alanine had been supplemented during the recovery period, suggesting a suppression of alanine *de novo* synthesis in this condition. However, plasma glutamine concentration did not demonstrate the expected increase in any condition during the second bout of exercise. This may be attributable to a reduction in muscle glycogen levels in the control conditions (van Hall et al. 1998), and a suppression of glutamine *de novo* synthesis following glutamine supplementation in the GP + GLN condition. It is interesting to note, that relative to the baseline concentration, no decrease in plasma glutamine concentration occurred during the recovery period in the GP + ALA & GLY condition. Although not statistically significant, the plasma concentration of glutamine in the GP + ALA & GLY condition was elevated above the control conditions during the second hour of the recovery period, suggesting that some degree of synthesis or reduction in splanchnic bed uptake of glutamine occurred. A suppression of hepatic gluconeogenesis will have occurred following the provision of glucose-polymer, resulting in a reduction in the uptake of hepatic gluconeogenic precursors such as alanine. Therefore, a portion of the ~76 % increase in plasma alanine concentration observed in the present study, following alanine supplementation, may be attributable to a reduction in the hepatic uptake of alanine. Furthermore, it is feasible that the provision of alanine in the GP + ALA & GLY condition may have promoted the synthesis of glutamine during the recovery period (Déchelotte et al. 1991), through a reversal of the alanine aminotransferase reaction, producing glutamate that can combine with ammonia to produce glutamine via the glutamine synthetase reaction. Furthermore, this notion is also supported by the tendency for a lower plasma ammonia concentration during recovery in the GP + ALA + GLY condition.

There seems to be a general agreement that during prolonged exercise skeletal muscle protein synthesis is reduced (Dohm et al. 1982; Rennie et al. 1981). Similarly, skeletal muscle protein breakdown is decreased, but is increased immediately after prolonged exercise (Carraro et al. 1990; Dohm et al. 1987). Neither phenylalanine or tyrosine are metabolised in skeletal muscle, therefore release of these amino acids into the plasma may be deemed indicative of muscle protein breakdown and these amino acids have been used as indicators of muscle protein breakdown in the literature (Blomstrand and Saltin, 1999; van Hall et al. 1999). However, it is important to consider the possibility that tissues other than skeletal muscle may play a role in exercise-induced proteolysis. It has been demonstrated that exercise-induced proteolysis also occurs in the splanchnic bed of postabsorptive humans (Felig and Wahren, 1971), with the contribution of the splanchnic bed (liver and gut) estimated to be ~40% of the whole body proteolysis during exercise (Wasserman et al. 1991; Williams et al. 1996; Halseth et al. 1998). However, it was outside the scope of the present study to directly determine protein turnover in the different organs or tissues of the body.

During the recovery period the plasma phenylalanine and tyrosine concentrations were significantly lower in the GP + GLN condition compared to both the control conditions and the GP + ALA & GLY condition. It has previously been demonstrated that glutamine inhibited protein breakdown in perfused rat skeletal muscle (MacLennan et al. 1988) and the present data lend support to this observation. Insulin has been suggested to play a major role in the stimulation of skeletal muscle protein synthesis (Newman et al. 1994; Millward, 1990; Kimball et al. 1994; Biolo et al. 1995), and also in the attenuation of skeletal muscle protein breakdown (Barrett and Gelfand, 1989; Svanberg et al. 1996). The concentration of insulin was increased after the provision of glucose-polymer in both glucose-polymer conditions, therefore it is possible to attribute a portion of the reduction in plasma phenylalanine and tyrosine concentration to the increased insulin concentration due to the provision of glucose-polymer i.e. insulin-induced hypoaminoacidemia (Barrett and Gelfand, 1989). However, a greater reduction in phenylalanine concentration and in the sum of phenylalanine and tyrosine concentration was observed in the GP + GLN condition compared to the GP + ALA & GLY condition, therefore the addition of glutamine appears to have exerted an additional inhibitory effect on protein breakdown, most probably in skeletal muscle (MacLennan et al. 1988). Furthermore, the increase in the summed plasma

concentration of those amino acids that cannot be metabolised in skeletal muscle, and hence are more indicative of muscle protein breakdown, was smaller in the GP + GLN condition than in the control conditions towards the end of the recovery period and during the second bout of exercise (a similar finding is observed compared with the GP + ALA & GLY condition when glycine is excluded). Taken together, these results suggest that the combination of both glucose-polymer (which increased insulin concentration) and glutamine (which possibly inhibited muscle protein breakdown) may have promoted a more positive protein balance during the recovery period.

The increase in plasma essential amino acid concentration in the last hour of the recovery period and during the second bout of exercise was greater during the control conditions than the glucose-polymer conditions, reinforcing the notion of increased protein loss in the control conditions. This notion is further supported by the greater increase in the sum of phenylalanine and tyrosine concentrations in the control conditions, compared to the glucose-polymer conditions in the last hour of the recovery period and during the second bout of exercise.

The ingestion of glucose in addition to amino acids caused a marked hyperglycemia and hyperinsulinemia in both the GP + GLN and GP + ALA & GLY conditions. The elevation in plasma glucose concentration reached a peak in both conditions 30 min after the ingestion of the first drink. Although there was no difference in the amount of glucose ingested between the GP + GLN and GP + ALA & GLY conditions, the peak in plasma glucose concentration, and indeed the plasma glucose concentration throughout recovery, was greater in the GP + GLN condition. This was mirrored by the serum insulin response during the first 90 min of recovery, which tended to be higher in the GP + GLN condition compared to the GP + ALA & GLY condition. It has been suggested that the consumption of protein may enhance the insulin response (Spiller et al. 1987), and indeed that the ingestion of individual amino acids may also augment the insulin response (Bertrand et al. 1995). Furthermore, the ingestion of protein in combination with carbohydrate has been shown to augment the insulin response (Spiller et al. 1987; Tarnopolsky et al. 1997; van Hall et al. 2000a; Jentjens et al. 2001). It is noteworthy, that despite the augmentation of the insulin response following the ingestion of protein in combination with carbohydrate after exercise, no further increase in muscle glycogen synthesis was observed (van Hall et al. 2000a; Jentjens et al.

2001). The results of the present study make it difficult to differentiate whether the additional insulin response in the GP + GLN condition is due to the higher glucose concentration or due to the amino acid itself. However, the latter would appear more likely if recent research is considered (van Hall et al. 2000b).

The plasma NEFA concentration decreased rapidly during recovery, from its elevated concentration at the end of the first exercise bout, in the glucose-polymer conditions. Conversely, an increase in plasma NEFA concentration during recovery was observed in the control conditions. The decrease in plasma NEFA concentration in the glucose-polymer conditions during recovery is most probably due to the inhibition of lipolysis by insulin, as the increase in serum insulin concentration was parallel to a decrease in plasma NEFA concentration. It must be recognised that the concentration of NEFA in plasma only represents a balance between the rate of mobilisation by lipolysis in adipose tissue and their oxidation by skeletal muscle, a measurement of plasma glycerol concentration would provide a better index of the rate of fatty acid mobilisation as glycerol is released from adipose tissue during lipolysis and is neither phosphorylated nor recycled. However, although plasma glycerol concentrations were not determined in the present study, the increase in plasma NEFA concentration during the placebo conditions, in the absence of any increase in serum insulin concentration, would suggest that the suppression of lipolysis mediated by insulin is indeed responsible for the decrease in plasma NEFA concentration.

There was a tendency for the plasma NEFA concentration to be suppressed to a greater extent during the GP + GLN condition compared to the GP + ALA & GLY condition. This added suppression is most likely due to the increased serum insulin response observed during the GP + GLN condition, but may be partly due to the reported inhibitory effect that an elevation in plasma glutamine concentration has upon lipolysis (Cersosimo et al. 1986; Déchelotte et al. 1991). However, during the second bout of exercise, the more rapid increase in the plasma NEFA concentration in the glucose-polymer conditions is presumably attributable to the rapid decline in insulin concentration over this time period.

During prolonged submaximal exercise, the oxidation of BCAA is increased, and ammonia is produced in skeletal muscle via a series of transamination and deamination

reactions. The greater increase in plasma ammonia concentration in the control conditions in the second bout of exercise compared to the glucose-polymer conditions is most probably related to the muscle glycogen content at the end of the 4 h recovery period. After the first bout of exercise, both muscle and liver glycogen stores will be reduced (Vøllestad et al. 1992). In addition, it is expected that there will be a negligible increase in the muscle glycogen resynthesis rate during the recovery period in the control conditions (Ivy et al. 1988b), and therefore it is expected that muscle glycogen content will remain substantially reduced in the control conditions at the end of the 4 h recovery period. Furthermore, it is expected that despite the provision of glucose-polymer in the glucose-polymer conditions, glycogen concentration will be reduced compared to normal levels after only 4 h recovery.

The exercise-induced activation of the branched-chain α -keto acid dehydrogenase complex (BCKADH) is enhanced in the glycogen-depleted state (Wagenmakers et al. 1991), with leucine oxidation being suppressed by oral glucose supplementation (Bowtell et al. 2000). Therefore, one may expect greater BCAA oxidation and hence ammonia production in the second bout of exercise in those conditions where muscle glycogen content is lower. Indeed, in the control conditions, where it is expected that glycogen concentration will have been substantially depleted, a decrease was observed in plasma BCAA concentration, whereas an increase was observed in both the glucose-polymer conditions. This may suggest an increased BCAA extraction and oxidation by skeletal muscle during the second bout of exercise in the control conditions.

The subjective sensations of fatigue that accompany prolonged exercise are generally considered a result of the events occurring in the exercising muscles or cardiovascular system. There is also evidence to suggest that the signals that arise in the periphery are modulated by events occurring within the central nervous system; generally referred to the 'central fatigue'. One hypothesis proposes that an increased concentration of the brain neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) is associated with the onset of fatigue. 5-HT has been linked to a variety of psychological responses including lethargy, arousal, sleepiness and mood, therefore alterations in the concentration of 5-HT in the brain may modulate muscular fatigue or perceived exertion during exercise. Amino acids do not readily enter across the blood-brain barrier and are transported by

means of a carrier or transporter. The large neutral amino acid (LNAA) transporter is a saturable carrier that transports six amino acids across the blood-brain barrier: tryptophan, tyrosine, phenylalanine and the BCAA (leucine, isoleucine and valine). Of particular importance in this context, is the fact that the apparent affinity of the LNAA transporter, and therefore the relative rate of transport into the brain of each individual amino acid, is dependent upon the concentration of each amino acid relative to its competitors. Therefore, a change in the plasma concentration ratio of free tryptophan:BCAA may increase the rate of tryptophan entry into the brain. This ratio could conceivably increase during prolonged exercise due to an increased uptake to the BCAA by skeletal muscle with a concomitant mobilisation of free fatty acids which can displace the tryptophan bound to albumin (McMenamy and Oncley, 1958) thereby increasing the free tryptophan concentration. This would lead to an increase in the free tryptophan:BCAA ratio which would conceivably lead to an increased entry of tryptophan into the brain. Tryptophan is a precursor for the synthesis of 5-HT.

This putative indicator of central fatigue, the plasma tryptophan:BCAA ratio (Blomstrand et al. 1997), was significantly greater in the GP + GLN condition at exhaustion in the second bout of exercise. Whether or not this increase in the plasma tryptophan:BCAA ratio contributed to an earlier onset of fatigue is unclear and the mechanism behind the increase in the tryptophan:BCAA ratio in the GP + GLN condition is unclear (although largely related to the greater increase in tryptophan concentration). However, recent evidence would suggest that the central fatigue hypothesis is a rather tenuous one, as under controlled laboratory conditions, no benefit has been gained from supplementing BCAA during exercise (Varnier et al. 1994; van Hall et al. 1995a; Verger et al. 1994; see Review of Literature section 2.4.4 for fuller discussion).

During the recovery period, the initial increase in plasma glucose concentration was greater in the GP + GLN condition compared to the GP + ALA & GLY condition and control conditions. There are several possible reasons for this; 1) a decrease in skeletal muscle glucose uptake, 2) an increase in hepatic glucose export, 3) an increase in renal gluconeogenesis (Stumvoll et al. 1998). It is expected that muscle glucose uptake will be increased in previously exercised muscle after glucose ingestion (Richter, 1996) due to an increase in GLUT-4 translocation. However, the addition of glutamine to the

glucose-polymer solution may have induced a synergistic effect, increasing the production of glucosamine, a product of glucose metabolism via the hexosamine biosynthetic pathway (Marshall et al. 1991b). In rat skeletal muscle, glucosamine has been shown to induce insulin resistance by causing an impairment of the translocation of GLUT-4 from an insulin-sensitive intracellular pool to the sarcolemmal membrane (Baron et al. 1995). The amination of fructose-6-phosphate to form glucosamine-6-phosphate catalysed by glutamine-fructose-6-phosphate amidotransferase (GFAT), with glutamine acting as the amido group donor, is the rate-limiting step in the hexosamine biosynthetic pathway (Buse et al. 1995; Marshall et al. 1991b; Marshall et al. 1991c; Crook et al. 1995; McClain and Crook, 1996). An increase in intracellular glucose and glutamine concentration, induced through the ingestion of the glucose-polymer and glutamine drink, may have increased flux through this rate-limiting step leading to increased production of glucosamine. An impairment of GLUT-4 translocation in skeletal muscle would serve to reduce skeletal muscle glucose uptake, which in turn may limit muscle glycogen synthesis despite any insulin-induced activation of glycogen synthase. Indeed, recent *in vitro* work has demonstrated that the addition of glutamine to rat skeletal muscle incubated with ^{14}C labelled glucose was able to reduce muscle glucose uptake (Sawada et al. 1998), suggesting that the provision of a combination of glucose and glutamine may indeed modulate skeletal muscle glucose uptake differently than when provided individually. It is unlikely that hepatic glucose output was increased during the recovery period after exercise when depleted hepatic glycogen stores are at least partially replenished, particularly as systemic glucose availability was increased by oral glucose. Finally, although an increase in gluconeogenesis provides a possible explanation for the increase in plasma glucose concentration during the recovery period in the GP + GLN condition, this is unlikely as the supplementation of glucose-polymer is likely to suppress gluconeogenesis.

Glutamine has previously been shown to be an effective substrate for hepatic glycogen synthesis in 72 h fasted rats (Mouterde et al. 1992), when hepatic glycogen concentration is substantially reduced, a situation that could also occur after prolonged exercise (Hultman and Nilsson, 1971). In hepatocytes, the inward transport of glutamine (via hepatic system N), due to an increase in the systemic concentration, causes an intracellular accumulation of glutamine, particularly due the Na^+ -coupled transport process (Häussinger et al. 1990). This increase in substrate flux across the cell

membrane, due to an increase in substrate concentration, will modify cellular osmolality and result in an increase in cell volume (i.e. cell swelling) (Lang et al. 1998). Therefore, both hepatocyte swelling and an increase in hepatic glutamate concentration (Meijer et al. 1992) may stimulate hepatic glycogen synthesis via a stimulation of glycogen synthase phosphatase leading to an activation of hepatic glycogen synthase (Lavoine et al. 1987). This may be independent or additive to any cell swelling and glycogen synthesis mediated by insulin (Häussinger et al. 1994). It has been suggested that the cytoskeleton architecture, in particular transmembrane integrins, may be responsible for transducing the mechanical stimuli (i.e. cell swelling) into chemical signals, which evoke metabolic responses (i.e. glycogen synthesis) (Ingber, 1997).

Furthermore, liver glucose uptake occurs via the non-insulin dependent transporter GLUT-2, which is not affected by glucosamine in the same manner as the skeletal muscle GLUT-4 transporter. Therefore, it is expected that liver glycogen synthesis will be increased in the GP + GLN condition, not only due to an increase in the hepatic glutamine concentration, but also due to an augmented liver glucose uptake due to a reduction in muscle glucose uptake. Indeed, it has recently been reported that the ingestion of glucose-polymer and glutamine promotes the storage of carbohydrate outside of skeletal muscle, the most feasible site being the liver (Bowtell et al. 1999). Therefore, the effect of consuming glutamine and glucose-polymer during recovery from exercise has the potential to increase glycogen synthesis in both the liver and skeletal muscle. Further 'indirect' support for this hypothesis is gained from the observation that carbohydrate oxidation rates after 10 min of the second bout of exercise were greater in the GP + GLN condition compared to the control conditions, suggesting increased carbohydrate storage, a phenomenon not observed in the GP + ALA & GLY condition. Unfortunately, in order to elucidate whether the addition of glutamine to the glucose-polymer caused a greater increase in liver glycogen storage during the recovery period, liver biopsies, ¹³C labelling or proton NMR would be required. At present it is impossible to directly confirm or refute this hypothesis.

The higher blood lactate concentration during recovery in the GP + GLN condition may also lend support to increased hepatic glucose metabolism via an increased flux through the 'indirect pathway' of glycogen synthesis (Magnusson and Shulman, 1991). Glucose may be passed through the liver to the peripheral tissue (e.g. muscle) where it is

converted to lactate, which is then transported back to the liver to serve as a gluconeogenic substrate (Wehmeyer et al. 1994). The G-6-P generated by gluconeogenesis can be used to regenerate glycogen stores in the liver. Presumably, the higher blood lactate concentration observed in the GP + GLN condition reflects the conversion of glucose to lactate in the peripheral tissues. However, if consideration is given to glucosamine-induced reduction in muscle glucose uptake, then the contribution that skeletal muscle may make to the conversion of glucose to lactate in muscle may be limited.

It is also interesting to note that in the final hour of recovery there is a rapid drop off in both plasma glucose and glutamine concentration in the GP + GLN condition. Intuitively, one would expect a drop off in both plasma glucose and glutamine concentration during this period since the glucose-polymer and glutamine were not consumed in the final hour of recovery. However, only a 26 % decrease was observed in the plasma glucose concentration in the last hour of recovery in the GP + ALA & GLY condition, whereas a 48% decrease in plasma glucose concentration was observed in the GP + GLN condition during the same period. The coincidence of a decrease in plasma glutamine concentration and an exaggerated decrease in plasma glucose concentration in the GP + GLN condition may be due to a compensatory increase in the rate of glucose uptake by the muscle or a decrease in the hepatic export of glucose. The former being the more likely explanation if flux through the hexosamine biosynthetic pathway is reduced during this period, due to a reduction in glutamine concentration and thus available amido donor. It has been reported that the effects of glucosamine are evident ~30 min after infusion in rats (Baron et al. 1995), with the intrinsic activity of GLUT-4 being reduced within 2 h of glucosamine infusion (Hawkins et al. 1999). In the present study, a slightly longer time course would be expected for activation of GFAT and glucosamine production after the ingestion of glucose-polymer and glutamine, to allow for the uptake of both substrates into the muscle cell and form glucosamine via flux through the hexosamine biosynthetic pathway. However, it is quite feasible for the effects of glucosamine to be prevalent during the second half of the recovery period and during the second bout of exercise.

Furthermore, it is quite feasible that an increased extraction of glucose and conversion to glycogen by the liver during the recovery period in the GP + GLN condition, gave

rise to an increase in hepatic glycogenolysis and export of glucose during the second bout of exercise. In addition, although the time course of the effects of glucosamine in human skeletal muscle are unclear, it is conceivable that during the second bout of exercise there may have been prevailing effects of glucosamine resulting in impaired glucose uptake into skeletal muscle, which jointly may account for the higher plasma glucose levels observed during this period in the GP + GLN condition.

In both the glucose-polymer conditions, where glucose-polymer was ingested during the 4 h recovery period, no difference was observed in cycle time to exhaustion compared to the control conditions. The ingestion of 1.0 g CHO body mass⁻² h⁻¹ during 4 h of recovery in comparison with a placebo solution has previously been shown to improve endurance running capacity in a subsequent bout of exercise (Fallowfield et al. 1995). The failure to observe a difference in endurance cycling capacity between the control and glucose-polymer conditions in the present study compared to this study may be related to the carbohydrate dose, the mode of exercise, or the sensitivity of endurance capacity to detect difference between conditions.

Reproducibility of protocol

It has been argued that the use of a correlation technique to assess the difference between two methods is inappropriate and can be misleading (Bland and Altman, 1986). Instead, a 'limits of agreement' approach provides a more qualitative method of assessing the difference between two methods. The calculated 95% limits of agreement of the present data are 3.5 ± 17.0 min, suggesting that a given subject's cycle time to exhaustion in another cycle to exhaustion should lie within -13.5 and $+20.5$ min of the first cycle to exhaustion. This range is not acceptable if one is attempting to assess the efficacy of a supplement where it is expected that only small changes in cycle time to exhaustion will be observed.

Although the present data do not display heteroscedasticity (i.e. the greater the cycle time to exhaustion the larger the difference between cycle times to exhaustion), it is apparent that the data is skewed by two subjects (signified by triangular data points; Fig. 4.1). Indeed, if these two subjects are removed, the variation in cycle time to exhaustion is reduced to $7.2 \pm 2.7\%$ (range: 2.3 – 16.5%). Given the small sample

number in the present study, the effect of individual data has a significant bearing on the group data. If the same 'limits of agreement' calculations are applied to five subjects (signified by square data points; Fig. 4.1), the 95% limits of agreement become -1.3 ± 5.4 min, equating to a second cycle time to exhaustion of between -6.7 and $+4.1$ min, relative to the first cycle time to exhaustion. These limits of agreement appear more acceptable in the present context, however small improvements in endurance capacity may not be detectable, suggesting that cycle time to exhaustion may not be sufficiently sensitive.

Previously, a large degree of individual variability has been associated with cycle time to exhaustion (Jeukendrup et al. 1996; McLellan et al. 1995) which may negate any true differences in endurance capacity. Therefore, it may be argued that endurance capacity is not a sensitive enough measure to detect any beneficial effect that may have been gained from the supplementation of both glucose-polymer and glutamine. In this context, the present study was able to demonstrate that that in terms of physiological measurements, no difference was observed between the two placebo conditions. However, this does not necessarily infer true reproducibility (Bland and Altman, 1986).

Many investigators assume that the reproducibility of cycling time to volitional fatigue is satisfactory, and the use of time to volitional fatigue as an index of the efficacy of a particular intervention is widespread (Coyle et al. 1983; Casey et al. 2000; Collomp et al. 2000; Morrison et al. 2000). In the present study, with regard to cycle time to exhaustion, 5 of the 7 subjects demonstrated good reproducibility, or low variability, with test-retest variability of $\sim 7\%$ of their first cycle to exhaustion. In the 2 subjects who demonstrated poor reproducibility, or high variability, their test-retest variability was $\sim 56\%$. Using a limits of agreement approach to assess reproducibility (Bland and Altman, 1986), based on the 5 subjects demonstrating good reproducibility, it was estimated that their second cycle to exhaustion would be between -6.7 to $+4.1$ min of their first cycle to exhaustion. It may be argued that an increase in the number of subjects may help elucidate whether cycle time to exhaustion is indeed an appropriate index. However, despite the data being skewed by these two subjects the mean within-subject test-retest variability was $\sim 21\%$ (2.3% - 72.1%). Previously, the reproducibility

of submaximal cycling time to exhaustion in trained cyclists has been reported to be poor, with the coefficient of variation for five rides to exhaustion ranging from 17.4% to 39.5% (mean 26.6%) in one study (Jeukendrup et al. 1996), and from 2.8 to 31.4% in another (McLellan et al. 1995). Therefore, the results of the present study are comparable, if not better than previous research.

In conclusion, the results from this study suggest that that the consumption of glutamine in combination with glucose-polymer during recovery from exhaustive exercise was effective in maintaining plasma glucose levels in a subsequent bout of exercise, which may be attributable to an increase in liver glycogen resynthesis. In addition, the results suggest that skeletal muscle protein breakdown was reduced post exercise following glucose-polymer and glutamine supplementation during recovery from exercise. However, the observed effects of glucose-polymer and glutamine supplementation did not extend endurance cycling capacity in a subsequent bout of exercise.

CHAPTER 5

EFFECT OF GLUTAMINE SUPPLEMENTATION DURING RECOVERY ON EXERCISE METABOLISM IN A SUBSEQUENT BOUT OF EXERCISE

5.1 INTRODUCTION

There is strong evidence from *in vitro* work, that insulin stimulates protein synthesis. However, this anabolic effect has been difficult to reproduce *in vivo* (Gelfand and Barrett, 1987). Previous experiments have demonstrated that plasma amino acid concentrations also have a role in the stimulation of amino acid accretion as protein in peripheral tissues (Lundholm et al. 1987). Furthermore, the addition of insulin to an amino acid mixture was unable to further increase skeletal muscle protein synthesis in rats (Mosoni et al. 1993). Therefore, amino acid availability may represent an important factor in protein balance, with several possible mechanisms to explain both the stimulation of skeletal muscle protein synthesis and decreased protein breakdown in humans (Garlick and Grant, 1988; Borel et al. 1997; McNulty et al. 1993). In particular, certain amino acids are able to stimulate protein synthesis and decrease protein breakdown in skeletal muscle by mechanisms that are not related to their role as components of protein structures (Svanberg et al. 1996).

In this context, the non-essential amino acid glutamine, the most abundant free amino acid in the body, appears to have a role in the regulation of protein metabolism. Considerable evidence suggests that a positive correlation exists between positive whole-body protein balance and both the size of the intramuscular glutamine pool (MacLennan et al. 1987; MacLennan et al. 1988; Jepson et al. 1988) and plasma glutamine concentration (Watt et al. 1992; Hankard et al. 1998). The existence of a relationship between glutamine levels and protein turnover rates has led to the use of glutamine in both enteral and parenteral nutrition (Khan et al. 1991; Hardy et al. 1992; Smith, 1997; Morlion et al. 1998) to promote positive protein balance. It has been suggested that glutamine becomes a conditionally 'essential' amino acid in critical illness (Hall et al. 1996; Lacy and Wilmore, 1990). Therefore, the first aim of this study was to investigate whether the supplementation of glutamine post exercise exerts

a controlling influence on whole-body protein balance, assessed indirectly via plasma amino acid concentration changes.

Glutamine has also been shown to be an important gluconeogenic precursor (Nurjhan et al. 1995), with the proposal that a 'glucose-glutamine' cycle analogous to the 'glucose-alanine' cycle exists in humans (Perriello et al. 1995). Glutamine infusion in postabsorptive humans led to a seven-fold increase in the conversion of glutamine into plasma glucose, demonstrating the significance of glutamine as a gluconeogenic precursor (Perriello et al. 1997). Therefore, in situations where the availability of glucose is reduced, such as in the post exercise state, the availability of gluconeogenic precursors such as glutamine, or indeed alanine, may allow for a better maintenance of glucose levels. Therefore, the post exercise provision of glutamine and alanine may provide the necessary substrate for the kidneys and liver, respectively (Stumvoll et al. 1998), to generate glucose in order to maintain homeostasis.

The provision of glutamine has been shown to stimulate glycogen synthesis both in skeletal muscle myotubes (Low et al. 1996), in rat skeletal muscle (Scislowski et al. 1989), and in rat liver cells (Lavoine et al. 1987; Mouterde et al. 1992). The mechanism is thought to be the activation of glycogen synthase via cell swelling induced by increased uptake of this amino acid (Baquet et al. 1990; Low et al. 1996). More specifically, it has been suggested that the cytoskeleton architecture, in particular transmembrane integrins may be responsible for transducing the mechanical stimuli (i.e. cell swelling) into chemical signals which evoke metabolic responses (i.e. glycogen synthesis) (Ingber, 1997; Low and Taylor, 1998).

An increase in the plasma concentration of glutamine, either through oral provision or intravenous administration, will lead to an increase in intramuscular glutamine concentration due to the rapid uptake of glutamine into skeletal muscle via System N^m (Varnier et al. 1995). Indeed, it has been demonstrated that the intravenous infusion of glutamine after exhaustive exercise is able to increase skeletal muscle glutamine content, resulting in a greater degree of muscle glycogen resynthesis than an infusion of an isoenergetic and isonitrogenous mixture of alanine and glycine (Varnier et al. 1995). It was suggested that the increase in skeletal muscle glycogen content was mediated via glycolytic mechanisms rather than gluconeogenic mechanisms as no increase in skeletal

muscle glycogen content was observed following infusion of glycine and glucogenic alanine.

An increase in both liver and skeletal muscle glycogen synthesis, either directly via glycogenic processes, or indirectly through gluconeogenesis, would result in a better maintenance of blood glucose concentration and a supply of readily available fuel for exercise. Indeed, an increase in systemic glucose circulation has been reported to delay the onset of fatigue during prolonged cycling exercise (Coyle et al. 1983). Furthermore, the rate of glucose utilisation is reported to be increased during cycling exercise compared to running exercise (Derman et al. 1996), due to a greater glucose utilisation of the smaller active muscle mass involved in cycling (Richter et al. 1988). These findings would suggest that systemic glucose concentration may play an important role in the aetiology of fatigue during prolonged cycling exercise. Therefore, the second aim of this study was to determine whether the supplementation of glutamine during recovery from prolonged exercise could extend endurance capacity in a subsequent bout of cycling exercise.

Plasma glutamine concentration has been shown to decline following prolonged submaximal exercise (Parry-Billings et al. 1987), reducing the availability of glutamine to tissues that utilise this substrate e.g. liver, kidneys, gut and cells of the immune system. Glutamine synthesis and efflux in skeletal muscle is increased in catabolic situations (Muhlbacher et al. 1984; Babij et al. 1986a; Babij et al. 1986b), reducing the availability of glutamine to take part in glycogenic processes within skeletal muscle itself. Therefore, the supplementation of glutamine during recovery from exercise should provide an exogenous source of the substrate, thus allowing these tissues and cells to function normally.

Furthermore, the provision of an individual amino acid, glutamate, the deamination product of glutamine, has been shown to enhance the insulin response (Bertrand et al. 1995), which could conceivably increase skeletal muscle protein synthesis (Newman et al. 1994). In addition, given the important role that insulin plays in governing skeletal muscle glycogen synthesis, the supplementation of glutamine may prove anabolic in the post exercise state not only in terms of protein metabolism, but also in terms of glycogen metabolism.

The use of glutamine is common within the clinical setting, both in parenteral and enteral nutrition. More recently, within the sports setting glutamine has been used by athletes in an attempt to boost the immune system post exercise. However, the use of glutamine for its glycogenic and gluconeogenic properties post exercise has rarely been advocated and has only been assessed in a few studies (Varnier et al. 1995; Bowtell, 1997; Bowtell et al. 1999; van Hall et al. 2000b).

Inherently linked to the previous study (Chapter 4), the underlying aim of the present study was to gain an understanding of the potential anabolic properties of glutamine alone. In the previous study, a combination of glucose-polymer and glutamine was ingested, both of which promote positive protein balance (Roy et al. 1997) and glycogen synthesis (Varnier et al. 1995). Therefore, the purpose of this second study was to allow us to delineate the effects of glutamine ingestion alone from any possible synergistic effects that may occur from ingesting glutamine in combination with glucose-polymer.

5.2 METHODS

Subjects. Seven well-trained, healthy male subjects participated in this study. Their mean (\pm SEM) age, height, body mass and $\dot{V}O_{2\max}$ were 26.4 ± 2.2 y, 180.5 ± 1.7 cm, 77.4 ± 2.1 kg and 4.66 ± 0.18 l.min⁻¹ (60.4 ± 2.6 ml.kg⁻¹.min⁻¹), respectively.

Preliminary tests. Subjects reported to the laboratory approximately 1 week before the experiment and undertook two preliminary tests in order to determine: (i) the oxygen cost of submaximal cycling, and (ii) maximal oxygen uptake ($\dot{V}O_{2\max}$). The protocols for these tests have been described elsewhere (see Chapter 3). Subjects were fully familiarised with the experimental procedures used during the experimental conditions.

Protocol of the study. Subjects were instructed to consume their habitual diet and refrain from exercise or strenuous physical activity for 48 h prior to each experiment. On arrival at the laboratory on the morning of the experiment, the overnight fasted subject rested in a supine position and a cannula was inserted into an antecubital vein and a resting blood sample was obtained. The experimental procedure utilised in this

study was a standard 'recovery protocol' similar to a protocol adopted previously in the literature (Fallowfield and Williams, 1997) and in the previous study, consisting of two bouts of exercise separated by a 4 h passive recovery period (Fig. 5.1).

Subjects performed a bout of glycogen depleting exercise (EX1), as described previously (Chapter 3), which was immediately followed by a 4 h passive recovery period, during which they consumed one of the assigned solutions allocated by systematic rotation in a double-blind fashion. They then immediately undertook a cycle to exhaustion at $\sim 70\% \dot{V}O_{2\max}$ (EX2) (Fig. 5.1) during which subjects were offered no fluid.

Expired air collections were taken prior to the commencement of EX1, then at 30 min, 60 min and 80 min during EX1. During recovery, collections were taken at 30 min intervals, with a collection being taken every 10 min and at exhaustion during EX2 (Fig. 5.1). Venous blood samples were then taken after 30 min, 60 min and at cessation of EX1. During recovery, samples were taken at 60, 75, 90, 105, 120, 180 and 240 min, with a subsequent sample being taken at exhaustion during EX2 (Fig. 5.1).

The two bouts of exercise (EX1 and EX2) were separated by a 4 h controlled recovery during which the subjects remained within the laboratory and their activity levels were minimal. After completion of EX1 subjects were rested for ~ 10 min before their post exercise nude bodyweight was measured. Any excess sweat on the subjects skin was towelled off prior to their weight being recorded.

Subjects received one of two drinks during the 4 h recovery period; they consumed either $0.3 \text{ g}\cdot\text{kg}^{-1}$ body wt L-glutamine (Sigma-Aldrich Chemicals, Dorset, UK) (GLN) dissolved in $31.25 \text{ ml}\cdot\text{kg}^{-1}$ body wt solution or an isoenergetic and isonitrogenous mixture of L-alanine and L-glycine (ALA & GLY) (Sigma-Aldrich Chemicals, Dorset, UK) dissolved in $31.25 \text{ ml}\cdot\text{kg}^{-1}$ body wt solution. Subjects received 780 ml of solution in the first drink with the remaining solution being divided into 5 equal aliquots. Subjects consumed the first of the six drinks after 30 min of recovery, after the first expired air collection, and then every 30 min thereafter. The last drink was administered at the 3 h point during recovery to minimise the possibility of gastro-

intestinal stress in the forthcoming exercise bout. Urine voided during the recovery period was collected and a cumulative volume recorded.

Prior to EX2, subjects post-recovery nude body weight was recorded in order to calculate rehydration status. Subjects then completed a 5 minute warm up at 60% $\dot{V}O_{2max}$, prior to commencing EX2. During EX2, subjects were instructed to cycle to volitional exhaustion at a workrate equivalent to $\sim 70\%$ $\dot{V}O_{2max}$, in order to determine their endurance capacity in terms of cycle time to exhaustion. Subjects were given no indication of the time completed during EX2. Exhaustion was determined using the definition described previously (Chapter 4, page 96). Subjects indicated when they could only continue for one more minute, at which point the final expired air collection and blood sample was taken.

Dry bulb temperatures within the laboratory during the main experimental trial were 21.1 ± 0.2 °C and 20.1 ± 0.2 °C for the GLN and ALA & GLY conditions respectively. Relative humidity within the laboratory during the main experimental trial was 55.4 ± 0.6 % and 55.8 ± 0.7 % for the GLN and ALA & GLY conditions respectively. No statistical difference was observed in relative humidity between conditions.

Analysis. Expired air samples were collected and analysed as previously described (Chapter 3). Venous blood samples were analysed for blood lactate and glucose concentration (using an automated analyser; YSI 2300 STATPLUS, see Chapter 3), plasma ammonia and amino acid concentration, serum insulin and NEFA concentrations, and haemoglobin concentration and haematocrit values, as previously described (Chapter 3). Percentage changes in plasma volume were calculated as previously described (see Chapter 3).

Pre-exercise energy intake. The energy intake of subjects during the 48 h prior to the main trial are presented in Table 5.1. No statistical differences were observed between conditions in the pre-exercise diet.

Table 5.1 Energy content and percentage of the primary dietary nutrients of the pre-exercise diet for both conditions (mean \pm SEM).

	ALA & GLY	GLN
Energy (MJ/day)	12.8 \pm 0.9	13.2 \pm 0.9
Carbohydrate (%)	54.0 \pm 3.6	52.0 \pm 5.0
Fat (%)	30.4 \pm 3.7	33.8 \pm 4.3
Protein (%)	14.7 \pm 1.2	14.0 \pm 0.9

NB. Data presented is mean of 2 days diet

Statistical analysis. The data were analysed by two-way analysis of variance (ANOVA) for repeated measures (time X condition). Cycle time to exhaustion data were analysed using a paired t-test. When the ANOVA resulted in a significant *F* ratio, Fisher's post hoc test was used to locate differences between means. Statistical significance was accepted at the 5% level ($P < 0.05$). Results are presented as means \pm SEM.

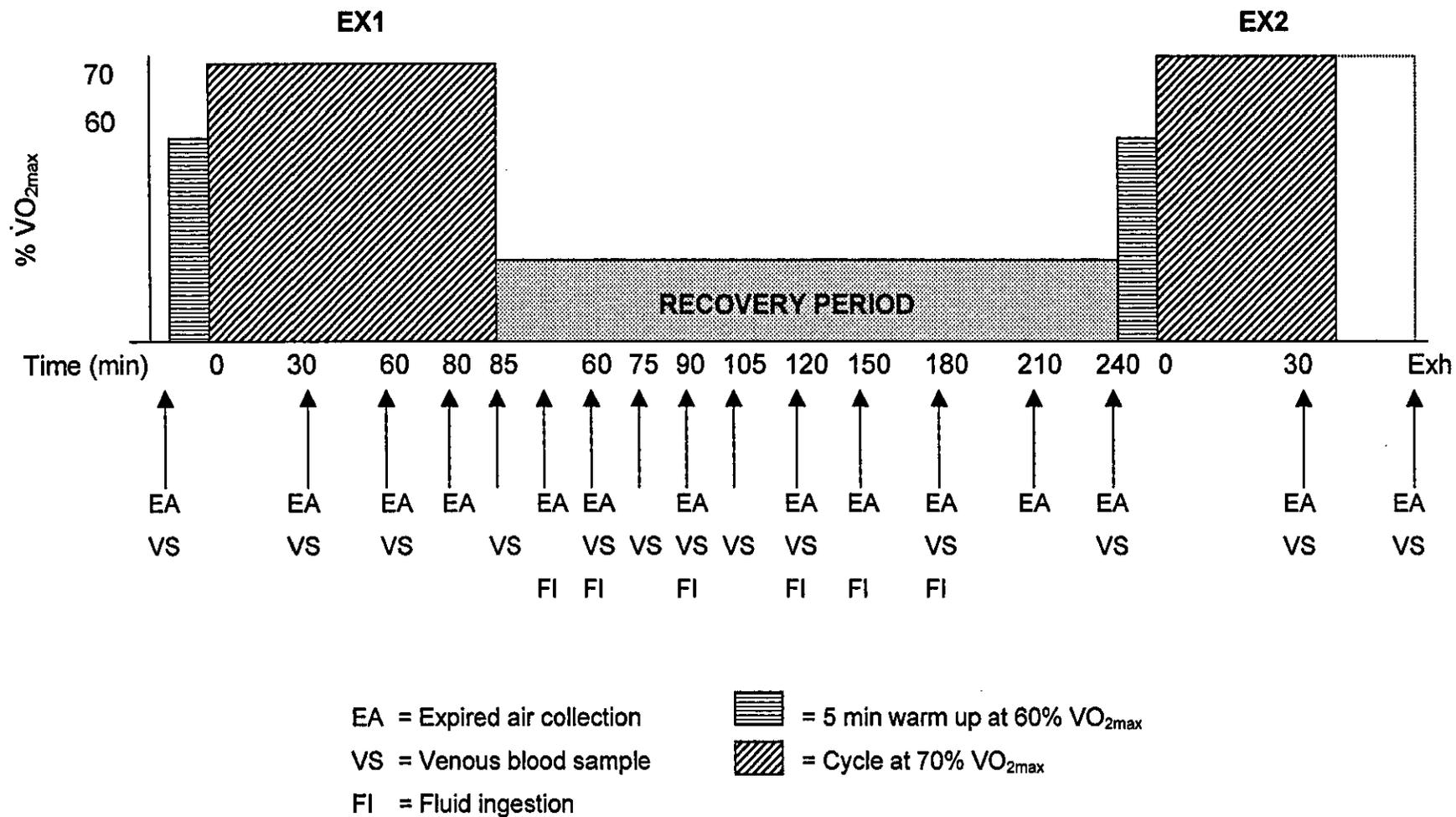


Fig. 5.1 Schematic illustration of the experimental protocol

5.3 RESULTS

Cardiorespiratory and exercise performance. Pulmonary oxygen uptake ($\dot{V}O_2$), expired minute ventilation (data not shown), carbohydrate oxidation and fat oxidation were all increased during exercise ($P < 0.05$), but there were no differences between conditions (Table 5.4). Mean power output was not different between conditions in either EX1 (ALA & GLY: 232 ± 1 ; GLN: 229 ± 2 , watts) or EX2 (ALA & GLY: 226 ± 5 ; GLN: 225 ± 4 , watts).

