FACTORS INFLUENCING AMMONIA METABOLISM DURING PROLONGED EXERCISE IN MAN

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ABSTRACT

The studies that comprise this thesis were undertaken to obtain further information on ammonia/ammonium (NH_3) metabolism during submaximal exercise and how it might be influenced by (1) exercise intensity and duration, (2) carbohydrate ingestion and (3) branched-chain amino acid (BCAA) feeding. A total of 25 subjects participated in these three studies. The first study utilised active, untrained subjects who cycled at approximately 38 and 70% peak oxygen consumption ($\dot{V}O_2$peak) for 40 min. These workrates represented low intensity (LI) and moderate intensity (MI) exercise, respectively. The other studies involved endurance trained cyclists or triathletes. The second study required subjects to cycle at approximately 65% $\dot{V}O_2$peak for 120 min with, or without, the ingestion of carbohydrate. In the BCAA study, subjects cycled for 60 min at 70% $\dot{V}O_2$peak with, or without, prior BCAA supplementation. In all trials blood samples were taken from a forearm vein and analysed for metabolites and insulin. Muscle biopsies were obtained at rest and during exercise. These samples were subsequently analysed for various metabolites.

The results from this thesis demonstrated that NH_3 accumulates in muscle and plasma throughout LI and MI exercise. This accumulation during exercise was best explained by an increase in skeletal muscle NH_3 production. The principal sources of muscle NH_3 production during submaximal exercise may be net adenosine monophosphate (AMP) deamination and/or amino acid catabolism. This thesis demonstrated the exercise-induced increase in a marker of net AMP deamination (i.e., muscle inosine monophosphate) was considerably less than NH_3, suggesting that the primary source of NH_3 production was amino acid catabolism rather than net AMP deamination. The present thesis also found that BCAA ingestion elevated plasma NH_3 concentration, while carbohydrate ingestion lowered muscle and plasma NH_3 levels. These changes in tissue NH_3 content occurred without any, or only a minor, alteration in net AMP deamination in the contracting muscle. These data provided further evidence that amino acid degradation was the significant source of NH_3 in active muscle during submaximal exercise. The exercise-induced alterations in muscle amino acid content reported in this thesis provided additional supporting evidence for this hypothesis. No consistent relationship

between plasma insulin concentration and muscle and plasma NH_3 concentration was observed during submaximal exercise. This finding suggests that insulin is not linked to, or has a minor role in regulating, NH_3 production in active muscle. Finally, this thesis demonstrated that at least some of the muscle NH_3 production was derived from net AMP deamination during MI exercise however, no evidence of AMP deamination was found during LI exercise.

DECLARATION

This dissertation reports original, previously unpublished observations conducted in the Departments of Chemistry and Biology and Physical Education and Recreation, Victoria University of Technology. Additional data were also collected in the Department of Physiology, The University of Melbourne. This dissertation is the result of the work performed solely by the author with the following exceptions. Help was obviously required for sample collection. In addition, the plasma data from eight of the thirteen subjects reported in chapter 5 were collected by D. Noonan as part of her Masters thesis. These data are included to add greater statistical power.

Rodney J. Snow

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Finally, but most importantly, I wish to thank my wife Andrea and my two wonderful children, Timothy and Samantha, for supporting my academic pursuits. Hopefully, the pain was worth the gain? Only the future can tell.

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PREFACE

Results reported in this dissertation which have been presented at scientific meetings are:

Snow, R., M. Carey, C.Stathis, M. Febbraio, and M. Hargreaves. Carbohydrate ingestion attenuates muscle and plasma ammonia accumulation during prolonged exercise. Clin. Sci. 87: 29, 1994.

Snow, R., M. Carey, J. Proietto, C. Stathis, and M. Hargreaves. BCAA feeding and ammonia metabolism during prolonged exercise. Cumberland College of Health Sciences Conference, Sydney, September, 1995.

Snow, R., M. Carey, J. Proietto, C. Stathis, and M. Hargreaves. Influence of exercise duration on ammonia metabolism during low intensity exercise. Cumberland College of Health Sciences Conference, Sydney, September, 1995.

ABBREVIATIONS

ADP	Adenosine diphosphate
ADP_f	Free adenosine diphosphate
AdL	Adenylosuccinate lyase
AdoD	Adenosine deaminase
AdSS	Adenylosuccinate synthetase
AICAr	5 Amino-4-imidazolecarboxamide riboside
Ala	Alanine
AMP	Adenosine monophosphate
AMPd	Adenosine monophosphate deaminase
AMP_f	Free adenosine monophosphate
ANOVA	Analysis of variance
Arg	Arginine
Asp	Aspartate
Asn	Asparagine
ATP	Adenosine triphosphate
ATP(E)	Adenosine triphosphate measured enzymatically
ATP(H)	Adenosine triphosphate measured by HPLC
BCAA	Branched-chain amino acids
BCAAT	Branched-chain amino acid transaminase
BCOA	Branched-chain oxo acids
BCOADH	Branched-chain oxo acid dehydrogenase
CO_2	Carbon dioxide
CoA	Coenzyme A
CP	Creatine phosphate
Cr	Creatine
dw	Dry weight
EAA	Essential amino acids

EC	Energy charge
F-actin	Filamentous actin
FT	Fast twitch
FTR	Fast twitch red
FTW	Fast twitch white
F-1,6-P_2	Fructose-1,6-bisphosphate
GDH	Glutamate dehydrogenase
GDP	Guanosine diphosphate
Gln	Glutamine
GlnS	Glutamine synthetase
Glu	Glutamate
Gly	Glycine
GTP	Guanosine triphosphate
H^+	Hydrogen ion
H_2O	Water
His	Histidine
HPLC	High performance liquid chromatography
Hx	Hypoxanthine
Ile	Isoleucine
IMP	Inosine monophosphate
Ino	Inosine
l	Litre
La	Lactate
Leu	Leucine
LI	Low intensity
Lys	Lysine
Km	Michaelis-Menten constant
Met	Methionine

MI	Moderate intensity
min	Minute
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NH_3	Ammonia/ammonium
Orn	Ornithine
PCA	Perchloric acid
pH	Negative $\log arithm_{10}$ of the hydrogen ion concentration
Phe	Phenylalanine
Pi	Inorganic phosphate
PNC	Purine nucleotide cycle/cycling
RER	Respiratory exchange ratio
SE	Standard error of the mean
Ser	Serine
ST	Slow twitch
TAN	Total adenine nucleotides
TCA	Tricarboxylic acid
TCAI	Tricarboxylic acid cycle intermediates
Thr	Threonine
Tot AA	Total amino acids
Val	Valine
$\dot{V}O_2$	Oxygen consumption
$\dot{V}O_2max$	Maximal oxygen consumption
$\dot{V}O_2peak$	Peak oxygen consumption
ww	Wet weight

CHAPTER ONE

INTRODUCTION

The production of ammonia/ammonium (NH_3[1]) by active skeletal muscle was first documented about seventy years ago (Parnas 1929). Since this discovery it has been established that there are several biochemical reactions which may produce NH_3 in contracting skeletal muscle (Terjung and Tullson 1992). A major biochemical reaction producing NH_3 in intensely contracting muscle is the net deamination of adenosine monophosphate (AMP) by AMP deaminase (AMPd). Furthermore, the deamination of amino acids in contracting muscle may also contribute substantial quantities of NH_3, especially during submaximal exercise (Graham et al. 1987, MacLean and Graham 1993; MacLean et al. 1994, 1996a; van Hall et al. 1995a; Wagenmakers et al. 1990, 1991). The NH_3 produced from amino acids may occur by the action of glutamate dehydrogenase (GDH) and/or purine nucleotide cycle (PNC) activity. The relative importance of GDH and PNC activity is unknown and is the source of much debate.

Researchers have examined some of the factors which may influence the production of muscle NH_3 from net AMP and/or amino acid catabolism. These factors include exercise mode (Bouckert and Pannier 1995; Jensen-Urstad et al. 1993; Wilkerson et al. 1975), training status (Dudley et al. 1987; Dudley and Terjung 1985a; Green et al. 1991, 1992, 1995; Graham et al. 1995b; Stathis et al. 1994), muscle fibre type (Dudley et al. 1983; Graham et al. 1987; Jansson et al. 1987; Norman et al. 1988; Sahlin et al. 1989), oxygen supply (Graham et al. 1987; Sahlin and Katz 1989; Wolfe et al. 1987; Young et al. 1987) , gender (Itoh and Ohkuwa 1993) and temperature (Febbraio et al. 1994; Snow et al. 1993). The present thesis focuses on the influence of exercise intensity, exercise duration and exogenous substrate supply on muscle NH_3 metabolism.

The rate of muscle NH_3 production is quite high during short-duration, high intensity exercise (>90% maximal oxygen consumption; $\dot{V}O_2max$). It is clear that net AMP catabolism provides most, if not all, the NH_3 produced during this type of activity (Graham et al. 1990; Katz et al. 1986a; Stathis et al. 1994). During moderate intensity (MI; 60-80%

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[1] In this thesis NH_3 represents the sum of ammonia and ammonium. When a specific form is referred to, the terms "ammonia" and "ammonium" will be used.

$\dot{V}O_2$max) exercise significant muscle NH_3 production also occurs. Net AMP catabolism, however, is probably not the major source of NH_3 during this type of activity (MacLean et al. 1994, 1996a; Wagenmakers et al. 1991; van Hall et al. 1995a), at least prior to the onset of fatigue (Broberg and Sahlin 1989; Sahlin et al. 1990c). Consequently, amino acid catabolism appears to be the major source of NH_3 under these conditions. At present, it is unclear whether contracting muscle produces NH_3 during low intensity (LI; ≤ 50% $\dot{V}O_2$max) exercise. One study has reported an increased muscle NH_3 efflux and arterial NH_3 concentration (Eriksson et al. 1985) during LI exercise, while another has not (Katz et al. 1986a). The reason(s) for the conflicting findings are not readily apparent and require further examination. If it was established that muscle NH_3 production occurred during LI exercise, the most likely source of this metabolite would be amino acid catabolism. This contention is based on reports which demonstrate that amino acid oxidation occurs during low work rates (Knapik et al. 1991; Wolfe et al. 1982, 1984), whereas no evidence of net AMP catabolism has been reported (Katz et al. 1986a; Sahlin et al. 1989).

The influence of substrate supply on muscle NH_3 metabolism during prolonged MI exercise has also been examined. The substrates which have been studied include carbohydrate (e.g., muscle glycogen content and carbohydrate ingestion), lipid and branched-chain amino acids (BCAA). Normal versus high, pre-exercise muscle glycogen content does not affect muscle NH_3 production during MI exercise (MacLean et al. 1991, 1992). The effects of low versus normal, pre-exercise muscle glycogen content results in either an increase (Broberg and Sahlin 1989) or no change (van Hall et al. 1996) in muscle NH_3 production during prolonged submaximal exercise. Only one study (van Hall 1996) has examined the effect of carbohydrate ingestion during MI exercise on plasma NH_3 concentration. van Hall (1996) reported that the plasma NH_3 concentration was not affected by this treatment. No studies have examined the influence of carbohydrate ingestion on muscle NH_3 metabolism during exercise. An increased plasma free fatty acid concentration has been shown to reduce muscle NH_3 efflux during 60 min of leg kicking exercise (Graham et al. 1991). The mechanism causing this reduction has not been examined.

Several studies have investigated the influence of BCAA ingestion on plasma NH_3 concentration during MI, two legged cycling exercise (MacLean and Graham 1993; Nemoto et al. 1996; van Hall et al. 1995b Wagenmakers et al. 1990). These studies found that the exercise-induced elevations in plasma NH_3 concentration were augmented when BCAA were ingested. In an effort to elucidate the mechanisms causing this elevation, MacLean et al. (1994, 1996a) examined the effect of BCAA ingestion on muscle NH_3 metabolism during single-leg kicking exercise. These authors established that BCAA feeding increased NH_3 production in contracting muscle. It is unclear, however, whether the results obtained from the single-leg kicking exercise experiments can be extrapolated directly to two-legged cycling exercise. The circulatory and endocrine responses are markedly different between the two types of activity (Andersen and Saltin 1985; Graham et al. 1991; Kjaer et al. 1991; Rowell 1988). Furthermore, the magnitude of net muscle protein degradation may also be different between the two exercise modes (van Hall 1996). No study has investigated the effect of BCAA feeding on muscle NH_3 metabolism during two-legged cycling.

The intention of this introduction was to highlight a number of research problems requiring further investigation. As a consequence, the aims of this dissertation were:

1. To establish whether muscle NH_3 production occurs during LI exercise and the effect that exercise duration may have on this phenomenon;
2. To compare and contrast the biochemical processes associated with muscle NH_3 metabolism during LI and MI exercise;
3. To examine the influence of carbohydrate ingestion on muscle NH_3 metabolism during prolonged MI exercise;
4. To determine the effect of BCAA feeding on muscle NH_3 metabolism during two-legged MI cycling exercise.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 INTRODUCTION

This review of literature focuses on the factors which influence skeletal muscle ammonia metabolism during exercise. The review begins with an overview of the important biochemical pathways involved with skeletal muscle NH_3 metabolism. The discussion then focuses on the cellular location, kinetics and regulation of the enzymatic reactions involved with muscle NH_3 production. The review then follows with a description of the influence of exercise on skeletal muscle NH_3 production and removal, with an attempt to ascertain the role of the various biochemical reactions in these processes. As part of this discussion various factors influencing NH_3 metabolism during exercise are addressed.

2.2 THE BIOCHEMICAL PATHWAYS OF AMMONIA METABOLISM IN SKELETAL MUSCLE

2.2.1 An Overview

The skeletal muscle NH_3 production rate is low at rest. In addition, quiescent muscle is involved in the removal of NH_3 from the circulation (Broberg and Sahlin 1989; Eriksson et al. 1985; Katz et al. 1986a). In contrast, when skeletal muscle is active the NH_3 production rate rises markedly and NH_3 is released into the extracellular fluid (Broberg and Sahlin 1989; Eriksson et al. 1985; Katz et al. 1986a).

Production of NH_3 by active skeletal muscle may arise from the catabolism of both AMP and amino acids. Catabolism of AMP can occur via two pathways (Fig. 2.1). First, AMP can be deaminated by AMPd to produce inosine monophosphate (IMP) and NH_3 (Lowenstein 1972). This reaction is the first in a series of reactions that make up the PNC (Fig. 2.2; Lowenstein 1972) and is generally considered to be the major contributor to muscle NH_3 production during intense exercise (Graham et al. 1990; Jansson et al. 1987; Katz et al. 1986a; Stathis et al. 1994). Most of the IMP produced is subsequently reaminated within the muscle by the remaining enzymes of the PNC, while a small proportion enters a degradation pathway forming inosine (Ino), hypoxanthine (Hx), xanthine and uric acid (Fig. 2.1; Stathis et al. 1994). Secondly,

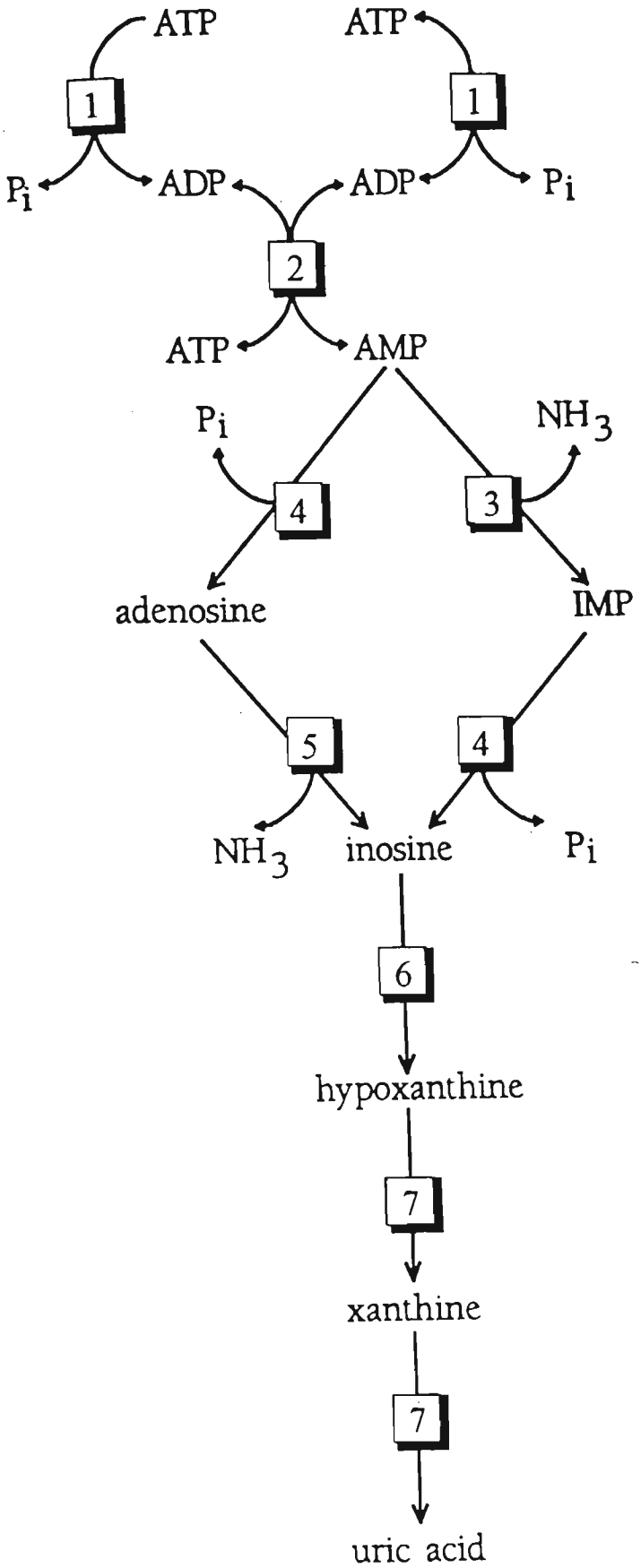

Fig. 2.1: Pathways of adenine nucleotide degradation in skeletal muscle. The enzymes involved in the reactions are: 1. myosin ATPase, 2. adenylate kinase, 3. AMP deaminase, 4. 5' nucleotidase, 5. adenosine deaminase, 6. purine nucleoside phosphorylase, 7. xanthine oxidase.

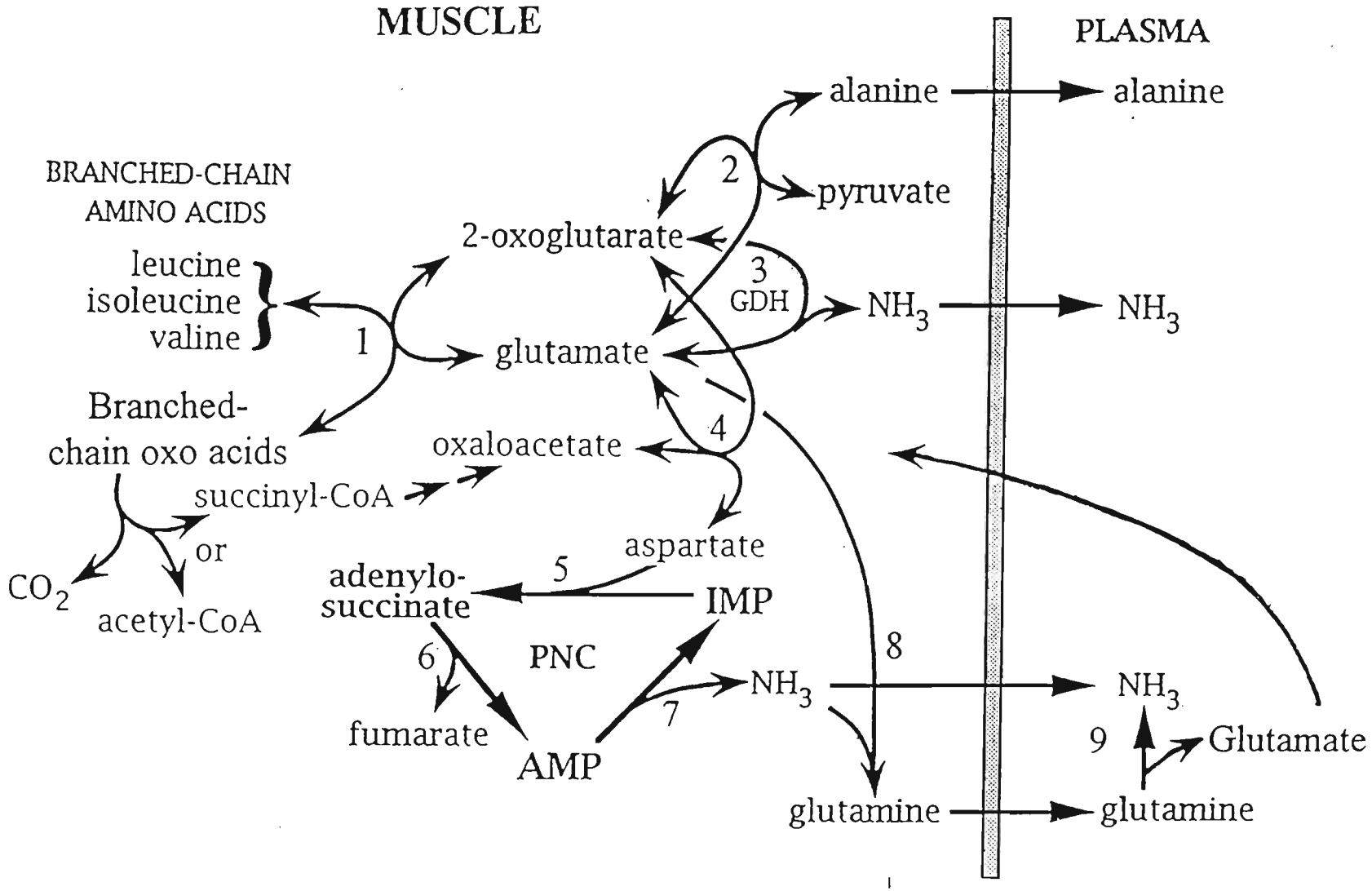

Fig. 2.2: Metabolic reactions coupling branched-chain amino acid degradation with ammonia metabolism in muscle.

1. Branched-chain amino acid transaminase, 2. alanine aminotransferase, 3. glutamate dehydrogenase, 4. aspartate aminotransferase, 5. adenylosuccinate synthetase, 6. adenylosuccinate lyase, 7. AMP deaminase, 8. glutamine synthetase, 9. glutaminase. (Adapted from Terjung

AMP can be dephosphorylated by 5'-nucleotidase to form adenosine and inorganic phosphate (Pi; Bockman and McKenzie 1983; MacLean et al. 1996b; Rubio et al. 1973; Tullson and Terjung 1992). The adenosine may subsequently be deaminated by adenosine deaminase (AdoD) to produce Ino and NH_3 (Fig. 2.1; Sabina et al. 1984; Schopf et al. 1986; Tullson and Terjung 1990; Zoref-Shani et al. 1987). The production of NH_3 by AdoD in contracting muscle is considered to be relatively minor (Terjung and Tullson 1992).

Amino acid catabolism is another potential NH_3 producing source in contracting skeletal muscle. Definitive proof that the catabolism of amino acids produces free NH_3 is lacking; however, indirect evidence strongly indicates that it occurs (Graham et al. 1987; MacLean et al. 1994; MacLean and Graham 1993; Wagenmakers et al. 1990). The most likely NH_3 producing amino acids are those which may be oxidised by muscle. These include alanine (Ala), glutamate (Glu), aspartate (Asp), leucine (Leu), isoleucine (Ile) and valine (Val) (Goldberg and Chang 1978).

It should be noted that the free amino acid pool is small and relatively constant (Layman et al. 1994). Consequently, the amino acids catabolised during exercise must come from a net breakdown of protein. This has been demonstrated to occur predominantly within liver and muscle (Dohm et al. 1981). The exercise-induced increase in the net breakdown of protein within these tissues probably arises from a decreased rate of protein synthesis (Booth and Watson 1985; Bylund-Fellenius et al. 1984; Dohm et al. 1980) and an increased rate of protein degradation (Kasperek et al. 1982; Kasperek and Snider 1985, 1989; Rennie et al. 1981). In muscle, however, the increase in degradation appears to be isolated to the non-contractile proteins, whereas contractile protein degradation rates seem to be depressed (Dohm et al. 1980, 1987; Kasperek and Snider 1989; MacLean et al. 1994).

Although muscle is capable of oxidising several amino acids, there is evidence which suggests that skeletal muscle preferentially catabolises BCAA (i.e., Val, Leu and Ile) (Dohm 1986; Gelfand et al. 1986). BCAA, however, do not contribute directly to free NH_3 production because these amino acids undergo transamination, catalysed by branched-chain aminotransferase (BCAAT), before their carbon chains are oxidised. In the BCAAT

reaction, the amino group is transferred from the BCAA to 2-oxoglutarate to form Glu and its respective branched-chain oxoacid (BCOA; see Fig 2.2). The fate of Glu determines whether the catabolism of BCAA can produce free NH_3.

There are many possible fates for Glu within a muscle fibre some of which lead to removal of free NH_3 rather than its formation (see Fig 2.2). Glu may be involved in many transamination reactions, in particular that catalysed by Ala aminotransferase which produces Ala and 2-oxoglutarate (Goldberg and Chang 1978; Wolfe et al. 1984). Furthermore, Glu may combine with NH_3 to form glutamine (Gln) a reaction catalysed by glutamine synthetase (Rowe 1985). Gln and Ala efflux from contracting muscle fibres may be large and considerably in excess of their occurrence in muscle proteins indicating that these amino acids may act as major nitrogen transporters (Goodman and Ruderman 1982). Some of the Gln released from the muscle fibres may be catabolised by glutaminase to produce Glu and NH_3 in adjacent endothelial cells (Willhoft et al. 1993), thus contributing to whole muscle NH_3 release (Wagenmakers et al. 1990). Alternatively, Glu may be oxidatively deaminated by GDH to produce 2-oxoglutarate, reduced nicotinamide adenine dinucleotide (NADH) and ammonium. The significance of the NH_3 producing role of GDH is currently unclear (see section 2.2.5.1). In another transamination reaction, the amino nitrogen of Glu may be transferred to oxaloacetate to form Asp and 2-oxoglutarate. This Asp may be catabolised in the PNC where free NH_3 is produced (Lowenstein and Goodman 1978). The operation of the PNC effectively deaminates Asp using guanosine triphosphate (GTP) as an energy source to produce fumarate, NH_3 and guanosine diphosphate (GDP) without any net change in IMP and AMP concentrations. There is debate about whether the PNC operates as a cycle within a contracting fibre or whether the deamination occurs during exercise and reamination during recovery (Lowenstein 1990; Meyer and Terjung 1980). This debate will be addressed in a later section of this review (section 2.2.2.4).

2.2.2 Enzymes of the Purine Nucleotide Cycle

2.2.2.1 AMP Deaminase

Introduction. AMPd is found in a number of tissues but is markedly more active (approximately 20-90 fold) in skeletal muscle (Conway and Cooke 1939; Ogasawara et al.

1982). The enzyme was first described in rabbit skeletal muscle by Schmidt (1928) and consists of four subunits with molecular weights between 68,000-80,000 Daltons (Ogasawara et al. 1982). The *in vitro* maximal activity of AMPd in normal human skeletal muscle homogenates ranges between 45-610 $mmol.kg^{-1}$ wet weight (ww).min^{-1} (DiMauro et al. 1980; Fishbein et al. 1993b; Norman 1994; Norman et al. 1994). The maximal *in vivo* activity, estimated from the rate of muscle IMP accumulation during high intensity exercise, is in the order of 3-4 $mmol.kg^{-1}$ ww.min^{-1} (Stathis et al. 1994). The large range of AMPd activity determined *in vitro* has been attributed to the differential expression of two alleles of the AMPd gene predominantly expressed in muscle (AMPd1) (Norman 1994). One of the alleles is believed to result in the production of a mutant, non-functional protein, whilst the other produces a normal product (Morisaki et al. 1992). Individuals who are homozygous for the normal allele display a high level of AMPd activity, heterozygous expression elicits intermediate levels and very low activity levels (i.e., AMP deficient) are found in individuals who are homozygous for the mutant gene (Norman 1994). Many studies on animals have reported values for *in vitro* skeletal muscle AMPd activity within the range published for humans (Fishbein et al. 1993a; Goodman and Lowenstein 1977; Lowenstein 1972; Schultz and Lowenstein 1976; Winder et al. 1974). The regulation of AMPd is complex and may involve substrate availability, isozyme variation, allosteric regulation, phosphorylation, proteolysis and myosin binding.

Substrate Concentration. The concentration of substrate, free AMP (AMP_f), should have a marked effect on AMPd activity since the calculated AMP_f concentration in skeletal muscle (approximately 1 $\mu mol.l^{-1}$)(Tullson and Terjung 1991) is several orders of magnitude lower than the concentration required to elicit half maximal velocity of human (Michaelis-Menten constant (Km) = 0.6-1.0 $mmol.l^{-1}$) skeletal muscle AMPd (Kaletha and Nowak 1988; Makarewicz and Stankiewicz 1974; Ogasawara et al. 1982). On the basis of these data, an elevation in AMP_f during muscle contraction is probably a major factor leading to an increase in AMPd activity. There are several reactions which result in the production of AMP; however, the reaction catalysed by adenylate kinase [i.e. 2 adenosine diphosphate (ADP) $\leftrightarrow$ AMP + adenosine triphosphate (ATP)] is the most important during muscle activity (Zeleznikar et al. 1990). Adenylate kinase is mainly found within the I band,

with some presence at the M-line of the sarcomere (Wegmann et al. 1992). An increase in AMP production occurs even at low exercise intensities and the rate of its production increases with elevations in work rate (Zeleznikar et al. 1990). As a consequence, a greater concentration of AMP_f probably occurs at higher exercise intensities and is associated with elevations in the rate of ATP turnover (Sahlin et al. 1990b).

Allosteric regulation. Allosteric regulation is another factor which controls AMPd activity, but our understanding of the precise regulation *in vivo* is far from complete. Many *in vitro* studies have investigated the allosteric regulation of the enzyme. Some authors (Raffin and Thebault 1991; Ronca-Testoni et al. 1970a) have reported that an increase in free ADP (ADP_f) acts to enhance AMPd activity. In contrast, others (Coffee and Solano 1977; Wheeler and Lowenstein 1979) have found that ADP, on its own, has little effect on AMPd activity. The reason for the discrepancy is not readily apparent, but may be attributed to differences in species, enzyme proteolysis during purification and/or inadequate purification resulting in some ADP remaining bound to the enzyme (Raffin and Thebault 1991). In the presence of inhibitors [e.g., ATP, GTP, Pi and creatine phosphate (CP)], however, ADP acts to remove the inhibition and thereby activates the enzyme (Ronca-Testoni et al. 1970a; Wheeler and Lowenstein 1979). At least two studies (Coffee and Solano 1977; Solano and Coffee 1978) have demonstrated that AMPd is markedly activated by a decrease in energy charge [EC=(ATP+0.5ADP)/(ATP+ADP+AMP)] within the physiological range suggesting that one of the major roles of this enzyme was to maintain the EC within the cell. Wheeler and Lowenstein (1979) used concentrations of various compounds (ATP, GTP, ADP, H^+, Pi) designed to simulate the conditions in resting and contracting muscle and examined their combined effect on AMPd activity. The experiment demonstrated that AMPd activity was enhanced in the simulated contraction condition compared with the resting state.

Increases in skeletal muscle hydrogen ion (H^+) concentration should lead to an elevated enzyme activity *in vivo,* since AMPd has a pH optimum of 6.5 (Makarewicz and Stankiewicz 1974; Ogasawara et al. 1982; Ronca-Testoni et al. 1970b). A decrease in muscle pH is, however, not essential for an increase in AMPd activity since significant IMP production has been observed in stimulated muscle poisoned by a glycolytic inhibitor

(Dudley and Terjung 1985b), and in contracting muscle of human patients with phosphorylase deficiency (Sahlin et al. 1990a, 1995).

Proteolysis. It has been demonstrated that rat and rabbit skeletal muscle AMPd is subject to limited proteolysis (Gross et al. 1994; Marquetant et al. 1989; Ronca et al. 1994) and that this process may be involved with regulating myosin binding (Marquetant et al. 1989) and/or the enzyme's kinetic properties (Ronca et al. 1994; Tullson et al. 1996b). The influence of proteolysis on the enzyme kinetic properties differs markedly between rat and rabbit and this has been attributed to isozyme differences between the species (Ronca et al. 1994). The possibility that human AMPd activity is regulated by limited proteolysis has not been examined.

Phosphorylation. In recent years another mechanism regulating AMPd activity in rat skeletal muscle has been discovered (Tovmasian et al. 1990). This mechanism involves the phosphorylation of the enzyme by the action of protein kinase C. Tovmasian et al. (1990) established that this process occurred *in vitro* and that phosphorylation had no influence on the maximal activity of the enzyme, but resulted in a 3 fold increase in the affinity for its substrate. The enhancement of substrate affinity probably results through phosphorylation of an active-site amino acid residue(s) (Thakker et al. 1993). Indirect evidence suggests that AMPd phosphorylation may also occur in fish white muscle since total AMPd activity was greatest in muscle which was extracted in an homogenising medium containing inhibitors of phosphatases and kinases (Lushchak and Storey 1994). The existence of this regulatory mechanism needs to be established in other species, as does its physiological importance.

Isozymes. There have been four AMPd isozymes identified in humans. These have been designated M, L, E1 and E2, since they are predominantly located in skeletal muscle, liver and erythrocytes, respectively. The M isoform is encoded by the AMPd1 gene, L by AMPd2 gene and both E1 and E2 result from alternative transcripts from the AMPd3 gene (Gross 1994; Mahnke-Zizelman and Sabina 1992). Alternative splicing in the AMPd1 and AMPd2 gene predicts multiple forms for the M and L isoforms as well (Morisaki et al. 1993; Van den Bergh and Sabina 1995). Isozymes for AMPd have also been described in other species (Ogasawara et al. 1978, 1983; Raggi et al. 1975; Thompson et al. 1992) and

these have been designated A, B and C (see below). The human isozymes M and L are immunologically similar to the rat isozymes A and B, respectively (Ogasawara et al. 1982). The E1 and E2 isozyme are immunologically similar to each other, whereas the E1 isozyme is immunologically different from the C isoform (Ogasawara et al. 1982). It has been demonstrated that the genes, which code for the M and A isoform, show a high degree of conservation of sequence and structural organisation (Sabina et al. 1990). This provides further evidence that the structure of the M and A isozyme are quite similar.

Two distinctively different isozymes (A-skeletal muscle isoform and C-cardiac isoform) have been isolated in the skeletal muscle of adult rats (Raggi et al. 1975; Thompson et al. 1992) and rabbits (Raggi et al. 1975; Ogasawara et al. 1983). Rat and rabbit fast twitch (FT[2]) muscle contains mostly isozyme A and very little, if any, isozyme C. In contrast, slow twitch (ST) muscle from these species contain both isoforms (Raggi et al. 1975; Thompson et al. 1992). The proportion of the isoforms in ST muscle is species dependent with rat, cat, monkey, toad and mice displaying 60-85% of their total muscle AMPd activity in the A isoform, whereas rabbit has 30-55% (Fishbein et al. 1993b; Raggi et al. 1975). Thompson et al. (1992) using an immunofluorescent isozyme localization technique, reported that the A isozyme was found within rat ST muscle fibres along with a small proportion of the C isozyme. Most of the C isozyme, however, was associated with non-fibre elements such as blood vessels and erythrocytes (Thompson et al. 1992).

To date, most of the evidence suggests that human skeletal muscle fibres contain a substantial activity of the M isozyme in conjunction with a small presence of isoform E. Early research (Raggi et al. 1975) reported a substantial presence of two forms of AMPd in the pectoralis major of humans, however, these results were not confirmed several years later (Ogasawara et al. 1982). In recent years, with the use of various antibody techniques, it has been established that at least 90% of the AMPd activity in human muscle homogenates can be attributed to the M isoform, with the remaining activity accounted for by the presence of the E isoform (Fishbein et al. 1993b). Moreover, it has been demonstrated that the M isoform is predominantly located in type II, with some presence in

[2] In this thesis, FT and ST will be used to classify muscle fibre types in animals. Human muscle fibre types, however, are classified as type II and type I, respectively.

type I fibres. The E isoform is present within type I fibres as well as other extramyocyte locations (Vankuppevelt et al. 1994).

Clearly the structural properties of the isozymes present in human muscle are different. Yamada et al. (1992) has demonstrated that the homology between the AMPd3 and AMPd1 complementary DNA is 64% and the similarity of the predicted amino acid sequence of the isozymes is 60% (Yamada et al. 1992). These structural differences presumably account for a divergence in regulatory properties. In the presence of 2 mmol.l^{-1} ATP, isozyme E1 (Km=0.5 mmol.l^{-1}) has a slightly lower Km for AMP than isozyme M (Km=0.6), whilst the pH optimum of E1 (pH=7) is considerably higher than M (pH=6.4)(Ogasawara et al. 1982). Based on these data it is possible that the E1 isozyme may be active in human type I muscle fibres in conditions which are not as conducive to M isozyme activity.

In rabbit skeletal muscle, the C isoform displays a higher Km (0.8-1.3 mmol.l^{-1}) than the A (0.4-0.6 mmol.l^{-1}) (Raggi et al. 1975; Raggi and Ranieri-Raggi 1987). Although the pH optimum is similar for both isozymes, the C isoform is prone to greater inhibition in the pH range 6.5-6.0 (Raggi and Ranieri-Raggi 1987). The isolated isozymes from rabbit muscle display differences in activity levels when assayed *in vitro* with physiological levels of ADP and GTP (Raggi and Ranieri-Raggi 1987). Similarly, with unphysiologically low levels of ATP the isozymes exhibit differences in activity. When assayed at pH 7.1, the C isoform is stimulated with increasing ATP concentration whereas the A isoform is markedly inhibited (Raggi and Ranieri-Raggi 1987). Despite these differences in regulatory properties, Solano and Coffee (1978) reported that both forms of rabbit muscle AMPd were activated in a similar fashion by a drop in EC.

The C isoform, unlike the A, does not interact with myosin (Shiraki et al. 1979a). Immunohistochemical staining for the C isozyme in rat ST muscle displays a faint, yet clear, banding pattern in longitudinal fibre sections (Thompson et al. 1992). This suggests that the C isoform binds to protein. It is possible that the C isoform binds to titin, which is a protein filament attached to the end of the thick filaments (Koretz et al. 1993; Podlubnaya 1992).

Myosin Binding. AMPd has been shown to be a persistent minor contaminant of myosin preparations (Byrnes and Sueltar 1965; Kalchar 1947). It was this observation that prompted Ashby and Frieden (1977) to examine the nature of this interaction. These authors established that purified AMPd, obtained from rabbit muscle, formed a complex with subfragment 2 of the heavy meromyosin molecule with a ratio of about 2 mol of enzyme per mol of myosin fragment. These results have been confirmed (Barshop and Frieden 1984) and similar results have also been found for breast muscle from chickens (Ashby et al. 1979). In contrast, rat muscle AMPd was reported to bind to light meromyosin in a ratio of 1 mol of AMPd per 3 mol of myosin (Shiraki et al. 1979a). The difference in binding pattern may be attributed to species differences. Studies examining AMPd and myosin interactions in human skeletal muscle have not been conducted. The actual ratio of enzyme per myosin fragment does not hold when native thick filaments are studied (Koretz 1982) and this is probably related to steric hinderance. Two studies (Ashby et al. 1979; Cooper and Trinick 1984) have demonstrated that AMPd binds primarily at the edge of the A-band of the sarcomere in chicken and rabbit muscle. This situation may hold true for human and rat muscle (at least the A form) since AMPd antibody staining produces a clear transverse banding pattern in longitudinal muscle fibre sections (Thompson et al. 1992; Vankuppevelt et al. 1994).

The binding of AMPd to myosin and myosin fragments increases the activity of the enzyme (Ashby and Frieden 1978; Rundell et al. 1992b; Shiraki et al. 1979b) and influences the effect of some allosteric regulators (Ashby and Frieden 1978; Barshop and Frieden 1984; Lushchak and Storey, 1994; Rundell et al. 1992b; Shiraki et al. 1979b). In addition, Rundell et al. (1992b) have demonstrated that bound AMPd has 10-20 fold higher affinity for substrate at physiological concentrations of AMP compared with the free enzyme. This finding suggests that binding allows for a higher rate of AMP deamination in contracting fibres. Such results question the physiological relevance of studies which have examined the regulation of AMPd *in vitro* without the presence of myofibrillar proteins.

