The Effect Of Branched-Chain Amino Acid Ingestion on Physical Performance During Prolonged Exercise.

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Abstract

It has been hypothesized that an increase in the ratio of plasma tryptophan (TRP) to branched-chain amino acid (BCAA) concentrations may mediate an increase in cerebral serotonin synthesis, through an increased cerebral tryptophan uptake. It is postulated that the increased brain serotonin content may induce central fatigue during prolonged exercise. Until present, this postulate had not been subject to rigorous scientific testing during prolonged exercise. Therefore the aim of this study was to investigate whether ingesting a BCAA supplement during prolonged exercise improves physical performance and central fatigue. The use of such a supplement during prolonged exercise could then be expected to have a large effect on performance. Eight trained cyclists (VO_2 max = 61.9 ± 4.3 ml O_2/kg/min) ingested, in random order, a drink containing either 10% carbohydrate (CHO), 10% CHO and 0.16% branched-chain amino acid (BCAA) or 0.16% BCAA. Every hour, for the duration of the exercise (4 hours, 55% VO_2 max) blood samples were analysed for amino acids, ammonia, free fatty acids, glycerol, glucose and insulin concentrations. Urine was analysed for urea and creatinine concentrations. Heart rate, oxygen consumption (VO_2), respiratory exchange ratio (RER) and rating of perceived exertion were also analysed. Thereafter, subject's 40km time trial performance and RPE was assessed on a Velodyne windtrainer. Central fatigue following the time trial was quantified using the Sternberg reaction-time paradigm. The serum concentration of the BCAA's declined as a result of the exercise, in the BCAA only trial. Tryptophan concentration, however, did not change during the exercise. The serum TRP:BCAA ratio increased (0.16 ± 0.06 to 0.20 ± 0.10; $p \leq 0.05$) in the CHO trial only. The BCAA trial differed from the two trials in which CHO was ingested because plasma ammonia and glucose concentrations did not increase, while free fatty acids (FFA's) and glycerol concentrations increased significantly ($p \leq 0.05$). The lower RER in the BCAA trials suggests a higher proportion of fat was oxidised in these trials, compared to the other two trials. Cycling performance, over a 40km time trial, (CHO = 68.59 ± 6.02; CHO + BCAA = 68.00 ± 3.01; BCAA = 69.43 ± 5.35 min/sec), ratings of perceived exertion, submaximal or maximal heart rates, and mental performance were not different between trials. Data from this study appears to refute the thesis hypothesis that an increase in serum TRP:BCAA decreases physical performance and central fatigue, during prolonged exercise.

Declaration

I declare that this dissertation is my own unaided work, it is being submitted for the degree of Master of Science, MSc (MED) Exercise Science, to the University of Cape Town, Medical School, Cape Town. Neither the whole work or any part therefore has been submitted before for any degree or examination in this or in any other university.

____

_____________ day of _____________, 1996.

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List of Abbreviations

° C	degrees Celsius
5-HIAA	5-hydroxyindoleacetic acid
ANOVA	analysis of variance
BCAA	branched-chain amino acids
BCKA	branched-chain keto-acids
CHO	carbohydrate
CoA	Co-enzyme A
EDTA	ethylenediamine tetraacetate
et al.	and others
FFA	free fatty acids
Fig	figure
GC	gas chromatography
Hb	Haemoglobin
Hct	Haematocrit
HPLC	high performance liquid chromatography
in vivo	in the body
kJ	kilojoule
k_m	affinity constant
km	kilometer(s)
LNAA	large neutral amino acid
MCE	mercaptoethanol
OPA	*o*-pthaldialdehyde
PEP	phosphoenolpyruvate
PNC	purine nucleotide cycle
psi	pounds per square inch
RER	respiratory exchange ratio
RPE	rate of perceived exertion
SE	standard error
TCA	tricarboxylic acid
TRP	tryptophan
TRP:BCAA	serum tryptophan to branched-chain amino acid ratio
TT	time trial
viz.	namely
VO_2	oxygen consumption
VO_2 max	maximal oxygen consumption
vol.	volume

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Chapter 1 - Motivation and Aims of the Study

1.1 Introduction:

For many years physiologists have used physical exercise as a model to study many perturbations of homeostasis. Fatigue following prolonged exercise, in particular, has enjoyed much attention as it serves as a model to investigate many physiological mechanisms. Fatigue is a complex phenomenon, and is defined physiologically as the inability to maintain power output. Fatigue may also occur, however, as a result of trauma and its aetiology is therefore as important to the clinician as it is to the elite athlete (Newsholme *et al.*, 1992).

The inability to maintain a particular power output over time, results from continuous muscle movement during prolonged endurance exercise. This phenomenon, known as fatigue, may be initiated by any one of a number of different processes, either central or peripheral, and may result in a decline in the maximal velocity of muscle contraction, a reduction in peak isometric force, tremor or in an inability to sustain an expected submaximal force or velocity (Green, 1987).

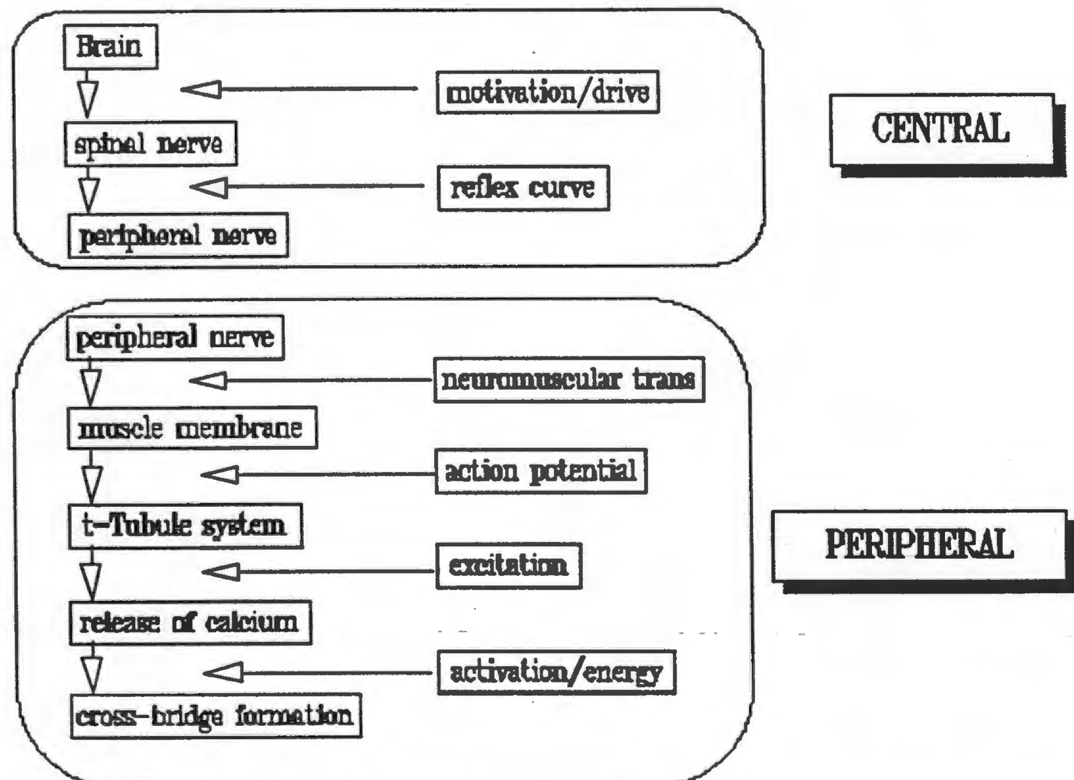


FIGURE 1.1 *Current summary of the proposed "command chain" initiating muscular contraction in man and the possible sites at which fatigue may occur (from Edwards, p3, 1981). (neuromuscular trans = neuromuscular transmission).*

The aetiology of fatigue has long been perceived as a failure somewhere in the 'command chain' that results in a muscle contraction. This 'command chain' is initiated in the brain where the subconscious decision to produce a muscle contraction is made, and it ends with the production of force, as a result of cross bridge interaction in the myofibrils of the muscle recruited (Edwards, 1983, Fig 1.1). Figure 1.1 illustrates that centrally, fatigue may occur either from a decline in motivation or drive, occurring in the brain, or from a decrease in the activity of spinal reflexes. Peripheral fatigue occurs in the muscle, and one of four possible mechanisms have been implicated in its production: i) a decline in neuromuscular transmission, between nerves and muscle, ii) a reduced activity of action potentials at the T-tubules, iii) decreased responsiveness of the T-tubules to excitation, or iv) failure of released calcium to produce muscular contraction. It has been suggested that the initiation of fatigue may be a protective mechanism, preventing irreparable muscle damage (Noakes, 1988).

Green (1987) suggests that in certain instances fatigue may be initiated in the brain itself or within the central nervous system. In this case fatigue is brought about by a modification in supraspinal centre output, either by neurally mediated afferent feedback from muscle or by the passage of blood-borne substances across the blood brain barrier (Green, 1987).

In accordance with this it has recently been proposed that an alteration in blood amino acid concentration may cause fatigue (Parry-Billings *et al.*, 1990, Blomstrand *et al.*, 1991; Bailey *et al.*, 1992; Wilson and Maughan, 1992). These authors propose that the decline in plasma branched-chain amino acids (BCAA) during prolonged exercise, leads to an alteration in the tryptophan (TRP) to BCAA ratio (TRP:BCAA). As a result of this alteration in TRP:BCAA, additional tryptophan is transported into the brain through the blood-brain barrier (Yuwiler *et al.*, 1977), and the increased cerebral tryptophan is then hydroxylated and decarboxylated into serotonin (Fernstrom, 1974; Blomstrand *et al.*, 1991). Consequently, serotonin content in certain brain neurones increases (Parry- Billings *et al.*, 1990; Newsholme *et al.*, 1991), and it is this increased serotonin concentration that is thought to increase the athlete's perception of effort (Newsholme *et al.*, 1991), and thereby impair performance during prolonged exercise.

It is further believed that the increased serotonin concentration may also induce mental fatigue, decreasing the ability to concentrate. This hypothesis has already been described in the lay press (Pine, 1983; Berg, 1983), and as a result athletes are

aware of the possibility that diet may have profound effects on behaviours as varied as mood, sleep, physical and mental performance.

The theory, that an increased serum TRP:BCAA, and an increased cerebral serotonin concentration might cause fatigue has, however, not been subject to rigorous scientific testing. To date studies investigating this hypothesis have either been conducted in the field, where stringent controls were not possible (Blomstrand *et al.*,1991) or in the laboratory where serotonin concentrations were pharmacologically altered (Bailey *et al*,. 1992; Wilson and Maughan, 1992; Bailey *et al.*, 1993).

Many questions are, however, still unanswered and we therefore felt that a carefully controlled laboratory investigation was required to further investigate the hypothesis (For a complete description of these issues see section 2.3)

1.2 Aims of the thesis.

The following study was designed to answer the following questions:

i) Do serum BCAA concentrations decrease during prolonged endurance exercise as has been previously described in the literature?
ii) Does the ingestion of a BCAA supplement during prolonged exercise prevent the decrease in serum BCAA concentration?
iii) Does the maintenance of the serum TRP:BCAA during prolonged exercise influence either physical or mental performance?

Another important component of this study was to develop the techniques necessary to rapidly and accurately analyse serum for amino acid concentrations, using high performance liquid chromatography (HPLC).

1.3 Thesis hypothesis.

In this thesis the following hypothesis was tested: During prolonged submaximal exercise serum BCAA concentrations decrease, causing an increase in serum TRP:BCAA, which decreases power output and mental alertness. Ingesting a BCAA supplement during the prolonged exercise maintains the serum TRP:BCAA, power output and mental alertness, as measured by a 40 km time trial and by the Sternberg reaction-time paradigm.

Chapter 2- Literature Review

The following review describes the evolution of the hypothesis and pertinent amino acid chemistry. This is followed by a description of alterations in plasma/serum TRP:BCAA concentrations induced by diet and exercise, and finally those studies that have specifically addressed the hypothesis will be described.

2.1 Amino Acids & Serotonin:

2.1.1 Introduction.

Twenty amino acids are common, and may be grouped according to charge and polarity. The variable component (R group) of an amino acid confers certain characteristics to the molecule, and it is on the basis of this R group that the amino acid is classified (see Table 2.1). When the R group ionises, the amino acid is either basic (if positively charged), or acidic (if negatively charged). The ionic and polar nature of amino acids is important as it provides us with the ability to separate these molecules using high performance liquid chromatography (HPLC). This will become relevant in chapter 3 when the technique of the HPLC system is discussed.

Branched-chain amino acids

The BCAA's (valine, isoleucine and leucine) are three of ten essential amino acids that cannot be synthesised by the human body, and are therefore required in the diet. Metabolism of the BCAA's is unique among amino acids and particular interest has been paid to the possibility that:

i) Branched-chain amino acids, through competition with the large neutral amino acids (especially tryptophan) for transport across the blood-brain barrier, appear to play a role in controlling brain amino acid concentrations and thereby the synthesis of amino acid-derived neurotransmitters, such as serotonin, dopamine and norepinephrine (Pardridge, 1977),
ii) The initial enzymes in the catabolism of BCAA's are unregulated, that is, reaction kinetics are governed purely by enzyme and substrate concentrations (Harper *et al.*, 1984), and
iii) The BCAA's appear to exert specific regulatory effects on tissue protein degradation and synthesis which may have clinical relevance (Harper *et al.*, 1984).

Biochemistry of tryptophan

The aromatic amino acid tryptophan, like the BCAA's, is essential and cannot be synthesised by the human body. In addition, tryptophan is the least abundant of all amino acids found in food. At rest, some 80% (McMenamy and Oncley, 1958; Fernstrom & Wurtman, 1974b) to 90% (Chaouloff, 1991) of the plasma tryptophan is found bound to the plasma protein albumin. The hydroxylation and decarboxylation of tryptophan in the brain leads to the production of 5-hydroxytryptamine (serotonin).

TABLE 2.1 *The essential amino acids and their classification.*

Amino acid	Classification
1) Valine	Branched-chain
2) Leucine	Branched-chain
3) Isoleucine	Branched-chain
4) Threonine	Hydroxyl
5) Methionine	Sulphur
6) Phenylalanine	Aromatic
7) Tryptophan	Aromatic
8) Histidine	Basic
9) Lysine	Imino
10)Arginine	Basic

Biochemistry of serotonin.

Serotonin is a ubiquitous neurotransmitter found in the certain brain neurones (hypothalamus and basal ganglia). It has been implicated in a number of physiological and behavioural processes in vertebrates, but does not appear to be essential for any of them (Jacobs and Azmitia, 1992). This is explained by the fact the expansive system of serotonergic neurones exert a tonic modulatory effect on its widespread targets.

REGULATION OF SEROTONIN

It appears that there are at least two pools of serotonin within the pre-synaptic terminals (Aprison and Hingten, 1972). One is firmly bound and increases directly through synthesis of this amine. The second is a smaller labile-bound pool which directly affects the amount of transmitter liberated in the synaptic junction or deaminated by monoamine oxidase in the mitochondria. As the amine is released by the labile-bound pool, it exerts its effect as a free pool, until it is inactivated through diffusion and uptake into the pre-synaptic terminal or the post-synaptic terminal or is desensitised. The physiologically effective serotonin pool is the sum of both the free and labile-bound pools. The predominant action of serotonin is related to motor activity and to the wake-sleep-arousal cycle of vertebrates (Jacobs and Azmitia, 1992).

THE ROLE OF SEROTONIN IN SLEEP

Serotonin plays an important role in the initiation of sleep in experimental animals. Studies have illustrated how the loss of serotonin in the brain, either by ablation of the raphe nuclei or by administration of serotonin receptor blockers, results in the loss of slow wave sleep time, which is proportional to the decline in serotonin concentrations (Aprison and Hingten, 1972). Ashley and Leathwood (1983) hypothesised that a 500mg dose of tryptophan in humans, which increased the TRP:BCAA was sufficient to alter brain serotonin concentrations. The influence this dose of tryptophan had on the nature of sleep patterns was investigated in humans (Pollet and Leathwood, 1983). Despite the superficiality of this study, with subjective scores assigned to the night's sleep these authors demonstrated a decreased latency and a better night's sleep following the ingestion of tryptophan. Young (1986) has stated that an increase in the serum concentration of serotonin produces a feeling of tiredness and sleepiness in both man and experimental animals. This is supported by Wurtman (1983), who has shown that the ingestion of a single dose of tryptophan significantly increased self reported levels of fatigue/inertia and decreased levels of vigour/activity. On the basis of these observations Newsholme *et al.* (1991) suggested that an increase in the concentration of serotonin, in certain areas of the brain, could increase the sensitivity of the brain to fatigue. It follows therefore that any process resulting in an increased cerebral serotonin concentration could induce central fatigue.

2.1.2 Plasma amino acid concentrations.

In the present study serum amino aids concentrations were determined, while most other authors refer to plasma concentrations.

Resting plasma amino acid concentrations reflect the balance between the utilisation of amino acids and their input from:

i) Release of dietary amino acids from ingested proteins from the hepatic portal system into the systemic blood supply,
ii) Amino acid release from, and protein turnover, in tissues and
iii) The uptake of amino acids from the blood to the tissues where they are used for protein synthesis, or deaminated to urea in the liver (Harper, 1968).

Total plasma amino concentrations vary rhythmically during the course of a 24 hour day (Feign *et al.*, 1967). The mechanism for this rhythmicity is unclear, but is

probably related to changes in amino acid pool sizes as a result of dietary and/or hormonal changes (Wurtman *et al.* 1968).

Changes in BCAA concentration

Under normal conditions plasma amino acid concentrations in humans tend to be proportional to protein intake (Frame, 1958), and the nutritive value of an ingested protein depends upon its absorption, in the form of amino acids. While ingested plasma amino acid concentrations may be ten-fold greater than the amounts of amino acid present in the extracellular space, plasma amino acid concentrations fluctuate by only 50% (Scriver *et al.*, 1971). The authors suggest that liver and skeletal muscle play a central role in the maintenance of constant plasma amino acid concentrations.

Of the total amino acid release from extrahepatic tissues, 50% is alanine and glutamine (Harper *et al.*, 1984). This alanine forms the primary vehicle of amino acid nitrogen (NH_2) transport from peripheral tissues to the liver (Scriver *et al.*, 1971). In the liver, this amino acid is deaminated to form urea and the resultant pyruvate is converted to glucose via gluconeogenesis, in the muscle (Goldstein and Newsholme, 1976).

Regulation of BCAA metabolism

Following a meal high in protein plasma BCAA concentrations increase and tend to remain high while other amino acids fall to control levels after the increased protein intake. This suggests that the BCAA degrading enzymes do not adapt to the high BCAA levels and that their capacity for oxidising BCAA's is exceeded during periods of increased protein intake. Calculations by Harper *et al.* (1984) however suggest that it is unlikely that these enzymes become limiting. For example, BCAA intake of a 150g rat increases by 5000 μmol when the dietary protein intake increases by 20 to 30%, over standard dietary levels, yet the increase in the body's BCAA pool is only 100 μmol. These authors suggest that some factor, other than a limited oxidative capacity is responsible for the elevated BCAA pools in rats adapted to a high protein intake.

Insulin appears to play an important role in plasma BCAA regulation. Intravenous insulin infusion stimulates leucine uptake and oxidation by peripheral muscles.

Amburand *et al.* (1982) found that insulin infusions elevated plasma BCAA concentrations in dogs moderately starved for up to two weeks. Lowered circulating

insulin and higher glucagon concentrations, seen in starvation, produces a decrease in plasma BCAA concentration. Periods of starvation can also alter the rate of protein degradation, resulting in higher BCAA levels (Pozefsky *et al.*, 1976).

Changes in tryptophan and serotonin concentrations

In contrast to the BCAA's, tryptophan concentrations are less likely to fluctuate as this amino acid is not taken up into skeletal muscle (Harper *et al.*, 1984), not even under the influence of insulin (Fernstrom and Wurtman, 1974a). Plasma tryptophan concentrations show a circadian rhythm, with the peak at midnight and the nadir in the early morning, and brain serotonin concentrations exhibit similar, but delayed rhythms (Fernstrom and Wurtman, 1974b).

Brain serotonin concentrations are also influenced by meal content (Fernstrom, 1974; Berg, 1983), through changes in plasma tryptophan. While a meal high in carbohydrate has no effect on total plasma tryptophan, but increases cerebral serotonin, a high protein meal decreases both plasma and cerebral serotonin concentration (Fernstrom and Wurtman, 1972; Fernstrom, 1974; Pérez-Cruet *et al.*, 1974; Wurtman, 1983). The insulin released following a high carbohydrate meal increases tissue absorption of most amino acids, including the BCAA's, but as insulin does not increase muscle absorption of tryptophan, the total plasma tryptophan content does not change. The resultant decrease in plasma BCAA concentration effects an increase in tryptophan transport into the brain and an acceleration of brain serotonin synthesis. However, the opposite occurs following a protein meal, where plasma BCAA concentrations increase, thereby decreasing the transport of tryptophan into the brain for serotonin synthesis (see amino acid transport - section 2.15). Hence, cerebral serotonin content mirrors changes seen in plasma tryptophan concentrations.

2.1.3 Amino acid transport across cell membranes

Amino acids are actively transported across the cell membranes of most tissues via carriers. These protein macromolecules, which extend across the entire membrane, are specific for the different classes of amino acids. Active transport of amino acids from the mucosal to the serosal side of enterocytes occurs in the small intestine (Larsen *et al.*, 1964; Nixon and Mawer, 1970). Active transport also occurs in muscle and the brain

Intestine

In the small intestine 5 distinct amino acid transport systems have now been identified for neutral, basic, acidic, iminoglycine and n-methylated (for example, sarcosine and betaine) amino acids. Each depends on the sodium concentration gradient, from the extracellular to the intracellular fluid space, to 'drive' the uphill transport of amino acids against their concentration gradient (Adibi, 1970). Similar carriers occur in the blood-brain barrier, but the affinity of the carriers for amino acids is between 10 and 20 times higher than the intestinal carriers.

Muscle

Very little work has been conducted on the kinetics of amino acid uptake into muscle. Pardridge (1977), however, has shown that the muscle amino acid carriers are similar to those in the gut, that is, low affinity, high capacity.

Brain

At the blood-brain barrier an amino acid is transported by one of three carrier systems. Each carrier protein has a specific affinity for either neutral, basic or acidic amino acids. Large neutral amino acids are transported by the 'L system' carrier, and uptake is stereospecific, with the L-enantiomer having the greatest affinity (Oldendorf, 1973). This carrier is in equilibrium, that is, it does not concentrate the amino acids against a concentration gradient and is sodium independent. The k_m values approximate plasma levels, which means that the rate of neutral amino acid transport across the blood-brain barrier is likely to be sensitive to changes in plasma amino acid concentration (Harper *et al.*, 1984). Phenylalanine, tyrosine, leucine, isoleucine, methionine, tryptophan, histidine, valine, threonine, cysteine, glutamine, asparagine and serine are transported by this carrier. Newsholme and Leech (1984) have shown that the L-system carrier has the highest affinity for leucine, isoleucine, valine, phenylalanine and tyrosine. The other two basic and acidic amino acid carriers show no affinity for these amino acids (Oldendorf and Szabo, 1976).

Transport of tryptophan across the blood-brain barrier

Tryptophan transport into the brain depends on the extent to which it is bound to plasma albumin (McMenamy, 1965), and this has important consequences in altering tryptophan's transport. The existence of this "albumin-bound" reservoir of tryptophan in the bloodstream gives this amino acid a competitive advantage over other neutral amino acids with respect to transport from the blood stream into the

brain (Fernstrom & Wurtman, 1974a). For example, following carbohydrate ingestion the plasma insulin concentration increases and promotes the absorption of all amino acids, except tryptophan, into peripheral tissues. As a result of this phenomenon the competition between tryptophan and the other neutral amino acids for transport into the brain is decreased and that is thought to increase the concentration of cerebral tryptophan and serotonin synthesis.

Competition for transport

Competition exists between the BCAA's and tryptophan, at the blood-brain barrier (Fig 2.1). It is still debated though, whether it is the free or total plasma tryptophan concentration that determines its transport into the brain.

