Stimulation of muscle ammonia production during exercise following branched-chain amino acid supplementation in humans

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1. This study examined the effects of a large (308 mg kg^{-1}) oral dose of branched-chain amino acids (BCAAs) on muscle amino acid and ammonia (NH_3) metabolism during 90 min of dynamic knee extensor exercise (64 ± 2% of maximum workload).

2. BCAA supplementation resulted in a 4-fold increase in the arterial BCAA level (from 373 to 1537 μM, $P < 0.05$) and a 1.5-fold increase in the intramuscular BCAA level (from 3.4 ± 0.2 to 5.2 ± 0.5 mmol (kg dry weight)$^{-1}$, $P < 0.05$) by the onset of exercise. Over the 90 min exercise period, the exercising muscle removed a total of 7104 ± 2572 μmol kg^{-1} of BCAAs. In contrast, in the control trial, there was a total release of 588 ± 86 μmol kg^{-1} ($P < 0.05$) of BCAAs.

3. The total release of NH_3 over the 90 min exercise period was 2889 ± 317 μmol kg^{-1} ($P < 0.05$) in the control trial and 4223 ± 552 μmol kg^{-1} ($P < 0.05$) in the BCAA trial. Similarly, the total release of alanine and glutamine was 1557 ± 153 and 2213 ± 270 μmol kg^{-1}, respectively, for the control trial and 2771 ± 178 and 3476 ± 217 μmol kg^{-1}, respectively, for the BCAA trial.

4. The lactate release and arterial lactate values were all consistently lower in the BCAA trial than in the control trial. The net production of lactate (intramuscular shifts + total release) was lower ($P < 0.05$) in the BCAA trial (49.9 ± 11.4 mmol kg^{-1}) than in the control trial (64.0 ± 11.7 mmol kg^{-1}).

5. It is concluded that: (1) the administration of BCAAs can greatly increase their concentration in plasma and subsequently their uptake by muscle during exercise, and (2) long-term exercise following BCAA administration results in significantly greater muscle NH_3, alanine and glutamine production, as well as lower lactate production, than is observed during exercise without BCAA supplementation. These data strongly suggest that BCAAs are an important source of NH_3 during submaximal exercise and that their contribution to NH_3, alanine and glutamine production can be significantly altered by changes in BCAA availability.

In recent years a number of studies (MacLean, Spriet, Hultman & Graham, 1991; Wagenmakers *et al.* 1991; MacLean, Graham & Saltin, 1994) have challenged the suggestion that the ammonia (NH_3) produced during a submaximal exercise bout originates from the breakdown of AMP, as one of the steps of the purine nucleotide cycle (PNC) (Broberg & Sahlin, 1989). In this step, AMP is deaminated to inosine monophosphate (IMP) and NH_3 by AMP deaminase. A major reason for this challenge is the fact that the primary function of this reaction is to help maintain the energy state of the cell (Lowenstein, 1990). This is accomplished by removing AMP and allowing the adenylate kinase reaction ($2ADP \rightleftharpoons ATP + IMP$) to move in the direction of ATP production. There is little doubt that this pathway is of considerable importance during high intensity exercise when the ATP/ADP ratio becomes compromised. However, during a prolonged submaximal exercise bout when the adenine nucleotide pool is well maintained (MacLean *et al.* 1991, 1994), it is most likely that NH_3 production from this source is limited.

In contrast, it has been suggested that the deamination of branched-chain amino acids (BCAAs) in muscle may be a source of NH_3 production (MacLean & Graham, 1993*a*;