Approximately 15 to 30% of AMPd is bound to myosin at rest in human muscle (Rush et al. 1995; Tullson et al. 1995) and the percent bound in resting rat muscle is in the vicinity of 5-30% (Ashby et al. 1979; Parra and Pette 1995; Rundell et al. 1991, 1992a,

1993, Shiraki et al. 1981). The fact that some AMPd always remains bound raises the possibility that at least some of the enzyme is primed ready to act on any increase in AMP_f concentration. With intense contractile activity there is a rapid and marked increase in the amount of the enzyme bound in rat skeletal muscle (Rundell et al. 1992a, 1993; Shiraki et al. 1981). An increase in AMPd binding precedes an increase in AMP deamination rate as judged by an increase in IMP concentration in stimulated (Rundell et al. 1992a) and resting rat muscle when exposed to an uncoupler of oxidative phosphorylation (Rundell et al. 1991). This indicates that binding plays a significant role in the regulation of the enzyme in rodents. Unlike rat muscle, no increase in AMPd binding occurs during prolonged submaximal exercise (Rush et al. 1995), or in intensely contracting human skeletal muscle (Rush et al. 1995; Tullson et al. 1995). These data provide evidence that enzyme binding does not contribute to the regulation of AMPd in humans.

Relatively few studies have examined the factors which regulate binding of the enzyme to myosin and these are likely to be different between species (see above). In the rat, muscle contraction alone is insufficent to cause an increase in binding. Muscles stimulated at an intensity which induces an energy demand that can be comfortably met by aerobic processes does not display an increase in enzyme binding (Rundell et al. 1992a). The increase in AMPd binding in rodent muscle may be regulated by changes in the EC of the muscle fibre. This hypothesis is supported by the observations that normal and elevated levels of ATP dissociate the enzyme-myosin complex *in vitro* (Ashby et al. 1979; Marquetant et al. 1989). Moreover, limited proteolysis of the N-terminal region of AMPd reportedly reduces its affinity to myosin (Marquetant et al. 1989). Proteolysis of the isolated enzyme has been observed when incubated in muscle extract, demonstrating that proteolysis may occur in muscle *in vivo* (Gross et al 1994). Shiraki et al. (1979a), using rats, demonstrated that AMPd binding to myosin was unaltered by pH in the range 6.5-7.5. In contrast, others have found that AMPd binding to myosin fragments was pH sensitive in chicken (Ashby et al. 1979) and rabbit muscle (Barshop and Frieden 1984). In these species, less AMPd-myosin complex formed as the pH became more alkaline. Rundell et al. (1992a) demonstrated that increased AMPd binding occurred in stimulated rat muscle

poisoned with a glycolytic inhibitor. These data indicate that, at least in this species, a decrease in pH was not essential for binding to take place.

Muscle fibre type. The maximal in vitro activity of AMPd in rodent, rabbit, cat, and mouse FT muscles is 2-4 fold higher compared with ST muscles (Fishbein et al. 1993a; Ogasawara et al. 1983; Raggi et al. 1969; Ren and Holloszy 1992; Rundell et al. 1992a; Winder et al. 1974). However, a recent study (Fishbein et al. 1993a) has demonstrated that there was very little difference in maximal AMPd activity between the FT and ST muscles of the pig and Rhesus monkey. Similarly, Norman (1994) reported that there was no systematic difference between maximal AMPd activity in type I and II fibres in humans.

Rates of AMP deamination are higher in type II compared with type I human muscle fibres during exercise (Jansson et al. 1987; Norman et al. 1988; Sahlin et al. 1989). Unlike human type I fibres, contracting rodent ST fibres do not normally deaminate AMP (Meyer et al. 1980; Meyer and Terjung, 1979), but are capable of doing so during prolonged stimulation in the absence of blood flow (Tullson et al. 1990; Whitlock and Terjung 1987). In contrast, rodent FT muscle readily deaminates AMP provided the contraction conditions are of sufficient intensity (Dudley and Terjung 1985a; Meyer et al. 1980; Meyer and Terjung 1979).

The greater accumulation of IMP in type II fibres in exercising humans is probably related to differences in enzyme activation and the recruitment pattern of the different muscle fibres. This hypothesis is supported by the work of Norman (1994) who reported there was no difference in adenine nucleotide catabolism between fibre types when non-stimulated biopsy samples were incubated for one or two hours in a low oxygen environment. The different activation of the enzyme in the various fibres types during contractile activity may be related to the ATP turnover rate and the capacity of these fibres to meet the metabolic demand (Dudley and Terjung 1985a; Whitlock and Terjung 1987). Investigations of energy metabolism in single human fibres during electrical stimulation (Greenhaff et al. 1993; Soderlund et al. 1992) have shown that the ATP turnover rate is higher in type II fibres and yet these fibres' capacity to sustain high rates of ATP synthesis is less than type I fibres. The high ATP turnover rate during intense exercise may lead to rapid, transient increases in ADP_f and AMP_f providing substrate and relief of allosteric

inhibition of the enzyme (see sections on subtrate concentration and allosteric regulation). In support of this contention, Katz et al. (1986b) observed a significant positive correlation between the calculated *in vivo* AMPd activity and ATP turnover rate in human subjects performing isometric contraction to fatigue.

The fact that rat FT muscle may display high rates of AMP deamination but ST fibres exhibit minimal rates of deamination may also be explained by fibre type differences in ATP turnover rate and the metabolic capacity of these fibres. Since there appear to be differences in AMPd regulation between rat and human skeletal muscle (e.g., presence of isozymes and myosin binding) other explanations may also exist. For example, differences in the allosteric regulation of the various AMPd isoforms present in rat ST muscle (but not in human) may explain the data (see section on isozymes). Moreover, the possibility that the AMPd isoform C does not bind to myosin may also account, at least in part, for the fibre type differences since this isoform constitutes about 20-30% of the total activity in rat ST fibres (Fishbein et al. 1993b; Raggi et al. 1975). It should be noted, however, that most of the isozyme C is not located within muscle fibres (Thompson et al. 1992), which casts doubt that the presence of this isozyme can explain the different rates of AMP deamination in rat FT and ST muscle. Alternatively, the differences may be explained by a reduced number and/or binding affinity of the AMPd binding sites on the myofibrillar A bands in ST muscle (Ren and Holloszy 1992; Rundell et al. 1992a). The different levels of IMP formation in the ST versus the FT fibres in rats are unlikely to be accounted for by differences in *in vitro* maximal activity, since only a small percentage (<3%) of this activity is actually used *in vivo*. In support of this contention, studies have demonstrated that large reductions in the maximal AMPd activity in rat FT muscle, to a level found in ST fibres, had little influence on the magnitude of AMP deamination normally found during contractile activity (Ren and Hollozsy 1992; Tullson et al. 1996a).

2.2.2.2 Adenylosuccinate Synthetase

Adenylosuccinate synthetase (AdSS) catalyses the first reaction in the reamination segment of the PNC. Specifically, AdSS catalyses the following reaction.

$$\text{IMP} + \text{Asp} + \text{GTP} \rightarrow \text{adenylosuccinate} + \text{GDP} + \text{Pi}$$

AdSS exists as two isozymes M and L, however, only the M form of the enzyme is found in skeletal muscle (Stayton et al. 1983; Van den Berghe et al. 1992). This isozyme consists of a dimer, with a molecular weight of approximately 104,000 Daltons (Ogawa et al. 1977) and maximal *in vitro* activity in rodent skeletal muscle of 0.74 $mmol.kg^{-1}$ $ww.min^{-1}$ (Goodman and Lowenstein 1977). To date, no studies have examined the kinetic and regulatory properties of AdSS obtained from human skeletal muscle. Estimates of *in vivo* IMP reamination rates based on the removal of IMP after high intensity exercise in man (Graham et al. 1990; Sahlin et al. 1978; Sahlin and Ren 1989) suggest that the activity of AdSS is about 0.1 $mmol.kg^{-1}$ $ww.min^{-1}$. The activity of AdSS is 2.5-20 fold higher in skeletal muscle than other tissues (Muirhead and Bishop 1974; Weber et al. 1977) and is the rate limiting reaction of the PNC (Lowenstein 1990). The purified rabbit skeletal muscle enzyme has a pH optimum at 6.6-6.8 (Fischer et al. 1978; Muirhead and Bishop 1974; Ogawa et al. 1977), whilst the crude enzyme has an optimum around 7.1 (Davey 1961). Several authors report (Goodman and Lowenstein 1977; Matsuda et al. 1977; Muirhead and Bishop 1974; Ogawa et al. 1977) that mammalian skeletal muscle AdSS has a Km for IMP in the range 0.2-0.7 $mmol.l^{-1}$. The IMP concentration in resting human muscle is approximately 0.02 $mmol.l^{-1}$ (Sahlin et al. 1990c; Spencer et al. 1992; Stathis et al. 1994). Therefore increases in IMP, which are observed during submaximal exercise (Norman et al. 1988), would be expected to elevate AdSS activity (Manfredi and Holmes 1984). The Km of AdSS for Asp is 0.25-0.36 $mmol.l^{-1}$ and for GTP between 0.004-0.38 $mmol.l^{-1}$ (Goodman and Lowenstein 1977; Matsuda et al. 1977; Muirhead and Bishop 1974; Ogawa et al. 1977). The reported concentrations of Asp and GTP in human skeletal muscle are 0.3-1.03 $mmol.l^{-1}$ (Bergstrom et al. 1985; MacLean et al. 1991) and 0.2 $mmol.l^{-1}$ (Sabina et al. 1980), respectively. These data indicate that Asp and GTP are generally near saturating levels and therefore probably play an insignificant role in AdSS regulation *in vivo*.

Inhibition of AdSS by near physiological concentrations of GDP (Lowenstein 1972; Muirhead and Bishop 1974), Pi (Ogawa et al. 1977), CP (Stayton et al. 1983) and fructose-1,6-bisphosphate (F-1,6-P_2; Ogawa et al. 1976) have been demonstrated. During exercise the increase in muscle Pi and the fall in CP tend to offset each other, therefore

these compounds may not be significant regulators of AdSS in contracting muscle. It has been reported that F-1,6-P_2 concentrations increase in human skeletal muscle during intense exercise (Katz and Lee 1988), and consequently this phosphorylated hexose may significantly inhibit AdSS in contracting fibres. The fact that concentrations of adenylosuccinate and purine di- and mono-nucleotides are required to be above that normally found in muscle fibres to inhibit AdSS (Muirhead and Bishop 1974; Ogawa et al. 1977) indicates that these compounds are not major regulators of the enzyme *in vivo*. Conflicting data exist about the inhibition of the enzyme by IMP. Stayton et al. (1983) argue that the AdSS activity is inhibited by IMP concentrations greater than 0.26 mmol.l^{-1}. Goodman and Lowenstein (1977) also report that AdSS is inhibited by 50% at IMP concentrations of 2 mmol.l^{-1}. In contrast, Manfredi and Holmes (1984) found no evidence of AdSS inhibition with IMP concentrations up to 4 mmol.l^{-1}. In fact, these authors observed that AdSS activity was progressively elevated with increases in IMP up to this level. The extent of substrate inhibition on AdSS activity *in vivo* may be estimated by determining the rate of IMP removal following intense contractile activity, since most of the accumulated IMP is reaminated after high intensity exercise. Data from several human studies (Graham et al. 1990; Sahlin et al. 1978; Sahlin and Ren 1989), indicate that the rate of IMP removal is uninfluenced by high concentrations of IMP and is therefore unlikely to inhibit AdSS activity within muscle. In addition, during prolonged submaximal exercise, prior to the onset of fatigue, IMP levels only increase to 0.08-0.23 mmol.l^{-1} in mixed human muscle (Sahlin et al. 1990c; Spencer et al. 1991, 1992). Such levels are unlikely to inhibit AdSS even if the enzyme is inhibited by IMP *in vivo*. There is evidence indicating that AdSS is inhibited by GTP levels greater than 0.1 mmol.l^{-1} (Stayton et al. 1983). Measurements of GTP concentration in contracting human muscle have not been conducted, however, data from mouse muscle indicate that GTP concentrations do not fall unless the muscle is under extreme metabolic stress (Stayton et al. 1983). This implies that GTP probably acts to inhibit AdSS in human muscle during submaximal exercise. Further research is required to ascertain whether GTP is a significant regulator of AdSS in active muscle.

AdSS binds to purified F-actin, actin tropomyosin complexes, reconstructed thin filaments and myofibrils, but not myosin (Manfredi et al. 1989; Ogawa et al. 1978). Manfredi et al. (1989), further postulate that effectively all the AdSS within the myocyte is bound to the myofibrils. The binding capacity of the enzyme to F-actin was not influenced by pH in the range 5.5-7.0 and unlike AMPd (see above) the kinetic properties of AdSS were not altered as a result of binding (Ogawa et al. 1978). The close association of AdSS with the myofibrils provides further evidence that the enzymes of the PNC are compartmentalised within the myofibrillar network presumably allowing efficient flux through the cycle.

2.2.2.3 Adenylosuccinate Lyase

Adenylosuccinate lyase (AdL) is a bifunctional enzyme catalysing one of the reactions of *de novo* adenine nucleotide synthesis and also catalysing the second reaction in the reamination leg of the PNC (Lowenstein 1990). The latter reaction occurs as follows:.

Adenylosuccinate $\rightarrow$ AMP + Fumarate

Isozymes of AdL have not been characterised but there is evidence which suggests AdL exists in human tissue in at least two forms (Van den Berghe et al. 1992). The native enzyme purified from rat skeletal muscle is a tetramer with a molecular weight of 200,000 Daltons (Casey and Lowenstein 1987). In rodent muscle AdL has a maximal *in vitro* activity of 0.61-1.75 $mmol.kg^{-1}ww.min^{-1}$ at 38°C (Schultz and Lowenstein 1976; Winder et al. 1974). Human muscle has slightly lower activity with values around 0.44-0.55 $mmol.kg^{-1}ww.min^{-1}$ (Jaeken and Van den Berghe 1984; Van den Berghe and Jaeken 1986). Winder et al. (1974) reported that AdL activity was highest in the most oxidative muscle fibres of the rat. In contrast to the other enzymes of the PNC, very little is known about AdL. This lack of information probably stems from an inability to obtain a stable form of the purified enzyme (Casey and Lowenstein 1987). In 1987, Casey and Lowenstein managed to purify the enzyme from rat skeletal muscle and established conditions in which the enzyme was stable. These authors discovered that the purified enzyme diplays hyperbolic kinetics and has a Km for adenylosuccinate of 1.5 $\mu mol.l^{-1}$. Since adenylosuccinate levels in resting skeletal muscle are undetectable (Goodman and Lowenstein 1977), an increase in substrate availability would be expected to activate the

enzyme. This notion is supported by the data of Manfredi and Holmes (1984). These authors demonstrated that AdL activation was primarily associated with elevations in substrate concentration.

2.2.2.4 Purine Nucleotide Cycling

Evidence that the three enzymes AMPd, AdSS and AdL may operate as a cycle was first provided by Kalchar and Rittenburg (1947) who reported that rat skeletal muscle incorporates labelled nitrogen into the 6-amino group on the purine ring 50 times more strongly than the other nitrogens of the purine molecule. This increased labelling of the 6-amino group could only occur if the PNC was operating. Other nitrogen labelling studies have since confirmed these initial results (Gorski et al. 1985; Newton and Perry 1957, 1960). Operation of the PNC has also been demonstrated using *in vitro* biochemical assay techniques (Lowenstein 1972; Manfredi and Holmes 1984). Since the establishment of the existence of the PNC in muscle, much debate has centred around whether the deamination and reamination reactions occur simultaneously or out of phase, with deamination occurring during contraction and reamination in recovery (Lowenstein 1990; Tullson and Terjung 1991). The resolution of this question is important (see below) and may depend on a number of factors including the intensity of exercise, muscle fibre type, species differences and experimental techniques.

Operation of the PNC does not appear to occur during very mild exercise intensities in rat muscle (Meyer and Terjung 1980). Indirect evidence suggests that this may also be the case in humans (Katz et al. 1986a). Moreover, most of the experimental evidence strongly indicates that the reamination leg of the PNC does not operate during short duration, high intensity exercise. Under these exercise conditions several studies have demonstrated that the fall in muscle ATP concentration is matched by an equimolar increase in IMP and NH_3 (Jansson et al. 1987; Katz et al. 1986a,b; Meyer and Terjung 1979; Stathis et al. 1994). In addition, Meyer and Terjung (1980) used an inhibitor of IMP reamination (hadacidin, inhibitor of AdSS) and demonstrated no further increase in IMP accumulation in tetanically stimulated (100 Hz) rat gastrocnemius muscles compared with controls. In contrast, two studies (Flanagan et al. 1986; Swain et al. 1984), have reported that the PNC may operate in mouse and rat muscle during intense contraction conditions.

These authors reported that an inhibitor of AdL (5 amino-4-imidazolecarboxamide riboside, AICAr) resulted in an increased accumulation of adenylosuccinate in mouse and rat gastrocnemius muscles tetanically stimulated *in situ* compared with saline treated controls. It should be noted, however, that treatment with AICAr may be an inappropriate means of inhibiting IMP reamination because it reduces systemic blood pressure (Foley et al. 1989). Thus it would be expected to lower perfusion pressure to the muscle thereby influencing muscle metabolism.

Several studies (Aragon and Lowenstein 1980; Hood and Parent 1991; Meyer and Terjung 1980) have reported the occurrence of IMP reamination during moderate, prolonged *in situ* stimulation (0.8-10 Hz) of rat FT muscle. Meyer and Terjung (1980), using the drug hadacidin, provided evidence that PNC occurs in rat fast twitch white (FTW) muscle during 30 min of stimulation at moderate stimulation frequencies (i.e., 3 and 5 Hz). These authors, however, demonstrated that the reamination of IMP was attributable to FTW muscle fibres which were no longer contracting. This finding may also explain the data obtained by Aragon and Lowenstein (1980). Based on this evidence it was concluded that the two legs of the PNC operated out of phase, even during mild contraction conditions (Meyer and Terjung 1980).

It should be recognised, however, that the studies which have utilised hadacidin to investigate the operation of the PNC are incapable of detecting cycling rates below approximately 7 $nmol.min^{-1}.g$ ww (Tullson and Terjung 1991). This probably represents only a minor limitation of the technique since this rate may account for about 10% of the NH_3 released from the muscle during submaximal exercise (Broberg and Sahlin 1989; van Hall et al. 1995a). A more important limitation, however, is that the degree of inhibition of IMP reamination by hadacidin may be dependent upon muscle fibre type. Tullson et al. (1996a) have recently reported that hadacidin totally inhibited IMP reamination in rat FTW muscle during 9 min of recovery from high intensity exercise. In contrast, within the fast twitch red (FTR) muscle fibres there was about a 35% decrease in the IMP content in the presence of hadacidin during a similar period of recovery. This decrease could not be accounted for by an increased accumulation of IMP degradation products. These data

suggest that IMP reamination was only partially inhibited in these fibres, consequently hadacidin may be an inappropriate drug to study the extent of PNC in FTR muscle.

A study which supports the notion that the PNC operates in contracting fibres was conducted by Hood and Parent (1991). They employed a more intense stimulation frequency than had been previously undertaken (i.e., 10 Hz) and observed significant IMP reamination in both FTW and FTR fibres during 60 min of stimulation. At the end of the stimulation period the muscle tension produced was 28% of the initial. This decline was attributed primarily to fatigue in the FTW fibres. Therefore, in agreement with Meyer and Terjung (1980), Hood and Parent (1991) suggested that IMP reamination was occurring in FTW fibres which were no longer contracting. It was further concluded, however, that most of the FTR fibres must have been contracting in order to produce the remaining tension. Consequently, the reamination leg of the PNC was probably operating in contracting FTR fibres. This possibility is supported by the finding that this fibre type accumulates more IMP during 4 min of treadmill running when exposed to hadacidin compared with control conditions (Meyer et al. 1980). In addition, the highest rate of IMP reamination during recovery from strenuous exercise is observed in FTR muscle (Tullson et al. 1996a). In fact, the rate of IMP deamination (1.25 $mmol.kg^{-1}$ ww) in FTR fibres was only twice the rate of reamination (0.64 $mmol.kg^{-1}$ ww; Tullson et al. 1996a). These data establish the possibility that IMP reamination may keep pace with an appreciable rate of IMP production during exercise.

To date, the operation of the PNC in contracting human fibres has not been examined. There is, however, indirect evidence to suggest that it may function during submaximal exercise in man (Broberg and Sahlin 1989). Furthermore, the data obtained from experimental animal models may not be applicable to human skeletal muscle since the regulation of AMPd activity appears to be different (see section 2.2.2.1). Very little information also exists about the regulation and kinetics of the enzymes catalysing the reamination legs of the PNC in human muscle compared with other species (see sections 2.2.2.2 and 2.2.2.3).

As mentioned previously, the net effect of one complete sequence of the PNC reactions results in the deamination of Asp with no net change in AMP and IMP muscle

contents. Additionally, GTP is consumed to provide energy for the cycle and fumarate is produced. If both the deamination and reamination arms of the PNC occur concurrently within contracting muscle fibres a potentially important pathway exists for the deamination of amino acids allowing the resultant carbon chains to be oxidised. Moreover, the production of fumarate has the potential of expanding the tricarboxylic acid cycle intermediates (TCAI; Aragon and Lowenstein 1980) and this could be important in supporting oxidative metabolism (Scislowski et al. 1982).

2.2.3 Enzymes Involved With An Alternative Pathway of AMP Catabolism

2.2.3.1 5'-Nucleotidase

5'-Nucleotidase catalyses the dephosphorylation of both AMP and IMP as depicted by the following reactions.

$$AMP + H_2O \rightarrow adenosine + Pi$$

$$IMP + H_2O \rightarrow Ino + Pi$$

The total maximal *in vitro* activity of human skeletal muscle 5'-nucleotidase is reported to be about 5.0 μmol.g protein^{-1}.min^{-1} (Schopf et al. 1986). This rate is about 250 fold less than maximal *in vitro* AMPd activity. Factors influencing the regulation of 5'-nucleotidase include isozyme variation, cellular location, allosteric modulation and substrate supply.

At least two isozymes, a membrane bound ecto-enzyme and a soluble cytosolic enzyme, have been found in skeletal muscle (Camici et al. 1985; Frick and Lowenstein 1976; Tullson and Terjung 1992). In rat skeletal muscle the activity of membrane bound ecto-enzyme is about 10-fold higher than the soluble cytosolic form (Tullson and Terjung 1992). Despite this marked difference in maximal activities the relative contribution of these isozymes to adenosine and Ino production is unclear. The cytosolic isoform may be more important since most of the IMP and AMP production occurs within the cytosol. A significant involvement of the ecto-enzyme in the intracellular nucleoside production in skeletal muscle cannot be discounted, however, since there is some evidence to suggest that this form of the enzyme may gain access to the sarcoplasm (Stanley et al. 1980).

In vitro studies of the purified ecto-enzyme have demonstrated some possible differences in their regulation. Camici et al. (1985) have reported that nucleoside di and tri

phosphates are powerful inhibitors of the 5'-nucleotidase ecto-enzyme in guinea-pig skeletal muscle. On the other hand, the cytosolic 5'-nucleotidase found in cardiac muscle is activated by ATP, ADP and decreasing EC, and is inhibited by Pi (Itoh et al. 1986). Furthermore, the two cytosolic isozymes identified in cardiac muscle appear to be activated by the adenine nucleotides and inhibited by Pi to different extents (Truong et al. 1988). Studies examining the existence of two cytosolic forms of the enzyme and their allosteric regulation in skeletal muscle have not been conducted.

The two soluble cytosolic enzymes in cardiac muscle have differing affinities for AMP and IMP (Truong et al. 1988). If these two isoforms also exist in skeletal muscle, the supply of substrates may play an important role in the *in vivo* regulation of the isozymes. It is generally accepted that AMP_f and IMP concentrations rise during high intensity exercise. During intense contraction conditions, however, the AMP favouring isozyme activity is likely to be curtailed. This probably occurs because the very high activity of AMPd effectively deprives the nucleotidase access to AMP_f (Tullson et al. 1990). In contrast, the IMP favouring 5'-nucleotidase is more likely to be exposed to elevated levels of its substrate, since other enzymes which utilise IMP do not have very high levels of activity.

In the rat, the total soluble 5'-nucleotidase activity is higher in soleus muscle when compared with other muscle groups (Tullson and Terjung 1992). Tullson and Terjung (1992) argue that the pattern of soluble 5'-nucleotidase activities among muscle fibre sections suggests that ST muscle is more likely to form nucleosides and bases. In contrast, FT muscle retain their purine metabolites as nucleotides. This argument has been supported by the findings from other experiments conducted on cat and rat skeletal muscle (Arabadjis et al. 1993; Bockman and Mckenzie 1983). As yet, there are no data available on the activity of soluble cytosolic 5'-nucleotidase activity in the different fibre types of humans.

2.2.3.2 Adenosine Deaminase

The adenosine produced by the action of 5'-nucleotidase may be deaminated by AdoD to produce Ino and NH_3.

$$\text{adenosine} \rightarrow \text{Ino} + NH_3$$

The maximal activity of the enzyme determined in homogenates of human skeletal muscle is quite low and ranges between 0.5-1.7 $\mu mol.g\ protein^{-1}.min^{-1}$ (Schopf et al. 1986) or about

0.12 μmol.g $ww^{-1}.min^{-1}$ (Ma and Magers 1975). There are three isoforms of the enzyme, two of which (A and C form) are found in human skeletal muscle (Ma and Magers 1975). There are no major differences in the kinetic properties of these isozymes with the Km for adenosine approximately 60 $\mu mol.l^{-1}$ and the optimal pH around 7 (Ma and Magers 1975). The concentration of adenosine in human muscle is normally below the Km (e.g., 0.10-20 $\mu mol.l^{-1}$; MacLean et al. 1996b; Sabina et al. 1984). Therefore, any increase in substrate supply should lead to an increase in AdoD activity. Data from rat skeletal muscle indicate that ST muscle contains about twice the level of AdoD activity than FT fibres (Newsholme et al. 1985). There is evidence that ST fibres are more likely to produce NH_3 from this reaction (Arabadjis et al. 1993; Hara et al. 1989).

2.2.4 Enzymes Involved With Branched-Chain Amino Acid Catabolism

2.2.4.1 Branched-chain Amino Acid Transaminase

BCAAT catalyses the first, but reversible, step of BCAA catabolism. BCAAT is a pyridoxal phosphate-dependent enzyme which accepts all three BCAA as substrates (Harper et al. 1984; Ichihara and Koyama 1966; Taylor and Jenkins 1966). There are various isoforms of the enzyme found in different tissues (Ichihara et al. 1973). Human and rodent skeletal muscle, however, have one isozyme (Goto et al. 1977; Kadowaki and Knox 1982). In both humans and rodents, BCAAT has relatively high activity in cardiac muscle and kidney, intermediate activity in skeletal muscle and low activity in liver tissue (Cappuccino et al. 1978; Goto et al. 1977; Ichihara and Koyama 1966; Kadowaki and Knox 1982; Shinnick and Harper 1976). The maximal *in vitro* activity of BCAAT in human skeletal muscle is about 0.16 $mmol.kg^{-1}ww.min^{-1}$ (Goto et al. 1977). When the approximate mass of the tissues found within the body is taken into consideration, skeletal muscle has the largest BCAAT capacity (Krebs and Lund 1977; Shinnick and Harper 1976). Henriksson et al. (1986) reported that the maximal *in vitro* BCAAT activity was similar between rabbit FT and ST muscle. These data indicate, that at least in this species, the capacity to transaminate BCAA's may not be dependent upon muscle fibre type.

The intracellular location of BCAAT is unclear. Snell and Duff (1985) reported that BCAAT was found predominantly within the mitochondria of rat skeletal muscle, irrespective of muscle fibre type. In contrast, other studies have found that the enzyme was

located within the cytosol and mitochondria of rat skeletal muscle fibres (Cappuccino et al. 1978; Hutson 1988; Odessey and Goldberg 1979). In addition, Hutson et al. (1988) found that the enzyme distribution between the cytosol and mitochondria differed across muscle fibre types since all the BCAAT activity was found within mitochondria of ST oxidative fibres, whereas 70% was found in the cytosol of FT glycolytic fibres (Hutson 1988). Differences in fractionation techniques and utilisation of inappropriate marker enzymes to identify intracellular locations may explain some of the conflicting results (Snell and Duff 1985). It is difficult, however, to reconcile the conflicting findings of Hutson (1988) and Snell and Duff (1985).

BCAAT has a pH optimum between 8.2-8.6 (Ichihara and Koyama 1966; Odessey and Goldberg 1979; Taylor and Jenkins 1966) and is inhibited by physiological levels of calcium (Odessey and Goldberg 1979). ATP and ADP levels are not involved in the regulation of the enzyme (Odessey and Goldberg 1979). The main amino-acceptor in the BCAAT reaction is considered to be 2-oxoglutarate and the Km for the rodent skeletal muscle enzyme is 0.1-0.15 $mmol.l^{-1}$ (Odessey and Goldberg 1979). This is about twice the concentration of 2-oxoglutarate normally found in resting rat muscle (Aragon and Lowenstein 1980) and is markedly higher than that found in nonactive human muscle (Graham and Saltin 1989; Katz et al. 1990). Studies examining the 2-oxoglutarate concentration required to produce half maximal velocity for BCAAT in human muscle have not been conducted. Furthermore, 2-oxoglutarate concentrations in contracting human muscle have been reported to both increase (Katz et al. 1990) and decrease (Graham and Saltin 1989), making it extremely difficult to ascertain the influence of this substrate on BCAAT activity during exercise *in vivo*. The Km values for the BCAA's are higher in rat (e.g., 0.4-1.3 $mmol.l^{-1}$; Kadowaki and Knox 1982; Odessey and Goldberg 1979) and humans (e.g., 1-5 $mmol.l^{-1}$; Goto et al. 1977) than the normal muscle concentration of these amino acids in these species (<0.3 $mmol.l^{-1}$; Bergstrom et al. 1985; Hutson et al. 1978). Consequently, any elevation in muscle BCAA content should lead to an increase in the rate of transamination *in vivo*. Researchers have either observed no change (Bergstrom et al. 1985; Rennie et al. 1981; van Hall et al. 1995a), or an increase (MacLean et al. 1991, 1994) in muscle BCAA concentration during submaximal exercise.

Since BCAAT catalyses a reversible reaction, an increase in muscle Glu and BCOA concentration would tend to lead to the formation of BCAA. Muscle Glu concentration decreases with submaximal exercise (Bergstrom et al. 1985; Graham and Saltin 1989; MacLean et al. 1991; Rennie et al. 1981) tending to drive the reaction toward BCOA formation. Elevated BCOA concentrations in human muscle have been reported during exercise of increasing intensity (Fielding et al. 1986) which should drive the BCAAT reaction towards amino acid formation if adequate Glu was available. During LI exercise (30% $\dot{V}O_2max$) in humans, it has been demonstrated that reamination takes place, however, the production of BCOA exceeds that of BCAA (Wolfe et al. 1984).

2.2.4.2 Branched-Chain Oxoacid Dehydrogenase

Branched-Chain Oxoacid Dehydrogenase (BCOADH) is a multienzyme complex located on the inner surface of the inner mitochondrial membrane (Harper et al. 1984; Odessey and Goldberg 1979). BCOADH catalyses the irreversible oxidative decarboxylation of the three BCOA. The basic reaction for each BCOA is as follows.

$$\text{Ketoleucine} + NAD^+ + CoA \rightarrow \text{isovalerylCoA} + NADH + CO_2$$

$$\text{Ketovaline} + NAD^+ + CoA \rightarrow \text{isobutyrylCoA} + NADH + CO_2$$

$$\text{Ketoisoleucine} + NAD^+ + CoA \rightarrow \text{3-methyl-butyrylCoA} + NADH + CO_2$$

Importantly, the removal of BCOA by the BCOADH reaction probably results in an increased flux through BCAAT in the direction of BCOA and Glu formation (Hood and Terjung 1991; May et al. 1987). As a consequence, the potential for free NH_3 production from Glu catabolism may also increase. Cytosolic BCOA traverse the inner mitochondrial membrane to the dehydrogenase location by a specific transporter (Hutson and Rannels 1985). The mitochondrial location of BCOADH suggests that muscle fibres with high oxidative capacity may be best suited to catabolise BCOA. Human muscle contains about 60% of the total body BCOADH activity, while rat muscle contains 10-30% (Khatra et al. 1977; Wagenmakers and Veerkamp 1982). This indicates that a greater proportion of BCOA may be oxidised in human, compared with rat muscle. The maximal *in vitro* activity of BCOADH in human skeletal muscle is approximately 20 $\mu mol.kg^{-1}$ $ww.min^{-1}$ (Wagenmakers et al. 1989, 1991) and constitutes the rate limiting step for BCAA oxidation in this tissue (May et al. 1987; Odessey and Goldberg 1979; Shinnick and Harper 1976).

The activity of BCOADH in skeletal muscle is highly regulated and is exerted via a number of mechanisms. The first of these is by a reversible phosphorylation (inactivation)-dephosphorylation (activation) mechanism (Harris et al. 1982; Miller et al. 1988; Odessey 1980; Parker and Randle 1980) which alters the maximal activity of the enzyme but not the affinity for its substrates (Odessey 1980). A recent study by Hood and Terjung (1991) examined the importance of dephosphorylation of BCOADH on the decarboxylation rate of BCOA in the perfused rat hindlimb. These authors reported that dephosphorylation of BCOADH resulted in an activation of the enzyme 13-26 fold but only increased BCOA decarboxylation by 3-4 fold. They concluded that phosphorylation-dephosphorylation of the dehydrogenase plays an important, but not exclusive role in modulating rates of BCAA metabolism in skeletal muscle.

Phosphorylation of BCOADH is catalysed by BCOADH kinase which is tightly associated with the dehydrogenase (Shimomura et al. 1990a). BCOADH kinase is inhibited *in vitro* by a number of compounds (Paxton and Harris 1984). Some of the physiologically more important include ADP, acetoacetyl coenzyme A (CoA), and the three BCOA, with ketoleucine being the most potent inhibitor of the three (Harris et al. 1982; Lau et al. 1982; Paxton and Harris 1984; Randle et al. 1984). Results from a number of studies (Aftring et al. 1986; Block et al. 1987a) indicate that an increase in BCOA supply may also inhibit the kinase *in vivo*. These studies demonstrated that an increase in plasma BCAA results in a marked elevation in the active state of BCOADH within resting skeletal muscle of the rat. Recently, this phenomenon has been observed in human muscle (van Hall et al. 1996).

Dephosphorylation of BCOADH is catalysed by the loosely bound BCOADH phosphatase (Damuni et al. 1984; Damuni and Reed 1987). The purified phosphatase is inhibited *in vitro* by products of the oxidation of BCOA, acetyl CoA, CoA and other acyl CoA compounds at physiological concentrations (Damuni et al. 1984; Damuni and Reed 1987). These data suggest that an increased concentration of the products of BCOA oxidation within muscle may lead to the inactivation of BCOADH by inhibition of the phosphatase. Furthermore, there is evidence which demonstrates that the phosphatase is also inhibited by various nucleoside tri and diphosphates. This, however, is probably

physiologically unimportant since the inhibition is completely reversed by 2 $mmol.l^{-1}$ magnesium (Damuni et al. 1984; Damuni and Reed 1987).

In addition to control by phosphorylation, BCOADH activity is modulated by end-product inhibition. Products of the BCOADH reaction, branched-chain acyl CoA derivatives and NADH, are inhibitors of the complex (Odessey 1980). The ratios of acyl CoA to CoA and NADH to oxidised nicotinamide adenine dinucleotide (NAD^+) probably influence the flux of BCOA through the dehydrogenase reaction since CoA and NAD^+ activate the enzyme *in vitro* (Odessey 1980). Furthermore, since muscle BCOA concentrations fall near or below the Km, an increase in substrate supply should lead to an increased rate of oxidation by the dehydrogenase *in vivo* (Hutson 1986).

Muscle BCOADH activity may also be influenced by the availability of substrates other than amino acids, in particular BCAA (see above). Starvation for 4 days increases (Aftring et al. 1988), while 3 days of starvation leads to no change or a decrease (Aftring et al. 1988; Wagenmakers et al. 1984) in the activation state of BCOADH in rat muscle. Furthermore, a 24 hour fast results in an increase (Shimomura et al. 1995), or no change (Kasperek and Snider 1987), in resting rat muscle BCOADH activity. An increase in Leu oxidation by rat muscle homogenates has been reported after 5 days of starvation (Paul and Adibi 1976). Interestingly, the activation of the enzyme is markedly increased during exercise in the fasted compared with the fed state (Kasperek and Snider 1987).

Studies, using *in vitro* techniques, have found an increased rate of rat muscle BCOA oxidation when supplied with some lipids (e.g., hexanoate or octanoate) (Buse et al. 1972; Paul and Adibi 1976; Spydevold 1979; Spydevold and Hokland 1981). In contrast, Leu oxidation rates were unaltered when rat muscle homogenates were incubated with palmitate and were inhibited when incubated with decanoate (Paul and Adibi 1976). These data suggest that the effect of fatty acids on *in vitro* Leu oxidation rates is dependent upon the fatty acid carbon chain length. Studies utilising whole-body isotopic tracer techniques report a decreased rate of Leu oxidation when fatty acids (octanoate or Intralipid) are infused into resting dogs (Beaufrere et al. 1985; Tessari et al. 1986). Unfortunately, these studies cannot identify the organs responsible for this inhibition. This is important since *in vitro* experiments indicate that octanoate may inhibit, or activate, BCAA oxidation

depending upon the tissue examined (see Beaufrere et al. 1985). An increase in glucose and pyruvate availability has been shown to inhibit BCOA oxidation in incubated rat diaphragm muscle (Buse et al. 1972, 1976), however, glucose had no affect when rat gastrocnemius homogenates were used (Paul and Adibi 1976). Pyruvate has also been reported to inhibit BCOA oxidation in isolated rat muscle mitochondria (Spydevold and Hokland 1981).

Several hormones may alter the activity of muscle BCOADH. Insulin may inhibit BCOA oxidation indirectly due to its action on protein synthesis and degradation. As a consequence, the availability of free amino acids for oxidation may decrease (Hutson et al. 1978). Insulin, however, does not increase the activation state of BCOADH (Aftring et al. 1988; Block et al. 1987b). Buse et al. (1973) reported that adrenaline and glucagon stimulated BCAA oxidation in muscle of fasted, but not fed rats. These authors also observed that the stimulatory effects of these hormones could be suppressed by the presence of glucose or pyruvate. Acute glucocorticoid administration, to levels found in stressed rats, increases the activation state of BCOADH in resting rat muscle, while chronic (5 days) exposure elevates both the total activity and the proportion of the enzyme in the active state (Block et al. 1987b). Consistent with this enhanced muscle enzyme activity, whole-body Leu tracer experiments have found an increased Leu oxidation after 5 days of corticosteroid treatment in humans (Beaufrere et al. 1989). Insulin administered in addition to glucocorticoids, partially blocked the latter hormones effect on BCOADH activation (Block et al. 1987b).

A small percentage (approximately 5%) of the dehydrogenase is active in resting skeletal muscle in rats and humans, however, the enzyme becomes markedly more active in contracting muscle in both species (Fujii et al. 1994; Hood and Terjung 1991; Kasperek et al. 1985; Kasperek 1989; Kasperek and Snider 1987; Rush et al. 1995; Shimomura et al. 1990b; van Hall et al. 1996; Wagenmakers et al. 1984, 1989, 1991). Furthermore, up to a point, the greater the intensity (Kasperek and Snider 1987) and duration (Kasperek et al. 1985; Rush et al. 1995) of submaximal exercise the higher the activation state of the enzyme.