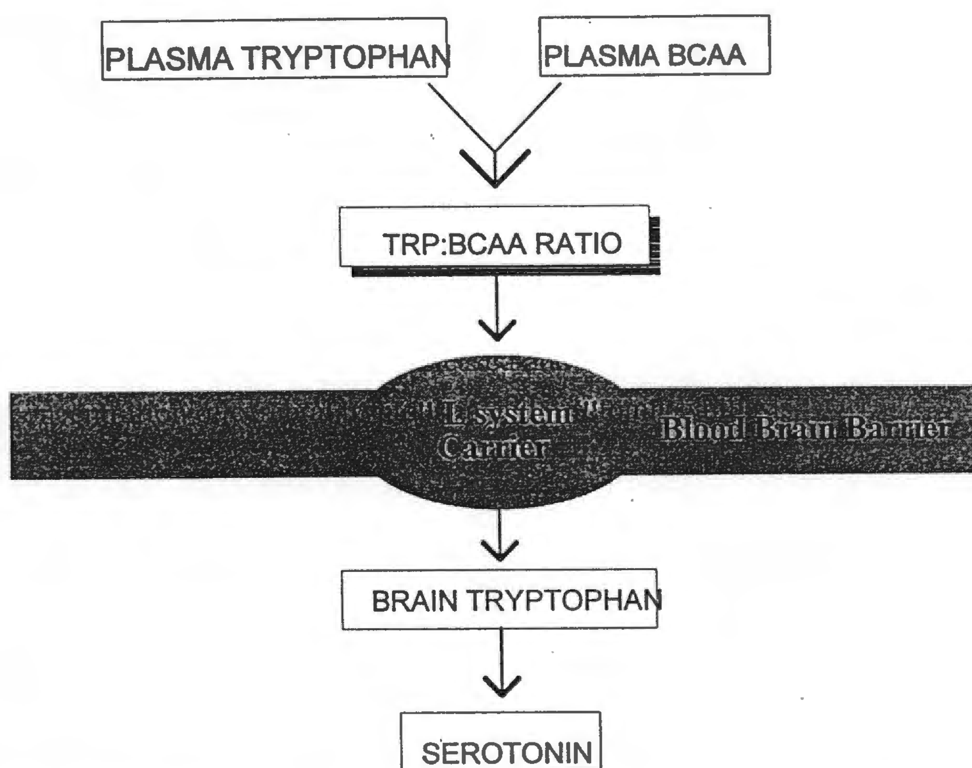


FIGURE 2.1 *Competition exists at the level of the blood-brain barrier between the BCAA's and tryptophan for entry into the brain. Any tryptophan transported into the brain is converted into serotonin in a dose-dependent manner. Any mechanism altering serum TRP:BCAA is thought to influence the cerebral serotonin concentration (based on Yuwiler et al., 1977).*

Chaouloff *et al.* (1986) showed in rats running for two hours at 20 m/min, that although total plasma tryptophan concentration did not change, plasma free tryptophan concentration increased as a result of competition with FFA for albumin binding sites. Nor was there any difference in the concentrations of the other large neutral amino acids (LNAA). Brain tryptophan concentration, however, increased by

80% implying that free, rather than total plasma tryptophan concentration, dictates the change in cerebral tryptophan concentration. Similar changes have been described by Knott and Curzon (1972), for changes in plasma free and total tryptophan concentrations. The findings of Knott and Curzon (1972) were disputed, however, by Fernstrom *et al.* (1976), who succeeded in altering cerebral tryptophan concentrations through dietary manipulation. Specifically, rats were fed either carbohydrate and fat (CHO + FAT) or merely carbohydrate (CHO). There was no difference in brain tryptophan concentrations, even though those rats which ate CHO + FAT had 2½ times higher plasma free tryptophan concentrations than rats that just ate CHO. The total correlation between brain tryptophan and plasma free tryptophan concentrations ($r=0.23$) was significantly lower than the correlation between brain tryptophan and total plasma tryptophan concentrations ($r=0.87$). The authors therefore concluded that it is the total rather than the free plasma tryptophan concentration that determines brain tryptophan concentrations. It is further postulated that the L-system carriers may possess a higher affinity for plasma tryptophan than tryptophan has for albumin and tryptophan is stripped from albumin as it passes through the cerebral circulation (Fernstrom *et al*,. 1976; Yuwiler *et al.*, 1977). It would appear from the conflicting literature that both free and total tryptophan concentrations influence tryptophan's transport into the brain, and total plasma tryptophan can, therefore, influence cerebral serotonin concentrations.

Summary

Amino acid transport relies upon the presence of a transport macromolecule to effect its passage across membranes. Competition between the large neutral amino acids (LNAA), most notably tryptophan, valine, isoleucine and leucine, affects the concentration of these amino acids reaching the brain. Certain conditions result in a decline in BCAA concentrations, thereby increasing the amount of tryptophan entering the brain. The increased cerebral tryptophan concentration leads to an increased cerebral serotonin production.

2.1.4 Metabolism of amino acids in muscle.

Upon being taken up into the muscle BCAA's are either used for protein synthesis or deaminated to yield branched-chain keto-acids (BCKA) and glutamate. BCKA's are then oxidatively decarboxylated in the tricarboxylic acid cycle (fig 2.2).

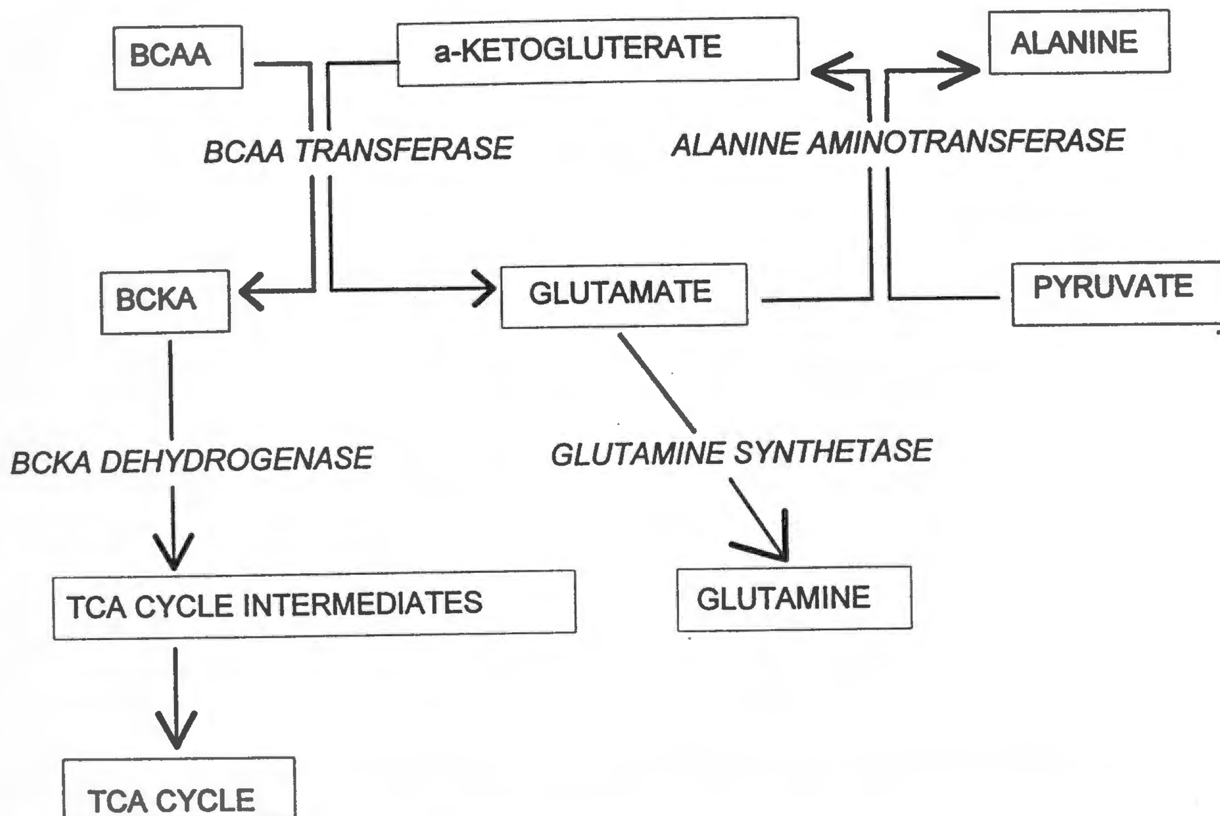


FIGURE 2.2 *The breakdown of amino acids to alanine and the corresponding keto-acids. Alanine is converted into glucose in the liver, and the keto-acids into energy by the tricarboxylic cycle.*

BCAA transferase

BCAA transferase is the enzyme that catalyses the conversion of leucine, isoleucine and valine into -ketoisocaproate, -ketomethylvalerate and -ketoisovalerate, respectively (Harper *et al.*, 1984).

THE REGULATION OF BCAA TRANSFERASES

The rate of transamination is dependent only upon the concentrations of enzyme and substrate. Because BCAA transferase has a relatively high k_m for valine, compared to isoleucine and leucine, valine is converted into its BCKA less rapidly than either leucine or isoleucine (Pardridge, 1977). Transamination rates may be reduced by a build-up of BCKA's, which occurs, for example, during metabolic acidosis where a decreased flux of BCKA from the mitochondria is observed (Block and Buse, 1990). Metabolic acidosis occurs in exercise which can influence the rate at which BCAA's are oxidised.

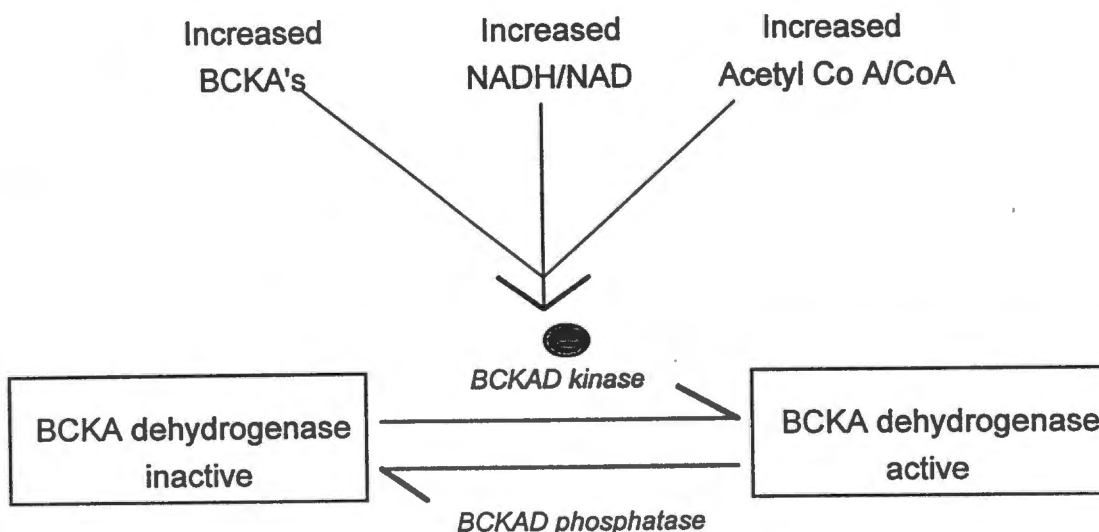


FIGURE 2.3 *Regulation of the BCKA dehydrogenase enzyme, by phosphorylation/dephosphorylation* (Shimomura *et al.*, 1990).

BCKA dehydrogenase

The BCKA's are first oxidatively decarboxylated by BCKA dehydrogenase, a multi-enzyme complex located on the inner surface of the inner mitochondrial membrane. This enzyme complex is similar to the pyruvate dehydrogenase enzyme (Block and Buse, 1990), and is the rate-limiting step in BCAA metabolism (Harper *et al.*, 1984; Block and Buse, 1990).

REGULATION

Like pyruvate dehydrogenase , BCKA dehydrogenase is tightly regulated through a reversible phosphorylation (deactivation) - dephosphorylation (activation) mechanism, allowing for fine control in enzyme activity (fig 2.3). Activity of the enzyme complex is increased during exercise (Shimomura *et al.*, 1990; Wagenmakers *et al.*, 1991) and during periods of net protein breakdown, resulting from increased glucocorticoid activity (Block and Buse, 1990), and decreased with carbohydrate loading prior to exercise (Wagenmakers *et al.*, 1991).

Factors affecting the in *vivo* activity of the enzyme (see fig 2.3) may include the NADH/NAD, acetyl CoA/CoA ratios and keto-acid substrate availability. An increase in the NADH/NAD and acetyl CoA/CoA ratios inhibits BCKA activity by I) decreasing activation of the enzyme, and ii) by slowing the active BCKA dehydrogenase enzyme (Shimomura *et al.*, 1990).

2.1.5 Tryptophan and serotonin metabolism.

Unlike BCAA's, tryptophan is not metabolised in skeletal muscle, but it does enter the brain (Yuwiler *et al.*, 1977). In the brain tryptophan is first hydroxylated to form 5-hydroxytryptophan, and then decarboxylated to produce 5-hydroxytryptamine or serotonin. Tryptophan hydroxylation is unsaturated at physiological levels, the reaction rate being controlled by substrate availability (Fernstrom, 1974), and therefore any change in the plasma tryptophan concentration will be reflected in the cerebral serotonin concentration (Pardridge, 1977). Intravenous injections of tryptophan rapidly increase plasma and brain tryptophan concentrations and brain serotonin levels (Segura and Ventura, 1988).

The principle metabolite of serotonin is 5-hydroxyindoleacetic acid (5-HIAA). This breakdown product is formed in a 2 step process involving a deamination by monoamine oxidase to yield an aldehyde intermediate, and then an oxidation to 5-HIAA through the action of aldehyde dehydrogenase.

Serotonin receptors

Receptors for serotonin each have their own unique pharmacological, behavioural and anatomic profiles (Jacobs and Azmitia, 1992). Serotonin receptors belong to three main families, viz. 5-HT_1, 5-HT_2 and 5-HT_3, and within each a number of subgroups exists.

All three receptor families are present to differing extents in the brain. The 5-HT_{1A} receptor is the neuronal cell body autoreceptor and is responsible for some of the post-synaptic actions of serotonin, including the descending pain modulating reticulo spinal tracts. The 5-HT_{1D} receptor is the axon terminal autoreceptor and is presumed to be involved in the inhibition of further release of serotonin from axon terminals. The 5-HT_2 family is post-synaptic, and is responsible for many of the behavioural effects of serotonin release and has been shown to be involved in the action of the major hallucinogenic drugs. Finally 5-HT_3 receptors are found in the limbic areas where they may be involved in anxiety and psychotic reactions (Jacobs and Azmitia, 1992).

The early literature on the post-synaptic effects of serotonin is frequently contradictory. Investigators initially expected to find a primary action of serotonin in the central nervous system, either excitatory (increasing the activity of postsynaptic

target neurones) or inhibitory (decreasing target neuron activity), this however, was simplistic and now a more consistent and detailed picture is emerging.

SEROTONIN'S INHIBITORY ROLE

The primary action of the serotonergic nervous system, particularly in the fore-brain is inhibitory, predominately mediated via the $5\text{-}HT_{1A}$ receptor. Serotonin itself may also auto inhibit further serotonin release via the $5\text{-}HT_{1D}$ receptors (Jacobs and Azmitia, 1992).

SEROTONIN'S EXCITATORY ROLE.

Excitation occurs predominantly in the brainstem and spinal cord. Excitation involves mostly motorneuron activation and the effect of serotonin is to amplify the responsiveness of target cells to incoming stimuli. Pharmacological analyses have identified the $5\text{-}HT_2$ receptors as being responsible for these effects (Jacobs and Azmitia, 1992).

Changes in brain serotonin content

In humans, alterations in brain serotonin content during exercise cannot be assessed, for the following reasons:

i) Serotonin metabolites in the blood are a poor predictor of brain serotonin synthesis as 90% of all serotonin is formed in the gut and, for the same reasons,
ii) Plasma 5-hydroxyindole acetic acid (5-HIAA) concentrations have been shown to be an unreliable predictor of brain serotonin turnover (Fernstrom, 1974), and while cerebrospinal 5-HIAA may be a better predictor of central nervous system activity than plasma 5-HIAA, it is not feasible to measure CSF levels in an exercise protocol.

The behavioural and physiological changes resulting from alterations in cerebral serotonin turnover, therefore, have to be extrapolated from studies on animals.

2.2 Plasma amino acid alterations during exercise:

Physical exercise has a profound effect upon plasma, muscle and liver concentrations of amino acids. Following prolonged exercise large amounts of nitrogen are excreted, resulting in a negative nitrogen balance, which implies that catabolism of protein occurs during exercise. In addition, the participation of amino acids in the energetics during exercise is demonstrated through an increased plasma

concentration of ammonia and urea (MacLean et *al.*, 1991) and in an increased concentration of alanine (Brodan *et al.*, 1976) released by active muscle, following the exercise bout. The purpose for this catabolism is at this stage disputed, and the various arguments will be discussed.

Much attention has focused on the effects of exercise on plasma amino acid concentrations (Brodan *et al.*, 1976; Décombaz *et al.*, 1979) and it has been speculated how these changes in plasma amino acids might affect various central nervous system neurotransmitter levels. Unfortunately these studies are difficult to compare as large discrepancies in the effect of exercise on plasma amino acid concentrations have been demonstrated. These differences appear to be related to intensity and duration of the exercise bout. A summary of the effects of exercise on plasma amino acid concentrations in humans can be seen in Table 2.2 (pg. 19).

Effects of duration & intensity of exercise on plasma amino acid concentrations

Okamura *et al.* (1987) investigated the effects of high intensity exercise on plasma amino acid concentration in rats. In spite of an increase in the plasma BCAA concentration, TRP:BCAA also increased after exercise.

Following 20 mins. of low intensity exercise in healthy human subjects both total plasma tryptophan and plasma BCAA concentrations decreased producing a 13% decrease in the TRP:BCAA ratio. Plasma alanine and glutamine also increased significantly over resting values (Brodan *et al.*, 1976). Exhaustive high intensity exercise in the same subjects produced a significant increase in plasma BCAA concentration, while total tryptophan concentrations fell, and the TRP:BCAA ratio did not change. Again both alanine and glutamine concentrations increased significantly, compared to resting levels.

Blomstrand *et al.* (1991) investigated the changes in plasma amino acid concentration during prolonged exercise. In a 30km cross country both the BCAA's and total tryptophan concentrations fell significantly, but the TRP:BCAA ratio increased as BCAA concentrations fell more than the tryptophan concentration (see table 2.2). Similar results were obtained by the same group in two studies, with subjects completing 42.2km (Blomstrand *et al.*, 1988;1991) where the TRP:BCAA ratio increased significantly. In a similar study Davis *et al.* (1992) exercised subjects at 68% VO_2 max for over three hours. This produced a large increase in plasma free tryptophan, a moderate increase in plasma total tryptophan and no change in BCAA concentrations. As a result the TRP:BCAA increased by 9.8%. Similar results are

achieved following prolonged exercise in rats (Acworth *et al.*, 1986). In both trained and untrained rats the plasma ratio of TRP:BCAA increased, resulting in an increased cerebral tryptophan concentration.

Ultra-endurance exercise, however, has an even more profound effect on the plasma amino acid concentrations. Décombaz and co-workers (1979) found that, following a 100km competitive run, concentration of most amino acids decreases by between 35 and 85% of their resting values. The total BCAA concentration fell by 63%, while the total amino acid content declined by only 17%. However, as plasma tryptophan was not measured it is not possible to calculate the change in the TRP:BCAA ratio. These authors also found that the urinary excretion of all amino acids was lowered after the race, mediated through a reduced renal clearance.

The decline of the plasma BCAA concentration is possibly linked to the fact that the activity of the BCKA dehydrogenase enzyme is enhanced during exercise (Hood and Terjung, 1987; Shimomura *et al.*, 1990). This increase in the BCKA dehydrogenase activity has prompted some investigators to suggest that BCAA's might contribute significantly to the energy demands of the organism during periods of starvation or endurance exercise (Dohm *et al.*, 1977) (see section 2.21).

Inconsistencies in the changes of plasma BCAA's following exercise

Despite the evidence demonstrating the decrease in plasma BCAA concentrations during exercise, there are data which disagree. For example, Dohm *et al.*(1981) showed, in rat experiments, that plasma BCAA concentrations following exhaustive exercise (either swimming or running) increases. In addition, Hagg *et al.* (1982) showed that two hours of mild leg exercise in human subjects, failed to produce a change in the plasma BCAA concentrations. The authors explain this by the fact that protein degradation increased and the utilisation of leucine for protein synthesis decreased markedly. They also suggested that these processes occurred at a rate greater than amino acid utilisation, which resulted in an unchanged plasma amino acid concentration (Hagg *et al.* 1982). However, the reasons for these discrepancies are unclear, but they may result from a combination of the following:

- EXERCISE PROTOCOL

It is important to consider the type of exercise involved when making conclusions about the effect of exercise on plasma amino acid concentrations, as plasma BCAA oxidation appears to be linked to the intensity of the exercise. For example, exhaustive exercise at 75% of VO_2 max, for 1½ to 2 hours, in six healthy, young

men failed to decrease the BCAA levels (MacLean *et al.*, 1991), whereas BCAA concentration decreased by 63%, following a 100km run at approximately 60% VO_2 max (Décombaz *et al.*, 1979).

- **TRAINING**

Training, too seems to play a role in the plasma concentrations of amino acids, and their oxidation during exercise. Einspahr and Tharp (1989) investigated the amino acid responses of a highly trained group and a sedentary control group of humans, following a bout of intense exercise. The trained group had higher plasma isoleucine, leucine and tyrosine concentrations at rest, and their plasma alanine concentrations post-exercise were significantly higher those that of the sedentary control group.

Henderson *et al.* (1985) investigated the training effects on leucine oxidation in rats, using constant leucine infusion. Leucine oxidation was 40% higher in the trained group during both exercise and rest. The results show that whole body leucine turnover is increased by training and that leucine oxidation is increased by both training and a single bout of exercise.

Effect of exercise on serum tryptophan concentrations

The influence of exercise on plasma tryptophan concentrations is less clear-cut. This is because exercise impacts differently on free and bound tryptophan. Plasma free tryptophan increases during prolonged exercise, as a result of the increased plasma FFA concentration, which displaces tryptophan bound to albumin (Fernstrom and Wurtman,1974a).

Exercise (running for 1-2 h at 20 m/min) causes time-dependent increases in the plasma free tryptophan concentrations and brain tryptophan concentrations in the rat, and this may lead to an increased metabolism of cerebral serotonin, as suggested by elevated levels of 5-HIAA (Chaouloff *et al.*, 1985).

Effect of exercise on plasma serotonin concentrations

One of the first studies to demonstrate an increase in cerebral serotonin following both acute and chronic exercise, in rats, was conducted in the early '70's (Barchas and Freedman, 1973). More recently other investigators have also demonstrated that brain serotonin content increases following endurance exercise in the rat (Brown *et al.*, 1979; Chaouloff *et al.*, 1985; Acworth *et al.* 1986). These authors measured both plasma and cerebral tryptophan concentrations, and found a positive relationship between brain and plasma tryptophan concentrations. They suggested that plasma tryptophan concentration may serve as an indicator of brain tryptophan concentration.
In humans, the possibility that increases in the concentration of tryptophan in the brain will elevate the level of brain serotonin has to be assumed (Fernstrom & Wurtman, 1971). Support for that assumption comes from the work of Wurtman (1983) that showed that a single dose ingestion of 500mg of tryptophan, in a group of healthy young men, produced a significant increase in their self-reported fatigue/inertia and a decrease in their vigour/activity rating, as measured by the POMS (Profile Of Mood States) test.