Heart rate increased during both bouts of exercise, however there were no differences between conditions (Table 5.2). The average heart rate during EX1 was 152 ± 4 b \cdot min $^{-1}$ and 150 ± 4 b \cdot min $^{-1}$ in the ALA & GLY and GLN conditions respectively. The average heart rate during exercise EX2 was 167 ± 2 b \cdot min $^{-1}$, and 165 ± 2 b \cdot min $^{-1}$ in the ALA & GLY and GLN conditions respectively.

Cycle time to exhaustion was 38.4 ± 4.3 min and 35.0 ± 1.6 min in the ALA & GLY and GLN conditions respectively. Cycle time to exhaustion was not significantly different between conditions.

Plasma glucose and lactate data. Plasma glucose concentration decreased during EX1 in both conditions and was significantly lower ($P < 0.05$) than basal after 60 min of EX1 in the ALA & GLY condition (Fig. 5.2). During the recovery period no difference was observed in plasma glucose concentration between conditions. During EX2, plasma glucose concentration decreased in both conditions, with the decline much greater in the ALA & GLY condition (-16.8 %) compared to the GLN condition (-3.0 %) relative to the concentration at the end of the recovery period, this difference being significantly different between conditions ($P < 0.01$).

In both conditions, plasma lactate concentration increased to a similar extent ($P < 0.05$) during both bouts of exercise (EX1 and EX2), but there were no differences between conditions (Fig. 5.3). There was a tendency for plasma lactate concentration to be higher during the recovery period in the ALA & GLY condition, however no difference was observed between conditions.

Serum insulin and plasma NEFA data. A similar decrease was observed in serum insulin concentration ($P < 0.05$) during exercise in both conditions (Fig. 5.4). During recovery, serum insulin concentration increased sharply in both conditions, with the peak in serum insulin concentration occurring after 75 min of recovery in both conditions and being greater in the ALA & GLY condition ($P < 0.01$). No statistically significant difference was observed between conditions during the recovery period at any other time point during the recovery period. However, the area under the serum insulin curve during the recovery period was greater ($P < 0.05$) in the ALA & GLY condition than the GLN condition (ALA & GLY: 1357 ± 103 ; GLN: 1162 ± 86 , $\mu\text{IU}\cdot\text{mL}\cdot\text{h}^{-1}$).

Plasma NEFA concentration initially decreased during EX1 and then increased sharply at the end of EX1 in both conditions (Fig 5.5). During recovery plasma NEFA concentration demonstrated a slight increase over the 4 h period in both conditions, with the concentration in the GLN condition at the end of the recovery period being significantly greater ($P < 0.05$) than the basal value. However, no significant differences were observed between conditions during the recovery period in plasma NEFA concentration. During EX2, plasma NEFA concentration increased to a similar extent in both conditions.

Plasma ammonia and amino acid data. Plasma ammonia concentration increased ($P < 0.05$) to a similar extent (ALA & GLY: $+579 \pm 72$ %; GLN: $+574 \pm 91$ %) during EX1 in both conditions, with no difference were observed between conditions (Fig. 5.6). During recovery, plasma ammonia concentration remained slightly elevated above basal levels in both conditions. During EX2, the increase in plasma ammonia concentration was slightly higher in the GLN condition (ALA & GLY: $+478 \pm 88$ %; GLN: $+526 \pm 146$ %), however no statistically significant difference was observed between conditions.

After glutamine ingestion, plasma glutamine concentration in the GLN condition was elevated significantly ($P < 0.05$) compared to the ALA & GLY condition throughout the recovery period (Fig. 5.7). In addition, after glutamine ingestion, plasma glutamate (Fig. 5.8) and plasma aspartate concentration (Table 5.3) increased significantly ($P <$

0.05) during the recovery period compared to the ALA & GLY condition. Plasma methionine (Table 5.3) concentration was significantly lower ($P < 0.05$) during the second hour of the recovery period after glutamine ingestion.

Plasma phenylalanine concentration had a tendency to be higher and plasma tyrosine concentration a tendency to be lower during the recovery period after glutamine ingestion (Table 5.3). In the second bout of exercise, no difference was observed in the plasma phenylalanine concentration, whereas plasma tyrosine concentration was lower ($P < 0.05$) in the GLN condition compared to the ALA & GLY condition.

Plasma alanine (Fig. 5.9) and glycine (Fig. 5.10) concentrations increased significantly ($P < 0.01$) after the ingestion of the alanine and glycine solution compared to the GLN condition, and plasma glycine concentration remained elevated in the second bout of exercise. In addition, the plasma concentration of serine and threonine was significantly increased ($P < 0.01$) during the recovery period after the ingestion of alanine and glycine (Table 5.3).

During the recovery period and during the second bout of exercise, the sum of plasma BCAA was greater ($P < 0.05$) following GLN ingestion (Fig. 5.11). However, no statistical difference was observed during this time in the sum of the essential plasma amino acids (Fig. 5.8).

The plasma tryptophan:BCAA ratio was not different between conditions during the experimental period (Table 5.4). The sum of the non-metabolised plasma amino acids (Thr, Met, Phe, Lys, Gly, & Tyr), the sum of the non-essential plasma amino acids (Ala, Arg, Asp, Asn, Glu, Gln, Gly, Ser, & Tyr) and the sum of all amino acids were higher at all time points during the recovery period in the ALA & GLY condition ($P < 0.05$) (Table 5.4).

Carbohydrate and fat oxidation rates. During EX1, respiratory exchange ratio, carbohydrate oxidation rate and fat oxidation rate increased in both conditions relative to the basal value (Table 5.2). During the first 30 min of recovery, the carbohydrate oxidation rate decreased and the fat oxidation increased in both conditions relative to the basal value. The respiratory exchange ratio was decreased relative to the basal value

in both conditions throughout the recovery period. However, no statistically significant differences were observed in respiratory exchange ratio, carbohydrate oxidation rate and fat oxidation rate during EX1 and the recovery period between conditions.

During the second bout of exercise, there was a tendency for carbohydrate oxidation to be greater and fat oxidation to be lower in the ALA & GLY condition in the initial period of exercise (Table 5.2). However, no statistically significant differences were observed in respiratory exchange ratio, carbohydrate oxidation rate and fat oxidation rate during the second bout of exercise.

Rehydration, plasma volume and urine output data. Rehydration during the recovery period was not different between conditions (ALA & GLY: 94.5 ± 6.1 %; GLN: 99.3 ± 6.0 %; see Chapter 3 for calculation). Plasma volume decreased during both bouts of exercise and increased during the recovery period, however there was no difference between conditions (data not shown). Total urine output during the recovery period was not different between conditions (ALA & GLY: 2261 ± 154 ml; GLN: 2022 ± 172 ml).

Table 5.2 Oxygen uptake ($\dot{V}O_2$), $\% \dot{V}O_{2\max}$, heart rate (HR), respiratory exchange ratio (RER), carbohydrate oxidation rate (CHO Ox) and fat oxidation rate (Fat Ox) at rest and during the first exercise bout in the ALA & GLY and GLN conditions.

	Condition	Basal	EX1		
			30 min	60 min	85 min
$\dot{V}O_2$ (l.min ⁻¹)	ALA & GLY	0.30 ± 0.02	3.39 ± 0.13*	3.40 ± 0.13*	3.47 ± 0.15*
	GLN	0.32 ± 0.01	3.36 ± 0.16*	3.37 ± 0.14*	3.40 ± 0.15*
% $\dot{V}O_{2\max}$	ALA & GLY	6.6 ± 0.5	72.7 ± 0.8*	73.0 ± 1.2*	74.4 ± 1.6*
	GLN	6.9 ± 0.3	72.0 ± 1.4*	72.5 ± 1.5*	72.9 ± 1.2*
HR (b.min ⁻¹)	ALA & GLY	—	139 ± 6	159 ± 6†	162 ± 7†
	GLN	—	145 ± 5	157 ± 6†	161 ± 6†
RER	ALA & GLY	0.88 ± 0.02	0.93 ± 0.01*	0.92 ± 0.02*	0.92 ± 0.02*
	GLN	0.88 ± 0.02	0.93 ± 0.01*	0.92 ± 0.01*	0.92 ± 0.01*
CHO Ox (g.min ⁻¹)	ALA & GLY	0.21 ± 0.03	3.06 ± 0.30*	3.00 ± 0.36*	3.10 ± 0.38*
	GLN	0.22 ± 0.02	3.06 ± 0.28*	2.99 ± 0.24*	2.96 ± 0.21*
Fat Ox (g.min ⁻¹)	ALA & GLY	0.06 ± 0.01	0.43 ± 0.08*	0.46 ± 0.10*	0.45 ± 0.10*
	GLN	0.07 ± 0.01	0.41 ± 0.07*	0.45 ± 0.06*	0.47 ± 0.06*

Values are means ± SE for 7 subjects. * $P < 0.05$ vs. basal value; † $P < 0.05$ vs. 30 min (EX1)

Table 5.2 (cont'd) Oxygen uptake ($\dot{V}O_2$), $\% \dot{V}O_{2max}$, heart rate (HR), respiratory exchange ratio (RER), carbohydrate oxidation rate (CHO Ox) and fat oxidation rate (Fat Ox) during the recovery period in the ALA & GLY and GLN conditions.

		RECOVERY							
	Condition	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
$\dot{V}O_2$ (l.min ⁻¹)	ALA & GLY	0.36 ± 0.01	0.37 ± 0.02	0.39 ± 0.02	0.37 ± 0.02	0.36 ± 0.02	0.35 ± 0.02	0.35 ± 0.01	0.34 ± 0.01
	GLN	0.37 ± 0.01	0.39 ± 0.02	0.39 ± 0.02	0.37 ± 0.02	0.36 ± 0.02	0.36 ± 0.01	0.36 ± 0.02	0.35 ± 0.02
% $\dot{V}O_{2max}$	ALA & GLY	7.9 ± 0.6	8.1 ± 0.6	8.5 ± 0.6	7.9 ± 0.4	7.9 ± 0.5	7.6 ± 0.5	7.6 ± 0.4	7.4 ± 0.4
	GLN	7.9 ± 0.4	8.4 ± 0.5	8.4 ± 0.6	7.9 ± 0.4	7.9 ± 0.4	7.7 ± 0.6	7.8 ± 0.4	7.4 ± 0.4
HR (b.min ⁻¹)	ALA & GLY	—	—	—	—	—	—	—	—
	GLN	—	—	—	—	—	—	—	—
RER	ALA & GLY	0.73 ± 0.02*	0.82 ± 0.02*	0.82 ± 0.02*	0.81 ± 0.02*	0.79 ± 0.02*	0.81 ± 0.02*	0.80 ± 0.01*	0.76 ± 0.01*
	GLN	0.73 ± 0.01*	0.85 ± 0.01*	0.82 ± 0.02*	0.79 ± 0.01*	0.81 ± 0.01*	0.82 ± 0.02*	0.81 ± 0.02*	0.75 ± 0.02*
CHO Ox (g.min ⁻¹)	ALA & GLY	0.03 ± 0.02	0.17 ± 0.04	0.17 ± 0.03	0.14 ± 0.04	0.11 ± 0.03	0.14 ± 0.02	0.13 ± 0.02	0.06 ± 0.01
	GLN	0.03 ± 0.02	0.22 ± 0.02	0.16 ± 0.02	0.11 ± 0.02	0.14 ± 0.02	0.16 ± 0.03	0.15 ± 0.02	0.05 ± 0.03
Fat Ox (g.min ⁻¹)	ALA & GLY	0.17 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.13 ± 0.02	0.14 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.15 ± 0.01
	GLN	0.17 ± 0.01	0.10 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.13 ± 0.02	0.11 ± 0.01	0.12 ± 0.02	0.15 ± 0.02

Values are means ± SE for 7 subjects. * $P < 0.05$ vs. basal value.

Table 5.2 (cont'd) Oxygen uptake ($\dot{V}O_2$), $\% \dot{V}O_{2max}$, heart rate (HR), respiratory exchange ratio (RER), carbohydrate oxidation rate (CHO Ox) and fat oxidation rate (Fat Ox) during the second bout of exercise in the ALA & GLY and GLN conditions.

	Condition	EX2		
		10 min	20 min	Exh
$\dot{V}O_2$ (l.min ⁻¹)	ALA & GLY	3.44 ± 0.13*	3.46 ± 0.13*	3.42 ± 0.11*
	GLN	3.38 ± 0.14*	3.50 ± 0.18*	3.34 ± 0.18*
$\% \dot{V}O_{2max}$	ALA & GLY	73.9 ± 1.1*	74.5 ± 1.1*	73.6 ± 1.7*
	GLN	72.5 ± 1.4*	75.0 ± 1.8*	71.8 ± 3.1*
HR (b.min ⁻¹)	ALA & GLY	165 ± 6	168 ± 6†	170 ± 5†
	GLN	165 ± 6	167 ± 7	170 ± 6†
RER	ALA & GLY	0.91 ± 0.01	0.91 ± 0.02	0.90 ± 0.02
	GLN	0.90 ± 0.01	0.90 ± 0.02	0.91 ± 0.02
CHO Ox (g.min ⁻¹)	ALA & GLY	2.91 ± 0.27*	2.91 ± 0.35*	2.71 ± 0.35*
	GLN	2.61 ± 0.22*	2.76 ± 0.29*	2.80 ± 0.40*
Fat Ox (g.min ⁻¹)	ALA & GLY	0.52 ± 0.07*	0.53 ± 0.09*	0.59 ± 0.11*
	GLN	0.61 ± 0.07*	0.61 ± 0.08*	0.52 ± 0.11*

Values are means ± SE for 7 subjects. * $P < 0.05$ vs. basal value.

Table 5.3. Plasma amino acid concentrations at rest and during exercise in the ALA & GLY and GLN conditions.

Amino Acid	Condition	Basal	EX1			RECOVERY						EX2	
			30 min	60 min	85 min	60 min	75 min	90 min	105 min	120 min	180 min	240 min	Exh
Valine	ALA & GLY	193 ± 11	187 ± 8	173 ± 13 [*]	171 ± 11 [*]	143 ± 13 ^{**}	164 ± 13 ^{**}	153 ± 12 ^{**}	156 ± 9 ^{**}	139 ± 11 ^{**}	136 ± 4 ^{**}	144 ± 8 ^{**}	171 ± 11 [*]
	GLN	215 ± 13 ^a	220 ± 12 ^a	210 ± 16 ^a	205 ± 13 ^a	183 ± 12 ^{**a}	178 ± 14 ^{**}	167 ± 9 ^{**}	155 ± 5 ^{**}	165 ± 10 ^{**a}	162 ± 9 ^{**a}	174 ± 11 ^{**a}	192 ± 17 [*]
Isoleucine	ALA & GLY	54 ± 3	54 ± 3	49 ± 5	47 ± 4 [*]	37 ± 4 ^{**}	41 ± 4 ^{**}	39 ± 4 ^{**}	40 ± 4 ^{**}	35 ± 4 ^{**}	37 ± 3 ^{**}	39 ± 3 ^{**}	52 ± 4
	GLN	63 ± 3 ^a	65 ± 4 ^a	60 ± 5 ^a	58 ± 3 ^a	50 ± 3 ^{**a}	48 ± 3 ^{**a}	45 ± 2 ^{**}	43 ± 1 ^{**}	46 ± 3 ^{**a}	48 ± 2 ^{**a}	53 ± 2 ^{**a}	60 ± 5 ^a
Leucine	ALA & GLY	104 ± 4	102 ± 6	93 ± 9	91 ± 8	70 ± 9 ^{**}	82 ± 9 ^{**}	77 ± 9 ^{**}	78 ± 8 ^{**}	68 ± 9 ^{**}	68 ± 5 ^{**}	71 ± 5 ^{**}	97 ± 7
	GLN	113 ± 8	119 ± 9 ^a	113 ± 10 ^a	108 ± 7 ^a	95 ± 7 [*]	91 ± 7 ^{**a}	85 ± 6 ^{**}	78 ± 4 ^{**}	84 ± 6 ^{**a}	86 ± 4 ^{**a}	94 ± 7 ^{**a}	109 ± 10
Tyrosine	ALA & GLY	48 ± 2	50 ± 4	55 ± 5	55 ± 6	44 ± 6	46 ± 3	43 ± 3	45 ± 2	40 ± 3	36 ± 4	34 ± 3 [*]	44 ± 4
	GLN	38 ± 4 ^a	34 ± 6 ^a	33 ± 5 ^a	35 ± 5 ^a	30 ± 4 ^a	28 ± 3 ^a	30 ± 5	30 ± 5 ^a	27 ± 4	25 ± 3	23 ± 2	27 ± 3 ^a
Phenylalanine	ALA & GLY	41 ± 1	40 ± 2	42 ± 3	43 ± 3	33 ± 5 ^{**}	38 ± 3	36 ± 3	37 ± 3	33 ± 3 ^{**}	33 ± 3 ^{**}	34 ± 3 [*]	45 ± 3
	GLN	42 ± 2	46 ± 2 ^c	47 ± 3 [*]	47 ± 1 [*]	42 ± 2 [*]	41 ± 2	39 ± 2	37 ± 2	39 ± 2 ^a	41 ± 1 ^a	43 ± 2 ^a	49 ± 2 ^{**}
Tryptophan	ALA & GLY	28 ± 5	33 ± 4	34 ± 4 [*]	34 ± 4	29 ± 3	27 ± 4	26 ± 3	24 ± 3	24 ± 3	24 ± 4	23 ± 4	32 ± 4
	GLN	41 ± 3 ^a	45 ± 4 ^a	46 ± 3 ^a	45 ± 2 ^a	38 ± 2 ^a	37 ± 3 ^a	35 ± 2 ^a	29 ± 2 ^{**}	32 ± 3 ^{**a}	32 ± 1 ^{**a}	32 ± 2 ^{**a}	35 ± 2 ^a
Lysine	ALA & GLY	127 ± 7	115 ± 10	120 ± 15	118 ± 14	92 ± 17 ^{**}	109 ± 13	103 ± 17 [*]	104 ± 13	86 ± 14 ^{**}	78 ± 9 ^{**}	83 ± 12 ^{**}	98 ± 11 [*]
	GLN	117 ± 14	135 ± 14	127 ± 10	114 ± 6	112 ± 9	101 ± 8	110 ± 12	89 ± 10 [*]	85 ± 8 ^{**}	88 ± 7 [*]	88 ± 9 [*]	94 ± 9
Ornithine	ALA & GLY	58 ± 3	54 ± 3	52 ± 4	49 ± 4 [*]	41 ± 6 ^{**}	44 ± 2 [*]	46 ± 6 ^{**}	51 ± 5	43 ± 6 ^{**}	38 ± 6 ^{**}	45 ± 5 ^{**}	43 ± 5 ^{**}
	GLN	39 ± 5 ^a	42 ± 5 ^a	39 ± 4 ^a	36 ± 3 ^a	36 ± 4	34 ± 3 ^a	35 ± 3 ^a	32 ± 5 ^a	30 ± 3 ^a	30 ± 3 ^a	30 ± 2 ^a	30 ± 3 ^a
Methionine	ALA & GLY	26 ± 1	29 ± 1	28 ± 2	27 ± 2	22 ± 3 [*]	25 ± 2	23 ± 2	24 ± 1	21 ± 1 ^{**}	20 ± 1 ^{**}	21 ± 2 ^{**}	25 ± 2
	GLN	25 ± 1	28 ± 2	28 ± 2	29 ± 1 [*]	23 ± 1	21 ± 1 ^a	19 ± 1 ^{**a}	20 ± 1 ^{**a}	20 ± 1 ^{**}	18 ± 1 ^{**}	19 ± 1 ^{**}	22 ± 1
Threonine	ALA & GLY	118 ± 9	120 ± 8	117 ± 9	112 ± 11	141 ± 8 ^{**b}	151 ± 7 ^{**b}	140 ± 9 ^{**b}	135 ± 9 ^{**b}	114 ± 8 ^b	108 ± 9 ^b	98 ± 5 ^{**b}	89 ± 7 ^{**}
	GLN	114 ± 5	119 ± 7	115 ± 8	114 ± 6	97 ± 6 ^{**}	94 ± 7 ^{**}	88 ± 7 ^{**}	87 ± 7 ^{**}	86 ± 5 ^{**}	79 ± 6 ^{**}	80 ± 6 ^{**}	91 ± 7 ^{**}
Glycine	ALA & GLY	213 ± 16	224 ± 22	208 ± 25	201 ± 28	665 ± 103 ^{**b}	841 ± 106 ^{**b}	733 ± 57 ^{**b}	669 ± 62 ^{**b}	578 ± 52 ^{**b}	535 ± 52 ^{**b}	453 ± 51 ^{**b}	328 ± 36 ^b
	GLN	243 ± 34	261 ± 33	240 ± 31	231 ± 26	176 ± 19	165 ± 15	154 ± 18	151 ± 14 [*]	144 ± 11 [*]	134 ± 12 [*]	149 ± 15 [*]	177 ± 14
Histidine	ALA & GLY	57 ± 4	55 ± 5	59 ± 8	53 ± 6	45 ± 7 [*]	54 ± 6	52 ± 7	51 ± 5	45 ± 7 [*]	44 ± 4 [*]	41 ± 6 ^{**}	49 ± 4
	GLN	53 ± 3	63 ± 4	58 ± 3	58 ± 2	61 ± 2 ^a	53 ± 2	57 ± 2	49 ± 3	53 ± 3	55 ± 1 ^a	51 ± 2	56 ± 3
Aspartate	ALA & GLY	14 ± 1	15 ± 2	14 ± 1	14 ± 1	16 ± 1 [*]	16 ± 1	15 ± 1	14 ± 1	13 ± 1	13 ± 1	12 ± 1	14 ± 1
	GLN	14 ± 1	15 ± 1	15 ± 1	15 ± 1	23 ± 1 ^{**a}	24 ± 1 ^{**a}	22 ± 1 ^{**a}	19 ± 1 ^{**a}	20 ± 1 ^{**a}	18 ± 1 ^{**a}	16 ± 1 [*]	15 ± 1
Asparagine	ALA & GLY	36 ± 2	36 ± 1	35 ± 2	34 ± 2	40 ± 2 [*]	42 ± 1 ^{**}	39 ± 2	37 ± 2	33 ± 1	33 ± 2 [*]	31 ± 1 ^{**}	30 ± 2 ^{**}
	GLN	35 ± 1	35 ± 2	34 ± 1	33 ± 1	30 ± 1 ^{**a}	29 ± 1 ^{**a}	27 ± 1 ^{**a}	27 ± 1 ^{**a}	27 ± 1 ^{**a}	26 ± 1 ^{**a}	28 ± 1 ^{**a}	30 ± 1 ^{**}
Serine	ALA & GLY	123 ± 5	135 ± 11	120 ± 7	116 ± 6	141 ± 5 ^{**b}	158 ± 9 ^{**b}	160 ± 12 ^{**b}	152 ± 11 ^{**b}	144 ± 9 ^{**b}	150 ± 15 ^{**b}	150 ± 10 ^{**b}	138 ± 8
	GLN	115 ± 10	112 ± 15 ^a	108 ± 9	106 ± 9	85 ± 7 ^{**}	79 ± 6 ^{**}	78 ± 6 ^{**}	75 ± 6 ^{**}	74 ± 5 ^{**}	69 ± 5 ^{**}	79 ± 5 ^{**}	96 ± 10 ^a
Arginine	ALA & GLY	80 ± 4	85 ± 4	83 ± 5	82 ± 5	77 ± 6	76 ± 4	69 ± 5 ^{**}	69 ± 5 ^{**}	62 ± 4 ^{**}	60 ± 4 ^{**}	61 ± 4 ^{**}	61 ± 7 ^{**}
	GLN	80 ± 5	87 ± 4	85 ± 5	81 ± 4	79 ± 5	76 ± 4	73 ± 7	70 ± 4 [*]	68 ± 4 ^{**}	61 ± 5 ^{**}	66 ± 7 ^{**}	73 ± 6 [*]

Values are means ± SD for 7 subjects, expressed in μmol/l. ^{*}different to basal value ($P < 0.05$), ^{**}different to basal value ($P < 0.01$), ^adifferent to ALA & GLY condition ($P < 0.05$), ^bdifferent to GLN condition ($P < 0.01$).

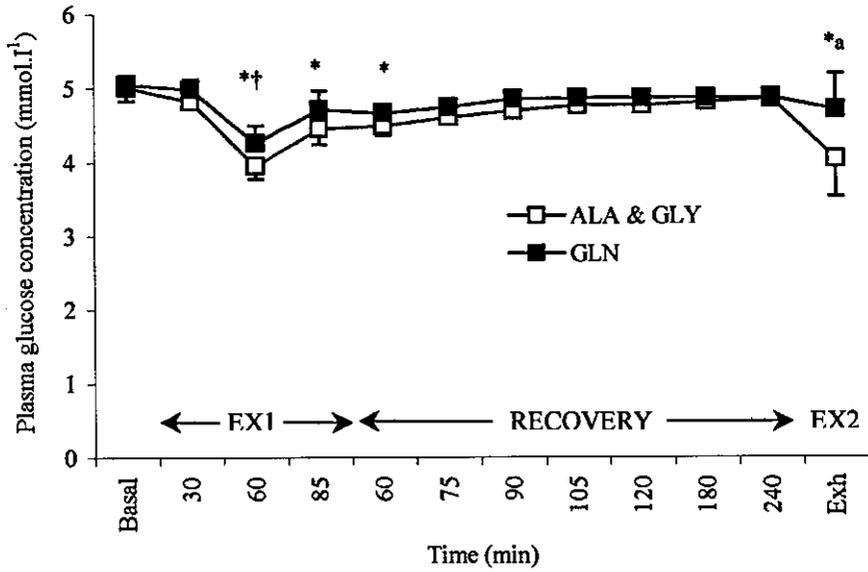
Table 5.4. Summed plasma amino acid concentrations at rest and during exercise in the ALA & GLY and GLN conditions.

Condition	Basal	EX1			RECOVERY							EX2	
		30 min	60 min	85 min	60 min	75 min	90 min	105 min	120 min	180 min	240 min	Exh	
Tryp:BCAA	ALA & GLY	0.08 ± 0.02	0.10 ± 0.01	0.11 ± 0.01 ^a	0.11 ± 0.01 ^a	0.12 ± 0.02 ^{**}	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01
	GLN	0.11 ± 0.01 ^a	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01 ^a	0.12 ± 0.01 ^a	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
n-m AA	ALA & GLY	578 ± 19	578 ± 31	570 ± 46	556 ± 50	997 ± 112 ^{**b}	1213 ± 119 ^{**b}	1078 ± 80 ^{**b}	1014 ± 75 ^{**b}	872 ± 66 ^{**b}	810 ± 68 ^{**b}	723 ± 68 ^{**b}	629 ± 46 ^b
	GLN	572 ± 46	623 ± 43	591 ± 44	571 ± 29	479 ± 25	451 ± 20 ^a	441 ± 29 ^a	403 ± 29 ^{**}	402 ± 11 ^{**}	386 ± 24 ^{**}	403 ± 21 ^{**}	461 ± 23 ^a
AAA	ALA & GLY	117 ± 6	124 ± 6	131 ± 10	131 ± 9	101 ± 14	107 ± 10	105 ± 8	106 ± 7	97 ± 8	89 ± 9	91 ± 10	121 ± 8 ^b
	GLN	114 ± 6	124 ± 10	126 ± 9	128 ± 6	110 ± 4	106 ± 5	105 ± 7	96 ± 8	99 ± 7	98 ± 4	98 ± 6	111 ± 4
NEAA	ALA & GLY	1348 ± 29	1584 ± 59 ^{**}	1582 ± 95 ^{**}	1527 ± 97 ^a	2117 ± 189 ^{**b}	2468 ± 206 ^{**b}	2205 ± 90 ^{**b}	2161 ± 102 ^{**b}	1846 ± 70 ^{**b}	1759 ± 120 ^{**b}	1576 ± 93 ^{**b}	1534 ± 83
	GLN	1469 ± 80	1837 ± 82 ^{**a}	1893 ± 111 ^{**a}	1762 ± 67 ^{**a}	1644 ± 36	1640 ± 48	1507 ± 88	1419 ± 50	1381 ± 45	1263 ± 58	1233 ± 74 ^a	1448 ± 72
Tot. AA	ALA & GLY	2160 ± 32	2370 ± 75 ^a	2348 ± 144	2272 ± 137	2767 ± 223 ^{**b}	3204 ± 238 ^{**b}	2900 ± 149 ^{**b}	2860 ± 119 ^{**b}	2454 ± 105 ^b	2392 ± 152 ^b	2175 ± 131 ^b	2234 ± 107
	GLN	2289 ± 104	2717 ± 91 ^{**a}	2737 ± 142 ^{**a}	2575 ± 69 ^{**a}	2381 ± 54	2338 ± 72	2188 ± 108	2038 ± 56	2022 ± 47	1903 ± 76 ^a	1897 ± 95 ^a	2187 ± 115

Values are means ± SE for 7 subjects, expressed in μmol/l. n-m AA, sum of non-metabolised amino acids (Thr, Met, Phe, Lys, Gly & Tyr); AAA, sum of aromatic amino acids (Try, Tyr & Phe); NEAA, sum of non-essential amino acids (Ala, Arg, Asp, Asn, Glu, Gln, Gly, Ser, & Tyr); Tot. AA, sum of all amino acids. ^adifferent to basal value ($P < 0.05$), ^{**}different to basal value ($P < 0.01$), ^adifferent to ALA & GLY condition ($P < 0.05$), ^bdifferent to GLN condition ($P < 0.01$).

BCAA, sum of branched-chain amino acids (Leu, Iso, Val) and EAA, sum of essential amino acids (His, Iso, Leu, Lys, Met, Phe, Thr, Try, & Val) are shown in Fig. 5.11 & 5.12 respectively.

Fig. 5.2 Plasma glucose concentration during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.

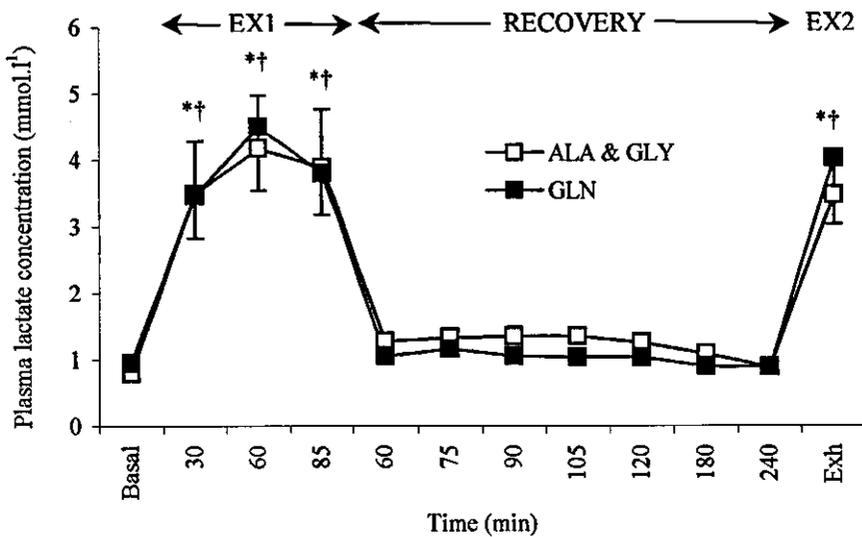


* = significantly different to basal in ALA & GLY conditions ($P < 0.01$)

† = significantly different to basal in GLN condition ($P < 0.01$)

a = significantly different to ALA & GLY condition ($P < 0.01$)

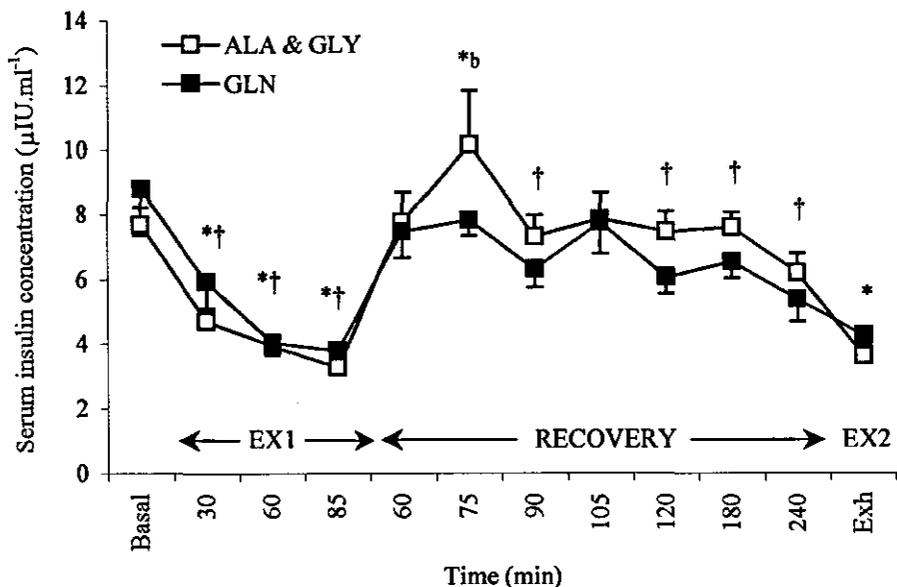
Fig. 5.3 Plasma lactate concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal in ALA & GLY condition ($P < 0.001$)

† = significantly different to basal in GLN condition ($P < 0.001$)

Fig. 5.4 Serum insulin concentration during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.

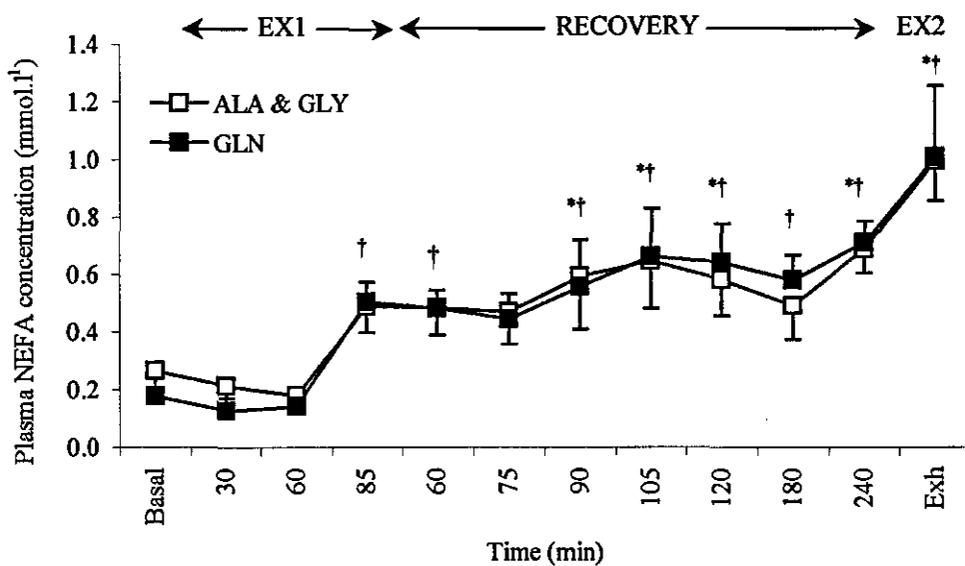


* = significantly different to basal in ALA & GLY condition ($P < 0.01$)

† = significantly different to basal in GLN condition ($P < 0.01$)

b = significantly different to GLN condition ($P < 0.01$)

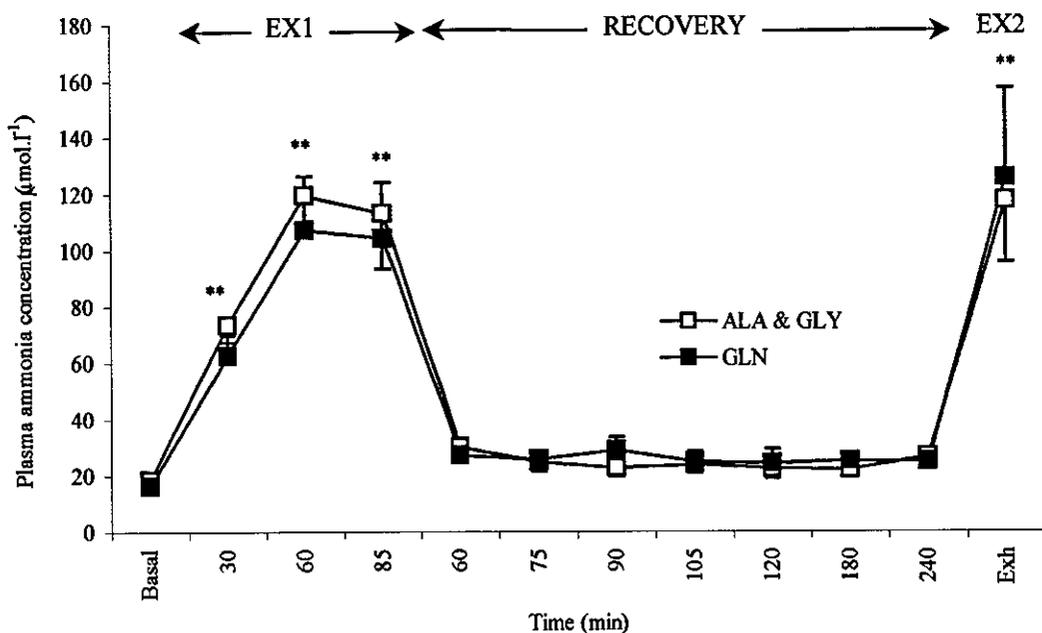
Fig. 5.5 Plasma NEFA concentration during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal in ALA & GLY condition ($P < 0.05$)

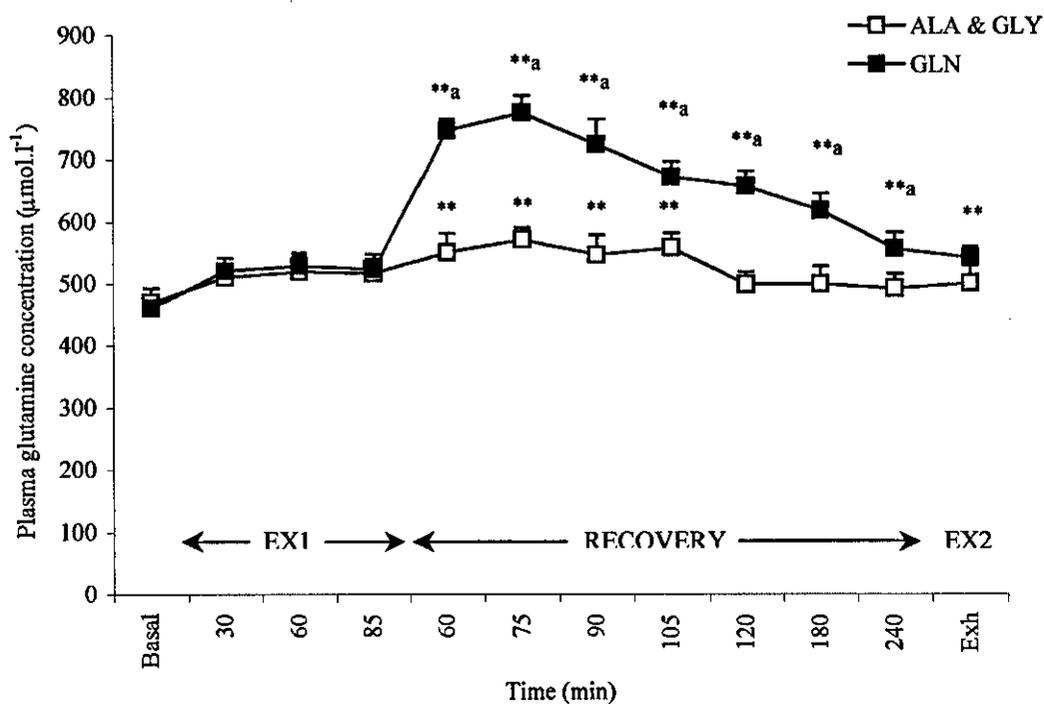
† = significantly different to basal in GLN condition ($P < 0.05$)

Fig. 5.6 Plasma ammonia concentration during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.