The major regulatory mechanisms inducing dephosphorylation of BCOADH in contracting muscle are currently unknown. Studies investigating this problem have yet to

measure changes in the intramitochondrial concentrations of possible regulators of the dehydrogenase such as ATP, ADP, BCAA, BCOA, H^+, acetyl CoA/CoA and NAD^+/NADH, thereby making it difficult to interpret the data (Graham and MacLean 1992). Nevertheless, changes in muscle adenine nucleotides have been found to correlate strongly with the level of BCOADH activation in one study (Kasperek 1989), but not in others (Shimomura et al. 1990b, 1993). Some studies have indicated that elevations in muscle BCAA and BCOA concentrations may be a major regulator increasing BCOADH activity in contracting muscle (Shimomura et al. 1990b, 1993). In contrast, others (Kasperek 1989) have suggested that BCOA and BCAA concentrations are probably unimportant during exercise since changes in these muscle metabolites correlate poorly with enzyme activation. Recently, van Hall et al. (1996) found that elevations of intramuscular BCAA concentration lead to BCOADH activation at rest, but could not account for the activation induced by exercise. Interestingly, these authors also found that the effects of exercise and enhanced muscle BCAA concentration on the dehydrogenase activation were additive. In addition, they reported that a low pre-exercise glycogen content augments the exercise-induced BCOADH activity without an increase in muscle BCAA content. Based on these data, van Hall and colleagues (1996) concluded that there must be different mechanisms to activate the enzyme.

2.2.5 Enzymes Involved With Glutamate and Ammonia

2.2.5.1 Glutamate Dehydrogenase

As previously discussed, the oxidation of BCAA within muscle does not directly lead to the production of free NH_3 since the amino nitrogen is donated to 2-oxoglutarate to form Glu. It is the fate of this Glu which determines the extent of NH_3 production from BCAA catabolism. Glu may be oxidatively deaminated by the enzyme GDH to produce free NH_3 and 2-oxoglutarate. The GDH reaction is as follows.

$$\text{Glu} + NAD^+ \leftrightarrow \text{2-oxoglutarate} + \text{NADH} + \text{ammonium}$$

GDH is found primarily within the mitochondrial matrix (Kovacevic and McGivan 1983) and its action has been linked with BCAAT resulting in a transdeamination of BCAA to form NH_3 and BCOA (see Fig. 2.2; Graham et al. 1994).

The activities of the dehydrogenase are high in the liver and kidneys, intermediate in brain, and low in skeletal muscle (Copenhaver et al. 1950; Frieden 1965). In addition, there appears to be no difference in the structure and regulation of GDH from tissue to tissue within the same species (Smith et al. 1975). The reaction catalysed by GDH is reversible, however the equilibrium of the reaction greatly favours the synthesis of Glu (Smith et al. 1975; Williamson et al. 1967). The dehydrogenase from animal tissues is inhibited *in vitro* by GTP and activated by ADP (Frieden 1965; Frieden and Colman 1967; Palaiologos and Felig 1976; Smith et al. 1975). GDP is also an inhibitor of the enzyme (Fisher 1973; Yielding and Tomkins 1961). More recent results (McCarthy and Tipton 1984) have shown that the inhibition of GDH by GTP is overcome by the addition of magnesium indicating that this nucleotide may have little influence over the enzyme *in vivo*. Interestingly, Leu and Ile concentrations, within the physiological range, activate GDH in the purified form (Yielding and Tomkins 1961) and within skeletal muscle mitochondria (Palaiologos and Felig 1976; Zhou and Thompson 1996).

Maximal GDH activity in rat and human muscle, when corrected to 37°C (assuming a factor of 1.8/10°C), is reportedly between 1-3.2 $mmol.kg^{-1}$ $ww.min^{-1}$ (Holloszy et al. 1970; Lowenstein 1972; Wergedal and Harper 1964; Wibom et al. 1992; Wibom and Hultman 1990; Williamson et al. 1967). A higher GDH activity has been reported in rabbit ST, compared with FT, muscle (Henriksson et al. 1986). Several authors have argued that GDH activity is negligible (Broberg and Sahlin 1989; Lowenstein 1972), however, the work of other researchers has clearly demonstrated that these maximal rates are theoretically sufficient to account for the muscle NH_3 production during submaximal exercise (Graham and MacLean 1992; MacLean et al. 1994).

Despite this evidence, the importance of GDH as a significant NH_3 producing reaction is clouded by a number of issues. First, the measurement of maximal GDH activity in muscle has been conducted in the direction of Glu formation. Schultz and Lowenstein (1976) have reported that maximal GDH activity in rat brain, measured in the direction of 2-oxoglutarate formation is 11-fold slower than that measured in the direction of Glu production. If this relationship holds for skeletal muscle, the capacity of GDH to produce NH_3 may be markedly lower than other studies have indicated. Secondly, the direction in

which the reaction proceeds within contracting fibres *in vivo* is currently unclear. Changes in the mitochondrial substrates and cofactor concentrations probably determine the direction of the reaction (Graham and MacLean 1992; Terjung and Tullson 1992). The concentrations of the reactants have been determined on muscle homogenates obtained from resting and exercising dogs and humans (Graham and Saltin 1989; Katz et al. 1990; Wolfe et al. 1987). This information, however is of little use in predicting the direction of the reaction since mitochondrial concentrations are required (Katz 1988). Thirdly, isolated muscle mitochondria, when incubated in the presence of Glu, do not normally liberate NH_3 from Glu (Lowenstein 1972). Glu catabolism proceeds in these cases via a transamination reaction involving oxaloacetate with the resultant production of Asp (Borst 1962; de Haan et al. 1967; Krebs and Bellamy 1960; Van Waarde and De Wilde-van Berge Henegouwen 1982). These data suggest that extramitochondrial Glu is preferentially directed away from access to GDH.

2.2.5.2 Glutamine synthetase

GlnS is a cytosolic enzyme (Herzfeld 1973; Kovacevic and McGivan 1983) which catalyses the reversible formation of Gln from Glu and NH_3 coupled to the hydrolysis of ATP.

$$\text{Glu} + \text{ammonia} + \text{ATP} \leftrightarrow \text{Gln} + \text{ADP} + \text{Pi}$$

The equilibrium of the reaction strongly favours Gln production (Meister 1984) and therefore GlnS acts to remove free NH_3 within the cell (see Fig 2.2). In comparison to other tissues, the activity of GlnS in rat skeletal muscle is not very high (i.e., 3.8-40 μmol.g w.w.$^{-1}$.hr^{-1}; Durschlag and Smith 1985; Iqbal and Ottaway 1970; King et al. 1983; Rowe 1985), but is sufficient to account for the rate of Gln release from contracting muscle (MacLean et al. 1994; van Hall et al. 1995a). Furthermore, due to the large mass of muscle it is probable that this tissue is the major site of Gln synthesis (Cooper 1988). Immunocytochemical staining for GlnS in rat plantaris muscle has shown that the enzyme is present in all fibre types with slow oxidative fibres staining most intensely (Willhoft et al. 1993). In contrast, Falduto et al. (1992) determined the *in vitro* activity of the enzyme in various muscles of the rat and concluded that its activity was highest in FTW muscle, intermediate in FTR, and lowest in SO fibres. The reason for the conflicting findings requires further examination.

The enzyme from rat muscle has a pH optimum of 7.2 (Iqbal and Ottaway 1970; Rowe 1985; Wu 1963). In addition, it has an apparent Km for ATP, Glu and ammonium of about 1.0, 5.0, and 0.20 mmol.l^{-1}, respectively (Iqbal and Ottaway 1970; King et al. 1983; Rowe 1985). Typically, the substrate concentrations of GlnS in skeletal muscle are above their Km indicating that substrate supply probably has a minor influence on the regulation of the activity of the synthetase. High, unphysiological levels of BCAA activate the enzyme (King et al. 1983). On the other hand, GlnS activity is inhibited by an increase in physiological levels of Pi (Iqbal and Ottaway 1970; Rowe 1985) and ADP (Rowe 1985) indicating that Gln formation probably occurs more slowly *in vivo* when muscle fibres are metabolically stressed.

2.2.5.3 Glutaminase

Glutaminase is a mitochondrial enzyme (Swierczynski and Makarewicz 1978) which catalyses the irreversible hydrolytic deamidation of Gln to produce Glu and ammonium.

$$Gln + H_2O \rightarrow Glu + ammonium$$

The maximal activity of the phosphate-dependent form of the enzyme in rat muscle homogenates is low (1.3-21.1 μmol.g w.w^{-1}.hr^{-1}; Durschlag and Smith 1985; Kelso et al. 1989; Swierczynski et al. 1993; Willhoft et al. 1993) in comparison with other NH_3 producing enzymes. This suggests that this reaction is probably only a minor source of NH_3 in contracting muscle. Importantly, the maximal activity of human skeletal muscle glutaminase is about 4-fold lower than that found in the rat (Swierczynski et al. 1993), indicating that it plays even less of a role in man. Recent evidence suggests that most of this activity may be associated with connective and endothelial tissue rather than the muscle fibres since immunofluoresence staining of the enzyme in sliced rat muscle sections was greatest in extramyocyte tissue, low in slow oxidative fibres and non-existent in the remaining fibre types (Willhoft et al. 1993). Other data have established the existence of a relatively large glutaminase activity associated with endothelial tissue (at least 20-fold greater than skeletal muscle; Leighton et al. 1987). These data support the suggestion of Wagenmakers et al. (1990) that some NH_3 in the venous blood draining active muscle may actually leave the muscle fibres in the form of Gln, but enter the plasma as NH_3 after being

metabolised by the endothelial cells (see Fig 2.2). The extent and significance of this process is unknown.

2.2.5.4 Aminotransferases

Most amino acids can be converted to their respective oxoacids by aminotransferase reactions in which the amino group from one amino acid is transferred to the oxoacid of another. The reactions catalysed by the aminotransferases are close to equilibrium. Aminotransferases are generally found in both the mitochondria and cytosol (Newsholme and Leech 1983).

BCAAT has been discussed in an earlier section of this review (see section 2.2.4.1). Other transaminases, which may be of importance in NH_3 metabolism, are Asp and Ala aminotransferase. Asp aminotranferase catalyses the transfer of the amino group from Glu to oxaloacetate to form 2-oxoglutarate and Asp. This reaction may supply Asp to the PNC facilitating the production of NH_3 (see section 2.2.2.4). Asp aminotransferase exists as two isozymes, one located within the mitochondria and the other in the cytosol (Rej and Horder 1974). These isozymes display different immunological and kinetic characteristics (Morino et al. 1964; Rej and Horder 1974). The activities of Asp aminotransferase in cytosolic and mitochondrial fractions of untrained human quadricep femoris muscle are 27 and 40 mmol.kg $ww^{-1}.min^{-1}$ (determined at 30 °C), respectively (Schantz et al. 1986). These activities are higher in endurance trained subjects (i.e., 39 and 59 mmol.kg $ww^{-1}.min^{-1}$; Schantz et al. 1986). Similar results have been reported for skeletal muscle of trained and untrained rats (Ji et al. 1987). A slightly higher enzyme activity has been found in rabbit ST, when compared with FT muscle (Henriksson et al. 1986). The *in vitro* Asp aminotransferase activity data indicate that the capacity of skeletal muscle to produce Asp markedly exceeds the capacity of the PNC to deaminate it. It should be noted however, that the direction in which the reaction proceeds during muscle activity is currently unclear. During recovery from intense stimulation there is strong evidence that Asp aminotransferase acts to produce Asp, since there is a considerable incorporation of labelled nitrogen from Leu into the muscle adenine nucleotide pool (Gorski et al. 1985).

Ala aminotransferase catalyses the transfer of the amino group from Glu to pyruvate to produce 2-oxoglutarate and Ala. Since this enzyme is involved with Glu

metabolism it has the potential to alter muscle NH_3 metabolism. In rat gastrocnemius muscle the enzyme activity is evenly distributed between the mitochondrial and cytosolic compartments (Mole et al. 1973). However, in muscle extracts from the whole rat hindlimb the cytosolic activity was 4-fold greater than that found within the mitochondria (Ji et al. 1987). The maximal *in vitro* activity in homogenates of rodent gastrocnemius muscle was 22.7 $mmol.kg^{-1}ww.min^{-1}$ (determined at 30 °C)(Mole et al. 1973). Ala aminotransferase activity increases almost twofold in response to endurance training (Ji et al. 1987; Mole et al. 1973). The enzyme activity is about three fold greater in rabbit ST compared with FT muscle (Henriksson et al. 1986), and approximately two fold greater in rat FTR than FTW muscle (Viru et al. 1994).

2.3 AMMONIA PRODUCTION DURING EXERCISE

2.3.1 Introduction

It has been known for many years that active skeletal muscle produces NH_3 (Parnas 1929). Since this initial discovery many studies have examined various factors influencing the production of NH_3 in contracting muscle. This section of the review will discuss this literature.

2.3.2 Exercise Intensity

During high intensity exercise (>90% $\dot{V}O_2max$) there is a marked increase in human muscle NH_3 production resulting in a large accumulation of NH_3 within the contracting fibres (Graham et al. 1990; Katz et al. 1986a,b; Stathis et al. 1994). The levels of NH_3 in resting muscle range between 0.5-1.4 $mmol.kg^{-1}$ dw (Graham et al. 1990; Katz et al. 1986a,b; Stathis et al. 1994) and following high intensity exercise muscle NH_3 content may reach 9.0 $mmol.kg^{-1}$ dw (Stathis et al. 1994). The fact that during short duration, high intensity exercise the fall in total adenine nucleotide (TAN) pool is matched by an increase in muscle IMP (Jansson et al. 1987; Sahlin et al. 1978; Stathis et al. 1994) and NH_3 (Katz et al. 1986a; Stathis et al. 1994) provides strong evidence that the major source of NH_3 production during this type of exercise is AMP deamination via AMPd activity. Furthermore, several studies (Di Mauro et al. 1980; Fishbein et al. 1978; Sinkeler et al. 1985, 1986, 1988) report that AMPd deficient individuals accumulate very small amounts of NH_3 in the plasma during high intensity forearm exercise under ischaemic conditions

compared with normal controls. Since increases in plasma NH_3 during exercise are caused primarily by an increased efflux from contracting muscle (Eriksson et al. 1985), these data provide further evidence that AMPd activity is the dominant NH_3 producing reaction during high intensity exercise.

During moderate exercise intensities (60-80% $\dot{V}O_2max$) muscle NH_3 content also increases (Broberg and Sahlin 1989; Febbraio et al. 1994; Graham et al. 1987; MacLean et al. 1991, 1994, 1996a) but to a smaller extent when compared with high intensity exercise. Several studies have reported that during non-fatiguing exercise at moderate intensities the TAN pool is unchanged from rest (Febbraio et al. 1994; Sahlin and Ren 1989; Sahlin et al. 1990c) and the content of IMP may only increase marginally above resting levels (Febbraio et al. 1994; MacLean et al. 1994). The fact that NH_3 accumulation and release from contracting muscle is much greater than IMP accumulation, indicates that net AMP deamination is not the major source of NH_3 during moderate exercise intensities. This statement is further supported by studies of AMPd deficient individuals who elicit a normal (Gross and Gresser 1993) or slightly reduced (Wagner et al. 1991) plasma NH_3 response during prolonged, submaximal exercise when compared with control subjects. There is, however, a lack of definitive evidence that net AMP deamination, via the action of AMPd, is not the major source of NH_3 during submaximal exercise. Studies of AMPd deficient individuals cannot exclude the possibility that sufficient residual activity of the enzyme remains. Furthermore, Tullson and Terjung (1992) speculate that some of the contracting muscle fibres may produce IMP and subsequently fatigue. Once these fibres no longer produce tension IMP may be reaminated, while other fibres are recruited to maintain the work rate. According to this scenario AMP deamination would be responsible for NH_3 production in contracting muscle which cannot be detected by biochemical analysis of mixed-muscle.

There is a good deal of support for the proposition that the major source of NH_3 production during MI exercise is the degradation of amino acids, in particular BCAA. The biochemical pathway responsible for this production remains unclear. Skeletal muscle is known to be the principal site of removal of circulating BCAA at rest and during exercise (Ahlborg et al. 1974; Gelfand et al. 1986; Rennie et al. 1981). Furthermore, skeletal muscle

BCOADH is activated as a result of exercise, indicating that BCAA oxidation within this tissue is increased (see section 2.5.1.2).

Very few studies have examined NH_3 metabolism during LI exercise (≤50% $\dot{V}O_2$max). At present there is uncertainty about whether NH_3 production occurs in contracting muscle during such circumstances. Eriksson et al. (1985) reported that muscle NH_3 efflux and arterial plasma NH_3 concentration increased during 15 min of cycling at 35% $\dot{V}O_2$max. Gass et al. (1991) found elevated plasma NH_3 levels in forearm venous blood during 40 min of cycling at 50% $\dot{V}O_2$max in untrained but not trained men. In contrast, Katz et al. (1986a) found no increase in muscle and arterial NH_3 concentration or muscle NH_3 efflux after 10 min of cycling at 50% $\dot{V}O_2$max. Interestingly, studies which have investigated the influence of exercise intensity on circulating NH_3 levels have also produced quite varied results. The reported minimum relative work rate required to produce an elevation in NH_3 within this fluid compartment ranges between 30-70% $\dot{V}O_2$max (Babij et al. 1983a; Buono et al. 1984; Denis et al. 1989; Eriksson et al. 1985; Greenhaff et al. 1991; Katz et al. 1986a; Wilkerson et al. 1977). These discrepancies may be caused by differences in exercise protocol, tissue sampling sites, nutritional state, muscle fibre type composition and/or training status.

During LI exercise, contracting muscle displays no evidence of metabolic stress since intramuscular concentrations of ATP, TAN, IMP and lactate are not different from resting values (Katz et al. 1986a; Sahlin et al. 1989). Although not definitive evidence, these data strongly suggest that if muscle NH_3 production occurs during these exercise intensities it is unlikely to result from net AMP deamination. A more likely source of NH_3 in these circumstances would be amino acid catabolism since it has been established that protein degradation and amino acid oxidation occur during LI exercise (Carraro et al. 1994; Knapik et al. 1991; Wolfe et al. 1982, 1984).

2.3.3 Exercise Duration

Studies examining NH_3 metabolism have demonstrated that, when the submaximal exercise intensity is reasonably high, there is a progressive increase in muscle NH_3 accumulation (Graham and Saltin 1987; MacLean et al. 1991; van Hall et al. 1995a), muscle NH_3 efflux (Broberg and Sahlin 1989; Graham et al. 1991) and plasma NH_3

concentration (Graham et al. 1991; MacLean and Graham 1993; Sahlin et al. 1990c; Snow et al. 1993) as a result of increasing exercise duration. The evidence suggests that in the early to mid-stages of prolonged submaximal exercise the source of NH_3 is predominantly amino acid catabolism since net AMP deamination is small (Norman et al. 1987; Sahlin et al. 1990c). With the onset of fatigue, however, an increased contribution to NH_3 production from net AMP deamination is likely to occur since marked increases in muscle IMP concentration have been observed (Broberg and Sahlin 1989; Norman et al. 1987; Spencer et al. 1991, 1992).

2.3.4 Substrate Supply

Several studies have manipulated the metabolic conditions in an attempt to alter muscle amino acid catabolism and NH_3 production during moderate exercise intensities. These manipulations have included BCAA feeding (MacLean et al. 1994, 1996a; MacLean and Graham 1993; van Hall et al. 1995b; Wagenmakers et al. 1990), alterations in carbohydrate supply (Broberg and Sahlin 1989; Czarnowski et al. 1995; Greenhaff et al. 1991; MacLean et al. 1992; Spencer et al. 1991, 1992; Wagenmakers et al. 1990, 1991) and Intralipid® infusion (Graham et al. 1991).

2.3.4.1 Carbohydrate

Several experiments have examined the influence of muscle glycogen concentration on human muscle NH_3 metabolism during prolonged exercise. Broberg and Sahlin (1989) reported that muscle NH_3 accumulation and efflux was markedly higher during a second bout of submaximal cycling exercise to fatigue when compared with an initial bout. The second exercise trial commenced approximately 75 min after the first, and was begun with markedly lower muscle glycogen levels. Using an experimental design which required trials on separate occasions, van Hall et al. (1995a) examined the influence of low pre-exercise muscle glycogen content on muscle NH_3 metabolism during 90 min of single-leg kicking. In contrast with the findings of Broberg and Sahlin (1989), van Hall et al. (1995a) found that muscle NH_3 production was not influenced by low pre-exercise glycogen levels. These conflicting data may be explained by differences in the experimental design and/or exercise mode. Interestingly, in a follow up study (van Hall et al. 1996), it was reported that the activation of BCOADH was two-fold greater at the end of 90 min of exercise in the leg

which began exercise in a glycogen depleted state. These data suggest that the activation of muscle BCOADH is not necessarily linked to elevated rates of muscle NH_3 production.

MacLean et al. (1991a,b) found no differences in muscle or plasma NH_3 accumulation when exercise was begun with average, compared with high, levels of muscle glycogen. These data support the notion that pre-exercise muscle glycogen levels have no influence on muscle NH_3 production. In contrast, studies of patients who are deficient in enzymes required for carbohydrate catabolism (Bertocci et al. 1993; Mineo et al. 1990; Sahlin et al. 1990a; Riley et al. 1993), observed that muscle NH_3 production was enhanced. In addition, Norman et al. (1988) reported that muscle IMP concentration only increased significantly in glycogen depleted fibres during 60 min of exercise. These data indicate that muscle NH_3 production, via net AMP deamination, was related to the glycogen content of the contracting fibres. Mixed-muscle analysis also revealed that during exercise the IMP content was relatively high in glycogen depleted states (Broberg and Sahlin 1989; Norman et al. 1987; Spencer et al. 1991, 1992).

Few studies have examined the effect of carbohydrate ingestion on human NH_3 metabolism during prolonged exercise. In a study conducted by Wagenmakers et al. (1991) subjects cycled for two hours either in a muscle glycogen depleted or in a glycogen loaded condition. In the depleted state only water was taken, whereas in the loaded trial a carbohydrate supplement was ingested throughout exercise. The exercise intensity was similar at the start of the two trials but had to be reduced in the depleted/non-supplemented trial in order for the subjects to complete the two hours of cycling. The authors reported a reduced plasma NH_3 concentration early in exercise in the glycogen loaded/carbohydrate supplemented trial suggesting that an enhanced carbohydrate supply attenuated muscle NH_3 production. This reduction was attributed to an inhibition of muscle amino acid catabolism since no exercise-induced net muscle AMP deamination was observed in either trial. Furthermore, the enhanced carbohydrate supply abolished the exercise related increase in BCOADH activity. The interpretation of these data needs to be treated cautiously since muscle sampling was performed after 120 min of exercise, and yet the plasma NH_3 differences occurred during the first 20 min of activity. Moreover, these results are made more difficult to interpret since the exercise intensities were different

between the trials at the end of 120 min of cycling. The study conducted by Wagenmakers et al. (1991) was unable to determine the sole effect of carbohydrate ingestion on muscle NH_3 metabolism, since muscle glycogen content was also manipulated.

Recently, van Hall (1996) performed a study which examined the influence of carbohydrate ingestion on plasma NH_3 concentration during prolonged, fatiguing exercise at a workload alternating between 50 and 80% workload maximum. These authors reported that plasma NH_3 concentration was not affected by carbohydrate ingestion throughout the exercise period. It is important to point out that in this study; 1. the subjects began exercise 75 min after eating a standardised breakfast, 2. the plasma glucose concentration never differed between treatments during exercise, and 3. the order of the trials was not randomised since the carbohydrate ingestion trial was conducted at least 3 days after the control. One, or all, of these factors may have influenced the plasma NH_3 results.

Spencer et al. (1991) reported that the accumulation of IMP content was attenuated in contracting muscle when carbohydrate was ingested during exercise. These data indicate that carbohydrate ingestion may reduce muscle NH_3 production from net AMP deamination (Spencer et al. 1991). In this experiment (Spencer et al. 1991) muscle was sampled in the carbohydrate ingestion trial at the time corresponding to fatigue in the control trial. It would be of interest to examine the influence of carbohydrate ingestion on muscle NH_3 metabolism prior to the onset of fatigue.

2.3.4.2 Branched-chain amino acids

BCAA supplementation is known to increase the BCAA concentrations in the blood and increase the delivery of these amino acids to contracting muscle (MacLean and Graham 1993; MacLean et al. 1994, 1996a; van Hall et al. 1996). Additionally, BCOADH, the rate limiting enzyme in the oxidation of BCAA in muscle, is activated to a greater extent during exercise when fed BCAA compared with controls (van Hall et al. 1996). Since BCAA supply to muscle is elevated, and the oxidation of these amino acids is increased, it is possible that BCAA feeding leads to an increase in muscle NH_3 production. The first study to investigate this possibility was conducted by Wagenmakers et al. (1990). These authors found that BCAA ingestion by McArdle's patients resulted in enhanced plasma NH_3

concentration during cycling exercise. More recently, several studies have examined the influence of BCAA feeding (BCAA doses ranged between 77-335 $mg.kg^{-1}$ body weight) on plasma NH_3 concentration during exercise in normal subjects (MacLean and Graham 1993; Nemoto et al. 1996; van Hall et al. 1995b). All the studies reported an elevated plasma NH_3 concentration during exercise as a result of BCAA ingestion, however, these authors were unable to precisely determine the mechanism(s) responsible. In an effort to elucidate the mechanism(s), MacLean et al. (1994, 1996a) have performed two studies examining the effect of BCAA feeding on NH_3 metabolism during dynamic knee extensor exercise. In the first study, a BCAA dose of 77 $mg.kg^{-1}$ body weight had no effect on intramuscular NH_3 accumulation, total release of NH_3 and total muscle NH_3 production. BCAA ingestion only induced a higher muscle NH_3 release when compared with control towards the end of the 60 min exercise period. When total muscle NH_3 production was added to total muscle Gln release, it was concluded that muscle NH_3 production was enhanced by BCAA feeding. This conclusion was convincingly supported by the results of a follow up study (MacLean et al. 1996a) in which a larger BCAA dose was given (308 $mg.kg^{-1}$ body weight). The studies by MacLean et al. (1994, 1996a) have also clearly demonstrated that the augmented muscle NH_3 production observed with BCAA ingestion was probably not due to an increased net AMP deamination.

2.3.4.3 Lipids

The only study to date which has examined the influence of fatty acid supply and muscle NH_3 metabolism during exercise was conducted by Graham et al. (1991). They found that an elevation of arterial free fatty acid levels resulted in a reduced rise in arterial NH_3 concentration and rate of muscle NH_3 release toward the end of 60 min of leg kicking. These authors speculated that muscle NH_3 production was decreased due to a fatty acid induced inhibition of amino acid catabolism. No direct evidence has been obtained to confirm this hypothesis.

2.3.5 Training

2.3.5.1 Endurance training

Endurance training reduces NH_3 production following short duration (3 min) *in situ* stimulation of rodent FTR (Dudley et al. 1987) and FTW (Dudley and Terjung 1985a)

muscle. Similarly, Constable et al. (1987) reported a reduction in muscle NH_3 content in trained rat plantaris muscle following 3 and 8 min of stimulation. In all of these training studies the reduction in muscle NH_3 content has been attributed to an attenuated NH_3 production due to a decreased activation of AMPd. The fact that the IMP content of the stimulated muscle was reduced after training (Dudley and Terjung 1985a; Dudley et al. 1987) provides strong support for this contention. These endurance training studies have also provided other evidence that a training-induced increase in oxidative capacity allows the contracting muscle to better match the rate of ATP degradation with ATP synthesis, thus explaining the reduced activation of AMPd.

To date, there are no endurance training studies directly examining human muscle NH_3 metabolism. Green and coworkers (1992, 1995) have reported that short-term endurance training attenuates muscle IMP content during prolonged submaximal exercise. These data suggest that NH_3 production from net AMP deamination is decreased by training, however, no measurements of muscle NH_3 and Gln content or efflux were undertaken to confirm this possibility. Interestingly, Green et al. (1992) found a reduced IMP accumulation in contracting muscle after training with no evidence of an increase in oxidative capacity. Green (1996) suggests that the reduced net AMP deamination following short-term training may result from an improved cardiovascular regulation, a decreased sympathoadrenal response and/or an increased mitochondrial sensitivity.

From the above evidence it seems clear that endurance training reduces net AMP deamination in active human muscle during prolonged MI exercise, thus leading to an attenuation of NH_3 production from this source. The effect that endurance training may have on human muscle NH_3 production from amino acid catabolism has yet to be established. There is little doubt that training increases the capacity of skeletal muscle to oxidise amino acids. For example, the maximal *in vitro* enzyme activities associated with muscle amino acid catabolism are increased with endurance training. These include Ala and Asp aminotransferases (Ji et al. 1987; Mole et al. 1973; Schantz et al. 1986), BCOADH (Shimomura et al. 1995) and GDH (Wibom and Hultman 1990). Furthermore, early studies using Leu isotopes established that Leu oxidation in rodent muscle slices was augmented by training (Askew et al. 1979; Dohm et al. 1977). Finally, trained rats display an increased

whole-body Leu oxidation during exercise (Dohm et al. 1977; Henderson et al. 1985). None of these data, however, establish that amino acid oxidation is actually elevated in contracting muscle. When such a study was conducted (Hood and Terjung 1987b), no increase in rat muscle Leu oxidation was observed during contractions. In fact, for a given oxygen consumption ($\dot{V}O_2$), trained muscle actually oxidised less Leu. Interestingly, this study also reported that the rates of Leu transamination were similar during contraction, irrespective of training status. These data suggest that the rate of muscle Glu production from Leu transamination was unaltered by training. This is important since the fate of the Glu amino nitrogen is a major determinant of the magnitude of NH_3 production from amino acid catabolism.

Several studies have investigated the influence of endurance training on human plasma NH_3 concentration. Short duration work protocols demonstrate no training-induced changes in plasma NH_3 concentration for a given relative workload (Denis et al. 1989; Hurley et al. 1984; Lo and Dudley 1987), whereas it is lower after training at the same absolute workload (Lo and Dudley 1987). In prolonged steady-state exercise (60-80% $\dot{V}O_2$max), endurance training lowers the plasma NH_3 concentration even at the same relative intensity (Denis et al. 1989; Green et al. 1991). A similar finding was reported when endurance trained subjects were compared with untrained individuals (Gass et al. 1991). All these data suggest that training reduces muscle NH_3 production during prolonged maximal exercise, however, no direct confirmation of this possibility has been undertaken. Recently, Graham et al. (1995b) compared muscle NH_3 and Gln efflux rates in endurance trained and untrained subjects during 3 hours of dynamic leg kicking. Interestingly, no differences were observed between the groups, suggesting that training may not reduce muscle NH_3 production. Further research into this problem is warranted.

2.3.5.2 Sprint training

Very little is known about the effects of sprint training on NH_3 metabolism. Stathis et al. (1994) reported that seven weeks of sprint training decreased the accumulation of muscle NH_3 content during a 30 s 'all-out' sprint. This reduction was attributed to a decreased net AMP deamination since the drop in the muscle TAN pool and increase in IMP were attenuated after training. This occurred despite an enhanced sprint performance

following training. The training-induced decrease in net AMP deamination is best explained by an improved match between ATP synthesis and degradation requiring a reduced activation of AMPd (Stathis et al. 1994). Hellsten-Westing (1993) reported a decreased maximal *in vitro* AMPd activity after sprint training which may contribute, at least in part, to the reduced rate of AMP deamination. Sprint training either reduces the plasma NH_3 concentration in recovery from sprinting (Snow et al. 1992) or results in very little alteration (Stathis et al. 1994). The reason for the discrepancy is not readily apparent. To date, no studies have examined the influence of sprint training on muscle amino acid metabolism and its interaction with NH_3 production.

2.3.6 Muscle fibre type

Studies directly ascertaining the production of NH_3 in various fibre types in human muscle have not been conducted due to technical difficulties. As mentioned previously (section 2.2.2.1), the magnitude of net AMP deamination is more marked in type II compared with type I human muscle fibres during intense exercise (Jansson et al. 1987; Sahlin et al. 1989). These data suggest that NH_3 production, via the action of AMPd, is greater in human type II muscle fibres during this type of exercise. In support of this contention, Dudley et al. (1983) found an inverse relationship between the percentage of type I fibres in the vastus lateralis muscle and increases in venous blood NH_3 levels following short duration, intense exercise. Such a relationship probably explains why sprint athletes achieve higher peak blood NH_3 levels after supramaximal exercise compared with endurance athletes (Hageloch et al. 1990; Itoh and Ohkuwa 1990).

It seems, however, that a relationship between fibre type and NH_3 may only be found when the exercise duration is short and the work rate is intense. For example, Graham et al. (1987) determined the relationship between NH_3 accumulation in muscle and blood with muscle fibre type. These relationships were determined during 30 min of cycling at 75-80% $\dot{V}O_2max$. These authors found that blood NH_3 concentration was only inversely related to type I fibres at 15 min, while muscle NH_3 concentration was related to type IIa fibres after 5, but not 30, min of exercise. Graham et al. (1987) concluded that type IIa fibres were only a major factor in producing NH_3 during the early stages of submaximal exercise and that type I fibres may contribute significantly to NH_3 production

when the exercise duration is prolonged. Norman et al. (1988) have demonstrated that at least some of this NH_3 production may be derived from net AMP deamination, since significant IMP accumulation was found in glycogen depleted type I fibres. Although speculative, it is likely that the NH_3 produced in type I fibres results primarily from amino acid catabolism.

2.3.7 Oxygen Supply

Breathing a hyperoxic gas mixture (60% O_2-40% N_2) during 30 min of submaximal cycling exercise at 75% $\dot{V}O_2$max had no effect on muscle and blood NH_3 concentrations compared with normoxic conditions (Graham et al. 1987). In contrast, Wolfe et al. (1987) reported that the accumulation of muscle NH_3 content was significantly increased after 10 min of 3 Hz stimulation of canine gastrocnemius muscle when the animals breathed hyperoxic (100% O_2), compared with normoxic, gas mixtures. The reason for the discrepancy is not readily apparent and requires further investigation.

One study (Young et al. 1987) has examined the influence of acute (<1 day) and chronic (13 days) exposure to high altitude (4,300 m) on plasma NH_3 accumulation during 30 min of exercise at 73% of the environmental specific $\dot{V}O_2$max ($\dot{V}O_2$max was significantly lower at altitude compared with sea level). These authors reported that the exercise-induced increase in plasma NH_3 concentration was similar between the sea level and acute altitude exposure tests. After chronic altitude exposure, however, the plasma NH_3 values were markedly attenuated in comparison to the other trials. The mechanism(s) causing this reduction has not been studied. Although muscle NH_3 production was not determined, Sahlin and Katz (1989) studied the effect of hypoxaemia on contracting muscle TAN degradation and IMP accumulation during short term LI exercise (5 min at 50% normoxic $\dot{V}O_2$max). Hypoxaemia resulted in an enhanced accumulation of IMP during exercise indicating that muscle NH_3 production from net AMP deamination was augmented in this condition.

2.3.8 Exercise Mode

Blood NH_3 concentration is higher during the latter stages (Bouckert and Pannier 1995) and following (Wilkerson et al. 1975) incremental maximal cycle ergometer exercise compared with a similar protocol involving treadmill running. The mechanism(s) causing

this difference has not been examined. In contrast, no differences in blood NH_3 concentration have been observed between running and cycling during 15 min of submaximal exercise at 65% $\dot{V}O_2$max (Bouckert and Pannier 1995).

Jensen-Urstad et al. (1993) have demonstrated a greater release of muscle lactate and NH_3 during 20 min of arm cranking compared with leg exercise at the same relative exercise intensity (i.e., 60% $\dot{V}O_2$peak for arm and leg exercise, respectively). These authors attributed this result to a higher metabolic stress and greater net AMP deamination during arm exercise, despite the activity being performed at the same relative intensity as during leg exercise. It is unclear why there is a greater metabolic stress during arm exercise. Jensen-Urstad et al. (1993) postulated that the distribution of oxygen to the arm tissue may be inadequate, although no supportive evidence for this hypothesis was provided.

2.3.9 Gender

Very few studies have examined the influence of gender on NH_3 metabolism and exercise. Itoh and Ohkuwa (1993) observed a greater peak blood NH_3 concentration in males compared with females after supramaximal exercise. These authors attributed this increase to a greater active muscle mass and/or a larger active type II fibre mass in the males. Such an explanation appears reasonable, because for a given muscle mass there is no sex related difference in the depletion of the muscle ATP pool during high intensity exercise (Bodin et al. 1994).

No study has compared NH_3 metabolism during submaximal exercise in males and females. Several researchers have reported that protein metabolism was lower during submaximal exercise in females compared with males (Phillips et al. 1993; Tarnopolsky et al. 1990, 1995). In addition, it seems that protein utilisation may also vary with the phase of the menstrual cycle (Lamont et al. 1987). These data suggest that muscle NH_3 production may be attenuated in females during submaximal activity and the magnitude of this reduction may be related to the menstrual cycle.

2.3.10 Temperature

Submaximal, cycling exercise in a hot environment (40°C) results in an increase in muscle (Febbraio et al. 1994; Snow et al. 1993) and plasma NH_3 (Snow et al. 1993) concentration compared with exercise performed at 20°C. At least in trained endurance

athletes, this occurs without any evidence of an increase in net AMP deamination within the contracting muscle (Febbraio et al. 1994). Febbraio et al. (1994), therefore, speculated that most of the increase in NH_3 production during submaximal exercise in the heat resulted from an augmented muscle amino acid catabolism. A marked increase in muscle NH_3 content was observed when supramaximal exercise was performed after pre-heating a leg (Febbraio et al. 1996). Under these circumstances, the elevation in NH_3 production was associated with an increased degradation of the TAN pool. These data demonstrate that net AMP deamination is augmented by elevations in muscle temperature during very intense exercise. An enhanced ATP degradation with increased muscle temperature has also been found during intense isometric contractions (Edwards et al. 1972).

2.4 AMMONIA REMOVAL DURING EXERCISE

2.4.1 Introduction

As previously discussed, exercise results in an increased muscle NH_3 production. Consequently, active muscle requires mechanisms to attenuate the accumulation of this toxic molecule. The strategies utilised to remove NH_3 involve its transport across the sarcolemma and its incorporation into amino acids which may subsequently exit the cell. This section of the review discusses the literature pertaining to these mechanisms.

2.4.2 Transport of Ammonia from Muscle

At rest, muscle is involved with the removal of NH_3 from the circulation with leg uptake rates about 2-3 $\mu mol.min^{-1}$ (Bangsbo et al. 1996; Eriksson et al. 1985; Katz et al. 1986a). During exercise, active muscle releases NH_3 with the magnitude varying with the exercise intensity and duration. At low exercise intensities NH_3 efflux rates are about 2-4 $\mu mol.min^{-1}$ (Eriksson et al. 1985; Katz et al. 1986a), and this rate increases to about 300 $\mu mol.min^{-1}$ during very intense contractions (Eriksson et al. 1985; Graham et al. 1990; Katz et al. 1986a). Immediately exercise ceases, the muscle NH_3 efflux rate falls and returns to resting values after 20-30 min of recovery (Graham et al. 1990). Several studies have demonstrated that only about 10-25% of the NH_3 produced by intensely contracting muscle is actually released during exercise and recovery (Graham et al. 1990; Katz et al. 1986a). The remainder is presumably incorporated into amino acids and adenine nucleotide resynthesis. Muscle NH_3 efflux rates increase within a few min after the onset of MI

exercise (MacLean et al. 1994, 1996a; van Hall et al. 1995a). If the submaximal exercise intensity is about 60-70% $\dot{V}O_2max$, efflux rates will increase to a new steady-state level of between 40-80 $\mu mol.min^{-1}$ (Graham et al. 1995b; MacLean et al. 1996a; van Hall et al. 1995a). However, if the exercise intensity is more severe, muscle NH_3 efflux rates will continue to increase throughout the exercise period, reaching around 100-110 $\mu mol.min^{-1}$ (Broberg and Sahlin 1989; Graham et al 1991).

There are a number of potential factors which may influence the transport of NH_3 across the sarcolemma. These include the NH_3 and pH gradients across the membrane, the membrane potential, ammonium's access to protein channels (in particular potassium channels), the number of opened channels, and the duration of their opening.

Discussion of NH_3 movement across muscle cell membranes traditionally follows the theory of nonionic diffusion (Mutch and Banister 1983). This theory states that NH_3 moves as ammonia according to the ammonia partial pressure gradient, because the membrane permeability of this species is higher than ammonium. The proportion of the NH_3 in the protonated and non-protonated form is dependent upon the pH. If this theory was correct this would lead to a calculated (using the Henderson-Hasselbach equation) intramuscular to extracellular NH_3 distribution ratio of about 2 at rest, and 4 immediately after intense exercise. Graham et al. (1990) determined the NH_3 concentration of muscle and plasma and calculated this ratio to be approximately 24 at rest and 9 after exercise. Since there was little agreement between the theoretical and actual ratios, the importance of nonionic diffusion in regulating the NH_3 distribution across the sarcolemma has been questioned (Graham et al. 1995a).