USE OF DRUGS TO MODIFY BRAIN SEROTONIN CONTENT

A number of studies have used drugs to investigate the potential influence of raised cerebral serotonin content on exercise performance. Bailey *et al.* (1992) investigated the influence of a serotonin agonist on exercise performance. An injection of m-

TABLE 2.2 *Effects of exercise on plasma amino acid concentrations in humans.*

Exercise	Intensity*	Duration (hours)	Total TRP (% Δ)	Free TRP (% Δ)	BCAA (% Δ)	TRP: BCAA	Reference
Incremental	High	Fatigue	-13	NM	9	-2	Brodan *et al.* 1976
100 Km	Medium		NM	NM	-63		Décombaz *et al.* 1979
30 Km	Medium	< 2.83	-15	NM	-39	17	Blomstrand *et al.* 1991
42.2 Km	Medium	< 3.5	-6	NM	-23	13	Blomstrand *et al.* 1991
42.2 Km	Medium	<4.7	4	59.5	-24	20	Blomstrand *et al.* 1988
68% VO_2 max	Medium	3.15	14	86.4	-4	10	Davis *et al.* 1992
1.5 W/Kg	Low	0.33	-18	NM	-8	-13	Brodan *et al.* 1976

NM = not measured
*Subjective rating of the exercise intensity of each study.

chlorophenyl piperazine (m-CPP), which produces an increased serotonergic activity, resulted in a decreased endurance exercise performance, in treadmill trained rats (Bailey *et al.*, 1992). It is not known, however, what effect m-CPP might have had on metabolic and cardiovascular processes in the periphery, and these effects may have overridden any effects of serotonin acting on the central nervous system.

The adverse effect of an increased serotonergic activity on exercise tolerance has also been demonstrated in humans (Wilson and Maughan, 1992). In this study the authors exercised recreational athletes to exhaustion on a cycle ergometer at 70% of VO_2 max, after administering paroxetine (20mg), a serotonin uptake inhibitor. Following the paroxetine administration endurance was decreased from 116 mins. to 94 mins. ($p \leq 0.05$). The fact that these were recreational athletes, however, is relevant as it has been shown that the state of training is an important modulator of BCAA oxidation (Einspahr and Tharp, 1989; Henderson *et al.*, 1985).

THE EFFECT OF TRAINING ON BRAIN SEROTONIN CONCENTRATIONS

The effect of endurance training on the brain serotonin content in rats was investigated by Brown *et al.* (1979). Rats ran on a treadmill were exercised for 30 mins., 5 days/wk for eight weeks. In most areas of the brain cerebral serotonin content increased in the exercise trained group compared to the sedentary group. The authors speculated that an adaptation to training leads to an increased cerebral level of serotonin, and this serves as a neurotransmittal link to increased incorporation of amino acid within muscle, as well as decreased appetite drive and sensitivity to pain.

2.2.1 BCAA skeletal muscle oxidation.

BCAA's are the only amino acids to be oxidised by skeletal muscle and other peripheral tissues (Newsholme and Leech, 1984). Uptake of BCAA by skeletal muscle occurs via two pathways; uptake for oxidation is sodium independent while uptake for incorporation into muscle protein is sodium dependent. Although BCAA oxidation in the liver occurs at a rate faster than that of muscle, the muscle remains the major site of BCAA degradation due to its large mass (Harper *et al.*, 1984). The rate of leucine oxidation in muscle is limited by its rate of transport from the intracellular pool .

Exercise induced changes in oxidation of BCAA

Many studies have outlined the nature of the oxidation of leucine in skeletal muscle in both rats and humans. The majority of these studies have been performed in exercising subjects.

During mild exercise (30% of VO_2 max) Wolfe and co-workers (1982) demonstrated that human whole body leucine oxidation increased by 150%, from resting values. These results were duplicated more recently where exercise at 45% VO_2 max increased leucine oxidation by 160%, from 20 ± 5 mol/kg/h at rest to 52 ± 17 mol/kg/h during exercise (Knapik *et al.*, 1991). Leucine oxidation, at rest, in these subjects was further enhanced by a 3½ day fast. White and Brooks (1981) have shown that in rats the rate of leucine oxidation is low at rest and this increases in proportion to the metabolic rate (VO_2). In addition, Dohm and co-workers (1977), have demonstrated that the $^{14}CO_2$ production in trained, compared to untrained rats, increases significantly after [^{14}C]-leucine injection. Moreover, the enzymes involved in leucine oxidation, in the muscle adapt to endurance training similarly to the enzymes involved in carbohydrate and fat catabolism.

Finally, BCAA oxidation has been shown to increase following an elevation of plasma free fatty acid concentration, and decreases in response to increased plasma glucose concentrations (Odessey and Goldberg, 1972). This is therefore especially important during exercise where the plasma FFA increases and plasma glucose concentrations decrease.

Teleology for BCAA oxidation

The rationale for skeletal muscle BCAA oxidation remains a subject of much debate. Although it is generally accepted that carbohydrates - and to a lesser extent fats - are the primary source of energy during exercise. Protein catabolism has, however, been proposed as an important source of energy, especially in the absence of other fuels. Brooks (1987) has stated that amino acids when oxidised by muscle can contribute significantly to the energy production during prolonged exercise, and this permits the maintenance of high muscular power outputs. Other authors have shown that between 5 and 10% of the fuel for prolonged exercise may be derived from amino acids (White and Brooks, 1981; Lemon and Mullin, 1980). Dohm and colleagues (1977) however, have calculated that 8.4 kJ of energy could have been derived from protein catabolism, in the trained rat. This amounted to 37% of the calculated energy expenditure during an exercise bout. Amino acids may also play important

anapleurotic functions in sustaining the whole metabolic apparatus during exercise and recovery.

Results from a number of studies on protein dynamics during starvation may provide important clues on the nature of amino acid oxidation during exercise. Adibi *et al.* (1975;) stated, for example, that increased transamination together with the stimulated oxidation, enhances the ability of skeletal muscles to utilise BCAA's as a metabolic fuel during periods of caloric deficiency. In other words it is conceivable that amino acids may play an important role in the provision of energy during prolonged endurance exercise when the availability of alternative energy sources becomes low.

There are other possible explanations for the observed increase in amino acid oxidation during exercise. Felig and Wahren (1971), for example, noticed that large amounts of alanine are released from skeletal muscle following the uptake of BCAA's. They proposed a glucose-alanine cycle , whereby gluconeogenic substrate was shuttled to the liver. This theory proposes that when amino acids are oxidised, muscle

pyruvate - produced from glucose via glycolysis - is transaminated into alanine. Alanine is released from the muscle and is transported to the liver. Alanine release is the rate-limiting step for glucose production in starved humans (Felig *et al.*, 1970).

Goldstein and Newsholme (1976) modified the proposed cycle somewhat, suggesting that both the carbon skeleton and amino groups are supplied by glutamate, the amino acid produced from a transaminase reaction (Fig 2.4). In the muscle, BCAA's are converted by an amino acid transaminase into a BCKA. The product is then converted into oxaloacetate, in the tricarboxylic acid cycle. Oxaloacetate is then converted into phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxylase, which is converted into pyruvate. Pyruvate is then transaminated into alanine, receiving the amino group from glutamate.
According to these authors this metabolic scheme explains how certain amino acids are oxidised by muscle. So, in the absence of glucose, certain amino acids are converted into alanine in muscle. This alanine, in addition to its role as a gluconeogenic substrate, may provide an alternative for the removal of nitrogen from the muscle so preventing an exercise induced hyperammonemia. This occurs in the conversion of glutamate to α-ketogluterate with the production of alanine. The

nitrogen is transferred from the glutamate to pyruvate, which is converted into alanine, and in this way a nitrogen molecule is removed from the muscle.

Goldstein and Newsholme's alternative scheme has been met with widespread approval as many researchers believe that amino acid contribution to energy production is low and this therefore better explains the rationale for amino acid oxidation (Hood and Terjung, 1987; Poortmans *et al.*, 1991).

Hood and Terjung (1987), for example, maintain that convincing data on the contribution of BCAA's to overall energy requirements are lacking and that the best estimates are that less than 5% of the energy needs of the muscle could be met by leucine oxidation under physiological conditions. Because exercise intensity and duration differed in these trials it is difficult to compare studies and more work is required to elucidate the role of protein as an energy source during exercise.

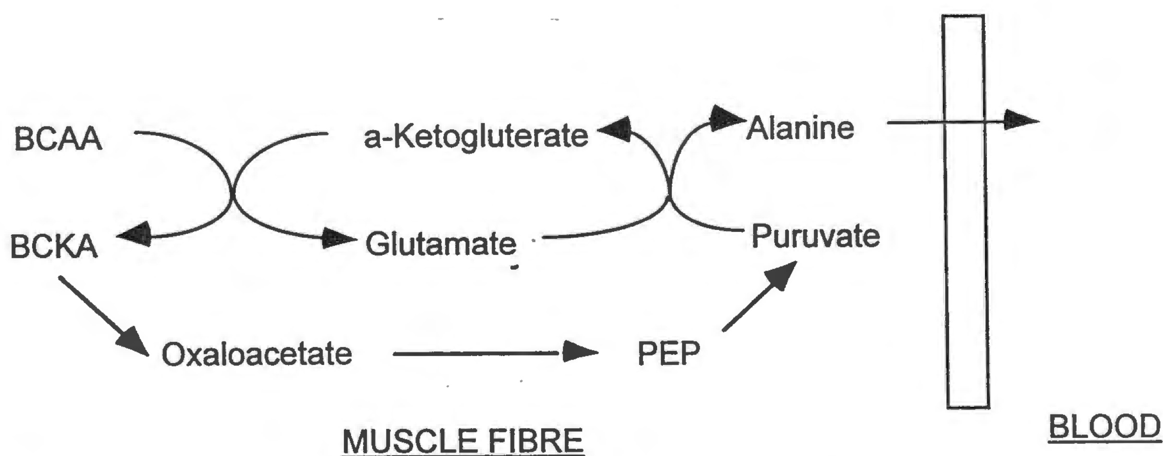


FIGURE 2.4 *A revised scheme for gluconeogenesis, according to Goldstein and Newsholme (p556, 1976). Here both the carbon skeleton and the amino group are derived from the deamination of an amino acid.*

Poortmans *et al.* (1991) also dispute the claim that proteins are oxidised for energy, and they suggest a more subtle role for the oxidation of branched-chain amino acids. These authors maintain that an increased BCAA uptake by the muscle may explain the decreased plasma BCAA level following endurance exercise. Because plasma total tryptophan concentrations did not change, an increased quantity of tryptophan is transported across the blood-brain barrier. This cerebral tryptophan is then converted into serotonin.

In light of the above it does seem reasonable to suggest that the oxidation of amino acids occurs to provide a communications link between the muscle and the brain, and this then has important consequences for feeding behaviour and in the sleep-arousal cycle. It seems unlikely, however, that the sole reason for the oxidation of amino acids is to alter brain serotonin concentration during long periods of physical activity. Rather, it is more likely that *in vivo,* neurotransmitter production and gluconeogenesis are the primary objective of BCAA oxidation and energy production is probably an important by-product during periods of substrate insufficiency (for example, exercise and starvation).

2.3 Summary of studies addressing the thesis hypothesis:

Many studies have attempted to investigate the effect of BCAA supplementation on exercise performance in both rats and humans. These studies have either altered cerebral serotonin indirectly through nutritional intervention, or they have altered brain serotonin pharmacologically. The results and conclusions of these studies will be discussed.

Indirect intervention.

The influence of BCAA supplementation on exercise performance, has been tested on human subjects in the field (Blomstrand *et al.*, 1991). Subjects received either a BCAA supplement or a placebo drink throughout a standard marathon (42.2km) road race. There was no significant difference between the performance times, until the authors arbitrarily subdivided the groups into fast and slow runners. Running performance in the marathon was improved for slower runners (3:05 - 3:30 hours) when BCAA's were ingested during the race, but there were no significant effects in the faster runners (<3:05 hours). An assessment of mental fatigue was measured by the Stroop colour word test (Stroop 1935). Mental performance improved post exercise, in the BCAA supplemented group only. Results from this study indicate that both mental and physical performance, in slower runners, improved after ingestion of a BCAA supplement.

A number of questions still remain, however, as this investigation was not properly controlled. As it was a field study a number of external variables may have influenced the results. Motivation, for example, would most definitely have differed between athletes, the study design, in spite of using large sample sizes (n=193), used a single cross-over method in which each athlete was tested on one occasion only. The post-exercise evaluation was completed only 1-2 hours after completion of the race, during which time many of the changes may have been reversed. Division into

fast and slow runners, too, is contentious, as there does not appear to be any basis to the method of division. Likewise, conclusions from these experiments may be confused by any peripheral effects that these nutritional interventions may have had (Wagenmakers *et al.*, 1991).

A recent well controlled study by Davis *et al.* (1992) investigated the effects of carbohydrate drinks on the oxidation of plasma BCAA's and on plasma free tryptophan, in humans. Plasma concentration of the BCAA's, following the ingestion of a carbohydrate drink, were decreased compared to those following the placebo, and there was an increase in TRP:BCAA. Davis *et al* (1992) suggest that the combination of carbohydrate and BCAA's increase skeletal muscle uptake of the amino acids due to an increased insulin concentration. In this study the cyclists receiving carbohydrate exercised for a significantly longer period than those receiving the placebo. However, there was no statistical difference in blood glucose levels, indicating that plasma glucose was not the factor limiting exercise performance. The authors conclude that central fatigue may have influenced cycling performance, but no measure of central fatigue was made in these athletes.

Pharmacological alteration of brain serotonin

Exercise performance in rats was investigated after the administration of m-chlorophenyl piperazine (mCPP), a serotonin agonist. Rats received either a placebo or varying concentrations of mCPP (Bailey *et al.*, 1992). At the greatest concentration of the drug, which increased brain serotonin concentration the most, the rats run time to exhaustion on the treadmill was significantly decreased. The authors conclude that these results provide evidence that increased brain serotonin results in a reduction in endurance performance. However, the other effects of mCPP on exercise tolerance are not known, and it is likely that this drug adversely affects motor function (Wilson and Maughan, 1992). In a subsequent study Bailey *et al.* (1993), again demonstrated a decreased run-time to exhaustion, in rats, following administration of quipazine dimaleate, a serotonin agonist. In addition they showed an improved exercise tolerance following the administration of a serotonin antagonist. They have demonstrated that pharmacological interventions produce changes in the performance of exercising rats. However, it is still not known what effects these drugs may have had on the peripheral musculature, and this obscures their results.

Wilson and Maughan (1992) used a serotonin re-uptake inhibitor (paroxetine) to investigate the effects of an increased serotonergic activity on exercise performance

in humans. These authors exercised seven recreational cyclists (age = 43 ± 6 years), at 70% VO_2 max, until exhaustion, and time to fatigue was significantly less after ingestion of paroxetine. The authors also measured metabolic and cardiorespiratory variables, none of which were different between the two trials. The authors interpret this to indicate that it is unlikely that the difference in exercise performance is mediated by peripheral effects, secondary to an altered activity of central serotonin pathways. This study, too, does not completely resolve all the questions, as it is not applicable to competitive athletes, for the following reasons: Subjects were recreational cyclists, and the effect of serotonin on performance is different depending on whether subjects are trained or not (Blomstrand *et al.*, 1991). The level of training also affects the accumulation of cerebral serotonin. In addition, it is not known what effect paroxetine might have on muscle function.

2.4 Mental Fatigue:

Introduction

Fatigue occurs either peripherally or centrally (see Fig 1.1) As most of the research effort to date on fatigue after prolonged, submaximal exercise has been directed at the periphery, very little is known about central fatigue, and few testable hypotheses exist. One hypothesis is that an increased serotonin concentration may influence an athlete's concentration and may lead to an increased perception of fatigue (Blomstrand *et al.*, 1988). Mental overload occurs when a person is required to perform beyond the limits of his or her resources. The scope and importance of fatigue during work or exercise is vast as it has implications for reasons of safety, efficiency and health. This type of fatigue may also be important in those sports requiring a great deal of concentration, for example, motor racing or even cycling. No single test exists for the purpose of measuring mental fatigue, and the reaction-time paradigm as described by Sternberg (1966) was used in the present study as it is an appropriate test to monitor acute changes in cognitive function (Hosstetler, personal communication). This test was selected over the Stroop Colour and Word Test as it has a very low error rate, there is no learning effect following repeated exposure , it uses letters which are familiar and discriminable (Sternberg; 1966) and each test takes only 2½ minutes, compared to the 15 minutes of the Stroop CWT (Newsholme *et al.*,1991). It is important to do the test immediately following the exercise bout as changes in mental performance are slight, and are likely to return to resting levels rapidly once the exercise has been completed.

Quantification of mental fatigue

Newsholme and co-workers (1991) were the first to document the anecdotal report that athlete's felt more aware and alert following the ingestion of BCAA's during prolonged exercise. They used the Stroop colour and word test (CWT) to quantify this subjective observation. According to the authors the Stroop CWT was chosen as it provides a useful tool in the study of neurophysiological and cognitive processes. Following a 30km cross-country race, performance on the Stroop test improved in most subjects consuming a BCAA drink. This preliminary data indicates that BCAA administration may help to offset mental fatigue, that is, if levels of plasma BCAA's can be maintained at resting values, a drop in the level of concentration might be prevented during prolonged endurance exercise (Blomstrand *et al.*, 1991).

2.5 Summary of the Literature:

From the above discussion it can be seen that several studies show that plasma BCAA concentrations decrease during prolonged exercise. Those studies that dispute this finding followed exercise protocols which were shorter and more intense. The literature concerning the change in tryptophan during prolonged exercise is less clear, as total plasma tryptophan does not always increase. However, it is clear that free tryptophan does indeed rise, following the increase in plasma FFA during prolonged exercise. This results in an increase in TRP:BCAA (Table 2.2), producing an increase in cerebral serotonin concentrations. Human cerebral serotonin concentrations cannot be measured, and its metabolites do not provide an accurate estimate of changes in the brain, however, data from rat studies indicate that alterations in plasma tryptophan concentrations, or an increased TRP:BCAA, result in an increased cerebral tryptophan which leads to an increased serotonin concentration in the brain.

Although there is no direct evidence that an increased cerebral serotonin content causes an increase in the amount of amine released into the synapses, it can be assumed that "since an increased amount of neurotransmitter will probably be released whenever the neurones that store serotonin are excited, it appears likely that brain function will be modified" (Fernstrom and Wurtman, 1974a, pg. 84). Changes in physiology and behaviour may also be indicative of alterations in brain serotonin release. Wurtman (1983) for example, found that a single dose ingestion of tryptophan in a group of healthy young men caused a significant increase in their

self-reported fatigue/inertia and a significant decrease in their vigour/activity ratings according to the POMS (Profile Of Mood States) test. Although it appears that an increased plasma TRP:BCAA is causally linked to a decline in mental and physical performance, after prolonged exercise, this theory has not been tested adequately in a well controlled laboratory experiment.

2.6 Scope of this thesis.

The first aim of the study was to develop an exercise protocol, in humans, where serum BCAA concentrations decreased, as a result of the prolonged exercise bout, causing an increased TRP:BCAA. The second aim was to prevent the increase in TRP:BCAA by having the subjects ingest a BCAA supplement during exercise. Physical and mental performance, and various cardiorespiratory and haematological variables were measured. This model was then used to examine the thesis hypothesis.

Before embarking on the study a method to analyse the plasma amino acid concentration had to be devised. The HPLC was selected as this device allows for the rapid and sensitive determination of plasma amino acids (Turnell and Cooper, 1982). An important part of this project was therefore to develop the HPLC system to validate its ability to accurately measure amino acid concentrations.

Chapter 3 - High performance liquid chromatography: Method development and troubleshooting

3.1 Introduction:

At the outset of this investigation it was decided that one of the preliminary tasks would be the development of an application to analyse serum amino acids using high performance liquid chromatography (HPLC). HPLC was a technique new to this laboratory, and a large amount of time was therefore required to validate both the quantification and qualification of the method.

3.2 Chromatography.

Chromatography is the separation of a chemical mixture into its individual components. The term encompasses a number of different applications and can be used to separate, isolate and quantify most substances, measure molecular weight of polymers and to characterise different aspects of the structure of substances.

3.3 High performance liquid chromatography.

High performance liquid chromatography, which has been in use since the 1960's, is now the most powerful and widely used chromatographic technique. It is largely utilised by research institutions and the pharmaceutical industry to analyse proteins, peptides, amino acids, sugars, nucleotides, catecholamines, vitamins, drugs and toxins. In the last 15 years particularly, much has been learned about the basis of HPLC separations and how they can be improved by varying experimental conditions.

Advantages of HPLC

For many years gas chromatography (GC) was the method of choice for analysis of unknown samples. HPLC has, however, superseded GC as the method of choice for the analysis of many compounds. This is as a result of the increased accuracy and precision of high performance liquid chromatography, which displays coefficients of variation of 0.1 to 0.5%. In addition a large number of different applications are possible, HPLC's are relatively simple to operate (once method development is complete), sample preparation is simple and analysis is rapid. Finally, HPLC is extremely sensitive and can achieve good quantification and qualification in the

femtomole (10^{-15}) range, as long as sample preparation and degassing is suitably performed.

Disadvantages of HPLC

The major disadvantage of using HPLC is that, in spite of the simplicity of the operational basics, method development and troubleshooting are demanding and time-consuming.

For an HPLC run the sample is prepared by removing the matrix, (a collection of interfering compounds), and dissolving it in a liquid solvent. This sample is then introduced to a column under high pressure. Components are separated into discrete bands following interactions with the mobile and stationary phases. The stationary phase consists of an immobile packing material within the column, while the mobile phase comprises a liquid solvent containing the sample. Depending on the application, one (isocratic system) or more (gradient system) different solvents may be required to effect an optimal separation.

3.4 Chromatographic Theory:

The crucial element of an HPLC application is the separation process. This occurs in a column, a narrow bore (0.46mm) stainless steel tube, varying in length, typically from 7 to 30cm, packed with small (3-10mm) silica particles. The silica is coated with a particular substances to affect a specific separation. As the sample moves through the column the components interact differently with the stationary phase. This results in component bands traveling down the column at different velocities.

The aim of a chromatographic separation is to achieve good retention and therefore good resolution between eluting component bands, as well as reducing band spreading (fronting and tailing) of peaks. This is achieved through the manipulation of many parameters.

Resolution.

As components proceed at different velocities through a column they separate and begin to broaden. An optimal separation will seek to separate each component sufficiently within a reasonable time. Resolution of a peak is a combination of both separation and time, and may be optimised by altering the column's selectivity and efficiency.

COLUMN SELECTIVITY

Selectivity is the ability of a certain set of experimental conditions to discriminate between two compounds. Selectivity can be optimised through alterations in column type (C8 to C18) and/or mobile phase (polarity, pH and temperature).

COLUMN EFFICIENCY

This may be defined as the ability of a particular column to prevent the spreading of a component of interest in the chromatogram. Efficiency can be enhanced by increasing column length and optimising the flow rate through the column.

3.5 Validation & Calibration:

The validation of any HPLC application is an important part of technique development. Because the system is extremely sensitive, small changes, can result in large variations in chromatographic output. Validation of the application used in this study revealed it to be accurate, precise, specific and reproducible. The application was validated using standard additives. Twenty microliters of a pure single standard was added to the standard cocktail and then to the sample matrix. The cocktail consisted of 500μl of each of the 17 amino acid standards, dissolved in 1L of double-distilled, deionised water. The sample matrix consisted of a precipitated blood sample, treated in the same manner (for details see section 4.27). Results between the spiked and unspiked chromatograms were then compared and analysed in terms of percent recovery. Precision of each chromatographic run was checked prior to each series of analyses, which consisted of 10 runs, during the calibration of the machine.

In the present study calibration was performed every morning on start-up, and then following every ten samples, by completing two chromatographic runs of the standard 'cocktail' solution. The coefficient of variation between runs and drift over time was calculated. If the coefficient of variation between calibration runs was greater than 3% another calibration was performed, ensuring a high degree of accuracy for calculation of unknown amino acid concentrations. Drift was determined by calculating the ratio between the internal standard (Methionine sulfone) and the leucine peak, and then comparing these ratios between calibrations. The average value of the ratio was 21.46% ± 1.98%. Again the difference was no greater than 3%. The method proved to be reproducible in that it showed less than 5% day-to-day variation.