** = significantly different to basal ($P < 0.01$)

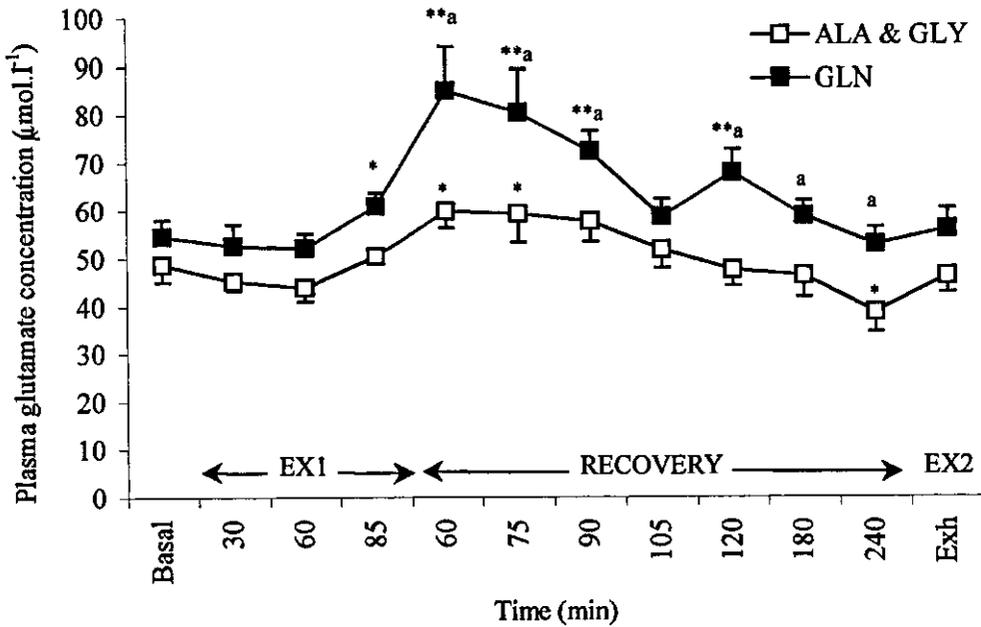
Fig. 5.7 Plasma glutamine concentration during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.



** = significantly different to basal ($P < 0.01$)

a = significantly different to ALA & GLY condition ($P < 0.01$)

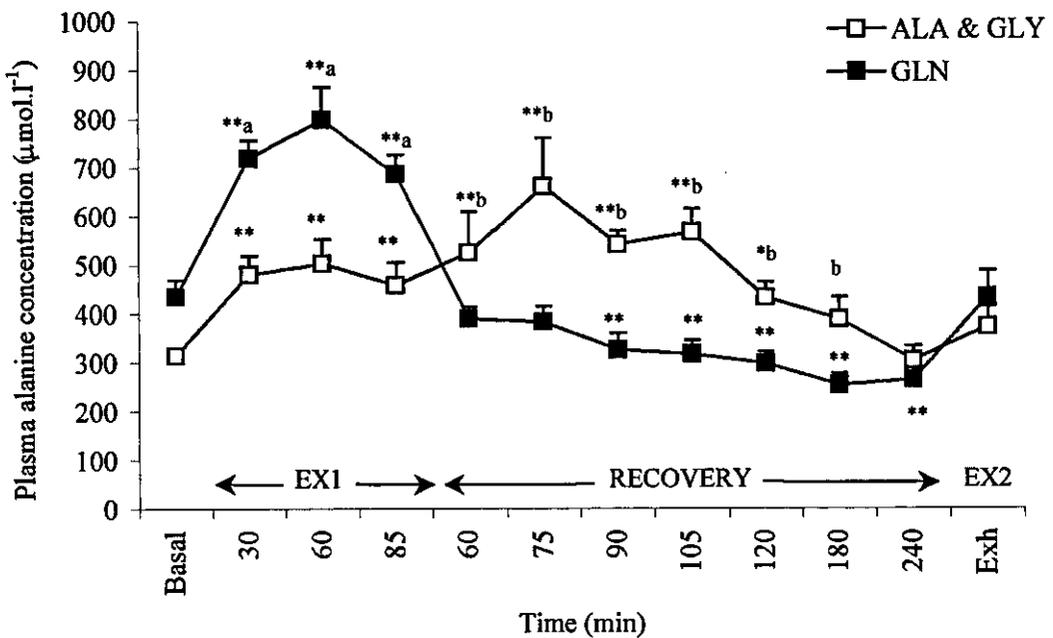
Fig. 5.8 Plasma glutamate concentration during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal ($P < 0.05$) ** = significantly different to basal ($P < 0.01$)

^a = significantly different to ALA & GLY condition ($P < 0.01$)

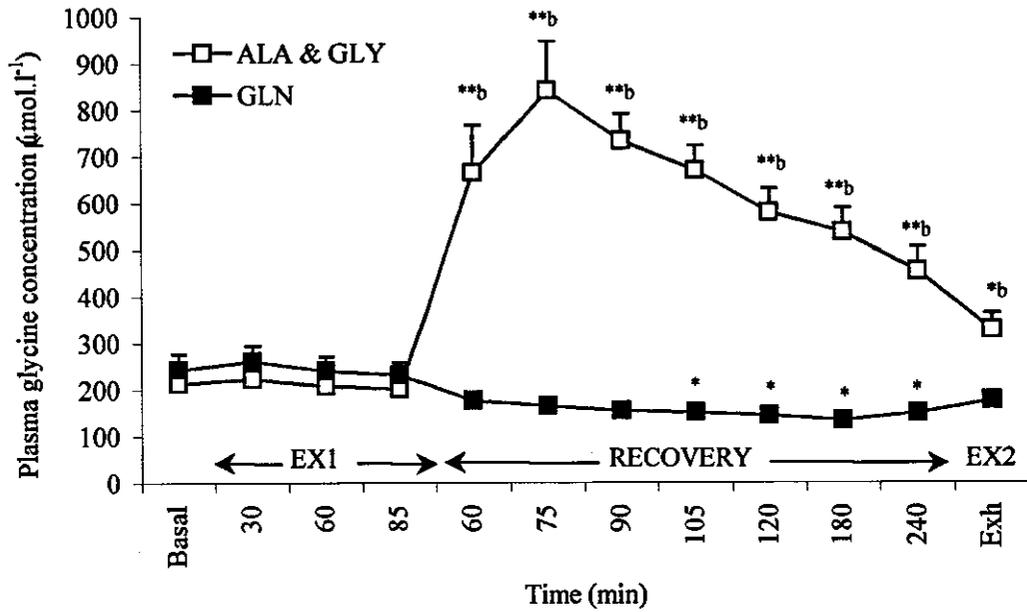
Fig. 5.9 Plasma alanine concentration during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal ($P < 0.05$) ** = significantly different to basal ($P < 0.01$)

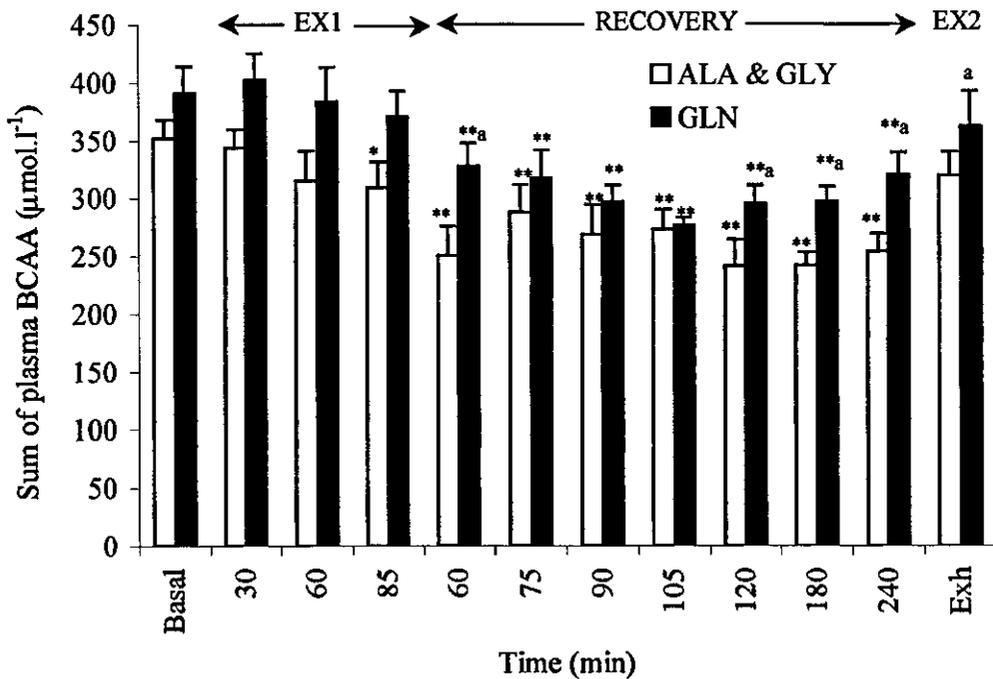
^a = significantly different to ALA & GLY condition ($P < 0.01$) ^b = significantly different to GLN condition ($P < 0.01$)

Fig. 5.10 Plasma glycine concentration during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.



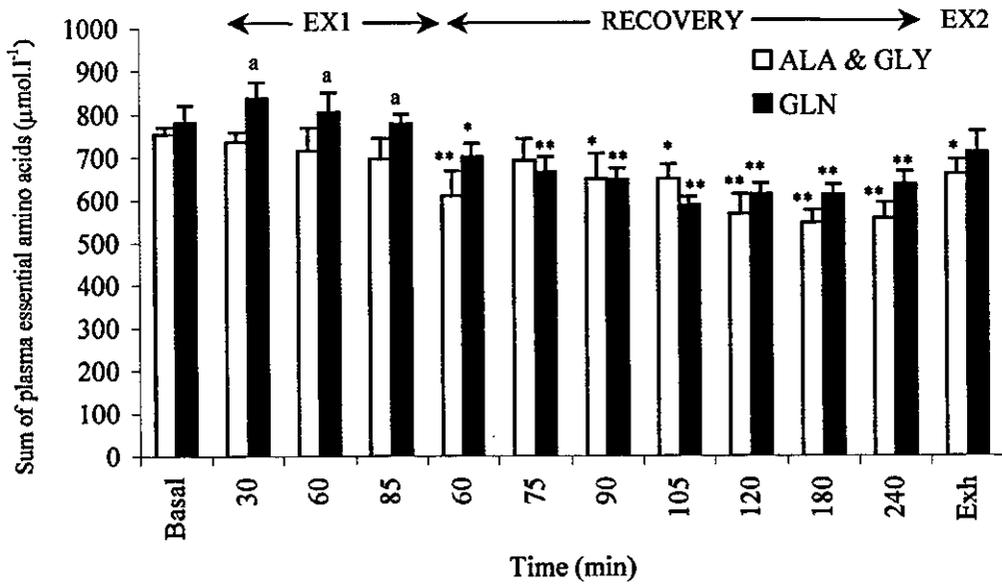
* = significantly different to basal ($P < 0.05$) ** = significantly different to basal ($P < 0.01$)
 b = significantly different to GLN condition ($P < 0.01$)

Fig. 5.11 Sum of plasma branched-chain amino acids (BCAA) during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal ($P < 0.05$) ** = significantly different to basal ($P < 0.01$)
 a = significantly different to ALA & GLY condition ($P < 0.01$)

Fig. 5.12 Sum of essential plasma amino acids during the experimental period in the ALA & GLY and GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal ($P < 0.05$) ** = significantly different to basal ($P < 0.01$)

a = significantly different to ALA & GLY condition ($P < 0.01$)

5.3 DISCUSSION

The main finding from the present study was that no difference was observed in cycle time to exhaustion between conditions in the second bout of exercise, despite a better maintenance of plasma glucose concentration during the second bout of exercise after the ingestion of glutamine during the recovery period.

The ingestion of glutamine in the recovery period was able to elevate plasma glutamine concentration at peak concentration by 64 ± 8 % relative to the basal value (ALA & GLY condition: 18 ± 6 %). This elevation in systemic glutamine concentration occurred despite the expected sequestration of approximately 55 % of the oral glutamine load by the splanchnic bed (Matthews et al. 1993; Haisch et al. 2000). It has been demonstrated that the intravenous infusion of glutamine is able to increase plasma glutamine concentration by ~ 70 % which resulted in a ~ 16 % increase in intramuscular glutamine concentration (Varnier et al. 1995). Therefore the increase in plasma glutamine concentration of ~ 65 % following oral glutamine ingestion in the present study is expected to have increased glutamine concentration in many tissues. Although not measured in this study, it is probable that the increase in plasma glutamine concentration will have increased glutamine concentration particularly in the liver, kidneys and skeletal muscle (Nurjhan et al. 1995; Stumvoll et al. 1996; Stumvoll et al. 1998).

In addition to the elevation in plasma glutamine concentration, the ingestion of glutamine increased the plasma glutamate concentration by 56 ± 14 %, relative to the basal value (ALA & GLY condition: 25 ± 8 %). This increase in plasma glutamate concentration is presumably due to glutamine-glutamate cycling, between the liver, kidneys, and skeletal muscle (Stumvoll et al. 1998).

It has been suggested that in normal postabsorptive humans 40-60 % of plasma glutamine disappearance is due to oxidation (Nurjhan et al. 1995; Perriello et al. 1997; Stumvoll et al. 1996), 10-20 % to gluconeogenesis (Nurjhan et al. 1995; Perriello et al. 1997; Stumvoll et al. 1996) and most of the remainder (~ 15 %) to protein synthesis (Perriello et al. 1997). However, it is feasible that the provision of exogenous

glutamine may alter the contribution of glutamine to these processes, for example increasing both gluconeogenesis (Nurjhan et al 1995) and skeletal muscle protein synthesis (MacLennan et al. 1988). Indeed, glutamine has been shown to increase liver glycogen synthesis in 72 h fasted rats, i.e., when glycogen is depleted (Mouterde et al. 1992). This scenario could also occur after a bout a prolonged submaximal exercise, when both skeletal muscle and liver glycogen levels has been shown to be considerably reduced (Casey et al. 2000). Therefore, it is conceivable that the better maintenance of plasma glucose concentration observed during the second bout of exercise in the GLN condition is indicative of an increased hepatic glycogen storage during the recovery period. This may be due to the provision of exogenous glutamine causing a more rapid increase in liver glycogen stores compared with intramuscular stores post exercise, as a larger proportion of glutamine is extracted on first pass by the splanchnic bed (Matthews et al. 1993; Haisch et al. 2000).

Therefore, during the second bout of exercise, the better maintenance of plasma glucose level is most probably related to an increased hepatic glucose output, although a reduction in skeletal muscle glucose uptake cannot be ruled out. When the results of the present study are compared to the previous study, it is feasible that a better maintenance of plasma glucose concentration is observed in both studies during the second bout of exercise following the ingestion of glutamine (glucose-polymer and glutamine in the previous study) during the recovery period. It is therefore conceivable that at least part of the maintenance in plasma glucose concentration observed in the second bout of exercise is attributable to glutamine ingestion during the recovery period, demonstrating the purported glucogenic or glycogenic properties of glutamine.

Despite a better maintenance of plasma glucose concentration in the second bout of exercise after the ingestion of glutamine in the recovery period, cycle time to exhaustion was not different between conditions. It has been reported that blood glucose concentration is an important factor in the aetiology of fatigue, particularly during cycling (Coyle et al. 1983; Derman et al. 1996). One may therefore have expected cycle time to exhaustion in the second bout of exercise to be greater after the ingestion of glutamine in the recovery period, but this was not observed. It appears that at least with the protocol employed in the present study, blood glucose concentration was not the crucial limiting factor in the aetiology of fatigue in the second bout of exercise.

Other factors including muscle glycogen concentration, may dictate cycle time to exhaustion during the second bout of exercise employed in the present protocol.

Recently, van Hall *et al.* demonstrated that a combination of glucose-polymer and glutamine solution consumed after exhaustive exercise does not increase skeletal muscle glycogen resynthesis, or activate glycogen synthase to a greater extent than glucose-polymer alone (van Hall *et al.* 2000a). However, no comparison was made with a glutamine only condition, therefore any effect that the ingestion of glutamine may have on skeletal muscle glycogen resynthesis, such as the increase demonstrated by Varnier and colleagues (Varnier *et al.* 1995), may have been masked by the co-ingestion of glucose-polymer. Indeed, the dose of glucose-polymer used by van Hall *et al.* ($0.8 \text{ g}\cdot\text{kg}^{-1}$) was sufficient to maximally stimulate glycogen synthase and achieve the maximal rate of muscle glycogen resynthesis (Ivy, 1988b). It would have been intriguing to have included a condition in which glutamine was ingested alone or in combination with a lower dose of glucose-polymer to examine the possible synergistic effects of glutamine and glucose-polymer supplementation during recovery from exercise.

During the determination of the plasma amino acid concentrations in the present study, problems were experienced with the autoinjector during the HPLC analysis. In particular the amino acids alanine, tyrosine, isoleucine, leucine and ornithine were affected. The basal concentration and the concentration during the first bout of exercise are significantly different between conditions, a period where no differences in the treatment of the subjects occurred, hence no statistical differences in the physiological measurements were expected. Therefore, the data obtained and subsequent discussion of the results for these particular amino acids must be treated with caution.

Despite problems with the determination of alanine concentration, the ingestion of alanine and glycine during recovery from exercise resulted in a significant increase in the concentration of both of these amino acids relative to the GLN condition during the recovery period. Plasma glycine concentration increased by ~300 % (at peak concentration; 45 min after ingestion) relative to basal concentration after alanine and glycine ingestion, with plasma glycine concentration falling by ~30 % at the same time point following glutamine ingestion. Plasma alanine concentration increased by ~100

% (at peak concentration; 45 min after ingestion) relative to basal concentration after alanine and glycine ingestion, with a plasma alanine concentration falling by ~10 % at the same time point following glutamine ingestion.

Following alanine and glycine supplementation, the area under the serum insulin curve was greater during the recovery period, suggesting an increase insulin response following the ingestion of these amino acids. Therefore, it would appear that either one or both of these amino acids has a greater insulinogenic effect than glutamine. Indeed, it has previously been reported in sheep that the intravenous infusion of both glycine and alanine individually increase the area under the plasma insulin curve to a greater extent than glutamine, with glycine invoking the greatest response (Kuhara et al. 1991). In addition, the infusion of these two amino acids also produced a greater glucagon response, suggesting that alanine and glycine ingestion individually may promote the secretion of both insulin and glucagon.

During the recovery period, there was a tendency for plasma tyrosine concentration to be reduced, conversely, there was a tendency for plasma phenylalanine concentration to be higher after glutamine ingestion. This is somewhat paradoxical as tyrosine is synthesised almost exclusively from phenylalanine via the phenylalanine hydroxylase reaction. Therefore, any increase in phenylalanine concentration should be reflected by an increase in tyrosine concentration, however in the present study this does not appear to be the case. This may be explained by a rapid increase in the rate of flux through the phenylalanine hydroxylase reaction following glutamine ingestion resulting in the rapid disappearance of tyrosine and the appearance of phenylalanine in plasma. However, in light of the potential problems experienced with the determination of tyrosine, these results should be treated with caution.

Both the sum of the non-essential plasma amino acids and the total amino acids were significantly higher during the recovery period in the ALA & GLY condition compared to the GLN condition. This difference is primarily due to the ingestion of the amino acid glycine, which is not metabolised in skeletal muscle and therefore remains present in the plasma for a prolonged period of time. Linked to the high prevailing plasma concentration of glycine is the synthesis of other amino acids. For example, serine is synthesised from glycine via the serine transhydroxymethylase reaction, and although

this reaction proceeds more often in the direction of glycine synthesis from serine, the ingestion and high plasma concentration of the non-metabolisable amino acid glycine appears to have forced the reaction towards serine production. Furthermore, it is feasible that the increase in glycine concentration explains the increase in threonine concentration, as the high concentration of glycine presumably forced the threonine aldolase reaction in the direction of threonine synthesis. However, glycine combines with acetaldehyde in this reaction, which in high concentrations is toxic, suggesting that this pathway of biosynthesis is less likely. In this respect, the increase in threonine concentration is difficult to explain.

Plasma asparagine concentration was also elevated during the recovery period following the ingestion of the alanine and glycine. This amino acid is synthesised primarily from aspartate via the asparagine synthetase reaction. However, no concomitant increase in plasma aspartate concentration was observed after the ingestion of the alanine and glycine, leaving the mechanism for the increase in asparagine concentration unresolved.

There was however, an increase in plasma aspartate concentration during the recovery period following the ingestion of glutamine. Aspartate is primarily derived from asparagine in the asparaginase reaction, however it is also possible for aspartate to be synthesised via the aspartate aminotransferase reaction ($\text{aspartate} + \alpha\text{-ketoglutarate} \leftrightarrow \text{oxaloacetate} + \text{glutamate}$). In the present study, the ingestion of glutamine increased the plasma concentration of both glutamine and glutamate, thereby providing readily available substrate for the aspartate aminotransferase reaction. Concurrently, the influx of glutamine derived carbons ($\text{glutamine} \leftrightarrow \text{glutamate} \leftrightarrow \alpha\text{-ketoglutarate}$) into the TCA cycle may have increased the availability of oxaloacetate. An increase in the availability of the substrates oxaloacetate and glutamate may therefore have increased flux through the aspartate aminotransferase reaction in the direction of aspartate formation.

Indeed, in isolated intestinal mucosal cells, glutamine carbon has been shown to be incorporated into aspartate (Fleming et al. 1997). The most likely pathway by which glutamine carbon could be incorporated into aspartate would be via efflux of intermediates from the TCA cycle. Therefore, if this mechanism is to explain the

increase in aspartate concentration following glutamine ingestion, this finding provides indirect evidence that carbon derived from glutamine can enter the TCA cycle and be removed at various points to be used in the biosynthesis of a variety of metabolites including glutamate, lactate, alanine, aspartate, citrulline, CO₂, proline, succinate, and ornithine (Fleming et al. 1997). Furthermore, the availability of glutamine appears to influence the rate of entry of glutamine carbons into the TCA cycle (see Chapter 6). Increasing the entry of glutamine into the TCA cycle in intestinal epithelial cells resulted in a decreased fractional oxidation of glutamine and an increased incorporation into synthetic products such as aspartate, lactate, alanine and succinate (Quan et al. 1998).

CHAPTER 6

EFFECT OF GLUTAMINE AND ORNITHINE α - KETOGLUTARATE SUPPLEMENTATION PRIOR TO EXERCISE ON TCA CYCLE INTERMEDIATE POOL SIZE AND OXIDATIVE ENERGY DELIVERY

6.1 INTRODUCTION

The tricarboxylic acid (TCA) cycle is the central site for interaction between carbohydrate, fat and amino acid metabolism in human skeletal muscle both at rest and during exercise. Acetyl units derived from glycolysis, the β -oxidation of fats and amino acid metabolism are oxidised during their passage through the TCA cycle. There is a nine-fold increase in the total content of the TCA cycle intermediates (TCAI) during the transition from rest to exercise at 75% $\dot{V}O_{2\max}$ (Sahlin et al. 1990). However, it is not clear whether this expansion is of any functional significance to oxidative energy production. It has been suggested that the increase in the total content of the TCA cycle intermediates may be necessary for optimal oxidative energy production during intense muscular contraction (Sahlin et al. 1990; Lee and Davis, 1979). The alternative view is that this expansion of the TCAI pool may merely reflect the increase in pyruvate availability resulting from the mismatch between the rate of pyruvate formation, due to the increase in glycolysis at the onset of exercise, and the rate of oxidation of acetyl units in the TCA cycle (Constantin-Teodosiu et al. 1999; Gibala et al. 1998).

It has also been suggested that a decrease in the total content of TCAI may reduce flux through the TCA cycle and the capacity to provide energy via oxidative metabolism (Sahlin et al. 1990). Therefore, it is expected that at fatigue the total content of the TCAI would be reduced, indeed, this is the case during prolonged submaximal exercise (Sahlin et al. 1990; Gibala et al. 1997b). However, this evidence is not sufficient to suggest a direct relationship between the total content of the TCAI and fatigue during prolonged submaximal exercise.

The TCAI pool size is determined by balance between the flux of carbon into and out of the TCA cycle (Lee and Davis, 1979). The increase in TCAI at the onset of exercise is thought to be primarily due to an increase in the rate of anaplerosis [replenishment of TCAI], attributable largely to an increase in flux through the alanine aminotransferase reaction (glutamate + pyruvate \leftrightarrow α -ketoglutarate + alanine) (Gibala et al. 1997a; Sahlin et al. 1990; Spencer et al. 1991b), with carbon entering the TCA cycle at the level of α -ketoglutarate. The significance of this 'anaplerotic' reaction is highlighted by the increase in muscle alanine and decrease in muscle glutamate content at the onset of moderate intensity exercise (Gibala et al. 1997a). One way in which to examine the functional significance of the TCAI pool size would be to attempt to further increase the expansion of the TCAI at the onset of exercise through nutritional intervention and examine the effect upon metabolism.

The content of the TCAI may also be affected by changes in muscle amino acid metabolism, as many of the TCA cycle intermediates take part in ancillary reactions involving amino acids. During conditions in which glycogen availability is limited, for example at the latter stages of prolonged exercise, branched-chain ketoacid dehydrogenase activity is increased (Wagenmakers et al. 1991), increasing BCAA oxidation. The first step in branched-chain amino acid (BCAA; leucine, isoleucine, valine) pathway is a reversible transamination reaction in which α -ketoglutarate acts as an amino group acceptor, forming glutamate and a branched-chain ketoacid. Therefore, an increase in BCAA oxidation may result in an increased rate of α -ketoglutarate 'drainage' from the TCA cycle (Wagenmakers et al. 1991; Wagenmakers et al. 1990). The implications of the removal of α -ketoglutarate from the TCA cycle are minimal as this 'drainage' is normally counteracted by the regeneration of this intermediate by the alanine aminotransferase reaction. However, during the latter stages of prolonged exercise when pyruvate availability for the alanine aminotransferase reaction is reduced, due to decreased glycogen or blood glucose, and ammonia levels are increased, the formation of glutamine is favoured (Goldberg and Chang, 1978). In this scenario, it has been suggested that the content of α -ketoglutarate may decrease, due to a net export of α -ketoglutarate as glutamine from the muscle. This may lead to a reduction in TCA cycle flux and the rate of oxidative energy production, which may have implications for the development of fatigue. This hypothesis was recently tested by supplementing

BCAA in the glycogen depleted state in an attempt to increase BCAA oxidation and reduce the muscle α -ketoglutarate content (Gibala et al. 1999b). The reduction in muscle α -ketoglutarate content was negligible, however only 15 min of moderate intensity exercise was performed; the scenario at fatigue may be different. We hypothesised that the provision of a nutritional supplement that could increase the muscle content of α -ketoglutarate may delay the fatigue process during prolonged exercise, particularly when glycogen levels are low.

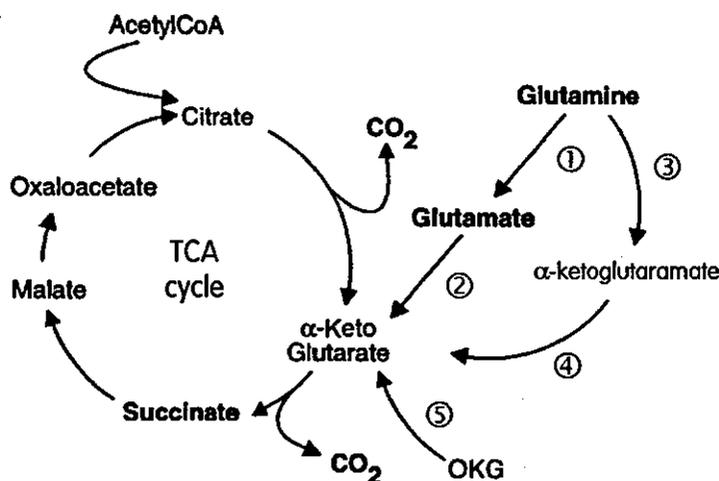


Fig 6.1 Proposed entry of carbon into TCA cycle. ① Conversion of glutamine to glutamate via the action of glutaminase or glutamine transaminase; ② Conversion of glutamate to α -ketoglutarate through the action of glutamate dehydrogenase; ③ Alternate conversion of glutamine to α -ketoglutaramate via the action of glutamine transaminase; ④ Alternate conversion of α -ketoglutaramate to α -ketoglutarate through the action of ω -amidase; ⑤ Proposed entry of α -ketoglutarate moiety of ornithine α -ketoglutarate (OKG).

It is feasible that carbon from the amino acid glutamine could enter the TCA cycle at the level of α -ketoglutarate (see Fig 6.1). Indeed, this has been demonstrated in intestinal epithelial cells (Quan et al. 1998), and should occur in skeletal muscle since all of the enzymes (glutaminase [EC 3.5.1.2], glutamine transaminase [EC 2.6.1.15], glutamate dehydrogenase [EC 1.4.1.2], ω -amidase [EC 3.5.1.3]) required to catalyse the conversion of glutamine to α -ketoglutarate are present (Wu et al. 1991; Rennie et al. 1992; Wibom et al. 1992; Swierczynski et al. 1993). Given the purported anabolic properties of glutamine (Lacy and Wilmore, 1990; MacLennan et al. 1988), the supplementation of this amino acid could also conceivably modulate liver, skeletal muscle and whole-body metabolism.

A more direct approach to provide carbon at the level of α -ketoglutarate would be to provide the substrate α -ketoglutarate itself (see Fig 6.1). Ornithine α -ketoglutarate (OKG) (a salt formed of two molecules of ornithine and one molecule of α -ketoglutarate) is a precursor of glutamine, arginine, proline, and polyamines, all of which have potent roles in the control of protein metabolism (for review see Le Boucher and Cynober, 1998). Furthermore, OKG administration has been shown to restore the intramuscular glutamine pool (Hammarqvist et al. 1990; Le Boucher et al. 1997). Glutamine synthesis is increased after OKG administration as ornithine and α -ketoglutarate share a common metabolic pathway in which all the steps depend upon enzyme reactions at equilibrium or uncontrolled unenzymatic reactions (see section 2.4, Chapter 2). This means that ornithine and α -ketoglutarate metabolism divert to other pathways when the common pathway is saturated, resulting in glutamine formation (Cynober, 1999). Therefore, in addition to modulating TCA cycle metabolism by providing the TCAI α -ketoglutarate, the ingestion of OKG may also promote protein retention.

The aims of the present study were therefore twofold. Firstly, to determine whether, under conditions of low glycogen availability, the consumption of glutamine or OKG influenced the TCAI pool size at rest and during exercise. Secondly, if successful, to investigate the effect of this expansion upon energy metabolism and exercise capacity in human skeletal muscle.

6.2 METHODS

Subjects. Seven healthy well-trained male subjects participated in this study. Their mean (\pm SEM) age, height, body mass and $\dot{V}O_{2\max}$ were 24.1 ± 1.2 y, 180.2 ± 2.0 cm, 80.0 ± 1.6 kg and 4.99 ± 0.15 l.min⁻¹ (62.5 ± 1.8 ml.kg⁻¹.min⁻¹), respectively.

Preliminary tests. Subjects reported to the laboratory approximately 1 week before the experiment and undertook two preliminary tests in order to determine: (i) the oxygen cost of submaximal cycling, and (ii) maximal oxygen uptake ($\dot{V}O_{2\max}$). The protocols

for these tests have been described elsewhere (see Chapter 3). Subjects were fully familiarised with the experimental procedures used during the experimental trials.

Protocol of the study. Subjects were instructed to consume their habitual diet and refrain from exercise or strenuous physical activity for 48 h prior to each experiment. On the afternoon before the experiment, subjects performed a bout of glycogen-depleting exercise, as described previously (Chapter 3). Prior to, and immediately after this bout of exercise, a finger prick capillary blood sample was obtained from each subject to determine both blood lactate and glucose concentrations (YSI 2300 STATPLUS lactate and glucose analyser) to ensure that blood lactate concentration was sufficiently reduced to prevent gluconeogenesis and glycogen resynthesis from lactate during recovery. Subjects then consumed a diet low in carbohydrate over the next ~18 h (see Chapter 3).

All food consumed after the glycogen-depleting exercise bout was prescribed for each subject and was identical before all trials for a given subject, providing $35 \pm 2\%$ carbohydrate, $56 \pm 2\%$ fat, and $11 \pm 2\%$ protein, ~6.0 MJ (Compeat 5.0 Diet Analysis Software, Carlston Bengston Consultants Ltd, USA), to ensure that muscle glycogen stores were reduced prior to the main exercise trial.

On arrival at the laboratory on the morning of the experiment, the overnight fasted subject rested in a supine position and a cannula was inserted into an anti-cubital vein and a resting blood sample was obtained. Subjects then consumed one of three solutions, in a double-blind manner, $5 \text{ ml}\cdot\text{kg}^{-1}$ body wt of an artificially sweetened placebo (CON), $0.125 \text{ g}\cdot\text{kg}^{-1}$ body wt L(+)-ornithine α -ketoglutarate (Laboratories Jacques Logeais, Paris, France) dissolved in $5 \text{ ml}\cdot\text{kg}^{-1}$ body wt of the artificially sweetened placebo (OKG), or $0.125 \text{ g}\cdot\text{kg}^{-1}$ body wt L-glutamine (Sigma-Aldrich Chemicals, Dorset, UK) dissolved in $5 \text{ ml}\cdot\text{kg}^{-1}$ body wt of the artificially sweetened placebo (GLN). The solutions were allocated by systematic rotation. After consumption of the assigned solution, subjects rested for 60 min, then immediately cycled at $70\% \dot{V}O_{2\text{max}}$ until volitional exhaustion. Exhaustion was determined using the definition as described previously (Chapter 4, page 96).

Approximately 15 min before exercise, the skin and fascia over the anterior aspect of one thigh was anaesthetised (Lignocaine, 1 % w:v, Antigen Pharmaceuticals, Ireland) and three small incisions were made to allow extraction of needle biopsy samples from the vastus lateralis muscle (Bergström, 1975). Needle biopsy samples were obtained immediately prior to exercise (60 min after consumption of the assigned solution), after 10 min of exercise and at exhaustion (see Chapter 3 for description of method). During the 1 h rest period, after consumption of the assigned solution, venous blood samples were taken every 20 min. During exercise, venous blood samples were taken at 10 min, and then every 15 min thereafter and at exhaustion. Expired air samples were collected, by Douglas bag for 6 min at rest, 20 and 40 min after consumption of the solution, and for 1 min every 15 min during exercise and at exhaustion.

Dry bulb temperature and relative humidity within the laboratory during the glycogen depleting exercise and the main experimental trial are presented in Table 6.1. No significant differences were observed in temperature and relative humidity between conditions.

Table 6.1 Dry bulb temperature (DBT) and relative humidity (RH) during the glycogen depleting exercise and the main experimental trial.

Condition	Glycogen depletion		Main trial	
	DBT (°C)	RH (%)	DBT (°C)	RH (%)
CON	21.4 ± 0.1	56.6 ± 2.8	20.8 ± 0.2	54.0 ± 2.6
OKG	21.5 ± 0.4	55.1 ± 3.5	20.6 ± 0.6	52.6 ± 1.8
GLN	21.2 ± 0.4	55.8 ± 2.4	20.5 ± 0.6	54.1 ± 1.8

Analysis. Expired air samples were collected and analysed as previously described (Chapter 3). Venous blood samples were analysed for whole blood lactate and glucose concentration, haemoglobin concentration and haematocrit values, plasma ammonia and amino acid concentration, serum insulin and NEFA concentrations, as previously described (Chapter 3). Percentage changes in plasma volume were calculated as previously described (see Chapter 3).

Muscle biopsy samples were immediately immersed in liquid nitrogen, removed from the needle and stored in liquid nitrogen until freeze-dried and stored at -70°C . At a later date the freeze-dried muscle sample was dissected free of visible blood and connective tissue, powdered and washed twice with petroleum ether to remove any fat (see Chapter 3 for full description). Muscle metabolites (ATP, PCr, Cr, lactate, pyruvate, citrate, succinate, malate, fumarate, glutamine and glutamate) were extracted (see Chapter 3 for full description) and determined enzymatically (see Appendix for each procedure).

Pre-exercise energy intake. The energy intake of subjects during the 48 h prior to the glycogen depletion protocol of the main trial are presented in Table 6.2. No differences were observed between conditions in the pre-exercise diet.

Table 6.2 Energy content and percentage of the primary dietary nutrients of the pre-exercise diet for all conditions (mean \pm SEM). Data presented is mean of 2 days diet.

	CON	OKG	GLN
Energy (MJ/day)	12.0 \pm 1.7	11.9 \pm 1.8	12.0 \pm 1.7
Carbohydrate (%)	65.3 \pm 3.1	64.9 \pm 3.2	64.9 \pm 3.2
Fat (%)	22.3 \pm 3.9	22.7 \pm 3.9	22.8 \pm 3.9
Protein (%)	12.5 \pm 0.6	12.5 \pm 0.6	12.5 \pm 0.6

Statistical analysis. The data were analysed by two-way analysis of variance (ANOVA) for repeated measures (time X condition). Cycle time to exhaustion data were analysed by one-way ANOVA. When the ANOVA resulted in a significant *F* ratio, Fisher's post hoc test was used to locate differences between means. Statistical significance was accepted at the 5% level ($P < 0.05$). Results are presented as means \pm SEM.

MS = Muscle sample
 VS = Venous blood sample
 FI = Fluid ingestion
 EA = Expired air collection

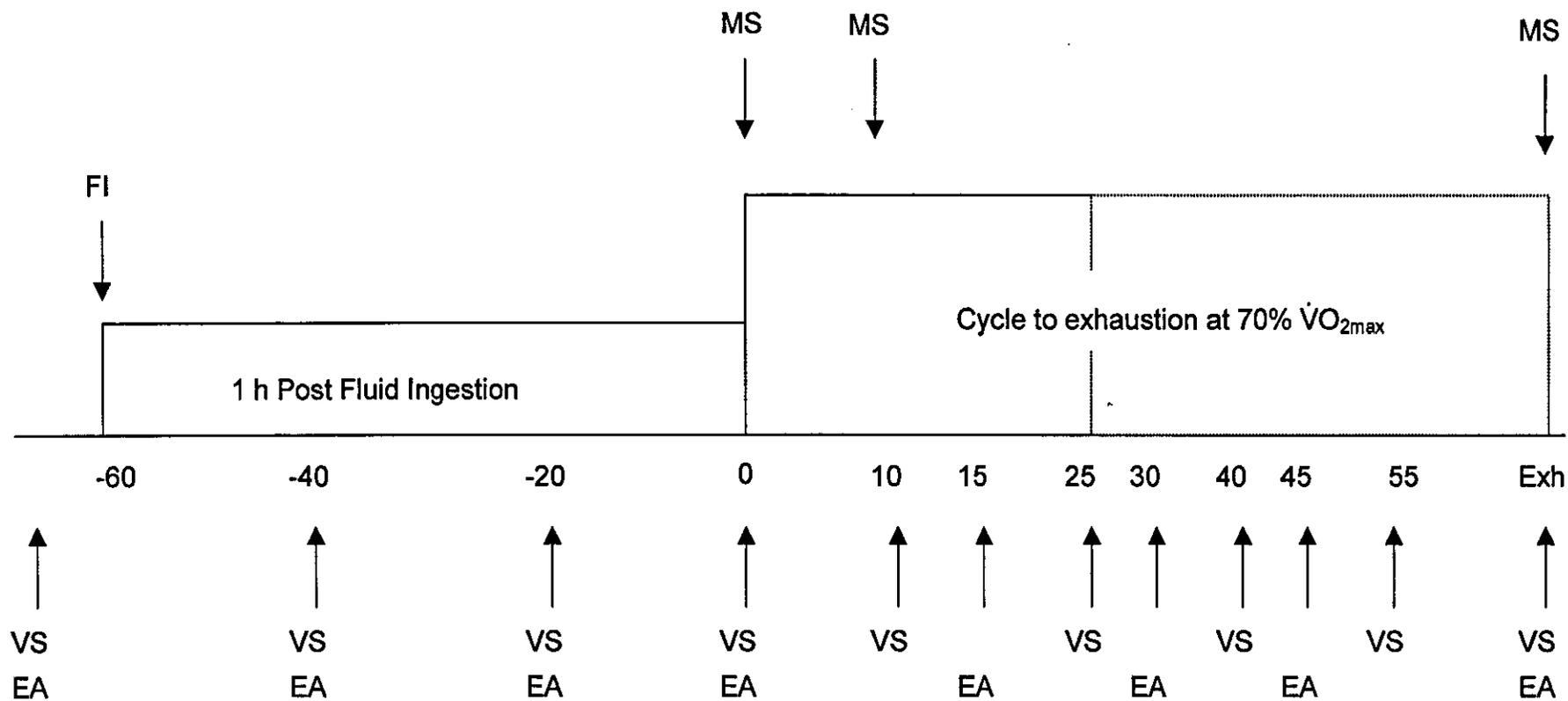


Fig. 6.2 Schematic illustration of the main experimental trial

6.3 RESULTS

No differences were observed between conditions in any of the measured physiological variables during the glycogen depleting exercise including blood lactate and glucose concentrations.