Although ammonia may be more permeable to the membrane than ammonium there is evidence which indicates that the latter may cross membranes via water filled channels (Blaty and Magleby 1984; Kleiner 1981; Knepper et al. 1989). This fact raises the possibility that the NH_3 distribution across the membrane may also be influenced by the membrane potential. Theoretical calculations, using the Nernst equation, indicate that if the NH_3 distribution was determined solely by the membrane potential the intra- to extracellular ratio would be about 25 (assuming a membrane potential of -85 mV) at rest (Wang et al. 1996), and this value would be expected to fall towards 14 (assuming a

membrane potential of -70 mV) at the end of exhaustive, intense exercise. These calculated ratios, are reasonable predictions of the ratios actually determined by Graham et al. (1990), indicating that the distribution of NH_3 across the sarcolemma may be influenced by the membrane potential. Recently, Wang et al. (1996) demonstrated that the NH_3 distribution across fish muscle membranes was affected by both the pH and electrical gradients. This finding provides further evidence that both species of NH_3 are permeable to the sarcolemma. Furthermore, these authors suggest that the NH_3 distribution across the membrane was predominantly determined by an interaction between pH and the electrical gradients.

Ammonium transport, via sodium/H^+ exchangers and sodium channels is unlikely to occur since experiments blocking these processes have not altered the NH_3 distribution across fish muscle membranes (Wang et al. 1996). Blaty and Magleby (1984), however, have found that ammonium can be transported across rat muscle membranes via potassium channels with a conductance ratio of one ammonium to ten potassium ions. Although speculative, this mechanism may provide an important route for ammonium transport since a large increase in potassium conductance via these channels occurs during contraction (Lindinger et al. 1995). It appears feasible that an increased efflux of ammonium through these channels may occur down the chemical gradient without an alteration in the membrane potential or pH gradient across the sarcolemma. A variation in the conductance of ammonium across the membrane is therefore, another factor influencing NH_3 transport which may need to be considered.

2.4.3 Muscle Alanine Synthesis and Efflux

Ala is produced in skeletal muscle either from the net degradation of proteins or *de novo* (Goldberg and Chang 1978). The latter involves the reaction catalysed by Ala aminotransferase (see section 2.2.5.3). Muscle does not possess an enzyme capable of producing Ala directly from the combination of free NH_3 and pyruvate (Ruderman and Berger 1974). Consequently, *de novo* Ala production can only be involved in removing NH_3 if Ala is synthesised from Glu, which was previously formed by utilising free NH_3. For example, Glu formed by the GDH reaction. As mentioned previously, the direction in which the GDH reaction proceeds during exercise is unclear (see section 2.2.5.1), therefore

the significance of Ala's role in removing NH_3 is also unknown. *De novo* Ala synthesis may be important, not because it removes NH_3, but because it attenuates NH_3 production. This could occur because Ala synthesis involves the consumption of Glu, thereby reducing the probability of Glu being involved in other NH_3 producing reactions.

Previous research has demonstrated that significant Ala synthesis occurs during exercise (Ahlborg et al. 1974; Carraro et al. 1994; Eriksson et al. 1985; Katz et al. 1986a; Sahlin et al. 1995; Wolfe et al. 1984). An increase in Ala accumulation within, and efflux from, contracting skeletal muscle has been observed by many researchers. The accumulation (Katz et al. 1986a; Sahlin et al. 1995) and increased release of Ala (Eriksson et al. 1985) from active muscle is directly related to the exercise intensity. Muscle Ala content may increase two fold (Katz et al. 1986a; Sahlin et al. 1995), while the release rate may increase to about 90-150 $\mu mol.min^{-1}$ during high intensity exercise (Eriksson et al. 1985; Felig and Wahren 1971; Katz et al. 1986a). During LI exercise, muscle Ala efflux increases as the duration of exercise progresses (Ahlborg et al. 1974). At higher submaximal exercise intensities the efflux rates either remain at a constant elevated level (Graham et al. 1991; MacLean et al. 1996a), or fall towards basal values (Graham et al. 1995b; MacLean et al. 1994; van Hall et al. 1995a). The peak increase in muscle Ala content occurs within the first few min of MI exercise (MacLean et al. 1994, 1996a; Sahlin et al. 1990c; van Hall et al. 1995a). After 60-90 min of MI exercise, muscle Ala content has returned to, or is approaching, pre-exercise levels (MacLean et al. 1994, 1996a; Sahlin et al. 1990c; van Hall et al. 1995a).

The synthesis of Ala within muscle is far in excess of the proportion of Ala found within skeletal muscle proteins implying that most of this synthesis involves a *de novo* process (Goldberg and Chang 1978). The carbon chain for *de novo* Ala production originates from carbohydrate sources (Caldecourt et al. 1985; Chang and Goldberg 1978, Spydevold 1976), while the amino group is obtained from BCAA (Chang and Goldberg 1978; Darmaun and Dechelotte 1991). It is commonly accepted that one function of Ala synthesis is to provide a non-toxic mechanism for transporting amino groups from the muscle to the liver.

2.4.4 Muscle Glutamine Synthesis and Efflux

Similar to Ala, Gln synthesis within muscle may occur from net protein degradation or via *de novo* synthesis utilising the GlnS reaction (see section 2.2.5.2). De novo synthesis of Gln requires the incorporation of one, possibly two, free NH_3 molecules and therefore constitutes a potentially important NH_3 removal mechanism. For Gln to remove two free NH_3 molecules, the amino group in the precursor Glu must have originated from free NH_3. In other words, the Glu must have been produced in the GDH reaction, a process which is associated with some uncertainty (see section 2.2.5.1).

There is little doubt that resting muscle is a major site of Gln synthesis and that a significant proportion occurs as a result of *de novo* processes (Goldberg and Chang 1978). The carbon skeleton for *de novo* Gln synthesis results from amino acids (Chang and Goldberg 1978; Wagenmakers et al. 1985) and possibly carbohydrate sources (Krebs 1975). The amino group of Gln may originate from BCAA (Darmaun and Dechelotte 1991). The source of the free NH_3, which is incorporated into Gln as the amide nitrogen, has not been identified.

There is some debate about whether an enhanced *de novo* Gln synthesis occurs in contracting muscle during exercise (Sahlin 1996), although several studies have provided indirect evidence that it does occur (Graham et al. 1995b; MacLean et al. 1994, 1996a; van Hall et al. 1995a). The debate still requires a more definitive resolution. Muscle Gln content remains constant with increasing exercise intensity (Katz et al. 1986a; Sahlin et al. 1995), while muscle Gln efflux increases only at high submaximal work rates (Katz et al. 1986a). During 20-180 min of MI exercise, muscle Gln content either increases above (Bergstrom et al. 1985) or remains at basal levels (Graham et al. 1995b; MacLean et al. 1994, 1996a; van Hall et al. 1995a; Sahlin et al. 1990c). Muscle Gln efflux is elevated above rest early in MI exercise averaging about 50-80 $\mu mol.min^{-1}$ (MacLean et al. 1994; van Hall et al. 1995a) and tends to fall towards basal levels, which are about 15 $\mu mol.min^{-1}$, during the latter stages of exercise (Graham et al. 1995b; MacLean et al. 1994, 1996a; van Hall et al. 1995a). The significant efflux of Gln from active muscle suggests that this amino acid, in conjunction with Ala, is a major transporter of nitrogen from this tissue (Graham et al. 1995b; MacLean et al. 1994, 1996a; van Hall et al. 1995a).

2.4.5 Ammonia Removal From The Circulation

Clearly, contracting muscle has the capacity to release relatively large quantities of NH_3 into the circulation. For example, in a study conducted by Graham et al. (1990), 1.1 mmol of NH_3 was released from muscle during 3 min of intense exercise and a further 3 min of recovery. In another study (Graham et al. 1995b), 11 mmol of NH_3 was released during 180 min of MI exercise. Assuming all the released NH_3 accumulated within the circulation, the blood NH_3 concentration would increase by 220 $\mu mol.l^{-1}$ immediately after sprinting, and by 2,200 $\mu mol.l^{-1}$ at the end of 180 min of submaximal exercise (assuming a blood volume of 5 l). Very few studies report increases in blood NH_3 concentration above 200 $\mu mol.l^{-1}$, with most research finding maximal elevations in the range between 100-150 $\mu mol.l^{-1}$ (Graham and MacLean 1992). These data clearly indicate that the NH_3 removal rate from the circulation must also increase during exercise.

Several processes act to remove NH_3 from the circulation during physical activity. Probably the major process involves a redistribution to other body fluids including the interstitial fluid (Sahlin 1996), resting muscle (up to 50 $\mu mol.min^{-1}$; Bangsbo et al. 1996), and the central nervous system (about 30 $\mu mol.min^{-1}$; Lockwood et al. 1979). The rate of this redistribution is proportional to the circulating NH_3 concentration (Bangsbo et al. 1996; Lockwood et al. 1979).

It has been reported that sweat contains significant amounts of NH_3 (Czarnowski and Gorski 1991; Czarnowski et al. 1992, 1995; Mitsubayashi et al. 1994), however, the source of this NH_3 remains unresolved. From the work of Czarnowski and Gorski (1991) it can be calculated that if all the NH_3 contained in the sweat was extracted from the plasma it would account for a plasma NH_3 removal rate of about 110 $\mu mol.min^{-1}$ during exercise at 80% VO_2max. This rate seems unlikely since NH_3 efflux from the exercising legs is only about 90 $\mu mol.min^{-1}$ at this exercise intensity (Eriksson et al. 1985). A similar conclusion is reached if the data from Czarnowski et al. (1995) is subjected to the same analysis. On this basis, it appears that a large proportion of the NH_3 excreted in the sweat is actually produced by the sweat glands and is not directly extracted from the plasma.

The rate of NH_3 removal by the splanchnic organs is about 12 $\mu mol.min^{-1}$ at rest and does not alter during exercise at intensities up to 80% VO_2max (Eriksson et al. 1985).

These data indicate that these organs play a minor role in removing NH_3 from the plasma, especially during intense submaximal exercise. It has been suggested that a considerable amount of NH_3 is excreted in the expired air (Graham et al. 1990). A recent preliminary report found that this was not the case (Colombani et al. 1996). Furthermore, a theoretical consideration of this matter by Sahlin (1996) also reached the conclusion that the lung was not a significant site for plasma NH_3 removal (<10 $\mu mol.min^{-1}$) during submaximal exercise.

2.5 SIGNIFICANCE OF MUSCLE AMMONIA PRODUCTION

It is clear that contracting muscle produces NH_3, however it is unclear what purpose, if any, this production serves within this organ. Some investigators (Lowenstein 1972) suggested that ammonia may act to buffer H^+ produced during exercise and/or stimulate glycolysis by activating phosphofructokinase. Several studies have demonstrated that the buffering role of ammonia is unlikely to be important during exercise (Dudley and Terjung 1985a; Katz et al. 1986b). Furthermore, sufficient evidence has been gathered to conclude that NH_3's role in activating phosphofructokinase is also minor (Graham et al. 1995a; Sahlin and Katz 1988).

It has also been proposed that NH_3 may directly cause muscle fatigue by inhibiting the rate of mitochondrial respiration, resulting in an increased reliance of anaerobic metabolism (Mutch and Banister 1983). This proposal was based on reports that mitochondrial respiration in brain and liver cells was decreased when these cells were exposed to varying concentrations of NH_3 (range 20 - 10,000 $\mu mol.l^{-1}$) (Hindfeldt and Siesjo 1970; Katunuma et al. 1966; McKann and Tower 1961; Worcel and Erecinska 1962). No study has investigated the effect of NH_3 on muscle fibre mitochondrial function. It is not known, therefore, whether the mitochondrial dysfunction observed in hepatic or neural tissue is also found in muscle.

Graham et al. (1995a), in an extensive review of the literature, argued that most of the experimental data indicates that NH_3 has no significant regulatory role in contracting skeletal muscle. This conclusion implies that muscle NH_3 production is simply a by-product of amino acid and/or AMP catabolism.

Finally, it should be noted that researchers have proposed that the hyperammonaemia observed during prolonged MI exercise may be linked to central nervous system dysfunction and the process of central fatigue (Banister and Cameron 1990; Mutch and Banister 1983). Unfortunately, definitive information coupling central dysfunction due to hyperammonaemia and fatigue during exercise is not available. The exercise-induced increase in circulating NH_3 concentration is most often below that required to produce overt neurological problems, suggesting that NH_3 is not a cause of central fatigue (Graham et al. 1995a; Terjung and Tullson 1992). It is unclear however, if the increases in plasma NH_3 concentration observed during exercise induce subtle changes in nervous system function which may lead to fatigue (Terjung and Tullson 1992). Further work is required to ascertain the importance of circulating NH_3 in the central fatigue process.

2.6 SUMMARY

NH_3 metabolism in active muscle has been extensively studied. Contracting muscle may produce NH_3 from AMP catabolism and/or amino acid degradation. AMP catabolism occurs predominantly via AMPd activity. The regulation of this enzyme is not fully understood but it involves factors such as substrate availability, phosphorylation, allosteric regulation, myosin binding and isozyme expression. It is clear that extensive AMP deamination occurs during short duration, intense exercise and this process may account for all the muscle NH_3 production in these circumstances. Net AMP deamination occurs when the ATP supply is exceeded by the ATP utilisation rate. Several factors are known to alter the net AMP deamination rate in contracting muscle. These include exercise intensity, duration and mode, substrate and oxygen supply, sprint and endurance training, muscle enzyme deficiencies, and environmental temperature.

There is evidence that most of the NH_3 produced during MI submaximal exercise is derived from amino acid catabolism, rather than net AMP deamination. The major amino acids oxidised by muscle are the BCAA and these are supplied by net hepatic or muscle protein degradation. It is unclear which biochemical pathways are responsible for the production of NH_3 from amino acids, however the two major candidates are the PNC and the GDH reaction. It is possible that Gln degradation by glutaminase, which is located

within the endothelial and connective tissue of muscle, may also contribute to whole-muscle NH_3 production.

The operation of the PNC in contracting muscle is controversial. Human skeletal muscle appears to have the enzymatic capacity to transfer amino groups from BCAA, at a fast enough rate, to supply sufficient Asp to the PNC to account for most of the NH_3 production observed during submaximal exercise. Further information is required in regards to the activity and regulation of the enzymes involved in the reaminating arm of the cycle in human muscle. It was noted that the regulation of the cycle may differ between species, therefore care is required when extrapolating the findings of animal studies to humans. In addition, the findings of studies using pharmacological interventions to examine the PNC, especially in rodent FTR muscle, are questionable and/or limited to too low an exercise intensity. The capacity of GDH to produce NH_3 from Glu degradation is also sufficient to account for the NH_3 production rates reported during submaximal exercise. Unfortunately, uncertainty remains about the direction the GDH reaction proceeds during muscular contractions.

The factors influencing muscle NH_3 production from amino acids have not been extensively studied. It seems that an increase in lipid supply inhibits, while an increased BCAA delivery enhances, muscle NH_3 production during MI exercise. The influence of muscle carbohydrate availability is currently unclear. There is evidence suggesting that the muscle NH_3 production from amino acids is greater when MI exercise is performed in the heat. Very little, or no information, is available on the effects of submaximal exercise intensities, exercise mode, gender or endurance training on muscle NH_3 production from amino acids.

The removal of NH_3 from active muscle probably involves the diffusion of ammonia and ammonium across the sarcolemma and the *de novo* synthesis of Gln. The NH_3 efflux from contracting muscle results in an increase in circulating NH_3 levels. The exercise-induced increase in plasma NH_3 concentration is attenuated by an elevated rate of removal. This process principally involves resting skeletal muscle and the central nervous system. The lungs and liver make a minor contribution to plasma NH_3 removal during MI exercise. The significance of NH_3 loss via the sweat is unclear.

CHAPTER 3

METHODS AND PROCEDURES

3.1 INTRODUCTION

This chapter presents the general methods and procedures used in the three studies presented in this thesis. Methods specific to each experiment are included in the appropriate experimental chapter. Throughout this thesis each study is referred to numerically as outlined below.

Study 1 - The influence of exercise intensity and duration on ammonia metabolism.

Study 2 - The effect of carbohydrate ingestion on ammonia metabolism during prolonged exercise.

Study 3 - Branched-chain amino acid feeding and ammonia metabolism during prolonged exercise.

3.2 SUBJECTS

The subjects used in this thesis were males aged between 18 and 35 years. In Studies 2 and 3 the subjects were endurance trained cyclists or triathletes. In the other study, the subjects were active but not specifically trained for cycling. All subjects were fully informed of the experimental procedures and signed an informed consent statement. The experiments were approved by either the Human Ethics Committee of Victoria University of Technology or the Human Research Ethics Committee at The University of Melbourne depending upon the location at which the experiment was conducted.

3.2.1 Subject Instructions

Subjects were instructed to refrain from strenuous exercise, caffeine, and alcohol 24 hours prior to all testing. In Studies 1 and 3 subjects were provided with food and drink which they consumed the day prior to testing. The total energy content of this diet was 13,500 kJ with 75% coming from carbohydrate. In Study 2, subjects were asked to record their food intake for the 24 hour period prior to trial 1 and to consume the same food the day prior to the second trial. Subjects presented to the laboratory on the morning of the trials after an overnight fast.

3.3 MEASUREMENT OF CARDIORESPIRATORY VARIABLES

3.3.1 Peak Oxygen Consumption

Peak oxygen consumption ($\dot{V}O_2$peak) tests were conducted on an electrically braked cycle ergometer (Excalibur, Lode; Studies 1 and 3) or a friction braked cycle ergometer (Monark Ergomedic 814E; Study 2). A heart rate monitor (Polar Sports Tester PE3000) was used to record heart rate. $\dot{V}O_2$peak was determined using open circuit spirometry (Study 2) and Douglas bags (Studies 1 and 3). During open circuit spirometry expired air was directed by a Hans-Rudolph valve and plastic tubing, into a mixing chamber and through a ventilometer (Pneumoscan 830). Aliquots of expired air were pumped from the mixing chamber through oxygen (Applied Electrochemistry S-3A) and carbon dioxide (Applied Electrochemistry CD-3A) analysers. These analysers were calibrated prior to each test with commercially (CIG, Australia) prepared gases of known concentration. When using Douglas bags, expired gases were directed, by a Hans-Rudolph valve and plastic tubing, into the bags. Aliquots of the bags' contents were analysed for oxygen and carbon dioxide using the aforementioned gas analysers, whilst volumes were determined by a Parkinson-Cowan ventilometer. Oxygen consumption was computed using standard equations (Consolazio et al. 1963).

All $\dot{V}O_2$peak tests consisted of a continuous incremental test. In Study 2 subjects began cycling at 100 watts (W), with the work rate increased by 50 W every 2 min for 10 min, and 25 W every min thereafter until volitional exhaustion. In the other studies, subjects cycled at either 100 or 150 W for 3 min and subsequently the work rate was increased by 50 W after 6 and 9 min. Work rates were increased by 25 W every min thereafter, until volitional exhaustion.

3.3.2 Submaximal Exercise

The relationship between steady state submaximal $\dot{V}O_2$ values for each individual and corresponding workloads was determined using a linear regression equation. From these equations workloads which would elicit the required exercise intensity were computed. Heart rate and expired air samples (collected using Douglas bags) were measured during submaximal exercise using methods described in 3.3.2.

3.4 BLOOD AND MUSCLE SAMPLING

Blood samples were obtained from an indwelling teflon catheter (Terumo 20G) inserted into a vein in the antecubital space. The catheter was kept patent by flushing with small amounts of heparinised saline (10 $IU.ml^{-1}$). Immediately prior to sampling a small volume of blood was drawn and discarded to ensure a fresh blood sample was obtained.

Muscle samples were obtained from the vastus lateralis muscle using the percutaneous needle biopsy technique (Bergstrom 1962) modified to include suction (Evans et al. 1982). An incision was made approximately 10 cm proximal to the lateral epicondyle of the femur after local anaesthetic (1% xylocaine) was injected into the site. A second incision was made 3 cm proximal to the first along the belly of the muscle. Where necessary, a further incision was made 3 cm proximal to the second. Biopsies were always obtained in sequence from the distal to proximal incision. Leg selection was random, and in the second trial the contralateral leg was biopsied. Muscle samples were quickly frozen and stored in liquid nitrogen. The estimated time between cessation of exercise and freezing of muscle was less than 20 seconds.

3.5 MUSCLE TREATMENT AND ANALYSIS

Muscle samples were divided into two portions which were weighed at -30°C. One portion (8-15 mg ww) was dissected into small pieces and placed into a microfuge tube (Eppendorf) and stored at -80°C until analysed for NH_3. These pieces were extracted at -20°C using 0.6 $mol.l^{-1}$ perchloric acid (PCA)-10% methanol, neutralised with potassium hydroxide and analysed for NH_3 by a flow injection technique (Svensson and Anfalt 1982) as described by Katz et al. (1986a). The second portion was freeze dried (Edwards, Modulyo) for 36 hours, weighed, powdered and apportioned for amino acid and other analyses. One portion (approximately 2 mg dw) was extracted according to the procedure of Harris et al. (1974). This extract was analysed enzymatically for ATP, creatine (Cr), creatine phosphate (CP) and lactate using fluorometric detection (Lowry and Passonneau 1972). In addition, reverse-phase high performance liquid chromatography (HPLC) was used to quantify ATP, ADP, AMP, and IMP using the methods of Wynants and van Belle (1985). This method was also used to analyse muscle Ino and Hx which are reported in Study 1 only. Separation was achieved with a Merck Hibar Lichrosphere 100 CH-18/2 250

mm x 4 mm column, using either a Bio-Rad Model 700 Chromatography Workstation (Study 2) or an ICI (Australia) HPLC Instrument (Studies 1 and 3). Another freeze dried muscle portion (Studies 1 and 3 only), about 1 mg in mass, was hydrolysed in 250 µl of 2 $mol.l^{-1}$ hydrochloric acid for two hours at 95°C. This extract was subsequently neutralised with 750 µl of 0.67 $mol.l^{-1}$ sodium hydroxide and stored at -80°C until analysed for glycogen using an enzymatic, fluorometric technique (Lowry and Passonneau 1972). In Studies 1 and 3, yet another freeze dried portion (about 2 mg) was extracted in ice cold 2.5% (w/v) sulphosalicylic acid (125 µl) for 10 min, spun (52,000 g for 2 min at 4°C) and the supernatant stored at -80°C until analysis. Immediately prior to analysis, 25 µl of each extract was diluted with 95 µl of 0.5 $mol.l^{-1}$ boric acid (pH=8.5) and 5 µl of 2 $mmol^{-1}$ phosphoserine (internal standard). This solution was used for the determination of free amino acids using a HPLC (ICI Australia), with automated precolumn derivatisation and fluorescence detection. The technique was a modification of the method described by Haynes et al. (1991). BCAA concentrations were calculated by addition of Ile, Leu, and Val, essential amino acids (EAA) by summing the BCAA, threonine (Thr), phenyalanine (Phe) and lysine (Lys), and total amino acids (total AA), by summing all measured amino acids. Taurine, proline and tyrosine were not reported since it was not possible to achieve reliable separation. Tryptophan was not analysed as the method required a precolumn derivatisation step which does not produce a fluorophore for this amino acid. In addition, muscle methionine (Met) concentrations could not be determined since these levels were below the detection limit of the method employed. Muscle EC was calculated in Studies 1 and 2 according to the formula (ATP+0.5ADP)/(ATP+ADP+AMP). Muscle metabolites, except for amino acids, glycogen (measured as glucose), lactate, Hx, Ino and NH_3 (due to their extracellular appearance), were adjusted to the peak total Cr (Cr+CP) for each subject.

3.6 BLOOD TREATMENT AND ANALYSIS

A portion of each blood sample was placed in fluoride heparin tubes. In Study 2 this blood was stored on ice for less than two hours until analysed for blood glucose concentration using an automated glucose oxidase method (YSI 23 AM analyser, Yellow Springs, OH). In the other studies this blood was immediately spun for 2 min at 15,000 g

and the plasma stored at -80°C prior to glucose analysis using the same glucose analyser. Some of these heparinised blood samples were spun by centrifugation and the plasma stored at -80°C for subsequent insulin analysis using a radioimmunoassay technique (Incstar, Stillwater, MN). A further portion of blood was placed into lithium heparin tubes. Some of this blood (500 µl) was directly mixed with 3 $mol.l^{-1}$ PCA, spun and the supernatant stored for blood lactate analysis. Alternatively, the blood was spun and 500 µl of plasma was mixed with 3 $mol.l^{-1}$ PCA, respun and the supernatant stored frozen until analysed for plasma lactate. Plasma or blood lactate was determined using an enzymatic, spectrophotometric technique (Lowry and Passonneau, 1972). In all studies, some of the blood from the lithium heparin tubes was spun and the plasma was placed into cryotubes and stored in liquid nitrogen until analysed for NH_3 and Hx. Plasma NH_3 was determined, within 72 hours of collection, using flow injection analysis (Svensson and Anfalt 1982), whilst Hx was measured on neutralised PCA extracts using a modification of a HPLC method described by Wynants and van Belle (1985). In Study 2, a 500 µl aliqout of lithium heparin treated plasma was mixed with 50 µl of 30% (w/v) sulphosalicylic acid. In the other studies 500 µl of plasma was mixed with 45 µl of 30% sulphosalicylic acid and 5 µl of 10 $mmol.^{-1}$ phosphoserine (internal standard). The deproteinised samples were spun and the supernatant stored at -80°C prior to amino acid measurement. Immediately prior to analysis 120 µl of each amino acid extract was diluted with 280 µl of 0.36 $mol.l^{-1}$ boric acid (pH=8.5). In the case of samples from Study 2, 5 µl of 10 $mmol^{-1}$ phosphoserine was also added. Plasma amino acids were analysed using the same HPLC technique as described for muscle amino acid determination. The circulating total AA, EAA and BCAA concentrations were calculated in Studies 1 & 3 as previously described, with the exception of Met, which was included in the determination of both total AA and EAA.

3.7 STATISTICAL ANALYSES

Analysis of variance (ANOVA) with repeated measures were used to compare data between trials and over time. Simple main effects analysis and Newman-Kuels post-hoc tests were used to locate differences when ANOVA revealed a significant interaction. Biomedical Data Processing (BMDP) statistical software packages were used to compute the ANOVA and simple main effects analyses. Where appropriate Student's t-test for paired

comparisons were also computed. Linear regression analyses were computed using Cricket Graph software. The level of probability to reject the null hypothesis was set at $P<0.05$. All data are reported as means ± the standard error of the mean (SE).

CHAPTER 4

THE INFLUENCE OF EXERCISE INTENSITY AND DURATION ON AMMONIA METABOLISM

4.1 INTRODUCTION

At present there are conflicting data regarding whether NH_3 production occurs in contracting skeletal muscle during LI (≤50% $\dot{V}O_2$max) exercise. Katz et al. (1986a) reported no increase in muscle and arterial plasma NH_3 concentration or muscle NH_3 efflux after 10 min of cycling exercise at 50% $\dot{V}O_2$max. These authors concluded that muscle and blood NH_3 levels only increased during strenuous exercise. In contrast, Eriksson et al. (1985) demonstrated that muscle NH_3 efflux and arterial plasma NH_3 concentration increased during 15 min of cycling at 35% $\dot{V}O_2$max. The reasons for the discrepancy are not readily apparent, but may be related to differences in the duration of exercise, nutritional state and/or muscle fibre type composition.

Several studies have demonstrated that during LI exercise contracting muscle displays no signs of metabolic stress since intramuscular concentrations of ATP, the TAN pool, IMP and lactate are not different from resting values (Katz et al. 1986a; Sahlin et al. 1989). Although not definitive evidence, these data suggest that if any muscle NH_3 production occurs during LI exercise it is unlikely to result from net AMP deamination. A more likely source of NH_3 in these circumstances would be amino acid catabolism since it has been established that net protein degradation and amino acid oxidation occurs during LI exercise (Carraro et al. 1994; Knapik et al. 1991; Wolfe et al. 1982, 1984).

The aim of this investigation, therefore, was to determine whether contracting muscle produced NH_3 during LI exercise and to establish the possible influence of exercise duration on this phenomenon. In addition, this experiment provided further information as to the likely source of NH_3 production during LI exercise and compared this source with the biochemical pathways being utilised during more intense exercise (i.e., 70% $\dot{V}O_2$max).

4.2 METHODS

Eight active males volunteered for this study. Their age, weight and $\dot{V}O_2$peak was 26.1 ± 1.3 yr, 78.4 ± 4.3 kg, and 3.99 ± 0.2 $l.min^{-1}$, respectively. $\dot{V}O_2$peak was determined according to the protocol described in chapter 3. Following this test, workloads

were selected which would elicit 35% (LI) and 70% (MI) $\dot{V}O_2$peak. On two separate occasions, separated by at least one week, each subject cycled at one of these work rates for 38 min. The first 2 min of exercise in each trial was used as a warm up. The workload in this period was 50 W and 100 W for the LI and MI exercise trials, respectively. The order of treatment was randomly assigned. Subjects consumed 250 ml of water just prior to, and after, 20 min of exercise in both trials. Heart rate was recorded at 5, 25 and 35 min during exercise. Expired gases were also collected in Douglas bags at these times. An electric fan was used to attenuate the exercise-induced thermal stress. Blood from an antecubital vein was sampled at rest, and at 10 min intervals of exercise. These samples were analysed for plasma NH_3, Hx, amino acids, glucose, insulin and lactate. Plasma amino acid and insulin analyses were not conducted at every sampling time. Muscle biopsies were obtained at rest, and immediately following 10 and 40 min of exercise. These samples were analysed for amino acids, NH_3, adenine nucleotides, IMP, Hx, Ino, Cr, CP, glycogen and lactate. Due to insufficient sample size the muscle amino acid and glycogen data are from seven and five subjects, respectively. A further problem was encountered with the analysis of Leu in one subject as coelution of this amino acid occurred with an unknown compound. Consequently, the muscle Leu and summary amino acid data are presented from six subjects.

4.3 RESULTS

4.3.1 Cardiorespiratory Data

The relative exercise intensities at which the two trials were conducted are shown in Fig. 4.1A. The LI trial was designed to elicit a relative $\dot{V}O_2$peak of 35%, however this value was approximately 38% during the initial stages, drifting upwards ($P<0.05$) to 41% towards the cessation of exercise. In the MI trial the relative $\dot{V}O_2$ peak was 69% after 5 min of exercise and increased ($P<0.05$) to approximately 73% during the latter stages of the exercise bout. As expected, the relative $\dot{V}O_2$peak was markedly different ($P<0.05$) between the trials at all measurement times. The mean heart rate after 5 min of exercise in LI was 108 $b.min^{-1}$ and this rate increased ($P<0.05$) moderately throughout the trial (see Fig. 4.1B). Heart rate was markedly elevated ($P<0.05$) in the MI trial with the mean rate

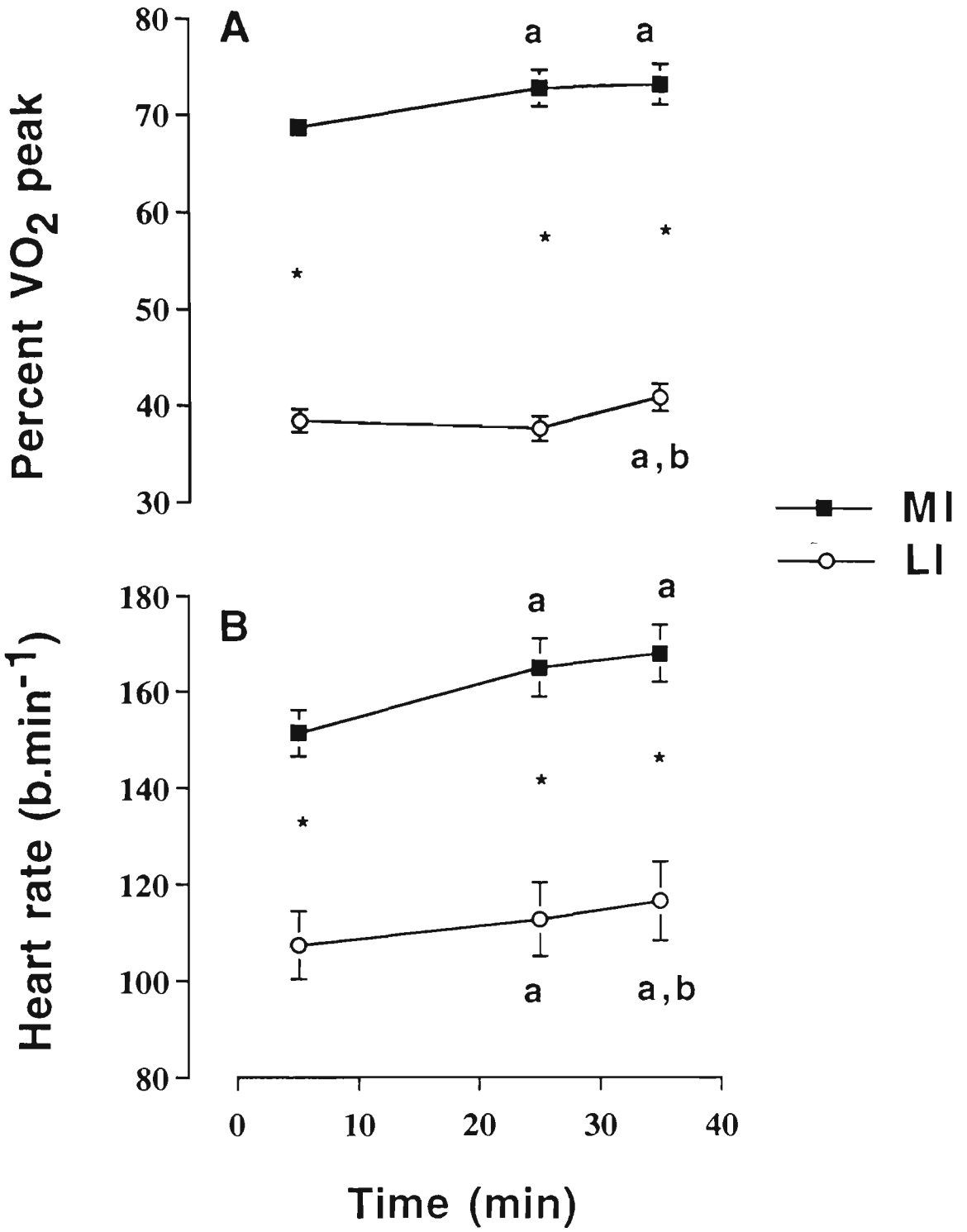

Fig. 4.1: The percent $\dot{V}O_2$peak (A) and heart rate (B) during 40 min of cycling at ≈ 40% (LI) and 70% (MI) $\dot{V}O_2$peak. Values are means ± SE, n=8. a different from 5 min ($P<0.05$), b different from 25 min ($P<0.05$), * difference between treatments, ($P<0.05$).

at 5, 25 and 35 min being 151, 165 and 168 $b.min^{-1}$, respectively. The heart rate during exercise was greater ($P<0.05$) in MI compared with LI.

4.3.2 Plasma Insulin, Glucose and Lactate

Plasma insulin concentrations were similar between trials at rest and early in exercise. After 40 min, however, the plasma insulin concentrations were lower ($P<0.05$) in MI compared with LI exercise (Fig. 4.2A). Plasma glucose levels were not different at rest or during exercise when comparing MI with LI (Fig. 4.2B). Plasma lactate concentrations were similar at rest between the two trials (Fig. 4.2C). Lactate concentrations in LI increased marginally ($P<0.05$) as a result of exercise; however, in MI the lactate values increased ($P<0.05$) about 6 fold. Consequently, there was a large difference ($P<0.05$) in the concentrations of this metabolite between trials. In both trials a plateau in plasma lactate concentration was observed during exercise .

4.3.3 Plasma NH_3 and Hypoxanthine

Similar plasma NH_3 and Hx concentrations were observed at rest between MI and LI (Fig.4.3A,B). In LI, no change in NH_3 concentration was observed during the first 20 min of exercise; however, about a 50% increase ($P<0.05$) from resting levels was noted after 30 and 40 min of activity. In MI, no increase in plasma NH_3 concentration occurred until 20 min and by 40 min the concentration of this metabolite was 400% above basal levels. NH_3 levels were markedly lower ($P<0.05$) throughout exercise in LI compared with MI. There was no exercise-induced increase in plasma Hx in LI. In contrast, in MI this metabolite increased ($P<0.05$) above resting values by 20 min of exercise and continued to accumulate throughout the remainder of the trial. As a consequence the plasma Hx concentration in MI was elevated ($P<0.05$) compared with LI at, and beyond, 20 min of exercise.

4.3.4 Plasma Amino Acids

The plasma amino data are summarised in Tables 4.1 and 4.2. No differences in plasma amino acid concentrations were observed between trials, with the exception of Ala. Plasma Ala levels were similar at rest and during the early stages of exercise between trials. However, at 20 and 40 min of exercise the Ala concentration was greater ($P<0.05$) in MI compared with LI. A significant main effect for time ($P<0.05$) was noted for some amino

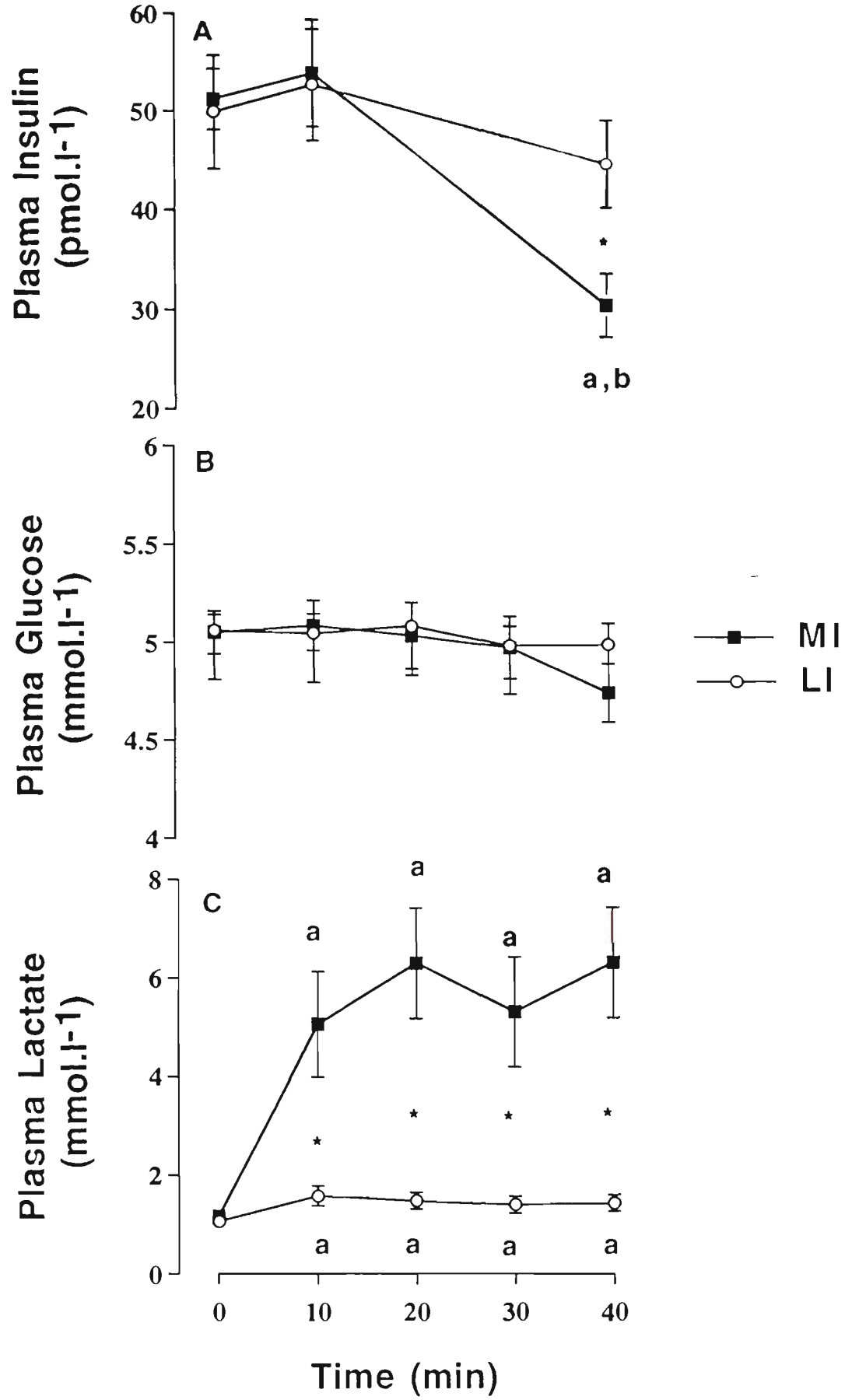

Fig. 4.2: Venous plasma insulin (A), glucose (B) and lactate (C) during 40 min of cycling at ≈ 40% (LI) and 70% (MI) $\dot{V}O_2$peak. Values are means ± SE, n=8. a different from 0 min ($P<0.05$), b different from 10 min ($P<0.05$), * difference between treatments ($P<0.05$).