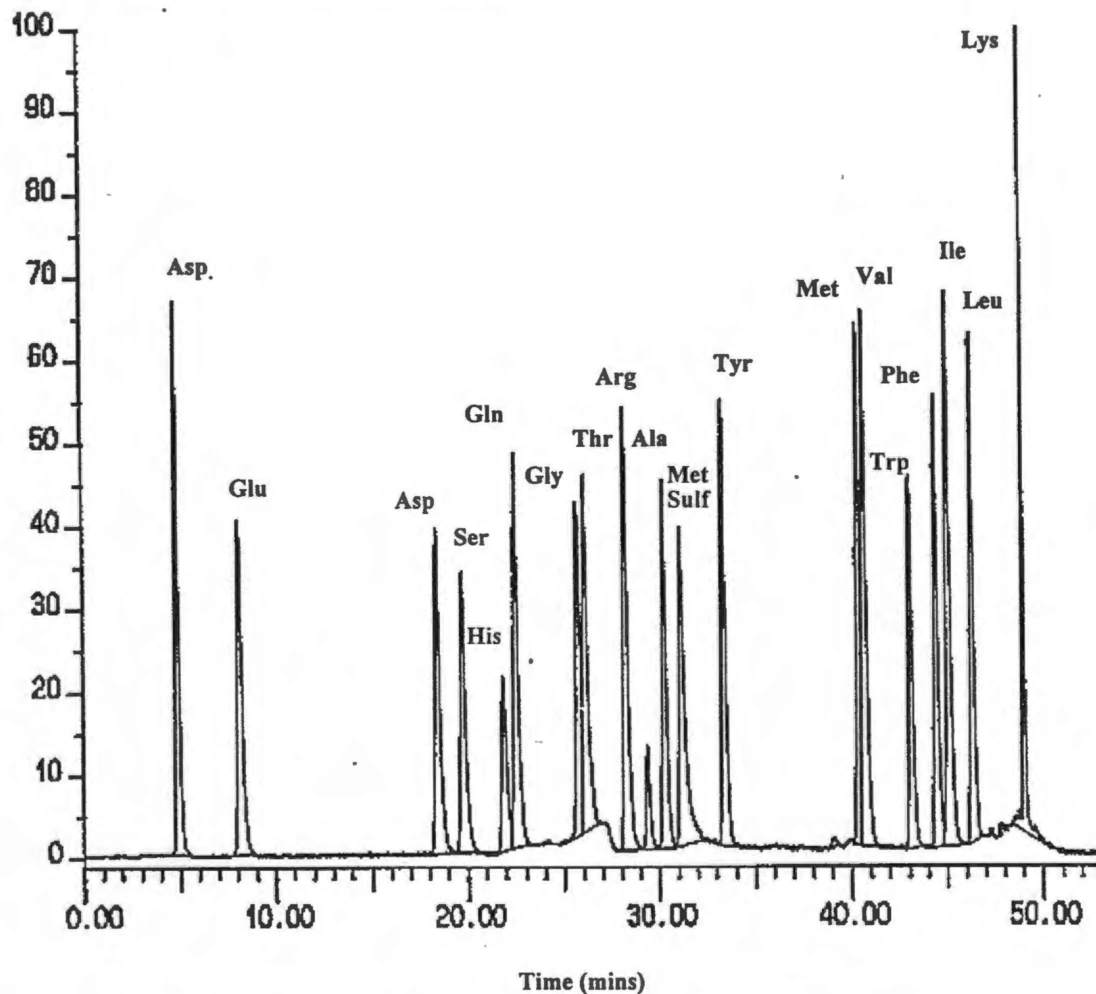


FIGURE 3.1 *An HPLC trace of the standard amino acid 'cocktail' solution, used for calibration of the machine (for abbreviations see Table 4.1).*

An internal standard was used to correct any small differences between runs. An internal standard is often preferred in HPLC as it reduces errors resulting from sample preparation, injection bias and technique. With internal standardisation a known amount of substance is added to both the standard and the unknown solutions. The quantification of all peaks is then related to the concentration of the internal standard.

The choice of an internal standard is an important one, and it must satisfy the following criteria:

i) it must be completely resolved from all peaks,
ii) should elute near the middle of the chromatogram,
iii) must be similar in concentration to the peaks of interest,

iv) should not react with any of the sample components, and
v) it must be pure and not appear naturally in the sample.

Because calibrations runs are time consuming it is important to determine an optimal interval for recalibration. In this study calibration was performed every ten samples as this represented a balance between time constraints without compromising accuracy or precision.

Other important considerations that had to be taken into account to maximise the reproducibility of the technique included the following:

STABILITY OF THE ANALYTE.

Decomposition of the samples over a period of time in the sample and/or standard will result in an underestimation of the amount of component present. Derivitisation of a sample includes the processes undertaken to render the components of interest visible to the detector attached to the HPLC. In the application used in this study, for example, *o*-pthaldialdehyde (OPA) was added to the plasma containing the amino acids. The OPA combines with the NH_2 group on each amino acid and renders it fluorescent. Any differences in component concentration over time suggests decomposition. Stability of the analyte was assessed by changing the length of time between derivitisation of the sample and between injection onto the column. The analyte did prove to be unstable with a decrease in fluorescence being observed over time. This is consistent with data of Cooper *et al.* (1984). In order to account for this a standardised time of one minute, between derivitisation and injection, was used to reduce variability.

STANDARD PURITY

Pure samples are crucial to ensure accurate quantification of the unknown. Standard solutions were constituted with double-distilled deionised water and high quality standards (Sigma Chemical Company, St. Louis, MO 63178). Standard solutions were made up to a large volume, divided into aliquots and then stored until required. Further it is possible that detector output can become non-linear in the upper range of solute concentration, and therefore the linearity of the analysis, within appropriate limits, was confirmed by injecting a series of standards varying from 50 to 150 percent of the expected working assay range. A regression line through the standard curve was not significantly different from zero ($r = 0.89$).

3.6 Problems encountered during method development:

The following is a brief synopsis of the problems encountered and how these were overcome. Both troubleshooting and method development are crucial skills required by a chromatographer, and much of the progress is made through trial and error.

Errors in the application may arise from a number of possibilities and may initially be difficult to pinpoint. However, with increased exposure to HPLC confidence and problem solving becomes easier and more rapid. Effective troubleshooting requires that only one variable is changed for a particular run so making the alteration unambiguous. This makes troubleshooting a time consuming process and method development often takes months or even years.

In attempting to solve a problem it is first necessary to identify the type of problem, and these typically include one of the following:

i) Leaks
ii) Pressure
iii) Quantitation or data quality
iv) Hardware, and
v) Chromatogram.

The following is a brief description of the problems encountered during method development in this study.

The standard solution was originally made up of the ten essential amino acids, as these were considered to be most important for this study. These were constituted singly, so that each amino acid's retention time could be calculated in the given application. At this point only the ten dietary essential amino acids were used, and the technique was refined for these. Each amino acid was injected singularly, in three

TABLE 3.1. *Differences in (Rt) and area or the BCAA's in successive chromatographic analyses (9th Sept. 1991).*

Amino Acid	Run #	Rt (mins.)	Peak Area
Valine	1	12.91	106298
	2	12.20	99970
	3	12.29	185659
Isoleucine	1	14.20	81361
	2	14.19	44006
	3	14.10	48180
Leucine	1	14.92	135646
	2	14.90	128941
	3	14.94	167216

Rt = Retention Time; Peak Area in arbitrary units

successive runs, and the resultant retention times (Rt) and peak areas were compared (Table 3.1). What was immediately obvious is that there was an unacceptable difference in both retention times and peak areas between runs.

The changes in retention time (Rt) may be due to a contribution of many factors (Table 3.2). During method development these all influenced the separation, preventing unambiguous peak identification.

PROBLEMS RELATED TO MOBILE PHASE AND REAGENTS

The appropriate choice of solvents is an important variable in effecting a good separation. Because sample components interact to different degrees with the stationary phase of the column the solvent must be sufficiently strong to remove well retained components but weak enough not to cause simultaneous removal of poorly retained compounds.

TABLE 3.2. *Effect of change in separation conditions on sample retention in reversed-phase HPLC (modified from Dolan and Snyder, 1989).*

Variable	Δ in Variable	Average Δ in Rt
Flow Rate	+1%	-1%
Temperature	+1°C	-(1-2%)
Mobile Phase Composition		
Organic Solvent	+1%v	-(5-10%)
pH	+0.01 unit	±(0-1%)

GRADIENT

Resolution was improved through the use of a gradient system. Here, by altering the contribution of two different mobile phases, having different strengths, throughout the run an optimal separation was achieved. Gradient elution is typically used in complex separations containing a number of particles having different characteristics (e.g. plasma amino acids). In this reversed-phase HPLC separation a polar (large water content) solvent was introduced at 100% and then the concentration of the non polar solvent (methanol or acetonitrile) was gradually increased to a point which results in complete elution of the components of interest. A large amount of time was spent optimising the gradient which is shown in Fig 4.1, pg. 54.

CONSTITUTION

In the preparation of a mobile phase the components should be measured individually and then combined, as the added volumes of two different liquids does not equal the sum of their individual volumes. Differences in mobile phase make-up will result in changes in pH and volume, leading to changes in retention times. After constitution mobile phases should be degassed. Air in the solvent may result in valve failure or in

flow rate or pressure inequalities. Filtration and degassing can be achieved simultaneously by attaching a specialized filtration flask to a vacuum pump. Solvents should be filtered through pores no larger than 0.45μm.

CONTAMINATION

Unless extreme care is taken mobile phase contamination will occur. Even extremely small changes in sample purity can result in large retention time differences between successive separations (Table 3.2). Mobile phase contamination occurs when errors are made in the manual preparation of solvents or if there are changes in the composition of the solvents (e.g. selective evaporation of a solvent or CO_2 uptake leading to changes in pH), which may occur overnight. In an effort to limit contamination glassware was washed in distilled water, mobile phases were constituted identically, sealed with parafilm, and solvents were stored at 4°C. This reduced the retention error significantly and resolved much of the error seen in Table 3.1. Additional variation between runs may have been due to changes in the composition of the derivatising agent, OPA. This reagent was later ordered already constituted from Sigma Chemical Company (Sigma Chemical Company., St. Louis, MO 63178).

ALTERATION IN PH

The pH of the mobile phase is crucial as a change of only 0.1 units may effect a change of 10% in retention time (Table 3.2). pH should always be measured in the aqueous phase only and then the organic modifier (e.g. acetonitrile) should be added. A method will usually specify the degree to which pH may vary, depending on the methods inherent sensitivity to pH. However, as a general rule pH should not deviate by more that 0.1 units between successive batches of mobile phase. Buffers are often used to stabilize the pH of the solution, but these can produce a number of problems related to the smooth operation of the equipment. When using buffers extreme care must be taken that it remains soluble in the organic modifier. This is especially true of gradient systems where the percentage of the organic modifier increases throughout the run. Often a buffer may precipitate out in very high concentrations of organic modifier and this blocks the stainless steel tubing, valves, injection ports and column frits.

EQUILIBRATION

Substantial changes in retention time between two successive chromatograms can result from incomplete equilibration. After running a gradient program the column

requires some time to re-equilibrate. Snyder *et al.* (1988) suggest that 10 to 20 column-volumes is usually sufficient to equilibrate a reversed-phase column. For a 10cm x 0.46 mm column, therefore, 10 to 20 ml of mobile phase A was required for equilibration. At a flow rate of 1.3 ml/min equilibration would take approximately 15 minutes. The time between successive injections was standardised (5-10 minutes), so that samples were introduced onto a column containing identical proportions of the mobile phases. Once an equilibration time was included in the method, ensuring column equilibration prior to each run (see Fig. 4.1, pg. 54) repeatability was further improved.

LEAKS AND FLOW RATE DISCREPANCIES

Changes in both flow rate and leaks are related. Because operating pressures are extremely high ±3000 psi leaks are likely to occur at any weaknesses, e.g. joints. Leaks may also occur where seals are worn in both the pumps and the mixer. Leaks leading to changes in flow rate are usually easily detected and easily solved.

COLUMN CONTAMINATION

With continued use column packing generally changes its composition. Particles may become irreversibly attached to the silica and part of the bonded phase may be removed through chemical attack. This results in long term changes in retention time. Toward the end of a column's life the retention time generally decreases and a loss of efficiency is seen, evidenced by band spreading, changes in peak area.

Another malady of prolonged use is the increase over time of back pressure. This is due to partial blockage of the inlet frit. This was cleaned by reversing the column, and then flushing with a strong solvent to remove the particles, or by replacing the inlet frit.

TEMPERATURE CHANGES

Temperature did affect the retention times in this application. As laboratory temperature increased throughout the day, retention times decreased, and then increased again towards evening as the laboratory temperature decreased. As column temperature was not regulated, and it was not possible to control lab temperature, the column was insulated using a foam rubber sleeve, removing the effects of temperature-related changes in retention time. However, because the ratio of retention times between peaks did not change, the effect of temperature induced changes was minimal and did not prevent accurate sample analysis.

In addition to the above, changes in the area of the peaks was observed. These errors in quantification may result as a combination of the following:

INJECTION TECHNIQUE

A manual injector has two modes, namely load and inject. In the 'load' position mobile phase passes directly from the pump to the column bypassing the injector loop. The constant volume loop is completely filled with sample, ensuring that an identical volume is injected, thereby reducing the variation. Once the loop has been completely filled the injector is turned to the 'inject' position, and the mobile phase from the pump is routed through the loop to the column. In this way sample is introduced easily and accurately into a high pressure system.

However, the correct technique is critical for the injector to operate to its potential. With the rheodyne manual injector the microsyringe is inserted until the tip hits the surface of the seal. The plunger is then depressed so filling the constant volume loop. Without removing the needle the injector is turned from 'inject' to 'load', the syringe is then withdrawn. Originally the application required a 5µl injection and the 20µl loop was only partially filled during each injection. This technique is difficult and often leads to volume inconsistencies, and should only be used with extremely concentrated samples where overload may occur. Once the procedure was modified to completely fill the loop consistency was greatly improved. Using this type of injector it is possible that cross-contamination occurs. That is, remnants of the previous sample remains in the injector and is introduced into the following chromatogram, producing spurious peaks. This was corrected by completely rinsing the injector with mobile phase A between runs.

SAMPLE OR STANDARD DEGENERATION

Large day-to-day variations in peak areas occurred as a result denaturation of calibration solutions, stored at room temperature. Once new solutions were divided into aliquots, sealed in 1.5ml reaction vials and frozen at -80 °C, reliability improved considerably. Standard solutions were thawed and vortexed mixed immediately prior to use.

The OPA derivitised amino acids are unstable at room temperature, and the derivitisation, therefore, had to be standardised. O-pthaldialdehyde was added to the sample and internal standard in a 1.5ml reaction vial. This was vortexed for ten

seconds, and after one minute injected onto the column, again improving repeatability.

QUENCHING

The application in this study used a fluorometric detector to quantify and qualify amino acid content of the eluent. The fluorometer increases both selectivity (only fluorescent species are observable) and sensitivity depending on the nature of the compounds and of the mobile phase. Fluorometers work on the principle that when a molecule absorbs energy it moves to a higher energy state. When returning to a more stable state they release energy in the form of light.

Fluorometric detectors measure changes in the fluorescence of the column eluent when it is exposed to selected wavelengths of light. Dissolved oxygen in the mobile phase results in quenching of the emitted fluorescence, so artificially decreasing component areas. Once all samples and mobile phases were degassed sufficiently some variability was removed (also improving pump performance). Degassing removes air bubbles resulting in a constant flow rate. Quantitation especially in a gradient system, was also improved as each band appeared exactly on the same point on the gradient in successive runs. Salt build-up and leaks will, for the same reason, interfere with quantitation.

ADDITIONAL CONCERNS

Over and above the difficulties already described additional problems were experienced. Although the application could satisfactorily resolve all ten essential amino acids in 30 minutes, it was decided that these ten would not satisfy all the requirements of the department. Therefore it was decided that a new technique should be developed and a new standard amino acid kit (LAA-21, Sigma Chemical Company, St. Louis, MO 63178) containing all 20 amino acids, was purchased. Using OPA derivitisation it was not possible to detect or quantify hydroxy-proline or proline, leaving 18 quantifiable amino acids.

TABLE 3.3. *Co-elution of amino acids using the technique of Turnell and Cooper (1982), in the present study.*

Amino Acids	Time
Glutamine & Histidine	3.5 mins.
Alanine & Arginine	6.3 mins.
Valine & Methionine	10.9 mins.

Once a standard 'cocktail' of these amino acids had been analysed a chromatogram of 14 peaks was produced. After spiking successive runs with the single amino acids it was determined that six amino acids were co-eluting (Table 3.3).

Believing that this poor resolution was a consequence of column degeneration the column was regenerated by flushing with the following solvents for five minutes each, in this order:

i) 50 ml HPLC-grade water at 55°C,
ii) 50 ml methanol,
iii) 50 ml acetonitrile,
iv) 30 ml tetrahydrofuran,
v) 25 ml methanol and
vi) 25 ml mobile phase.

This succeeded in reducing back pressure but had no effect on resolution. After much manipulation it was decided that given the current application it was not possible to resolve all 18 peaks, and a new application was implemented making use of a different combination of solvents (Turnell and Cooper, 1982). This immediately improved resolution but lengthened each run to an hour.

In order to improve resolution further (there continued to be overlap) the gradient program was systematically changed. After manipulation of both flow rate and gradient the separation was eventually optimised. Valine and methionine, however, continued to overlap. Analysis of samples from the present study on an amino acid autoanalyser (Beckmann Amino Acid Autoanalyser, Beckmann Instruments Inc., Brea, CA 92621) showed that methionine concentration does not change as a result of the exercise protocol. In addition methionine is only 5% of the concentration of valine (0.0015 mM vs. 0.26 mM), and the valine concentration is therefore expressed as the summed values of valine and methionine concentration throughout the experiment.

In a final attempt to improve resolution the length of the column was increased. This improved the efficiency of the column, resulting in narrower, better defined peaks.

3.7 Summary:

The application produced the trace shown in Fig 3.1, which is the trace for the standard cocktail solution used to calibrate the machine. In the analysis of the

sample the peaks for valine and methionine overlapped, but apart from this the trace was identical. Development of this application for analyzing plasma amino acids took far longer than anticipated though, largely as a result of a number of fundamental errors being committed throughout method development. However, the value of developing the technique from first principles enhanced my knowledge of the apparatus, and at completion I am confident that the technique was both precise and accurate (see Table 3.4). The specifics of the HPLC apparatus used in this experiment are described in detail in the methods on 52.

TABLE 3.4. *Calibration data for the HPLC method. Two calibrations were performed prior to analysis (see section 3.5)*

Number	Calibration 1	Calibration 2	Average
1	57 950	57 461	57 706
2	39 625	40 873	40 249
3	43 809	41 275	42 542
4	54 583	52 542	53 415
5	44 465	48 225	46 345
6	68 610	67 904	68 257
7	54 889	52 801	53 845
8	56 721	58 162	57 442

Experimental Section:

Chapter 4 - The Effect Of BCAA Ingestion on Physical Performance Following Prolonged Exercise

4.1 Introduction:

Previous studies have conclusively demonstrated that prolonged endurance exercise promotes the decline in plasma concentration of BCAA's, while plasma total tryptophan concentration either increases or does not change (Table 2.2). As a result, serum TRP:BCAA increases over time, resulting in an increased transport of tryptophan into the brain. Within the brain tryptophan is then hydroxylated and decarboxylated to form serotonin. Elevated levels of serotonin are, according to the hypothesis, thought to increase the perception of both physical and mental effort during prolonged exercise.

Certain studies have investigated the influence of altered serotonin concentrations on exercise performance, in both experimental animals and in humans. These studies have either manipulated brain serotonin indirectly, by altering plasma tryptophan concentrations (Fernstrom and Wurtman, 1974b, Okamura *et al.*, 1987), or drugs have been used to alter brain serotonin concentrations directly (Bailey *et al.*, 1992; Wilson and Maughan,1992; Bailey *et al.*, 1993). However, because of limitations in design and because of insufficient controls, results from these studies are inconclusive, and many questions, therefore, remain unresolved.

The present study investigated the effects of prolonged submaximal exercise on physical and mental performance in eight trained cyclists. The exercise protocol was designed to produce a decline in serum BCAA concentrations, and then to reverse this decline by administering a BCAA supplement. Finally, the effects of this supplementation on physical and mental performance were examined.

4.2 Materials and Methods:

4.2.1 Introduction.

To test the hypothesis of this thesis, eight trained cyclists participated in an exercise trial. Subjects rode for four hours at 55% of their VO_2 max, and then transferred to their own cycles, mounted on a windtrainer, where they completed a 40km time trial at maximal effort. Subjects ingested different drinks:

i) CHO,
ii) CHO + BCAA, and
iii) BCAA

during three randomised consecutive trials, one week apart. Physical performance was defined as the time taken to complete a 40km time trial, while mental performance was assessed before the exercise trial and after the time trial, using a computer-based Sternberg reaction-time test.

4.2.2 Subjects.

Eight trained male cyclists were selected for this study. All subjects were informed of the purpose and nature of this study, and the experimental protocol was approved by the Ethics and Research Committee of the University of Cape Town, Medical School.

In order to qualify for participation subjects were required to be currently training more than 250 km/week, for the past four weeks, and had to have completed a major local race (The 1992 Argus Cycle Tour) of 105 km in less than 3½ hours. Subject characteristics are presented in Table 4.2, pg. 56.

Body Composition

The percentage body fat, height and mass of the subjects was measured before the first trial. Body mass, in addition, was determined before all three trials, prior to urination. Since all subjects were weight stable, values reported in Table 4.2 are an average of three measurements. Bicep, tricep, subscapular and supra-iliac skinfolds were measured with Holtain skinfold calipers (Holtain Ltd., Crosswell, UK), and body density and percentage body fat was predicted using the equations of Durnin and Womersley (1974) and Brozek *et al.*(1963):

$$D = 1.1631 - (0.0632 \times \log sk) \qquad \text{(Durnin and Womersley,1974)}$$

&

$$\% \text{ Fat} = 100(\frac{4.570}{D} - 4.142) \qquad \text{(Brozek } et\ al.\text{, 1963)}$$

D= the predicted density of the body (g/ml), for males between the ages of 19.2 and 29.1 yr. of age, and log *sk* is the log of the sum of the 4 skinfold measurements.

4.2.3 Preliminary Tests.

VO_2 max

At least one week before the first trial, each subject performed an incremental exercise test to exhaustion to determine the maximal oxygen consumption (VO_2 max). In this trial, starting work load was 3.33 W/kg, and work rate was increased by 25 W every 2½ minutes. During the test subjects wore a noseclip and breathed inspired air via a Hans Rudolph #2700 non-rebreathing valve (Vacumed, Ventura) connected to a dry gas meter. Expired air was passed through a 15 litre baffled mixing chamber and a condensation coil. Exhaled air was drawn into oxygen (Ametek, S-3A/II, Thermox Instruments, Pittsburgh, PA) and carbon dioxide analyzers (Ametek, S-3A/II, Thermox Instruments, Pittsburgh, PA) at a constant flow-rate of 5ml/min by two flow pumps (Ametek, 12-0010, Thermox Instruments, Pittsburgh, PA). The analyzers were connected to sensors, which detect changes in O_2 and CO_2 concentrations (N-22M and P-61B, Thermox instruments, Pittsburgh, PA). VO_2, CO_2 production and RER were calculated using conventional formulae (Consolazio *et al.*1963).

Velodyne

Following the determination of VO_2 max, all subjects (n=6) who had not previously ridden the Schwinn Velodyne Cyclosimulator (Schwinn, Chicago, USA) windtrainer completed a familiarization ride. The Velodyne, an indoor trainer onto which a subject's own bicycle can be mounted, simulated road cycling. Work rate is adjusted by a load applied to the rear wheel. The work load applied takes into account the cyclist's weight and frontal surface area, in order to simulate cycling conditions as closely as possible.

The Velodyne was calibrated prior to each trial following a warm-up of at least thirty minutes. To calibrate each subject's bicycle was mounted to the trainer, a constant weight was placed on the rear wheel and the axle was tightened. The subject then

pedaled smoothly to a speed of 40 km/hr, stopped and allowed the speed to decrease below 8 km/hr. The rider again pedaled to 40 km/hr and stopped. Computer software then used these two sequences to calculate a speed to power calibration curve. Thereafter, the subject rode a 40km time trial at maximum effort. The work load set on the Velodyne has proved to be accurate to within ±5% (Kyle, 1991).

4.2.4 Experimental Protocol.

As meal content, prior to exercise, was likely to be important in this experiment the two meals prior to each exercise trial were standardised in order to eliminate any inter-individual differences. The meal eaten the night before each trial was standardised for each subject, that is, each subject selected a dinner, and an identical meal was eaten on the night before each subsequent trial. On the morning of the trial, three different isocaloric breakfasts were offered (see section D). The breakfasts content was designed by a dietitian and contained identical proportions of carbohydrate, fat and protein. Different breakfasts were available in order to allow each subject to select a meal palatable to himself.