Cardiorespiratory and exercise capacity data. Heart rate, pulmonary oxygen uptake, respiratory exchange ratio and expired minute ventilation (data not shown) showed main effects for time ($P < 0.05$); however, there were no significant differences between conditions (Table 6.3 & 6.3). The average heart rate during the glycogen-depleting exercise was $158 \pm 2 \text{ b.min}^{-1}$, $162 \pm 2 \text{ b.min}^{-1}$ and $157 \pm 2 \text{ b.min}^{-1}$ in the CON, OKG and GLN conditions respectively. The average heart rate during exercise in the main experimental trial was $169 \pm 3 \text{ b.min}^{-1}$, $172 \pm 3 \text{ b.min}^{-1}$ and $171 \pm 4 \text{ b.min}^{-1}$ in the CON, OKG and GLN conditions respectively.

Cycle times to exhaustion were $77.5 \pm 6.9 \text{ min}$, $63.7 \pm 5.4 \text{ min}$ and $84.4 \pm 9.3 \text{ min}$ in the CON, OKG and GLN conditions respectively. Cycle time to exhaustion was not significantly different between conditions.

Blood glucose and lactate concentration. Blood lactate concentration was not different during the 1 h rest period after the consumption of the assigned solution. Blood lactate concentration increased during exercise ($P < 0.05$), but there were no differences between conditions, despite a tendency for a higher concentration in the OKG condition during exercise (Fig. 6.3).

Blood glucose concentration was not different between conditions in the 1 h after ingestion of the assigned solution (Fig. 6.4). Blood glucose concentration increased slightly in all conditions during exercise, with a tendency for a higher concentration in the OKG condition throughout exercise and at exhaustion (Fig. 6.4).

Serum insulin and NEFA concentration. The serum concentration of insulin increased ($P < 0.05$) during the 1 h after consumption of the OKG and GLN solution compared to the CON condition (Fig. 6.5), with the concentration in the OKG condition still elevated

above the CON condition immediately prior to exercise ($P < 0.05$). During exercise, the serum insulin concentration decreased in all conditions, with no differences observed between conditions.

Serum NEFA concentration increased steadily during the 1 h after consumption of the solution in the CON condition compared to the basal value (Fig. 6.6). In contrast, a decrease was observed from basal values in the OKG and GLN conditions. During exercise, the serum NEFA concentration steadily increased in all conditions until exhaustion, with no differences between conditions observed.

Plasma ammonia and amino acid concentration. Plasma ammonia concentration was not different between conditions at any time point. At exhaustion, plasma ammonia concentration was significantly elevated ($P < 0.01$) in all conditions (Table 6.5).

The plasma concentration of both glutamine (Fig 6.7) and glutamate (Fig 6.8) was increased 1 h after consumption of the supplement in the GLN condition, compared to both the CON and OKG conditions ($P < 0.05$). Furthermore, the plasma concentration of glutamine remained elevated at 10 min of exercise in the GLN condition, compared to both the CON and OKG conditions (both $P < 0.05$). Plasma ornithine concentration was higher 1 h after ingestion of the supplement in the OKG condition (Fig. 6.9) and remained elevated throughout exercise in the OKG condition compared to both the CON and OKG conditions (both $P < 0.05$). Plasma alanine concentration significantly increased ($P < 0.05$) in all conditions during exercise relative to the basal value (Fig. 6.10). In addition, plasma alanine concentration was significantly higher in the GLN and OKG conditions compared to the CON condition 1 h after the consumption of the supplements and at 10 min of exercise. Plasma aspartate concentration increased significantly ($P < 0.05$) in the GLN condition 1 h after the consumption of the solution, compared to both the CON and OKG conditions (Table 6.5). Plasma arginine concentration was significantly elevated at 10 min of exercise in the OKG condition compared to the CON condition ($P < 0.05$).

No differences were observed over time or between conditions in the sum of the plasma branched chain amino acids (Iso, Val, & Leu). No differences were observed in the sum of the plasma concentration of the essential amino acids (His, Iso, Leu, Lys, Met, Phe,

Thr, Try, & Val) between conditions at any time point (Table 6.6). The sum of the plasma concentration of the non-essential amino acids (Ala, Arg, Asp, Asn, Glu, Gln, Gly, Ser, & Tyr) was significantly higher ($P < 0.05$) in the GLN condition compared to the CON condition 1 h after ingestion of the solution. In addition, the sum of the plasma concentration of the non-essential amino acids increased at exhaustion in all conditions, with no differences observed between conditions (Fig. 6.11). The sum of the plasma concentration of the aromatic amino acids (Try, Tyr & Phe) was significantly higher ($P < 0.05$) in the OKG condition 1 h after consumption of the solution and at 10 min exercise (Fig. 6.12). Total plasma amino acid concentration was significantly lower ($P < 0.05$) in the CON condition 1 h after consumption of the solution and at 10 min exercise (Table 6.6).

Muscle amino acid content. The intramuscular content of glutamine pre exercise was significantly higher ($P < 0.05$) after GLN supplementation (Table 6.7), compared with the CON and OKG conditions. Muscle glutamine content was not different between conditions at 10 min of exercise or at exhaustion. Intramuscular glutamate content decreased by ~60 % in all conditions after 10 min of exercise ($P < 0.05$) relative to the pre exercise value and was unchanged at exhaustion (Table 6.7). No difference was observed between conditions in muscle glutamate content at any time point.

TCAI content. The total content of the four measured TCAI (Σ TCAI; citrate, succinate, malate, and fumarate) was not different between conditions at rest (Fig. 6.13). The Σ TCAI at 10 min of exercise was greater than at rest in all conditions ($P < 0.05$), and was higher in the GLN condition (4.90 ± 0.61 mmol/kg dry wt) compared to the control condition (3.74 ± 0.38 mmol/kg dry wt, $P < 0.05$) and the OKG condition (3.85 ± 0.28 mmol/kg dry wt) (Fig. 6.13). The Σ TCAI decreased in all conditions at exhaustion, with no difference observed between conditions (CON: 3.16 ± 0.49 ; OKG: 3.49 ± 0.75 ; GLN: 3.41 ± 0.53 , mmol/kg dry wt). The decrease in the Σ TCAI at exhaustion was greatest in the GLN condition with the value at exhaustion being significantly lower than at 10 min of exercise (Fig. 1). The total content of the TCAI pre exercise was comprised of malate (~22%), succinate (~23%), citrate (~52%) and fumarate (~3%). At 10 min of exercise, the total content of the TCAI was comprised of malate (~42%), succinate (~31%), citrate (~22%) and fumarate (~5%). At exhaustion, the total content

of the TCAI was comprised of malate (~34%), succinate (~33%), citrate (~29%) and fumarate (~4%). There were no differences in any of the individual TCAI between conditions (Table 6.8).

Intramuscular metabolite content. No differences were observed in ATP content over time or between conditions. PCr content had declined to a similar extent after 10 min of exercise in all conditions (CON: from 89.5 ± 2.9 mmol/kg dm by 26 ± 6 %; OKG: from 93.5 ± 1.7 mmol/kg dm by 19 ± 7 %; GLN: from 88.2 ± 3.7 mmol/kg dm by 21 ± 6 %, Table 6.9). At exhaustion, there was a slight tendency for a further decrease in all conditions in PCr content relative to the 10 min value, however no difference was observed between conditions. Intramuscular lactate content was not different between conditions either pre exercise, at 10 min of exercise or at exhaustion (Table 6.9). Intramuscular pyruvate content increased in all conditions during first 10 min of exercise (CON: from 0.37 ± 0.06 mmol/kg dm by 70 ± 39 %; OKG: from 0.31 ± 0.06 mmol/kg dm by 134 ± 96 %; GLN: from 0.32 ± 0.05 mmol/kg dm by 171 ± 99 %, Table 6.9). Pyruvate content continued to increase in the CON condition such that the content at exhaustion was significantly different ($P < 0.05$) to the pre exercise value. No differences were observed between conditions at any time point.

Table 6.3 Oxygen uptake ($\dot{V}O_2$), % $\dot{V}O_{2max}$, heart rate (HR), respiratory exchange ratio (RER), carbohydrate oxidation rate (CHO Ox) and fat oxidation rate (Fat Ox) during the glycogen depleting exercise in the CON, OKG and GLN conditions.

	Condition	Exercise		
		30 min	60 min	85 min
$\dot{V}O_2$ (l.min ⁻¹)	CON	3.62 ± 0.12	3.58 ± 0.14	3.62 ± 0.16
	OKG	3.67 ± 0.12	3.65 ± 0.15	3.81 ± 0.19
	GLN	3.53 ± 0.10	3.77 ± 0.15	3.61 ± 0.11
% $\dot{V}O_{2max}$	CON	73.6 ± 1.8	72.7 ± 1.4	73.4 ± 2.1
	OKG	74.6 ± 0.8	74.1 ± 1.6	77.2 ± 2.4
	GLN	71.7 ± 0.7	76.7 ± 2.2	73.5 ± 1.2
HR (b.min ⁻¹)	CON	155 ± 4	160 ± 6	161 ± 5*
	OKG	159 ± 5	163 ± 7	165 ± 5*
	GLN	154 ± 3	159 ± 5	160 ± 5*
RER	CON	0.95 ± 0.01	0.93 ± 0.01	0.92 ± 0.01*
	OKG	0.96 ± 0.01	0.95 ± 0.01	0.94 ± 0.01*
	GLN	0.95 ± 0.01	0.93 ± 0.02	0.92 ± 0.02*
CHO Ox (g.min ⁻¹)	CON	3.45 ± 0.11	3.46 ± 0.12	3.38 ± 0.13
	OKG	3.71 ± 0.20	3.47 ± 0.22	3.41 ± 0.30
	GLN	3.53 ± 0.15	3.46 ± 0.19	3.30 ± 0.22
Fat Ox (g.min ⁻¹)	CON	0.32 ± 0.09	0.48 ± 0.07	0.47 ± 0.08*
	OKG	0.35 ± 0.05	0.37 ± 0.09	0.47 ± 0.08*
	GLN	0.36 ± 0.07	0.44 ± 0.06	0.45 ± 0.11*

Values are means ± SEM for 7 subjects.

* $P < 0.05$ vs. 30 min

Table 6.4 Oxygen uptake, %VO_{2max}, heart rate (HR), respiratory exchange ratio (RER), carbohydrate oxidation rate (CHO Ox) and fat oxidation rate (Fat Ox) during the main experimental trial in the CON, OKG and GLN conditions.

	Condition	Rest			Exercise				
		Basal	-40 min	-20 min	15 min	30 min	45 min	60 min	Exh
VO ₂ (l.min ⁻¹)	CON	0.34 ± 0.02	0.34 ± 0.01	0.36 ± 0.02	3.73 ± 0.16	3.89 ± 0.15	3.77 ± 0.18	3.91 ± 0.14	3.71 ± 0.24
	OKG	0.33 ± 0.01	0.32 ± 0.01	0.35 ± 0.01	3.73 ± 0.15	3.74 ± 0.15	3.76 ± 0.18	3.78 ± 0.23	3.64 ± 0.21
	GLN	0.33 ± 0.01	0.36 ± 0.01	0.36 ± 0.01	3.72 ± 0.14	3.71 ± 0.13	3.73 ± 0.15	3.52 ± 0.14	3.62 ± 0.19
%VO _{2max}	CON	6.8 ± 0.4	6.9 ± 0.2	7.2 ± 0.4	75.7 ± 1.6	79.4 ± 3.5	76.5 ± 1.7	78.2 ± 2.8†	75.1 ± 3.4
	OKG	6.7 ± 0.2	6.6 ± 0.3	7.2 ± 0.4	75.7 ± 1.7	76.0 ± 2.0	76.3 ± 2.1	74.8 ± 2.3	73.8 ± 3.0
	GLN	6.7 ± 0.3	7.2 ± 0.2	7.4 ± 0.2	75.5 ± 2.0	75.4 ± 1.5	75.8 ± 1.7	71.9 ± 1.3	73.3 ± 2.3
HR (b.min ⁻¹)	CON	—	—	—	158 ± 6	166 ± 6*	171 ± 6	176 ± 6	177 ± 6
	OKG	—	—	—	162 ± 5	171 ± 5*	175 ± 5	179 ± 7	179 ± 6
	GLN	—	—	—	157 ± 4	167 ± 4*	171 ± 4	174 ± 5	177 ± 5
RER	CON	0.78 ± 0.02	0.81 ± 0.02	0.80 ± 0.01	0.89 ± 0.01	0.88 ± 0.01	0.90 ± 0.03	0.91 ± 0.01	0.89 ± 0.02
	OKG	0.79 ± 0.02	0.80 ± 0.02	0.80 ± 0.02	0.90 ± 0.01	0.91 ± 0.02	0.90 ± 0.01	0.90 ± 0.02	0.90 ± 0.02
	GLN	0.77 ± 0.02	0.80 ± 0.02	0.80 ± 0.01	0.89 ± 0.01	0.89 ± 0.01	0.88 ± 0.01	0.89 ± 0.03	0.91 ± 0.02
CHO Ox (g.min ⁻¹)	CON	0.09 ± 0.03	0.13 ± 0.03	0.13 ± 0.02	2.72 ± 0.22	2.71 ± 0.18	2.96 ± 0.51	3.29 ± 0.39	2.93 ± 0.46
	OKG	0.10 ± 0.03	0.12 ± 0.03	0.13 ± 0.02	3.01 ± 0.26	3.09 ± 0.28	2.99 ± 0.34	3.02 ± 0.52	3.01 ± 0.47
	GLN	0.09 ± 0.02	0.12 ± 0.03	0.14 ± 0.02	2.74 ± 0.19	2.80 ± 0.11	2.62 ± 0.19	2.65 ± 0.18	3.13 ± 0.48
Fat Ox (g.min ⁻¹)	CON	0.13 ± 0.02	0.11 ± 0.01	0.13 ± 0.02	0.74 ± 0.08	0.83 ± 0.07 ^c	0.87 ± 0.06	0.67 ± 0.07	0.65 ± 0.09
	OKG	0.11 ± 0.02	0.11 ± 0.02	0.12 ± 0.01	0.69 ± 0.08	0.71 ± 0.06	0.71 ± 0.07	0.64 ± 0.06	0.58 ± 0.01
	GLN	0.13 ± 0.02	0.13 ± 0.02	0.13 ± 0.01	0.73 ± 0.07	0.70 ± 0.05	0.79 ± 0.09	0.67 ± 0.05	0.61 ± 0.26

Values are means ± SEM for 7 subjects. Heart rate was not recorded at rest. In all conditions, VO₂, %VO_{2max}, RER, CHO Ox and Fat Ox during exercise was significantly different to rest values ($P < 0.05$); * $P < 0.05$ vs 15 min exercise; ^c $P < 0.05$ vs GLN condition at same time point

Table 6.5 Plasma amino acid and ammonia concentrations during the experimental period in the CON, OKG & GLN conditions

	Condition	REST		EXERCISE	
		Basal	Pre ex	10 min	Exh
NH ₃	CON	23 ± 1	23 ± 4	32 ± 7	150 ± 35**
	OKG	28 ± 4	22 ± 5	42 ± 6	123 ± 24**
	GLN	21 ± 3	24 ± 4	28 ± 4	150 ± 18**
Valine	CON	212 ± 17	212 ± 13	200 ± 15	228 ± 13
	OKG	236 ± 21	223 ± 20	226 ± 14	216 ± 22
	GLN	237 ± 13	219 ± 14	216 ± 13	226 ± 11
Isoleucine	CON	71 ± 8	70 ± 6	68 ± 6	74 ± 6
	OKG	80 ± 7	77 ± 6	78 ± 5	75 ± 7
	GLN	82 ± 7	75 ± 7	74 ± 7	76 ± 5
Leucine	CON	128 ± 13	127 ± 11	121 ± 11	135 ± 9
	OKG	144 ± 13	137 ± 12	140 ± 7	133 ± 14
	GLN	145 ± 10	131 ± 11	130 ± 9	136 ± 8
Tyrosine	CON	54 ± 7	56 ± 7	53 ± 5	75 ± 13***
	OKG	61 ± 7	75 ± 7 ^{ac}	74 ± 10 ^{ac}	69 ± 8
	GLN	54 ± 8	51 ± 7	49 ± 7	65 ± 6 [‡]
Phenylalanine	CON	46 ± 5	50 ± 5	46 ± 7	64 ± 6***
	OKG	55 ± 6	56 ± 8	56 ± 5	59 ± 9
	GLN	54 ± 5	49 ± 6	50 ± 5	64 ± 8 [‡]
Tryptophan	CON	38 ± 6	37 ± 6	41 ± 6	45 ± 8
	OKG	45 ± 7	48 ± 5	44 ± 7	45 ± 9
	GLN	45 ± 7	39 ± 7	44 ± 7	51 ± 10
Lysine	CON	147 ± 14	148 ± 20	125 ± 18	161 ± 12
	OKG	165 ± 10	184 ± 23	197 ± 10	142 ± 20
	GLN	162 ± 13	157 ± 15	159 ± 17	151 ± 14
Methionine	CON	31 ± 3	32 ± 2	31 ± 3	44 ± 3**
	OKG	38 ± 3	44 ± 7 ^{ac}	46 ± 7 ^{ac}	42 ± 5
	GLN	36 ± 3	35 ± 3	36 ± 3	42 ± 2
Threonine	CON	121 ± 6	126 ± 6	119 ± 5	131 ± 7
	OKG	126 ± 9	132 ± 8	126 ± 7	128 ± 11
	GLN	133 ± 7	133 ± 4	126 ± 7	147 ± 17
Glycine	CON	240 ± 18	261 ± 30	254 ± 23	285 ± 29
	OKG	265 ± 20	271 ± 18	271 ± 17	275 ± 25
	GLN	276 ± 23	241 ± 12	241 ± 20	259 ± 37
Histidine	CON	72 ± 12	63 ± 10	59 ± 10	77 ± 8
	OKG	94 ± 12	85 ± 8	91 ± 7	82 ± 12
	GLN	88 ± 10	90 ± 9	90 ± 13	78 ± 8
Aspartate	CON	22 ± 2	23 ± 2	24 ± 1	28 ± 2**
	OKG	20 ± 2	20 ± 1	23 ± 2	24 ± 2 [‡]
	GLN	21 ± 2	30 ± 2 ^{**ab}	26 ± 3 ^{**b}	26 ± 2**
Asparagine	CON	42 ± 2	43 ± 2	42 ± 2	44 ± 2
	OKG	42 ± 12	45 ± 2	42 ± 1	43 ± 3
	GLN	42 ± 1	43 ± 1	41 ± 2	41 ± 2
Serine	CON	129 ± 6	127 ± 10	125 ± 7	133 ± 7
	OKG	137 ± 6	121 ± 4	121 ± 4	132 ± 8
	GLN	135 ± 8	135 ± 4	131 ± 5	141 ± 9
Arginine	CON	95 ± 8	105 ± 8	107 ± 6 ^b	112 ± 6**
	OKG	98 ± 4	114 ± 6**	119 ± 5**	107 ± 4 [‡]
	GLN	97 ± 6	115 ± 8**	116 ± 6**	110 ± 5**

Values are means ± SEM for 7 subjects, expressed in μmol/l. NH₃, ammonia. **P* < 0.05 vs. Basal, ***P* < 0.01 vs. Basal, †*P* < 0.05 vs. 10 min ex, ^a*P* < 0.05 vs. CON condition at same time point, ^b*P* < 0.05 vs. OKG condition at same time point, ^c*P* < 0.05 vs. GLN condition at same time point.

Table 6.6 Summed plasma amino acid concentrations during the experimental period in the CON, OKG & GLN conditions

	Condition	REST		EXERCISE	
		Basal	Pre ex	10 min	Exh
Tryp:BCAA	CON	0.10 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	0.11 ± 0.03
	OKG	0.11 ± 0.02	0.12 ± 0.02	0.10 ± 0.02	0.11 ± 0.02
	GLN	0.10 ± 0.02	0.10 ± 0.02	0.11 ± 0.02	0.12 ± 0.03
n-m AA	CON	585 ± 35	618 ± 54	575 ± 40	685 ± 36*
	OKG	648 ± 39	687 ± 51	696 ± 30 ^{a,c}	646 ± 46
	GLN	662 ± 38	615 ± 21	613 ± 30	663 ± 32
BCAA	CON	410 ± 37	409 ± 30	389 ± 32	438 ± 27
	OKG	461 ± 40	438 ± 37	445 ± 26	422 ± 43
	GLN	464 ± 29	425 ± 31	420 ± 28	438 ± 23
AAA	CON	138 ± 14	142 ± 13	140 ± 14	184 ± 13**
	OKG	161 ± 13	179 ± 16 ^{a,c}	174 ± 19 ^{a,c}	173 ± 22
	GLN	153 ± 11	139 ± 10	144 ± 10	180 ± 17*
EAA	CON	737 ± 59	738 ± 51	690 ± 59	824 ± 35
	OKG	838 ± 56	849 ± 67	864 ± 25	786 ± 75
	GLN	838 ± 38	797 ± 43	796 ± 46	834 ± 32
Tot. AA	CON	2358 ± 120	2431 ± 112 ^{b,c}	2474 ± 109 ^{b,c}	3056 ± 121 ^{***}
	OKG	2584 ± 154	2931 ± 170*	3133 ± 101**	3163 ± 230**
	GLN	2636 ± 98	2908 ± 123	2881 ± 124	3100 ± 50**

Values are means ± SEM for 7 subjects, expressed in µmol/l. n-m AA, sum of non-metabolised amino acids (Thr, Met, Phe, Lys, Gly & Tyr); BCAA, sum of branched-chain amino acids (Leu, Iso, Val); AAA, sum of aromatic amino acids (Try, Tyr & Phe); EAA, sum of essential amino acids (His, Iso, Leu, Lys, Met, Phe, Thr, Try, & Val); Tot. AA, sum of all amino acids.

* $P < 0.05$ vs. Basal, ** $P < 0.01$ vs. Basal, † $P < 0.05$ vs. 10 min ex, ^a $P < 0.05$ vs. CON condition at same time point, ^b $P < 0.05$ vs. OKG condition at same time point, ^c $P < 0.05$ vs. GLN condition at same time point.

Sum of non-essential amino acids (NEAA, Ala, Arg, Asp, Asn, Glu, Gln, Gly, Ser, & Tyr) are shown in Fig. 6.10

Table 6.7 Content of amino acids in human skeletal muscle at rest and during exercise after ingestion of the CON, OKG or GLN solution

	CON			OKG			GLN		
	Pre ex	10 min	Exh	Pre ex	10 min	Exh	Pre ex	10 min	Exh
Glutamine	30.1 ± 1.8	31.7 ± 1.6	33.2 ± 1.8*	29.6 ± 0.4	33.1 ± 1.6*	35.6 ± 1.9*	38.4 ± 1.1†	31.2 ± 1.3*	34.2 ± 1.6*
Glutamate	18.3 ± 0.9	7.5 ± 0.6*	7.4 ± 0.7*	19.1 ± 1.0	7.7 ± 0.5*	7.1 ± 0.6*	18.0 ± 1.3	7.3 ± 0.6*	7.6 ± 0.5*

Values are means ± SEM for 7 subjects, expressed in mmol/kg dry muscle. Exh, exhaustion. *P < 0.05 vs. Pre ex, †P < 0.05 vs. CON and OKG treatment

Table 6.8 Content of TCAI in human skeletal muscle at rest and during exercise after ingestion of the CON, OKG or GLN solution

	CON			OKG			GLN		
	Pre ex	10 min	Exh	Pre ex	10 min	Exh	Pre ex	10 min	Exh
Citrate	0.59 ± 0.06	0.78 ± 0.06	0.79 ± 0.10	0.57 ± 0.07	0.87 ± 0.08	0.88 ± 0.06	0.51 ± 0.07	0.90 ± 0.01	0.87 ± 0.08
Malate	0.25 ± 0.05	1.61 ± 0.23	1.09 ± 0.24	0.24 ± 0.03	1.63 ± 0.20	1.24 ± 0.35	0.24 ± 0.04	2.12 ± 0.22	1.29 ± 0.25*
Fumarate	0.03 ± 0.01	0.19 ± 0.03	0.14 ± 0.04	0.03 ± 0.01	0.17 ± 0.03	0.14 ± 0.04	0.04 ± 0.04	0.26 ± 0.05	0.13 ± 0.03
Succinate	0.25 ± 0.04	1.16 ± 0.13	1.14 ± 0.23	0.24 ± 0.03	1.17 ± 0.12	1.24 ± 0.36	0.23 ± 0.06	1.63 ± 0.36	1.13 ± 0.23

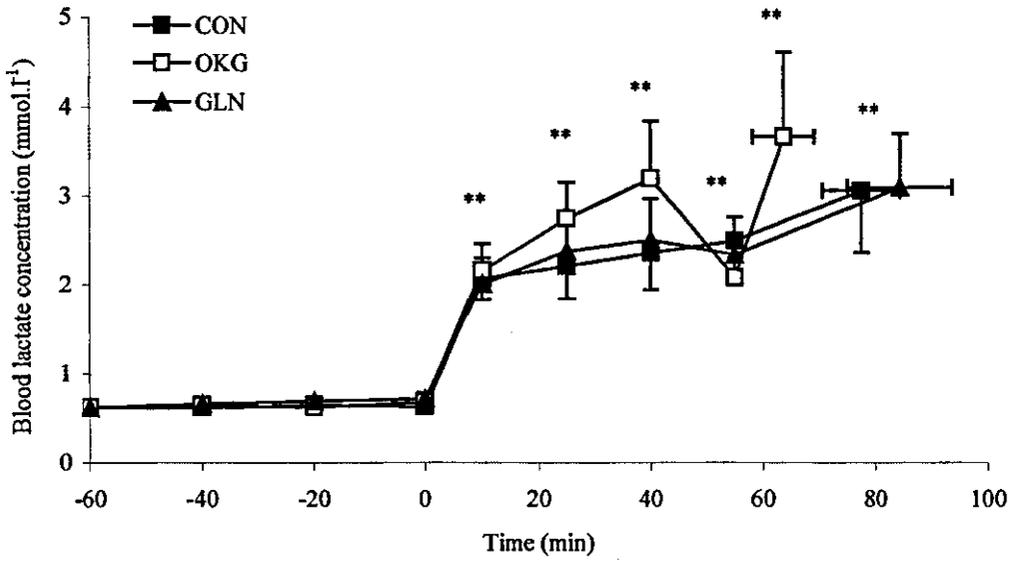
Values are means ± SEM for 7 subjects, expressed in mmol/kg dry muscle. Exh, exhaustion; TCAI, tricarboxylic acid cycle intermediates. For all intermediates, there was a main effect (P < 0.05) for time, such that 10 min and Exh > Pre ex. *P < 0.05 vs. 10 min.

Table 6.9 Metabolite content in human skeletal muscle at rest and during exercise after ingestion of the CON, OKG or GLN solution

	CON			OKG			GLN		
	Pre ex	10 min	Exh	Pre ex	10 min	Exh	Pre ex	10 min	Exh
ATP	26.5 ± 1.0	27.1 ± 2.4	27.4 ± 1.9	26.4 ± 1.0	26.5 ± 1.4	26.4 ± 1.4	25.0 ± 0.9	24.8 ± 1.0	25.6 ± 1.3
PCr	89.5 ± 2.9	65.5 ± 5.1*	63.0 ± 8.7*	93.6 ± 1.7	76.6 ± 7.6*	64.8 ± 11.2*	88.2 ± 3.7	69.4 ± 5.5*	59.6 ± 7.7*
Creatine	41.1 ± 1.8	60.5 ± 5.9*	74.1 ± 13.1*	40.6 ± 2.6	62.3 ± 8.2	79.7 ± 12.7*	35.5 ± 4.2	59.3 ± 7.1	73.2 ± 9.8*
Lactate	2.2 ± 0.1	6.7 ± 1.7	9.1 ± 3.5*	2.1 ± 0.1	6.0 ± 1.0	9.4 ± 3.5*	1.9 ± 0.1	7.1 ± 1.8	8.1 ± 3.0*
Pyruvate	0.37 ± 0.06	0.51 ± 0.05	0.62 ± 0.08*	0.31 ± 0.06	0.42 ± 0.09	0.47 ± 0.09	0.32 ± 0.07	0.60 ± 0.07*	0.61 ± 0.14*

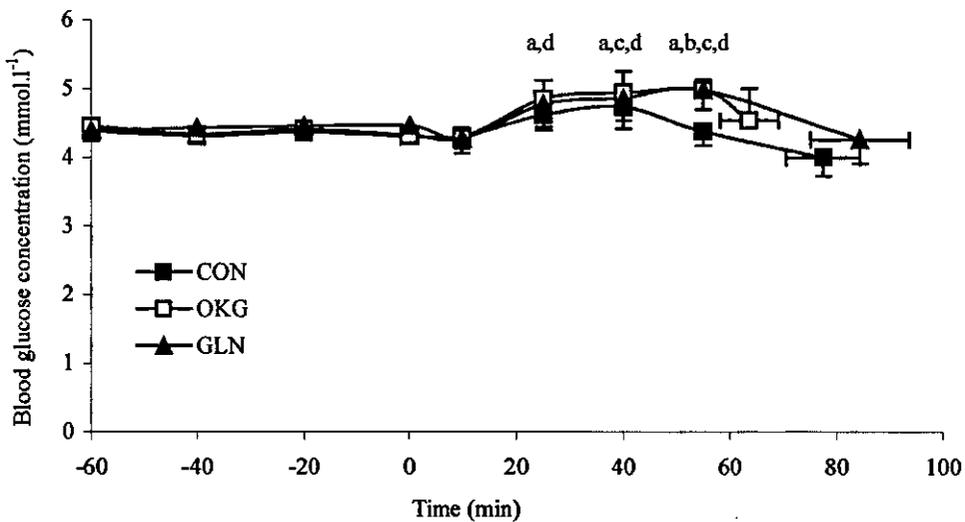
Values are means ± SEM for 7 subjects, expressed in mmol/kg dry muscle. Exh, exhaustion; ATP, adenosine triphosphate; PCr, phosphocreatine. *P < 0.05 vs. Pre ex.

Fig. 6.3 Blood lactate concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



** = significantly different to Basal value in all conditions ($P < 0.01$)

Fig. 6.4 Blood glucose concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



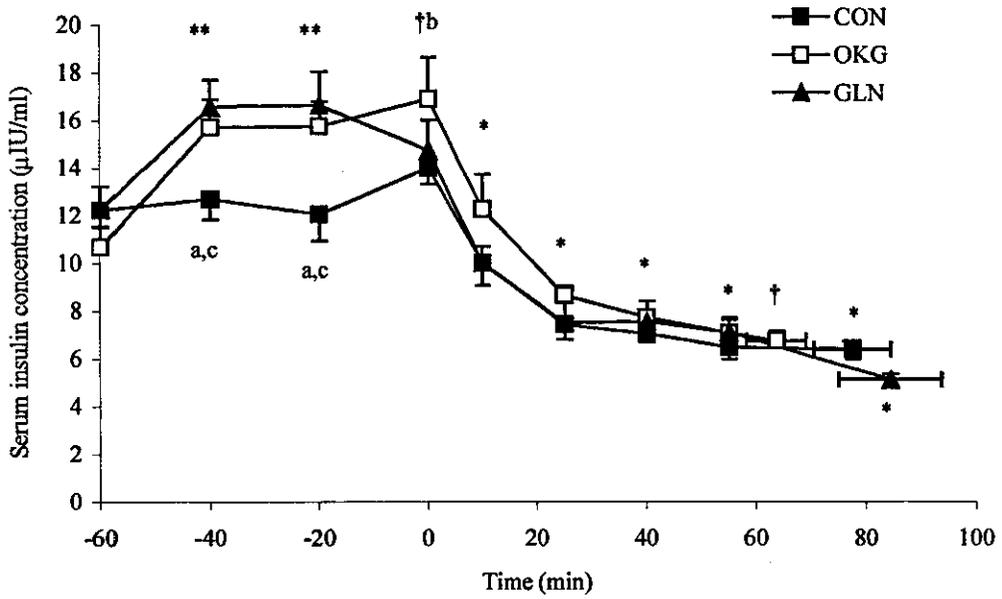
a = significantly different to Exh value in CON condition ($P < 0.05$)

b = significantly different to Exh value in OKG condition ($P < 0.05$)

c = significantly different to Exh value in GLN condition ($P < 0.05$)

d = significantly different to Basal value in GLN condition ($P < 0.05$)

Fig. 6.5 Serum insulin concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to basal value in CON & GLN conditions ($P < 0.05$)
 ** = significantly different to basal value in OKG & GLN conditions ($P < 0.01$)
 † = significantly different to basal value in OKG condition ($P < 0.01$)
 a, c = significantly different to OKG & GLN ($P < 0.05$)
 b = CON significantly different to OKG ($P < 0.01$)

Fig. 6.6 Serum NEFA concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.