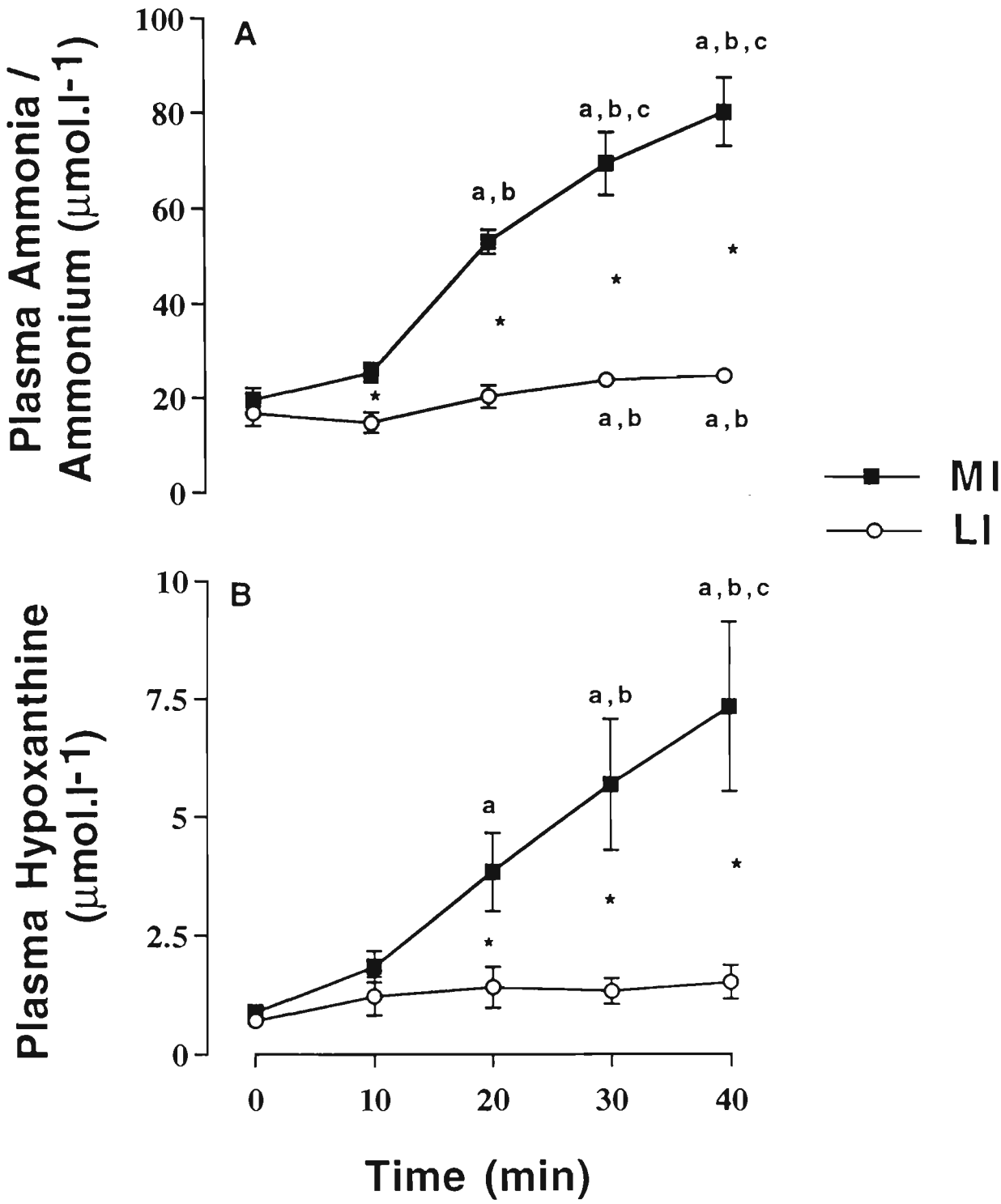

Fig. 4.3: Venous plasma ammonia/ammonium (A) and hypoxanthine (B) during 40 min of cycling at ≈ 40% (LI) and 70% (MI) $\dot{V}O_2$peak. Values are means ± SE, n=8. a different from 0 min ($P<0.05$), b different from 10 min ($P<0.05$), c different from 20 min ($P<0.05$), * difference between treatments ($P<0.05$).

	LI				MI			
	Rest	10 min	20 min	40 min	Rest	10 min	20 min	40 min
Asp	8.6 ± 0.5	9.8 ± 0.6	9.7 ± 0.5	10.1 ± 0.5	9.2 ± 0.6	8.7 ± 0.6	9.5 ± 0.6	10.8 ± 1.8*
Glu	15.0 ± 3.6	12.8 ± 3.3	18.1 ± 2.5	17.9 ± 1.6	15.5 ± 2.2	12.8 ± 2.8	20.0 ± 2.1	22.6 ± 1.6*
Asn	57.1 ± 5.5	58.8 ± 3.8	60.0 ± 3.5	61.4 ± 2.6	61.3 ± 4.1	58.1 ± 1.7	59.1 ± 4.2	61.1 ± 4.0
Gln	626 ± 30	648 ± 22	657 ± 25	671 ± 24	644 ± 32	644 ± 27	650 ± 30	678 ± 28
Ser	93.1 ± 6.8	95.3 ± 6.5	99.3 ± 6.0	102.2 ± 6.5	98.3 ± 7.7	95.2 ± 5.8	100.5 ± 6.6	102.8 ± 8.1
His	69.7 ± 5.0	75.1 ± 5.3	73.7 ± 4.1	72.6 ± 4.1	75.5 ± 5.4	78.0 ± 4.4	77.6 ± 5.6	79.0 ± 5.2
Gly	228 ± 16	221 ± 12	216 ± 11	223 ± 12	217 ± 10	220 ± 9	227 ± 10	228 ± 10
Thr	75.3 ± 6.0	76.2 ± 3.0	76.6 ± 3.4	77.3 ± 3.4	79.4 ± 5.6	74.9 ± 4.0	79.1 ± 5.1	81.4 ± 5.7
Ala	361 ± 25	381 ± 24	400 ± 26	387 ± 23	378 ± 21	405 ± 18	496 ± 24#	520 ± 30#
Arg	91.7 ± 6.6	93.8 ± 3.7	93.3 ± 4.3	94.5 ± 4.0	93.2 ± 5.9	94.8 ± 4.4	98.4 ± 4.7	98.7 ± 5.4
Val	216 ± 21	216 ± 14	216 ± 13	218 ± 12	222 ± 17	216 ± 11	222 ± 15	224 ± 13
Met	19.6 ± 1.6	21.0 ± 1.7	20.6 ± 1.6	20.6 ± 1.5	21.1 ± 1.9	21.0 ± 2.0	21.5 ± 1.7	22.1 ± 2.0
Ile	54.4 ± 4.9	54.3 ± 3.6	53.4 ± 2.9	53.1 ± 2.9	53.8 ± 3.5	53.6 ± 2.9	55.9 ± 3.1	55.4 ± 2.9
Leu	115 ± 10	116 ± 8	117 ± 8	115 ± 7	122 ± 8	118 ± 6	122 ± 7	124 ± 6
Phe	47.3 ± 3.8	49.2 ± 3.8	48.9 ± 4.1	49.9 ± 3.9	49.6 ± 3.9	50.2 ± 3.8	52.0 ± 3.5	53.8 ± 4.1
Orn	34.7 ± 4.2	36.6 ± 3.4	35.8 ± 2.7	36.2 ± 3.6	35.3 ± 2.8	38.4 ± 3.0	36.4 ± 3.1	38.2 ± 3.6
Lys	124 ± 13	139 ± 12	134 ± 9	131 ± 11	134 ± 15	146 ± 15	140 ± 14	143 ± 14*

Table 4.1: Plasma amino acid concentrations (in $\mu mol.l^{-1}$) at rest and during 40 min of cycling at ≈ 40% (LI) and 70% (MI) $\dot{V}O_2peak$. Values are means ± SE, n=8. * Significant time effect $P<0.05$; # denotes different from LI, $P<0.05$.

Time		Total AA	EAA	BCAA
Rest				
	LI	2243 ± 133	622 ± 66	494 ± 79
	MI	2376 ± 125	649 ± 62	512 ± 78
10				
	LI	2304 ± 87	634 ± 57	507 ± 77
	MI	2410 ± 76	644 ± 52	505 ± 72
20				
	LI	2329 ± 90	633 ± 52	504 ± 75
	MI	2467 ± 101	657 ± 57	515 ± 72
40				
	LI	2340 ± 81	630 ± 50	498 ± 71
	MI	2542 ± 116@	670 ± 59	521 ± 79

Table 4.2: Venous plasma amino acid concentrations (in $\mu mol.l^{-1}$) at rest and during 40 min of cycling at ≈ 40% (LI) or 70% (MI) $\dot{V}O_2peak$. Values are means ± SE n=8. AA, amino acid, EAA essential amino acids, BCAA, branched-chain amino acids. @ denotes significant treatment effect $P<0.05$.

acids including Asp, Glu, and Lys demonstrating that these amino acids increased in concentration as a result of exercise. The total AA, EAA and BCAA concentrations in the plasma for each trial are summarised in Table 4.2. There was no significant interaction for any of these variables; however, there was a significant treatment effect observed for total AA showing that the total AA concentration was higher ($P<0.05$) in the MI trial.

4.3.5 Muscle Metabolites

LI exercise did not influence ($P>0.05$) the content of the adenine nucleotides, TAN pool, IMP, Hx, Ino, EC, ATP/ADP ratio, lactate or glycogen within the contracting vastus lateralis muscle (Table 4.3). There was a small, yet significant ($P<0.05$), exercise-induced decrease in CP and a corresponding increase ($P<0.05$) in Cr. In addition, LI resulted in a progressive increase ($P<0.05$) in muscle NH_3 concentration so that after 40 min of cycling NH_3 levels were twofold greater than rest.

The concentration of the TAN pool was unaffected by 40 min of MI exercise. A significant treatment effect ($P<0.05$) for muscle ATP (measured enzymatically, E) and ADP content was found demonstrating that the mean values for ATP(E) and ADP, were lower and higher, respectively in the MI trial compared with LI. Similarly, a treatment effect ($P<0.05$) was also found for the ATP/ADP ratio and the EC. Both these variables were lower in the MI compared with LI exercise. The results obtained for ATP, analysed by the HPLC technique, tended to reflect the aforementioned results from the enzymatic analysis, although, no significant main effect for treatment was seen. ANOVA revealed a significant ($P<0.05$) time and treatment effect for muscle IMP content but no interaction ($P=0.06$) was achieved. The mean IMP concentration in the MI trial was greater than that found in LI. Muscle CP content decreased ($P<0.05$) as a consequence of MI exercise and reached values much lower ($P<0.05$) than found in LI. In contrast, Cr content increased ($P<0.05$) in the MI trial and was greatly elevated ($P<0.05$) when compared with LI. Similarly, muscle lactate and NH_3 concentrations increased ($P<0.05$) above resting levels in MI and both metabolites were in higher concentration ($P<0.05$) after 10 and 40 min of cycling than that observed in LI. Furthermore, glycogen content was reduced ($P<0.05$) throughout

	Rest	LI 10 min	40 min	Rest	MI 10 min	40 min
ATP(E)	26.3 ± 0.4	26.0 ± 0.7	25.9 ± 0.6	26.2 ± 0.7	25.2 ± 0.6	24.5 ± 0.9@
ATP(H)	23.9 ± 1.1	23.3 ± 1.0	23.9 ± 1.1	24.1 ± 0.6	23.1 ± 0.8	22.7 ± 1.0
ADP	1.97 ± 0.12	1.93 ± 0.13	1.97 ± 0.13	2.00 ± 0.11	2.19 ± 0.08	2.45 ± 0.13@
AMP	0.11 ± 0.02	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
TAN	26.0 ± 1.2	25.4 ± 0.9	25.9 ± 1.2	26.2 ± 0.6	25.4 ± 0.8	25.3 ± 1.0
ATP(H)/ADP	12.3 ± 0.5	12.5 ± 1.0	12.3 ± 0.5	12.3 ± 0.8	10.6 ± 0.5	9.4 ± 0.6@
EC	0.959 ± 0.002	0.956 ± 0.004	0.956 ± 0.002	0.958 ± 0.003	0.951 ± 0.002	0.946 ± 0.003@
IMP	0.08 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.12 ± 0.02	0.25 ± 0.09	0.41 ± 0.11@~
Ino	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01~
Hx	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.01*ab
CP	85.2 ± 1.8	75.1 ± 2.5a	73.2 ± 2.0a	84.8 ± 1.7	42.5 ± 5.3*a	38.5 ± 5.1*a
Cr	39.2 ± 3.5	49.3 ± 4.5a	51.1 ± 2.9a	39.7 ± 3.1	81.6 ± 6.0*a	85.9 ± 7.1*a
La	4.5 ± 0.5	6.0 ± 1.4	5.3 ± 1.1	5.0 ± 0.5	30.3 ± 6.0*a	23.7 ± 6.2*a
NH_3	0.44 ± 0.06	0.62 ± 0.09a	0.88 ± 0.08ab	0.47 ± 0.05	1.09 ± 0.16*a	1.55 ± 0.16*ab
Gly^	500 ± 23	472 ± 18	436 ± 22	469 ± 17	387 ± 17*a	246 ± 16*ab

Table 4.3: Muscle metabolites at rest and during 40 min of cycling at ≈ 40% (LI) and 70% (MI) $\dot{V}O_2$peak. Values are means ± SE, (n=8, ^except glycogen values n=5); Metabolite concentrations expressed in mmol.kg^{-1} dry weight (glycogen expressed as mmol.kg^{-1} glucosyl units dry weight); ATP(E), ATP analysed enzymatically; ATP(H), ATP analysed by HPLC; TAN, total adenine nucleotide pool= (ATP+ADP+AMP); EC, energy charge=(ATP+0.5ADP)/TAN; IMP, inosine 5'-monophosphate; Ino, inosine; Hx, hypoxanthine; CP, creatine phosphate; Cr, creatine; La, lactate; Gly, glycogen; NH_3, ammonia/ammonium; * different from same time point in LI $P<0.05$; @ significant treatment effect, $P<0.05$. ~significant time effect, $P<0.05$. a different from rest, $P<0.05$; b different from 10 min, $P<0.05$.

MI exercise. Consequently the content of this metabolite was lower after 10 and 40 min of exercise ($P<0.05$) in MI compared with LI.

The individual muscle amino acid data are summarised in Table 4.4. Exercise duration or intensity did not influence the muscle content of Ile, Leu and Orn. Muscle Gln, His, Gly, Thr, Arg, Val, Phe and Lys were elevated ($P<0.05$) with increasing exercise duration (i.e., significant main effect for time). Phe concentration was also higher ($P<0.05$) in MI compared with LI. Muscle Glu content decreased ($P<0.05$) with exercise in both trials, however, the reduction was more marked ($P<0.05$) in MI compared with LI. Muscle Ala levels were unaltered during LI but were elevated ($P<0.05$) as a result of exercise in MI. In the LI trial, Asn content fell ($P<0.05$) after 10 min of exercise and had returned to resting levels by 40 min. In contrast, during the MI trial there was no initial fall in Asn concentration and by 40 min the muscle content of this amino acid was actually greater ($P<0.05$) than that measured at rest and 10 min. Muscle Asp increased ($P<0.05$) with exercise duration and was higher ($P<0.05$) in the LI trial. The summary muscle amino acid data are shown in Table 4.5. The total AA content was not affected by exercise duration or intensity. In contrast, muscle EAA and BCAA content were influenced by exercise duration since these levels were greater ($P<0.05$) after 40 min compared with samples obtained at rest and 10 min of exercise.

4.4 DISCUSSION

4.4.1 Low Intensity Exercise

One of the major findings of this study was the observation that muscle and plasma NH_3 concentration increased during LI exercise (Table 4.3, Fig 4.3A). This supports the findings of Eriksson et al. (1985), but directly conflicts with Katz et al. (1986a). The reason(s) for the discrepancy is/are not clear since all three studies have utilised a similar type of subject, exercise mode and analytical techniques. The discrepancy cannot be explained by differences in exercise duration since muscle NH_3 content was increased after 10 min of exercise in the present study. This finding directly conflicts with that reported by Katz et al. (1986a).

The increase in muscle NH_3 content during LI must be due to a greater rate of muscle NH_3 production compared with NH_3 removal. NH_3 production in contracting

	LI			MI		
	Rest	10 min	40 min	Rest	10 min	40 min
Asp	0.78 ± 0.08	1.64 ± 0.18	1.72 ± 0.24	0.60 ± 0.04	1.05 ± 0.16	0.99 ± 0.16@~
Glu	6.43 ± 0.26	4.40 ± 0.40a	4.78 ± 0.36a	5.95 ± 0.24	2.72 ± 0.3*a	3.13 ± 0.38*a
Asn	0.90 ± 0.05	0.77 ± 0.04a	0.86 ± 0.04b	0.83 ± 0.04	0.89 ± 0.06*	0.99 ± 0.06ab
Gln	39.8 ± 1.3	37.8 ± 1.1	41.1 ± 1.3	40.8 ± 1.4	40.8 ± 2.3	43.5 ± 2.1~
Ser	1.71 ± 0.08	1.65 ± 0.09	2.22 ± 0.38	1.75 ± 0.16	1.72 ± 0.15	2.07 ± 0.10~
His	0.70 ± 0.11	0.76 ± 0.13	0.81 ± 0.13	0.73 ± 0.12	0.96 ± 0.12	0.95 ± 0.13~
Gly	3.21 ± 0.15	2.83 ± 0.15	3.34 ± 0.28	3.34 ± 0.21	3.03 ± 0.21	3.40 ± 0.19~
Thr	1.02 ± 0.04	0.94 ± 0.04	1.08 ± 0.06	1.00 ± 0.04	1.01 ± 0.04	1.19 ± 0.04~
Ala	5.52 ± 0.22	5.99 ± 0.21	6.12 ± 0.44	5.52 ± 0.34	9.90 ± 1.1*a	9.10 ± 0.53*a
Arg	1.04 ± 0.06	0.98 ± 0.05	1.09 ± 0.06	1.02 ± 0.06	1.06 ± 0.08	1.24 ± 0.06~
Val	0.75 ± 0.05	0.71 ± 0.05	0.79 ± 0.10	0.79 ± 0.07	0.77 ± 0.05	0.87 ± 0.05~
Ile	0.22 ± 0.01	0.20 ± 0.01	0.23 ± 0.04	0.24 ± 0.02	0.22 ± 0.01	0.25 ± 0.01
Leu+	0.43 ± 0.04	0.40 ± 0.03	0.45 ± 0.07	0.44 ± 0.06	0.45 ± 0.04	0.51 ± 0.03
Phe	0.19 ± 0.01	0.19 ± 0.02	0.22 ± 0.03	0.22 ± 0.02	0.22 ± 0.02	0.27 ± 0.01@~
Orn	0.45 ± 0.03	0.44 ± 0.03	0.58 ± 0.15	0.43 ± 0.04	0.45 ± 0.03	0.48 ± 0.03
Lys	1.78 ± 0.08	1.68 ± 0.10	1.90 ± 0.13	1.73 ± 0.11	1.79 ± 0.13	2.10 ± 0.12~

Table 4.4: Muscle amino acid concentrations at rest and during 40 min of cycling at ≈ 40% (LI) and 70% (MI) $\dot{V}O_2$peak. Values are means ± SE, (n=7, + except Leu values n=6); Metabolite concentrations expressed in mmol.kg^{-1} dry weight; * different from same time point in LI P<0.05; @ significant treatment effect, P<0.05; ~significant time effect, P<0.05; a different from rest, P<0.05; b different from 10 min, P<0.05.

Time		Total AA	EAA	BCAA
Rest				
	LI	64.9 ± 2.5	4.38 ± 0.22	1.39 ± 0.10
	MI	64.6 ± 2.9	4.42 ± 0.34	1.46 ± 0.17
10				
	LI	62.0 ± 2.0	4.17 ± 0.23	1.31 ± 0.10
	MI	67.8 ± 4.5	4.46 ± 0.29	1.46 ± 0.11
40				
	LI	67.5 ± 3.4	4.75 ± 0.47	1.50 ± 0.23
	MI	71.1 ± 4.0	5.15 ± 0.22 @	1.65 ± 0.10 @

Table 4.5: Muscle amino acid concentrations at rest and during 40 min of cycling at ≈ 40% (LI) or 70% (MI) $\dot{V}O_2$peak. Values are expressed in mmol.kg^{-1} dry weight n=6. AA, amino acid, EAA essential amino acids, BCAA, branched-chain amino acids. @ denotes significant time effect $P<0.05$.

muscle may occur as a result of AMP and/or amino acid catabolism. The source of NH_3 production during LI exercise is unlikely to be net AMP deamination, via AMPd activity, since the TAN pool was stable and there was no accumulation of muscle IMP, Ino or Hx (Table 4.3). Furthermore, there was no change in plasma Hx concentration (Fig.4.3B). Previous studies have also shown this to be the case (Katz et al. 1986a; Sahlin et al. 1989). There is the possibility that AMP catabolism via AdoD activity, may contribute to NH_3 production during prolonged exercise. The activities of soluble 5'-nucleotidase and AdoD are highest in ST rat muscle (Newsholme et al. 1985; Tullson and Terjung 1992). Moreover, ischemic contractions of ST and FTR fibres produce more adenosine and Ino than FTW fibres (Arabadjis et al. 1993). Since type I (i.e., ST) fibres are the only fibre type expected to be contracting during LI exercise this AMP catabolic pathway may therefore be a source of NH_3 production in the present study. The fact that there was no exercise-induced increase in muscle and plasma Hx or muscle Ino (Table 4.3, Fig 4.3B) during the LI exercise trial indicates that significant NH_3 production from AdoD activity is unlikely.

Muscle NH_3 production during LI exercise is therefore, best explained by an increase in muscle amino acid catabolism. An increase in amino acid oxidation during LI exercise has been demonstrated (Knapik et al. 1991; Wolfe et al. 1982,1984). Based on the present data, the increase in amino acid oxidation during LI exercise must occur despite adequate carbohydrate levels in muscle and plasma (Table 4.3, Fig 4.2B), and without a fall in plasma insulin (Fig 4.2A). A similar finding has been reported previously (Graham et al. 1991). Definitive proof that the catabolism of amino acids produces free NH_3 is lacking; however, indirect evidence strongly indicates that it occurs (Graham et al. 1987; MacLean et al. 1994, 1996a; MacLean and Graham 1993; van Hall et al. 1995a; Wagenmakers et al. 1990, 1991).

The muscle amino acid data of the present study was also able to provide indirect evidence that amino acid catabolism contributed to NH_3 production since changes in some muscle amino acid contents occurred during the LI exercise trial (Table 4.4). Unfortunately, the plasma amino acid data are difficult to interpret, since concentrations were determined from forearm venous blood. It has been demonstrated that a relatively constant arterial plasma amino acid profile occurs despite large changes in amino acid flux

across the contracting muscle (Graham et al. 1991, 1995a; MacLean et al. 1994). In the present study, the most marked muscle amino acid response occurred with Glu. The data indicate that within the first 10 min of LI exercise there was a 32% fall in Glu and this was maintained throughout the remainder of the exercise bout. A similar result has been reported previously (Katz et al. 1986a; Sahlin et al. 1995). This drop in Glu content is best explained by an increased utilisation since an enhanced muscle Glu uptake occurs during mild contraction conditions (Eriksson et al. 1985; Katz et al. 1986a). Moreover, an increase in muscle Glu production would also be expected to occur as a consequence of protein (Carraro et al. 1994) and other amino acid catabolism, in particular the BCAA (Knapik et al 1991; Wolfe et al. 1982, 1984). This is further supported by the observation of an elevated muscle EAA and BCAA content as the exercise duration progressed (Table 4.5). Exercise, in conjunction with an increase in muscle BCAA content, is known to activate BCOADH (Shimomura et al. 1990b, 1993; van Hall et al. 1996). The activation of this enzyme should promote Glu production via BCAAT activity (Hood and Terjung 1991; May et al. 1987).

As mentioned previously (Chapter 2, section 2.2.1), the fate of the amino group of Glu is the major determinant of the magnitude of NH_3 production from amino acid catabolism. N^{15}-Leu infusion studies indicate that during LI exercise the major fate of this amino group is the production of Ala via the Ala aminotransferase reaction (Wolfe et al. 1982, 1984). The methods employed in the present study and that of others (Ahlborg et al. 1974; Eriksson et al. 1985; Sahlin et al. 1995) are unable to confirm the results of the tracer studies since increases in muscle Ala concentration and muscle Ala efflux have not been found during short duration (<60 min), LI exercise. Glu may be converted to Gln by the action of Gln synthetase. In this reaction free NH_3 is consumed rather than produced. A lack of an increase in muscle Gln content (Table 4.4; Katz et al. 1986a; Sahlin et al. 1995) and muscle Gln efflux (Eriksson et al. 1985; Katz et al. 1986a) indicate that significant Glu consumption via the Gln synthetase reaction does not occur during the early stages of LI exercise. The fact that muscle Gln content increased during the latter stages of exercise (Table 4.4) suggests that some of Glu, in conjunction with NH_3, may be removed via this mechanism. Interestingly, the Asp concentration increased about 2 fold early in LI (Table

4.4) indicating that at least some of the nitrogen from Glu may be involved in Asp production via the Asp aminotransferase reaction.

The two pathways considered most likely to produce free NH_3 from Glu are the oxidative deamination of glutamate by GDH, and the transfer of the amino group from Glu to oxaloacetate to form aspartate which is subsequently deaminated in the PNC (Graham et al. 1995a). The reaction catalysed by glutaminase, however, cannot be excluded from a significant whole muscle NH_3 producing function (Wagenmakers et al. 1990). It is currently unclear which of these biochemical reactions is responsible for the production of NH_3 from amino acid catabolism. The data from the present study are unable to resolve this problem, however, some comments about the activity of the PNC and GDH reaction during LI exercise are worth noting.

Only two studies (Flanagan et al. 1986; Meyer and Terjung 1980) have directly examined the PNC during mild contraction conditions. These studies compared the accumulation of IMP in contracting rat muscle with or without exposure to pharmacological agents blocking IMP reamination. Flanagan et al (1986) reported that PNC operated however, since a marked decrease in muscle tension occurred in response to the drug, it was impossible to resolve the issue of whether the PNC operated in contracting fibres. Furthermore, their data have been questioned on the basis of the non-specific effects of the compound used to inhibit the cycle (i.e., AICAr). Moreover, Meyer and Terjung (1980) reported that the PNC did not operate during mild exercise conditions, but neither did they observe any evidence of NH_3 production. This absence of NH_3 production is in contrast to the findings of the present study. The conflicting findings raise the possibility that the PNC and NH_3 results obtained from rat muscle, especially during LI exercise, are not applicable to human muscle.

There are some notable differences in adenine nucleotide metabolism between human and rat muscle which indicate that species differences in PNC operation are possible. Unlike human type I fibres (Jansson et al. 1987; Norman et al. 1988; Sahlin et al. 1989), contracting rat ST fibres do not normally deaminate AMP (Meyer et al. 1980; Meyer and Terjung 1979). This indicates a marked difference between the species in the way the adenine nucleotide pool is regulated in this fibre type. The reason for this is unclear, but it

may be related to species differences in the regulation of AMPd activity. The predominant isoforms of AMPd in rat and human muscle are A and M, respectively (Ogasawara et al. 1978; Thompson et al. 1992; Vankuppevelt et al. 1994). These forms of AMPd appear to have similar immunological (Ogasawara et al. 1982), kinetic and regulatory behaviour. There is, however, one notable exception. AMPd binds to myosin in contracting rat, but not human, muscle (Rundell et al. 1992a, 1993; Rush et al. 1995; Shiraki et al. 1981; Tullson et al. 1995). The implications of this species difference in regulation is unclear. Perhaps more importantly there is a difference in the presence of a second form of AMPd in the ST fibres of both species. This isoform has been classified E in human muscle and C in rats (Ogasawara et al. 1982). These isoforms have different immunological, kinetic and regulatory characteristics (Ogasawara et al. 1982; Raggi et al. 1975; Raggi and Ranieri-Raggi 1987). Of note, the kinetic and regulatory characteristics of the E isozyme suggest that it may be active in conditions not too disimilar from resting muscle, unlike the M isozyme. Although speculative, the activity of the E isozyme may enable the operation of the PNC in human type I muscle fibres during LI contraction conditions. It should be noted, however, that the muscle metabolite data of the present study were unable to demonstrate any evidence of an increased AMPd activity during LI exercise.

The fact that individuals deficient in the M isoform of AMPd display a normal or slightly reduced plasma NH_3 accumulation during prolonged aerobic exercise (Gross and Gresser 1993; Wagner et al. 1991) suggests that NH_3 production may not be due to the action of AMPd and the operation of the PNC. There is, however, a lack of definitive evidence that this is the case since some residual AMPd activity remains in AMPd deficient patients (presumably by isoform E in type I muscle fibres), and this may be sufficient to account for the NH_3 production.

Terjung and Tullson (1992) have argued that during fairly intense submaximal exercise, some of the less oxidative fibres produce IMP and subsequently fatigue. Once these fibres no longer produce tension, IMP may be reaminated, whilst other fibres are recruited to maintain the work rate. According to this scenario, AMP deamination would be responsible for the NH_3 production in the contracting fibres and not deamination of Asp via the PNC. At the whole muscle level, however, it would appear that both AMP

deamination/IMP reamination were occuring simultaneously. Such a scenario may be occurring during MI exercise in the present study, but the extent to which it is operating remains unknown. On the other hand, if the PNC was operating during LI exercise, then a strong argument can be made that both legs of the cycle must be occurring within the contracting fibres. Studies have shown that type I fibres are the only muscle fibre type recruited during exercise at 40% $\dot{V}O_2$max (Gollnick et al. 1974; Sale 1987). Since, these fibres are fatigue resistant, it is unlikely that contracting type I fibres would accumulate IMP, then subsequently reaminate it after they stopped contracting.

The other possible source of muscle NH_3 production from amino acid catabolism is the oxidative deamination of Glu to produce 2-oxoglutarate and ammonium, catalysed by GDH. GDH is found primarily in the mitochondrial matrix (Kovacevic and McGivan 1983) and therefore, its activity should be highest in the fibres contracting during LI exercise (e.g., type I fibres). The maximal *in vitro* GDH activity in human muscle (Wibom et al. 1992; Wibom and Hultman 1990) is clearly sufficient to account for the rate of NH_3 accumulation in the muscle and plasma during LI exercise in the present study. Some authors (Graham et al. 1995a; Newsholme and Leech 1983) have postulated that the action of GDH is linked with BCAAT activity which results in a transdeamination of BCAA to form NH_3 and BCOA. It should be noted that the importance of GDH as a significant NH_3 producing reaction is clouded by a number of issues. The reader is referred to Chapter 2 (see section 2.2.5.1) for a detailed discussion of these matters.

4.4.2 Moderate Intensity Exercise

Muscle NH_3 metabolism during MI exercise has been studied extensively (Broberg and Sahlin 1989; Graham et al. 1987; MacLean et al. 1991, 1994; van Hall et al. 1995a). The exercise-induced increase in muscle and plasma NH_3 concentrations observed in the present study was, therefore, expected (Table 4.3, Fig. 4.3B). It was also expected that the accumulation of NH_3 in these tissues would be higher in the MI trial compared with LI. At least some of the increase in NH_3 production at the higher exercise intensity must be due to net AMP deamination since elevations in muscle IMP, Ino and Hx content and plasma Hx concentration occurred in the MI trial. Most studies (Green et al. 1995; Febbraio et al. 1994; MacLean et al. 1994; Sahlin et al. 1989; Spencer et al. 1991, 1992), but not all

(Sahlin et al. 1990c; Norman et al. 1987), have found a significant elevation in muscle IMP during MI non-fatiguing exercise. The mean increase in muscle IMP content in this trial was 0.29 mmol.kg^{-1}dw which is about 27% of the muscle NH_3 accumulation. If the accumulation of plasma NH_3 and Hx is taken into consideration, one can estimate that net AMP deamination accounts for no more than 20% of the total muscle NH_3 production. This value is in close agreement with that reported by Broberg and Sahlin (1989). It should be noted however, that this estimate ignores plasma and muscle NH_3 removal mechanisms and cannot account for any reamination of IMP in fatigued fibres. The latter may lead to a significant underestimation of the AMP deamination rate (Terjung and Tullson 1992) and therefore an overestimate of NH_3 production from amino acid catabolism. More sophisticated studies (MacLean et al. 1994, 1996a), have been better able to quantify muscle NH_3 removal and hence provide an improved estimate of actual NH_3 production. These authors calculate that net AMP deamination, as estimated from muscle IMP accumulation, can only account for 0.6-6.0% of the NH_3 produced in contracting muscle during 60-90 min of MI leg-kicking exercise.

Although net AMP deamination is probably underestimated by the measurement of AMP degradation products during MI exercise (Terjung and Tullson 1992), it is likely that most of the NH_3 production during MI exercise also results from amino acid catabolism. The rate of amino acid catabolism must increase with exercise intensity to account for the elevated muscle and plasma NH_3 concentration found in the MI trial compared with LI . Several studies (Babij et al. 1983b; Dohm et al. 1980; Felig and Wahren 1971; Henderson et al. 1985; Hood and Terjung 1987a,b; White and Brooks 1981) have demonstrated that this is probably the case. In the present study, muscle amino acid content was relatively insensitive to increases in exercise intensity, suggesting that such a measure is only a crude marker of amino acid catabolism. For example the levels of EAA, BCAA and total AA were not affected by exercise intensity (Table 4.5). There were a few muscle amino acids, however, which were affected by the work rate (Table 4.4). Muscle Asn, Phe and Ala were elevated, whilst Glu and Asp were reduced in MI compared with LI. Interestingly, Phe has been used as a marker of non-contractile protein degradation (Hood and Terjung 1994; Smith and Rennie 1990) since it is not subject to intermediary metabolism in muscle.

Consequently, the increase in the muscle Phe content in MI compared with LI provides indirect evidence that non-contractile protein degradation was greater at the higher exercise intensity.

As was the case for LI exercise, it is unclear which amino acid catabolic pathway(s) is/are responsible for muscle NH_3 production during MI exercise. Both GDH and the enzymes of the PNC appear to have the capacity to produce NH_3 at the rate at which this metabolite accumulates in muscle and plasma during MI exercise. Most of the arguments related to the role of GDH and PNC activity in NH_3 metabolism have already been discussed. There are however, a few further points to make. First, type IIa fibres will be recruited in addition to type I fibres during MI exercise. Type IIa fibres will probably have a smaller GDH and BCOADH activity due to their lower oxidative capacity. Furthermore, a greater proportion of the BCAAT activity may be present in the cytosol compared with type I fibres (Hutson 1988). This suggests that an increased Glu production, at least from BCAA catabolism, may occur within the cytosol of type IIa fibres. As mentioned previously (Chapter 2, section 2.2.5.1), extramitochondrial Glu may not gain access to GDH. This raises the possiblity that Glu catabolism is less likely to occur via GDH activity in type IIa, compared with type I fibres.

Second, two studies have directly examined the operation of the PNC during prolonged MI exercise using drugs to inhibit IMP reamination (Aragon and Lowenstein 1980; Meyer and Terjung 1980). Both studies provided evidence that the PNC operated in rat muscle, containing both FTR and FTW fibres, stimulated *in situ* at moderate stimulation frequencies (3-5 Hz). In addition, Meyer and Terjung (1980) demonstrated that at these frequencies, the PNC occurred only in rat FTW muscle and that the reamination of IMP was attributable to FTW fibres which were no longer contracting. Based on this evidence it was concluded that the two legs of the PNC operate out of phase during mild contraction conditions (Meyer and Terjung 1980). Thus, the cycle could not contribute to the deamination of Asp in contracting muscle. Interestingly, the operation of the PNC in contracting FTR fibres at stimulation frequencies greater than 5 Hz has not been directly assessed, however, there is some evidence that it may occur (Hood and Parent 1991). If more definitive evidence confirmed this observation, it then raises the possibility that the

PNC may be active in contracting human type IIa fibres. Finally, in contrast to LI exercise, the data clearly indicates that AMPd was active during MI exercise. This at least provides the opportunity for IMP reamination to take place.

4.4.3 Conclusion

In conclusion, the increases in muscle and plasma NH_3 accumulation observed in this study indicate that muscle NH_3 production occurs during LI exercise. Furthermore, NH_3 concentration in these tissues increased with increasing exercise duration. The source of NH_3 production during LI exercise appears to be exclusively amino acid catabolism as there was no evidence of net AMP deamination as judged by a constant muscle TAN pool and an absence of an exercise-induced increase in AMP degradation products in muscle or plasma. Although the major source of NH_3 production during MI exercise was probably amino acid catabolism, net AMP deamination was also contributing to the NH_3 pool. This study was unable to provide evidence as to which biochemical pathway(s) was/were responsible for NH_3 production from amino acid catabolism during MI or LI exercise.

CHAPTER 5

THE EFFECT OF CARBOHYDRATE INGESTION ON HUMAN AMMONIA METABOLISM DURING PROLONGED EXERCISE

5.1 INTRODUCTION

Muscle NH_3 production increases during prolonged exercise and the source of this production is likely to be AMP and/or amino acid catabolism (Terjung and Tullson 1992). Several studies (Broberg and Sahlin 1989; Czarnowski et al. 1995; MacLean et al. 1992, 1994; van Hall 1996; Wagenmakers et al. 1991) have manipulated carbohydrate availability to contracting muscle in an attempt to alter NH_3 production during prolonged exercise at moderate intensities. Experiments which have examined the effect of pre-exercise muscle glycogen content on NH_3 metabolism indicate that muscle NH_3 production is similar in muscle with normal compared with high glycogen contents (MacLean et al. 1991, 1992). The effect of low pre-exercise glycogen content on NH_3 metabolism is currently unclear since one study (Broberg and Sahlin 1989) reports that muscle NH_3 production is enhanced due to an increased AMP catabolism whilst another (van Hall et al. 1995a) found no change.

Wagenmakers et al. (1991) studied the influence of carbohydrate ingestion combined with altered pre-exercise muscle glycogen content during two hours of cycling exercise. The exercise bout commenced at 70-75% maximum workload. During the first 20 min of exercise, when the exercise intensities were identical, plasma NH_3 concentration was elevated in the subjects with reduced carbohydrate availability. This elevation was attributed to an increased muscle NH_3 production derived from amino acid catabolism, although no measurements of muscle NH_3 content or muscle NH_3 efflux were obtained to confirm active muscle as the source of the elevated plasma NH_3 concentration. In addition, no muscle measurements were made at this time to demonstrate that amino acid catabolism was the source of the NH_3 production. Finally, the study by Wagenmakers et al. (1991) was unable to determine the sole effect of carbohydrate ingestion on muscle NH_3 metabolism, since muscle glycogen content was also manipulated. Recently, van Hall (1996) conducted a study which examined the influence of carbohydrate ingestion on plasma NH_3 concentration during prolonged cycling exercise at alternating workloads of

50 and 80% workload maximum. These authors reported that plasma NH_3 concentration was not affected by carbohydrate ingestion throughout the exercise period. This study, however, was unable to determine if any changes in NH_3 metabolism occurred within contracting muscle as a result of carbohydrate ingestion. In fact, no studies have examined this specific question. Thus, the aim of the present study was to ascertain the effect of carbohydrate ingestion on muscle and plasma NH_3 metabolism during prolonged submaximal exercise. Furthermore, this study aimed to provide information on the influence of carbohydrate ingestion on the biochemical reactions involved with muscle NH_3 production.