Each trial was separated by one week, and was conducted at the same time on the same day of the week. This was an attempt to standardise conditions in the two days preceding the trial, for each subject. Subjects were also required to record their training during the three weeks and report any changes in volume or intensity.

Sternberg test

REVIEW OF THE LITERATURE

The Sternberg reaction-time test was selected to quantify mental fatigue following each exercise trial (Sternberg, 1966). The test was chosen over the Stroop colour word test, which had been used previously (Blomstrand *et al.*, 1989) as it is of shorter duration, and is therefore more sensitive to detect small reversible changes in reaction time, post-exercise (Hosstetler, personal communication). In addition, this test does not exhibit a learning effect and is characterized by a low error rate (Smith and Langolf, 1981). Sternberg (1966), used this test to demonstrate how symbolic information is retrieved from memory, via a serial comparison process. The test determines the time taken for a subject to judge whether a test symbol is contained within a memorized sequence. He found that reaction times increased linearly with the length of the sequence. This test may therefore be used to study how cognitive function (mental awareness) changes as a result of prolonged exercise. The

Sternberg test was deemed suitable for this purpose as it is both precise and well grounded in cognitive theory (Smith and Langolf, 1981).

The Sternberg reaction-time test uses letters of the alphabet as these are well learned and highly discriminable. A feature of this test is the low error rate which justifies the assumption that on a typical trial the subject's memory of the series presented is identical to the series presented by the test (Sternberg, 1966). In addition, the validity of the Sternberg test is fully proven by the fact that there is little or no learning effect and that only a short practice session is required by each subject prior to the test.

METHODOLOGY

Subjects performed the reaction-time test before each trial began and after the time trial in a closed silent laboratory. The test was performed on a microcomputer, using software developed by Mr. Frank Bokhurst of the Department of Psychology of the University of Cape Town.

In this task the subject is asked to memorize a sequence of either two or five letters. The test is classically performed with series containing one to six letters, but, it has been demonstrated that a test containing series of two and five letters is representative of the complete test (Smith and Langolf, 1981).

Subjects selected their own true and false key on the computer keyboard, which they pressed with different hands, and prior to each session subjects completed a three minute practice session.

Each test comprised the following:

i) An auditory warning signal indicating the beginning of each series.
ii) A series of either two or five letters. The length of each sequence was randomly chosen by the program. Each letter appeared on the screen for one second, immediately replaced by the subsequent letter.
iii) Following a delay of one second the test digit appeared, remaining on the screen for one second.
iv) Subjects were required to decide whether the test letter had appeared in the preceding sequence. Subjects responded as quickly as possible, by pressing the 'true' key if it had appeared in the sequence, and the 'false' key if it had not.

Each trial comprised 60 test sequences of which approximately 30 were two digits long and the remainder five digits.

Three mental performance measures are available as a result of this test:

i) memory scanning time,
ii) effect of response type, and
iii) Y intercept.

The effect of response type deals with the difference between a 'yes' and 'no' answer while the intercept deals with the latency before making a response. Both of these factors are 'hard-wired', that is, they are unalterable characteristics of brain function and are not affected in the short-term by perturbations such as exercise.

The reaction time pre- and post-exercise (a measure of mental fatigue), which is quantified by the memory scanning time (i) may be affected by both an acute (endurance exercise) or a chronic (mercury poisoning) intervention (Smith and Langolf, 1981).

Exercise Procedure

Following the Sternberg reaction-time test subjects urinated to empty their bladders. This pre-exercise urine sample was stored on ice until analysis five to six hours later. A cannula, attached to a stop cock, was then placed percutaneously into a medial antecubital vein. Blood samples were drawn into a syringe via the stop-cock, and the cannula flushed with saline. Heparin was not used to maintain patency of the cannula as this may have induced lipolysis, artificially elevating plasma free fatty acid concentrations. Cannulae remained patent for most subjects, on most trials (n=18), for the full hour between the collection of blood samples. In the event that the cannula became blocked, a new cannula was inserted, more medially, in the same vein.

SUBMAXIMAL EXERCISE

Subjects then began cycling on a stationary electronically braked cycle ergometer at 55% of their previously determined VO_2 max value. After an hour on the ergometer subjects were allowed a five minute rest, during which time they were permitted to urinate. This urine sample was collected together with any other produced over the remainder of the trial and the pooled urine sample formed the exercise-urine sample. Urinary urea and creatinine were determined in the pre- and during-exercise samples.

Subjects then rode for 55 minutes at which time they again rested for five minutes. This was repeated twice for a total exercise time of four hours. Blood was collected every hour, prior to the five minute rest, and then again, within five minutes, following completion of the 40km time trial. During the low intensity trial subjects were permitted to watch videos or listen to music. Each subject completed four hours at this intensity, after which time they moved to their own bicycle mounted on the Velodyne.

TIME TRIAL

The Velodyne was quickly calibrated and the subjects began a forty kilometer time trial, not less than ten and not more than fifteen minutes after finishing the four hour ride. Heart rate was measured every five seconds using a Polar Vantage pulse monitor (Polar USA, Stamford, CT).

Subjects were isolated in a small, temperature constant laboratory (22°C), for the time trial, where no external motivation was available. Subjects were aware of the current speed and distance covered on the Velodyne, and were therefore able to pace themselves accordingly.

Drinking Regimen

Each subject participated in three trials, one week apart, during which he received one of three different drinks per trial. Trial order was randomised, and all experiments were conducted in a single blind manner, with only the investigator aware of drink content.

Immediately preceding each trial, subjects received a 400 ml drink bolus, and then drank a further 100 ml every 15 minutes. In the time trial 100ml was given before starting, and a further 100 ml was given every 15 minutes until completion of the 40km time trial. The drink was supplied in all cases by the same investigator and no encouragement was given throughout the time trial.

The drinks consisted of the following:

i) **CHO** - 10% carbohydrate solution (Leppin glucose polymer v/v)
ii) **CHO/BCAA** - a 10% carbohydrate solution plus 0.156% branched-chain amino acid mixture.
iii) **BCAA** - a 0.156% branched-chain amino acid mixture.

The BCAA mixture contained the three branched-chain amino acids; valine, leucine and isoleucine in the proportion 3:2:1. This ratio was selected as it approximates the distribution of BCAA's in the blood (Frame, 1958). The drink contained, per litre, 781.5mg of val, 521.0mg of leu and 260.5mg of ile. Subjects therefore received 1.250g BCAA during the first hour and 0.625g per hour for the following three hours and the time trial. Drinks were chilled, were palatable and tasted similar enough to prevent identification. This was determined prior to the experiment in a small test using laboratory personnel (n=5). In addition, subjects were unaware of the possible drink content until completion of all three trials. The BCAA solution was sweetened with a sugar-free concentrate.

Additional Measurements

Expired air was collected from the subjects, every 20 minutes, for the determination of oxygen consumption (VO_2), and respiratory exchange ratio (RER). Ratings of perceived exertion (RPE), for general muscular fatigue was performed using the Borg scale (Borg, 1982) (Appendix B) and heart rate, recorded via telemetry on a Polar Vantage (Polar USA, Stamford, CT) cordless heart rate monitor, were noted, every 30 minutes. For the duration of the time-trial heart rate was recorded every five seconds, and this was later downloaded via a Polar interface (Polar USA, Stamford, CT) to a computer.

4.2.5 Blood Sampling.

Ten millilitres of blood per sample was drawn and this was decanted into four vacutainers containing different active ingredients. The first, containing heparin, was used for the determination of the haematocrit and haemoglobin concentration, the second containing ethylenediamine tetraacetate (EDTA) for the determination of ammonia concentration, the third, sodium fluoride for plasma glucose determination and the fourth promoted coagulation of the blood in order to obtain serum for the subsequent measurement of serum amino acids, free fatty acids, glycerol and insulin concentrations.

The heparinised blood was immediately placed on ice. The blood being used for the determination of ammonia and glucose concentrations was centrifuged at 1500 rpm for ten minutes. The supernatant for each sample was withdrawn, placed in sterile 1.5 ml reaction vials, sealed with parafilm and placed on ice. The fourth tube was allowed to coagulate at room temperature and then centrifuged at 1500 rpm for ten minutes. The supernatant was then treated as described above.

Later the same day haematocrit, haemoglobin and plasma ammonia concentrations were measured. The remaining blood was frozen at -80°C until later analysis.

4.2.6 Biochemical Assays.

Haematocrit

Whole heparinised blood was kept on ice, for between one and four hours, until haematocrit determination. For each sample two microcapillary tubes were filled, and sealed with putty at one end. Tubes were then placed in a microcentrifuge and spun at 11 500 rpm, for five minutes. Haematocrit was calculated as the ratio of red blood corpuscles to plasma volume.

Haemoglobin

Twenty microlitres of whole heparinised blood was added to 5ml of cold Drabkin's solution (Hainline, 1958) (Appendix C). This tube was then inverted a number of times and then allowed to stand for 20 minutes. Samples were then read at 540nm in the visible range on a spectrophotometer (Beckmann Instruments Inc, Brea, CA 92621). Haemoglobin concentrations were calculated by comparing resultant sample absorbances against a standard curve. The standard curve was derived, for each assay, by reading the absorbance of three standards (Sigma Chemical Company, St. Louis, MO 63178). Standards with concentrations of 50, 100 and 200 g/l were measured directly.

Plasma volume

Changes in plasma volume were calculated according to the method of Dill and Costill (1974). Plasma volume was calculated from values of haematocrit and haemoglobin concentrations, using the relations below. The subscripts B and A refer to before and after exercise respectively.

$$BV_A = BV_B \left(\frac{Hb_B}{Hb_A}\right)$$

$$CV_A = BV_A (Hct_A)$$

$$PV_A = BV_A - CV_A$$

BV = blood volume, CV = cell volume and PV = plasma volume.

Ammonia

Blood for this assay was collected into a vacutainer containing ethylenediamine tetraacetate (EDTA). Blood was mixed and centrifuged within 15 minutes. Ammonia assays were performed using a kit (#125857, Boehringer Mannheim, Mannheim, Germany). This assay was modified by placing 150µl of sample into a cuvette containing 0.75ml of buffer. This was mixed and allowed to stand for ten minutes, before reading the absorbance (A1). Six microlitres of suspension 2 (from the kit) was then added, and the absorbance was again read after ten minutes (A2), after which a further 6µl of suspension 2 was added. The final absorbance was then read after ten mins. (A3) and ammonia concentration was calculated as follows:

$$(A1 - A2) - (A2 - A3) = \Delta A_{\text{sample / blank}}$$

and

$$\Delta A_{\text{sample}} \times 959 = \text{Ammonia concentration } (\mu\text{mol / L})$$

(959 = Extinction co-efficient corrected for the dilution).

Glucose

Glucose concentrations were determined from plasma in which glycolysis had been terminated through the use of sodium fluoride. Glucose concentrations were determined in duplicate, on a glucose analyser 2 (Beckmann Instruments Inc, Brea, CA 92621). Prior to glucose concentration determination the analyser was calibrated, using an 8 mmol/l Beckmann standard (Beckmann Instruments Inc, Brea, CA 92621). The analyser was recalibrated after every 12 samples. Each sample was thawed once and thoroughly mixed prior to glucose concentration determination.

Other biochemical assays

Serum was also analysed for free fatty acid (Kit # 1383175, Boehringer Mannheim, Mannheim, Germany) and glycerol (Kit # 148270, Boehringer Mannheim, Mannheim, Germany) concentrations. Insulin concentration was determined using radio-immuno assay (Pharmacia Diagnostic, Insulin Kit, Uppsala, Sweden). Urea and creatinine concentrations were determined using a Beckmann multi-channel analyser (Beckmann Instruments Inc, Brea, CA 92621).

Calculation of TRP:BCAA ratio

This important parameter was calculated individually for each subject at each time point, as follows:

$$\text{TRP:BCAA} = \frac{[TRP]}{[VAL]+[LEU]+[ILE]} \text{ (mmol / l)}$$

4.2.7 High Performance Liquid Chromatography.

The concentrations of 17 amino acids were measured (Table 4.1) using an HPLC. Serum was collected and stored as described above at -80°C until analysis.

TABLE 4.1 *List of amino acids measured on the HPLC with their common abbreviations.*

Amino Acid	Abr.
1. Aspartate	ASP
2. Glutamate	GLU
3. Asparagine	ASN
4. Serine	SER
5. Histidine	HIS
6. Glutamine	GLN
7. Glycine	GLY
8. Threonine	THR
9. Arginine	ARG
10. Alanine	ALA
11. Tyrosine	TYR
12. Valine	VAL
13. Tryptophan	TRP
14 Phenylalanine	PHE
15. Isoleucine	ILE
16. Leucine	LEU
17. Lysine	LYS

Apparatus

A Gilson gradient HPLC system (Gilson Medical Electronics, Villiers-le-Bel, France) was used for the determination of serum amino acid concentrations. Samples were injected through a rheodyne valve #7125 (Rheodyne Inc. Cotati, CA 94928), fitted with a 20µl loop. A 250 x 4.6 mm internal diameter analytical column, pre packed with 5µm diameter Hypersil C18, was used. No guard column was used, but all samples and reagents were thoroughly filtered with 0.45µm pore size filter (Millipore Intertech Inc., Bedford, MA 01730). Detection of derivitised amino acids was achieved with a Gilson 121 fluorometer, excitation wavelength 380nm and emission cut-off filter at 450nm. Data was analysed using Gilson software (Gilson Medical Electronics, Villiers-le-Bel, France) on a microcomputer.

Reagents

All standard amino acid powders were obtained from Sigma Chemical Company (St. Louis, MO 63178). Acetonitrile, methanol, proprionic acid (HPLC grade) and di-

sodium hydrogen phosphate (uniVAR grade) were obtained from Saarchem Pty Ltd. (Observatory, Cape Town 7925). Double-distilled, deionised water was used in the preparation of all solvents, reagents and standard solutions.

OPA/MCE REAGENT

This reagent (# P-0532) was bought already constituted from Sigma Chemical Company (St. Louis, MO 63178). This reduced inter-assay variability due to possible differences in make-up of the derivatising agent. The *o*-pthaldialdehyde/2-mercaptoethanol solution was stored at ± 4°C.

AMINO ACID STANDARDS

A standard cocktail solution of 17 amino acids was mixed to calibrate the HPLC. Five hundred μmol of each amino acid was dissolved in 1L of water. This solution was then divided into aliquots, placed in 1.5 ml reaction vials, sealed with parafilm and frozen at -80°C.

INTERNAL STANDARD

Methionine sulfone was selected as the internal standard. 15μmol of this amino acid was dissolved in water, and the solution was then divided into aliquots, sealed and stored at -80°C.

PRECIPITATION REAGENT

Serum protein was precipitated using an acetonitrile reagent. This reagent was made up daily by adding 10μl of mercaptoethanol to 5 ml acetonitrile.

SOLVENTS

In a gradient system two solvents are used to facilitate the separation of the components of interest. In this application the solvents are constituted as follows;

- Solvent A: water/stock sodium proprionate/acetonitrile (72/20/8 by vol.) pH=6.50 ± 0.1 at room temperature.
- Solvent B: water/acetonitrile/methanol (45/30/25 by vol.)

The stock solution contains, per litre, 350 mmol di-sodium hydrogen phosphate and 250 mmol proprionic acid (ρ=0.990-0.995 kg/L). All solvents were filtered with 0.45μm Millipore cellulose filters (Millipore Intertech Inc., Bedford, MA 01730) and degassed thoroughly in a vacuum. The gradient program is shown in figure 4.1.

Procedure

Both filtered standards and samples were stored at -80°C and thawed immediately before analysis.

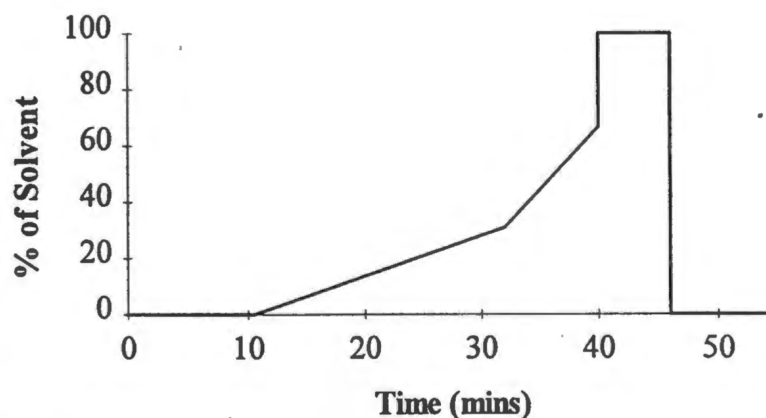


FIGURE 4.1 *The gradient profile for the determination of serum amino acid concentration. An isocratic hold of solvent A is maintained for 10.5 minutes, and then B is gradually introduced until at 40 mins. 100% of B is maintained for 5 minutes. At 45 mins. 100% of A is reintroduced, re-equilibrating the column.*

DERIVITISATION

Each sample was derivitised by adding 150μl of the acetonitrile precipitation mixture to 20μl of the sample / standard solution. 20 μl of methionine sulfone, the internal standard was then added. This was vortex mixed and centrifuged at 11 500 rpm for two minutes to remove the protein precipitate. Twenty microlitres of the supernatant was then pipetted into a separate tube and 100μl of the OPA/MCE reagent was added. This was vortex mixed for ten seconds and exactly one minute after mixing the sample was injected, using a 5 μl syringe (SGE A-rn, Victoria 3134, Australia). Three times the loop's volume was injected to ensure that the 20μl loop was completely filled. The sample was introduced slowly and evenly to prevent the creation of air bubbles, which alters sample volume. Finally, the delay between derivitisation and injection was constant, at one minute, as OPA derivatives tend to be unstable at room temperature in these solvents (Turnell and Cooper, 1982).

QUANTITATION

Amino acids are identified through their retention times relative to the internal standard, methionine sulfone. During method development a standard solution was used to identify peaks. This standard 'cocktail', containing all the amino acids in equal concentrations of 500μmol/L, produces a chromatogram with 17 peaks. The identity of each peak was, however, at this stage unknown, and an additional 17 runs were therefore required to identify each peak. In each of the 17 runs an additional 20μl of one of the amino acids was added. This 'spiking' resulted in one extra-large peak, the identity of which was known. In this way each peak in the chromatogram

was identified, and then because retention times remained constant, with respect to the internal standard, qualification was possible.

QUANTIFICATION

The area under each peak is proportional to the amount of the component in the sample, which allows the calculation of the concentration of each amino acid. Area under the curve for each peak was calculated by the Gilson Software (Gilson Medical Electronics, Villiers-le-Bel, France). A number of calibration runs were performed and from this a standard curve was produced. Using this curve the concentration of each amino acid was calculated. Concentrations of each amino acid are expressed relative to the area of the internal standard to ensure that differences in both injection volume and derivitisation are accounted for.

Operation

High performance liquid chromatography is an extremely sensitive technique. To ensure accuracy and precision it is therefore necessary to perform a calibration before attempting to analyse samples. Each calibration run was performed in the same manner as a sample run, with the exception that the sample was replaced by a standard 'cocktail' solution containing the amino acids of interest (see Table 4.1). The calibration solution was treated identically to the sample and information from the two runs is averaged and used to create a standard curve. If a coefficient of variation greater than 5% existed between two calibrations the calibration was repeated. Following the calibration a maximum of 12 samples were run before recalibration.

4.2.8 Statistics.

Samples were recorded throughout the low intensity ride and then upon completion of the time trial. Even though finishing time was different for all subjects, these are reported as 300 minutes in all graphs and tables, to allow for comparisons between subjects. Statistical tests were processed using Statgraphics statistical software package (STSC inc., Rockville, Maryland 20852), on an IBM compatible 386-SX personal computer. A two-way repeated measures analysis of variance (ANOVA) was performed to detect changes over time in biochemical, performance and psychological variables on different treatments. A one way repeated measures analysis of variance (ANOVA) was used to assess differences in cycling time between trials (Snedecor and Cochran, 1980). A paired T-test was used to determine differences between measurements pre- and post-exercise. When overall significance

occurred, a Scheffé's post-hoc test was performed to detect specific differences (Snedecor and Cochran, 1980). Statistical significance was accepted at the 5% level ($p \leq 0.05$). Levine's test for homogeneity of variance (Snedecor and Cochran, 1980) was performed on the physical performance and on the Sternberg data, to confirm that variances were equal, this being one of the assumptions for the use of parametric statistics. All data are expressed as means ± standard error of the mean. Standard error bars are consistently shown on only two of the three curves on each graph. This was done as no differences existed in any of the standard errors for any of the curves (see Appendix A for raw concentrations), and it permitted improved visualization of the data.

4.3 Results:

All Figures and raw data not included in the text may be found in Appendix A.

4.3.1 Subjects.

TABLE 4.2 *Demographic data for the eight test subjects.*

Variable	Mean ± SE
Age (yrs.)	25.0 ± 3.3
Height (cm)	182.1 ± 5.9
Weight (kg)	79.5 ± 7.0
% Fat	10.1 ± 2.1
VO_2 max (ml O_2/kg/min)	61.9 ± 4.3

Body fat % (10.1 ± 2.1%) and VO_2 max (61.9 ± 4.3 ml/kg/min) in this study group was normal for a trained athletic population (McArdle, *et al.*, 1991). None of the subjects reported a significant change in their training volumes or intensity for the duration of the study. In addition, in an attempt to standardise pre-trial intake each subject was required to eat the same dinner and breakfast the evening and morning before the trial. All subjects adhered to this directive as confirmed by pre-exercise questionnaires (see Section 4.24).

4.3.2 Amino Acids.

The serum concentration of many of the amino acids changed during prolonged endurance exercise. In addition the concentration of certain serum amino acids changed differently depending upon treatment. All amino acid concentrations are expressed as a percentage of the resting value as there were large intra and inter-individual differences in absolute resting values. By expressing the concentration as a percentage these differences are minimized and variation is reduced. Absolute values may, however, be found in Appendix A.

Serum amino acid concentrations.

NO CHANGE IN AMINO ACID CONCENTRATION

The concentrations of aspartate, histidine, phenylalanine (all in Appendix A - Table A.1), glutamine, glutamate (Fig. 4.2 B) and tryptophan (Fig. 4.2 A) did not change over time for the duration of the test. Nor were there any differences in amino acid concentration between the three trials.