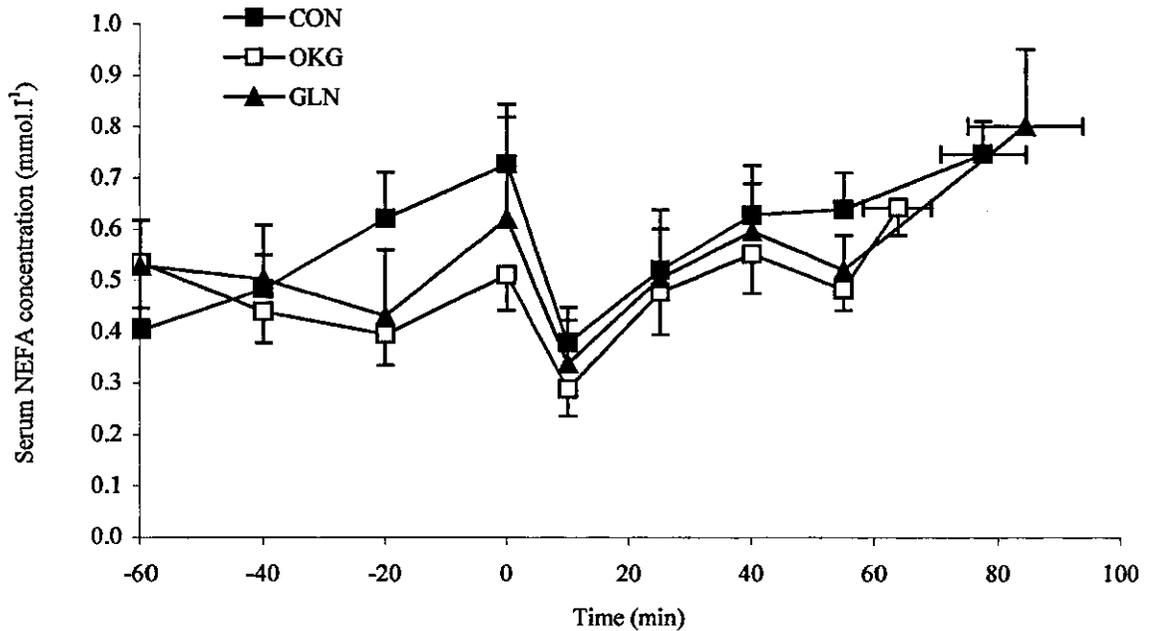
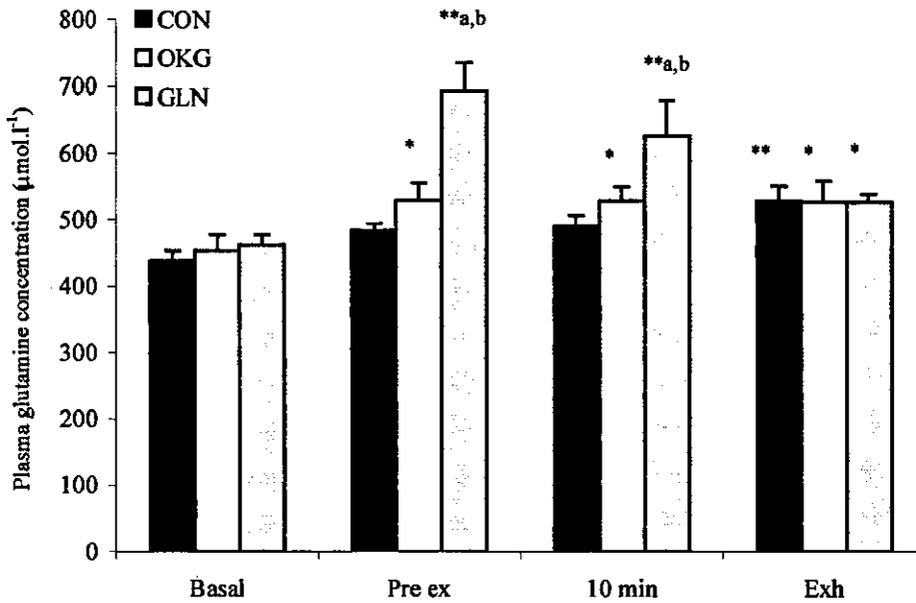
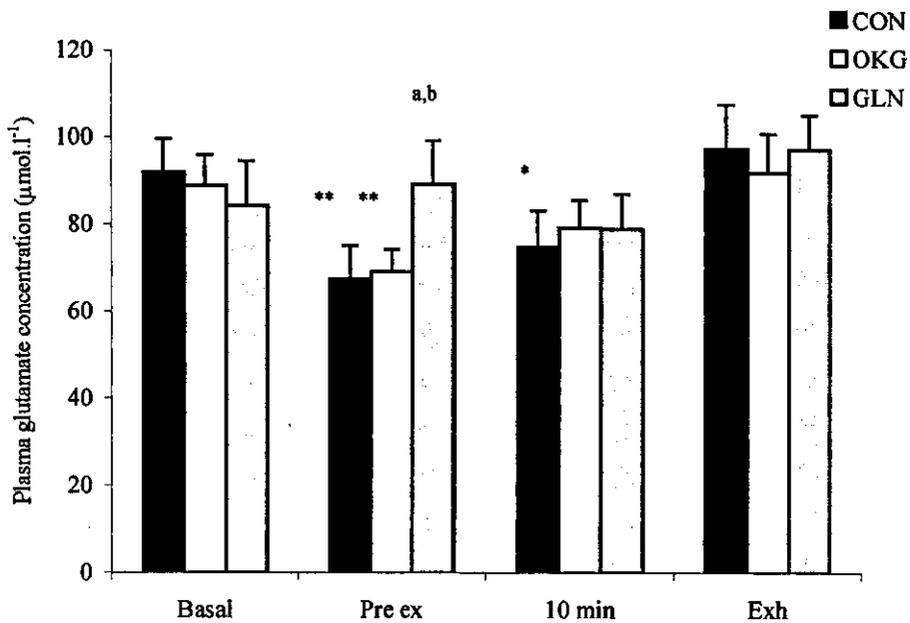


Fig. 6.7 Plasma glutamine concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



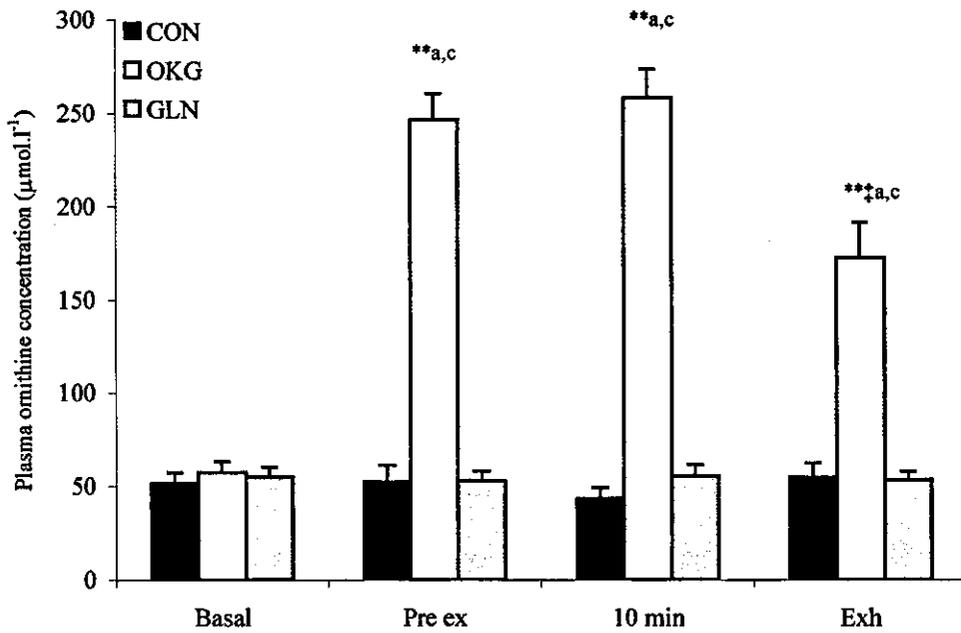
* = significantly different to Basal value ($P < 0.05$)
 ** = significantly different to Basal value ($P < 0.01$)
 a,b = significantly different to CON and OKG condition ($P < 0.05$)

Fig. 6.8 Plasma glutamate concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



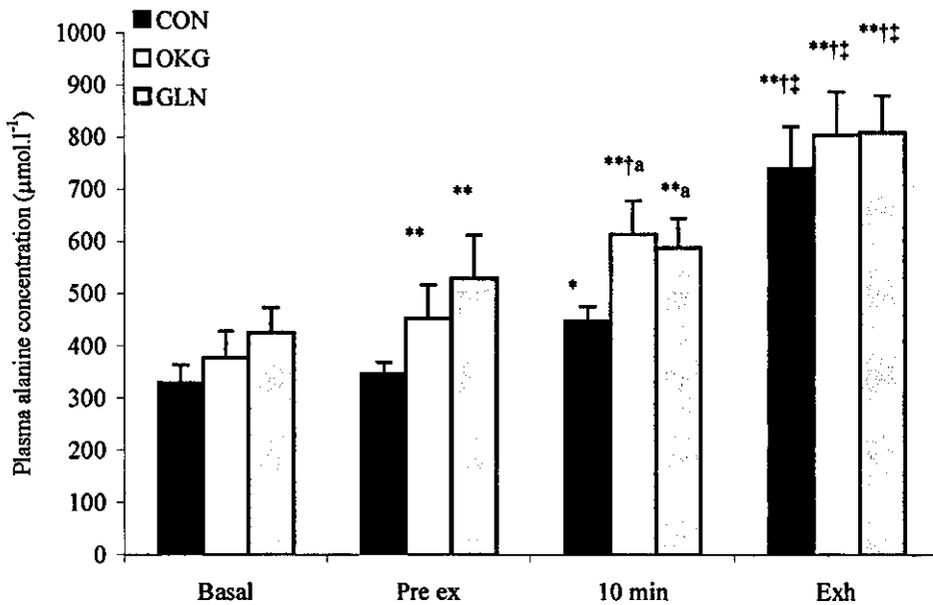
* = significantly different to Basal value ($P < 0.05$)
 ** = significantly different to Basal value ($P < 0.01$)
 a,b = significantly different to CON and OKG condition ($P < 0.05$)

Fig. 6.9 Plasma ornithine concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



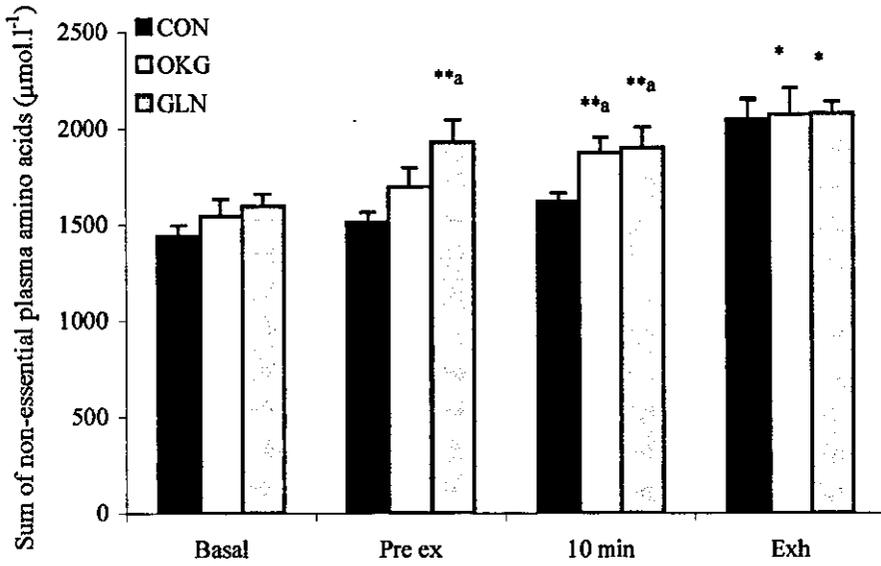
** = significantly different to Basal value ($P < 0.01$)
 † = significantly different to 10 min value ($P < 0.05$)
 a,c = significantly different to CON and GLN condition ($P < 0.05$)

Fig. 6.10 Plasma alanine concentration during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



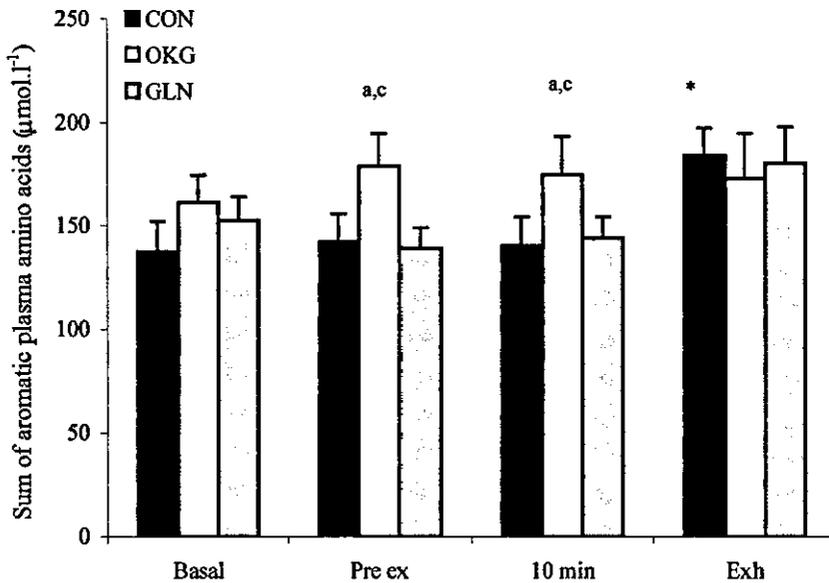
* = significantly different to Basal value ($P < 0.05$)
 ** = significantly different to Basal value ($P < 0.01$)
 † = significantly different to Pre ex value ($P < 0.05$)
 ‡ = significantly different to 10 min value ($P < 0.05$)
 a = significantly different to CON condition ($P < 0.05$)

Fig. 6.11 Sum of non-essential amino acids during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



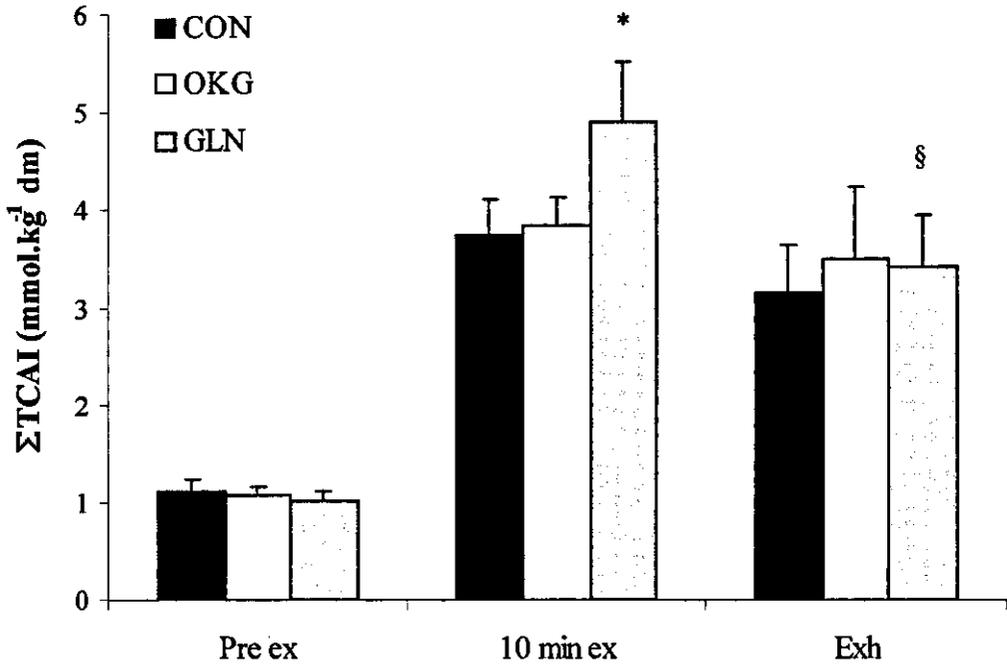
** = significantly different to Basal value ($P < 0.01$)
a = significantly different to CON condition ($P < 0.05$)

Fig. 6.12 Sum of aromatic amino acids during the experimental period in the CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



* = significantly different to Basal value ($P < 0.01$)
a,c = significantly different to CON and GLN condition ($P < 0.05$)

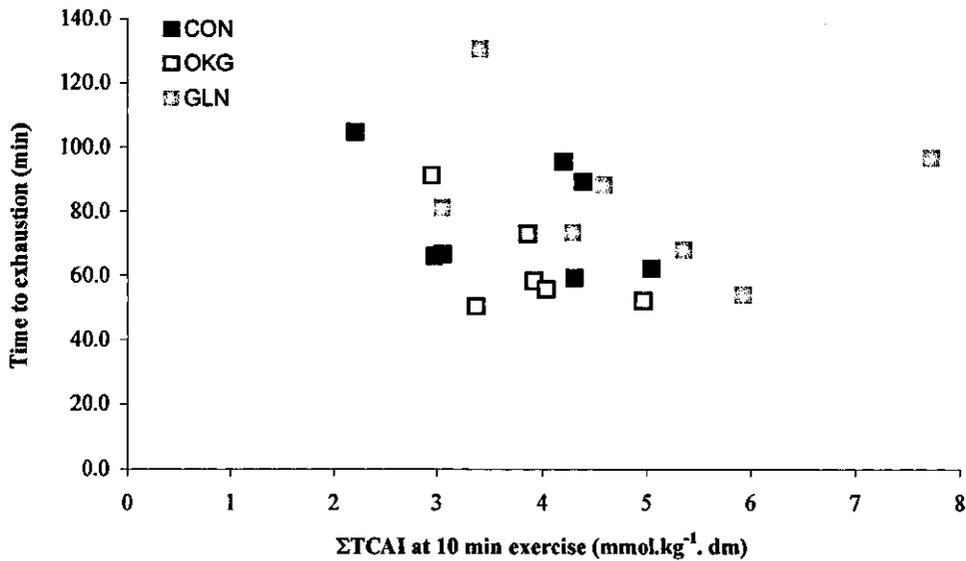
Fig. 6.13 Total muscle content of the 4 measured tricarboxylic acid cycle intermediates (Σ TCAI) citrate, succinate, malate, and fumarate, at rest and during exercise for CON, OKG and GLN conditions. Values are means \pm SEM for 7 subjects.



* $P < 0.05$ vs. CON at same time point

§ $P < 0.05$ vs. 10 min ex

Fig. 6.14 Relationship between Σ TCAI at 10 min exercise and time to exhaustion. Values are for 7 subjects, except for OKG (6 subjects).



6.4 DISCUSSION

The main findings from the present investigation were that the consumption of glutamine 1 h prior to exercise markedly increased the total content of the TCAI in human skeletal muscle after 10 min of moderate intensity cycling exercise. However, despite this further increase in the TCAI pool size, no sparing of PCr or reduction in muscle lactate accumulation was observed during this initial period of exercise. Taken together, these findings suggest that since the rate of mitochondrial respiration is reflected by the extent of phosphocreatine breakdown, at the onset of exercise (Hultman et al. 1967b), oxidative energy production does not appear to be limited by the size of the TCAI pool. Secondly, no difference was observed in the total content of the TCAI at exhaustion or in cycle time to exhaustion between conditions suggesting that the size of the TCAI pool is not an important factor in the aetiology of fatigue during this type of exercise.

In the present study, our aim was to increase the TCAI pool size through nutritional intervention. Glutamine ingestion resulted in an amplification of TCAI pool expansion during the first 10 min of exercise, however the decline in PCr content and accumulation in muscle lactate content were similar in all conditions. Glutamine is readily taken up into skeletal muscle via the high capacity, sodium-dependent system N^m (Ahmed et al. 1993), resulting in the elevated muscle glutamine content in the GLN condition relative to the CON and OKG conditions 1 h after consuming the supplements.

There are several pathways by which α -ketoglutarate may derive from glutamine and hence increase the total content of the TCAI. Glutamine that enters skeletal muscle can be deaminated to form glutamate and then α -ketoglutarate through the action of glutaminase (glutaminase I) and glutamate dehydrogenase, respectively. Alternatively, glutamine may be deaminated to form α -ketoglutaramate and then α -ketoglutarate through the action of glutamine transaminase (glutaminase II) and ω -amidase, respectively. The enzymes responsible for the conversion of glutamine to α -ketoglutarate have all been shown to be present in skeletal muscle (Wu et al. 1991;

Rennie et al. 1992). In the present study, glutamine ingestion resulted in an increase in both plasma and muscle glutamine concentration.

Despite the greater elevation in the TCAI pool size at 10 min of exercise after glutamine ingestion, compared to the OKG or CON conditions, it appears that ATP production via oxidative phosphorylation was not enhanced, since neither PCr depletion or muscle lactate accumulation was different between conditions. This finding would appear to suggest that TCAI pool size does not limit oxidative energy production at the onset of exercise. Indeed, it has been suggested that the availability of acetyl units (Constantin-Teodosiu et al. 1999; Gibala and Saltin, 1999a; Howlett et al. 1999; Constantin-Teodosiu et al. 1999) or some other factor such as muscle oxygenation may limit the extent of oxidative energy production at the start of exercise.

It is noteworthy, however, that after pharmacological activation of the PDC with dichloroacetate (DCA), a decrease was observed in PCr utilisation at 2 min of moderate intensity exercise (Howlett et al. 1999). It is possible therefore that taking a muscle biopsy at 10 min of exercise resulted in an equilibration between conditions in phosphocreatine utilisation, whereas an earlier sampling point may have resulted in a difference in PCr utilisation between conditions. Furthermore, although the TCA cycle ostensibly refers to the mitochondrial matrix, most of the TCAI and the enzymes that catalyse anaplerotic reactions are present in both cytosolic and mitochondrial compartments. At present, analytical techniques allow content and activity to be measured only in whole muscle homogenate. It is conceivable that the mitochondrial content may not be reflected by whole muscle homogenate and that differences may exist between skeletal muscle fibre types.

At exhaustion, the total content of the TCAI was decreased from that at 10 min of exercise in all conditions. The greatest decrease was observed in the GLN condition, so that TCAI content was not different between conditions at exhaustion. In addition, no difference was observed in cycle time to exhaustion between the three conditions. Very few studies have examined the total content of the TCAI during moderate intensity exercise to exhaustion in humans (Sahlin et al. 1990; Spencer et al. 1991c; Gibala et al. 1997b). The present study is in agreement with previous findings (Gibala et al. 1997b), demonstrating a decrease in the TCAI pool size at exhaustion, relative to the expansion

in the pool observed during the initial minutes of exercise. These findings could be interpreted to suggest that, as no difference was observed in TCAI content at exhaustion between conditions, the TCAI content may decrease to a limiting value at which fatigue occurs.

Although no difference was observed in cycle time to exhaustion between the three conditions, there was a tendency for increased exercise capacity in the GLN condition ($P = 0.063$ vs. OKG). It would have been interesting to measure the total content of the TCAI in the GLN condition at the exhaustion time point in the other conditions, however due to the double-blind design employed this was not feasible in the present study. It is feasible that at this time point the total content of the TCAI in the GLN condition may be elevated above the other conditions, and it is only at exhaustion that total content of the TCAI are similar (Fig. 6.13). In this respect, it is conceivable that a critical level in the total content of the TCAI may exist during prolonged moderate exercise, and a decrease below this level may play a part in the aetiology of fatigue. This may explain why, at exhaustion, no difference was observed between conditions in the total content of the TCAI. Only two studies have measured the total content of the TCAI at exhaustion during prolonged moderate exercise (Sahlin et al. 1990; Gibala et al. 1997b), and a reduction in the total content of the TCAI was observed at exhaustion in both studies.

In the present study, glutamine was provided as a single bolus prior to exercise, thus an elevation of plasma and muscle glutamine content was not observed beyond 10 min of exercise, hence the TCAI pool size decreased rapidly (from 4.90 ± 0.61 mmol/kg dry wt at 10 min exercise to 3.41 ± 0.53 mmol/kg dry wt at exhaustion [net decrease CON: 0.58 ± 0.59 ; OKG: 0.30 ± 0.59 ; GLN: 1.49 ± 0.74 mmol/kg dry wt). It would be interesting to supplement glutamine throughout exercise to determine whether the elevation in TCAI pool size could be maintained during exercise and examine the effect upon exercise capacity.

However, an alternative explanation arises if the relationship between the TCAI and endurance capacity is examined in detail. There is a large degree of intra- and inter-subject variation, failing to demonstrate any relationship between TCAI content (at 10

min exercise) and fatigue during prolonged exercise (Fig. 6.14). This would appear to imply that there is no relationship between TCAI content and fatigue, implying that TCAI pool size is not an important factor in the aetiology of fatigue. It appears that the aetiology of fatigue during prolonged moderate intensity exercise is multi-factorial, with factors such as the supply of acetyl units to the TCA cycle or increases in the products of ATP hydrolysis (i.e. ADP, AMP or Pi) being of greater importance than the TCAI pool size.

It is acknowledged that the measurements of substrate-level phosphorylation in the present study (i.e. PCr degradation and muscle lactate accumulation) provide only indirect measurements of the rate of TCA cycle flux and oxidative energy production. However, it was beyond the scope of the study to obtain more direct measurements of TCA cycle flux, which would have required arterio-venous O₂ difference measurements. Furthermore, the technique of nuclear magnetic resonance (NMR) has also shown potential in determining TCA cycle activity (Cohen and Bergman, 1997; Jeffrey et al. 1999; Jucker et al. 1998; Robitaille et al. 1993) which was also beyond the scope of the present study.

Previous studies using DCA to pharmacologically increase the active fraction of PDC, have demonstrated an increase in intramuscular acetyl-CoA and acetylcarnitine (Constantin-Teodosiu et al. 1999; Howlett et al. 1999) with a concomitant reduction in the TCAI pool size at rest, due to a diversion of pyruvate away from anaplerosis (Constantin-Teodosiu et al. 1999; Gibala and Saltin, 1999a). At the onset of exercise the TCAI pool size was not different, however an enlarged pool of acetyl units was readily available, allowing oxidative metabolism to be enhanced leading to a sparing of both phosphocreatine and glycogen and a reduction in muscle lactate accumulation (Timmons et al. 1996). The lesser reliance upon substrate level phosphorylation during this initial period of exercise was possibly due to an increased flux through PDC at the onset of exercise (Howlett et al. 1999) or an accumulation of acetyl groups prior to exercise, 'priming' the TCA cycle with readily available 'fuel' (Constantin-Teodosiu et al. 1999). In terms of oxidative energy delivery, these findings have prompted the notion that the delivery of acetyl units to the TCA cycle is of greater importance than an increase in the total content of the TCAI. In the present study, two strategies were employed to examine the relationship between TCAI pool size and oxidative energy

metabolism. Firstly, to feed carbon, derived from either glutamine or OKG supplements, into the TCA cycle at the level of α -ketoglutarate. Secondly, to deplete the muscle glycogen stores in order to reduce the availability of pyruvate for anaplerotic reactions, so that the influx of α -ketoglutarate into the TCA cycle could be more readily observed. It was hypothesised that this nutritional intervention, as opposed to pharmacological intervention (DCA), would allow the TCAI pool size to be manipulated without altering acetyl unit availability. Therefore, it was hoped that these strategies would allow oxidative energy production to be examined in the presence of an increased TCAI pool size, but with similar acetyl unit availability.

Previously, it has been suggested that anaplerosis is dependent upon pyruvate availability (Constantin-Teodosiu et al. 1999). We therefore employed a bout of exercise followed by a low-carbohydrate diet on the day prior to the experiment to ensure that intramuscular and hepatic glycogen content was reduced prior to exercise. Glycogenolysis and hence the rate of pyruvate formation is reduced in the glycogen-depleted state. The aim of this strategy was to reduce pyruvate related anaplerosis thus allowing the effect of the nutritional intervention on alternative anaplerotic pathways to be observed more readily. Although muscle glycogen content was not measured in the present study, the bout of glycogen depleting exercise employed has previously been shown by our group to reduce muscle glycogen content to 13 mmol glycosyl U/kg wet weight (~55 mmol glycosyl U/kg dry weight) (Bowtell et al. 1999). It is likely therefore that, in combination with the low-carbohydrate diet, muscle glycogen stores were substantially reduced. The diet and exercise regimen were replicated for each trial and the metabolic response during the glycogen-depleting exercise bout was identical between trials confirming that the physiological status of each subject was similar prior to each trial.

It has been reported that approximately 50% of an enterally delivered dose of glutamine is sequestered on first pass through the splanchnic bed (Matthews et al. 1993; Hankard et al. 1995). However, in the present study, the oral provision of glutamine was able to increase plasma glutamine at the peak concentration by 51%, demonstrating that a substantial portion of the oral load escaped utilisation by the gut mucosal cells and uptake by the kidneys and liver. Due to this increase in systemic glutamine

concentration, glutamine will be readily available to be taken up into skeletal muscle via the high capacity, sodium-dependent system N^m . In skeletal muscle, glutamine can undergo a series of reactions to form α -ketoglutarate that can enter the TCA cycle. In addition, plasma ornithine concentration increased by 345% 1 hour after consumption of the OKG solution, suggesting that at least the ornithine component of the oral dose of OKG also escaped utilisation by the splanchnic bed.

The increase in the TCAI pool size was concomitant with a decrease in intramuscular glutamate content in all conditions, presumably asserting the prime importance of glutamate as an anaplerotic precursor via the alanine aminotransferase, glutamate dehydrogenase or ω -amidase reaction in the process of anaplerosis. Furthermore, the ingestion of glutamine, prior to exercise, enhanced the expansion of the TCAI at 10 min of exercise (Fig. 1). Intramuscular glutamine and plasma glutamine concentrations decreased in the GLN condition in addition to the decrease in muscle glutamine content during the initial 10 min of exercise, presumably accounting for the larger expansion in the TCAI pool. These factors suggest that carbon derived from glutamine was able to enter the TCA cycle, presumably at the level of α -ketoglutarate, and was rapidly converted into other TCAI, namely succinate, malate and fumarate, during the initial period of exercise.

Gibala et al. (Gibala et al. 1997b) demonstrated that the sum of citrate, succinate, malate, and fumarate accounts for $\geq 85\%$ of the total TCAI, both at rest and during exercise in humans, thus we are confident that our data represents an accurate quantitative and qualitative index of the total TCAI pool. α -ketoglutarate content was not measured in the present study, however, previous studies have not observed an increase in this TCAI during the initial minutes of moderate exercise (Gibala et al. 1997a; Gibala et al. 1997b; Graham and Saltin, 1989). This phenomenon has been linked to the equilibrium between α -ketoglutarate and glutamate via the glutamate dehydrogenase reaction, thus α -ketoglutarate content may be influenced by the decrease in glutamate content during the initial minutes of exercise (Gibala et al. 1997b; Sahlin et al. 1990; van Hall et al. 1999; Sahlin et al. 1990; van Hall et al. 1999). (Gibala et al. 1998) A disproportionate increase in the content of succinate, malate and fumarate has been observed during exercise in the present and previous studies (Constantin-

Teodosiu et al. 1999; Gibala et al. 1998). It has been demonstrated that an increase in α -ketoglutarate content causes a rapid activation of the α -ketoglutarate dehydrogenase complex (Bunik et al. 1991), therefore, α -ketoglutarate derived from exogenous glutamine is likely to be rapidly converted to intermediates situated in the 'second span' of the TCA cycle. In particular, there appears to be a greater increase in the content of malate in comparison with the rest of the TCAI at 10 min of exercise in the present study. The malate dehydrogenase reaction (L -MDH; oxaloacetate + NADH + H⁺ \leftrightarrow malate + NAD⁺), one possible pathway for malate formation, appears to favour malate formation as the K_m for oxaloacetate is reported to be 10 times lower than for malate (Bergmeyer, 1974). If this is the case, a reduction in oxaloacetate content may limit TCA cycle flux by reducing the availability of substrate for citrate formation, despite an adequate supply of acetyl-CoA.

In addition to the increase in intramuscular glutamine content after GLN supplementation, an increase was observed in plasma glutamate concentration 1 h after GLN supplementation. It is likely that glutamine-glutamate cycling in the splanchnic bed resulted in the higher plasma glutamate level (Curthoys and Watford, 1995; Haisch et al. 2000). Plasma glutamate concentration decreased at 10 min of exercise, suggesting that glutamate was taken up into skeletal muscle and fed into the alanine aminotransferase reaction (glutamate + pyruvate \leftrightarrow α -ketoglutarate + alanine) to increase the total TCAI pool size, which has been observed in previous studies (Gibala et al. 1997b; Sahlin et al. 1990), particularly in the glycogen depleted state (van Hall et al. 1999). Indeed, it has been reported that resting skeletal muscle is able to act as a sink for excess circulating glutamate (Graham et al. 2000), and given the central role that glutamate holds in amino acid metabolism is thus able to modulate human skeletal muscle metabolism. However, it has been reported (Rennie, 1996a) that the glutamate transporter in skeletal muscle (System X_{AG}) has a low capacity (80 nmol·g⁻¹·min⁻¹). The kinetics of this transporter do not appear to adequately explain the vast concentration difference that is maintained between the muscle and blood (muscle > blood) (Bergström et al. 1974), nor the uptake of glutamate from the blood into the muscle despite this gradient. Furthermore, it has been reported that under conditions in which glutamine concentrations fall, the capacity of this transporter is elevated (Low et al.

1994). More work is required in this area to fully understand the kinetics of glutamate transport.

Despite increasing the intramuscular glutamine pool after glutamine ingestion, there appears to be some discrepancy in the increase that one may expect to observe in the expansion of the TCAI pool. An ~ 7 mmol/kg dw decrease was observed in intramuscular glutamine content in the GLN condition, in addition to an ~ 11 mmol/kg dw decrease in the intramuscular glutamate pool during exercise. If all glutamine/glutamate carbon were exclusively channelled into expansion of the TCA cycle intermediate pool, then the extent of the TCAI expansion would have been ~ 4 -fold higher. Furthermore, during the CON and OKG conditions the net decline of glutamine and glutamate pools (~ 9 mmol/kg dw), still far exceeds the expansion observed in the TCAI pool (~ 3 mmol/kg dw).

It is possible to speculate on the potential pathways by which the apparent 'excess' glutamine/glutamate carbon may be disposed. During the GLN condition, it is feasible that the elevated intramuscular glutamine pool drives its own deamination to form glutamate catalysed by glutaminase. However, this is not a simple substrate driven mechanism related to the K_m of glutaminase (4.65 mM in cultured rat skeletal muscle cells; Smith et al. 1984), since exercise must be taken into consideration. There are two possible explanations: first, the decline in glutamate precedes that of glutamine and 'pulls' the conversion of glutamine to glutamate due to the elevated glutamine content and the resultant shift in the reaction equilibrium. Secondly, there is an exercise-induced activation of glutaminase. The former is the most likely explanation since the conversion of glutamine to glutamate does appear to occur in the other two conditions and there is negligible evidence to suggest that glutaminase is activated during exercise. However, the processes that account for the large decline in intramuscular glutamate content at the start of exercise are more difficult to determine. Certainly, the alanine aminotransferase reaction is an important factor in to this decrease (glutamate + pyruvate \leftrightarrow α -ketoglutarate + alanine) (Gibala et al. 1997a). It is thought that at the start of exercise the reaction is driven by the excess pyruvate present due to the rapid stimulation of glycolysis and delayed activation of pyruvate dehydrogenase complex. Certainly plasma alanine concentration was higher during the GLN than the CON condition indicating increased alanine synthesis, if one assumes that splanchnic uptake

of alanine was not different between conditions. Furthermore, as the intracellular compartmentation of the glutamate is yet to be determined, it is feasible that mitochondrial glutamate is more extensively depleted thus limiting the aminotransferase reactions, since these take place largely in the mitochondria. In addition, there is also evidence of glutamate dehydrogenase activity ($\text{glutamate} + \text{NAD}^+ \rightarrow \alpha\text{-ketoglutarate} + \text{NH}_3 + \text{NADH}$) in human skeletal muscle (Wibom et al. 1992; Starritt et al. 1999) which may contribute to the reduction in the glutamate pool at the onset of moderate intensity exercise.

Glutamate is also a key amino group donor in many other reactions. There are two possibilities by which the smaller than 'expected' increase in the TCAI pool might be explained. Firstly, drainage of TCAI to take part in the plethora of other reactions in which the TCAI are involved. Secondly, glutamate and α -ketoglutarate participate in other reactions with no net production of α -ketoglutarate e.g. transamination. The production of aspartate via the aspartate aminotransferase (EC 2.6.1.1) reaction, which is present in human skeletal muscle (Schantz and Henriksson, 1987; Lindena et al. 1986), is an example of the former, with oxaloacetate acting as the amino group ($\text{oxaloacetate} + \text{glutamate} \leftrightarrow \text{aspartate} + \alpha\text{-ketoglutarate}$). The drainage of oxaloacetate (OAA) is balanced by the production of α -ketoglutarate, hence a decrease in glutamate occurs with no net production of TCAI. Certainly in the present study, plasma aspartate was elevated during exercise and to a greater extent in the GLN condition and hence this pathway may provide a partial explanation for the 'disappearance' of glutamate carbon.

Furthermore, the synthesis of glutamine in exercising skeletal muscle is another pathway by which glutamate carbon may be 'lost' from the muscle. Glutamine efflux from skeletal muscle increases significantly from resting levels ($3 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{kg ww}^{-1}$) during moderate intensity exercise ($\sim 70 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{kg ww}^{-1}$) (Gibala et al. 1998). However, it is unlikely that glutamine synthesis occurred to any great extent during the GLN condition since it has been demonstrated that exogenous glutamine suppresses *de novo* glutamine production (Hankard et al. 1995).

The elevation in plasma aspartate in the present study following glutamine ingestion is similar to that seen with monosodium glutamate supplementation (Graham et al. 2000; Stegink et al. 1987), presumably produced via the aspartate aminotransferase reaction (oxaloacetate + glutamate \leftrightarrow aspartate + α -ketoglutarate). It is interesting to note however, that although plasma glutamate concentration was elevated in the present study, intramuscular glutamate concentration was not, whereas both plasma and intramuscular glutamate concentration were elevated by monosodium glutamate ingestion (Graham et al. 2000). It is feasible that this may be due to differences in the intracellular compartmentation of the glutamate carbon in the two studies. Monosodium glutamate will be taken up slowly into the muscle cytosol via the System X_{AG} transporter. However, glutamine is rapidly taken up by the system N^m transporter and then converted to glutamate within the mitochondria since glutaminase is exclusively mitochondrial, as are the majority of aminotransferase enzymes. Cytosolic glutamate is therefore less available and to some extent accumulates, since mitochondrial glutamate uptake is slow. However, mitochondrial glutamate is rapidly utilised in a multitude of reactions and hence does not accumulate.

Ingestion of OKG did not enhance TCAI expansion at the onset of exercise either directly via entry of α -ketoglutarate into the TCA cycle or indirectly via the elevation of the muscle glutamate or glutamine pool. In fact, the ingestion of OKG elicited intramuscular responses that to some extent mirrored the response observed in the control condition. It is possible therefore that despite successfully increasing plasma ornithine concentration and presumably also plasma α -ketoglutarate concentration (Cynober et al. 1990), the substrate was not effectively taken up by the skeletal muscle. The splanchnic bed may have sequestered a large portion of the OKG given by the oral route (particularly the α -ketoglutarate component), as reported previously in starved rats (Ziegler et al. 1992), which may have resulted in the amount of α -ketoglutarate entering the skeletal muscle being too low to affect metabolism.

It is unclear however, whether the α -ketoglutarate component of OKG would follow the same time course, since α -ketoglutarate and ornithine interact resulting in a different metabolic pattern to that observed when subjects receive ornithine (as hydrochloride) or α -ketoglutarate (as a calcium salt) individually (Cynober et al. 1990). Indeed, previous

studies in burn-injured rats have shown that OKG counteracts the trauma-induced decrease of the intramuscular glutamine pool after 48 h, when administered enterally in three boluses daily ($5 \text{ g}\cdot\text{kg}^{-1}$ day; Vaubourdolle et al. 1991). This suggests that OKG may elicit anabolic effects over a greater time course than in the present study. Furthermore, the majority of studies have investigated the effect of OKG supplementation in hypercatabolic states, employing animal models or human subjects. When the muscle glutamine pool is reduced it has been shown that the administration of OKG to humans post-surgery effectively restores the muscle glutamine pool (Wernerman et al. 1990), presumably due to OKG acting as a precursor to glutamine (Cynober, 1999). However, in the present study, at rest, no increase was observed in muscle glutamine content in the OKG condition compared to the CON condition. The effect of OKG upon muscle glutamine may therefore only be evident when the muscle glutamine pool is previously depleted, which was not the case in the present study.

Theoretically, providing the substrate α -ketoglutarate would be the most direct way of attempting to introduce carbon into the TCA cycle in human skeletal muscle. However, in the present study, the oral provision of OKG did not increase the content of the TCAI. There are two possible explanations for the absence of any effect of OKG upon the TCAI and glutamine pool size. First, the ingested α -ketoglutarate component was sequestered by the splanchnic bed, as suggested previously. Secondly the transport of α -ketoglutarate into skeletal muscle is not efficient enough to increase α -ketoglutarate content. Given that the infusion of α -ketoglutarate has been shown to increase the intramuscular α -ketoglutarate content (Roth et al. 1991), it is more likely that the oral delivery of OKG resulted in the uptake of the α -ketoglutarate moiety by the splanchnic bed, which would substantially reduce the amount of α -ketoglutarate available to enter the muscle. This is supported by the fact that when OKG is given parenterally, effectively avoiding the effect of 'first pass' sequestration of the α -ketoglutarate moiety by the splanchnic bed observed when provided enterally (Ziegler et al. 1992), it is equally as effective as α -ketoglutarate or glutamine in restoring the muscle glutamine pool (Vinnars et al. 1990). However, in the study by Vinnars et al. (1990) the two groups (OKG and α -ketoglutarate) were not identical in terms of α -ketoglutarate intake, precluding any firm conclusion.

It has previously been demonstrated that OKG and glutamine exert their metabolic influence in different target tissues (Ziegler et al. 1992). OKG was shown to interact most in the splanchnic region, predominantly in the liver, demonstrated by an increase in OKG metabolites (proline, arginine, glutamate). Furthermore, the higher blood glucose levels during exercise in the present study provide further support for the notion that the splanchnic bed sequestered the OKG load during the OKG condition. This suggests an increased metabolism of gluconeogenic amino acids, in particular proline, which although not measured in the present study, has been shown to increase rapidly in response to an OKG load (Cynober et al. 1990).

The concentrations of both tyrosine and phenylalanine have previously been used as an indicator of muscle protein breakdown (Blomstrand and Saltin, 1999), with an increase in their appearance representing an increase in muscle protein breakdown, based upon the fact that phenylalanine has no significant role other than as a component of proteins and a precursor to tyrosine. An increase in plasma phenylalanine concentration was observed at exhaustion in all conditions, therefore suggesting an increase in muscle protein breakdown. The increase in plasma tyrosine concentration, and parallel increase in the sum of the aromatic amino acids and non-metabolised amino acids, in response to the ingestion of OKG is difficult to explain. No concomitant increase was observed in plasma phenylalanine concentration after OKG ingestion in the present study suggesting that muscle protein degradation was not increased. Indeed, it has been reported that plasma phenylalanine concentration is actually reduced after OKG ingestion (Cynober et al. 1984). Therefore the mechanism for this increase in tyrosine concentration in the present study remains to be elucidated. On the basis of these findings it is impossible to determine the effect of OKG upon protein turnover. Although, the increase in tyrosine concentration would seem to suggest a catabolic rather than anabolic influence.

In the glycogen depleted state it has been suggested that branched chain amino acid (BCAA) oxidation is increased, resulting in a reduction in α -ketoglutarate content, due to α -ketoglutarate acting as an amino group recipient in the BCAA aminotransferase reaction (Wagenmakers et al. 1991). It would therefore be expected to observe a decrease in plasma BCAA concentration during exercise as they are taken up by skeletal muscle for oxidation. However, no change in the sum of the plasma BCAA was

observed over time in any condition. One explanation may be that the increase in BCAA oxidation was matched, and somewhat masked by an increase in proteolysis, which is supported by the increase in plasma phenylalanine concentration at exhaustion in both the CON and GLN conditions.

The increase in plasma arginine concentration at 10 min of exercise in the OKG condition is most likely due to the elevated ornithine concentration, since ornithine is the precursor to arginine in the urea cycle. Furthermore, although not statistically different to the other conditions, there was a tendency for the increase in blood lactate concentration to be greater during exercise after OKG ingestion. This may also infer an increased metabolism in the splanchnic bed, principally in the liver, which would be in agreement with previous results suggesting that orally provided OKG is primarily trapped in splanchnic areas (Ziegler et al. 1992).

The increase in plasma alanine concentration at exhaustion in all conditions was somewhat surprising, although the increase observed at 10 min of exercise is commonly seen (Gibala et al. 1997a). Alanine is produced in skeletal muscle intramitochondrially primarily through the transamination of pyruvate (alanine aminotransferase reaction (AAT): $\text{glutamate} + \text{pyruvate} \leftrightarrow \alpha\text{-ketoglutarate} + \text{alanine}$) (Chang and Goldberg, 1978b; Spydevold, 1976), and plasma alanine is derived primarily from the export of this alanine from the skeletal muscle (Consoli et al. 1990). In addition, alanine may be synthesised in muscle from the amino groups of other amino acids, derived through proteolysis (Felig and Wahren, 1971; Garber et al. 1976b). It is somewhat paradoxical to observe a peak in plasma alanine concentration at exhaustion, muscle glycogen levels would be low at this time, and thus muscle pyruvate availability reduced, leading to less formation of alanine. However, during the latter stages of prolonged submaximal exercise, particularly in the glycogen depleted state as in the present study, BCAA transamination will be increased (Gibala et al. 1999b) giving rise to an increased number of amino groups which may be used for alanine synthesis (Felig and Wahren, 1971; Garber et al. 1976b). This may therefore partly explain the unexpected increase in plasma alanine concentration.