5.2 METHODS

This study was conducted in two parts. Initially, eight endurance trained men volunteered to exercise and have cardiorespiratory and blood samples taken (see Declaration). Subsequently, two of these subjects and a further three subjects volunteered to undertake the same experimental protocol except muscle samples were also obtained. The common data obtained for the two subjects participating in both parts of the experiment were averaged, resulting in n=11. It should be noted that there were some plasma measurements made in the first part of the study (n=8) but not the second (n=5), and vice versa. This accounts for the variation in the number of subjects from which the mean data are presented.

The mean age, weight and $\dot{V}O_2$peak (n=11) of the subjects were 27.7 ± 1.6 yrs, 69.8 ± 1.7 kg and 4.40 ± 0.08 $l.min^{-1}$, respectively. At least one week prior to the trials each subject performed an incremental maximal exercise test on a Monark cycle ergometer according to the procedure outlined in Chapter 3. Subsequently, the subjects were studied during two hours of cycle ergometer exercise, at a workload requiring 65% $\dot{V}O_2$peak, on two occasions at least one week apart. An electric fan was used to attenuate the exercise-induced thermal stress. During one trial (CHO) subjects ingested 250 ml of an 8% carbohydrate-electrolyte solution at the onset of exercise and every 15 min thereafter; in the other (CON) they consumed an equal volume of a sweet placebo.

Heart rate, respiratory exchange ratio (RER) and $\dot{V}O_2$ were monitored at 30 min intervals during each trial (n=11). Expired gases were collected using Douglas bags.

Carbohydrate oxidation was estimated using the $\dot{V}O_2$ and RER data. Blood was sampled from a vein in the antecubital space at rest and after 30, 60, 90 and 120 min of exercise. Two ml of blood from each sample were placed in a fluoride heparin tube and stored on ice for glucose analysis (n=11). Immediately after blood glucose analysis the resting, 60, and 120 min samples were spun by centrifugation and the plasma stored at -80°C until analysed for insulin (n=8). A further portion of the drawn blood sample was immediately placed into ice-cold 3 $mol.l^{-1}$ PCA, spun and the extract stored for later blood lactate analysis (n=8). In addition, blood was immediatedly placed into lithium heparin tubes, mixed and spun. The resulting plasma was analysed for NH_3 (n=11), lactate (n=5), amino acids (n=5) and Hx (n=5). Amino acid analysis was not conducted on the 60 min blood sample. Muscle biopsies (n=5) were obtained at rest, and after 30 and 120 min of exercise. These samples were analysed for ATP, ADP, AMP, TAN, IMP, lactate, NH_3, CP, and Cr. The ATP/ADP ratio and EC were also calculated (see Chapter 3). Muscle amino acid analysis was not conducted due to insufficient sample size.

5.3 RESULTS

5.3.1 Cardiorespiratory Data

Mean $\dot{V}O_2$ and heart rate were not different between the trials (Table 5.1). Carbohydrate oxidation and the RER were not different at any time (data not shown); however, the mean carbohydrate oxidation and RER during exercise were higher in CHO compared with CON ($P<0.05$; Table 5.1).

5.3.2 Blood and Plasma Metabolites

All the plasma metabolite concentrations were similar at rest between trials. Blood glucose and plasma insulin concentrations were higher ($P<0.05$) at all sampling time points during exercise in CHO compared with CON (Fig. 5.1A,B). Plasma (data not shown) and blood lactate concentrations increased with exercise (Fig. 5.1C) but were not different during exercise between treatments. Plasma NH_3 levels increased ($P<0.05$) during the two hours of exercise in both trials. Furthermore, the NH_3 concentration was lower ($P<0.05$) in CHO compared with CON after 60, 90 and 120 min of exercise (Fig. 5.2A). Similarly, plasma Hx concentrations increased ($P<0.05$) during exercise in both trials (Figure 5.2B),

	CON	CHO
VO_2 ($l.min^{-1}$)	2.92 ± 0.08	2.86 ± 0.09
RER	0.84 ± 0.01	0.87 ± 0.01*
Carbohydrate oxidation ($g.min^{-1}$)	1.69 ± 0.10	1.96 ± 0.07*
Heart Rate ($b.min^{-1}$)	150 ± 3	149 ± 1

Table 5.1: Mean oxygen consumption ($\dot{V}O_2$), respiratory exchange ratio (RER), carbohydrate oxidation and heart rate during two hours of cycling at ≈ 65% $\dot{V}O_2$peak with (CHO) and without (CON) carbohydrate ingestion. Values are means ± SE, n=11. * denotes different from CON, $P<0.05$.

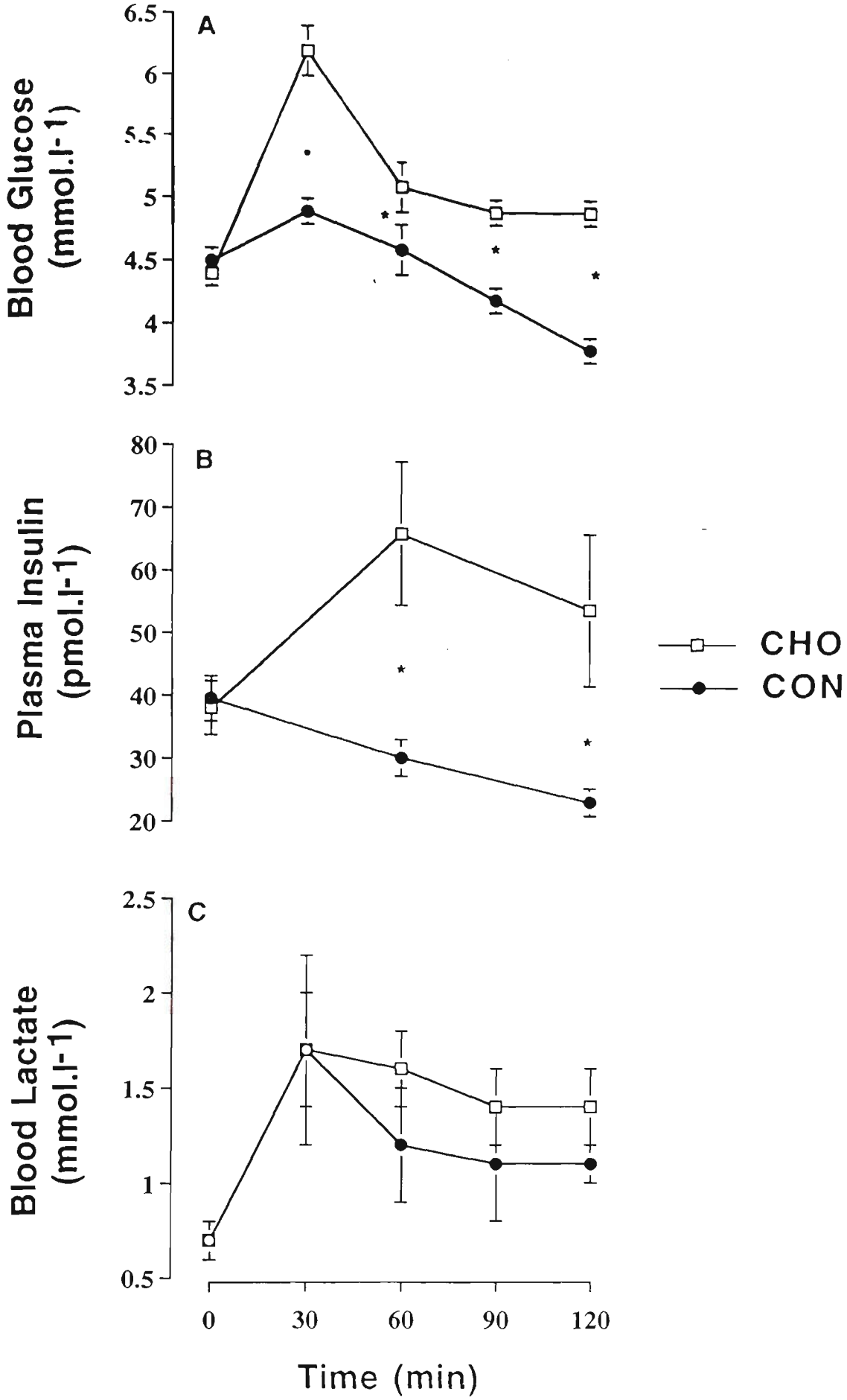

Fig. 5.1: Venous blood glucose^{+} (A), plasma insulin (B) and blood lactate (C) during 120 min of cycling at ≈ 65% $\dot{V}O_2$peak with (CHO) and without (CON) carbohydrate ingestion. Values are means ± SE, n=8, ($^{+}$ n=11),* different from CON, $P<0.05$.

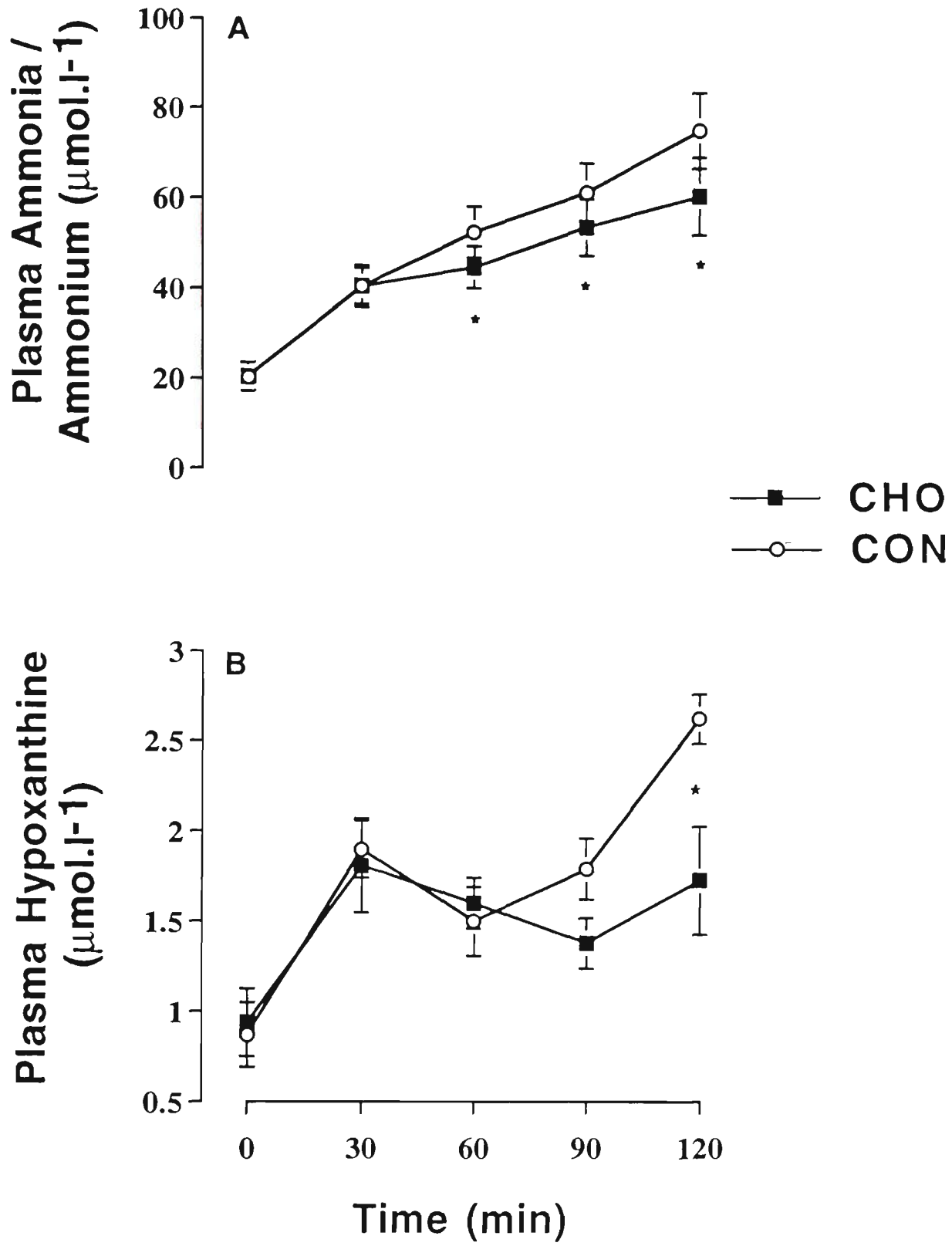

Fig. 5.2: Venous plasma ammonia/ammonium (A) and plasma hypoxanthine $^{+}$ (B) during 120 min of cycling at ≈ 65% $\dot{V}O_2$peak with (CHO) and without (CON) carbohydrate ingestion. Values are means ± SE, n=11 ($^{+}$ n=5), * different from CON, $P<0.05$.

however, after 120 min of exercise the Hx concentration was lower ($P<0.05$) in CHO versus CON. The plasma amino acid values are summarised in Table 5.2. No treatment by time interaction was observed for any of the amino acids; however, a significant main effect for treatment ($P<0.05$) was obtained for Leu and Phe such that the concentrations of these amino acids were lower in CHO compared with CON. In addition, a significant time effect ($P<0.05$) was observed for several amino acids. Thr, Val, Ile and Leu concentrations were reduced, whereas Ala, Asp and Phe increased during exercise compared with resting values.

5.3.3 Muscle Metabolites

None of the metabolites in resting muscle were different between treatments. Muscle ATP, ADP, AMP, TAN and ATP/ADP contents were not different between the trials at any time (Table 5.3). The EC, however, was higher ($P<0.05$) in the CHO trial compared with CON (significant treatment effect). CP levels were reduced as a result of exercise and the CP concentration was higher ($P<0.05$) in CHO after 30 min of exercise when compared with CON; however, no differences were observed between trials after 120 min (Table 5.3). Muscle Cr values followed a trend opposite to that seen with CP. There was no significant interaction between treatment and time for muscle IMP (Table 5.3). There was however, a significant main effect for time with IMP concentration at 120 min being higher ($P<0.05$) than resting and 30 min values. Muscle NH_3 increased ($P<0.05$) throughout exercise in CON. In CHO, only at 120 min was muscle NH_3 concentration higher ($P<0.05$) than the resting and 30 min levels. The latter two means were not different. Muscle NH_3 concentration after 120 min of exercise was lower ($P<0.05$) in CHO versus CON (Table 5.3).

5.4 DISCUSSION

5.4.1 Muscle and Plasma NH_3 Metabolism During the First 30 min of Exercise

Muscle NH_3 content increased 2.6 fold ($P<0.05$) during the first 30 min in the CON trial and 1.6 fold in the CHO trial, however the latter was not significant ($P>0.05$). Furthermore, plasma NH_3 levels increased ($P<0.05$) approximately 2 fold early in exercise in both trials. This increase in plasma NH_3 concentration is generally attributed to an elevated efflux from contracting muscle (Eriksson et al. 1985). Taken together, these data

	CON				CHO			
	Rest	30 min	90 min	120 min	Rest	30 min	90 min	120 min
Asp	13.6 ± 0.6	14.3 ± 0.7	17.0 ± 1.6	17.5 ± 1.8	13.2 ± 0.9	13.7 ± 0.9	16.4 ± 1.9	15.5 ± 1.8#
Glu	22.7 ± 4.6	25.8 ± 2.4	26.6 ± 3.1	28.1 ± 3.8	18.1 ± 4.7	22.5 ± 4.2	29.1 ± 3.8	28.2 ± 2.8
Asn	66.9 ± 2.3	68.0 ± 1.1	68.4 ± 2.5	65.7 ± 3.0	66.7 ± 2.2	70.1 ± 3.8	66.4 ± 3.9	65.5 ± 4.0
Gln	637 ± 39	662 ± 20	685 ± 26	681 ± 30	627 ± 28	642 ± 25	629 ± 19	639 ± 26
Ser	114 ± 9	115 ± 6	115 ± 5.2	107 ± 5	118 ± 4	122 ± 5	124 ± 10	119 ± 3
His	51.5 ± 10.6	53.8 ± 7.9	49.4 ± 4.5	51.4 ± 5.1	37.4 ± 6.6	48.8 ± 6.4	48.9 ± 4.4	50.3 ± 4.2
Gly	206 ± 11	211 ± 12	205 ± 20.5	186 ± 11	190 ± 11	206 ± 10	209 ± 11	207 ± 11
Thr	88.0 ± 6.9	83.1 ± 4.4	80.2 ± 5.3	76.4 ± 5.3	86.2 ± 1.4	89.4 ± 2.4	83.5 ± 3.1	82.8 ± 2.6#
Ala	394 ± 21	469 ± 19	426 ± 28	394 ± 34	370 ± 24	491 ± 18	461 ± 20	450 ± 18#
Arg	100 ± 3	110 ± 7	114 ± 8	106 ± 13	94 ± 7	98 ± 6	89 ± 6	90 ± 6
Val	231 ± 20	231 ± 11	211 ± 9	216 ± 12	247 ± 17	237 ± 20	206 ± 11	195 ± 9#
Met	19.1 ± 2.4	18.4 ± 1.7	19.9 ± 1.4	20.1 ± 2.0	15.7 ± 0.8	17.2 ± 1.1	17.0 ± 2.1	17.3 ± 1.7
Ile	54.5 ± 8.0	56.2 ± 3.9	50.5 ± 3.1	52.9 ± 3.7	55.7 ± 8.5	53.3 ± 6.7	41.8 ± 3.4	38.7 ± 2.7#
Leu	130 ± 8	133 ± 7	129 ± 12	126 ± 9	117 ± 17	116 ± 14	100 ± 6	88 ± 4#@
Phe	44.6 ± 1.4	52.4 ± 2.1	57.4 ± 3.6	69.3 ± 8.4	39.9 ± 6.6	43.4 ± 5.1	44.1 ± 2.9	45.0 ± 4.0#@
Orn	39.9 ± 6.3	43.1 ± 6.9	37.9 ± 4.8	41.8 ± 6.3	31.7 ± 4.8	30.9 ± 3.2	29.0 ± 3.1	28.2 ± 2.8
Lys	139 ± 2	167 ± 11	137 ± 11	145 ± 12	133 ± 17	143 ± 12	130 ± 6	136 ± 10

Table 5.2: Venous plasma amino acid concentrations (in $\mu mol.l^{-1}$) at rest, and during two hours of cycling at ≈ 65% $\dot{V}O_2peak$, with (CHO) and without (CON) carbohydrate ingestion. Values are means ± SE; n=5. # Significant main effect for time ($P<0.05$); @ Significant main effect for treatment ($P<0.05$).

	CON			CHO		
	Rest	30 min	120 min	Rest	30 min	120 min
ATP(E)	27.3 ± 1.1	26.4 ± 0.3	25.8 ± 0.8	26.5 ± 1.1	28.2 ± 0.6	26.2 ± 1.2
ATP(H)	25.7 ± 1.3	27.3 ± 0.9	24.9 ± 1.0	25.6 ± 1.5	27.7 ± 2.3	25.9 ± 1.6
ADP	1.93 ± 0.13	2.26 ± 0.19	2.22 ± 0.19	1.95 ± 0.15	2.10 ± 0.23	2.06 ± 0.17
AMP	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
TAN	27.7 ± 1.4	29.7 ± 1.0	27.3 ± 1.2	27.7 ± 1.5	29.9 ± 2.5	28.0 ± 1.7
ATP(H)/ADP	13.5 ± 0.65	12.3 ± 0.81	11.5 ± 0.80	13.3 ± 0.80	13.5 ± 1.10	12.7 ± 0.40
EC	0.964 ± 0.002	0.956 ± 0.002	0.954 ± 0.002	0.962 ± 0.002	0.964 ± 0.004	0.958 ± 0.002@
IMP	0.05 ± 0.01	0.06 ± 0.01	0.14 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.12 ± 0.04
CP	88.0 ± 2.4	67.5 ± 3.4	58.5 ± 2.6	86.3 ± 5.2	79.2 ± 3.0*	68.3 ± 4.2
Cr	43.6 ± 0.6	62.2 ± 1.4	73.2 ± 4.0	45.4 ± 3.6	52.4 ± 2.3*	62.5 ± 4.1
La	6.5 ± 0.7	9.7 ± 2.2	7.7 ± 1.5	5.7 ± 1.3	7.0 ± 1.2	9.4 ± 1.8
NH_3	0.42 ± 0.10	1.11 ± 0.22	2.07 ± 0.23	0.61 ± 0.12	0.95 ± 0.07	1.51 ± 0.21*

Table 5.3: Muscle metabolites at rest and after 30 and 120 min of cycling at ≈ 65% $\dot{V}O_2$peak with (CHO) and without (CON) carbohydrate ingestion. Values are means ± SE, (n=5); Metabolite concentrations expressed in mmol.kg^{-1} dry weight; ATP(E), ATP measured enzymatically; ATP(H), ATP measured by HPLC; TAN, total adenine nucleotide pool = (ATP+ADP+AMP); CP, creatine phosphate; Cr, creatine; La, lactate; EC, energy charge =(ATP+0.5ADP)/TAN; IMP, inosine 5'-monophosphate; NH_3, ammonia/ ammonium; * different from CON ($P<0.05$), @ significant treatment effect ($P<0.05$).

indicate that muscle NH_3 production was elevated even during the initial stages of prolonged exercise, irrespective of treatment. Several other studies have also reported significant muscle NH_3 production early in submaximal exercise (Graham et al. 1987; MacLean et al. 1991; van Hall et al. 1995a).

The source of the muscle NH_3 production early in submaximal exercise appears to be predominantly amino acid catabolism, since no changes in the muscle TAN pool and IMP were observed (Table 5.3). In addition, only a small increase ($P<0.05$) in Hx, a degradation product of IMP, was found in the plasma (Fig. 5.2B). It is likely that this increase in Hx occurred in the transition from rest to exercise since concentrations of this metabolite did not rise during the subsequent 60 min of cycling. The present study was unable to provide direct evidence that amino acid catabolism was the primary source of the NH_3; however the results from other studies strongly suggest that this is the case (MacLean et al. 1994, 1996a; van Hall et al. 1995a; Wagenmakers et al. 1991). The principal reactions believed to produce NH_3 from the degradation of amino acids in contracting muscle are the PNC and the GDH reaction (Graham et al. 1995a; Terjung and Tullson 1992). Wagenmakers et al. (1990) also suggest that glutaminase may also be involved, however, the importance of this reaction has not been established. The data from the present study are unable to add any further information regarding which reaction(s) is/are making a major contribution to muscle NH_3 production during submaximal exercise.

Since no differences ($P>0.05$) in muscle and plasma NH_3 were observed between trials early in submaximal exercise (Fig. 5.2A; Table 5.3) it may be concluded that carbohydrate ingestion has little influence on muscle NH_3 metabolism under these conditions. This lack of an effect occurs despite marked elevations in blood glucose (Fig. 5.1A) and plasma insulin (Fig. 5.1B). Insulin is known to promote protein synthesis (Jefferson 1980) and inhibit protein degradation (Castellino et al. 1990; Fryburg et al. 1990) and therefore, may be expected to reduce amino acid catabolism. Based on the present results there appears to be no relationship, at least for the first 30 min, between insulin and muscle NH_3 production. Similar findings have been reported previously (Study 1; Graham et al. 1991). The finding that carbohydrate ingestion does not influence NH_3 metabolism early in exercise is probably not surprising since plasma glucose utilisation

contributes a relatively small proportion of the energy cost at this stage of exercise (Coggan 1991; Coyle et al. 1986). Early in exercise most of the carbohydrate energy source is derived from muscle glycogen and its utilisation is unaffected by carbohydrate ingestion during prolonged exercise (Coyle et al. 1986; Flynn et al. 1987; Hargreaves and Briggs 1988). In addition, McConell et al. (1994) found that glucose uptake was not enhanced by carbohydrate ingestion in trained subjects during the first 30 min of cycling exercise at 69% VO_2max. These data suggest that the carbohydrate supply within contracting muscle fibres was similar early in exercise in the present study, irrespective of carbohydrate ingestion. Consequently, muscle NH_3 metabolism would not be expected to change.

Although amino acid catabolism best explains the major source of muscle NH_3 production early in exercise, a contribution from net AMP deamination, at least in theory, cannot be totally disregarded (Terjung and Tullson 1992). It is possible that some of the less oxidative fibres produce IMP and subsequently fatigue. Once these fibres no longer produce tension most of the IMP may be reaminated whilst other fibres are recruited to maintain the work rate. According to this scenario, AMP deamination would be responsible for the NH_3 production in the contracting fibres and not deamination of Asp via the PNC. At the whole muscle level, however, it would appear that both AMP deamination and IMP reamination were occurring simultaneously and NH_3 production would be incorrectly attributed to amino acid catabolism. Although theoretically possible, this process is unlikely to occur during the early stages of exercise in the present study. Studies have demonstrated that muscle NH_3 accumulation and efflux occurs even during mild contraction conditions when only type I muscle fibres are expected to be recruited (Eriksson et al. 1985; Study 1). In addition, type IIa fibres recruited to maintain the work rate in the present study would be expected to have a high aerobic capacity as a consequence of endurance training (Jansson and Kaijser 1977) and therefore, would be expected to be relatively fatigue resistant. Furthermore, endurance trained muscle is less likely to utilise the AMPd pathway as judged by an attenuated muscle IMP accumulation (Dudley and Terjung 1985a; Green et al. 1995). Finally, reasonable levels of glycogen would be expected in the contracting muscle fibres after 30 min of submaximal exercise

(Ball-Burnett et al. 1991; Febbraio et al. 1994) therefore, these fibres are not likely to be fatigued.

5.4.2 Muscle NH_3 Metabolism Between 60-120 min of Exercise

The major finding of this study is that carbohydrate ingestion resulted in a lower ($P<0.05$) muscle NH_3 concentration in the latter stages of the exercise bout compared with CON (Fig 5.2A & Table 5.3). This may have occurred as a result of an inhibition of muscle NH_3 production and/or enhanced muscle NH_3 removal.

Several studies have demonstrated that plasma glucose becomes an important source of carbohydrate for the contracting muscle as the exercise duration progresses beyond 60-90 min (Coggan 1991; Coyle et al. 1986). Importantly, carbohydrate ingestion can enhance glucose uptake (McConell et al. 1994) and better maintain muscle carbohydrate oxidation toward the latter stages of prolonged exercise (Coyle et al. 1986; Spencer et al. 1991). As mentioned previously, carbohydrate ingestion during continuous, prolonged cycling exercise does not alter muscle glycogen utilisation (Coyle et al. 1986; Flynn et al. 1987; Hargreaves and Briggs 1988). Therefore, the maintenance of carbohydrate oxidation in such circumstances must result from an increased plasma glucose utilisation. Although carbohydrate oxidation was not different at any time between the trials, the mean carbohydrate oxidation rate was higher in the CHO trial ($P<0.05$) compared with CON (Table 5.1). These data indicate that muscle carbohydrate oxidation rates were higher as a result of carbohydrate ingestion during exercise.

The predominant source of muscle NH_3 production during the final hour of exercise was unlikely to be net AMP deamination, since the accumulation of muscle IMP, a product of the deamination reaction, was small (<10%) when compared with the magnitude of intramuscular NH_3 accumulation. A similar conclusion has been made by others (MacLean et al. 1991, 1996a). Such a statement may require qualification, since it is possible that fibres which are near fatigue produce IMP and NH_3 and then cease contracting (Terjung and Tullson 1992). As mentioned previously, these fibres may then reaminate most of the IMP during recovery whilst other fibres are recruited to maintain the work rate. Consequently, NH_3 production may be incorrectly attributed to amino acid catabolism rather than net AMP deamination. As exercise duration progresses this scenario becomes

more tenable since an increasing number of type I and type IIa muscle fibres become glycogen deplete (Ball-Burnett 1991; Norman et al. 1988) and produce IMP (Norman et al. 1988). The extent to which net AMP deamination may contribute to NH_3 production, via the mechanism proposed by Terjung and Tullson (1992), is unknown.

Muscle IMP content was similar after 120 min of exercise between trials. This may suggest that carbohydrate ingestion did not affect net AMP deamination and hence, NH_3 production from this source. This may not be the case, however, since muscle EC was reduced in the CON versus CHO trial ($P<0.05$; Table 5.3), and plasma Hx elevated ($P<0.05$; Fig. 5.2B) in CON compared with CHO after 120 min of exercise. Although these changes were small, these latter results indicate that net AMP deamination may actually have been greater towards the end of exercise in CON compared with CHO. As a consequence, this elevated net AMP deamination may partially account for the markedly augmented muscle NH_3 levels observed at the end of the CON trial.

Although equivocal, the data from the present study suggests that the changes in net AMP deamination are too small to account for the attenuated muscle NH_3 content observed during the CHO trial. Most of the reduced muscle NH_3 content is therefore likely to result from a reduction in muscle NH_3 production caused by an enhanced carbohydrate availability inhibiting muscle amino acid catabolism. The data from the present study are unable to provide direct evidence to support this proposition. The lower plasma Leu and Phe concentrations found in the CHO trial compared with CON ($P<0.05$; Table 5.2) may indicate a decreased release of these EAA's into the plasma due to a reduction in tissue protein degradation (Graham et al. 1995a, Smith and Rennie 1990).

Previous research indicates that there is a link between carbohydrate availability, protein degradation and amino acid catabolism. Davies et al. (1982) demonstrated that glucose ingestion during exercise attenuated Leu oxidation as measured by isotopic $^{13}CO_2$ production from ^{13}C-Leu. In addition, Lemon and Mullin (1980) reported that sweat urea nitrogen levels, a marker of protein catabolism, are markedly lower after prolonged exercise begun in the glycogen loaded compared with the glycogen depleted state. Similarly, van Hall (1996) found an increase in net muscle protein degradation during exercise begun with a glycogen depleted leg compared with a leg containing normal

glycogen content. Greater increases in the active form of the rate limiting enzyme of BCAA oxidation (BCOADH) have also been observed in human skeletal muscle when carbohydrate supply is low (van Hall et al. 1996; Wagenmakers et al. 1991). In addition, McArdle's disease patients display a greater percentage of this enzyme in the active form both at rest, and during exercise, compared with normal individuals (Wagenmakers et al. 1987). Buse et al. (1972, 1976) have also demonstrated that the presence of glucose and pyruvate inhibited BCOA oxidation in incubated rat diaphragm muscle. Interestingly, carbohydrate ingestion increases the concentration of glucose in contracting muscle during prolonged submaximal exercise (Spencer et al. 1991). The above data strongly indicate that elevations in muscle carbohydrate availability may suppress net protein degradation and amino acid catabolism. This inhibition may explain, most of, the attenuated muscle NH_3 accumulation observed in the present study.

The attenuation of muscle NH_3 content observed toward the latter stages of exercise in the CHO trial compared with CON may also be explained, at least in part, by an enhanced incorporation of the NH_3 produced by muscle into the formation of Glu and Ala. Ala is one of the principal nitrogen carriers released from active skeletal muscle (Felig and Wahren 1971; Wahren et al. 1976). It should be noted, however, that in order for Ala to remove free NH_3, the amino group from Glu must have also originated from free NH_3. The major reaction involving the fixation of NH_3 to form Glu is catalysed by GDH. Although the direction in which the GDH reaction proceeds is unknown, several authors (MacLean and Graham 1993; MacLean et al. 1996a) argue that in contracting muscle the reaction favours NH_3 production rather than removal. If this is the case, muscle Ala production is not likely to enhance free NH_3 removal. Alternatively, there is the possibility that *de novo* Ala production may act to reduce muscle NH_3 production by removing Glu. Consequently, this may reduce the availability of Glu for other NH_3 producing reactions. Previous research has demonstrated that pyruvate, derived from glycolysis, is used in the Ala aminotransferase reaction to produce Ala (Chang and Goldberg 1978; Odessey et al. 1974; Spydevold 1976). Moreover, carbohydrate ingestion has been shown to elevate muscle Ala content during prolonged submaximal exercise (Spencer et al. 1991), suggesting that muscle Ala production is enhanced when the carbohydrate supply is

augmented. The fact that carbohydrate ingestion had no effect ($P>0.05$) on forearm, venous plasma Ala concentration indicates that the mechanisms outlined above may not explain the reduced muscle NH_3 content in the CHO trial (Table 5.2). Such a conclusion, however, must be treated with caution since the interpretation of plasma amino acid data is far from straight forward (see Graham et al. 1991).

5.4.3 Plasma NH_3 Metabolism Between 60-120 min of Exercise

The lower ($P<0.05$) plasma NH_3 concentration observed during the second hour of exercise in CHO compared with CON (Fig. 5.2A) must be due to an attenuated rate of NH_3 entry into, and/or an enhanced rate of NH_3 removal from, the plasma. Previous research has demonstrated that there is a marked increase in NH_3 efflux from contracting muscle (Eriksson et al. 1985; Katz et al. 1986a; Graham et al. 1991; MacLean et al. 1994, 1996a) and that this is the primary cause of exercise-induced increase in plasma NH_3 concentration (Eriksson et al. 1985). Muscle NH_3 efflux is influenced by the concentration gradient across the sarcolemma, which is in part, determined by the intramuscular NH_3 concentration. Muscle NH_3 content was reduced ($P<0.05$) after 120 min of exercise in CHO compared with CON (Table 5.3). Although speculative, this observation indicates that there may have been a concomitant decrease in muscle NH_3 efflux in CHO compared with CON, at least at this time, and perhaps throughout the last hour of exercise. Such a mechanism may explain the reduced plasma NH_3 levels. This study cannot exclude the possibility that the plasma NH_3 concentration was reduced in the CHO trial due to an increased plasma NH_3 removal rate, however the responsible mechanism(s) is/are not readily apparent.

In contrast with the present plasma NH_3 findings, van Hall et al. (1996) recently reported that carbohydrate ingestion did not affect plasma NH_3 concentration during exhaustive exercise when alternating between 50 and 80% maximum workload. The experimental protocols conducted by the present study and van Hall were not identical. The two studies utilised different exercise intensities, pre-exercise nutrition states and rates of carbohydrate ingestion. Each of these differences on their own, or in combination, may explain the conflicting findings.

5.4.4 Conclusion

Carbohydrate ingestion had no effect on muscle and plasma NH_3 concentration during the first 30 min of exercise but, resulted in a reduction in the concentration of this metabolite in both tissues during the second hour. The major source of muscle NH_3 production throughout the entire exercise period in both trials appears to be amino acid catabolism since net AMP deamination was small. Muscle NH_3 production from net AMP deamination may have decreased toward the end of exercise as a consequence of carbohydrate ingestion. Since the influence of carbohydrate ingestion on net AMP deamination appears to be small it is unlikely to account for the relatively large reductions in muscle NH_3 content observed in the CHO trial. A reduction in muscle NH_3 production in the CHO trial is, therefore, best explained by an inhibition of muscle amino acid catabolism. Although speculative, the decrease in muscle NH_3 content may also be due, at least in part, to an increase rate of muscle Ala production in the CHO trial. The attenuated plasma NH_3 concentration observed toward the later stages of exercise with CHO ingestion is probably the result of a decrease in muscle NH_3 efflux as a consequence of the reduced muscle NH_3 content. This study cannot exclude the possibility that plasma NH_3 was attenuated in the CHO trial due to an enhanced NH_3 removal rate from the circulation, however, the mechanism for such a phenomenon is not obvious.

CHAPTER 6

BRANCHED-CHAIN AMINO ACID FEEDING AND AMMONIA METABOLISM DURING PROLONGED EXERCISE

6.1 INTRODUCTION

There are two potential major sources of NH_3 in skeletal muscle during prolonged exercise. The first involves the deamination of AMP to IMP and NH_3 catalysed by AMPd (Lowenstein 1972). The second involves the deamination and release of NH_3 from amino acids, in particular BCAA (Graham and MacLean 1992; MacLean and Graham 1993; MacLean et al. 1991). While the relative contribution of each pathway to NH_3 production during prolonged exercise is controversial (Broberg and Sahlin 1989; Graham and MacLean 1992), there is increasing evidence that BCAA catabolism may play a significant role (MacLean and Graham 1993; MacLean et al. 1994, 1996a). BCAA ingestion is known to increase BCAA concentrations in the blood and to increase the delivery of these amino acids to contracting muscle (Blomstrand et al. 1996; MacLean and Graham 1993; MacLean et al. 1994). Furthermore, BCAA feeding (77-335 mg of BCAA.kg^{-1} body weight) increases plasma NH_3 concentration during two-legged cycling exercise (MacLean and Graham 1993; Nemoto et al. 1996; van Hall et al. 1995b). MacLean et al. (1994; 1996a) have conducted two studies investigating the mechanism(s) causing the elevated plasma NH_3 levels during exercise when fed BCAA. In the first study, a BCAA dose of 77 mg.kg^{-1} body weight had no effect on intramuscular NH_3 accumulation, total release of muscle NH_3 and total muscle NH_3 production during 60 min of single-leg kicking exercise. When total muscle NH_3 production was added to total muscle Gln release to gain a more realistic assessment of the magnitude of free NH_3 production, MacLean et al. (1994) reported that NH_3 production was enhanced by BCAA ingestion. In the second study (MacLean et al. 1996a) subjects ingested a larger BCAA dose (308 mg.kg^{-1} body weight) and performed single-leg kicking exercise for 90 min. This study clearly demonstrated that BCAA ingestion enhanced muscle NH_3 production.

The single-leg kicking model is excellent for examining the mechanisms by which BCAA ingestion may influence NH_3 production and release from contracting human muscle. The results from this model, however, may not be directly applicable to activity

involving a large muscle mass since the cardiovascular and endocrine responses are dependent upon the mass of the contracting musculature (Andersen and Saltin 1985; Graham et al. 1991; Kjaer et al. 1991; Rowell 1988). Furthermore, the magnitude of net muscle protein degradation may be considerably higher using the single-leg kicking model when compared with two-legged exercise (van Hall 1996). No studies have examined the influence of BCAA feeding on muscle NH_3 metabolism during whole body exercise (e.g., two-legged cycling) and therefore, this experiment aimed to examine this phenomenon.

6.2 METHODS

Seven endurance trained cyclists volunteered for this study. Their age, weight, and $\dot{V}O_2$peak was 24.7 ± 1.6 yr, 74.7 ± 3.6 kg and 4.78 ± 0.13 $l.min^{-1}$, respectively. Each subject cycled for 58 min at approximately 70% $\dot{V}O_2$peak on two separate occasions. In order to allow for the rest-exercise transition a 2 min warm up at 100 W was completed in each trial before increasing the workload to the appropriate level. Prior to exercise the subjects ingested either a placebo (lemon flavoured, uncarbonated artificially sweetened soft drink) or BCAA (100 $mg.kg^{-1}$ body weight) mixed with the placebo fluid. These treatments were assigned using a cross over design in a double-blind fashion. The placebo (CON) and BCAA supplements (BCAA) were administered in two equal oral doses 60 and 30 min before the onset of exercise. Oxygen consumption and heart rate were determined during exercise at 10, 25, 45, and 55 min. The subjects consumed 250 ml of water after 20 and 40 min of cycling. An electric fan was used to attenuate the exercise-induced thermal stress. Venous blood samples were obtained immediately prior to ingestion, just before exercise and every 15 min throughout exercise. Muscle biopsies were taken from the vastus lateralis before and immediately after exercise. Plasma was analysed for amino acids, insulin, lactate, NH_3, Hx and glucose according to the methods outlined in Chapter 3. Muscle samples were analysed for amino acids, NH_3, ATP, ADP, Cr, CP, IMP and lactate content using the methods described previously (see Chapter 3). Unfortunately, muscle AMP content was unable to be determined in this study since, for some unknown reason, AMP coeluted from the HPLC column with another compound. Furthermore, due to insufficient muscle sample obtained from one individual the muscle amino acid data are reported from six subjects.

6.3 RESULTS

6.3.1 Cardiorespiratory Data

The mean $\dot{V}O_2$, relative $\dot{V}O_2$, heart rate and RER were not different ($P>0.05$) between across the trials (Table 6.1).