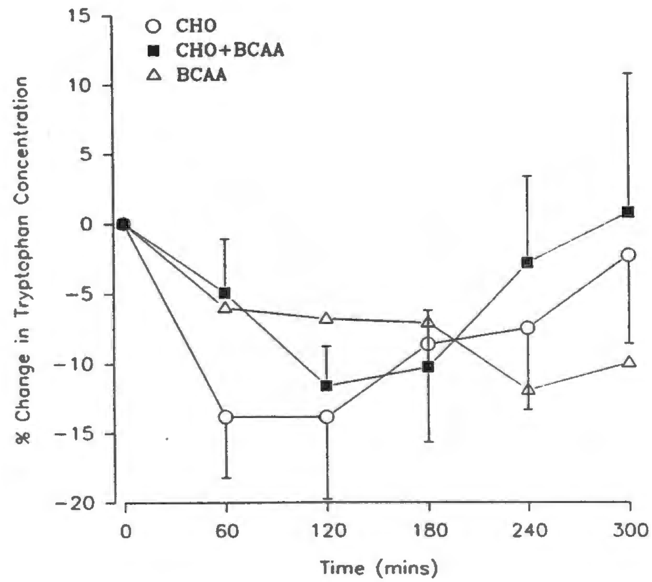


FIGURE 4.2A *The percentage change in tryptophan*

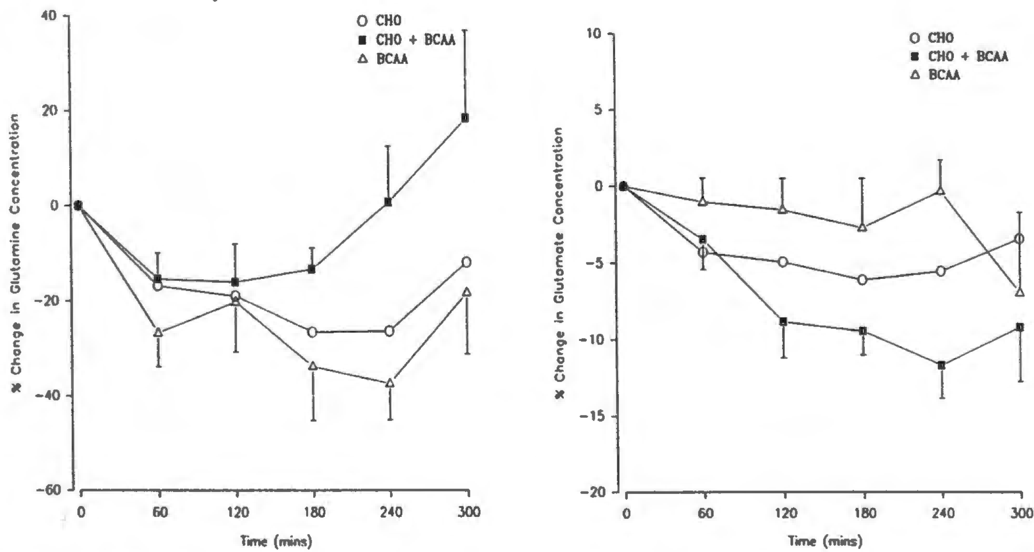
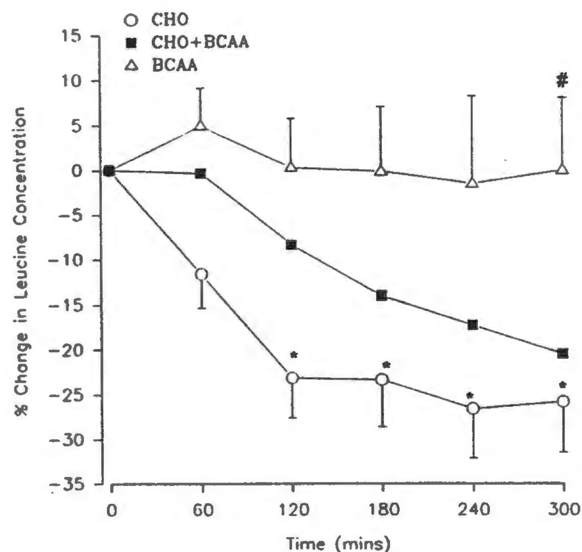
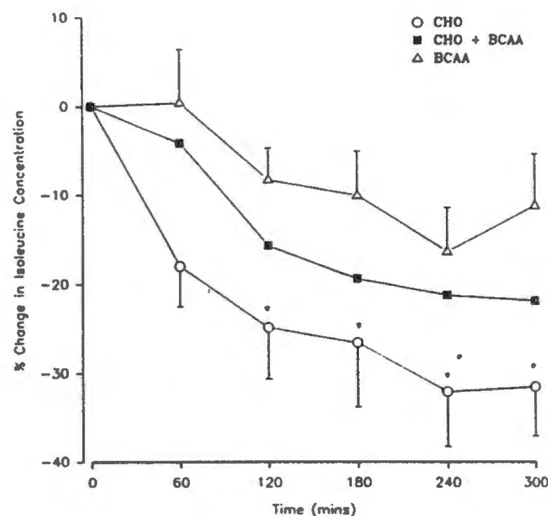


FIGURE 4.2B *Serum glutamine and glutamate concentrations do not change in any of the trials.*

INCREASE IN AMINO ACID CONCENTRATION

No significant increases in concentration were noted for any of the amino acids.





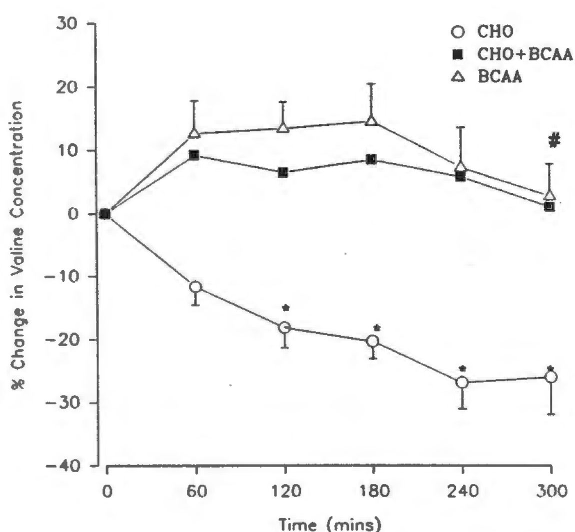


FIGURE 4.3 S*erum BCAA (leu, ile and val) concentrations during prolonged exercise. * denotes a significant difference in amino acid concentration, in the CHO trial, compared to the resting values in that trial (p≤0.01), and the # denotes a significant difference between the CHO only trial and the other two trials at 300 mins. (p≤0.05).*

DECREASE IN AMINO ACID CONCENTRATION

The concentration of asparagine, glycine, threonine, arginine, alanine, tyrosine, valine, isoleucine, leucine and lysine decreased significantly, in at least one of the three trials, over the duration of the exercise test (p≤0.05).

Arginine and tyrosine decreased significantly only in the CHO + BCAA trial, while lysine decreased in both the CHO + BCAA and the BCAA trial.

In the CHO only trial the concentrations of all three BCAA's - valine, isoleucine and leucine - decreased progressively for the duration of the trial, and this became significant at 120 mins. of low intensity exercise, in all three trials (p≤0.01). In addition, all three BCAA concentrations post-exercise were significantly lower in the CHO trial, compared to the other two trials containing the BCAA supplement. In the CHO + BCAA trials serum BCAA concentrations were not significantly different from the resting values in any of the trials.

Ratio of TRP:BCAA

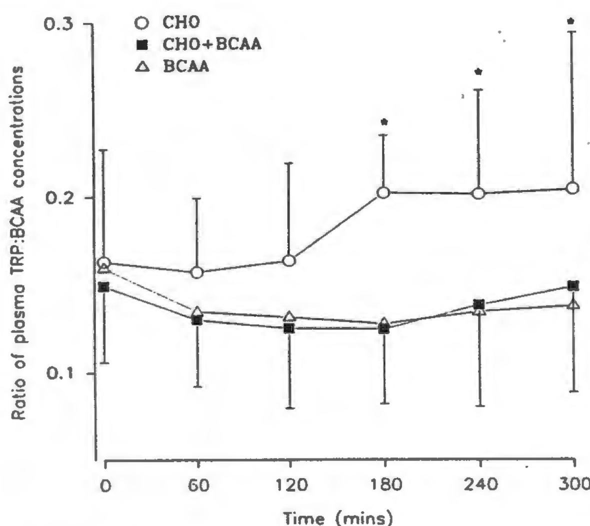


Fig 4.4 shows that the TRP:BCAA ratio increases in the CHO trial, but does not change in the CHO + BCAA and BCAA trials. This difference becomes significant at 180 minutes and this maintained for the remainder of the trial.

FIGURE 4.4 *Change in TRP:BCAA over the duration of the exercise trial. * denotes a significant difference in the CHO trial compared to resting values and to the other two trials ($p \leq 0.05$).*

4.3.3 Physical Performance

The time taken to complete a 40km time trial was not significantly different whether subjects ingested CHO only, CHO + BCAA or BCAA's only (Table 4.3). Time was measured on the Velodyne, in minutes and seconds.

TABLE 4.3 *Mean 40km time trial time under three test conditions.*

Trial	Time (mins. : secs)
CHO	68:59 ± 6:02
CHO+BCAA	68:00 ± 3.02
BCAA	69:44 ± 5.39

4.3.4 Blood Variables.

Serum amino acid and serum glycerol and ammonia concentrations displayed a large inter-individual difference. For this reason these variables have been expressed as a percentage change. However, the absolute and the normalized values have been presented for scrutiny (Appendix A).

Haematocrit and Haemoglobin

Mean resting haematocrit values were 44.3% ± 2.3%, 44.6% ± 1.2% and 45.1% ± 1.6% for the CHO, CHO + BCAA and BCAA trials respectively. Haematocrit increased significantly over the five hours of the trial, while haemoglobin concentrations increased similarly in all trials, until completion of the time trial. Haemoglobin values were used to calculate the change in plasma volume over the duration of the trial (Dill and Costill, 1974).

Plasma volume decreased similarly in all three trials. The differences between trials (-8.4 ± 0.2 CHO, -9.4 ± 0.1 CHO + BCAA & -6.3 ± 0.3 BCAA) were non-significant, and for this reason the concentrations of the blood-borne variables have not been corrected for changes in plasma volume.

Ammonia

Ammonia concentration increased significantly in both the CHO + BCAA and the CHO trial ($p \leq 0.01$). At 300 minutes plasma ammonia accumulation is significantly greater in the CHO and CHO + BCAA trials compared with the BCAA trial ($p \leq 0.01$). The ammonia values in the CHO and the CHO + BCAA trials were also significantly greater than the resting values following the time trial ($p \leq 0.01$).

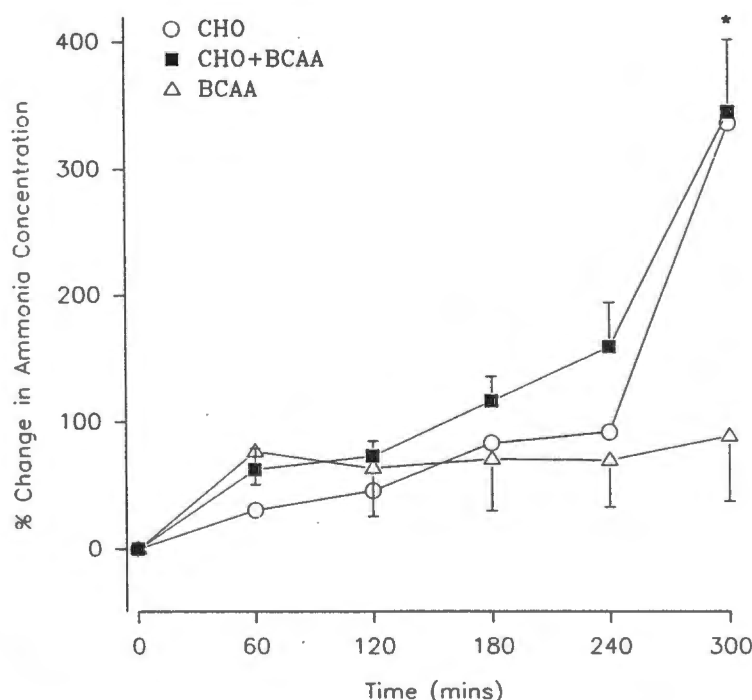


FIGURE 4.5 *Percentage change in plasma ammonia concentration. Values are calculated as a percentage of the resting values. * denotes a significant difference compared to the BCAA trial and to resting values ($p \leq 0.01$)*

Glucose

Glucose concentration remained constant throughout submaximal exercise, for all three trials, but rose significantly , compared to resting values, in the CHO and CHO + BCAA trials following the time trial ($p \leq 0.01$). Also, glucose concentration was significantly greater in the CHO and CHO + BCAA trials than that in the BCAA trial at 300 mins. ($p \leq 0.05$).

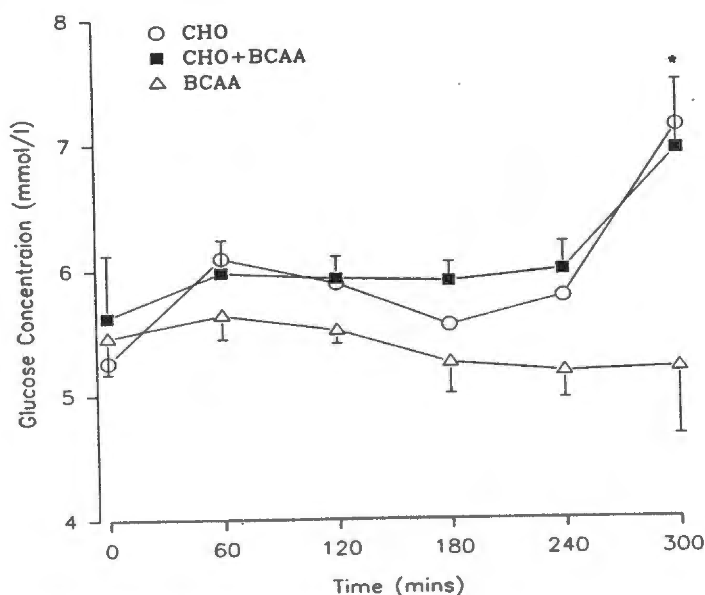


FIGURE 4.6 *Plasma glucose concentration. * denotes significant increases above resting values ($p \leq 0.01$), and above the BCAA trial ($p \leq 0.05$).*

Insulin

The insulin concentrations decreased similarly from resting values for all three trials, becoming significant at 120 minutes ($p \leq 0.05$). No differences exist between any of the trials for the decline in insulin concentrations.

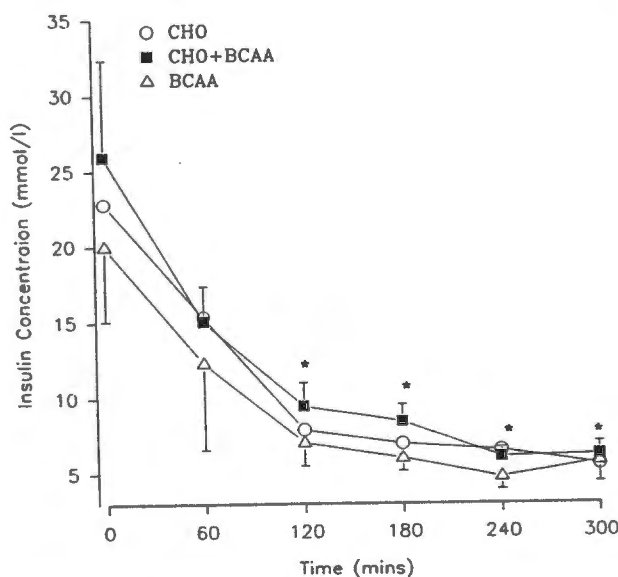


FIGURE 4.7 *Serum insulin concentration decreased significantly in all three trials. * denotes a significant difference in all three trials compared to resting values.*

Free Fatty Acids

Serum free fatty acid concentrations increased with time ($p<0.05$), in all three trials compared to resting values (Fig 4.8). Following the time trial FFA concentrations in the BCAA trial were significantly higher than those in the CHO and CHO + BCAA trials ($p<0.01$).

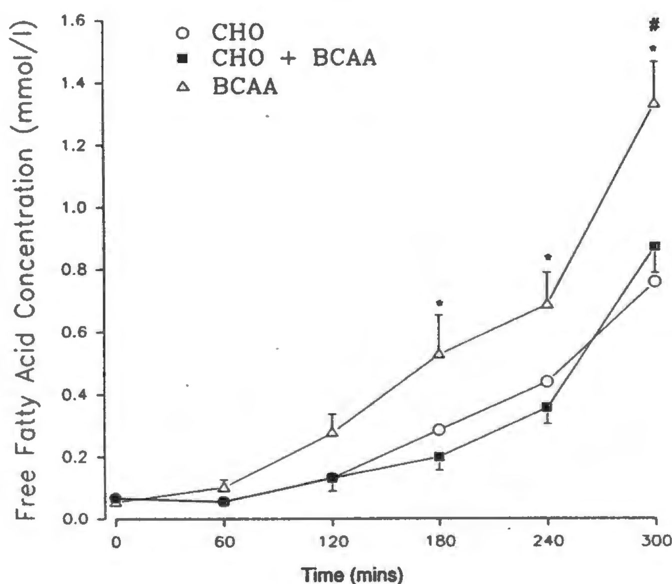


FIGURE 4.8 *Serum free fatty acid concentrations. * indicates a significant difference between FFA's in all three trials compared to resting values ($p\leq0.05$). # denotes significance at 300 mins. between the BCAA trial and the other two ($p\leq0.01$).*

Glycerol

The percentage change in serum glycerol is shown in Fig 4.9. From 180 minutes onwards glycerol levels increased above resting values, in the BCAA trial only ($p\leq0.01$). Glycerol concentrations did not change in the CHO and CHO + BCAA trials, with regard to resting values. The percentage change in glycerol was also significantly increased in the BCAA trial, compared to the other two, at 300 mins. ($p\leq0.05$).

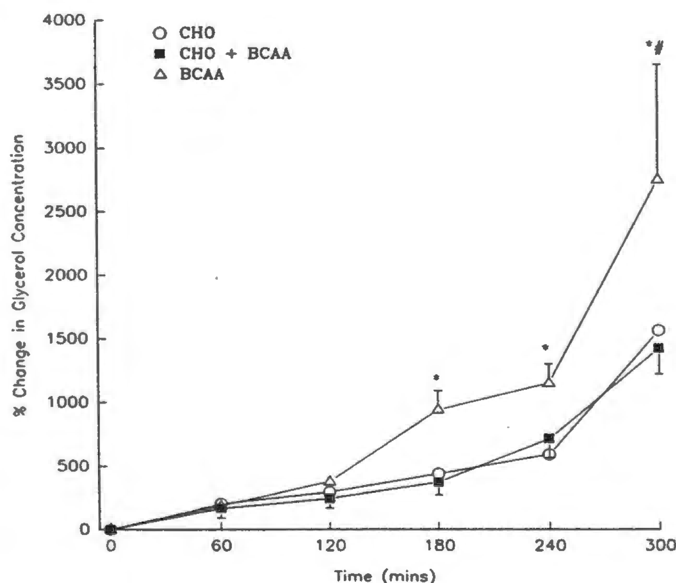


FIGURE 4.9 *The percentage change in glycerol concentration. * indicates a significant difference in all three trials from resting values ($p\leq0.01$), and # indicates a difference between the BCAA trial at 300 mins. compared to the CHO and CHO + BCAA trials ($p\leq0.05$)*

Urea and Creatinine

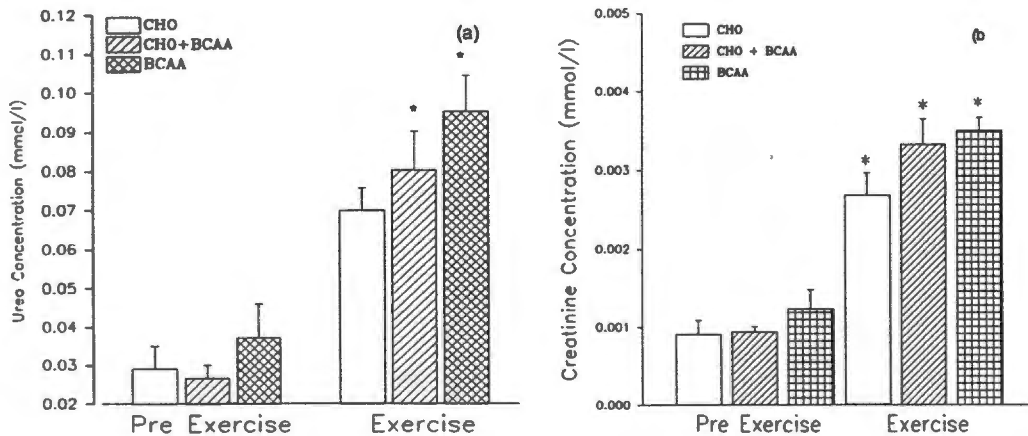


Figure 4.10 *Urea and creatinine levels in urine. * indicates significance between pre- and during exercise values (p≤0.05).*

The urinary urea and creatinine concentrations were significantly increased in the exercise sample compared to the pre-exercise sample. The increase, for urea, was significant for the CHO + BCAA and BCAA trials only (p≤0.05). While urinary creatinine concentration increased significantly in the exercise sample, in all three trials. No differences occurred between the three trials for either urea or creatinine.

4.3.5 Sternberg Test .

The response to the test sequence is either 'true' (test digit appeared in the sequence) or 'false' (digit does not appear). It has been shown that there is no difference in reaction time, between 'true' and 'false' responses (Smith and Langolf, 1981), and data from these has been combined.

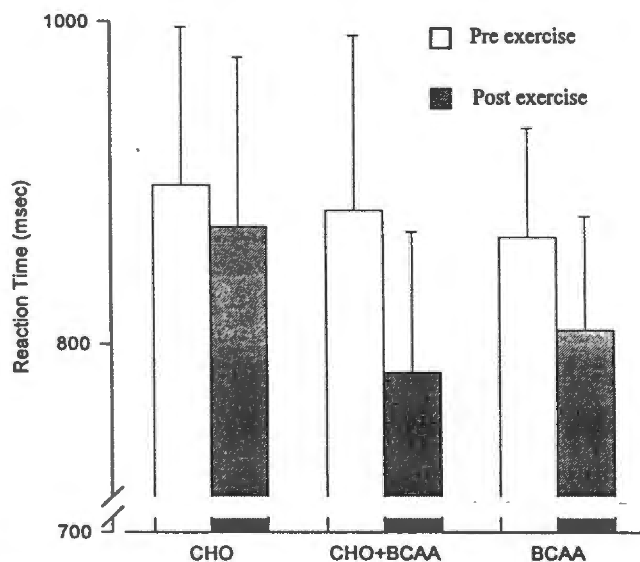


FIGURE 4.11 *Changes in reaction time following prolonged exercise. Data shown is for time taken to react to a series containing five digits (see text).*

The change in reaction time is the only parameter of the three discussed previously (memory scanning time - see pg. 46) that changes

exercise), and this parameter was measured both pre- and post-exercise. Fig 4.11 shows the effect of prolonged exercise on reaction times, which can be interpreted as short-term memory. Pre-exercise reaction time is indicated with an open bar, while reaction times following the time trial are shown with hatched bars. No significant differences were found between the pre- and post-exercise mental performance on the Sternberg reaction-time test. There does however appear to be a trend towards a faster reaction times post-exercise, especially in the BCAA-containing trials, see Fig 4.11. The large inter-individual differences, produce large standard deviations, which prevents the data from reaching significance. As described by Smith and Langolf (1981) the error rate was very low, with all subjects making fewer than 3 mistakes per 60 item sequence (5%). However, one must be cautious in interpreting results from a small population (n=8) as large inherent differences in scanning time exists between individuals.

4.3.6 Cardiorespiratory Data.

Oxygen consumption (%VO_2 max) was similar throughout the submaximal exercise for all trials (Fig 4.12). VO_2 fluctuated between 48 and 59% of VO_2 max, however, this difference was non significant between trials over the duration of the trial.

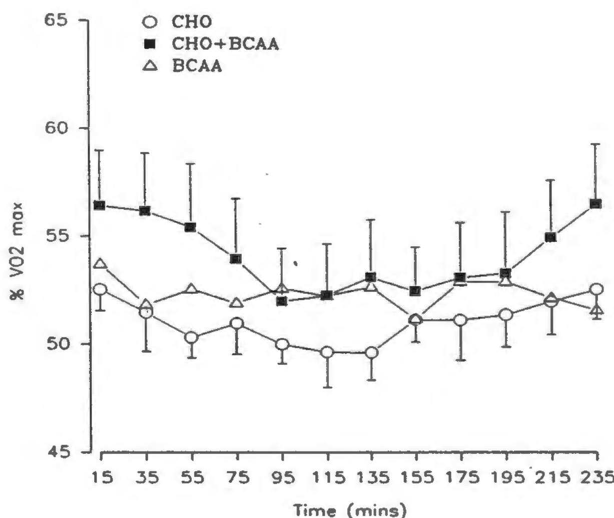


FIGURE 4.12 *Oxygen consumption throughout the low intensity trial. Values are a percentage of the VO_2 max.*

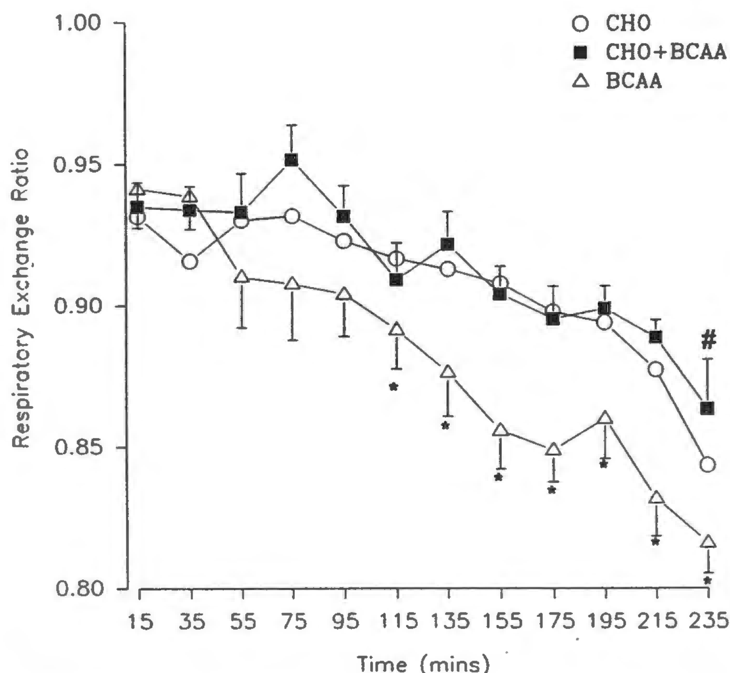


FIGURE 4.13 *The respiratory exchange ratio measured for the duration of the low intensity exercise bout. * indicates a significant decrease in RER for the BCAA trial compared to rest and to the CHO and CHO + BCAA trials($p \leq 0.05$), and # indicates a significant decrease compared to resting values, at 235 minutes in the other two trials ($p \leq 0.05$).*

Respiratory exchange ratio

The respiratory exchange ratio (RER), measured every twenty minutes for the duration of the low intensity exercise, but not during the time trial, was significantly lower in the BCAA trial, compared to the CHO + BCAA and CHO trials, after 115 minutes (Fig 4.13). Also at 115 minutes RER had decreased significantly from the resting values, in the BCAA trial. A significant decrease was only achieved in the other two trials by 235 minutes.