MacLean *et al.* 1994). It is well established that muscle amino acid oxidation increases during submaximal exercise (Hagg, Morse & Adibi, 1982; Wolfe, Goodenough, Wolfe, Royle & Nadel, 1982; Dohm, 1986). Similarly, it has been shown that muscle readily removes BCAAs from the circulation and when their concentration is increased their removal is increased (Gelfand, Glickman, Jacob, Sherwin & DeFronzo, 1986). Therefore, it is reasonable to speculate that by increasing the circulating levels of BCAAs during exercise it may be possible to increase muscle BCAA utilization and influence NH_3 production. A recent study by MacLean *et al.* (1994) tested this hypothesis and found that exercise following BCAA administration resulted in a significantly greater net muscle NH_3 production than that obtained during exercise without BCAA supplementation. However, in that study a relatively small (77 mg kg^{-1}) oral dose of BCAAs was given and the largest differences in NH_3 production occurred at the end of exercise. Therefore, by increasing exercise duration and the dose of BCAAs, it may be possible to promote even greater NH_3 production and establish a clear link between amino acid metabolism and NH_3 production. The purpose of the present study was to examine the effects of a large (308 mg kg^{-1}) dose of BCAA on muscle amino acid and NH_3 metabolism during exercise.

METHODS

Subjects

The experimental protocol was approved by the Ethics Committee at the Karolinska Institute and written consent was obtained from five healthy male subjects who were informed of the purposes and risks of the study. The subjects were aged 23–30 years (mean ± S.E.M., 26·6 ± 1·3 years), weighed 65–85 kg (71·6 ± 3·8 kg) and measured between 173 and 189 cm in height (180·6 ± 2·8 cm). The control leg quadriceps muscle mass was 2·80 ± 0·08 kg and consisted of 50 ± 4% type I muscle fibres, 44 ± 1% type IIA muscle fibres and 6 ± 3% type IIB muscle fibres. The quadriceps muscle mass for BCAA trials was 2·85 ± 0·05 kg and consisted of 50 ± 6% type I muscle fibres, 42 ± 6% type IIA muscle fibres and 8 ± 7% type IIB muscle fibres.

Experimental protocol

Prior to the experiment the subjects were familiarized with the Krogh ergometer modified for one-legged knee-extensor exercise as previously described (Andersen & Saltin, 1985). The subjects performed an incremental leg exercise test with their dominant leg to determine the peak power of the knee extensors and this ranged from 55 to 80 W (65·0 ± 4·5 W). The subjects then reported to the laboratory after an overnight fast. Teflon catheters were inserted below the inguinal ligament into the femoral artery and vein of the leg to be exercised and advanced proximally so that the tips of the arterial and venous catheters were located approximately 2 cm proximal and 2 cm distal to the inguinal ligament, respectively. The subjects were moved to the exercise apparatus and rested while baseline (0 min) arterial and venous blood samples were drawn simultaneously and a muscle biopsy obtained from the vastus lateralis with suction. Blood flow was determined by the constant thermal dilution technique (Andersen & Saltin, 1985).

The subjects exercised by kicking at 63·9 ± 1·7% of their one-legged peak power output for 90 min. Arterial and venous blood samples were taken 5, 30, 60, 75 and 90 min after the start of exercise and blood flow determinations were made immediately after each blood sample. Muscle biopsies were obtained after 5 and 90 min of exercise and expired air was collected after 30, 60 and 90 min of exercise. Heart rate was monitored throughout the experiment and recorded. The selection of the dominant or non-dominant leg was randomized.

Following the 90 min exercise period, the subjects rested supine for 90 min, during which time a catheter was placed into the contralateral femoral vein. At the end of the rest period simultaneous arterial and venous blood samples were taken prior to BCAA administration (time point −45 min). The subjects then consumed a 308 mg kg^{-1} supplement of BCAAs administered in three doses. The first and second doses (154 and 77 mg kg^{-1}, respectively) were administered 45 and 20 min prior to the onset of exercise, while the final dose (77 mg kg^{-1}) was given 5 min into the exercise bout. The 500 mg capsules of the commercially available BCAA supplement (Quest) were reported to contain only the three BCAAs: 220 mg L-leucine, 150 mg L-valine and 130 mg L-isoleucine. This was confirmed by dilution of the capsules in water and analysis by high performance liquid chromatography (HPLC; as described later). Following the 45 min supplementation period the subjects exercised the second leg for 90 min at the same work intensity as the first leg. Blood, muscle and cardiorespiratory samples were obtained at the same time points as in the first trial.