6.3.2 Plasma Insulin and Metabolites

The statistical analyses revealed no significant treatment or treatment by time interaction ($P>0.05$) for the plasma insulin, lactate (Fig. 6.1A,C) or Hx (Fig 6.2A) data. A significant main effect for time ($P<0.05$), however, was observed for these metabolites. The plasma lactate and Hx levels were stable during the 60 min rest period in both trials and increased ($P<0.05$) above resting values during exercise. In contrast, the circulating insulin levels increased during rest and fell during exercise ($P<0.05$). Plasma glucose concentration was similar at rest prior to the initial ingestion of fluid, but were higher ($P<0.05$) 60 min after this point in CON compared with BCAA (Fig. 6.1B). No differences ($P>0.05$) were observed between treatments for glucose during exercise. A main effect for treatment was observed for plasma NH_3 ($P<0.05$) demonstrating that BCAA ingestion resulted in an elevated circulating NH_3 level compared with CON (Fig. 6.2B). In addition, a main effect for time revealed that the plasma NH_3 concentration was stable at rest and increased ($P<0.05$) throughout exercise. Plasma BCAA concentration was similar at rest prior to treatment but was 2-3 fold higher ($P<0.05$) than CON in the BCAA trial after ingestion of the supplement (Fig. 6.3). Although the BCAA concentration fell ($P<0.05$) during exercise in the BCAA trial, the plasma BCAA concentration remained above CON levels ($P<0.05$) throughout the entire exercise period. The results for the individual plasma amino acids are summarised in Table 6.2. Apart from the individual BCAA's, there were no differences ($P>0.05$) in the concentration of the other plasma amino acids between the trials. The total AA and EAA concentration were not different ($P>0.05$) prior to supplementation, however, after ingestion these concentrations were higher ($P<0.05$) in the BCAA compared with CON (Table 6.3). These differences were maintained for at least the first 30 min of exercise in the case of total AA, and throughout the entire exercise period when referring to EAA. Both the elevation in plasma total AA and EAA in the BCAA trial were accounted

	CON	BCAA
$\dot{V}O_2$(l.min^{-1})	3.24 ± 0.11	3.21 ± 0.10
%$\dot{V}O_2$peak	67.9 ± 1.6	67.0 ± 1.4
RER	0.98 ± 0.03	0.96 ± 0.02
HR (b.min^{-1})	155 ± 6	156 ± 6

Table 6.1: Mean oxygen consumption ($\dot{V}O_2$), relative oxygen consumption (%$\dot{V}O_2$peak), respiratory exchange ratio (RER), and heart rate (HR) during 60 min of cycling with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. Values are means ± SE, n=7.

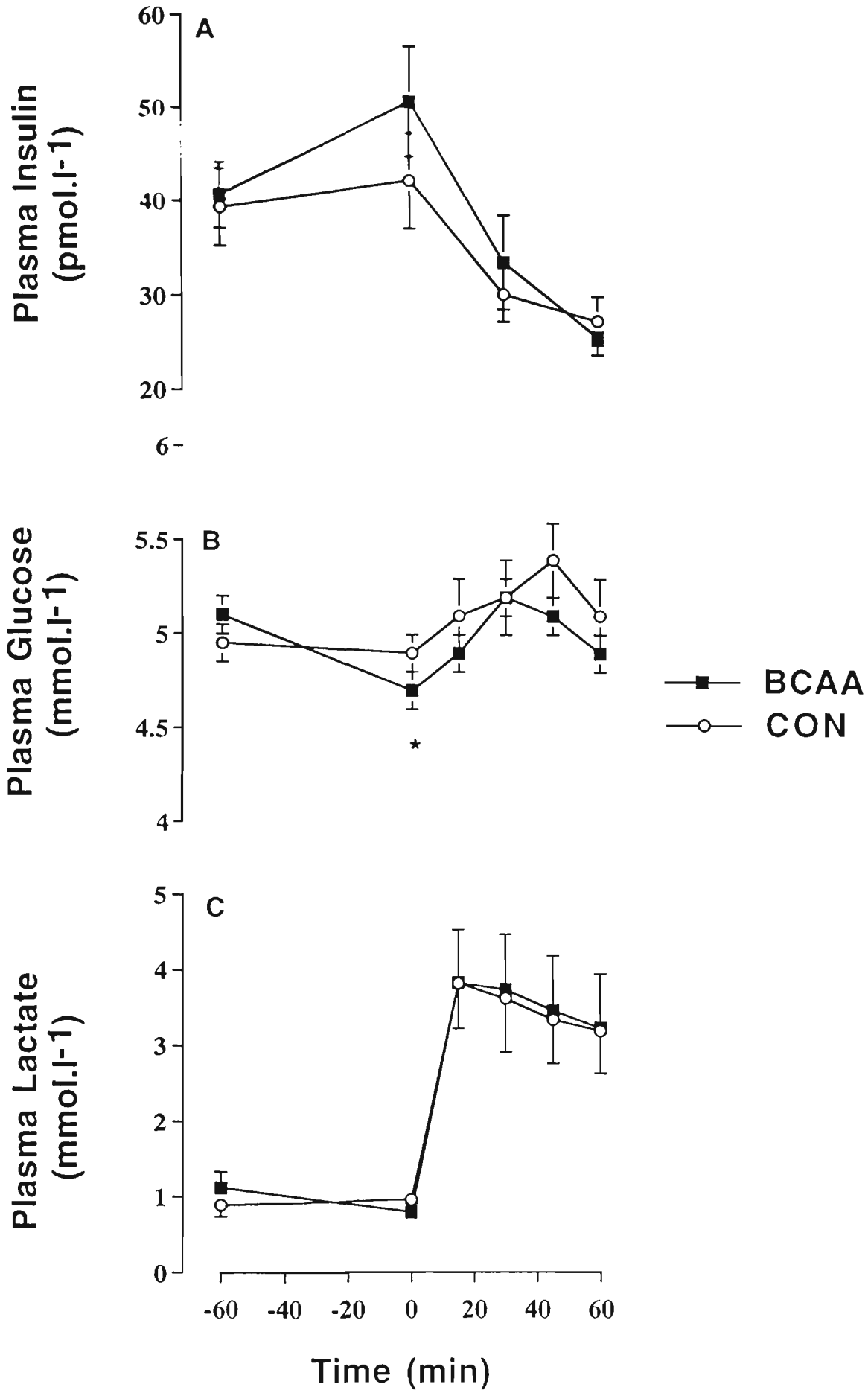

Fig. 6.1: Venous plasma insulin (A), glucose (B) and lactate (C) at rest and during 60 min of cycling exercise at ≈ 70% $\dot{V}O_2$peak with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. Values are means ± SE, n=7. * different from CON, $P<0.05$.

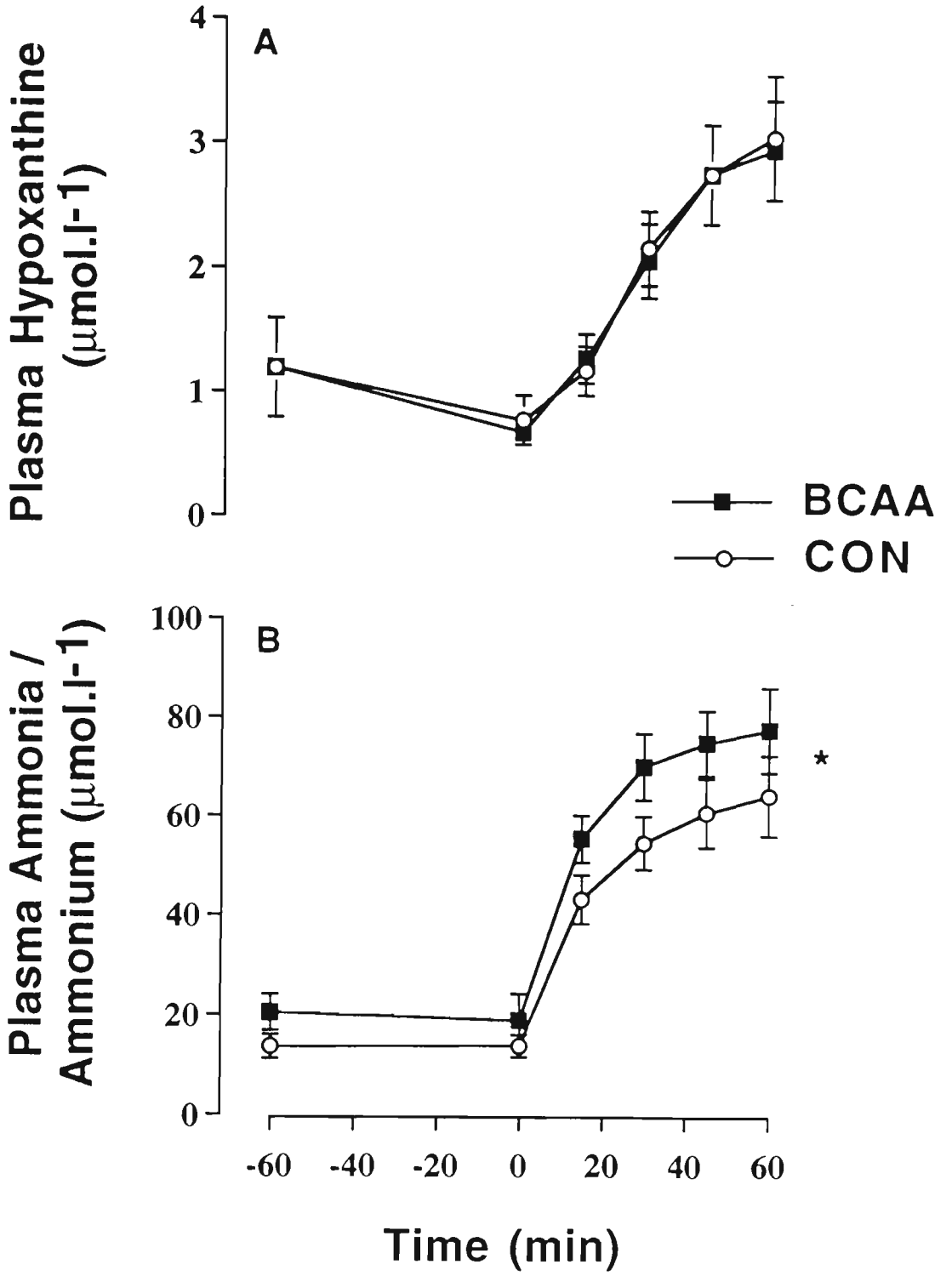

Fig. 6.2: Venous plasma hypoxanthine (A), and ammonia/ammonium (B) at rest and during 60 min of cycling exercise at ≈ 70 %$\dot{V}O_2$peak with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. Values are means ± SE, n=7. * main effect for treatment, $P<0.05$.

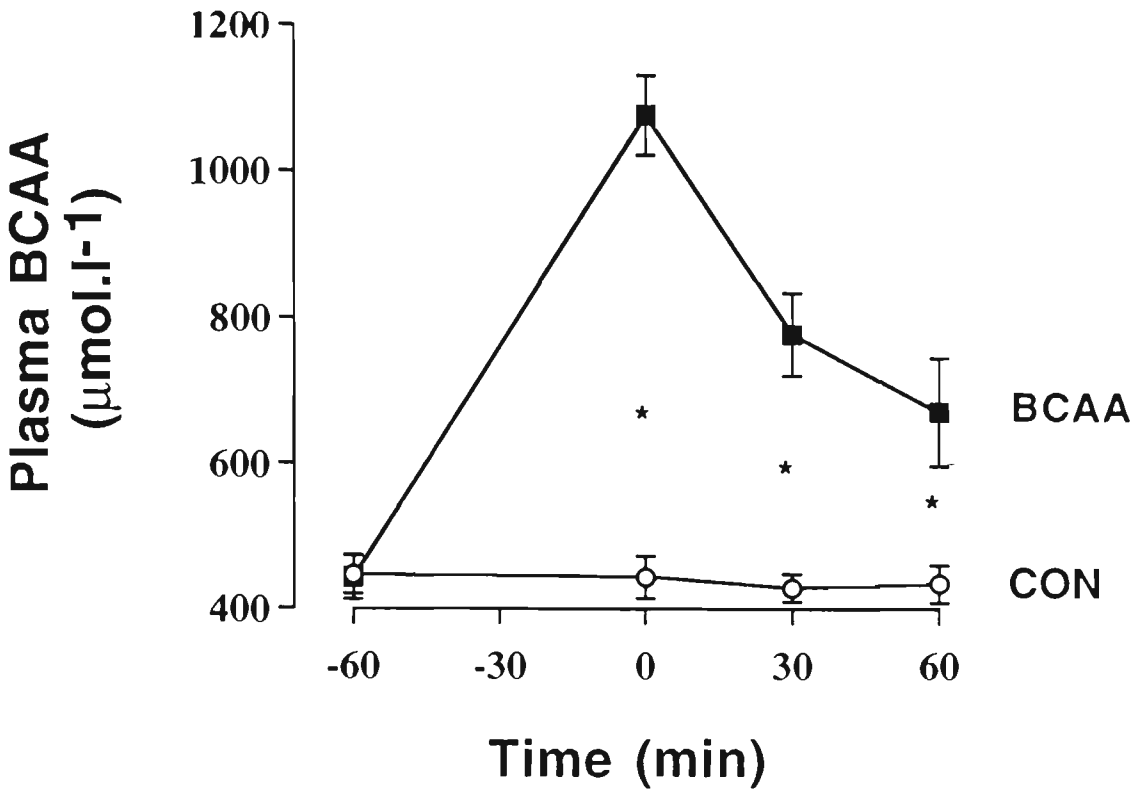

Fig. 6.3: Venous plasma BCAA concentration at rest and during 60 min of cycling exercise at ≈ 70 %$\dot{V}O_2$peak with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. Values are means ± SE, n=7. * Different from CON, $P<0.05$.

	CON				BCAA			
	-60 min	0 min	30 min	60 min	-60 min	0 min	30 min	60 min
Asp	15 ± 1	15 ± 1	14 ± 1	15 ± 2	14 ± 1	15 ± 1	14 ± 1	15 ± 1
Glu	13 ± 2	10 ± 1	20 ± 2	21 ± 3	17 ± 2	11 ± 2	18 ± 2	19 ± 2*
Asn	65 ± 3	66 ± 3	59 ± 2	63 ± 2	61 ± 3	65 ± 3	56 ± 1	60 ± 5*
Gln	679 ± 26	715 ± 43	685 ± 26	709 ± 37	660 ± 29	748 ± 41	725 ± 40	735 ± 40*
Ser	96 ± 7	97 ± 6	95 ± 7	97 ± 8	94 ± 6	103 ± 7	91 ± 6	93 ± 6
His	58 ± 9	68 ± 10	68 ± 10	68 ± 13	58 ± 7	57 ± 10	52 ± 9	46 ± 7
Gly	237 ± 17	238 ± 20	236 ± 20	240 ± 22	236 ± 23	224 ± 22	227 ± 23	229 ± 23
Thr	77 ± 5	76 ± 4	73 ± 4	76 ± 5	76 ± 4	76 ± 3	69 ± 3	71 ± 4*
Ala	397 ± 28	382 ± 33	505 ± 30	515 ± 29	397 ± 18	402 ± 23	516 ± 31	514 ± 30*
Arg	111 ± 9	107 ± 13	112 ± 15	117 ± 20	115 ± 18	135 ± 20	144 ± 27	131 ± 25
Val	244 ± 13	237 ± 11	230 ± 8	233 ± 10	241 ± 15	485 ± 27@	373 ± 17@	333 ± 25@
Met	27 ± 4	25 ± 3	24 ± 3	27 ± 3	26 ± 4	28 ± 5	28 ± 6	26 ± 4
Ile	59 ± 7	54 ± 3	56 ± 3	54 ± 3	57 ± 5	242 ± 15@	156 ± 17@	140 ± 30@
Leu	149 ± 14	154 ± 20	144 ± 14	149 ± 16	145 ± 13	353 ± 24@	251 ± 28@	199 ± 27@
Phe	53 ± 4	54 ± 5	55 ± 5	60 ± 6	55 ± 6	55 ± 5	58 ± 9	62 ± 12
Orn	49 ± 11	47 ± 7	47 ± 7	48 ± 7	48 ± 9	52 ± 9	56 ± 12	65 ± 22
Lys	148 ± 14	173 ± 19	161 ± 12	167 ± 13	133 ± 12	153 ± 13	156 ± 12	163 ± 21*

Table 6.2: Venous plasma amino acid concentrations (in $\mu mol.l^{-1}$) at rest and during 60 min of cycling at ≈ 70% $\dot{V}O_2$peak with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. Values are means ± SE; n=7. @ Different from CON, $P<0.05$. * Main effect for time $P<0.05$.

Time		Total AA	EAA	BCAA	EAA-BCAA	AA-BCAA
-60 min						
	CON	2475 ± 92	757 ± 45	447 ± 27	310 ± 23	2028 ± 73
	BCAA	2435 ± 117	734 ± 47	443 ± 30	291 ± 20	1991 ± 92
0						
	CON	2519 ± 132	771 ± 51	444 ± 29	326 ± 25	2075 ± 106
	BCAA	3193 ± 180&	1393 ± 69@	1081 ± 55@	313 ± 19	2113 ± 127
30						
	CON	2585 ± 104	742 ± 32	429 ± 19	313 ± 17	2156 ± 92
	BCAA	2986 ± 182@	1089 ± 73@	780 ± 57@	310 ± 21	2206 ± 138
60						
	CON	2658 ± 138	765 ± 42	435 ± 26	330 ± 20	2222 ± 118
	BCAA	2892 ± 219	995 ± 105@	673 ± 75@	322 ± 34	2219 ± 154

Table 6.3: Venous plasma amino acid concentrations (in $\mu mol.l^{-1}$) at rest and during 60 min of cycling at ≈ 70% $\dot{V}O_2$peak with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. AA, amino acid, EAA, essential amino acids, EAA - BCAA, EAA minus BCAA, AA - BCAA, total AA minus BCAA. Values are means ± SE, n=7. @ Significantly different from CON, $P<0.05$.

for by the increase in BCAA since, AA-BCAA and EAA-BCAA were not different between treatments (Table 6.3).

6.3.3 Muscle Metabolites

The muscle metabolite data are summarised in Table 6.4. ANOVA revealed no significant interaction or main effect for treatment for any of the measured muscle metabolites. With the exception of ATP (H, determined using HPLC), there was a significant main effect for time ($P<0.05$) for the remaining muscle metabolites. Exercise, therefore, resulted in an increase in lactate, NH_3, Cr and IMP content and led to a reduction in CP, ATP (E, determined enzymatically), ATP(H)/ADP ratio and glycogen.

A summary of the muscle amino acid contents is shown in Table 6.5. As expected, the resting BCAA levels increased ($P<0.05$) about 2 fold after BCAA ingestion and remained above ($P<0.05$) CON throughout exercise. The EAA content of resting muscle was elevated ($P<0.05$) in the BCAA trial compared with CON, however no differences were observed after 60 min of exercise. The latter finding resulted from an exercise-induced increase ($P<0.05$) in EAA in the CON but not the BCAA trial. Although no differences ($P>0.05$) were observed between the trials for the total AA content, EAA-BCAA and AA-BCAA, an exercise-induced increase ($P<0.05$) was observed for each of these variables. The increase in intramuscular AA-BCAA content was due to elevations ($P<0.05$) in Asp, Gln, Ser, His, Gly, Thr, Ala, Arg, Phe, and Lys, whilst the rise in EAA-BCAA was accounted for by increases in Thr, Phe, and Lys (Table 6.6). Apart from Val, Ile, and Leu, the ingestion of BCAA had no effect ($P>0.05$) on the concentrations of the other individual muscle amino acids measured in this study.

6.4. DISCUSSION

The results from this study demonstrated that BCAA ingestion markedly elevated ($P<0.05$) the BCAA levels in muscle (Table 6.5) and plasma (Fig. 6.3) at rest and after 60 min of two-legged cycling exercise compared with CON. Similar findings have been observed by others (Blomstrand et al. 1996; MacLean and Graham 1993; MacLean et al. 1994, 1996a; van Hall 1996). Despite an increase in muscle BCAA content at rest and

	CON		BCAA	
	Rest	Exercise	Rest	Exercise
ATP(E)	24.7 ± 1.2	24.0 ± 1.1	25.7 ± 0.6	24.4 ± 1.0 #
ATP(H)	25.5 ± 1.4	25.5 ± 1.6	26.3 ± 1.6	24.4 ± 1.8
ADP	2.22 ± 0.12	2.47 ± 0.11	2.14 ± 0.09	2.18 ± 0.08
ATP(H)/ADP	11.6 ± 0.7	10.4 ± 0.6	12.3 ± 0.7	11.1 ± 0.5 #
IMP	0.03 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.08 ± 0.01 #
CP	78.1 ± 4.4	46.4 ± 4.4	76.2 ± 4.5	42.8 ± 5.4 #
Cr	37.3 ± 2.3	69.0 ± 4.5	39.1 ± 2.8	72.6 ± 4.7 #
La	3.7 ± 0.7	8.6 ± 1.5	3.6 ± 0.3	10.4 ± 1.5 #
NH_3	0.39 ± 0.08	1.07 ± 0.11	0.31 ± 0.05	1.10 ± 0.12 #
Gly	557 ± 64	284 ± 60	500 ± 50	287 ± 52 #

Table 6.4: Muscle metabolites at rest and after 60 min of cycling at ≈ 70% $\dot{V}O_2$peak with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. Values are means ± SE, (n=7). Metabolite concentrations expressed in mmol.kg^{-1} dry weight (glycogen expressed as mmol.kg^{-1} glucosyl units dry weight). ATP(E), ATP analysed enzymatically; ATP(H), ATP analysed by HPLC; IMP, inosine 5'-monophosphate, CP, creatine phosphate; Cr, creatine; La, lactate; Gly, glycogen; NH_3, ammonia/ammonium; # significant time effect, $P<0.05$.

Time		Total AA	EAA	BCAA	EAA-BCAA	AA-BCAA
Rest						
	CON	67.5 ± 3.4	4.48 ± 0.22	1.60 ± 0.07	2.88 ± 0.19	65.9 ± 3.4
	BCAA	70.4 ± 2.6	6.30 ± 0.29*	3.28 ± 0.09*	3.02 ± 0.25	67.2 ± 2.5
Exercise						
	CON	78.0 ± 2.5	5.57 ± 0.22+	1.70 ± 0.10	3.87 ± 0.25	76.3 ± 2.6
	BCAA	77.4 ± 3.1@	6.00 ± 0.44	2.29 ± 0.2*+	3.71 ± 0.30@	74.5 ± 3.1@

Table 6.5: Muscle amino acid concentrations at rest and during 60 min of cycling at ≈ 70% $\dot{V}O_2$peak with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. Values are means ± SE and are expressed in mmol.kg^{-1} dry weight, n=6. AA, amino acid, EAA, essential amino acids, EAA - BCAA, EAA minus BCAA, AA - BCAA, total AA minus BCAA. * Different from CON, $P<0.05$; + different from rest, $P<0.05$; @ significant time effect, $P<0.05$.

	CON		BCAA	
	Rest	Exercise	Rest	Exercise
Asp	0.65 ± 0.05	1.44 ± 0.20	0.66 ± 0.06	1.33 ± 0.12@
Glu	6.81 ± 0.59	3.86 ± 0.29	6.88 ± 0.20	3.58 ± 0.24@
Asn	0.88 ± 0.04	1.22 ± 0.09	0.96 ± 0.08	1.19 ± 0.09
Gln	41.4 ± 3.0	45.4 ± 2.5	40.9 ± 1.9	44.4 ± 2.2@
Ser	1.77 ± 0.10	2.57 ± 0.20	1.91 ± 0.10	2.42 ± 0.13@
His	1.11 ± 0.13	1.39 ± 0.12	1.06 ± 0.07	1.44 ± 0.11@
Gly	3.34 ± 0.13	4.19 ± 0.09	3.52 ± 0.22	4.07 ± 0.10@
Thr	0.98 ± 0.05	1.39 ± 0.07	1.03 ± 0.07	1.31 ± 0.10@
Ala	6.16 ± 0.47	10.44 ± 0.81	6.71 ± 0.30	11.24 ± 0.83@
Arg	0.95 ± 14	1.29 ± 17	1.00 ± 0.10	1.24 ± 0.13@
Val	0.87 ± 0.03	0.95 ± 0.05	1.41 ± 0.04	1.24 ± 0.08#
Ile	0.23 ± 0.02	0.23 ± 0.02	0.74 ± 0.03*	0.39 ± 0.04*+
Leu	0.45 ± 0.05	0.46 ± 0.03	1.07 ± 0.07*	0.66 ± 0.06*+
Phe	0.24 ± 0.01	0.31 ± 0.02+	0.25 ± 0.01	0.26 ± 0.02
Orn	0.44 ± 0.06	0.58 ± 0.06	0.57 ± 0.09	0.48 ± 0.04
Lys	1.66 ± 0.19	2.17 ± 0.22	1.75 ± 0.20	2.14 ± 0.21@

Table 6.6: Muscle amino acid concentrations at rest and after 60 min of cycling at ≈ 70% $\dot{V}O_2$peak with (BCAA) or without (CON) ingestion of a branched-chain amino acid supplement. Values are means ± SE, (n=6). Metabolite concentrations expressed in mmol.kg^{-1} dry weight, * different from CON, P<0.05; + different from rest, P<0.05; @ significant time effect, P<0.05; # significant treatment effect, P<0.05.

during exercise, no difference in muscle NH_3 content was observed between trials (Table 6.4). It may be concluded, therefore, that the difference between the rate of muscle NH_3 production and removal was not altered by an increased muscle BCAA content in the present study. This result supports the findings of MacLean et al. (1994) who found that BCAA ingestion had no effect on muscle NH_3 content after 60 min of leg kicking exercise. In a follow up study, however, MacLean and coworkers (1996a) were able to demonstrate a higher muscle NH_3 content after a large dose of BCAA (three fold greater than the present study) following 90 min at a similar exercise intensity.

The three fold increase in muscle NH_3 levels observed during exercise in both trials (Table 6.4) means that muscle NH_3 production rates must have exceeded removal rates. In the absence of muscle efflux data, or an isotopic tracer technique, it is not possible to definitively conclude what was happening to muscle NH_3 production and removal rates during exercise in the present study. Results from previous research (Broberg and Sahlin 1989; Graham and MacLean 1992; Katz et al. 1986a), however, demonstrate that both rates increase as a result of submaximal exercise, with the rate of production exceeding removal at moderate exercise intensities.

With the exception of muscle BCAA content, no differences were observed between trials for any other muscle metabolite. Of particular interest is a lack of difference in the potential intramuscular markers of NH_3 production such as IMP and Glu. Furthermore, intramuscular markers of free NH_3 removal such as Gln and possibly Ala (see below) were also not different between the trials. At first glance these data suggest that BCAA ingestion had no effect on muscle NH_3 metabolism during exercise. Such an interpretation is probably incorrect since MacLean et al. (1994, 1996a) also observed that BCAA ingestion had no affect on various intramuscular markers of NH_3 metabolism, but found an enhanced efflux of Gln, Ala and NH_3 from contracting muscle after BCAA ingestion. When muscle metabolite and efflux data are considered together, as in the studies conducted by MacLean et al. (1994, 1996a), a totally different conclusion about the influence of BCAA ingestion on NH_3 metabolism may be reached. That is, BCAA ingestion most likely increases muscle NH_3 production and removal rates compared with CON.

Statistically, only a main effect for treatment was demonstrated for the plasma NH_3 data. Strictly interpreted, this means that the BCAA ingestion effect was not expressed during exercise alone, but during rest and exercise. However, ANOVA applied to the resting values before and after BCAA and placebo ingestion indicated that none of these values were different ($P>0.05$). In contrast, ANOVA applied to the exercise data revealed a significant difference between the treatments ($P<0.05$). It is likely therefore, that BCAA ingestion augmented the plasma NH_3 concentration during exercise only. With this in mind, the higher plasma NH_3 concentration found in the BCAA trial compared with CON (Fig 6.2B) supports the proposition that muscle NH_3 efflux rates were greater when fed BCAA. During exercise the major contributor to the circulating NH_3 pool is active muscle (Broberg and Sahlin 1989; Eriksson et al. 1985). Removal of plasma NH_3 by inactive muscle increases during exercise (Bangsbo et al. 1996). An increased removal by the central nervous system would also be expected (Lockwood et al. 1979). In addition, splanchnic NH_3 removal rates remain at basal levels (Eriksson et al. 1985). Taken together these data indicate that the rates of NH_3 entry into, and removal from, the plasma increase during exercise. As MacLean and Graham (1993) have argued, there is no reason to think that BCAA ingestion would decrease the plasma NH_3 removal rate. The elevated plasma NH_3 concentration observed in the BCAA trial is, therefore, best explained by an enhanced efflux of NH_3 from the contracting muscle.

Interestingly, if NH_3 efflux rates are enhanced by BCAA ingestion then this occurs even though the NH_3 concentration gradient across the sarcolemma was probably reduced. This conclusion is based on the observations that muscle NH_3 contents were similar (Table 6.4; MacLean et al. 1994) but circulating plasma NH_3 levels were higher (Fig. 6.2B; MacLean et al. 1994) during exercise following BCAA ingestion compared with CON. In addition, muscle blood flow, which aids in the reduction of NH_3 in the muscle interstitial fluid (thus is an important factor influencing muscle efflux rates), was not altered by BCAA feeding (MacLean et al. 1994, 1996a). Apart from the concentration gradient across the sarcolemma and muscle blood flow there are a few other factors which determine muscle NH_3 efflux rates. Firstly, NH_3 efflux may be influenced by the pH gradient across the sarcolemma since ammonia may diffuse across the lipid bilayer portion of the cell

membrane unlike ammonium (Kliener 1981; Wang et al. 1996). During endurance exercise changes in pH across the membrane are small (Fitts 1994), therefore alterations in NH_3 efflux via this mechanism are unlikely to account for the probable higher efflux rate in the BCAA trial. The relatively minor accumulation of muscle and plasma lactate (Table 6.4 and Fig. 6.1C) observed in the present study support this contention. Secondly, muscle NH_3 efflux is affected by the magnitude of the muscle membrane potential with depolarisation enhancing ammonium efflux (Kliener 1981; Wang et al. 1996). Significant depolarisation of the resting membrane potential is unlikely to occur in endurance trained individuals during prolonged submaximal exercise since the intramuscular potassium content remains constant and increases in plasma potassium concentration are small (Madsen et al. 1993). Furthermore, it is unlikely that BCAA feeding influences NH_3 efflux by this mechanism. Thirdly, ammonium efflux across cell membranes may occur via potassium channels (Blaty and Magelby 1984; Knepper et al. 1989). This rate should be determined by the electrochemical gradient for ammonium and competition for the channels with potassium. As mentioned above, the chemical gradient appears to be less favourable for ammonium efflux via this mechanism in the BCAA trial. In addition, the electrical gradient and competition with potassium for the channels is probably not influenced by BCAA ingestion during prolonged submaximal exercise. Since the aforementioned mechanisms appear unhelpful, the proposed higher rate of NH_3 efflux in the BCAA trial may be best explained by an indirect pathway which involves an increased incorporation of NH_3 into the production of Gln within the contracting muscle fibres. As a consequence, Gln efflux from these fibres may increase (MacLean et al. 1994, 1996a) and subsequently result in an elevated catabolic rate of Gln by glutaminase within the muscle endothelial and connective tissue (Willhoft et al. 1993). Glutaminase degrades Gln to produce Glu and NH_3, the latter may then enter the circulation and thus contribute to an increased muscle NH_3 efflux (Wagenmakers et al. 1990) despite a decreased NH_3 concentration gradient across the sarcolemma.

As indicated by the results of the present study and demonstrated previously by others (MacLean et al. 1994, 1996a), BCAA ingestion results in an increase in muscle NH_3 production. It is logical to conclude that the source of this additional NH_3 is derived from

an increased BCAA catabolism within the contracting muscle. The fact that muscle BCAA content decreased by about 1 mmol.kg^{-1}dw (i.e., 16 μmol.kg^{-1}.min^{-1}) during exercise ($P<0.05$) in the BCAA trial (Table 6.5), whilst no change was observed in the CON trial provides indirect evidence that an enhanced rate of BCAA removal occurred as a result of BCAA ingestion. This contention is further supported by data from studies which demonstrate only a small rate of BCAA release from contracting muscle under control conditions (mean varies between 0-15 μmol.kg^{-1}.min^{-1}; MacLean et al. 1994, 1996a; van Hall et al. 1996) or following BCAA ingestion using a similar dose to that used in the present study (1-2 μmol.kg^{-1}.min^{-1}; MacLean et al. 1994). Large doses of BCAA (>300 mg.kg^{-1} body weight) result in net BCAA uptake into contracting muscle (MacLean et al. 1996a; van Hall et al. 1996). Further support for the argument that intramuscular BCAA removal was enhanced following BCAA ingestion is the probability that BCAA production from muscle protein degradation was similar between trials. For example, no differences were observed for plasma or muscle EAA-BCAA and AA-BCAA concentrations (Table 6.3 and Table 6.5) between treatments. Furthermore, Phe, which has been used as a marker of non-contractile muscle protein degradation (Hood and Terjung 1994; Smith and Rennie 1990) was not different between trials (Table 6.6). It should be acknowledged, however, that there was a tendency ($P=0.06$) for the contracting muscle Phe content to be greater in CON compared with BCAA. Any conclusions based on the muscle Phe data, therefore, need to be treated with some caution. Previous studies have reported that BCAA ingestion may suppress (MacLean et al. 1994) or result in no change in endogenous muscle protein breakdown during contraction (Blomstrand et al. 1996; MacLean et al. 1996a).

The decrease in muscle BCAA content observed in the BCAA trial is best explained by BCAA degradation since the other major fate of BCAA, incorporation into muscle proteins, is likely to be reduced during exercise (Hagg et al. 1982; Rennie et al. 1981; Wolfe et al. 1982). Moreover, active skeletal muscle is a site of BCAA oxidation (Hood and Terjung 1987a,b). The first step in the degradation of BCAA is the BCAAT reaction in which the amino groups from the BCAA are transferred to 2-oxoglutarate to produce Glu and BCOA. Subsequently, the BCOA may be oxidised with the rate limiting step in this pathway catalysed by BCOADH (May et al. 1987; Odessey and Goldberg 1979; Shinnick

and Harper 1976). Activation of this mitochondrial enzyme is thought to increase flux through the BCAAT reaction in the direction of Glu and BCOA formation (Hood and Terjung 1991; May et al. 1987). It is the fate of Glu which determines the magnitude of NH_3 production from BCAA catabolism and this issue will addressed later. The regulation of BCOADH is complex, however, the major regulatory mechanism probably involves a phosphorylation / dephosphorylation cycle with dephosphorylation causing activation. Several rodent studies (Aftring et al. 1986; Block et al. 1987a) and one human experiment (van Hall et al. 1996) have demonstrated that an enhanced intramuscular BCAA supply leads to an increased activation of the enzyme in resting and contracting muscle.

As noted above, the BCAA catabolic pathway begins with a transamination reaction such that the amino nitrogen from BCAA is used to synthesise Glu. Therefore, an increase in muscle BCAA catabolism should result in an increase in Glu production from this source. The data from the present study were unable to confirm whether this occurred. The exercise-induced decrease in muscle Glu content in both trials demonstrates that the rate of Glu removal was greater than production (Table 6.6). Furthermore, the difference between the two rates must have been similar between the trials since the fall in muscle Glu was almost identical.

Glu has several fates within contracting muscle. The possible major reactions producing NH_3 from Glu are catalysed by GDH and the coupling of the Asp aminotransferase reaction with PNC activity (Graham et al. 1995a). There is some debate about which of these pathways is most important (Broberg and Sahlin 1989; MacLean et al. 1994). Unfortunately, the data from the present study are unable to resolve this conflict. Nevertheless, the present NH_3 and amino acid data and that of others (MacLean and Graham 1993; MacLean et al. 1994, 1996a; Wagenmakers et al. 1990) indicate that BCAA feeding stimulates one or both of these pathways in contracting muscle. As mentioned previously, glutaminase which is present in the endothelial and connective tissue of skeletal muscle (Willhoft et al 1993) may also play a role in whole muscle NH_3 production (Wagenmakers et al. 1990).

There are a few reactions involving Glu and the removal of NH_3. One such reaction is catalysed by GlnS and involves the coupling of free NH_3 to Glu to produce Gln.

Although no differences in muscle or plasma Gln concentration were observed between trials (Tables 6.2 and 6.6), an increase occurred in both tissues during exercise, indicating that Gln production was stimulated in the present study. As previously discussed, studies capable of measuring muscle Gln content and efflux have reported that BCAA ingestion increases Gln production above control levels (MacLean et al. 1994, 1996a). Since at least one free NH_3 molecule is incorporated into Gln, an increased *de novo* Gln production represents NH_3 production, in addition to an exercise-induced increase in muscle NH_3 content and efflux. Consequently, Gln production needs to be determined to gain a more complete estimate of total muscle NH_3 production. Unfortunately, the techniques used in the present study were not capable of measuring muscle Gln production and therefore the influence of BCAA ingestion on NH_3 metabolism is probably underestimated by the present study. As Gln contains two nitrogen groups, it is possible for Gln production to represent the removal of two free NH_3 molecules provided that the synthesis of the precursor Glu involved an incorporation of free NH_3. Such a reaction is catalysed by GDH in which ammonium is combined to 2-oxoglutarate to produce Glu. Although the direction in which the GDH reaction proceeds is unclear, some authors argue that the principal direction of the GDH reaction during contraction is towards NH_3 production (see above), rather than its removal (MacLean et al. 1996a). If this is correct, Gln may only remove one NH_3 molecule from the cell. If GDH acts in the direction of NH_3 production then the synthesis of Ala, via the Ala aminotransferase reaction, probably does not involve the removal of free NH_3. Consequently, Ala synthesis and release from contracting muscle represents the removal of nitrogen but not additional free NH_3. Therefore, the determination of Ala production may not be necessary to ascertain total muscle NH_3 production. The data from the present study indicate that muscle Ala production increased during exercise in both trials (Table 6.6). Any conclusions about the influence BCAA feeding may have on this production rate however, would be highly speculative. An increased muscle Ala accumulation or release above normal levels has been observed during exercise in some, (Blomstrand et al. 1996; Maclean et al. 1996a), but not all (MacLean et al. 1994), studies.

The results from the present study also demonstrate that at least some of the muscle NH_3 production during exercise must have been derived from net AMP deamination, since a small, yet significant, increase in muscle IMP content was observed (Table 6.4). This contention is further supported by the small exercise-induced increase in plasma Hx concentration found during both trials (Fig. 6.2A). The measurement of plasma Hx provides an extracellular marker of adenine nucleotide degradation and therefore an indirect clue to the extent of IMP production. Further evidence of the magnitude of the degradation of the TAN pool can be gleaned from the measurement of muscle ATP content. The results from the present study are inconsistent in that intramuscular ATP content determined by HPLC analysis indicates no exercise-induced ATP reduction, whilst the results from the enzymatic analysis suggest that a small decrease in the ATP pool occurred (Table 6.4). The reason for the discrepancy is difficult to reconcile. Nevertheless, it is reasonable to suggest that if the muscle ATP stores were reduced during exercise the extent of this decrease was small. The exercise-induced increase in muscle IMP content could only account for 5% of the increase in muscle NH_3 content and provides further indirect evidence that the major source of NH_3 production during prolonged submaximal exercise is unlikely to be net AMP deamination. It should be noted that NH_3 production from AMP deamination is probably underestimated by measuring IMP accumulation in mixed-muscle samples at the exercise intensity used in the present study. It is possible that fibres which produce IMP may cease contracting due to fatigue and subsequently reaminate it during recovery, whilst other fibres are recruited to maintain the work rate (Terjung and Tullson 1992). Even though such a process may have occurred, it is unlikely to explain the probable increase in muscle NH_3 production as a consequence of BCAA ingestion. As expected (MacLean et al. 1994, 1996a), BCAA ingestion did not affect the muscle IMP and ATP contents (Table 6.4) or plasma Hx accumulation (Fig 6.2A) in the present experiment. These findings suggest that the rate of net AMP deamination, and consequently NH_3 production from this source, was similar between the trials. If this was the case, net AMP deamination cannot account for the probable increase in NH_3 production during exercise following BCAA ingestion.