Rating of perceived exertion

Perceived exertion was measured in both the low intensity exercise and in the time trial. RPE increased throughout exercise, in all three trials (Fig 4.14). Perceived exertion increased above resting values after 180 minutes in all three trials, and increased further in the time trial, in all three treatments. There was no difference in RPE, between trials, that is as a result of ingested drink.

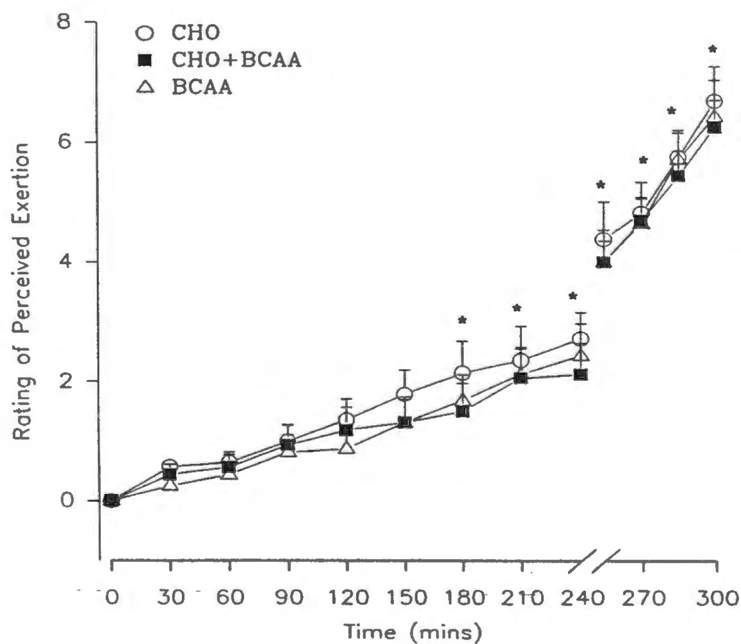


FIGURE 4.14 *RPE in both the low intensity exercise and time trial. The break in the y-axis indicates the transition to the time trial. * indicates a significant increase in RPE in all three trials compared to resting values ($p \leq 0.05$).*

Heart rate

The heart rate was similar for each treatment and ranged between 121 bpm and 136 bpm. Heart rate at 215 minutes, increased significantly above the first measured heart rate at fifteen minutes ($p \leq 0.05$), for all three trials. Heart rates, between treatments, were similar at each time point (Fig 4.15). In addition, no significant differences occurred for the average and maximal heart rates achieved during the time trial (Fig 4.16).

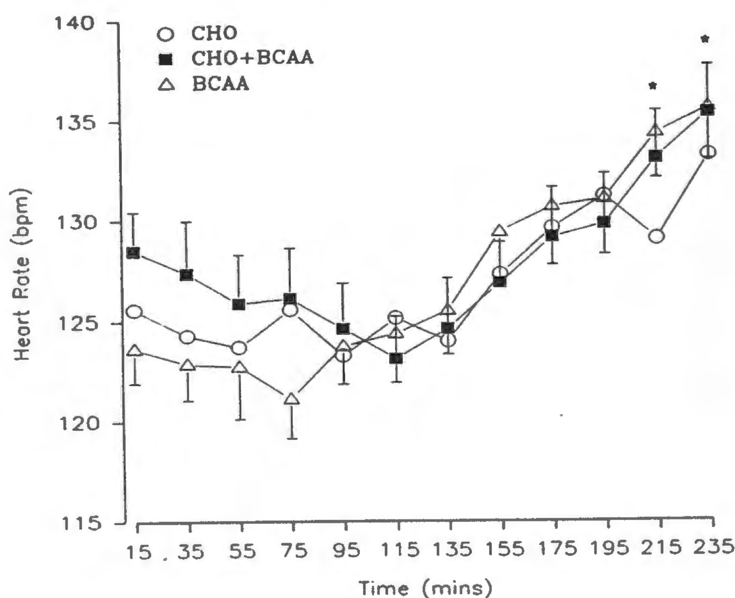


FIGURE 4.15 *Heart rate during the low intensity trial, was measured every 20 mins.. * indicates a significant increase compared to the first reading at 15 mins. (p≤0.05).*

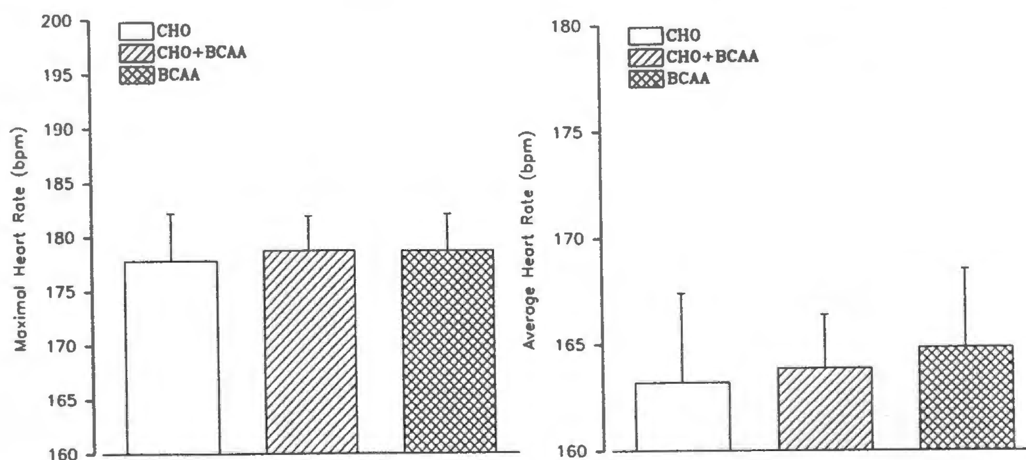


FIGURE 4.16 *Average and maximal heart rates during the time trial.*

4.4 Discussion:

The aim of this study was to investigate the hypothesis that an increase in the serum tryptophan to branched-chain amino acids ratio during prolonged exercise might impair physical performance, through the development of central fatigue. None of the previous studies investigating this hypothesis in humans, has been sufficiently rigorous to test the hypothesis completely. In addition, we attempted to quantify mental performance both pre- and post-exercise, as it has previously been shown that mental performance improves following the ingestion of a BCAA supplement (Blomstrand *et al.*, 1991).

Exercise induced change in TRP:BCAA ratio

In order to test the hypothesis that an increased serum tryptophan could lead to central fatigue, the exercise had to be designed so that the serum TRP:BCAA ratio increased, producing a increased serum tryptophan, following prolonged exercise. It was also important that the TRP:BCAA did not change following ingestion of a BCAA supplement, so preventing an increase in the cerebral tryptophan concentration.

This was achieved as the TRP:BCAA ratio in the CHO only trial increased significantly (Fig 4.4), suggesting an increased tryptophan transport into the brain (Blomstrand *et al.*, 1989; Parry-Billings *et al.*, 1990). In the CHO + BCAA and BCAA trials however, the TRP:BCAA ratio did not change, for the duration of these trials. This suggests that an increased tryptophan transport into the brain occurred, resulting in an increased brain serotonin concentration in the CHO only trial but not in the other two.

The change in TRP:BCAA was brought about by a change in the serum BCAA concentrations only, as total serum tryptophan concentrations did not change in any of the three trials (Fig 4.2). Average serum BCAA concentrations fell by between 26% (leucine) and 31% (isoleucine) (Fig 4.3) in the trial where a 10% carbohydrate-only (CHO) supplement was ingested ($p \leq 0.05$).

Total serum tryptophan was measured in this study as there is still a degree of doubt as to whether it is serum total or free tryptophan that determines the passage of tryptophan into the brain (Knott and Curzon, 1972; Fernstrom *et al.*, 1976; Chaouloff *et al.*, 1986). Previous studies investigating tryptophan metabolism, have measured either free or total serum tryptophan concentration with different results. For

example, Chauoloff *et al.* (1986), measured plasma total tryptophan concentration, following prolonged exercise in rats. No change in total TRP:BCAA (0.30 to 0.28) occurred, but brain tryptophan concentrations increased by 80%. In another rat experiment, Blomstrand *et al.* (1989), demonstrated a 2 to 3- fold increase in plasma free tryptophan with an associated increase in brain serotonin concentration and metabolism. Similar results have been seen in humans (Davis *et al.*, 1992), where these authors measured both free and total tryptophan concentrations, following exercise, in a protocol similar to that of the present study (cycle at 68% VO_2 max for up to 255 mins.). A five- to seven-fold increase in plasma free tryptophan concentration occurred while serum total tryptophan did not change. The plasma free TRP:BCAA increased seven-fold in the placebo trial.

It can be inferred from these data that the change in the TRP:BCAA ratio is in fact an underestimation of the change in the free TRP:BCAA ratio in this study which occurred in the present study.

Physical performance

Despite an increased serum TRP:BCAA in the CHO trial, the time taken to complete a 40km time trial was not different compared to the other trials where serum TRP:BCAA did not change.

There are two general explanations for the lack of an improved physical performance in this study:

i) either the thesis hypothesis is invalid and an increase in serum TRP:BCAA, and consequent increased cerebral serotonin concentrations, does not decrease power output following prolonged endurance exercise, or,
ii) the protocol was not appropriate to test the hypothesis.

These data suggest that the protocol did induce the necessary changes in serum amino acid concentrations (fig 4.4). In the CHO trial total BCAA concentration decreased by approximately 30% and the serum TRP:BCAA ratio increased in the CHO trial (25.13%), while the ratio decreased in the CHO + BCAA (-0.48%) and in the BCAA trial (-13.66%). It has been suggested however that a difference of 20-30% in the BCAA concentration is insufficient to test the hypothesis fully, and a change of between 8- and 16-fold in the serum TRP:BCAA ratio is required to induce changes in cerebral serotonin (Davis, personal communication). To resolve this issue

therefore, an exercise protocol is required that would induce such large changes. On the basis of this it is not possible to invalidate the hypothesis.

If we assume that the protocol did induce a sufficiently large decrease in the BCAA concentrations to increase the serum TRP:BCAA ratio, then the reason for the lack of a physical or mental performance difference, is the result of an invalid hypothesis. This could be due to:

i) In spite of an increased serum TRP:BCAA, brain serotonin concentration did not increase, or
ii) Central fatigue did not result from an increased cerebral serotonin concentration, or
iii) The nature of the high intensity exercise overrides the central fatigue caused by an increased cerebral serotonin concentration.

Number (iii) can be disproved from data in this study, as there is no difference in the RPE for the duration of the low intensity exercise. If the high intensity exercise that was responsible for the lack of a response, it could be expected that the RPE would be higher in the CHO only trial compared to the other two. This was however not the case as seen in Fig 4.14.

It must therefore be concluded that either;
i) the change in the serum TRP:BCAA ratio was insufficient to cause an increase in brain serotonin,
ii) central fatigue did not result from an increased cerebral serotonin concentration.

Further research is required to determine which of these scenarios is responsible for the results obtained in this study.

Mental performance

Since no test has been designed specifically for the measurement of mental fatigue (Hosstetler, personal communication), the Sternberg reaction-time test was used. This test measures mental performance and it was assumed that any decrease in mental performance would reflect the presence of mental fatigue.

Reaction times on the Sternberg test did not change following the prolonged exercise, and we must conclude that mental performance did not change as a result of the exercise. However large inter-individual differences may have masked changes in

reaction time (See section 4.24). Blomstrand *et al*, (1991), showed an improvement in mental performance post-exercise, following BCAA ingestion. These authors, however, made use of a different test (the Stroop colour and word association test) which does not measure reaction time. We chose the Sternberg test in this study as a measure of mental performance as we believed it would be more sensitive to the small changes in mental performance expected, post-exercise. In the study of Blomstrand *et al*. (1991) the mental performance test was only conducted between one and two hours after a standard marathon of 42.2 km. In the present study, however, the test was conducted immediately (within 5 minutes) after the time trial. The differences in the design of the two studies may explain the differences in the results.

Improved mental performance following BCAA ingestion

Only one other study has previously shown an ergogenic effect of ingested BCAA's during exercise (Blomstrand *et al*., 1991). This study differed from the present study in that subjects ran a standard marathon (42.2km), and the performance criteria was the time taken to complete the race, which in all cases was less than 3½ hours. Unfortunately the authors arbitrarily subdivided the experimental group into slower and faster runners, and improvement was only seen in the slower runners. This arbitrary division has no foundation and the results are therefore disputable.

We may speculate that the slower runners in the study of Blomstrand *et al*. (1991) were less highly trained than the faster runners. This would have influenced the results as the state of training significantly influences the oxidation of BCAA's (Einspahr and Tharp, 1989). Dohm *et al*. (1977) showed that the enzymes involved in leucine oxidation, in rat muscle, adapt to endurance training in a manner similar to those of fat and carbohydrate catabolism, that is activity of these enzymes increase and BCAA oxidation is increased. Moreover, the trained rats had a less positive nitrogen balance than control rats, indicating that increased protein breakdown occurs in trained rats. This is presumably an important training adaptation in which amino acids are able to provide an alternative energy source (Dohm *et al*., 1977).

Trained athletes exhibit a number of important changes with regard to the metabolism of BCAA's:

i) The oxidation of BCAA's in trained subjects is higher due to an increased uptake into the brain which is controlled by an increased enzyme activity, but also by changes in insulin concentration and by skeletal muscle uptake, which

in turn is regulated through muscle enzyme activity (Henderson *et al.*, 1985). However the concentration of the three BCAA's is significantly higher in the plasma of trained compared to untrained individuals (Einspahr and Tharp, 1989), and the TRP:BCAA ratio is therefore not necessarily altered, and

ii) The influence of the enhanced muscle enzyme activity is usually overshadowed by the practice of carbohydrate loading. Typically this is practiced by the majority of endurance athletes prior to major competition (McArdle *et al.*, 1991). The intention of this practice is to maximally load the muscle with glycogen to prevent the development of hypoglycaemia during competition. A major effect of this "carbo loading" is to reduce the activity of the BCKA dehydrogenase enzyme (Wagenmakers *et al.* 1991), which influences the degree to which BCAA's may be oxidised by the muscle.

In spite of there being no difference in the physical or mental performance between trials there was a significant difference in various metabolic parameters. Based on these differences it may be speculated that there might be some benefit associated with the ingestion of BCAA's, during prolonged exercise. In order to identify the potential benefits associated with the ingestion of BCAA's it is important to determine the source of energy in the three trials.

Metabolism during the trials

A surprising result was that for the duration of the BCAA trial, in spite of the absence of exogenous carbohydrate, cyclists were able to perform equally well compared to those trials where carbohydrate supplementation occurred. In the BCAA trial energy to maintain a high work rate was probably derived from a combination of the following substrates:

CARBOHYDRATE

Normoglycaemia was maintained for the duration of the exercise bout, in all three trials (Fig 4.6). The maintenance of a stable plasma glucose concentration of 5 mmol/l, was most likely due to the consumption of a pre-exercise breakfast (see appendix D).

In the CHO and CHO + BCAA trials plasma glucose concentration increased significantly following the time trial. This can be attributed to an increased liver glycogenolysis which continued post-exercise, and was reflected in the blood sample withdrawn within a few minutes after the time trial. This increase in plasma glucose concentration did not occur in the BCAA trial, possibly as a result of a decreased

hepatic glucose output. A reduced hepatic glucose output is due to an exhaustion of liver glycogen, which was utilised throughout the BCAA trial, resulting in a decreased glycogenolysis toward the end of the exercise bout. In those trials where carbohydrate supplementation occurred the exogenous CHO allowed liver glycogen sparing and hepatic glucose output was maintained towards the end of the trial.

In the BCAA trial it is likely that the majority of the plasma glucose was derived from activity of the glucose-alanine cycle in which alanine, a major gluconeogenic precursor, is produced. Alanine concentrations were the same in all three trials (Fig A.1), becoming significantly lower than resting values after 120 minutes. In order to accurately investigate the contribution of alanine to gluconeogenesis it will be important for future studies to follow changes in alanine turnover throughout the exercise bout.

At this stage we may only speculate about metabolic fluxes, because only serum concentrations were measured in this study. In the BCAA trial it is likely that an increased oxidation of BCAA's to the BCKA's occurred. Associated with this glutamate and pyruvate combine to produce α ketogluterate and alanine (Fig 2.2). Alanine is then transported to the liver, where glucose is synthesised. An increased gluconeogenesis in this trial might explain why no differences were seen in serum alanine, glutamate and glutamine concentrations. In addition, the expected increase in the ammonia concentration in this trial, due to an increased amino oxidation, may have been prevented by an increased amino acid conversion to glucose (Fig 4.5).

Serum insulin concentration decreased in all three trials post-exercise. This decline in serum insulin during prolonged exercise is expected (McArdle *et al.*, 1991). The decline in serum insulin, during exercise, is mediated through an increased α-adrenergic input to the β cells of the pancreas (McArdle *et al.* 1991), which overrides the usual stimulatory effects of plasma glucose and amino acid concentrations. Davis *et al.* (1992) found a gradual decrease in the insulin concentration over time, but insulin concentration was significantly higher in the two carbohydrate trials (subjects ingested 6% and 12% CHO solutions), compared to the placebo trial. In the CHO trials a significant decrease in the plasma concentration of the BCAA's occurred following the ingestion of two carbohydrate solutions (6% and 12%). These authors suggest that an increased insulin concentration is responsible for the increased uptake of amino acids. In the present study, though, insulin concentration did not differ significantly in any of the three trials, suggesting that another mechanism may be responsible for the decreased serum BCAA concentrations.

The discrepancy in the insulin response in this study and the study of Davis *et al.* (1992) may be related to the fact that subjects in this study where fed, while those in the study Davis *et al.* (1992) were fasted. Inconsistencies between the two studies did not appear to be to related to differences in exercise intensity (insulin decreased to a minimum during the low intensity exercise), or in exogenous carbohydrate supply, which was similar in both trials (10% in the present study vs. 6% & 12% in Davis *et al.* 1992).

It may be that some carbohydrate is required to oxidize amino acids, as a similar phenomenon has been shown to occur in fat oxidation where endogenous FFA's are not oxidised unless carbohydrate is available. It therefore appears that both fat and amino acid oxidation requires the presence of carbohydrate.

FAT

In the BCAA trial, endogenous fat stores were used to a greater extent to provide energy, compared to the other trials. This is evidenced by a higher serum free fatty acid (Fig 4.8) and glycerol (Fig 4.9) concentration in this group.

This is consistent with data from Davis *et al.* (1992) where FFA concentrations in the placebo trial increased significantly compared to the other two trials. In addition, RER in the present study was significantly lower in the BCAA trial ($p \leq 0.05$, Fig 4.13), compared to the other two trials, indicating a greater FFA oxidation.

PROTEIN

Serum BCAA concentrations in the CHO trial had fallen by more than 10% after the first hour of exercise (Fig 4.3). This decrease, in a trial where no exogenous amino acids were supplied, suggests that BCAA's are oxidised to some extent, even when alternate substrates are available. These amino acids contribute a certain amount of energy to maintain the work rate throughout exercise (Dohm *et al.*, 1977; Brooks, 1987). It has also been proposed that oxidation of amino acids serves a number of functions (see section 2.21), and for this reason they may still be oxidised in the presence of carbohydrate.

AMMONIA

The changes in plasma ammonia concentration are difficult to explain. Since the concentration of plasma ammonia, and not turnover, was measured it is not possible

to report on the rates of ammonia production and utilisation. In spite of this it appears that the ammonia concentration may provide some important clues concerning BCAA metabolism during exercise in trained individuals.

Plasma ammonia concentration following the time trial, increased significantly in both the CHO and CHO + BCAA trials, but not in the BCAA trial (see Fig 4.5). Because amino acids are the only source of exogenous energy in the BCAA trial, and as one of the by-products of amino acid oxidation is ammonia, it would be reasonable to assume that plasma ammonia concentrations would increase more in the BCAA trial, compared to the other two trials. This, however, did not occur.

Because plasma ammonia concentration is proportional to exercise intensity, a possible explanation for the lower plasma ammonia concentration in the BCAA trial might be that subjects were not able to cycle at a high intensity in this trial, and plasma ammonia concentrations failed to increase because of the decreased exercise intensity. This however did not occur, as physical performance, average and maximal heart rates were similar during the time trial, in all three trials (see Table 4.3, Fig 4.15 and Fig 4.16 (pg. 59 & 66) during the time trial. There must, therefore, have been an alteration in metabolism to account for this discrepancy.

During exercise ammonia is derived from two main sources;

i) The purine nucleotide cycle (PNC). Here the reaction that is relevant to the present study is the is the deamination of AMP to IMP with the concomitant release of NH_4^+ (Fig 4.17). It is unlikely that the ingestion of BCAA's would induce a change in the PNC (as shown in Fig 4.17), and this cannot therefore explain the differences in plasma ammonia concentration. The differences seen in Fig. 4.5 must then be explained by changes in the amino acid oxidation.

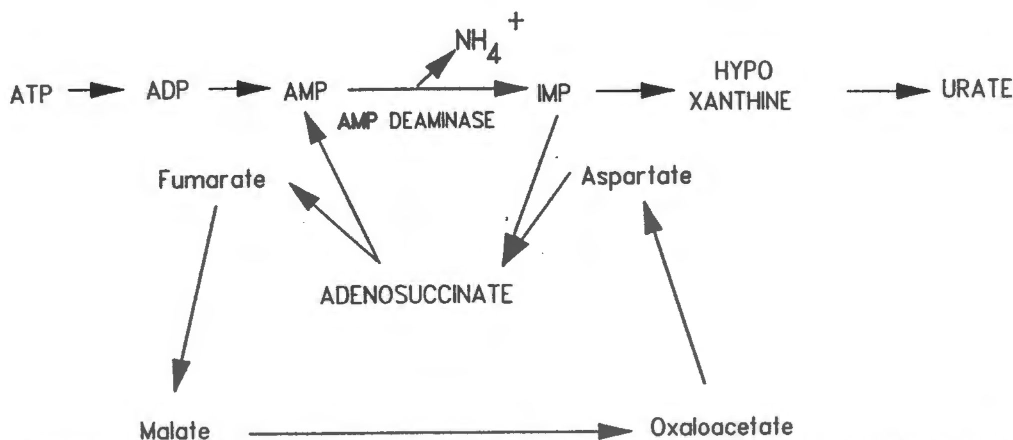


FIGURE 4.17 *A schematic representation of the formation of ammonia by the purine nucleotide cycle (Newsholme and Leech, p 384, 1984.)*

ii) In certain conditions ammonia may be derived from the oxidation of amino acids (Brooks, 1987). Ammonia production as a result of amino acid oxidation is less definite, however, and is a subject of some debate.