Inherently linked to the alanine concentration, it was also somewhat surprising to observe an increase in muscle pyruvate content at exhaustion, given the glycogen depleted state induced in the present study. This may be due to an increased recruitment of type II muscle fibres. It has previously been demonstrated during prolonged submaximal exercise that as muscle glycogen is depleted in type I fibres, there is an increase in the number of type II fibres recruited (Gollnick et al. 1973). It is therefore possible that the lowered glycogen levels led to an increase in the number of type II fibres recruited and thus an increase in glycolytic activity in these fibres leading to the increase in muscle pyruvate content during exercise.

In conclusion, it has been demonstrated that in the low glycogen state, the provision of glutamine 1 h prior to exercise was able to increase the TCAI pool expansion after 10 min of moderate intensity exercise. However, despite this further increase in the TCAI pool size, no sparing of phosphocreatine or reduction in muscle lactate accumulation was observed. This suggests that at the onset of exercise, oxidative energy production is not limited by the size of the TCAI pool, but some other factor, possibly muscle oxygenation or the availability of acetyl units to the TCA cycle may limit oxidative energy production during this period of exercise. Furthermore, no difference was observed in endurance capacity despite a greater elevation in the TCAI pool size after glutamine ingestion. However, TCAI pool size was not maintained following glutamine ingestion at exhaustion. Therefore, the data in the present study suggests a lack of a relationship between TCAI pool size and the fatigue process.

CHAPTER 7

GENERAL DISCUSSION

Introduction

Amino acids are involved in a wide array of physiological processes, some common to all 20 amino acids and others restricted to particular amino acids. In general, all amino acids are involved in protein turnover. Further to this, the intermediary metabolism of amino acids, within and beyond skeletal muscle, is linked to a wide variety of metabolic functions. The intracellular functions include maintenance of adenine nucleotide concentrations, *de novo* synthesis of purines and pyrimidine bases, the transfer of reducing equivalents in the mitochondria, anaplerotic maintenance of intermediates of the TCA cycle, and extracellular functions which include the provision of fuel for cells of the immune system, acid-base regulation, gluconeogenesis and ureagenesis. The non-essential amino acid glutamine has a role in almost all of these processes and in this respect has been described as 'conditionally-essential' (Lacy and Wilmore, 1990) particularly due to its role in gluconeogenesis (Nurjhan et al. 1995), cell swelling in both the liver (Häussinger et al. 1990) and skeletal muscle (Low et al. 1996) and as an important respiratory fuel for the cells of the immune system (Ardawi and Newsholme, 1983) and gut (Windmueller and Spaeth, 1974).

The aim of all of the studies presented in this thesis was to investigate the role of amino acid metabolism during exercise and during recovery from exercise in humans. Amino acids were supplemented during recovery from exercise or prior to exercise providing a model to examine the interaction between amino acid metabolism and carbohydrate metabolism both at the whole-body level (Chapters 4 & 5) and at cellular level (Chapter 6). In the first two studies presented in this thesis, the oral consumption of glutamine during recovery from exhaustive exercise, whether ingested in combination with a glucose-polymer or not, appeared to facilitate a better maintenance of glucose levels during a subsequent cycle to exhaustion. Despite this, cycle time to exhaustion was not different between conditions.

Research findings

The oral consumption of 0.3 g.kg^{-1} glutamine over a 4 h recovery period ($\sim 6 \text{ g}$ glutamine in total) was able to increase plasma glutamine concentration by $\sim 30 \%$ when consumed in combination with glucose-polymer, and by $\sim 60 \%$ when glutamine only was consumed. Therefore, it appears that the addition of 1.7 g.kg^{-1} glucose-polymer solution diluted the increase in plasma glutamine concentration by approximately half. The increase in plasma glutamine concentration is similar to that observed when 0.3 g.kg^{-1} glutamine and 1.7 g.kg^{-1} glucose-polymer were provided (Bowtell *et al.*, unpublished findings). In the third study, it was demonstrated that the oral consumption of 0.125 g.kg^{-1} glutamine ($\sim 10 \text{ g}$ glutamine) was able to increase plasma glutamine concentration by $\sim 50 \%$, with a corresponding increase muscle glutamine content ($\sim 30 \%$) compared to the control condition. Based on the findings of the third study, it is conceivable that the increase in plasma glutamine concentration observed in both the first and second studies of this thesis was sufficient to increase muscle glutamine concentration. By comparison, the intravenous infusion of glutamine after exhaustive exercise, which would be expected to increase plasma glutamine concentration to a greater extent than oral provision, was able to increase plasma glutamine concentration by $\sim 70 \%$, however muscle glutamine content increased by only $\sim 15\%$ (Varnier *et al.* 1995). The disparity in the increase in muscle glutamine content between the results of the third study and the work of Varnier *et al.* may be related to the extent that the intramuscular glutamine pool was depleted prior to glutamine administration. Indeed, basal muscle glutamine concentration was reduced to $\sim 30 \text{ mmol/kg}$ dry muscle in the third study (based on the CON condition value 1 hour post ingestion), whereas basal muscle glutamine concentration in the study by Varnier *et al.* was $\sim 55 \text{ mmol/kg}$ dry muscle, which suggests that skeletal muscle uptake of glutamine may be proportional to the size of the intramuscular glutamine pool following the exogenous provision of glutamine.

It is evident from both the first and second studies that the plasma concentration of serine, asparagine, threonine and ornithine, in addition to alanine and glycine, were increased in response to alanine and glycine ingestion. This is most probably due to a saturation of the normal metabolic pathways, causing reactions to be shifted in the opposite direction, leading to the biosynthesis of these amino acids. In the second study (Chapter 5), when alanine and glycine were consumed without glucose-polymer, the

plasma concentration of glycine increased approximately 3-fold, and the peak in concentration occurred 45 min after consumption. In the first study (Chapter 4), when alanine and glycine were consumed with glucose-polymer, the increase in plasma glycine concentration was of a similar order, but the peak in concentration occurred 150 min after consumption. Therefore, there appears to some effect of the addition of the glucose-polymer to the solution. This effect is mirrored in the plasma serine response, which is expected as serine can be synthesised from glycine via the serine transhydroxymethylase reaction. This may infer that the supplementation of glucose-polymer in addition to alanine and glycine reduces the rate of absorption in the gut or the uptake of glycine into the systemic circulation.

The combined results of the studies presented in this thesis demonstrate that the ingestion of glutamine is able to increase the plasma concentration of both glutamate and aspartate, in addition to glutamine. Furthermore, the plasma concentrations of phenylalanine, tyrosine and methionine were decreased in response to glutamine ingestion. The reduction in the plasma concentrations of phenylalanine, tyrosine and methionine may be indicative of a reduction in skeletal muscle protein breakdown. An increase in the concentration of these amino acids that are not metabolised in skeletal muscle is suggestive of an increase in skeletal muscle protein breakdown as these amino acids are only used for protein synthesis (van Hall et al. 1999; Blomstrand and Saltin, 1999). Therefore, a decrease in their concentration would suggest a decrease in skeletal muscle protein breakdown. Indeed a reduction in skeletal muscle protein breakdown has previously been observed following glutamine administration (MacLennan et al. 1988), with glutamine having a similar mode of action to that of insulin, inferring that glutamine does indeed have an anabolic affect.

The increase in plasma glutamate concentration observed following glutamine supplementation is most likely due to the deamination of glutamine to glutamate via glutaminase in the liver. Indeed, a degree of glutamate-glutamine 'cycling' appears to have occurred between the splanchnic bed, in particular the liver, and skeletal muscle giving rise to the increased plasma glutamate concentration following glutamine supplementation. It is apparent that the increase in plasma glutamate concentration also had a 'knock-on' effect, increasing plasma aspartate concentration. This was most

probably due to an increase in flux through the aspartate aminotransferase reaction (EC 2.6.1.1; glutamate + oxaloacetate + NAD⁺ ↔ α -ketoglutarate + aspartate + NADH).

In the first study (Chapter 4), the plasma free tryptophan:BCAA ratio increased during recovery and at exhaustion in the second bout of exercise in the glucose-polymer and glutamine condition. However, a similar response was not observed in the second study (Chapter 5) when glutamine alone was supplemented during recovery from exercise. It is feasible that when both glucose-polymer and glutamine are ingested together a different and synergistic metabolic response is invoked. Indeed, the supplementation of both glucose-polymer and glutamine was able to suppress the plasma NEFA concentration during both the recovery period and the second bout of exercise to a greater extent than when glucose-polymer and alanine and glycine were supplemented. However, the supplementation of glutamine alone was unable to produce a similar response during the second study when compared to the supplementation of alanine and glycine. The reduction in plasma NEFA concentration following glucose-polymer and glutamine ingestion is most probably related to the ability of glutamine to increase insulin concentration or to suppress lipolysis (Cersosimo et al. 1986). According to the central fatigue hypothesis, the reduction in plasma FFA concentration would have allowed increased binding of tryptophan to albumin, thereby decreasing the plasma free tryptophan concentration. Contrary to this, in the first study an increase in plasma free tryptophan concentration was observed during the second bout of exercise, which, in combination with the decrease in plasma BCAA concentration, increased the plasma free tryptophan:BCAA ratio. Therefore, the mechanism for this response is difficult to explain, although it appears to be related mostly to tryptophan biosynthesis.

The anabolic effect of ingesting carbohydrate (glucose-polymer) post exercise was clearly demonstrated in the first study. An increase was observed in both glucose and insulin concentrations in both the experimental conditions. The increase in insulin concentration following carbohydrate supplementation was such that the contribution from any amino acid was masked. However, when the results from the second study are considered, it is apparent that there is an increase in insulin concentration which is attributable to the ingestion of either alanine or glycine, or a combination of both (Kuhara et al. 1991). This would suggest that glutamine has less significant

insulinogenic properties than previously thought, in concurrence with recent findings demonstrating that other amino acids have greater insulinogenic properties than glutamine (van Loon et al. 2000).

It is surprising, however, that no difference was observed in cycle time to exhaustion between the control conditions (placebo) and the experimental conditions (glucose-polymer plus amino acids). Previous investigations by Blom *et al.* (Blom et al. 1987) and Ivy *et al.* (Ivy et al. 1988b) demonstrated that carbohydrate ingestion between 0.7 and 1.5 g·kg⁻¹ body wt·h⁻¹ after exhaustive exercise provided adequate stimulus for maximal muscle glycogen restoration over a 4 h period. However, carbohydrate ingestion greater than 0.7 g·kg⁻¹ body wt·h⁻¹ did not appear to provide an enhanced benefit in the restoration of muscle glycogen (Blom et al. 1987; Ivy et al. 1988b). In the first study, 1.7 g·kg⁻¹ body wt carbohydrate was ingested over the 4 h recovery period, equating to approximately 0.43 g·kg⁻¹ body wt·h⁻¹. This dose of glucose-polymer solution may have not been sufficient to fully activate the enzyme glycogen synthase and achieve maximal muscle glycogen resynthesis during this time period. Even if the maximal rate of muscle glycogen resynthesis was achieved (~6-7 mmol·kg⁻¹·h⁻¹ dry weight) by providing a greater glucose load, then over the 4 h recovery period there would have been at most an increase in muscle glycogen content of 28 mmol·kg⁻¹ dry weight. Even with an increase in muscle glycogen content of this order, it is questionable whether this would equate to an increase in endurance capacity in a subsequent bout of exercise.

However, one would intuitively expect that cycle time to exhaustion to be greater after the ingestion of a glucose-polymer solution during the recovery period after exercise. Theoretically, this would have promoted a greater resynthesis of both liver and skeletal muscle glycogen, thus providing a greater supply of energy for the second bout of exercise. However, it is possible that a trade off between the supplementation of glucose-polymer and the suppression of fat oxidation during exercise may have occurred. Certainly, carbohydrate oxidation was greater in the period following the ingestion of the glucose-polymer and glutamine solution compared to the control conditions. Furthermore, the hypothesised increase in both muscle and liver glycogen storage following glucose-polymer and glutamine supplementation during the recovery period would be expected to increase carbohydrate oxidation in the second bout of

exercise, thus increasing the rate of depletion of carbohydrate stores and placing a lesser demand upon fat as a fuel source. This may have partly accounted for the failure to observe a difference between conditions in exercise capacity.

The addition of a glucose-polymer solution to the amino acids in the first study made it difficult to delineate the effects of the ingestion of amino acids. However, some clear differences were observed between the experimental conditions and the control conditions that were attributable only to the ingestion of the amino acids. Furthermore, differences were observed between the two experimental conditions, suggesting that the individual amino acids or a combination of the amino acid and the glucose-polymer elicited an altered metabolic response. When the findings of the first (Chapter 4) and second (Chapter 5) studies are combined, it is clear that the ingestion of glutamine during recovery from exhaustive exercise is able to modulate glucose metabolism in a subsequent bout of exercise. In both the first and second studies of this thesis (Chapters 4 & 5), systemic glucose concentration in the second bout of exercise was either increased or maintained after glutamine supplementation, conversely a decrease was observed in systemic glucose concentration in the comparative condition. This finding is suggestive that glutamine may hold glucogenic or glycogenic properties, possibly through an increase in liver glycogen content or by acting as a gluconeogenic precursor, the former being more likely given previous findings (Mouterde et al. 1992; Bowtell et al. 1999). Despite this suggested increase in liver glycogen storage, no difference in cycle time to exhaustion was observed in either study. This is surprising since it has been demonstrated that fatigue during prolonged cycling exercise was delayed following an increase in systemic glucose concentration (Coyle et al. 1983). Furthermore, it has been reported that a greater rate of glucose utilisation occurs during prolonged cycling exercise compared to prolonged running exercise (Derman et al. 1996), most probably related to the observation that glucose uptake by a given muscle group is inversely related to the total amount of muscle that is exercising (i.e. greater glucose utilisation due to a smaller active muscle mass) (Richter et al. 1988). Therefore, during prolonged cycling exercise, systemic glucose concentration may affect the aetiology of fatigue.

Although systemic glucose concentration was maintained during the second bout of exercise following both glucose-polymer & glutamine and glutamine supplementation

in both the first and second studies respectively (Chapters 4 & 5), it is feasible that hypoglycaemia may have played a minor role in the aetiology of fatigue in this bout of exercise. Felig *et al.* (1982) have demonstrated that during cycling exercise to exhaustion at 60-65% $\dot{V}O_{2max}$, symptoms of hypoglycaemia are exhibited. Although, a higher intensity of exercise was utilised in the present studies, which may place a greater reliance upon blood borne fuel sources such as glucose, in neither study did systemic glucose at exhaustion reach clinically defined hypoglycaemic levels (< 2.5 mM), suggesting that hypoglycaemia may not have been the primary cause of fatigue. Indeed, Felig *et al.* were able to demonstrate that cycling exercise could be continued even in the presence of symptoms of hypoglycaemia. Therefore, the failure to observe a difference in cycle time to exhaustion between conditions in the first and second studies, may in part be related to the fact that cycle time to exhaustion (endurance capacity) may not be a sensitive enough tool to detect differences between conditions, as discussed in Chapter 4. There is also the possibility that the recovery period (4 h) was too short for the purported anabolic effects of glutamine to influence endurance capacity.

In light of recent evidence suggesting that the addition of glutamine to a glucose solution is unable to further increase skeletal muscle glycogen synthesis or increase the activation of skeletal muscle glycogen synthase compared to glucose alone (Wagenmakers *et al.* 1997; van Hall *et al.* 2000a), it would appear that the proven ergogenic effect of carbohydrate supplementation post exercise in skeletal muscle is not further enhanced by the addition of glutamine (van Hall *et al.* 2000b). However, Blom *et al.* (1987) have demonstrated that increasing the amount of glucose provided post exercise from 0.7 to 1.4 g.kg⁻¹ body wt.h⁻¹ does not cause a further increase in the rate of skeletal muscle glycogen synthesis. It is feasible therefore, that the glucose dose (0.8 g.kg⁻¹ body wt) provided by van Hall *et al.* (2000b) may have been great enough to maximally stimulate skeletal muscle glycogen synthase. Thus, any activation of glycogen synthase by glutamine would not be observed, if glutamine-stimulated glycogen synthesis were mediated via glycogen synthase activation and glycogen synthesis would not be increased.

The effect that the supplementation of glutamine alone may have on both hepatic and skeletal muscle glycogen synthase has not been examined *in vivo* in humans.

Furthermore, the extent to which glycogen synthase is activated by glutamine, both in the liver and skeletal muscle, may be determined by glycogen concentration. Indeed, the administration of glutamine alone post exercise has been shown to increase muscle glycogen content (Varnier et al. 1995). The effect observed, in absolute terms, was rather modest and responsible for no more than $2 \text{ mmol}\cdot\text{kg}^{-1}$ wet weight of glycogen per hour. Nevertheless, an increase was observed in muscle glycogen content following the provision of glutamine, which cannot be merely attributed to a gluconeogenic effect as the total amount of carbon provided as alanine and glycine, in the comparator condition, was equivalent to that in glutamine. Therefore, it appears that a reduction in glycogen content, may provide the stimulus for the activation of glycogen synthase following the administration of glutamine. It is feasible that the same response could be invoked in the liver following glutamine supplementation, if liver glycogen content has been reduced through a previous bout of exercise. Indeed, in rats whose liver glycogen content has been reduced through starvation, glutamine has been shown to be a good substrate for liver glycogen synthesis (Mouterde et al. 1992). Therefore, the provision of glutamine, a glycogenic substrate, may prove a suitable alternative source of carbon for glycogen synthesis in the glycogen depleted state in both the liver and skeletal muscle, if the availability of common glycogenic substrates is reduced.

It would have been interesting if Wagenmakers *et al.* (1997) or van Hall *et al.* (2000b) had included a glutamine only condition to determine the extent that glutamine itself may increase skeletal muscle glycogen content. Recent research has indicated that the addition of glutamine to glucose-polymer supplementation is unable to further increase skeletal muscle glycogen synthesis compared to glucose-polymer alone (Bowtell et al. 1999). However, in this study, whole-body carbohydrate storage was increased following glutamine and glucose-polymer supplementation, with the most feasible site of the increased storage being the liver. Therefore, it is plausible that the ingestion of glutamine and glucose-polymer post exercise may produce a synergistic effect, an effect not observed when either glutamine or glucose-polymer are consumed individually.

Further to this, it is an intriguing possibility that the production of glucosamine, following the consumption of glucose-polymer and glutamine, may increase liver glycogen content by increasing the availability of glucose to the liver, via a reduction in skeletal muscle glucose uptake due to an impairment of GLUT-4 translocation (Baron et

al. 1995). The results from the studies presented in this thesis provide some evidence to suggest that the supplementation of glutamine is able modulate metabolism in the liver. In particular, the elevated glucose levels during the second bout of exercise after glutamine supplementation may be indicative of increased hepatic glucose output, which in turn may be indicative of an increase in liver glycogen storage during the recovery period. However, given the complexity in measuring liver metabolism *in vivo*, it is difficult to determine whether the effect is glucogenic or glycogenic.

To date, liver glycogen content has never been assessed *in vivo* in humans following the consumption of either glucose-polymer and glutamine or glutamine. Until such data is obtained, which would involve MRS/NMR techniques or liver biopsies, the exact mechanism for the increase in systemic glucose concentration, observed in these studies following glutamine supplementation, will remain unresolved.

It is evident from the results of the third study (Chapter 6) that the supplementation of glutamine is able to alter skeletal muscle metabolism at a cellular level. The consumption of glutamine 1 h prior to exercise, in glycogen-depleted subjects, resulted in a further increase in the total concentration of the TCAI (i.e. promoting anaplerosis) after 10 min of submaximal exercise. For glutamine to interact with the TCA cycle, the catabolism of glutamine must have occurred. Although, no tracer technique was implemented to track the carbon skeleton of glutamine, the most logical and feasible route by which glutamine can enter the TCA cycle is by the two-step conversion to α -ketoglutarate. Glutamine that enters skeletal muscle can be deaminated to form glutamate and then α -ketoglutarate through the action of glutaminase (glutaminase I) and glutamate dehydrogenase or alanine aminotransferase, respectively. Alternatively, glutamine may be deaminated to form α -ketoglutaramate and then α -ketoglutarate through the action of glutamine transaminase (glutaminase II) and ω -amidase, respectively. It is noteworthy that the activity of either form of glutaminase (I or II) in human skeletal muscle has not been quantified, although there is some tenuous evidence that glutaminase I is present in both in rat (Ottaway, 1969; de Almeida et al. 1989) and human skeletal muscle (Swierczynski et al. 1993). The pathway by which glutamine degradation occurs is unclear. However, glutamine degradation is not inhibited by the presence of the glutaminase inhibitor 6-diazo-5-oxo-L-norleucine (DON) in rat skeletal

muscle (Wu et al. 1991), suggesting that the glutamine transaminase and ω -amidase pathway is most feasible in skeletal muscle, although it is conceivable that a species difference may exist. However, future research should attempt to determine the activity of both isoforms of glutaminase in human skeletal muscle so that the pathway of glutamine degradation may be resolved.

Despite effectively promoting anaplerosis at 10 min of submaximal exercise, following the ingestion of glutamine 1 h prior to exercise, the decrease in phosphocreatine and increase in muscle lactate content was similar in all conditions at 10 min exercise. This would appear to suggest that the enhanced increase in the TCAI observed during exercise was not important for oxidative energy delivery. Furthermore, despite increasing the total concentration of the TCAI at 10 min of exercise, no difference was observed at exhaustion, with cycle time to exhaustion no different between conditions. This suggests that the total concentration of the TCAI may not be important in the aetiology of fatigue during submaximal exercise.

Implications for athletes

The implications for athletes from results of the presented studies are related to the anabolic benefit that may be gained from the supplementation of glutamine. Athletes involved in endurance activities have primarily used glutamine as a post exercise 'ergogenic aid' to ensure optimal functioning of the immune system. Evidence from the studies presented in this thesis, would suggest that supplementation of glutamine during recovery from exercise or prior to exercise is able to modulate both whole body and cellular level metabolism. In particular, the provision of glutamine in the dose and pattern described in the studies reported in this thesis demonstrates that glutamine can invoke an anabolic environment in humans potentially allowing a more rapid recovery post exercise. However the proven anabolic effect of glucose-polymer supplementation must not be overlooked. The provision of glucose-polymer alone post exercise has been demonstrated to increase both liver and skeletal muscle glycogen resynthesis (Casey et al. 2000) and reduce skeletal muscle protein breakdown (Roy et al. 1997). For this reason, the consumption of glutamine post exercise is most probably best combined with glucose-polymer to achieve the maximal beneficial effect.

Future work

In light of the findings from the third study (Chapter 6), it would be interesting to further examine the role of the TCAI in the aetiology of fatigue. One possible way to study this would be to supplement glutamine during exercise in an attempt to increase the TCAI pool size in skeletal muscle, and take multiple biopsies whilst exercising to exhaustion. This would allow a better understanding of the role of the intermediates in the fatigue process, in addition to determining whether or not a critical level in the total content of the TCAI exists. Furthermore, it would also be intriguing to examine the effect of infusing dichloroacetate (DCA), to increase the supply of acetyl units to the TCA cycle, whilst providing a supply of either glutamine or pyruvate, both of which have proved capable of increasing the total content of the TCAI (Gibala and Saltin, 2000). This may provide an optimal environment for oxidative metabolism, which may reduce the lag in the onset of oxidative metabolism at the onset of moderate intensity exercise, with a lesser reliance on substrate level phosphorylation and which may also extend endurance capacity during submaximal exercise.

The studies in this thesis have been able to demonstrate that exercise is able to alter amino acid metabolism in humans and that the supplementation of amino acids during recovery from exercise can modulate exercise metabolism during a subsequent bout of exercise. Furthermore, it has been shown that amino acids in muscle do indeed occupy a central role in metabolism during exercise acting as precursors for the synthesis of TCA cycle intermediates. However, the physiological significance of increasing the TCA cycle intermediates may not be as important to TCA cycle flux and oxidative energy metabolism during exercise as previously thought.

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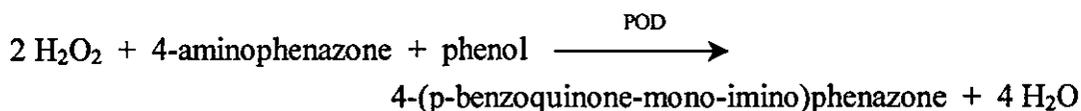
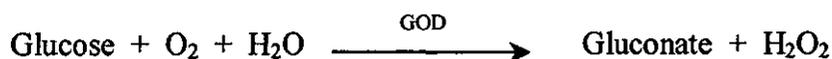
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APPENDIX A

PLASMA GLUCOSE

Principle

Plasma glucose concentration was determined using an automated analyser (Cobas Mira Plus, Roche Diagnostic Systems, Basal, Switzerland) employing an enzymatic colorimetric method using a commercially available kit (Boehringer Mannheim GmbH, No. 166 391). The GOD-PAP method for glucose determination was used in this kit. In the presence of peroxidase, the hydrogen peroxide formed affects the oxidative coupling of hydroxybenzoic acid and 4-aminopyridine to form a red-coloured quinoneimine derivative. The colour intensity is directly related to the glucose concentration and is measured photometrically at 500 nm.



GOD = glucose oxidase; POD = peroxidase

Reagents

	Reagent	Concentration/other details	Storage conditions
Assay solution	Phenol	11.0 mmol.l ⁻¹	+4 - 8°C
	4-aminophenazone	0.77 mmol.l ⁻¹	+4 - 8°C
Enzymes	GOD	18 U/ml	+4 - 8°C
	POD	1.1 U/ml	+4 - 8°C
Reagents	Water		Room temp

Method

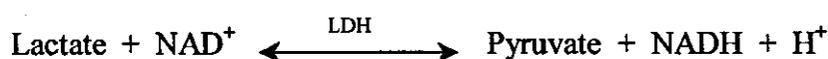
A calibration curve is run using a calibrator and appropriate quality controls (CFAS: calibrator for automated systems and Control Serum Precinorm N & U; all Boehringer Mannheim). Samples, standards and controls are pipetted in excess into cuvettes and loaded into the analyser. The analyser adds 300 µl of the assay solution containing both reagents and enzymes to 4 µl of sample, standard or control at 37°C. The absorbance of the cuvettes is then measured at 500 nm and the colour intensity is directly related to the glucose concentration relative to the calibration curve.

APPENDIX B

BLOOD LACTATE

Principle

Blood lactate concentration was determined manually using a fluorometric method (Maughan, 1982). The principle is based on a change in the oxidative state of NAD^+ , with the buffer containing and excess NAD^+ and of a high pH to ensure the reaction proceeds from left to right. A 20 μl sample was deproteinised in 200 μl 2.5 % perchloric acid.



LDH = lactate dehydrogenase; NAD = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide

Reagents

	Reagent	Concentration/other details	Storage conditions
Buffer (1.1 mol.l ⁻¹) (pH 9.0-9.5)	Hydrazine sulphate	6.5 g	Room temp
	Hydrazine hydrate	24.26 ml	Room temp
	EDTA	1.0 g	Room temp
Diluent	HCl	0.07 mol.l ⁻¹	Room temp
Cofactor	NAD	5.0 mmol.l ⁻¹	+4 - 8°C
Enzymes	Lactate dehydrogenase	(Boehringer 106 984)	+4 - 8°C
Reagents	Water		Room temp

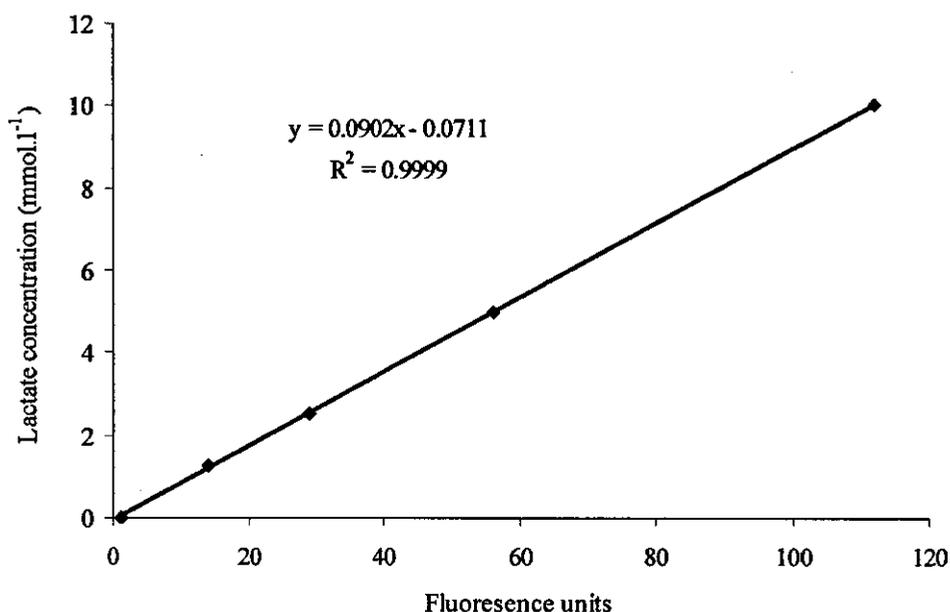
Method

1. Prepare hydrazine buffer by adding 6.5 g hydrazine sulphate, 24.26 ml hydrazine hydrate and 1.0 g EDTA to a volumetric flask and make up to 500 ml with distilled water. Check pH with pH meter (pH 9.0-9.5).
2. Prepare lactate diluent by adding 70 ml of 1 M HCl to a volumetric flask, make up to 1.0 L with distilled water.
3. Prepare buffer mixture by adding 10 μl LDH and 2.0 mg of NAD per 1.0 ml of buffer mixture (e.g. for 10 samples; 10 x 200 μl = 2.0 ml: 2.0 ml hydrazine buffer, 20 μl LDH and 4.0 mg NAD)

4. Pipette 200 μl of buffer mixture and 20 μl of standard, control (2.2 mM) or sample into Pyrex glass test tubes (75 x 10 mm). Samples should be defrosted thoroughly, mixed with Whirlimix and centrifuged for 3 min prior to use.
5. Prepare standard curve using standards of the following concentration: 0, 1.25, 2.5, 5.0, 10.0 (mmol.l^{-1}).
6. Mix solution in tubes thoroughly using a Whirlimix taking care not to displace any of the contents. Cover appropriately to prevent contamination.
7. Incubate for 30 min at room temperature.
8. Add 1.0 ml of lactate diluent to each tube to end reaction.
9. Read fluorescence (F1) of tubes using a fluorometer (Locarte Model LF 8-9, Locarte, London, UK).
10. Calculations:

The inverse relationship between fluorescence and lactate concentration of the lactate standards was determined using a software program by fitting a linear trendline (Microsoft Excel; see Figure B.1). Concentration of the lactate in the samples was then calculated from the equation for the relationship.

Figure B.1 Example standard curve for lactate.



Example calculation:

$$\text{Lactate (mmol l}^{-1}\text{)} = \text{F1} \times 0.0902 - 0.0711$$

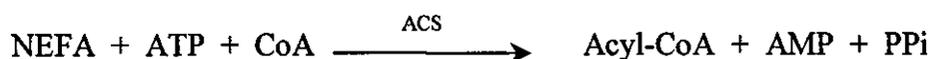
F1 = fluorescence of sample at the end of step 9.

APPENDIX C

SERUM AND PLASMA NON-ESTERIFIED FATTY ACIDS (NEFA)

Principle

Plasma and serum NEFA concentration was determined colorimetrically using a commercially available kit (Wako ACS-ACOD Method). NEFA when treated with acyl-CoA synthetase (ACS) in the presence of adenosine triphosphosphate (ATP), magnesium cations and CoA, form the thiol esters of CoA known as acyl-CoA as well as the by products adenosine monophosphate (AMP) and pyrophosphate (PPi). The acyl-CoA is oxidised by adding acyl-CoA oxidase (ACOD) to produce hydrogen peroxide which in the presence of added peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct with an absorption maximum of 550 nm. Therefore the amount of NEFA in the sample can be determined from the optical density measured at 550 nm.



Reagents

	Reagent	Concentration/other details	Storage conditions
Assay solution	Phosphate buffer ¹ (pH 6.9)	0.05 mol.l ⁻¹	+4 - 8°C
	Magnesium chloride ¹	3.0 mmol.l ⁻¹	+4 - 8°C
	MEHA ²	1.2 mmol.l ⁻¹	+4 - 8°C
	CoA ³	7 mg/vial	+4 - 8°C
	ATP ³	30 mg/vial	+4 - 8°C
	4-aminoantipyrine ³	3 mg/vial	+4 - 8°C
Enzymes	ACS ³	3 U/vial	+4 - 8°C
	AOD ³ (ascorbate oxidase)	30 U/vial	+4 - 8°C
	ACOD ⁴	132 U/vial	+4 - 8°C
	POD ⁴	150 U/vial	+4 - 8°C
Reagents	Water		Room temp

1 = contained in solvent A; 2 = contained in solvent B; 3 = contained in reagent A; 3 = contained in reagent B

Method

This assay is run using an automated analyser (Cobas Mira Plus, Roche Diagnostic Systems, Basal, Switzerland). Both plasma and serum NEFA concentration can be determined. To create the reagents required for the assay 10 ml of solvent A is added to reagent A and 7 ml of solvent B is added to reagent B.

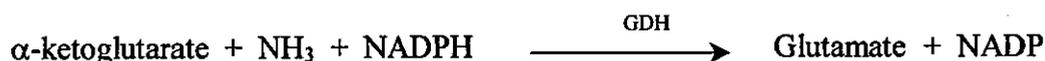
A calibration curve is run using the standard provided with the kit and an appropriate quality control. Samples, standards and controls are pipetted in excess into cuvettes and loaded into the analyser. The analyser adds 120 μ l of reagent A to 6 μ l of sample, standard or control and incubates at 37°C for 10 min. The analyser then adds 85 μ l of reagent B to the sample, standard or control and incubates for a further 10 min at 37°C. The optical density of the cuvettes is then measured at 550 nm. The NEFA concentration corresponding to the measured absorbance can be determined directly from the previously run calibration curve.

APPENDIX D

PLASMA AMMONIA (NH₃)

Principle

Plasma ammonia (NH₃) concentration was determined using a commercially available kit (Sigma 171-UV). The principle is based on the reductive amination of α -ketoglutarate, using glutamate dehydrogenase and reduced nicotinamide adenine dinucleotide phosphate. The decrease in absorbance at 340 nm due to oxidation of NADPH is proportional to the plasma ammonia concentration. The procedure is standardised by means of the millimolar absorptivity of NADPH, which is 6.22 at 340 nm. The reductive amination of α -ketoglutarate to form glutamate catalysed by GDH is coupled with the oxidation of NADPH to NADP on a molar equivalent basis.



GDH = glutamate dehydrogenase; NADPH = reduced nicotinamide adenine dinucleotide phosphate

Reagents

	Reagent	Concentration/other details	Storage conditions
Assay solution	2-oxoglutarate	3.4 mmol.l ⁻¹	+4 - 8°C
	NADPH	0.23 mmol.l ⁻¹	+4 - 8°C
Enzymes	Glutamate dehydrogenase	1200 U/ml (Sigma 170-4)	+4 - 8°C
	Phosphate buffer	pH 7.4	+4 - 8°C
	Glycerol	50 % w/v	+4 - 8°C
Reagents	Water		Room temp

Method

1. Label cuvettes for blank, standards and samples all in duplicate.
2. Pipette 500 μ l of assay solution into all cuvettes. To blank cuvette add 150 μ l water, to standard cuvette add 50 μ l of standard (29.4 μ M, 118 μ M and 294 μ M) and 100 μ l of water, to sample cuvettes add 150 μ l of plasma sample.
3. Cover cuvettes with Parafilm[®] and mix by gentle inversion.
4. Allow cuvette to equilibrate for approximately 3 min at room temperature.
5. Set zero on spectrophotometer using blank cuvette.
6. Read initial absorbance (A1) of each cuvette versus blank at 340 nm.

7. Add 5 μl of GDH to each cuvette. Mix by gentle inversion and wait approximately 5 min for reaction to complete.
8. Set zero on spectrophotometer using blank cuvette.
9. Read final absorbance (A_2) of each cuvette versus blank at 340 nm.
10. Calculation:

Plasma ammonia ($\mu\text{mol}\cdot\text{l}^{-1}$) =

$$((A_1 - A_2) \times 35.8) \times 58.8$$

A_1 = absorbance of sample and blank at the end of step 6.

A_2 = absorbance of sample and blank at the end of step 9.

APPENDIX E

SERUM INSULIN (RADIOIMMUNOASSAY)

Principle

¹²⁵I labeled insulin competes for a fixed time with insulin in the sample for sites on insulin-specific antibody. Because the antibody is immobilised on the wall of a polypropylene tube, decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled insulin. Counting the tube in a gamma counter then yields a value, which converts using a calibration curve to a measure of the insulin in the sample.

Method

1. Label four uncoated tubes for total count (TOT) and non-specific binding (NSB).
2. Label seven Insulin Ab-coated tubes in duplicate for calibrators of standard (calibration) curve (see table below).

Calibrator	Approximate μ IU/ml
A(MB)	0
B	5
C	15
D	50
E	100
F	200
G	400

MB = maximum binding

3. Pipette 200 μ l of the zero calibrator A into the NSB and A tubes, and 200 μ l of each remaining calibrator, control and sample into the labelled prepared tubes. Three different concentrations of control were used every 15 samples during each run.
4. Add 1.0 ml of ¹²⁵I Insulin to every tube and vortex taking care not to displace any of the contents.
5. Incubate for 18-24 h at room temperature (15-28°C) with the tubes covered appropriately (e.g. silver foil).
6. Decant thoroughly all contents of tubes, except for TOT tubes.
7. Count for one minute in a gamma counter (Cobra II, Packard Instruments)

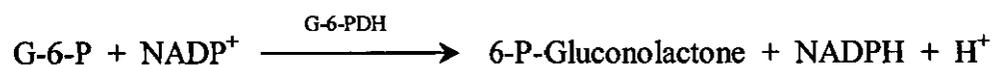
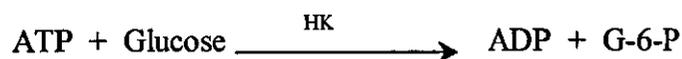
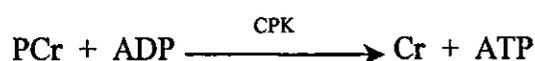
8. Insulin concentration of each sample was calculated using computer software (Cobra II, Packard Instruments) fitting an auto-spline curve to the calibration data, plotting concentration against percentage of ^{125}I Insulin bound.

APPENDIX F

MUSCLE ADENOSINE TRIPHOSPHATE (ATP) AND PHOSPHOCREATINE (PCr)

Principle

The content of ATP and PCr can be measured by the production of NADPH at 340 nm when sufficient NADP, glucose, ADP and glucose-6-phosphate dehydrogenase (G-6-PDH) are included in the assays, so that the reaction is limited by the availability of ATP and PCr.



ADP = adenosine diphosphate; Cr = creatine; CPK = creatine phosphokinase; HK = hexokinase; G-6-P = glucose-6-phosphate; G-6-PDH = glucose-6-phosphate dehydrogenase; NADP = nicotinamide adenine dinucleotide phosphate

Reagents

	Reagent	Concentration/other details	Storage conditions
Buffer (pH 7.5-7.6)	Triethanolamine (TEA)	(18.6 g·ml ⁻¹)	+4 - 8°C
	Magnesium acetate	(2.2 g·ml ⁻¹)	Room temp
	EDTA	(0.1 g·ml ⁻¹)	Room temp
Cofactor	NADP	(20.9 mg·ml ⁻¹)	+4 - 8°C
Enzymes	G-6-PDH	(Boehringer 127 035)	+4 - 8°C
	Hexokinase	(Boehringer 127 175)	+4 - 8°C
	Creatine phosphokinase	(Sigma C-3755)	-20°C
Reagents	Dithiothreitol (DTT)	(7.8 mg·ml ⁻¹)	+4 - 8°C
	Glucose	(22.5 mg·ml ⁻¹)	Room temp
	ADP	(5.1 mg·ml ⁻¹)	-20°C
	Water		Room temp

Working standards were prepared prior to analysis and stored at -20°C (ATP: 1.51 mmol/l; PCr: 2.11 mmol/l).