In the present experiment, BCAA ingestion appeared to have no measurable effect upon carbohydrate metabolism during exercise. This conclusion was based on the following observations. First, muscle glycogen contents were similar at rest and after 60 min of exercise between the trials (Table 6.4). Secondly, muscle (Table 6.4) and plasma lactate (Fig. 6.1C) concentrations were virtually identical between treatments. Thirdly, except for a small, yet significant, drop in resting plasma glucose after BCAA ingestion, there were no differences in the circulating glucose levels during exercise when the BCAA trial was compared with CON (Fig 6.1B). Finally, the RER measured during exercise was not influenced by BCAA ingestion (Table 6.1). Most of these findings have been reported previously (MacLean and Graham 1993; MacLean et al. 1994). Interestingly, Blomstrand et al. (1996) observed that BCAA ingestion during exercise resulted in muscle glycogen sparing when the activity was begun with low muscle glycogen content. The fall in resting plasma glucose concentration following BCAA ingestion has not been found previously (MacLean and Graham 1993; MacLean et al. 1994). This result was probably caused by the tendency for plasma insulin to increase to a greater extent after BCAA feeding than in CON (Fig 6.1A). An increase in plasma BCAA has been reported to stimulate insulin secretion (Castellino et al. 1987; Hutton et al. 1980). It should be noted that MacLean et al. (1996a) also reported that BCAA ingestion had no significant effect on muscle lactate accumulation, muscle lactate efflux or arterial plasma lactate concentration during 90 min of leg kicking exercise. When net lactate production (muscle lactate accumulation plus efflux) was calculated for the exercise period there was, however, a greater production in the CON versus the BCAA trial. The results from the present study were unable to confirm this finding because the experimental techniques required to provide confirmation were not utilised.

In summary, this study demonstrated that BCAA ingestion resulted in an increase in plasma and muscle BCAA at rest and during exercise. The increase in muscle BCAA content appeared to result in an elevation in BCAA catabolism during exercise. This factor best explains the probable increase in muscle NH_3 production in the BCAA trial. Furthermore, at least some of the NH_3 production occurring during exercise was derived from net AMP deamination, but this source of NH_3 was likely to be small and appeared not

to be influenced by BCAA ingestion. Consequently, these findings lead to the conclusion that amino acid catabolism, in particular BCAA catabolism, may be a source of muscle NH_3 production during prolonged submaximal exercise. Importantly, the magnitude of the contracting muscle mass, with associated cardiovascular and endocrine responses, appear not to influence this conclusion since the results from previous BCAA feeding studies, utilising single-leg kicking exercise, are similar to those in the present experiment.

CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS

7.1 INTRODUCTION

The following discussion explores the processes involved in muscle NH_3 metabolism during LI and MI prolonged exercise. In so doing, the discussion focuses primarily on the role of amino acid metabolism in an attempt to provide some insight into its significance and function in contracting skeletal muscle. As part of this process the influence of substrate availability on muscle amino acid catabolism during exercise is addressed. The overall aim of this chapter is therefore, to establish a link between amino acid metabolism and muscle NH_3 production.

7.2 LOW INTENSITY EXERCISE

During LI exercise (≈40% $\dot{V}O_2max$) muscle NH_3 production occurs (Study 1; Eriksson et al. 1985), presumably in type I fibres, as a consequence of amino acid catabolism (Study 1; Knapik et al. 1991; Wolfe et al. 1982, 1984). The production of NH_3 begins early (Study 1; Eriksson et al. 1985) and occurs throughout the entire exercise period (Study 1).

It remains unclear which biochemical reactions are responsible for the production of NH_3 from amino acids within contracting muscle. There is considerable debate about the contribution from the PNC and GDH reactions (Lowenstein 1990; Graham et al. 1995a). There is also the possibility that glutaminase may be important (Study 3; Wagenmakers et al. 1990). No evidence of significant AMPd activity has been observed during LI exercise (Study 1; Sahlin et al. 1989) indicating that the PNC may not be operating in contracting human skeletal muscle under such circumstances. In addition, studies of rat skeletal muscle suggest that the PNC does not operate during LI exercise (Meyer and Terjung 1980). These data provide a case for discounting PNC activity, however, to do so may be premature. The evidence obtained from human muscle is circumstantial and that from rodents is questionable, and possibly not applicable to humans (see Chapter 2 and Study 1).

Because the free amino acid pool is small and relatively constant, the amino acids utilised during exercise are predominantly obtained from the net degradation of proteins (i.e., protein degradation exceeds synthesis) (Carraro et al. 1994; Graham et al. 1995a).

There are many potential regulators of both protein synthesis and degradation. For example, protein synthesis is increased in response to growth hormone (Fryburg et al. 1991), insulin (Jefferson 1980; Marshal and Monzon 1989), thyroid hormones (Millward 1985), leucine, and other amino acids (Goldberg and Chang 1978; Watt et al. 1992) but decreased by the combined infusion of adrenaline, glucagon and cortisol (Wernerman et al. 1989), a reduced intake of dietary protein (Tawa and Goldberg 1992), depressed cellular energy state (Byelund-Fellinius et al. 1984), corticosteroids (Odedra and Millward 1982; Odedra et al. 1983) and exercise (Dohm et al. 1987). Moreover, protein degradation was increased in response to fasting (Fryburg et al. 1990), thyroid hormones (Millward 1985), glucocorticoids (Beaufrere et al. 1989; Simmons et al. 1984) and exercise (Balon et al. 1990), and attenuated by decreases in dietary protein (Tawa and Goldberg 1992), insulin (Castellino et al. 1990; Fryburg et al. 1990), leucine (Goldberg and Chang 1978; Nair et al. 1992) and medium chain triglycerides (Beaufrere et al. 1985). Graham et al. (1995a) argue that the net change in protein turnover during exercise most likely reflects a balance of these regulators.

During about an hour of LI cycling exercise, plasma insulin (Study 1; Ahlborg et al. 1974; Galbo 1983), adrenaline (Galbo et al. 1975), cortisol (Berger et al. 1977) and glucagon (Ahlborg et al. 1974; Berger et al. 1977) remain stable, while growth hormone increases (Berger et al. 1977). Furthermore, muscle and plasma Leu concentrations are also unchanged (Study 1; Eriksson et al. 1985). Similarly, the EC in the contracting muscle was unaltered (Study 1). These hormonal and metabolite data appear unable to explain the increased net protein degradation that occurs during LI exercise (Carraro et al. 1994). It should be recognised that the measurement of EC was conducted in mixed-muscle samples and includes the total adenine nucleotide content. Increases in ADP_f and AMP_f possibly occur in contracting muscle but such changes cannot be measured by current analytical techniques. Although speculative, an increase in net protein degradation may result from an increase in ADP_f and AMP_f. Interestingly, in a recent review, Layman et al. (1994) postulated that decreased protein synthesis in contracting muscle may result from inhibition of a protein initiation factor (eurokaryotic initiation factor 2). The inhibition of this factor is controlled via phosphorylation and this is achieved by the action of a protein kinase. This

enzyme is stimulated by glucagon, cyclic AMP and calcium, and inhibited by insulin. The possibility that calcium may inhibit protein synthesis is particularly appealing since the appropriate hormonal changes are minimal during LI exercise and calcium release from the sarcoplasmic reticulum is required for normal muscle contraction, irrespective of the exercise intensity.

The source of the amino acids catabolised during LI exercise is probably an increased hepatic and gut output (Ahlborg et al. 1974; Williams et al. 1996) and an increase in net muscle protein degradation (Ahlborg et al. 1974; Dohm 1986). It remains unclear which proteins are degraded within contracting muscle, however it is probably non-contractile protein since rates of contractile protein degradation are depressed during muscular activity (Dohm et al. 1980, 1987; Kasperek and Snider 1989, MacLean et al. 1994). The rate of amino acid catabolism during LI exercise is likely to increase to a greater extent if the exercise is performed for several hours (Ahlborg et al. 1974; Haralambie and Berg 1976). This is possibly related to a depletion of glycogen stores within the active type I muscle fibres (Gollnick et al. 1974).

It is interesting to speculate on the purpose of amino acid catabolism and its relationship to muscle NH_3 production. Clearly some of the catabolised amino acids provide an energy source to the contracting muscle. This is expected to be small (less than 5% of the total energy expenditure) compared with the energy supplied from fat and carbohydrate catabolism (Calles-Escandon et al. 1984; Wolfe et al. 1982). It is possible that the amino acid contribution to energy expenditure may become more important (e.g., 10% of total energy expenditure) when muscle carbohydrate supply is reduced (Lemon and Mullin 1980). It should be noted, however, that the glycogen stores of type I fibres are expected to be only moderately depleted after 40 min of LI exercise (Gollnick et al. 1974). Consequently, the energy contribution from amino acid oxidation is likely to be small and constant throughout the LI exercise bout performed in Study 1 of the present thesis. The NH_3 produced during LI exercise may therefore simply be an obligatory by-product of amino acid oxidation.

Muscle amino acid degradation may also result in the *de novo* production of Ala and Gln (Chang and Goldberg 1978; Spydevold 1976). An enhanced Ala production, via Ala aminotransferase activity, has been demonstrated using tracer methodology during LI exercise (Carraro et al. 1994; Wolfe et al. 1984). Furthermore, an increase in muscle Ala content and efflux has been observed during LI exercise by some researchers (Ahlborg et al. 1974; Felig and Wahren 1971; Katz et al. 1986a), but not by others (Study 1; Eriksson et al. 1985; Katz et al. 1986a; Sahlin et al. 1995). An increase in muscle Gln content or efflux has not been found during short duration, low work intensities (Study 1; Eriksson et al. 1985; Katz et al. 1986a; Sahlin et al. 1995) suggesting that an increased GlnS activity does not occur in these circumstances. However, when the exercise duration is more prolonged an increased rate of Gln synthesis may occur (Study 1).

De novo muscle Ala production may be important for several reasons. Firstly, Ala provides a non-toxic alternative to NH_3 for the transport of amino groups from muscle to the liver. Ala production achieves this role by removing Glu, reducing the possiblity of this amino acid being involved in other reactions, in particular NH_3 producing reactions. Furthermore, Ala production results in the concomitant synthesis of 2-oxoglutarate which is a tricarboxylic acid cycle intermediate (TCAI). The importance of TCAI synthesis is outlined below. Finally, once Ala exits the contracting muscle, its carbon skeleton may act as a gluconeogenic precursor (Ahlborg et al. 1974; Felig et al. 1970) and the nitrogen utilised to synthesize acute phase proteins (Carraro et al. 1990). Both of these processes occur within the liver.

Another important function of some catabolic reactions involving amino acids may be to help expand and maintain the pool of TCAI in order to augment aerobic metabolism (Aragon and Lowenstein 1980; Sahlin et al. 1990c). The anaplerotic reactions involving amino acids include the PNC, Ala aminotransferase and GDH. It has been suggested that the Ala aminotransferase reaction and reactions involving pyruvate and phosphoenolpyruvate are the major producers of TCAI (Sahlin et al. 1990c, 1995; Spencer et al. 1991). The anaplerotic role of GDH and the PNC is probably small (Sahlin et al 1990), but may account for most of the muscle NH_3 production during LI exercise. These reactions possibly increase in importance when muscle fibre carbohydrate supply is reduced

and amino acid availability is augmented (Ahlborg et al. 1974; Greenhaff et al. 1991; Sahlin et al. 1990c; Wagenmakers et al. 1990).

In contrast to the anaplerotic role that some amino acids may play, others may actually drain the TCA cycle. Wagenmakers et al (1990) provided evidence that muscle BCAA catabolism, due to the action of BCAAT, may reduce muscle TCAI content. An increase in Leu oxidation has been observed during LI exercise (Knapik et al. 1991; Wolfe et al. 1982, 1984). Furthermore, the GlnS reaction has the potential to drain TCAI from the TCA cycle (Goldberg and Chang 1978) since it involves the removal of Glu, an important substrate in other anaplerotic reactions (i.e., GDH and Ala aminotransferase). The utilisation of this reaction, however, appears quite small during LI exercise (see above). Any removal of TCAI from the TCA cycle may need to be counteracted by an increased activity of the anaplerotic reactions in order to maintain aerobic metabolism in the contracting muscle. As a consequence NH_3 could be produced.

7.3 MODERATE INTENSITY EXERCISE

The rate of muscle NH_3 production is greater during MI (≈70% VO_2max) compared with LI exercise (Study 1; Eriksson et al. 1985). The evidence presented in this thesis (Studies 1,2 and 3) and that of other research (MacLean et al. 1994, 1996a; van Hall et al. 1995a; Wagenmakers et al. 1991) indicates that the increase in NH_3 production is primarily the result of an enhanced amino acid catabolism. Again it remains unclear which reactions are producing NH_3, however, at moderate exercise intensities there is evidence that AMPd is active (Study 1, 2 and 3). This makes the operation of the PNC in contracting muscle more likely (see Chapter 2).

The muscle fibres recruited at moderate work rates are expected to be type I and type IIa (Gollnick et al. 1974; Sale 1987). This is in contrast to LI exercise when only type I fibres are contracting, at least for the first two hours of activity (Gollnick et al. 1974). It is probable that net AMP deamination is higher in glycogen depleted type I and type II fibres during prolonged, MI exercise (Norman et al. 1988) and this may partially explain (see below) the elevated NH_3 production observed at higher exercise intensities (Study 1).

It can only be a partial explanation since the predominant source of NH_3 during MI exercise is probably amino acid catabolism, not net AMP deamination. A comparison of the abilities of the various human muscle fibre types to utilise the PNC or GDH reaction has not been conducted. Although speculative, PNC activity may be greater in type IIa fibres, while the role of GDH may be more important in type I fibres. This speculation is based upon the probability that AMPd is activated to a greater extent in type IIa fibres compared with type I fibres (Jansson et al. 1987; Norman et al. 1988; Sahlin et al. 1989), while the maximal rate of IMP reamination is probably similar between the two fibre types (Tullson et al. 1996a). In contrast, since GDH is a mitochondrial enzyme (Kovacevic and McGivan 1983), the highest maximal activity is found in the mitochondrial rich ST fibres (Henriksson et al. 1986).

The augmented requirement for amino acids during MI, compared with LI, exercise is probably obtained from an increased whole-body net protein degradation (Butterfield 1987; Dohm et al. 1987; Lemon et al. 1984). This intensity-dependent increase may partially result from a hormonal and metabolic milieu favouring net protein degradation. For example, plasma insulin concentration falls during MI exercise (Study 1; Galbo 1983). In contrast, adrenaline (Galbo et al. 1975; Green et al. 1989), and cortisol (Davies and Few 1973) increase, while glucagon either increases (Galbo et al. 1975) or remains at pre-exercise levels (Green et al. 1991; Tarnpolsky et al. 1990). In addition, the EC within the contracting muscle undergoes a small, yet significant, fall (Study 1 and 2). It is not clear which organs are responsible for the augmented rate of protein degradation, however contributions are likely to come from the liver, gut and contracting skeletal muscle (i.e., non-contractile protein). Net protein degradation rates would be expected to increase during the few hours in which MI exercise can be sustained (Lemon and Nagle 1981) since this rate appears to be inversely related to carbohydrate availability (Lemon and Mullin 1980; van Hall et al. 1996).

As mentioned for LI exercise, there are a number of possible reasons to catabolise amino acids. The contribution that amino acid oxidation makes to the total energy expenditure during MI exercise in humans is about 4-8% (Lemon and Mullin 1980; Rennie et al. 1981). Hood and Terjung (1987a,b) reported that in an isolated rat hindlimb

preparation, the absolute rate of Leu oxidation increased when stimulated, however, the relative energy contribution from the oxidation of this amino acid (about 1%) was not altered by stimulation frequency. These data suggest that the relative importance of amino acid oxidation to muscle ATP production is similar, irrespective of the exercise intensity. At high submaximal work rates, however, carbohydrate stores are depleted more quickly (Study 1; Gollnick et al. 1974) which may result in an elevated energy contribution from amino acid oxidation when compared with low exercise intensities (Lemon and Mullin 1980). In further support of this argument, an increased activation of muscle BCOADH occurs as MI exercise progresses (Rush et al. 1995; Wagenmakers et al. 1989) and this seems to be inversely related to muscle carbohydrate availability (Wagenmakers et al. 1991; van Hall et al. 1996). An absolute increase in the rate of muscle amino acid oxidation requires an enhanced rate of amino nitrogen processing, a proportion of which may result in NH_3 production. This may explain, at least in part, the sustained, elevated muscle NH_3 production rates observed at higher work intensities in the present thesis (Study 1, 2 and 3).

Another reason for the occurrence of amino acid catabolism in contracting muscle may be to produce Ala and Gln. Although controversial (Sahlin et al. 1996), it is becoming more accepted that an increased *de novo* Gln production, via GlnS activity, may occur in active muscle during MI exercise (Graham et al. 1995b; MacLean et al. 1994, 1996a, van Hall et al. 1995a). This is in contrast to exercise at lower intensities when an elevated GlnS activity may only occur when the exercise duration is prolonged (see above). Obviously, one function of *de novo* Gln synthesis is to attenuate the rise in intramuscular NH_3 accumulation. In fact, Chang and Goldberg (1978) have demonstrated that an increase in muscle NH_3 content promotes Gln synthesis perhaps explaining why such a process has been more easily determined at higher work rates. In addition to an increase in Gln production, an augmented *de novo* muscle Ala production is known to occur with increasing work rates (Eriksson et al. 1985; Felig and Wahren 1971; Katz et al. 1986a; Sahlin et al. 1995). Muscle efflux of Ala (Eriksson et al. 1985; Katz et al. 1986a), and probably Gln (Katz et al. 1986a), occurs more quickly at higher exercise intensities. Therefore, there is an enhanced transport of nitrogen and carbon skeletons from muscle to

other sites within the body. The fate of the released Ala has already been discussed (see previous section). The released Gln may undergo a similar fate or, more importantly, it may be metabolised by cells of the gut, immune system or kidney (Hanson and Parsons 1980; Newsholme and Parry-Billings 1990; Tannen 1978; Windmueller and Spaeth 1980).

The carbon skeletons for Ala production are derived predominantly from carbohydrate sources (Caldecourt et al. 1985; Chang and Goldberg 1978; Spydevold 1976), whilst for Gln they may be obtained from a similar source (Krebs 1975) or from other amino acids (Chang and Goldberg 1978; Wagenmakers et al. 1985). Consequently, a reduction in carbohydrate supply, which occurs more rapidly in MI exercise, may compromise muscle Ala production (Sahlin et al. 1990c; Spencer et al. 1991,1992; van Hall et al. 1995a) thereby attenuating the removal of Glu. This could lead to an increased opportunity for Glu to participate in other reactions some of which may result in NH_3 production (e.g., GDH and/or PNC via Asp aminotransferase activity). The fact that carbohydrate ingestion attenuated muscle and plasma NH_3 levels during prolonged submaximal exercise (Study 2) may be explained, at least in part, by an enhanced ability to sustain muscle Ala production rates.

A further reason for muscle amino acid catabolism during exercise centres around the regulation of aerobic metabolism. An increase in exercise intensity requires a greater expansion of the TCAI content in order to cope with the elevated energy demands of the activity (Sahlin et al. 1995). An increased anaplerotic rate is therefore required and, although speculative, this may involve a more marked contribution from GDH and/or the PNC (Aragon and Lowenstein 1980; MacLean et al. 1996a; Sahlin et al. 1990c). These enhanced contributions may explain the elevated rate of muscle NH_3 accumulation observed at the higher exercise intensity in the present thesis (Study 1).

Moreover, a further increase in GDH and/or PNC activity may be required as the duration of the exercise progresses. Sahlin et al. (1990c) reported that the TCAI content of active skeletal muscle fell nearing the onset of fatigue when performing MI exercise. This was attributed to a reduced anaplerotic rate from reactions involving glycolytic intermediates due to a lack of carbohydrate. In support of this argument manipulations of the carbohydrate supply prior to, or during, MI exercise lead to a better maintenance of the

TCAI pool (Spencer et al. 1991, 1992) and this may therefore attenuate the need to utilise the PNC and/or GDH reaction. In addition, an enhanced carbohydrate supply may reduce the drain on the TCAI pool by inhibiting BCAA oxidation (Buse et al. 1972; Davies et al. 1982; Wagenmakers et al. 1991) and thus, further reduce the need to use the PNC and/or GDH. The fact that carbohydrate ingestion attenuated muscle and plasma NH_3 levels towards the latter stages of 120 min of exercise (study 2) with only a small increase in net AMP deamination also supports this contention.

An increase in muscle amino acid catabolism, in particular BCAA, is known to occur with increasing exercise intensity (Babij et al. 1983b; Dohm et al. 1980; Felig and Wahren 1971; Henderson et al. 1985; Hood and Terjung 1987a,b; White and Brooks 1981). This enhanced BCAA utilisation probably results from an intensity-dependent activation of BCOADH (Kasparek and Snider 1987) and may provide an increased drain on the muscle TCAI content placing further demands on the anaplerotic reactions to maintain the pool. Furthermore, this drain may be enhanced by the action of GlnS which increases in activity (see above) and appears to be involved in the removal of Glu produced by the BCAAT reaction (Darmaun and Dechelotte 1991). One, or both of these mechanisms may explain why BCAA ingestion results in an elevated muscle NH_3 production during moderate exercise intensities (Study 3, MacLean et al. 1994; 1996a).

Despite most of the NH_3 being derived from amino acid catabolism, this thesis clearly demonstrates that a small, yet significant, proportion of the NH_3 must also be produced from net AMP deamination during MI exercise. In endurance trained cyclists (Study 2) net AMP deamination became apparent sometime after 30 min and more likely towards the end of 120 min of exercise. Although speculative, this is best explained by a decreased carbohydrate supply in both type I and type II fibres (Norman et al. 1988). Spencer et al. (1991) have demonstrated that aerobic ATP synthesis rates are maintained in endurance trained muscle nearing fatigue despite a low muscle glycogen content and a fall in TCAI content. This however, was achieved at the cost of greater ADP_f (an allosteric activator of several enzymes in the TCA cycle; Hansford 1980) and AMP_f contents. As a consequence, AMPd was activated above the level of the IMP reaminating pathway, resulting in significant net AMP deamination.

In contrast, the active, non-specifically trained subjects examined in Study 1, displayed a two and four fold increases in mean IMP content after 10 and 40 min of exercise at 70% $\dot{V}O_2max$, respectively. Furthermore, an increase in IMP degradation products was found in muscle and plasma during the 40 min exercise period. These results are unlikely to be adequately explained by depletion of glycogen within the muscle fibres (Gollnick et al. 1974). A better explanation may relate to the training status of the subjects. Several studies have demonstrated that untrained subjects display a greater disturbance in the cellular milieu known to activate AMPd (Cadefau et al. 1994; Denis et al. 1991; Green et al. 1992, 1995) and consequently an enhanced net AMP deamination (Green et al. 1992, 1995) during submaximal exercise before, compared with after, endurance training. The mechanisms causing the attenuated rate of net AMP deamination after training may involve an improved metabolic control due to: (1) an increase in mitochondrial sensitivity, size and number; (2) attenuation of sympathetic drive; and/or (3) cardiovascular adaptations (Constable et al. 1987; Dudley et al. 1987; Dudley and Terjung 1985a; Green 1996). The early activation of AMPd in untrained subjects may of course be exacerbated by a reduction in muscle fibre glycogen content (Baldwin et al. 1996). Therefore, net AMP deamination may increase as the exercise duration progresses.

7.4 SUBSTRATE AVAILABILITY

The studies presented in this thesis (Study 1) showed that NH_3 production occurred shortly after the onset of exercise in subjects who had a plentiful supply of muscle glycogen and were euglycaemic. These data indicate that, under these circumstances, NH_3 production is not occurring as a result of a lack of carbohydrate but must be associated with other cellular processes (e.g., amino acid catabolism resulting in an expansion of the TCAI pool).

As mentioned previously, the results of the present thesis (Study 2) demonstrated that an enhanced carbohydrate supply to the contracting muscle attenuated muscle NH_3 production after about an hour of MI exercise. This attenuation is best explained by a reduction in muscle amino catabolism, although a decreased net AMP deamination cannot be excluded. How an augmented carbohydrate supply to the active muscle results in a reduction in muscle amino acid catabolism is unknown. One possibility is that an elevated

supply of carbohydrate attenuates the rate of net protein degradation in liver, gut and muscle. Consequently, there is a reduced availability of amino acids to be catabolised by the contracting muscle. A decrease in net protein degradation may occur because carbohydrate ingestion attenuates the rise in catabolic hormones (e.g., glucagon; Ahlborg and Felig 1976) and reduces the fall in insulin (an anabolic hormone) during prolonged MI exercise (Study 2; Galbo 1983). Furthermore, net muscle protein degradation may be reduced because the muscle EC is maintained at a higher level when carbohydrate is ingested during exercise (Study 2; Spencer et al. 1991). Byelund-Fellinus et al. (1984) demonstrated that the muscle protein synthesis rate was decreased when the muscle energy state was depressed.

In addition to attenuating the supply of amino acids, an enhanced carbohydrate supply may inhibit the muscle's capacity to oxidise amino acids, in particular BCAA (Buse et al. 1972; Davies et al. 1982). For example, Wagenmakers et al. (1991) demonstrated that the activation of muscle BCOADH was abolished during MI exercise when the carbohydrate supply was plentiful. How carbohydrate availability regulates the activation of BCOADH during exercise is not understood (van Hall et al. 1996).

The present thesis (Study 3) provided evidence that the ingestion of BCAA enhanced muscle NH_3 production during MI exercise. It seems clear that this increased production results from an augmented BCAA catabolism within the contracting muscle, since net AMP deamination was unaltered by BCAA feeding. The increased BCAA catabolism is best explained by an increased activation of BCOADH. van Hall et al. (1996) reported that BCAA ingestion caused an increase in BCOADH activation in resting muscle. Moreover, these authors demonstrated that the effects of BCAA ingestion and exercise on BCOADH activation were additive. An increased supply of BCAA to muscle probably activates the BCOADH by inhibiting BCOADH kinase as a consequence of the increase in muscle BCAA (Study 3) and BCOA contents (Aftring et al. 1986; Block et al. 1987a; van Hall et al. 1996). The regulation of BCOADH during exercise remains unclear (see Chapter 2).

7.5 FATE OF NH_3

The NH_3 produced within contracting muscle may have several fates some of which involve excretion of nitrogen from the body, while others involve nitrogen recycling. The

free NH_3 released from active muscle (Broberg and Sahlin 1989; Eriksson et al. 1985; Graham et al. 1991) is distributed to other sites where it may be involved in; (1) amino acid synthesis (Bangsbo et al. 1996; Cooper et al. 1979), (2) liver urea production (Wasserman et al. 1991) in preparation for excretion via urine and sweat (Calles-Escandon et al. 1984; Czarnowski and Gorski 1991; Lemon and Mullin 1980), and (3) excretion from the body in expired air (Sahlin 1996) and sweat (Czarnowski and Gorski 1991). It should be noted that several human studies have not found an increased urea production during, and in recovery from, prolonged exercise (Carraro et al. 1993; Wolfe et al. 1982, 1984). Carraro et al. (1993) suggested that the exercise-induced increase in urea loss via sweat and urine results from a depletion of the whole-body urea pool rather than an increased urea production. Finally, the NH_3 produced may be involved in Gln (Meister 1984), and possibly Ala synthesis within the active muscle. These amino acids may subsequently leave the muscle and be processed by other tissues (see above).

7.6 CONCLUSIONS

The major conclusions of this thesis were the following.

1. Skeletal muscle NH_3 production occurs during LI and MI exercise.
2. The source of NH_3 production during LI was exclusively amino acid catabolism.
3. Muscle NH_3 production occurred early in LI exercise suggesting that this production occurs, most likely within type I muscle fibres, even when muscle carbohydrate supplies were adequate.
4. During MI exercise, amino acid catabolism was the predominant NH_3 source, however, net AMP deamination was also contributing to the NH_3 production.
5. Carbohydrate ingestion attenuated muscle and plasma NH_3 concentrations toward the latter stages of 120 min of MI exercise. This relatively large decrease in NH_3 metabolism occurred with only a very small decrease in net AMP deamination, suggesting that most of the reduction in NH_3 production was due to an inhibition of muscle amino acid catabolism.

6. BCAA feeding did not result in an elevation of muscle NH_3 content at rest or exercise, however, plasma NH_3 concentration was increased. These data indicate that BCAA feeding caused an increased NH_3 production and efflux from contracting muscle. This augmented NH_3 production was best explained by an increased BCAA metabolism since no evidence of an enhanced net AMP deamination rate was observed.
7. Exercise mode appears to have little influence on the effect that BCAA feeding has on NH_3 metabolism, since the results from the two-legged cycling study presented in this thesis were similar to those previously reported for single-leg kicking exercise.
8. Muscle NH_3 production in contracting muscle does not appear to be related to circulating insulin concentrations.

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APPENDIX

The following paper was completed during the period of my doctoral studies.

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HEAT STRESS INCREASES AMMONIA ACCUMULATION DURING EXERCISE IN HUMANS

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SUMMARY

Seven men were studied during 40 min of exercise at 70% $\dot{V}_{O_2}$ peak, in an environmental chamber maintained at either 20 or 40 °C, to examine the effect of heat stress on ammonia metabolism during exercise. Heart rate and rectal and muscle temperatures were higher during exercise in the heat, while no differences were observed in pulmonary oxygen uptake or respiratory exchange ratio. Plasma ammonia levels and muscle ammonia accumulation were higher during exercise at 40 °C compared with 20 °C. Such metabolic alterations may be associated with reduced performance during exercise in the heat.

INTRODUCTION

Previous studies in humans have observed increased rates of muscle glycogenolysis (Fink, Costill & Van Handel, 1975) and muscle and blood lactate accumulation (Fink *et al.* 1975; Young, Sawka, Levine, Cadarette & Pandolf, 1985) during exercise in the heat. Similar results have been obtained in dogs who become hyperthermic during prolonged exercise (Kozlowski, Brzezinska, Kruk, Kaciuba-Uscilko, Greenleaf & Nazar, 1985). In the latter study, an increased muscle adenine nucleotide degradation, resulting in higher muscle ADP and AMP levels, was also observed during hyperthermic exercise. An increase in free ADP and AMP will activate AMP deaminase, resulting in the formation of inosine monophosphate (IMP) and ammonia, although these metabolites were not measured in the study by Kozlowski *et al.* (1985). To our knowledge, no studies have previously examined the effect of heat stress on ammonia metabolism during exercise in humans. Thus, the present study was undertaken.

METHODS

Seven healthy, active but untrained, male subjects, aged 23·6±1·9 years (mean±S.E.M.), with a mean body weight of 70·1±1·3 kg, agreed in writing to take part in this study after being informed of all risks and stresses associated with participation. The study was approved by the Victoria University of Technology Human Experimentation Committee. Peak pulmonary oxygen uptake ($\dot{V}_{O_2}$ peak), measured during cycling exercise to volitional fatigue, averaged 3·6±0·1 l min^{-1} (mean±S.E.M.). None of the subjects was specifically heat acclimated and the study was conducted between July and December, with the daily maximum temperature averaging 19·4±1·9 °C. Subjects reported to the laboratory in the morning after an overnight fast and having abstained from strenuous exercise, tobacco, alcohol and caffeine for at least 24 h. A catheter was positioned in a forearm vein for blood sampling and was kept patent by periodic flushing with 0·9% saline containing heparin (10 U ml^{-1}). Exercise was performed for 40 min on an electrically-

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braked bicycle ergometer, at a workload estimated to require 70% $\dot{V}_{O_2}$ peak (175±5 W), in an environmental chamber maintained at either 20 or 40 °C. Relative humidity was approximately 20% in both trials and there was no wind motion within the chamber. The order of the trials was randomized and they were conducted at least 1 week apart. A venous blood sample was obtained after 5–10 min of rest and after 10 and 40 min of exercise. Five millilitres of blood, placed in a lithium heparin tube, were spun and the plasma was frozen in liquid N_2 for subsequent ammonia determination using flow injection analysis (Svensson & Anfält, 1982). The coefficient of variation for repeated determinations in our laboratory is 5·7%. Immediately before and after exercise, muscle samples were obtained from the vastus lateralis using a percutaneous needle biopsy technique, with suction. These samples were immediately frozen in liquid N_2 for later ammonia analysis. Briefly, 5–12 mg of wet muscle were extracted as described by Katz, Broberg, Sahlin & Wahren, (1986). The sample was placed in a pre-cooled mixture of 0·6 M perchloric acid (PCA) and 30% methanol for 30 min at –20 °C. Ice-cold 0·6 M PCA was added and the sample placed on ice for 15 min, with frequent vortexing. After addition of ice-cold 1·8 M KOH, the sample was vortexed and spun at 50000 *g* for 2 min at 2 °C. The supernatant was analysed within 1 h of extraction by flow injection analysis (Katz *et al.* 1986). Within 10 s of obtaining the post-exercise muscle biopsy, muscle temperature was measured at a depth of 4 cm using a 25 g needle thermistor (YSI 524, Yellow Springs, OH, USA), inserted through the incision made for the muscle biopsy. Slightly more time was allowed for the measurement of the pre-exercise muscle temperature, but it was always measured within 30 s of the pre-exercise biopsy. Rectal temperature was monitored continuously during exercise by a thermistor (YSI 401, Yellow Springs, OH, USA) inserted to a depth of 10 cm beyond the anal sphincter. Expired gas samples were collected in Douglas bags at 10 min intervals during exercise. These samples were analysed for oxygen and carbon dioxide (Applied Electrochemistry S-3A and CD-3A analysers, Ametek, PA, USA) and the volume was measured using a Parkinson-Cowan gas meter, calibrated against a Tissot spirometer. Heart rate was monitored continuously by electrocardiography. No fluids were ingested during exercise in either trial. The data from the two trials were compared using analysis of variance for repeated measures, with significance accepted at the 0·05 level. Specific differences between means were located using the Student-Newman-Keuls *post hoc* test. All data are reported as means±S.E.M.

RESULTS

No differences were observed between the two trials in oxygen uptake and respiratory

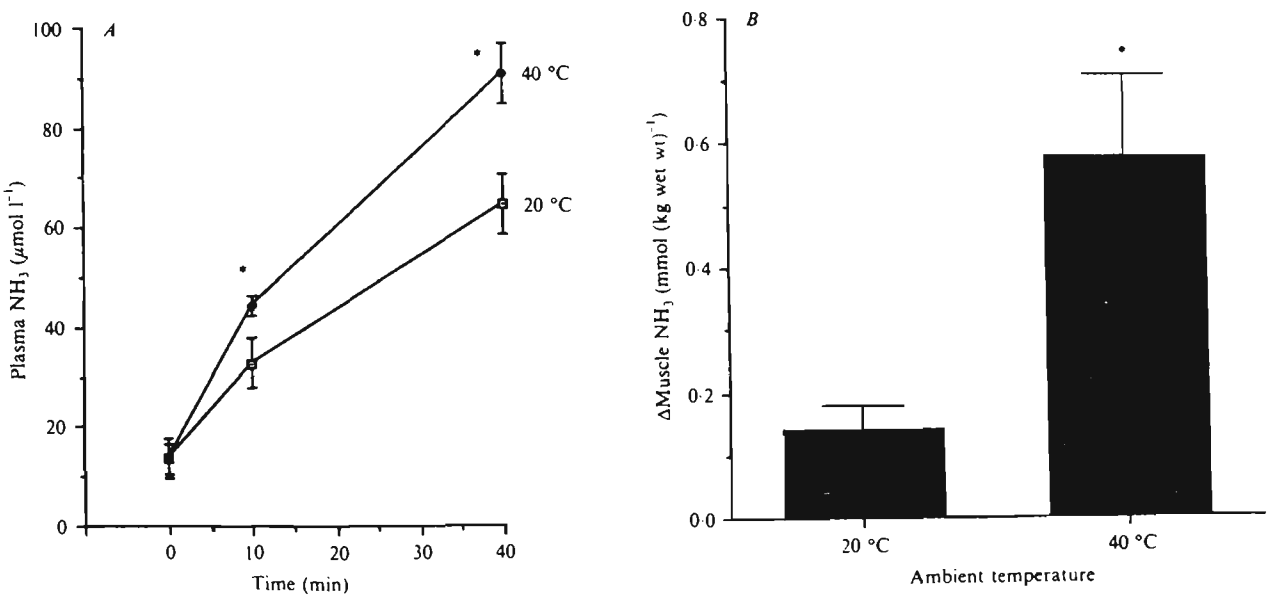

Fig. 1. *A*, plasma ammonia levels before and during exercise at 20 and 40 °C. *B*, increase (post *vs.* pre) in muscle ammonia during exercise at 20 and 40 °C. Values are means±S.E.M. (n = 7). * Significantly different from 20 °C value, $P < 0.05$.

exchange ratio, which averaged 2·42±0·10 and 2·47±0·12 l min^{-1} and 0·92±0·01 and 0·91±0·01 during exercise at 20 and 40 °C, respectively. In contrast, heart rate was higher ($P < 0·05$) throughout exercise in the heat, averaging 159±5 and 177±5 beats min^{-1} during exercise at 20 and 40 °C, respectively. No differences were observed between trials in either rectal or muscle temperature before exercise; however, 40 min of exercise at 40 °C resulted in higher ($P < 0·05$) rectal (38·7±0·1 *vs.* 38·1±0·2 °C) and muscle (39·5±0·2 *vs.* 38·3±0·2 °C) temperatures. No differences in resting plasma ammonia levels were observed between the two trials; however, plasma ammonia levels were higher ($P < 0·05$) during exercise at 40 °C (Fig. 1). Pre-exercise muscle ammonia levels were similar in both trials (0·48±0·10 mmol (kg wet wt)$^{-1}$. Muscle ammonia levels were higher ($P < 0·05$) after exercise at 40 °C (1·06±0·11) compared with 20 °C (0·62±0·09). The increase in muscle ammonia during exercise was higher ($P < 0·05$) in the hot environment (0·58±0·13 *vs.* 0·14±0·04 mmol (kg wet wt)$^{-1}$, Fig. 1).

DISCUSSION

The results of the present study indicate that heat stress increases plasma and muscle ammonia accumulation during exercise in humans (Fig. 1). The potential sources of ammonia production during exercise include AMP deamination and/or amino acid catabolism, although the relative importance of each of these mechanisms during submaximal exercise has not been clearly defined (Graham & MacLean, 1992). It is possible that amino acid catabolism could account for the higher plasma and muscle ammonia levels; however, it has been suggested that protein metabolism, as measured by urea excretion, is reduced during exercise in the heat (Dolny & Lemon, 1988). Increases in muscle adenine nucleotide degradation during contraction have been observed in humans (Edwards, Harris, Hultman, Kaijser, Koh & Nordesjö, 1972) and dogs (Kozlowski *et al.* 1985) when muscle temperature is elevated. The decrease in total adenine nucleotide concentration is likely to be due to activation of AMP deaminase, with production of IMP and ammonia. The increased muscle temperature following exercise at 40 °C in the present study, although relatively small ($\Delta = 1·2$ °C), may contribute, in part, to an increased ammonia production and the higher ammonia accumulation we have observed. Heat stress is also associated with increased adrenaline levels during exercise (Dolny & Lemon, 1988; Neilsen, Savard, Richter, Hargreaves & Saltin, 1990). Since adrenaline has been shown to increase IMP and ammonia levels in perfused rat muscle (Goodman & Lowenstein, 1977), an increase in circulating adrenaline, although not measured in the present study, could result in increased muscle and plasma ammonia. Finally, although it remains to be clearly established (Nielsen *et al.* 1990), a reduction in muscle blood flow during exercise in the heat may have influenced muscle ammonia metabolism. Reduced oxygen delivery could increase the reliance on anaerobic energy sources (Fink *et al.* 1975; Young *et al.* 1985), including the adenine nucleotides (Sahlin & Katz, 1989), resulting in increased ammonia production. Furthermore, a reduction in muscle blood flow may reduce the removal of ammonia from muscle, thereby resulting in increased intramuscular ammonia accumulation. While the most likely explanation for the increased plasma ammonia levels is increased ammonia production by, and release from, contracting skeletal muscle, decreased ammonia clearance is another possibility. Vasoconstriction in the splanchnic region and inactive muscle will occur during exercise

in the heat, although splanchnic ammonia uptake is not altered during exercise (Eriksson, Broberg, Bjorkman & Wahren, 1985).

In summary, we have observed higher plasma and muscle ammonia levels following exercise at 40 °C, compared with 20 °C, in untrained men. Such metabolic alterations could reflect increased muscle levels of free ADP and AMP, which may be associated with reduced performance during exercise in the heat.

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