However, given the data in this study, it is not possible to come to any conclusions regarding metabolism of ammonia in the BCAA trial, and further research is required to identify the dynamics of the fluxes in the ammonia concentrations in the three trials.

These findings do however have practical importance as a high concentration of plasma ammonia, during exercise, has been associated with fatigue (Banister and Cameron, 1990) and muscular cramps (Green, 1987). Therefore if plasma ammonia concentrations are decreased, as they are following BCAA ingestion, then fatigue may be delayed or muscle cramps may be prevented. This is discussed in more detail later (See potential benefits of BCAA supplementation).

Changes in urinary urea

During the exercise bout urinary urea excretion increased significantly in the CHO + BCAA and BCAA trials, suggesting increased protein degradation compared to the CHO trial where urea excretion did not increase. This suggests that oxidation of amino acids is enhanced in the two trials where exogenous BCAA's were supplied. This result, however, appears to be incompatible with the ammonia data; for example urea excretion does not change during exercise whereas plasma ammonia concentrations increase 3-fold in the CHO trial, while urea excretion increases and plasma ammonia concentration remains low in the BCAA trial.

Wolfe *et al.* (1982), have proposed that the indirect measurement of protein catabolism (urinary urea and creatinine) conceals many pitfalls, and this has lead to the confusion regarding the effects of exercise on protein metabolism. As a result of this, it is not possible, using urinary urea and creatinine concentrations, to accurately determine the source of the increased excreted nitrogen.

Potential benefits of BCAA supplementation

Even though there was no improvement in physical or mental performance in this study following the ingestion of BCAA's, it appears that there might still be some benefits associated with the ingestion of a BCAA supplement during prolonged endurance exercise.

DECREASED LIKELIHOOD OF CRAMPS

The low serum ammonia accumulation following intense exercise in the BCAA trial requires more attention as Brouns and co-workers (1990) have suggested that muscle cramps, during intense exercise, seems to be related to high ammonia levels and not potassium, lactate or glycogen concentrations. Moreover, elevated blood ammonia concentration is known to cause dysfunction in the central nervous system, either by acting directly on selected centres or by altering the brain permeability to certain amino acids involved in neurotransmission (Green, 1987). Furthermore, Banister and Cameron (1990) have suggested that a consequence of high plasma ammonia concentrations is that more ammonia crosses the blood-brain barrier, causing fatigue. Data from the present study seems to indicate that all of these effects may be reduced if BCAA's are ingested during exercise, since the serum ammonia concentration does not increase as exercise duration progresses. However, most athletes ingest carbohydrate during prolonged endurance exercise, in order to delay fatigue, as this has been shown to delay the depletion of liver glycogen. Therefore, studies will have to be conducted to determine whether the advantages of a lower serum ammonia concentration outweigh the disadvantage of lower liver glycogen stores, which may occur in the absence of exogenous carbohydrate.

IMPROVED IMMUNE SYSTEM FUNCTIONING

Glutamine is the primary fuel for macrophages and lymphocytes (Parry-Billings *et al.*, 1990). Most glutamine is produced in skeletal muscle, and it has been suggested that a function of BCAA oxidation is the production of glutamine for the

maintenance of the immune system (Parry-Billings *et al.*, 1990). Prolonged exercise, in the absence of exogenous carbohydrate or BCAA supplementation, causes plasma glutamine concentrations to decline, in both rats and humans (Parry-Billings *et al.*, 1990). In the present study, however, serum glutamine concentrations were the same for the duration of the exercise, in all three trials, as previously discussed.

Parry-Billings *et al.* (1990) propose that the decrease in plasma glutamine following repeated bouts of intense training may be responsible for the immunosuppressive effects of exercise. This hypothesis has implications for the overtrained athlete, where complete recovery does not occur following intensive training. This syndrome is difficult to quantify, and it is possible that the measurement of plasma amino acid concentration may aid in confirming a diagnosis of the overtrained athlete. Indeed, Parry-Billings *et al.* (1990), has demonstrated that the plasma glutamine concentrations are lower in overtrained athletes compared to trained athletes. It is further speculated that the decreased glutamine concentrations in the plasma of overtrained athletes may be linked to some of the symptoms of overtraining, for example, upper respiratory tract infections and impaired wound healing (Parry-Billings *et al.*, 1990). The change in glutamine concentrations, during and after prolonged exercise, should be studied further as it might provide an important application for the ingestion of BCAA's in overtrained athletes who have compromised immune systems.

Summary

This study has investigated the implications of consuming a branched-chain amino acid solution during prolonged exercise. Serum BCAA concentrations decreased, as expected during prolonged exercise when carbohydrate alone was ingested, causing an increase in serum TRP:BCAA. However, after ingesting a BCAA supplement without carbohydrate, serum BCAA concentrations and TRP:BCAA ratio did not change. Neither cycling performance in a 40km time trial, or mental performance as measured by the Sternberg test was different in this trial compared to the CHO + BCAA or CHO trials

The results of this study illustrate that in the absence of exogenous carbohydrate a greater proportion of fats were oxidised for energy during exercise. It is also likely that a greater proportion of amino acids were oxidised in the BCAA trial, to provide energy during low intensity prolonged exercise, followed by a short intense performance trial. This is in itself surprising because it is well accepted that exogenous carbohydrate prolongs time to fatigue. Data from this study suggests that

the ingestion of a BCAA supplement, sufficient to prevent the decline in serum amino acid concentration, did not delay central fatigue sufficiently to improve physical or mental performance. The BCAA supplement did however permit subjects to perform equally well in the 40km time trial, compared to when they ingested carbohydrate supplements, which is a surprising result.

It may be concluded that:

i) The thesis hypothesis is invalid, or

ii) The TRP:BCAA did not increase enough to increase cerebral serotonin sufficiently to have any affect on physical and mental performance, in the CHO only trial.

With the data from this study we are unable to satisfactorily resolve these questions, and it is with these conclusions in mind that future research should be directed.

4.5 Directions of future research:

Although, the nature of the interaction between BCAA's and exercise is now well understood (Acworth,1986, Block and Buse, 1990, Blomstrand *et al.*, 1988, Blomstrand *et al.*, 1989, Brodan, 1976, Décombaz *et al.*, 1979, Einspahr and Tharp, 1989, MacLean *et al.*, 1991, Newsholme *et al.*, 1991), certain questions remain unanswered.

i) It was speculated in this study that the TRP:BCAA change may not be sufficient to produce a large enough change in cerebral serotonin concentrations. Therefore a study must be conducted where increasingly larger doses of BCAA's are supplemented producing systematically larger changes in TRP:BCAA.

ii) The stable serum ammonia concentration after prolonged exercise in the BCAA trial has practical importance. Therefore a study investigating the rates of ammonia utilisation and production, following BCAA supplementation is required.

iii) Serum glutamine changes, after prolonged exercise, are also important as decreases in this amino acid may cause many symptoms of overtraining. If it can be shown that serum glutamine concentrations increase following BCAA

supplementation, it would prove to be an important therapy for overtrained athletes.

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Appendix A - Figures and Raw Data not included in text

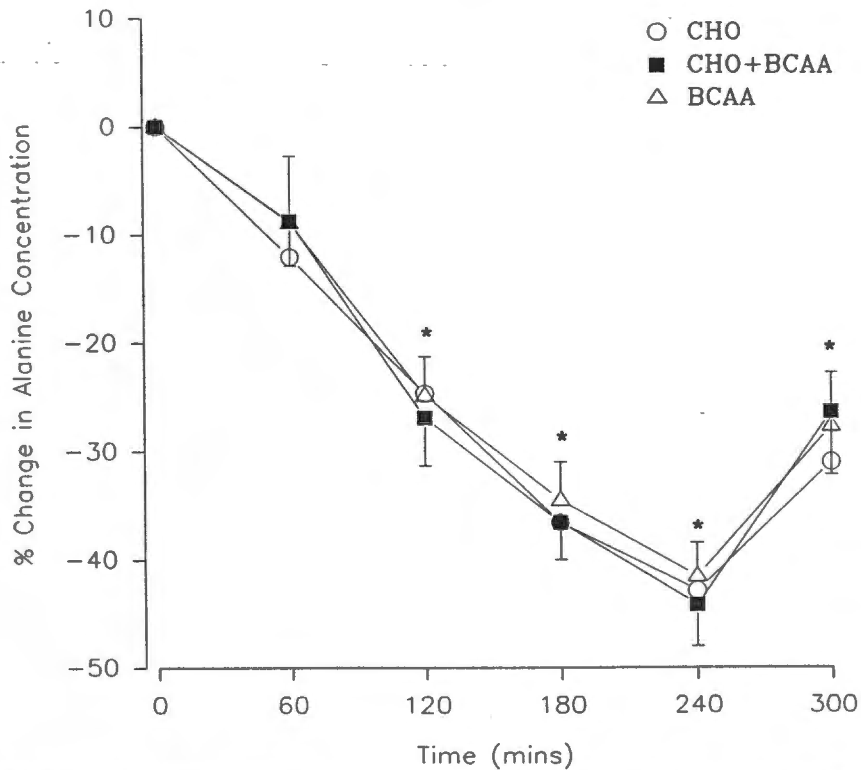

FIGURE A.1. *Percentage change in alanine concentration. Alanine concentration decreases in all three trials, and this becomes significant at 120 minutes (* = p≤0.05).*

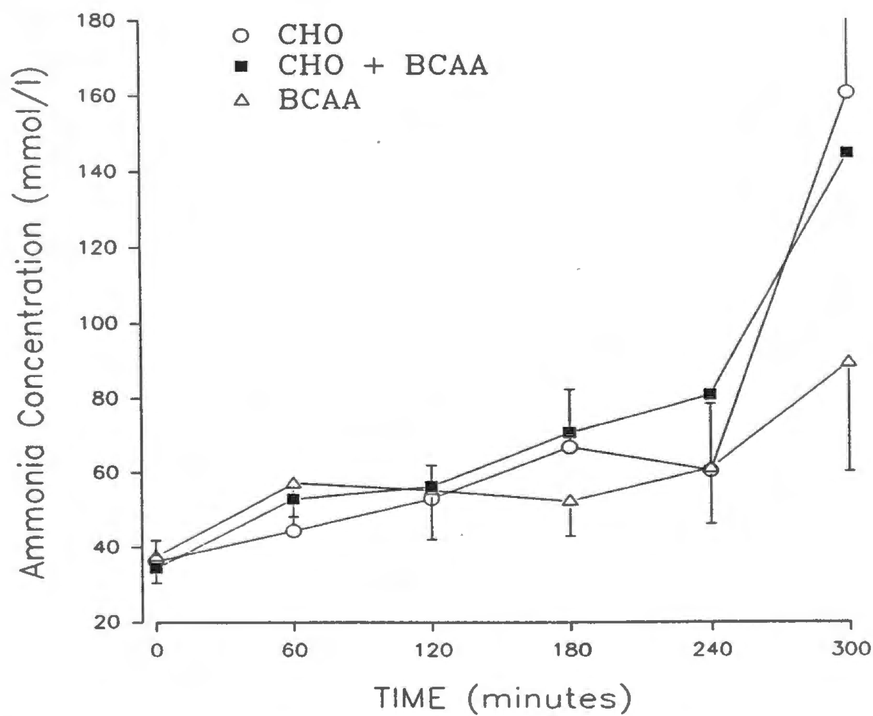

FIGURE A.2 *Absolute change in ammonia concentrations.*

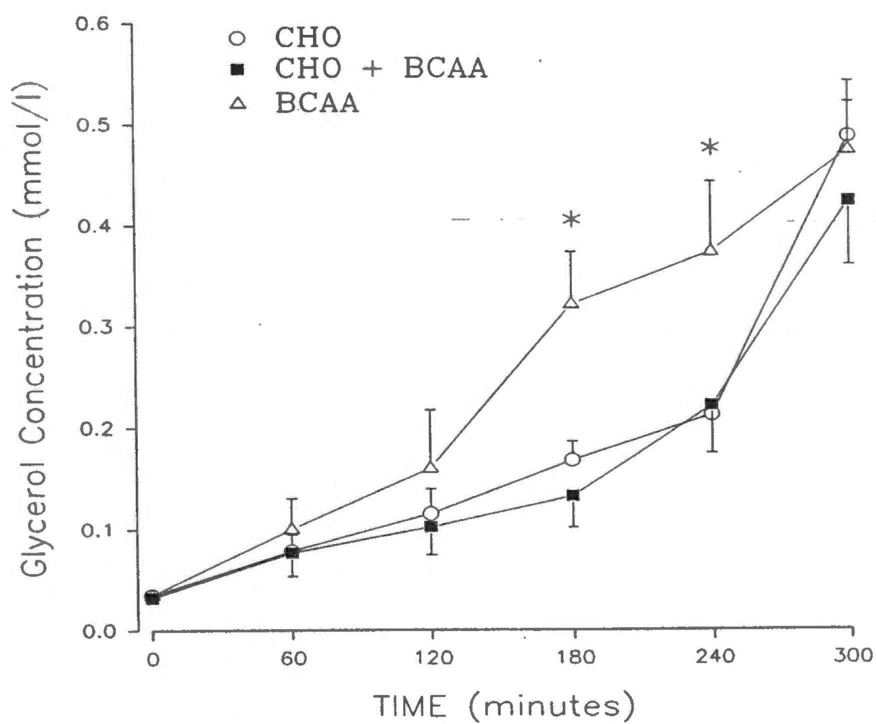

FIGURE A.3 *The absolute change in glycerol concentration. * indicates a significant difference in glycerol concentration in the BCAA trial compared to the two other trials p≤0.05).*

TABLE A.1 *Plasma amino acid concentrations (mmol/l) throughout a five hour cycle. Amino acid concentrations were measured in eight subjects completing three trials, while ingesting different drinks.*

Trial	Amino Acid	Time (minutes)					
		0	60	120	180	240	300
CHO	ASP	0.019 ± 0.012	0.013 ± 0.006	0.015 ± 0.008	0.013 ± 0.003	0.011 ± 0.004	0.013 ± 0.004
	GLU	0.034 ± 0.009	0.023 ± 0.004	0.022 ± 0.005	0.023 ± 0.006	0.022 ± 0.005	0.027 ± 0.008
	ASN	0.053 ± 0.015	0.043 ± 0.008	0.046 ± 0.022	0.037 ± 0.006	0.035 ± 0.003	0.032 ± 0.007
	SER	0.120 ± 0.018	0.112 ± 0.019	0.119 ± 0.027	0.106 ± 0.019	0.098 ± 0.009	0.095 ± 0.013
	HIS	0.079 ± 0.013	0.077 ± 0.019	0.071 ± 0.010	0.067 ± 0.012	0.069 ± 0.009	0.068 ± 0.010
	GLN	0.490 ± 0.059	0.472 ± 0.061	0.470 ± 0.067	0.460 ± 0.059	0.457 ± 0.058	0.475 ± 0.084
	GLY	0.234 ± 0.045	0.235 ± 0.050	0.184 ± 0.088	0.211 ± 0.045	0.198 ± 0.043	0.197 ± 0.045
	THR	0.162 ± 0.042	0.155 ± 0.040	0.119 ± 0.060	0.136 ± 0.020	0.136 ± 0.020	0.134 ± 0.028
	ARG	0.067 ± 0.014	0.058 ± 0.010	0.054 ± 0.008	0.052 ± 0.008	0.051 ± 0.009	0.054 ± 0.013
	ALA	0.475 ± 0.051	0.425 ± 0.064	0.358 ± 0.050	0.299 ± 0.046	0.269 ± 0.052	0.333 ± 0.103
	TYR	0.088 ± 0.027	0.075 ± 0.016	0.072 ± 0.012	0.066 ± 0.008	0.068 ± 0.010	0.082 ± 0.015
	VAL	0.450 ± 0.085	0.403 ± 0.071	0.373 ± 0.062	0.320 ± 0.150	0.332 ± 0.079	0.340 ± 0.085
	TRP	0.073 ± 0.016	0.063 ± 0.009	0.061 ± 0.010	0.065 ± 0.014	0.067 ± 0.013	0.069 ± 0.021
	PHE	0.082 ± 0.021	0.072 ± 0.015	0.067 ± 0.015	0.067 ± 0.013	0.066 ± 0.012	0.078 ± 0.020
	ILE	0.094 ± 0.033	0.077 ± 0.023	0.068 ± 0.017	0.068 ± 0.017	0.058 ± 0.015	0.066 ± 0.019
	LEU	0.181 ± 0.057	0.163 ± 0.045	0.134 ± 0.035	0.140 ± 0.028	0.122 ± 0.026	0.134 ± 0.041
	LYS	0.096 ± 0.026	0.081 ± 0.022	0.087 ± 0.022	0.086 ± 0.024	0.068 ± 0.007	0.071 ± 0.038
CHO+	ASP	0.010 ± 0.004	0.007 ± 0.002	0.009 ± 0.003	0.009 ± 0.003	0.011 ± 0.004	0.012 ± 0.004
BCAA	GLU	0.021 ± 0.006	0.018 ± 0.004	0.018 ± 0.004	0.018 ± 0.004	0.023 ± 0.010	0.028 ± 0.012
	ASN	0.048 ± 0.010	0.041 ± 0.008	0.036 ± 0.005	0.034 ± 0.005	0.034 ± 0.004	0.033 ± 0.005
	SER	0.120 ± 0.014	0.107 ± 0.014	0.103 ± 0.016	0.101 ± 0.015	0.098 ± 0.015	0.099 ± 0.016
	HIS	0.072 ± 0.010	0.071 ± 0.009	0.064 ± 0.008	0.064 ± 0.008	0.066 ± 0.010	0.067 ± 0.009
	GLN	0.518 ± 0.051	0.499 ± 0.051	0.471 ± 0.051	0.469 ± 0.057	0.457 ± 0.051	0.478 ± 0.062
	GLY	0.233 ± 0.056	0.221 ± 0.043	0.210 ± 0.044	0.201 ± 0.042	0.195 ± 0.034	0.165 ± 0.084
	THR	0.157 ± 0.028	0.150 ± 0.031	0.136 ± 0.028	0.130 ± 0.018	0.124 ± 0.015	0.105 ± 0.055
	ARG	0.064 ± 0.011	0.061 ± 0.011	0.055 ± 0.011	0.053 ± 0.011	0.052 ± 0.011	0.052 ± 0.013
	ALA	0.483 ± 0.063	0.438 ± 0.060	0.350 ± 0.051	0.304 ± 0.043	0.267 ± 0.042	0.358 ± 0.075
	TYR	0.084 ± 0.021	0.075 ± 0.018	0.065 ± 0.015	0.064 ± 0.017	0.067 ± 0.017	0.082 ± 0.021
	VAL	0.447 ± 0.080	0.486 ± 0.083	0.470 ± 0.059	0.477 ± 0.063	0.465 ± 0.070	0.447 ± 0.039
	TRP	0.067 ± 0.010	0.063 ± 0.009	0.059 ± 0.008	0.059 ± 0.008	0.064 ± 0.011	0.066 ± 0.018
	PHE	0.073 ± 0.014	0.066 ± 0.008	0.059 ± 0.008	0.059 ± 0.008	0.063 ± 0.010	0.081 ± 0.016
	ILE	0.094 ± 0.031	0.089 ± 0.023	0.077 ± 0.018	0.073 ± 0.018	0.070 ± 0.017	0.071 ± 0.014
	LEU	0.179 ± 0.053	0.177 ± 0.045	0.158 ± 0.027	0.149 ± 0.032	0.143 ± 0.024	0.139 ± 0.025
	LYS	0.106 ± 0.019	0.096 ± 0.015	0.075 ± 0.016	0.067 ± 0.015	0.072 ± 0.014	0.054 ± 0.026
BCAA	ASP	0.012 ± 0.005	0.010 ± 0.002	0.010 ± 0.003	0.010 ± 0.004	0.010 ± 0.004	0.013 ± 0.006
	GLU	0.028 ± 0.006	0.019 ± 0.004	0.021 ± 0.008	0.020 ± 0.008	0.017 ± 0.005	0.019 ± 0.009
	ASN	0.045 ± 0.007	0.041 ± 0.006	0.035 ± 0.004	0.033 ± 0.005	0.031 ± 0.004	0.030 ± 0.003
	SER	0.120 ± 0.013	0.111 ± 0.013	0.108 ± 0.017	0.102 ± 0.014	0.094 ± 0.013	0.090 ± 0.011
	HIS	0.074 ± 0.007	0.076 ± 0.015	0.071 ± 0.010	0.071 ± 0.008	0.074 ± 0.019	0.065 ± 0.008
	GLN	0.470 ± 0.058	0.465 ± 0.044	0.462 ± 0.045	0.456 ± 0.054	0.466 ± 0.045	0.441 ± 0.069
	GLY	0.212 ± 0.103	0.193 ± 0.085	0.210 ± 0.028	0.167 ± 0.071	0.188 ± 0.031	0.172 ± 0.031
	THR	0.134 ± 0.062	0.127 ± 0.060	0.142 ± 0.036	0.123 ± 0.057	0.131 ± 0.023	0.115 ± 0.020
	ARG	0.061 ± 0.011	0.057 ± 0.012	0.053 ± 0.008	0.050 ± 0.009	0.049 ± 0.006	0.054 ± 0.016
	ALA	0.443 ± 0.071	0.402 ± 0.060	0.331 ± 0.057	0.286 ± 0.047	0.258 ± 0.041	0.317 ± 0.057
	TYR	0.082 ± 0.018	0.073 ± 0.016	0.070 ± 0.014	0.069 ± 0.009	0.068 ± 0.010	0.078 ± 0.008
	VAL	0.461 ± 0.070	0.508 ± 0.060	0.507 ± 0.062	0.510 ± 0.062	0.481 ± 0.059	0.553 ± 0.205
	TRP	0.074 ± 0.012	0.068 ± 0.007	0.067 ± 0.009	0.065 ± 0.009	0.065 ± 0.007	0.066 ± 0.014
	PHE	0.075 ± 0.014	0.068 ± 0.009	0.065 ± 0.009	0.066 ± 0.010	0.064 ± 0.008	0.074 ± 0.011
	ILE	0.087 ± 0.027	0.085 ± 0.021	0.077 ± 0.019	0.073 ± 0.017	0.069 ± 0.016	0.073 ± 0.014
	LEU	0.174 ± 0.047	0.176 ± 0.030	0.159 ± 0.026	0.156 ± 0.025	0.157 ± 0.022	0.158 ± 0.020
	LYS	0.098 ± 0.011	0.093 ± 0.025	0.084 ± 0.019	0.078 ± 0.021	0.050 ± 0.024	0.071 ± 0.021

Appendix B - Borg Scale of Perceived Exertion

0	None
0.5	Very very little
1	Very little
2	Little
3	Moderate
4	Somewhat strong
5	Strong
6	
7	Very strong
8	
9	
10	Very very strong maximal

Appendix C - Reagents

Drabkin's solution.

- 0.2g KCN
- 0.2g $K_3Fe(CN)_6$
- 1.0g $NaHCO_3$.

This is constituted in one litre of double distilled deionised water.

HPLC reagents.

- Solvent A: water/stock sodium proprionate/acetonitrile (72/20/8 by vol.) pH=6.50 ± 0.1 at room temperature.
- Solvent B: water/acetonitrile/methanol (45/30/25 by vol.)

Appendix D - Breakfasts

Option 1

i) Banana shake, with 2 cups skim milk and 2 large bananas
ii) 4 slices whole wheat toast
iii) 3 teaspoons peanut butter

Option 2

i) 1 cup cooked oats, with 4 tablespoons raisins and half a cup skim milk
ii) 6 provitas and 3 teaspoons margarine
iii) Half tub fat free cottage cheese
iv) coffee with half a cup skim milk

Option 3

i) 1 cup fruit juice (unsweetened)
ii) 1 cup fruit salad (unsweetened)
iii) 1 cup low fat muesli
iv) 1 fat free, sugar free yoghurt
v) 1 whole wheat roll
vi) Half an avocado pear
vii) Cocoa with I cup skim milk

Each of the three breakfasts contain 823 kcal, 144g of carbohydrate, 28g of protein and 15g of fat.

The breakfasts contained 70% carbohydrate, 14% protein and 16% fat.

Each subject ate the same breakfast each morning preceding the exercise trial.