Method

1. Make up the reagent mixture, vortex and store at room temperature.
2. Dilute the following enzymes:
Hexokinase (HK). Dilute 1 part enzyme to 1 part water. Use 3 μl per cuvette. Vortex gently and keep on ice.
Creatine phosphokinase (CPK). Dissolve 15 $\text{mg}\cdot\text{ml}^{-1}$ in 0.5% NaHCO_3 + 0.05% Bovine Serum Albumin (BSA). Dilute 2 parts enzyme to 1 part water. Use 3 μl per cuvette. Vortex gently and keep on ice.
3. Pipette 350 μl of the reagent mix into each cuvette. Samples are run in batches of 10 + 1 blank.
4. Add 40 μl of water for the blank, 20 μl of ATP standard and 20 μl of PCr standard, or 40 μl sample. The muscle samples should be defrosted quickly in hot water, then vortexed and spun down (22,000 \times g, 3 min) before use.
5. Set the spectrophotometer to read samples at 340 nm, from 0.0-1.1 units absorbance.
6. Read initial absorbance (A1) at 30 sec intervals.
7. Add 3 μl of HK to each cuvette, agitate to mix. Incubate for 15 min at room temperature. Read intermediate absorbance at 30 sec intervals.
8. Add 3 μl of CPK to each cuvette, agitate to mix. Incubate for 30 min at room temperature. Read final absorbance (A2) at 30 sec intervals.
9. Calculations:

(1) ATP ($\text{mmol kg}^{-1} \text{ dm}$) =

$$((393 \times (A2-BI2) - 390 \times (A1-BI1))/6.22 \times 40) \times EF \times 1.25$$

(2) PCr ($\text{mmol kg}^{-1} \text{ dm}$) =

$$((396 \times (A2-BI2) - 393 \times (A1-BI1))/6.22 \times 40) \times EF \times 1.25$$

ATP: A1 and BI1 = absorbance of sample and blank at the end of step 6.

A2 and BI2 = absorbance of sample and blank at the end of step 7.

PCr: A1 and BI1 = absorbance of sample and blank at the end of step 7.

A2 and BI2 = absorbance of sample and blank at the end of step 8.

6.22 = molar extinction coefficient of NADPH at 340 nm (units are $\text{cm}^2\cdot\mu\text{mol}^{-1}$).

40 = sample volume (μl)

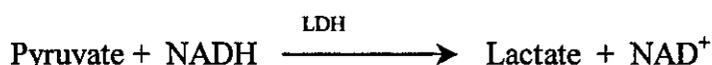
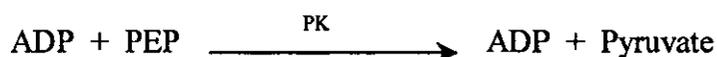
EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

APPENDIX G

MUSCLE CREATINE (Cr)

Principle

The content of Cr can be measured by the utilisation of NADH at 340 nm when sufficient NADH, PEP and ATP is included in the assay.



Cr = creatine; ATP = adenosine triphosphate; ADP = adenosine diphosphate; CPK = creatine phosphokinase; PEP = phosphoenolpyruvate; PK = pyruvate kinase; G-6-P = glucose-6-phosphate; LDH = lactate dehydrogenase; NADH = reduced nicotinamide adenine dinucleotide; NAD⁺ = nicotinamide adenine dinucleotide

Reagents

	Reagent	Concentration/other details	Storage conditions
Buffer (pH 9.0-9.1)	Glycine	(2.4 g 100·ml ⁻¹)	+4 - 8°C
	Magnesium acetate	(0.4 g 100·ml ⁻¹)	Room temp
Cofactor	NADH	(9.0 mg·ml ⁻¹)	+4 - 8°C
Enzymes	Creatine phosphokinase	(Sigma C-3755)	-20°C
	Lactate dehydrogenase	(Boehringer 107034)	+4 - 8°C
	Pyruvate kinase	(Boehringer 128155)	+4 - 8°C
Reagents	KCl	(15 g 100·ml ⁻¹)	+4 - 8°C
	ATP	(15.4 mg·ml ⁻¹)	-20°C
	PEP	(11.6 mg·ml ⁻¹)	-20°C
	Water		Room temp

Working standards were prepared prior to analysis and stored at -20°C (Cr: 3.34 mmol/l).

Method

1. Make up the reagent mixture, vortex and store at room temperature.
2. Prepare the CPK enzyme, by dissolving 15 mg ml⁻¹ in 0.5% NaHCO₃ + 0.05% Bovine Serum Albumin (BSA). Use 10 µl per cuvette. Vortex and keep on ice.
3. Pipette 450ul of the reagent mix into each cuvette. Samples are run in batches of 10 + 1 blank.
4. Add 30 µl of water for the blank, 30 µl of standard or 30 µl of extract. The muscle samples should be defrosted quickly in hot water, then vortexed and spun down (22,000 x g, 3 min) before use. Mix well.
5. Set the spectrophotometer to read samples at 340 nm, from 0.0-1.1 units absorbance.
6. Read initial absorbance (A1) at 30 sec intervals.
7. Add 10 µl of CPK to each cuvette, agitate to mix. Incubate for 20 minutes at room temperature.
8. Read final absorbance (A2) at 30 sec intervals.
9. Calculation:

Cr (mmol kg⁻¹ dm) =

$$(-1) \times ((490 \times (A2-BI2) - 480 \times (A1-BI1)) \times (EF \times 1.25)) / (6.22 \times 30)$$

A1 and BI1 = absorbance of sample and blank at the end of step 6.

A2 and BI2 = absorbance of sample and blank at the end of step 8.

6.22 = molar extinction coefficient of NADH at 340nm (units are cm²·µmol⁻¹).

30 = sample volume (µl)

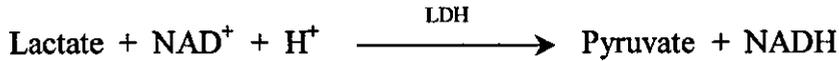
EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

APPENDIX H

MUSCLE LACTATE

Principle

Lactate was determined indirectly from changes in the absorbance of NADH, using a fluorometric assay. Calculation of lactate concentration was from an NADH standard curve, which was run in parallel with the samples.



LDH = lactate dehydrogenase; NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide

Reagents

	Reagent	Concentration/other details	Storage conditions
Buffer	2-amino-2-methyl-1-propranolol	1 M (9.5 ml or 8.9 g of powder in 100 ml ⁻¹)	+4 - 8°C
Cofactor	NAD^+	100 mM (70.0 mg·ml ⁻¹)	+4 - 8°C
Enzymes	Lactate dehydrogenase	(Boehringer 106 984)	+4 - 8°C
Reagents	Hydrazine	1 M (Dilute 5ml of 20M stock solution to 100 ml)	Room temp
	Water		Room temp

N.B. The assay is carried out at pH 10.0. At this pH beef heart lactate dehydrogenase is more stable than the skeletal enzyme.

Method

1. Prepare lactate standards:
Make up sodium lactate stock solution 500 μM (6.0 mg sodium lactate/100 ml water). Store at -20°C.
2. Prepare 2-amino-2-methyl-1-propranolol buffer 1 M pH 8.0. Set pH 10.0 with HCL 6 M. Store at -20°C.
3. Prepare the enzyme:
Dilute lactate dehydrogenase (beef heart) to 550 U/ml from stock 1250 U/ml, with Tris buffer 20 mM, pH 8.0 containing 0.02% albumin. Store on ice.
4. Make up the reagent mixture, vortex and store at room temperature.

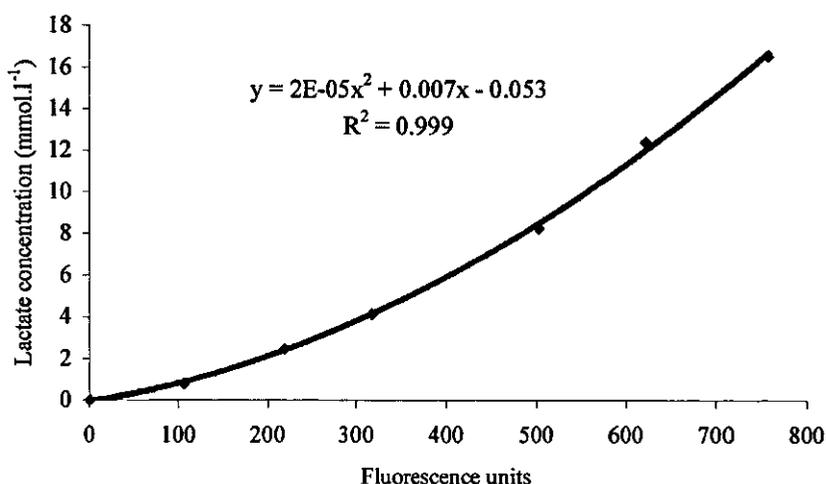
- Prepare the lactate standards by adding the following volumes of sodium lactate stock solution (500 μM) to the reagent buffer in cuvettes. Mix well and leave for 5 min at room temperature.

Sodium lactate (500 μM) stock (μl)	1	3	5	10	15	20
Reagent buffer (μl)	525	525	525	525	525	525
Water (μl)	74	72	70	65	60	55
[Lactate] μM	0.83	2.48	4.13	8.26	12.40	16.53

- Pipette 525 μl of reagent buffer, 65 μl water and 10 μl sample into each cuvette. The muscle samples should be defrosted quickly in hot water, then vortexed and spun down (22,000 \times g, 3 min) before use. Samples are run in batches of 10 + 1 blank. Mix well.
- Leave for 5 min at room temperature.
- Read initial fluorescence (F1) at 30 sec intervals (Excitation 340 nm/Emission 460 nm; Hitachi F2000, fluorescence spectrophotometer).
- Add 5 μl of LDH each cuvette, agitate to mix and incubate for 30 min at room temperature.
- Read final fluorescence (F2) at 30 sec intervals.
- Calculations:

The inverse relationship between fluorescence and lactate concentration was determined using a software program by fitting a second order polynomial trendline (Microsoft Excel; see Figure H.1). Concentration of lactate was then calculated from the (second degree) equation for the relationship.

Figure H.1 Example standard curve for lactate.



Example calculation:

Lactate ($\text{mmol kg}^{-1} \text{ dm}$) =

$$(-0.053 + 0.007 \times ((F2-F1)-(B12-B11)) + 0.00002 \times ((F2-F1)-(B12-B11)) \times ((F2-F1)-(B12-B11)) \times EF \times 1.25 \times 605)/10000$$

F1 and B11 = fluorescence of sample and blank at the end of step 8.

F2 and B12 = fluorescence of sample and blank at the end of step 10.

605 = total cuvette volume (μl).

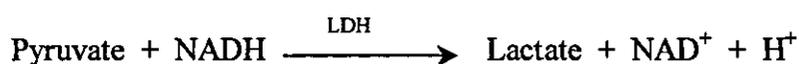
EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

APPENDIX I

MUSCLE PYRUVATE

Principle

Pyruvate concentration was determined indirectly from changes in NADH using a fluorometric assay. NADH oxidation by compounds other than pyruvate is minimised at pH 6.8-7.0, therefore a phosphate buffer is used. However, phosphate accelerates the destruction of NADH, therefore a blank, standard and sample must be run in parallel.



LDH = lactate dehydrogenase; NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide

Reagents

	Reagent	Concentration	Storage conditions
Buffer (pH 7.5-7.6)	K_2HPO_4	1 M (17.4 g·100 ml ⁻¹)	+4 - 8°C
	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	1 M (15.6 g·100 ml ⁻¹)	+4 - 8°C
Cofactor	NADH	5 M (20.9 mg·ml ⁻¹)	+4 - 8°C
Enzymes	Lactate dehydrogenase	(Boehringer 106 984)	+4 - 8°C
Reagents	Water		Room temp

Method

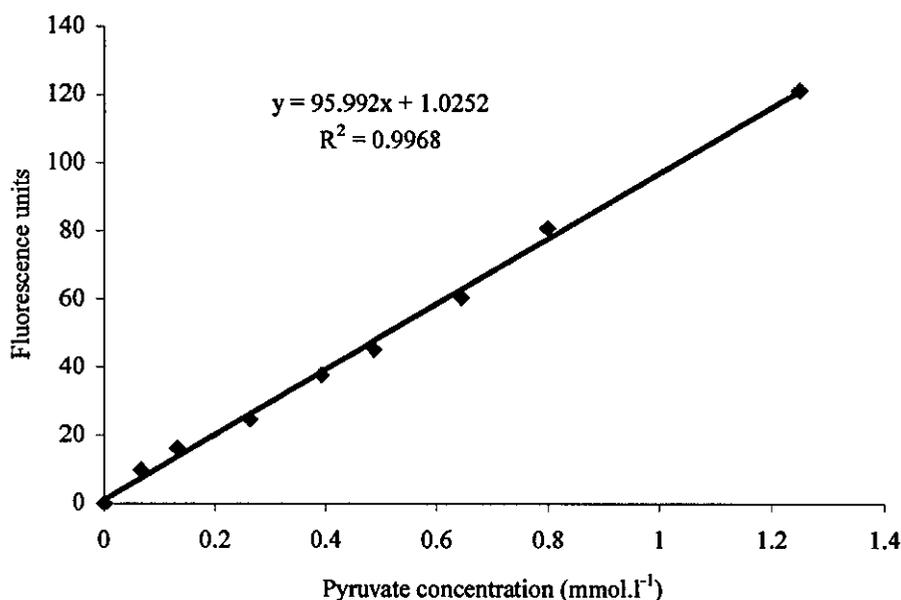
1. Prepare pyruvate standards:
Make up pyruvate stock solution 100 mM (11.0 mg pyruvate/ml water). Store at -20°C. Dilute the pyruvate standard to 20 μM prior to use.
2. Prepare phosphate buffers (K_2HPO_4 and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$).
3. Prepare the NADH solution:
Carbonate buffer (100 mM; pH 10.6); dissolve 0.85 g Na_2CO_3 and 0.17 g NaHCO_3 in 100 ml water. Dissolve NADH 3.5 mg/ml in carbonate buffer.
4. Prepare the enzyme:
Dilute lactate dehydrogenase stock (beef heart) (50 mg/10 ml or 5 mg/ml or 250 U/ml or 1250 U/ml) to 0.4 mg/ml with Tris buffer 20 mM, pH 8.0 containing 0.02% albumin (0.24 g·100 ml⁻¹ Tris with 2.0 mg albumin).
5. Prepare the pyruvate standards by adding the following volumes of pyruvate stock solution (20 μM) to the reagent buffer in cuvettes. Mix well.

Pyruvate (20 μ M) stock (μ l)	2	4	8	12	15	20	25	40
Reagent buffer (μ l)	600	600	600	600	600	600	600	600
[Pyruvate] μ M	0.066	0.132	0.263	0.391	0.487	0.644	0.798	1.247

6. Make up the reagent mixture, containing phosphate buffers, and titrate with NADH solution until the initial fluorescence is \sim 700-800 units. Store at room temperature.
7. Pipette 600 μ l of reagent buffer in each cuvette. Samples are run in batches of 10 + 1 blank.
8. Add 20 μ l of water for the blank or 20 μ l of sample. The muscle samples should be defrosted quickly in hot water, then vortexed and spun down (22,000 x g, 3 min) before use. Mix well.
9. Read initial fluorescence (F1) at 30 sec intervals (Excitation 340 nm/Emission 460 nm; Hitachi F2000, fluorescence spectrophotometer).
10. Add 1.5 μ l of LDH to each cuvette, agitate to mix and incubate for 10 min at room temperature.
11. Read final fluorescence (F2) at 30 sec intervals.
12. Calculations:

The relationship between fluorescence and pyruvate concentration was determined using a software program by fitting a linear trendline (Microsoft Excel; see Figure I.1). Concentration of pyruvate was then calculated using the slope from the equation of the relationship.

Figure I.1 Example standard curve for pyruvate.



Example calculation:

Pyruvate (mmol kg⁻¹ dm) =

$$(((F2-F1)-(B12-B11))/95.992) \times EF \times 1.25 \times 31.075/1000$$

F1 and B11 = fluorescence of sample and blank at the end of step 9.

F2 and B12 = fluorescence of sample and blank at the end of step 11.

31.075 = dilution factor (total cuvette volume (621.5 µl)/sample volume (20 µl)).

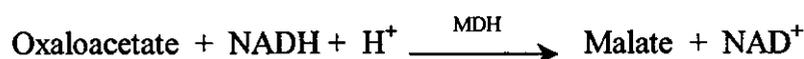
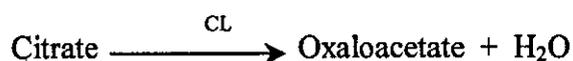
EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

APPENDIX J

MUSCLE CITRATE

Principle

Citrate concentration was determined indirectly from changes in NADH using a fluorometric assay.



MDH = Malate dehydrogenase; CL = citrate lyase; NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide

Reagents

	Reagent	Concentration	Storage conditions
Buffer (pH 8.0)	Tris Base	45 mM (0.55 g·100 ml ⁻¹)	+4 - 8°C
	EDTA	0.1 mM (3.7 mg·100 ml ⁻¹)	
	MgCl ₂ ·6H ₂ O	2.5 mM	
Stabilising Buffer (pH 7.4)	Triethanolamine	45 mM (0.23 g·25 ml ⁻¹)	+4 - 8°C
	MgCl ₂ ·H ₂ O	0.3 mM (1.5 mg·25 ml ⁻¹)	
	(NH ₄) ₂ SO ₄	0.5 mM (1.65 g·25 ml ⁻¹)	
Cofactor	NADH	10 mg · ml ⁻¹	+4 - 8°C
Enzymes	Citrate Lyase	(Boehringer 374 074)	+4 - 8°C
	Malate Dehydrogenase	(Boehringer 127 256)	+4 - 8°C
Reagents	Water		Room temp

Method

1. Prepare citrate buffer:

Add 0.55 g Tris Base, 3.7 mg EDTA and 51 mg MgCl₂·6H₂O to 100 ml water. Set to pH 8.0 with HCl.

2. Prepare stabilising buffer:

Add 0.23 g Triethanolamine, 1.5 mg MgCl₂·H₂O and 1.65 g (NH₄)₂SO₄ to 25 ml water. Set to pH 7.4 with NH₄OH. Stable for 24 h.

3. Prepare citrate standard:

Make up citrate stock solution (2 mM). Store at -20°C. Dilute the citrate standard to 100 µM prior to use.

4. Prepare the NADH solution:

Carbonate buffer (100 mM; pH 10.6); dissolve 0.85 g Na₂CO₃ and 0.17 g NaHCO₃ in 100 ml water. Dissolve NADH 10 mg/ml in carbonate buffer.

5. Prepare the enzyme:

Dilute 1 mg citrate lyase (CL) liophilysate 200µl stabilising buffer. Dilute 5 times with citrate buffer (without NADH) just prior to use.

6. Prepare the citrate standards by adding the following volumes of citrate diluted stock solution (100 µM) to the reagent buffer in cuvettes. Mix well.

Citrate (100 µM) stock (µl)	20	20	20	20	20	20	20	20
Water (µl)	1980	980	480	313.3	230	180	146.7	113.3
[Citrate] µM	1	2	4	6	8	10	12	15

13. Make up the reagent mixture: To 100 ml citrate buffer, add 5 µl of undiluted malate dehydrogenase (MDH) and 75 µl of NADH solution. Store at room temperature.

14. Pipette 500 µl of reagent buffer in each cuvette. Samples are run in batches of 10 + 1 blank.

15. To each cuvette add 75 µl of water for the blank, 75 µl of standard mixture or 75 µl of sample. The muscle samples should be defrosted quickly in hot water, then vortexed and spun down (22,000 x g, 3 min) before use. Mix well.

16. Read initial fluorescence (F1) at 30 sec intervals (Excitation 340 nm/Emission 460 nm; Hitachi F2000, fluorescence spectrophotometer).

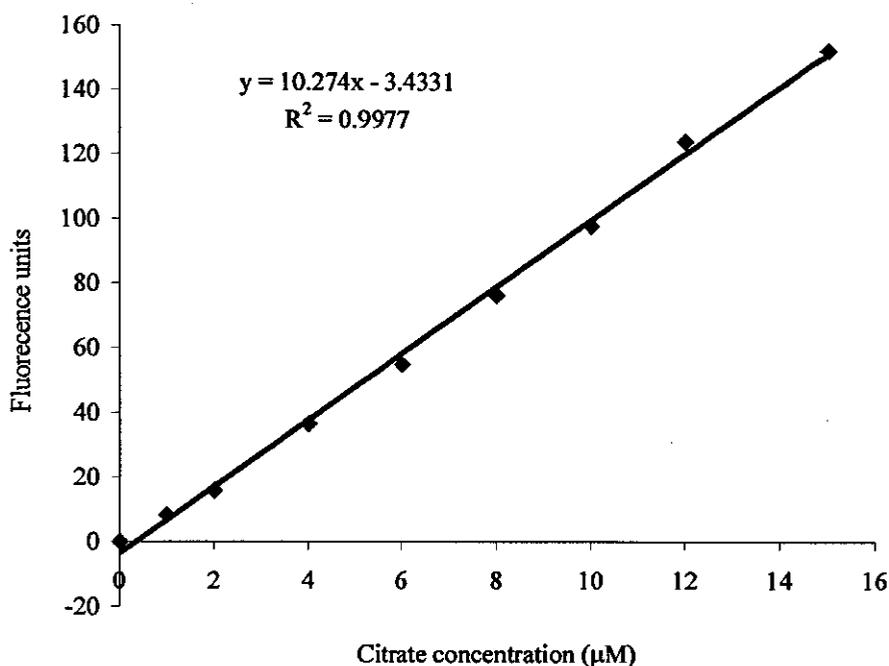
17. Add 10.0 µl of CL to each cuvette, agitate to mix and incubate for 15 min at room temperature.

18. Read final fluorescence (F2) at 30 sec intervals.

19. Calculations:

The relationship between fluorescence and citrate concentration was determined using a software program by fitting a linear trendline (Microsoft Excel; see Figure J.1). Concentration of citrate was then calculated using the slope from the equation of the relationship.

Figure J.1 Example standard curve for citrate.



Example calculation:

Citrate ($\text{mmol kg}^{-1} \text{ dm}$) =

$$\frac{((F2-F1)-(B12-B11))/10.274) \times EF \times 1.25}{1000}$$

F1 and B11 = fluorescence of sample and blank at the end of step 16.

F2 and B12 = fluorescence of sample and blank at the end of step 18.

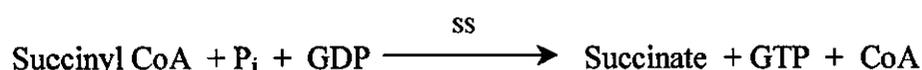
EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

APPENDIX K

MUSCLE SUCCINATE

Principle

Succinate concentration was determined indirectly from changes in NADH using a fluorometric assay.



SS = Succinyl CoA synthetase; NAD⁺ = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide

Reagents

	Reagent	Concentration	Storage conditions
Buffers (pH 7.4)	Triethanolamine * HCl	50 mM (0.93 g·100 ml ⁻¹)	+4 - 8°C
	EDTA	5 mM (0.19 g·100 ml ⁻¹)	
	MgCl ₂ *6H ₂ O	10 mM (0.2 g·100 ml ⁻¹)	Room temp
NADH buffer (pH 8.2)	Triethanolamine	0.1 M (1 g·100 ml ⁻¹)	+4 - 8°C
	K ₂ CO ₃	2.3 M (~8 g)	
Cofactor	NADH	2.4 mM (2.4 mg·ml ⁻¹)	+4 - 8°C
	CoASH	11 mM (8.45 mg·ml ⁻¹)	+4 - 8°C
	GTP	21 mM (11.3 mg·ml ⁻¹)	+4 - 8°C
	PEP	110 mM (51.21 mg·ml ⁻¹)	
Enzymes	Lactate Dehydrogenase	(Boehringer 106 984)	+4 - 8°C
	Pyruvate kinase	(Boehringer 128 155)	+4 - 8°C
	Succinyl-CoA synthetase	(Boehringer 161 543)	+4 - 8°C
Reagents	Water		Room temp

Method

1. Prepare TEA buffer:

Add 0.93 g Triethanolamine, 0.19 g EDTA and 0.2 g MgCl₂*6H₂O to 100 ml water.
Set to pH 7.4 with 1 M KOH.

2. Prepare TEA/K₂CO₃ buffer:

Add 1 g Triethanolamine to 100 ml water and titrate with K₂CO₃ until pH 8.2.

3. Prepare succinate standard:

Make up succinate stock solution 10 mM (11.8 mg succinic acid/ml water), adjust pH to 7.0 with 1 M KOH. Store at -20°C. Dilute the succinate standard to 50 μ M prior to use (50 μ l of 10 μ M stock with 9,950 μ l water).

4. Prepare the NADH solution:

Dissolve NADH 2 mg/ml in TEA/K₂CO₃ buffer.

5. Prepare the enzymes:

Lactate dehydrogenase (5 mg/ml), recommended 4.2 μ g/ml in cuvette. Pyruvate kinase (10 mg/ml) recommended 4.2 μ g/ml in cuvette. Succinyl-CoA synthetase (5 mg/ml), recommended 90 mU/ml in cuvette.

6. Prepare the succinate standards by adding the following volumes of diluted succinate stock solution (10 mM diluted to 50 μ M) to the reagent buffer in cuvettes.

Mix well.

Succinate (50 μ M) stock (μ l)	2	3	5	8	10	15	20	30
Water (μ l)	73	72	70	67	65	60	55	45
Reagent (μ l)	500	500	500	500	500	500	500	500
[Succinate] μ M	1.33	2.00	3.33	5.33	6.66	10.00	13.33	20.00

7. Make up the reagent mixture (For 1 sample): Add 1 ml TEA buffer, 10 μ l CoASH, 10 μ l GTP, 10 μ l PEP, 1 μ l lactate dehydrogenase (undiluted) and 0.5 μ l pyruvate kinase (undiluted). Titrate with NADH solution until initial fluorescence is ~800 units.

NB. The pyruvate contained within PEP and the samples causes erroneously high values. To overcome this, the reagent mixture is titrated with NADH to provide a high initial fluorescence, taking care not to reduce sensitivity. The reagent mixture containing PEP and NADH should be left for 15 min in the dark in order for the pyruvate contained within PEP to be reduced to lactate. Prior to use the fluorescence of the reagent mixture should be checked. Pipetted samples should be left for at least 10 min to allow pyruvate contained in samples to be reduced to lactate.

8. Pipette 500 μ l of reagent buffer in each cuvette. Samples are run in batches of 10 + 1 blank.

9. To each cuvette add 75 μ l of water for the blank, 75 μ l of standard mixture or 75 μ l of sample. The muscle samples should be defrosted quickly in hot water, then vortexed and spun down (22,000 x g, 3 min) before use. Mix well.

10. Read initial fluorescence (F1) at 30 sec intervals (Excitation 340 nm/Emission 460 nm; Hitachi F2000, fluorescence spectrophotometer).

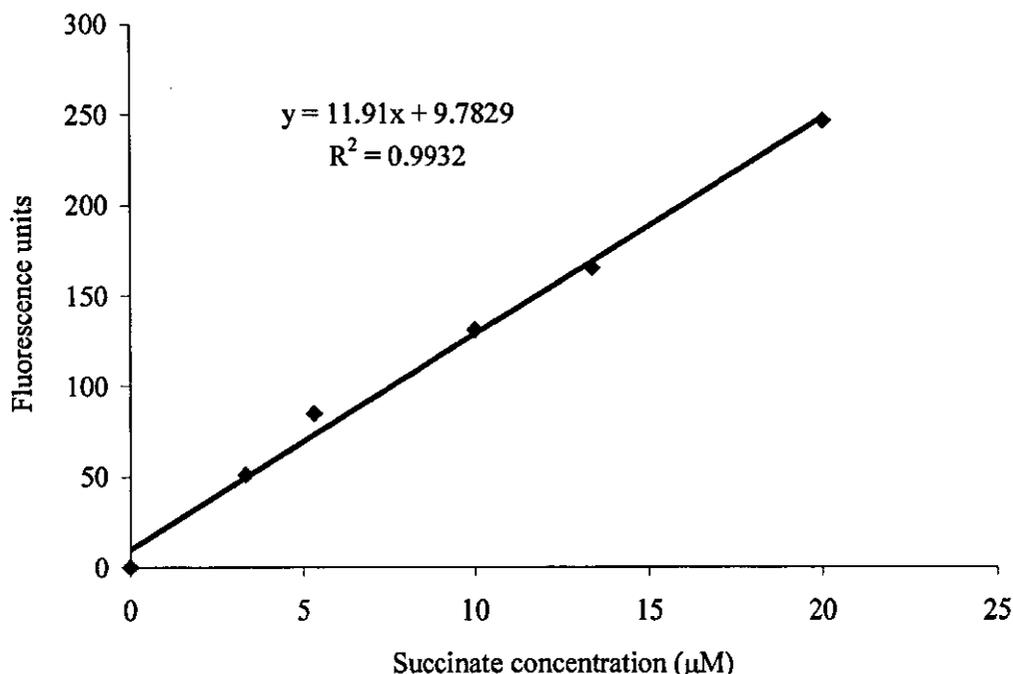
11. Add 2.5 μ l of succinyl-CoA synthetase to each cuvette, agitate to mix and incubate for 30 min at room temperature.

12. Read final fluorescence (F2) at 30 sec intervals.

13. Calculations:

The relationship between fluorescence and succinate concentration was determined using a software program by fitting a linear trendline (Microsoft Excel; see Figure K.1). Concentration of succinate was then calculated using the slope from the equation of the relationship.

Figure K.1 Example standard curve for succinate.



Example calculation:

Succinate ($\text{mmol kg}^{-1} \text{ dm}$) =

$$\frac{(((F2-F1)-(B12-B11))/11.91) \times EF \times 1.25}{1000}$$

F1 and B11 = fluorescence of sample and blank at the end of step 10.

F2 and B12 = fluorescence of sample and blank at the end of step 12.

EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

APPENDIX L

MUSCLE MALATE AND FUMARATE

Principle

Both malate and fumarate concentration was determined indirectly from changes in NADH using a fluorometric assay.



FM = Fumarase; MDH = Malate dehydrogenase; NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide

Reagents

	Reagent	Concentration	Storage conditions
Buffer (pH 9.0)	Hydrazine	1.0 M	-20°C
	EDTA	0.1 M (3.7 g·100 ml ⁻¹)	+4 - 8°C
	Acetylpyridine-adenine dinucleotide (APAD)	0.1 M (70 mg·ml ⁻¹)	-20°C
Stabilising Buffer (pH 8.0)	Tris	0.02 M (0.24 g·100 ml ⁻¹)	-20°C
Enzymes	Malate Dehydrogenase	(Boehringer 127 256)	+4 - 8°C
	Fumarase	(Sigma-F1757)	+4 - 8°C
Reagents	Water		Room temp

Method

1. Prepare malate and fumarate buffer:

Add 0.55 g Tris Base, 3.7 mg EDTA and 51 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to 100 ml water. Set to pH 8.0 with HCl.

2. Prepare stabilising buffer:

Tris buffer 0.02 M containing 0.02% albumin (0.24 g·100 ml⁻¹ Tris with 2.0 mg albumin). Set to pH 8.0 with 1 M HCl.

3. Prepare malate and fumarate standards:

Make up L-malate stock solution 100 mM (19.97 mg malic acid/ml water). Store at -20°C. Dilute the malate standard to 50 μM prior to use (5 μl of 100 mM stock to

10 ml water). Make up fumarate stock solution 100 mM (16.0 mg fumaric acid/ml water). Store at -20°C. Dilute the fumarate standard to 50 μ M prior to use (5 μ l of 100 mM stock to 10 ml water).

4. Prepare the enzymes:

Dilute malate dehydrogenase stock (5 mg/ml) 5 times with stabilising buffer (Tris buffer 0.02 M, pH 8.0, containing 0.02% albumin) until final concentration is 1 mg protein/ml. Dilute fumarase stock (2500 U) with stabilising buffer (Tris buffer 0.02 M, pH 8.0, containing 0.02% albumin) until final concentration is 1 mg protein/ml.

5. Prepare the malate and fumarate standards by adding the following volumes of diluted malate and fumarate stock solution (100 mM diluted to 50 μ M) to the reagent buffer in cuvettes. Mix well.

Malate (50 μ M) (μ l)	2	4	6	8	10	15	20	25
Fumarate (50 μ M) (μ l)	2	4	6	8	10	15	20	25
Water (μ l)	71	67	63	59	55	45	35	25
Reagent (μ l)	500	500	500	500	500	500	500	500
[Malate] μ M	0.173	0.345	0.518	0.691	0.864	1.295	1.727	2.159
[Fumarate] μ M	0.172	0.343	0.515	0.686	0.858	1.286	1.715	2.144

6. Make up the reagent mixture: To 100 ml water, add 10 ml of hydrazine 1.0 M, pH 9.0 (5 ml hydrazine hydrate ~20 M), 0.2 ml EDTA 0.1 M and 0.06 ml APAD 0.1 M. Store at room temperature.

7. Pipette 500 μ l of reagent buffer in each cuvette. Samples are run in batches of 10 + 1 blank.

8. To each cuvette add 75 μ l of water for the blank, 75 μ l of standard mixture or 75 μ l of sample. The muscle samples should be defrosted quickly in hot water, then vortexed and spun down (22,000 x g, 3 min) before use. Mix well.

9. Read initial fluorescence (F1) at 30 sec intervals (Excitation 340 nm/Emission 460 nm; Hitachi F2000, fluorescence spectrophotometer).

10. Add 4 μ l of malate dehydrogenase to each cuvette, agitate to mix and incubate for 25 min at room temperature.

11. Read intermediate fluorescence (F2) at 30 sec intervals.

12. Add 4 μ l of fumarase to each cuvette, agitate to mix and incubate for 25 min at room temperature.

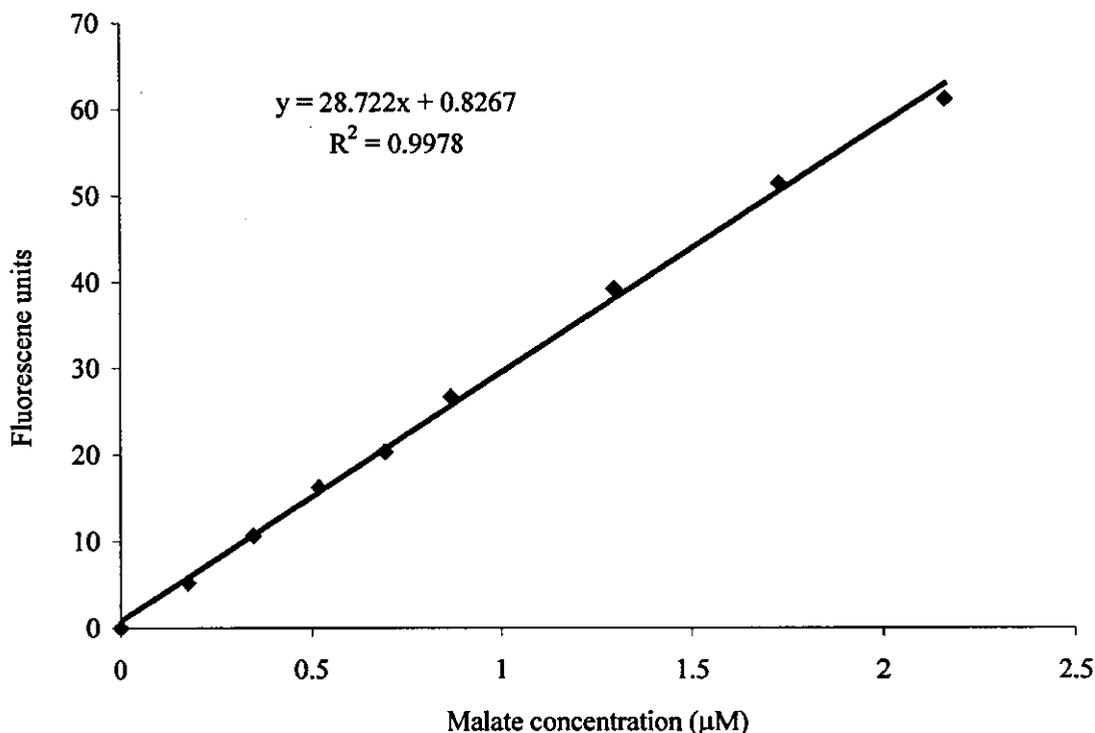
13. Read final fluorescence (F3) at 30 sec intervals.

14. Calculations:

The relationship between fluorescence and malate and fumarate concentration was determined using a software program by fitting a linear trendline (Microsoft Excel;

see Figures L.1 and L.2). Concentration of malate and fumarate was then calculated using the slope from the equation of the relationship.

Figure L.1 Example standard curve for malate.



Example calculation:

Malate ($\text{mmol kg}^{-1} \text{ dm}$) =

$$\left(\frac{(F2-F1)-(B12-B11)}{28.722} \right) \times EF \times 1.25 \times (579/75) / 1000$$

F1 and B11 = fluorescence of sample and blank at the end of step 9.

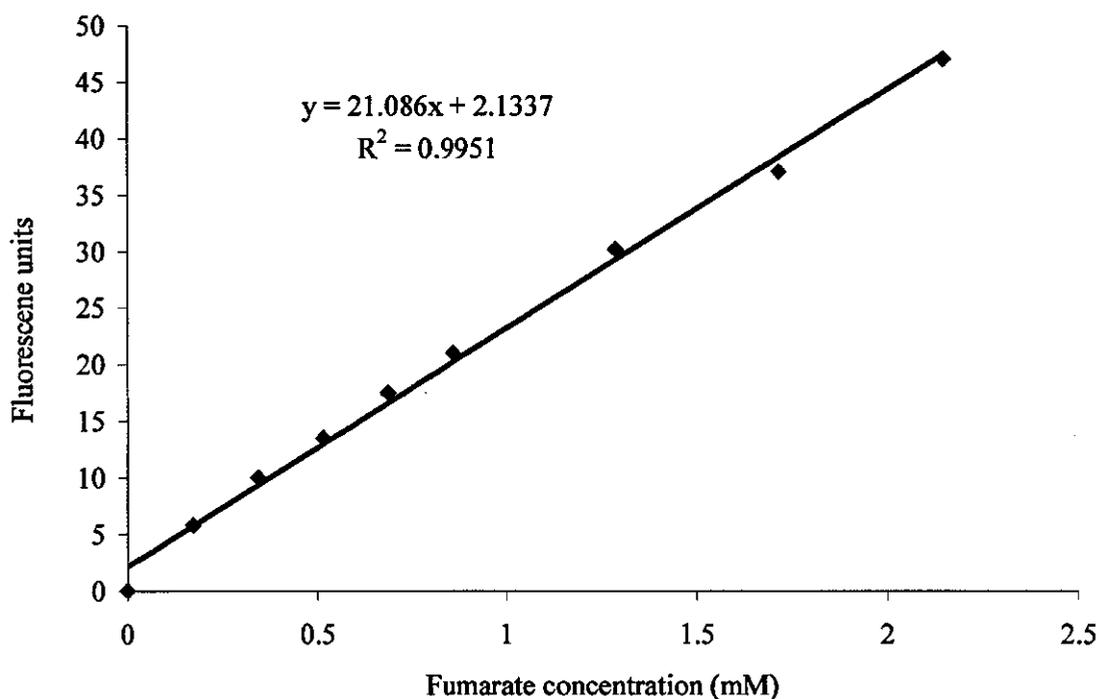
F2 and B12 = fluorescence of sample and blank at the end of step 11.

EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

579 = total volume in cuvette

75 = volume of sample added

Figure L.2 Example standard curve for fumarate.



Example calculation:

Fumarate ($\text{mmol kg}^{-1} \text{ dm}$) =

$$\frac{((F2-F1)-(B12-B11))/21.086}{1.25} \times EF \times \frac{583}{75} \times 1000$$

F1 and B11 = fluorescence of sample and blank at the end of step 11.

F2 and B12 = fluorescence of sample and blank at the end of step 13.

EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

583 = total volume in cuvette

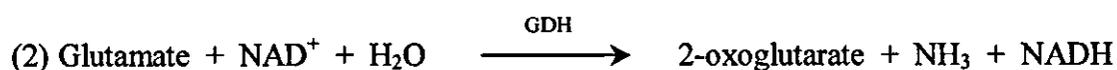
75 = volume of sample added

APPENDIX M

MUSCLE GLUTAMINE AND GLUTAMATE

Principle

Both glutamine and glutamate concentration was determined indirectly from changes in NADH using a fluorometric assay. The assay is performed in two steps; firstly the glutamine contained in the sample is converted to glutamate (1), in the second step glutamate concentration is determined by converting glutamate to 2-oxoglutarate (2) with a concomitant measurable change in NADH.



GDH = glutamate dehydrogenase; NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide

The equilibrium of reaction (2) favours glutamate formation. To overcome the unfavourable kinetics a high concentration of NAD^+ and low concentration of H^+ is used. ADP is used to activate glutamate dehydrogenase and to decrease its reactivity with other amino acids.

Reagents

	Reagent	Concentration	Storage conditions
Glutamine Buffer (pH 5.0) 0.2 M	Sodium acetate	18.44 g·1000 ml ⁻¹	Room temp
	Glacial acetic acid	3.74 ml·1000 ml ⁻¹	Room temp
Glutamate Buffer (pH 9.0) 1.1 M	Hydrazine sulphate	1.3 g·100 ml ⁻¹	Room temp
	Hydrazine hydrate	5.0 g·100 ml ⁻¹	Room temp
	EDTA (Na ₂)	0.2 g·100 ml ⁻¹	Room temp
Cofactors	ADP	100 mM (8.0 g·100 ml ⁻¹)	-20°C
	NAD	50 mM (33.0 mg·1 ml ⁻¹)	+4 - 8°C
Enzymes	Glutaminase	(Sigma-G8880)	+4 - 8°C
	Glutamate dehydrogenase	(Boehringer 127 710)	+4 - 8°C
Reagents	Water		Room temp

Method

1. Prepare glutamine buffer:
Add 18.44 g sodium acetate, 3.74 ml glacial acetic acid to 1000 ml water. Check pH 5.0 with pH meter.
2. Prepare glutamate buffer:
Add 1.3 g hydrazine sulphate, 5.0 g hydrazine hydrate and 0.2 g EDTA·Na₂ to 100 ml water. Check pH 9.0 with pH meter.
3. Prepare glutamine and glutamate standards:
Make up L-glutamine stock solution 100 mM (14.61 mg L-glutamine/ml water). Store at -20°C. Dilute the glutamine standard to 900 µM prior to use (90 µl of 100 mM stock in 10 ml water). Make up glutamate stock solution 100 mM (16.92 mg L-glutamic acid/ml water). Store at -20°C. Dilute the glutamate standard to 155 µM prior to use (15.5 µl of 100 mM stock in 10 ml water).
4. Prepare the enzymes:
Dissolve 25 U of glutaminase powder in 2.5 ml in 0.1 M acetate buffer (stable for several months). Use glutamate dehydrogenase undiluted (10 mg/ml in glycerol).
5. Prepare the glutamine and glutamate standards by adding the following volumes of diluted glutamine stock solution (100 mM diluted to 900 µM) and glutamate stock solution (100 mM diluted to 155 µM) to the reagent buffer in cuvettes. Mix well.

(1) For glutamine standards:

Glutamine (900 µM) (µl)	5	10	15	20	25	30	35	40
Water (µl)	45	40	35	30	25	20	15	10
Reagent (µl)	555	555	555	555	555	555	555	555
[Glutamine] µM	90	180	270	360	450	540	630	720

(2) For glutamate standards:

Glutamate (155 µM) (µl)	2	4	6	8	10	12	15	20
Water (µl)	18	16	14	12	10	8	5	0
Reagent (µl)	565	565	565	565	565	565	565	565
[Glutamate] µM	15.5	31.0	46.5	62.0	77.5	93.0	116.3	155.0

A separate set of standards is required for each step of the assay; the glutamine is converted to glutamate in the first step and then assayed for glutamate concentration along with the glutamate standards in the second step of the assay.

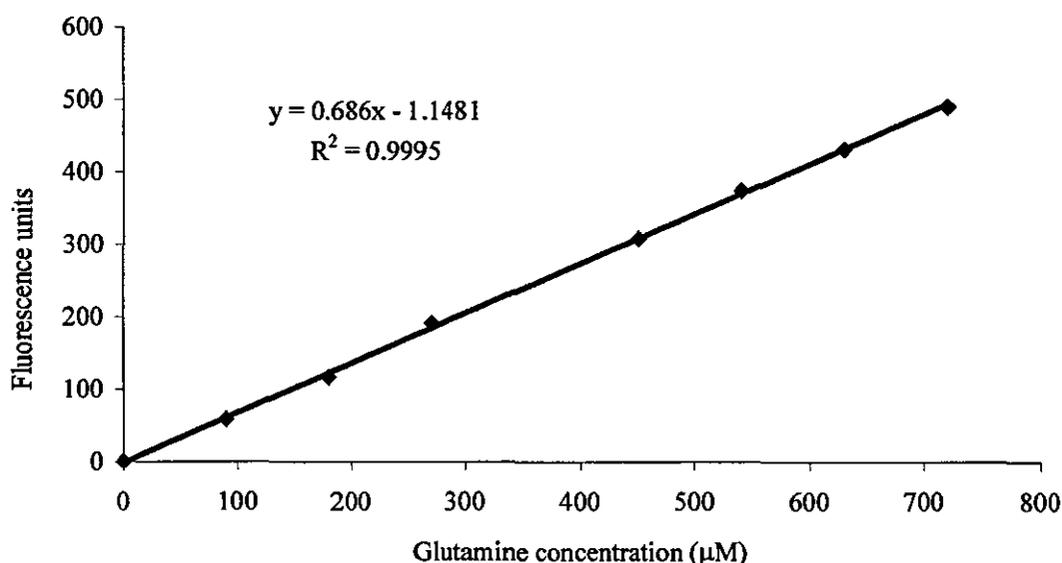
6. For glutamine assay: To a flip-top eppendorf add 100 µl of acetate buffer, 5 µl of glutaminase and 450 µl of water. Add 50 µl of water for the blank, 50 µl of standard mixture for the glutamine assay or 40 µl of sample. The muscle samples

should be defrosted quickly in hot water, then vortexed and spun down (22,000 x g, 3 min) before use. Mix well.

7. Incubate for 1 h at 37°C.
8. For glutamate assay: To each cuvette add 550 µl of hydrazine buffer, 10 µl of NAD solution and 5 µl of ADP solution. Add 20 µl of water for the blank or 20 µl of standard mixture for the glutamate assay. For the samples, add either 20 µl of hydrolysed sample (from the first step of the assay) or 20 µl of untreated sample. The muscle samples should be defrosted quickly in hot water, then vortexed and spun down (22,000 x g, 3 min) before use. Mix well.
9. Read initial fluorescence (F1) at 30 sec intervals (Excitation 340 nm/Emission 460 nm; Hitachi F2000, fluorescence spectrophotometer).
10. Add 5 µl of glutamate dehydrogenase to each cuvette (from both the first step and second step of the assay), agitate to mix and incubate for 25 min at room temperature.
11. Read final fluorescence (F2) at 30 sec intervals.
12. Calculations:

The relationship between fluorescence and glutamine and glutamate concentration was determined using a software program by fitting a linear trendline (Microsoft Excel; see Figures M.1 and M.2). Concentration of glutamine and glutamate was then calculated using the slope from the equation of the relationship.

Figure M.1 Example standard curve for glutamine.



Example calculation:

Glutamine ($\text{mmol kg}^{-1} \text{ dm}$) =

$$(((F2-F1)-(B12-B11)) \times EF \times 1.25)/(0.686 \times 1000))$$

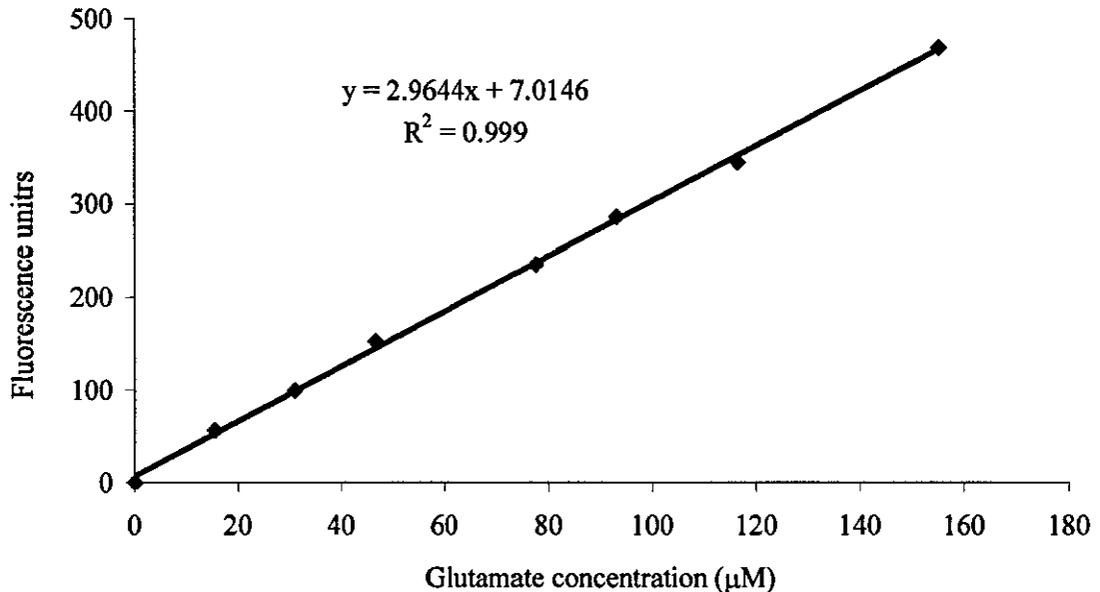
F1 and B11 = fluorescence of sample and blank at the end of step 9.

F2 and B12 = fluorescence of sample and blank at the end of step 11.

EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

N.B. To calculate final glutamine concentration, subtract the calculated glutamate concentration from the calculated glutamine concentration.

Figure M.2 Example standard curve for glutamate.



Example calculation:

Glutamate ($\text{mmol kg}^{-1} \text{ dm}$) =

$$(((F2-F1)-(B12-B11)) \times EF \times 1.25)/(2.9644 \times 1000))$$

F1 and B11 = fluorescence of sample and blank at the end of step 9.

F2 and B12 = fluorescence of sample and blank at the end of step 11.

EF = extraction factor (known volume of PCA added to muscle powder during extraction procedure/weight of sample in mg).

APPENDIX N

A comparison of different analytical methods for the determination of glucose and lactate concentrations in whole blood and plasma (unpublished paper)

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Abstract

The purpose of the present work was to compare the whole blood and plasma glucose and lactate concentrations obtained by different commonly used automated and manual analytical methods to determine whether results are quantitatively and qualitatively similar. Measurements of blood glucose and lactate concentration were made using a Yellow Springs Instruments (YSI) automated analyser and manual methods, and plasma glucose and lactate concentration using two automated analysers; the YSI analyser and the Cobas Mira Plus analyser. A high level of agreement was observed between all comparisons (Mean difference; blood glucose: -0.04 ± 0.15 mmol/L; blood lactate: 0.01 ± 0.05 mmol/L; plasma lactate: 0.01 ± 0.15 mmol/L), except plasma glucose concentration measured on the YSI analyser and the Cobas Mira Plus analyser (0.47 ± 0.16 mmol/L; 10% difference). The present work suggests that it cannot be assumed that glucose and lactate concentrations can be quantitatively compared when different analytical methods have been used.

Keywords: Level of agreement, glucose oxidase, exercise, manual and automated methods.

Introduction

A variety of methods have been employed for the determination of glucose and lactate concentrations both within the clinical and research setting. An accurate and precise method for the measurement of these metabolites is desirable, in each of these settings. Furthermore, it would be desirable that different methods of determination display a high level of agreement, so that data from different laboratories are comparable.

Blood glucose concentration declines immediately after the sample has been collected, with a concomitant increase in blood lactate concentration, due to the action of glycolytic enzymes within both erythrocytes and leukocytes. When a delay is expected between sample collection and analysis, pre-treatment of the blood sample is required, e.g. deproteinisation in perchloric acid to prevent glycolysis. This time consuming pre-treatment of samples and relatively lengthy manual assays for lactate and glucose concentration makes the automated analysis of fresh samples an attractive alternative. Furthermore, the rapid and accurate determination of both glucose and lactate concentrations is vital, particularly in the clinical environment.

Automated analysers such as the Yellow Springs Instruments glucose and lactate analyser allow the analysis of fresh blood or plasma samples without any pre-treatment of the sample. Manual methods of analysis generally require the deproteinisation and storage of the sample, with the glucose or lactate concentration being determined at a later date. This results in a longer analysis period. Alternatively, plasma can be analysed using an automated analyser (e.g. Cobas Mira Plus), which will reduce analysis time compared to performing the analysis manually. The latter methods

involve a longer delay before the glucose and lactate concentration data are available, thus limiting their suitability in the clinical setting.

Different methods of analysis for both glucose and lactate will not necessarily produce quantitatively the same results (Genter and Ipp, 1994). However, direct comparisons of results between studies are often made without consideration of any systematic difference in the analytical methods. Published studies that have compared different methods of analysis have, in general, applied unsuitable statistical analysis (i.e. correlation or regression) to assess the agreement between methods (Chua and Tan, 1978). A more appropriate statistical analysis of different methods has been suggested (Bland and Altman, 1986).

The purpose of the present work was to compare glucose and lactate data derived by different analytical methods in both plasma and blood. Measurements of blood glucose concentration were made using a Yellow Springs Instruments automated analyser and a colorimetric manual method. Plasma glucose concentration was measured using two automated analysers: the Yellow Springs Instruments analyser and the Cobas Mira Plus analyser. Comparisons of blood lactate concentration were made between the Yellow Springs Instruments analyser and a manual fluorometric method, and plasma lactate concentration using the Yellow Springs Instruments analyser and the Cobas Mira Plus analyser.

Method

Samples ($n = 168$) were obtained from healthy subjects completing a standard short-term exercise and recovery protocol (7 h protocol incorporating periods of rest and

exercise; Fallowfield et al. 1995). Samples were taken from normal postabsorptive subjects through an indwelling forearm venous cannula throughout the experimental period. After an overnight fast, subjects completed approximately 90 min of cycling exercise at 70 % of their maximal oxygen uptake ($\dot{V}O_{2max}$). After 30 min, subject completed 3 x 50 s efforts at twice the workload, followed by a further 45 min at the original intensity. After this initial exercise bout subjects recovered passively for 4 h. Subjects then performed a second bout of exercise which entailed cycling to exhaustion at 70 % $\dot{V}O_{2max}$. The experimental protocol was approved by the Ethical Committee of Loughborough University.

The concentration of glucose in whole blood was determined using both a Yellow Springs Instruments automated analyser (YSI) (Model 2300 STAT Plus, YSI, Ohio, USA) and a manual technique using a commercially available kit (GOD-Perid; Boehringer Mannheim, GmbH) employing an enzymatic (glucose oxidase) colorimetric method. The concentration of glucose in plasma was determined using the YSI analyser and another automated analyser (Cobas Mira Plus, Roche Diagnostic Systems, Basel, Switzerland) using a commercially available kit (GOD-PAP; Boehringer Mannheim, GmbH) employing an enzymatic (glucose oxidase) colorimetric method (*see* Table 1).

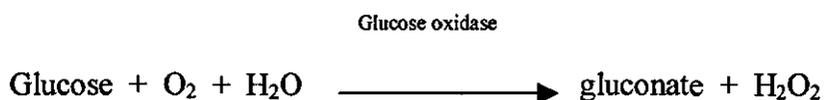
The concentration of lactate in whole blood was determined using both the YSI analyser and a manual technique employing an enzymatic fluorometric method (Maughan, 1982). The concentration of lactate in plasma was determined using the YSI analyser and the Cobas Mira Plus analyser using a commercially available kit (Boehringer Mannheim, GmbH) employing an enzymatic method (*see* Table 1).

Table 1. Comparison of analytical methods employed for the determination of glucose and lactate concentration.

	Analytical methods compared	
<i>Blood glucose</i>	YSI analyser	Manual spectrophotometric
<i>Blood lactate</i>	YSI analyser	Manual fluometric
<i>Plasma glucose</i>	Cobas Mira Plus analyser	YSI analyser
<i>Plasma lactate</i>	Cobas Mira Plus analyser	YSI analyser

Principles of Analysis

The conversion of glucose to gluconate and hydrogen peroxide, in a reaction catalysed by the enzyme glucose oxidase (EC 1.1.3.4), constitutes the basis for several widely used methods of glucose determination in either whole blood or plasma. The increase in gluconate and hydrogen peroxide being proportional to the concentration of glucose in the sample being analysed.



Both the manual and automated (Cobas Mira Plus) methods rely on the spectrophotometric determination of glucose.

Automated determination of plasma glucose concentration (Cobas Mira Plus)

A 4 ml aliquot was collected into a plastic tube containing ethylenediamine-tetra-acetic acid (EDTA) (Sarstedt, Leicester, UK) and was promptly centrifuged (6000 g, 15 min). After centrifugation, all samples were immediately stored at -20°C in untreated Eppendorf tubes. After a mean storage time of approximately 5 weeks, the plasma

samples were analysed for glucose concentration using the Cobas Mira Plus analyser. Plasma treated with EDTA has been shown not to interfere with any subsequent glucose analysis.

Plasma samples can be analysed using the Cobas Mira Plus (GOD-PAP method) since deproteinisation is not required. The presence of peroxidase, phenol and 4-aminophenazone in the reaction mixture results in the conversion of the hydrogen peroxide produced by the glucose oxidase reaction, to water and 4-(p-benzoquinone-mono-imino)phenazone. The intensity of the resultant change in colour is measured spectrophotometrically at 500 nm, and is directly proportional to the glucose concentration.

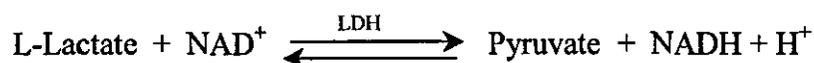
Manual determination of blood glucose concentration

A 20 µl sample of whole blood was deproteinised in 200 µl of perchloric acid to halt glycolysis and immediately centrifuged (12,000 g, 4 min). After centrifugation, all samples were stored at -20°C. Due to the relative instability of glucose, the supernatant was analysed firstly for blood glucose concentration (manual method) and then, after re-freezing, blood lactate concentration (manual method).

For the manual method of blood glucose analysis (GOD-Perid method), peroxidase and ABTS (di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) are introduced into the reaction mixture, resulting in the conversion of hydrogen peroxide to water and a coloured complex.. The intensity of the resultant colour change measured on a spectrophotometer at 610 nm (Cecil Instruments Model 393 Series 2, Cambridge, UK) is directly proportional to the glucose concentration.

Manual determination of blood lactate concentration

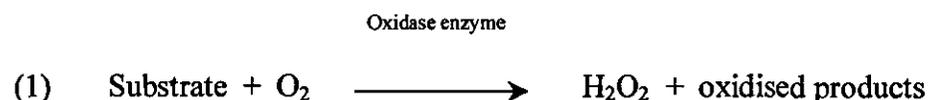
The method for the fluorometric determination of blood lactate concentration was based upon that of Maughan (1982). The addition of lactate dehydrogenase (EC 1.1.1.27) to the reaction mixture (pH 9.0) containing excess NAD^+ results in the production of reduced NAD^+ (NADH) causing a change in fluorescence, measured using a fluorimeter (Locarte Model LF 8-9; Locarte, London, UK).



Automated determination of both plasma and blood glucose and lactate concentration (Yellow Springs Instruments analyser)

A 1 ml aliquot was collected into an Eppendorf tube for whole blood lactate and glucose analysis using the YSI analyser. After analysis of whole blood and within 1 minute of collection, this aliquot was centrifuged and the subsequent plasma sample was then presented to the YSI analyser for lactate and glucose analysis.

The YSI analyser measured total (i.e. intracellular and extracellular using a cell-lysing agent) concentration of glucose and lactate in whole blood or plasma. Upon injection of the test sample (25 μl) into the chamber, the determination of either glucose and lactate concentration depends on the following principle:



The test substrate (glucose or lactate) is rapidly oxidised upon contact with the respective membrane (separate membranes for glucose and lactate) holding an

immobilised oxidase enzyme, producing hydrogen peroxide (H₂O₂). The H₂O₂ produced is oxidised at a platinum anode producing electrons:



A dynamic equilibrium is achieved when the rate of H₂O₂ production and the rate at which the H₂O₂ leaves the immobilised enzyme are constant. The electron flow is linearly proportional to the H₂O₂ concentration, and therefore to the glucose or lactate concentration of the test sample. The current generated is equated to the metabolite concentration by the use of internal standards, provided by the manufacturer.

Standards and Quality Controls

All assays using commercially available kits followed the manufacturers instructions for analysis. All buffers, standards and control sera were freshly prepared prior to analysis. The standards used during the manual blood lactate assay ranged from 0 mmol/L to 10 mmol/L, with a quality control of 2.2 mmol/L. The standard used in the manual blood glucose assay was provided by the manufacturer (0.505 mmol/L) and a deproteinised serum based quality control was used (Precinorm U, Boehringer Mannheim, GmBH; Assigned value: 5.71 mmol/L; Range: 4.86 – 6.57 mmol/L).

The YSI analyser was checked and calibrated daily according to manufacturers instructions using aqueous standards obtained from the manufacturer. An internal aqueous standard was used to calibrate the analyser (Lactate: 5 mmol/L; Glucose: 10 mmol/L), in addition to external aqueous standards to check linearity (Lactate: 30 mmol/L; Glucose: 50 mmol/L). A serum based quality control (Control Serum N,

Boehringer Mannheim, GmbH; Assigned values: Lactate: 1.27 mmol/L; Glucose: 4.44 mmol/L) was also used to check the analyser.

A variety of serum based quality controls were used to check the Cobas Mira Plus analyser (CFAS [calibrator for automated systems], Precinorm U, Precipath U; Boehringer Mannheim, GmbH and Control Serum N, Control Serum P; Roche Diagnostics; Range: Lactate: 1.14 – 5.04 mmol/L; Glucose: 3.94 – 16.0 mmol/L).

Between-assay means and coefficient of variations (CV) were determined for each analytical method:

4.26 mmol/L, CV 1.3 %, glucose – YSI analyser (serum based QC)

1.30 mmol/L, CV 2.2 %, lactate – YSI analyser (serum based QC)

4.57 mmol/L, CV 2.8 %, plasma glucose – Cobas Mira Plus analyser

4.64 mmol/L, CV 2.0 %, plasma lactate – Cobas Mira Plus analyser

2.22 mmol/L, CV 2.2 %, blood lactate – manual method

5.54 mmol/L, CV 1.3 %, blood glucose – manual method

Statistical methods

Agreement between methods was assessed using the method recommended by Bland and Altman (1986). In order to apply this method, differences in time over the experimental period were ignored. A two-way analysis of variance (ANOVA) for repeated measurements (time x method) was used to determine if any quantitative differences existed between methods. The interaction between time and method effects of the two-way ANOVA was used to examine whether there were qualitative differences between two given methods of analysis. A Pearson Product Moment

correlation was used to examine the relationship between methods. The 0.05 level was used for statistical significance, values are presented as means \pm SD.

Results

Glucose analysis – Plasma

A mean difference ($\sim 10\%$) of 0.47 ± 0.16 mmol/L (95 % confidence interval 0.11 – 0.82 mmol/L) was observed in plasma glucose concentrations measured using the two automated analysers (Cobas Mira Plus analyser and the YSI analyser) (Fig. 1). This difference between methods was significant ($P < 0.0001$) (Fig. 2), but, there was no interaction between method of analysis and time effects. Despite this difference between the two methods, a significant positive correlation was observed ($r = 0.9952$, $P < 0.01$).

Glucose analysis – Blood

There was no significant difference between the blood glucose concentration measured on the YSI analyser and by the manual method (-0.04 ± 0.15 mmol/L, 95 % confidence interval $-0.38 - 0.30$ mmol/L, $\sim 1\%$ difference). A significant positive correlation ($r = 0.9872$, $P < 0.01$) was observed between the two methods.

Lactate analysis - Plasma

The mean difference (Fig. 3) between the measurements for plasma lactate concentration made using the two automated analysers (YSI analyser and Cobas Mira Plus analyser) was 0.01 ± 0.15 mmol/L (95 % confidence interval $-0.32 - 0.33$ mmol/L, $\sim 0.6\%$ difference). No difference was observed between the two methods of analysis

using a two-way ANOVA (Fig. 4). A significant positive correlation was found between the measurements of plasma lactate ($r = 0.9967$, $P < 0.01$).

Lactate analysis - Blood

When comparing the measurements of blood lactate made on the YSI analyser and those made using the manual method, a mean difference of 0.01 ± 0.05 mmol/L (95 % confidence interval $-0.09 - 0.12$ mmol/L) (~ 1.3 % difference) was observed between the two methods, which was not statistically significant. Furthermore, a significant positive correlation was found between the measurements of blood lactate ($r = 0.9998$, $P < 0.01$).

Glucose and Lactate analysis - Plasma vs Blood

Plasma glucose concentrations were consistently ~ 14 % (0.58 ± 0.10 mmol/L, $P < 0.001$) higher than blood glucose concentration, both measured on the YSI analyser. A significant positive correlation ($r = 0.9919$, $P < 0.01$) was observed between the two measurements.

Plasma lactate concentrations were approximately 38 % (0.54 ± 0.47 mmol/L, $P < 0.05$) higher than blood lactate concentrations, both measured using the YSI analyser. However, a significant positive correlation was observed between plasma and blood lactate ($r = 0.9994$, $P < 0.01$).

Discussion

The use of the enzymatic fluorometric method to determine blood lactate concentrations has previously been shown to be both reliable and accurate (Maughan, 1982). Despite employing different principles of determination, the results from the present work demonstrate that the Yellow Springs analyser using immobilised enzyme technology is able to produce consistently similar measurements of blood lactate to that of the conventional manual method. Indeed, this finding is in accordance with previous research suggesting that the use of haemolysed blood samples with the YSI analyser, as in the present work, produces comparable lactate results with those obtained in deproteinised whole blood (Foxdal et al. 1992).

Of greatest interest with the present work is the disparity between the two automated analysers (the YSI analyser and the Cobas Mira Plus analyser) measurements of plasma glucose concentration. The plasma glucose concentration values from the Cobas Mira Plus are consistently ~10 % higher than the YSI analyser data. It has previously been reported that plasma glucose concentrations measured using YSI glucose analyser (Model 23AM) were lower than other glucose oxidase methods of plasma glucose concentration determination (Unwin et al. 1995). These authors suggested that the use of an aqueous calibrant with the YSI may not have been appropriate when plasma samples were presented, and may have resulted in lower plasma glucose measurements. Indeed, with the present work, when using an aqueous internal calibrator, the YSI analyser tended to return lower values for the serum based control. These values were approximately 3.6 % lower than the assigned value (Assigned value: 4.44 mmol/L; Mean YSI value: 4.28 mmol/L) for the quality control, but were still within the assigned range (4.00 – 4.88 mmol/L). It should be noted that the underlying difference in the

calibration of the two automated analysers is that a serum based calibrator was used with Cobas Mira Plus (CFAS), whereas the YSI analyser used aqueous standards. It is conceivable that this difference in calibration may have led to the difference observed in the plasma glucose concentrations between the two analysers.

The lack of an accepted 'gold standard' measurement of plasma glucose concentration makes any comparison between the two measurements difficult. The close agreement between the YSI analyser and the manual methods for both blood glucose and blood lactate measurement corroborates the use of these methods. The lack of agreement between the two methods for plasma glucose determination would suggest that the method employed by the Cobas Mira Plus may have produced elevated values for plasma glucose concentration. However, one cannot merely attribute the difference in measurement to the Cobas Mira Plus analyser itself as a close agreement was observed between the YSI analyser and Cobas Mira Plus analyser in the determination of plasma lactate concentration.

Several confounding factors exist, that may explain the observed difference between the two measurements of plasma glucose concentration. In the case of the YSI analyser, fresh samples were presented to the analyser, whereas stored samples (5 weeks at -20°C) were presented to the Cobas Mira Plus analyser. During the process of sample collection, it is possible that there may have been a delay in spinning the samples, furthermore, no glycolytic inhibitor (e.g. fluoride) was used in the tubes at any point in the process. These factors may have served to reduce the glucose concentration in the plasma sample presented to the Cobas Mira Plus analyser. In addition, the freezing process itself has been shown to decrease plasma glucose concentration in the sample,

with a linear relationship being observed between storage duration and the decrease the plasma glucose concentration (Giampietro et al. 1980). However, it has been demonstrated that the decrease in plasma glucose concentration due to freezing, is no greater after one month than one day, and is not prevented by the addition of fluoride to the samples (Clark et al. 1990).

However, the present data shows approximately 10 % higher values for the Cobas Mira Plus analyser than the YSI analyser. Therefore the disparity observed between the two methods may in fact be an underestimation of the true difference. Further support for the notion that the difference is related to the analytical method employed rather than differences in sample collection and storage, is supplied by the quality control data. The glucose concentration in the freshly prepared control sera was consistently 8 % higher when measured using the Cobas Mira Plus rather than the YSI analyser.

The plasma glucose concentrations measured by the Cobas Mira Plus were consistently 10 % higher than the YSI analyser throughout the experimental period. Indeed there was no interaction effect (method of analysis x time) was demonstrated using a two-way analysis of variance (Fig. 2), confirming that the data was qualitatively identical, although the plasma glucose concentrations were quantitatively different.

It is evident from the present work that the use of correlation as a technique for assessing agreement between two methods is inappropriate. This is highlighted by the fact that, in spite of a lack of agreement using the Bland and Altman method between the Cobas and YSI methods for analysing plasma glucose, a correlation coefficient of > 0.99 was demonstrated. The correlation coefficient is able to detect qualitative

differences, but in the present context was unable to detect quantitative differences between methods. This suggests that reliance upon such statistical techniques is inadequate. Bland and Altman (1986) have proposed more appropriate statistical techniques when making comparisons.

In agreement with previous findings (Holtkamp et al. 1975), plasma glucose concentrations were consistently 14 % higher than blood glucose concentrations. The difference between plasma and blood concentrations remained constant for the range of glucose values observed in the present work, suggesting that the proportion of glucose transported in plasma and erythrocytes remains consistent through the physiological range of glucose concentration.

Haemoglobin, the main constituent of erythrocytes, has a negative charge and thus attracts a greater number of H^+ ions than plasma. This results in a strong ionic difference between plasma and erythrocytes, also known as the Donnan equilibrium. Lactate is a strong anion, and therefore the greater accumulation of lactate in plasma than in erythrocytes counteracts the ionic difference between plasma and erythrocytes. In the present study, lactate concentrations were 38 % (0.58 (0.10 mmol/L) higher in plasma than in blood throughout the experimental period. Previously, plasma lactate concentrations were reported to be approximately 40 % higher than blood lactate concentrations (Foxdal et al. 1990), indicating an unequal distribution of lactate between plasma and erythrocytes, most probably due to the Donnan equilibrium (Harris et al. 1989).

The levels of agreement between different methods of analysis employed in the present work are generally good, indicating both accuracy and precision. This statement holds true for all comparisons except for the comparison of plasma glucose measured on the YSI analyser and the Cobas Mira Plus analyser. The reason for the disparity between the two methods of plasma glucose determination remains unresolved. However, it is evident that one cannot assume data derived using different analytical techniques to be quantitatively comparable.

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Figure Legends

Fig 1.

Bland-Altman plot of plasma glucose concentration measured by the YSI analyser and Cobas Mira Plus analyser. The difference between each pair of measurements is plotted against their mean ($n = 168$). Dotted line indicates the mean difference between the two methods (0.47 mmol/L).

Fig 2.

Plasma glucose concentrations measured by YSI analyser and Cobas Mira Plus analyser. Shaded blocks represent exercise periods at 70% VO_{2max} .

Fig 3.

Bland-Altman plot of plasma lactate concentration measured by the YSI analyser and Cobas Mira Plus analyser. The difference between each pair of measurements is plotted against their mean ($n = 168$). Dotted line indicates the mean difference between the two methods (0.01 mmol/L).

Fig 4.

Plasma lactate concentrations measured by YSI analyser and Cobas Mira Plus analyser. Shaded blocks represent exercise periods at 70% VO_{2max} .

Fig. 1

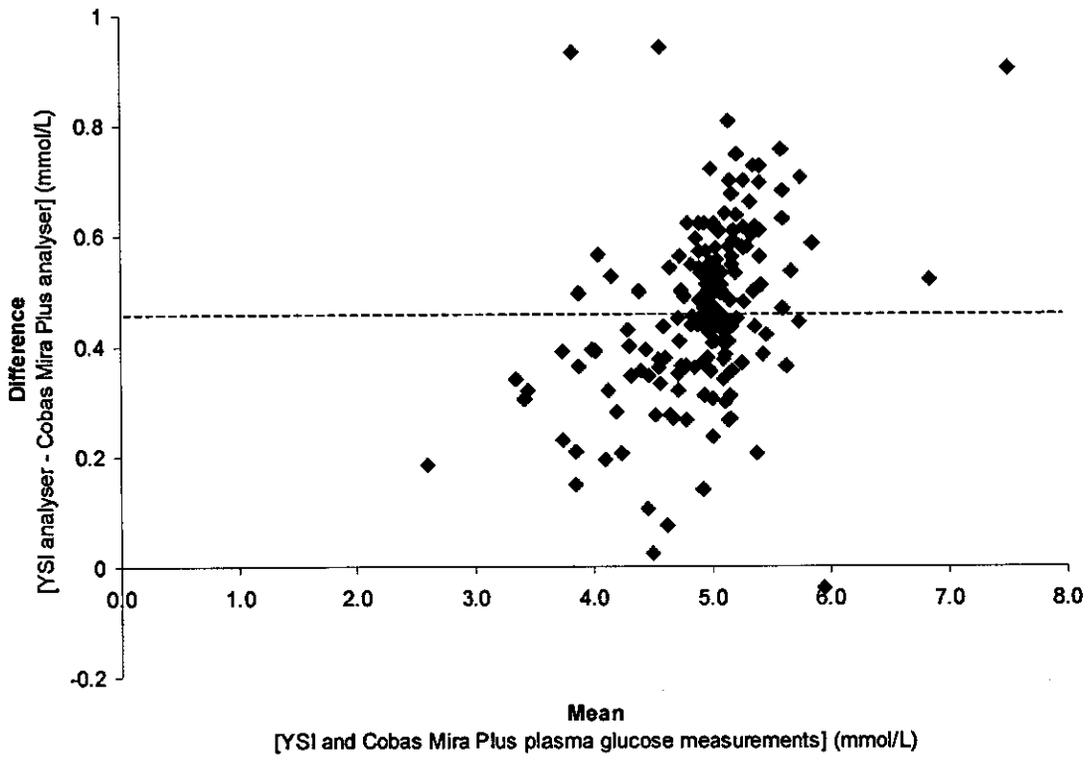


Fig. 3

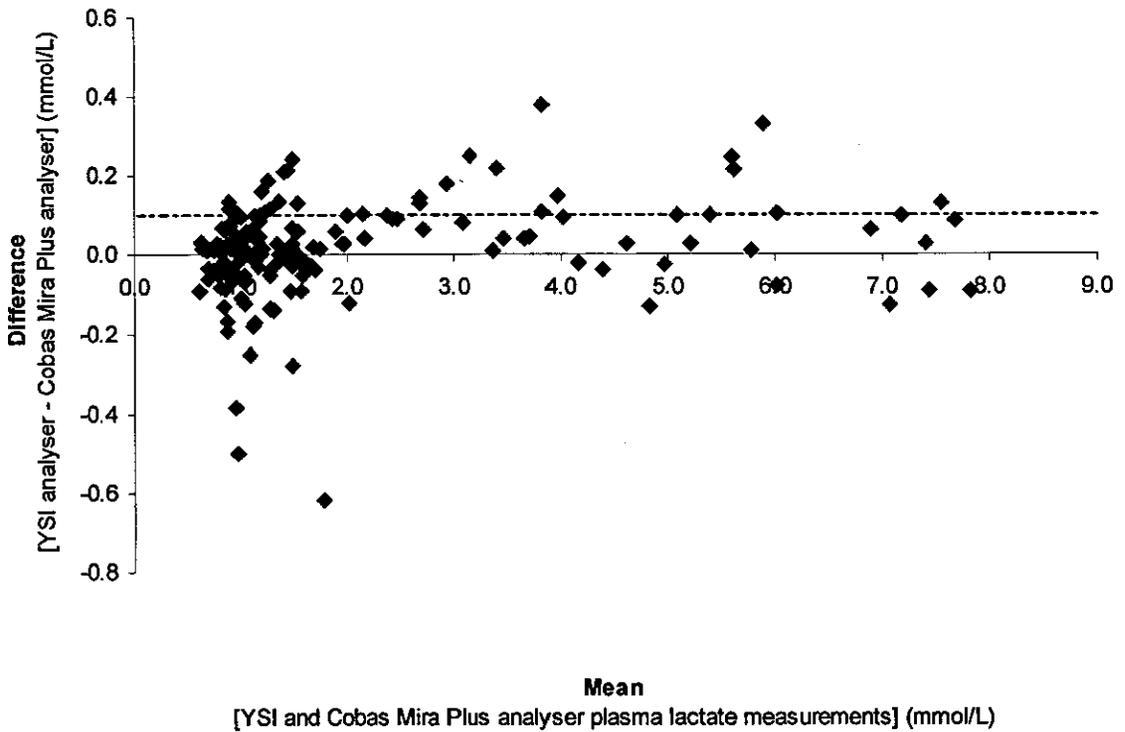


Fig. 2

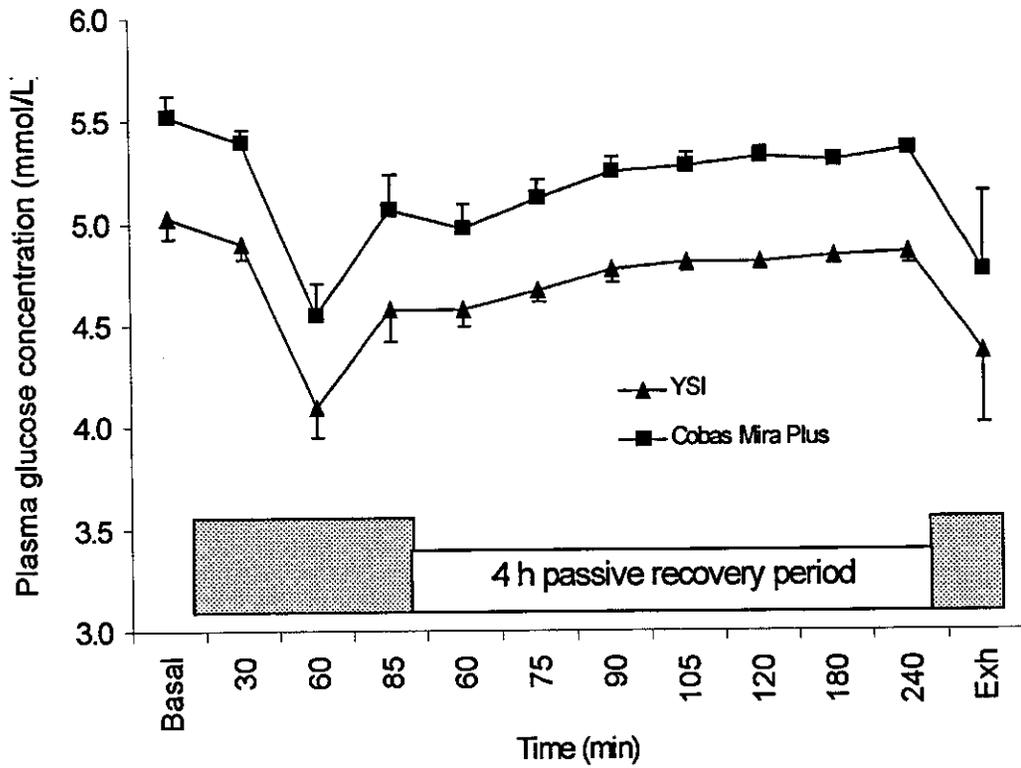


Fig. 4