Analyses

The fractions of expired O_2 and CO_2 were determined with Applied Electrochemical S-3A O_2 analyser and the Beckman LB-2 infrared CO_2 analyser, respectively. Expired volumes were determined with a Parkinson-Cowan volumeter. The analysers were calibrated with known gas concentrations and the volumeter was calibrated with a Tissot spirometer.

Blood samples for plasma were drawn with syringes treated with heparin, while blood for serum were drawn with untreated syringes. One hundred microlitres of heparinized blood was quickly added to 500 μl of 0·3 M $HClO_4$. These samples and the remaining arterial and venous blood samples were all immediately centrifuged and the supernatant was collected and stored at −80 °C. The whole-blood extracts were analysed enzymatically (Bergmeyer, 1974) in triplicate for glucose and lactate using a fluorometer. Plasma was analysed enzymatically in triplicate for NH_3 using a fluorometer (Kun & Kearney, 1974). Amino acids were analysed in duplicate by prior derivatization with phenyl-isothiocyanate (Heinrikson & Meredith, 1984) and HPLC. Serum was analysed enzymatically in duplicate for glycerol (Bergmeyer, 1974) using a fluorometer and in duplicate for free fatty acids (FFA; Wako FFA kit No. 990-75401) using a Beckman Du-70 spectrophotometer. Arterial haematocrit was determined by high-speed centrifugation to document changes in plasma volume.

The muscle biopsies taken at rest had a small piece removed for fibre type determination (Saltin, Henriksson, Nygaard, Jansson & Andersen, 1977). They were then immediately frozen in liquid N_2, removed from the needle and stored at −80 °C. A 5–8 mg portion of the frozen biopsy was weighed at −20 °C, extracted in 3 M $HClO_4$ for 20 min and neutralized with 2 M $KHCO_3$. The neutralized extract was immediately assayed for NH_3 by the method of Kun & Kearney (1974) using a fluorometer. All samples for the experiment were analysed at the same time and analysis was complete within 1 h of neutralization. The remaining extract was used for lactate determination (Bergmeyer, 1974).

The remainder of the frozen muscle sample was freeze dried and

dissected free of visible blood, connective tissue and other non-muscle elements. A 2–3 mg portion of this sample was homogenized for 1 min in 100 μl of deionized water (Milli Q) and then centrifuged for 3 min. The supernatant was used for the determination of free amino acids using the method of Heinrikson & Meredith (1984) and HPLC. A 1·5–2 mg portion of freeze-dried muscle was extracted in 1 ml of 2 M HCl and incubated for 2 h at 85–90 °C. The muscle–acid misture was weighed before and after incubation to document any possible fluid loss due to evaporation. Following incubation the extract was neutralized with 1 ml of 2 M NaOH and centrifuged for 15 min at 15000 r.p.m. Duplicate samples of supernatant were taken for the fluorimetric determination of glycogen using an enzymatic glucose assay (Bergmeyer, 1974). The remainder of the muscle was extracted with 0·5 M $HClO_4$ (1·0 mM EDTA), neutralized with 2·2 M $KHCO_3$ and analysed for ATP, ADP, AMP and inosine monophosphate (IMP) by HPLC (Sellevold, Jynge & Aarstad, 1986). A portion of the extract was used for the enzymatic determination of phosphocreatine (PCr) and creatine (Cr) as described by Harris, Hultman & Nordesjo (1974). Muscle metabolite contents were corrected to total Cr and expressed per kilogram of dry muscle. Both muscle NH_3 and lactate are expressed per kilogram wet muscle.

Calculations

Thigh volume was calculated by using the thigh length, three circumferences and three skinfold measurements (Jones & Pearson, 1969) and muscle mass was estimated from a regression equation (Andersen & Saltin, 1985). The total adenine nucleotide (TAN) pool was calculated by summing ATP, ADP and AMP. The uptake and/or release of O_2, glucose, lactate, NH_3, FFA and amino acids was calculated by multiplying the blood or plasma flow by the arteriovenous difference in concentration and was expressed per kilogram of muscle. The total exchange of lactate, NH_3 and amino acids was estimated for each subject by averaging the exchange between consecutive sample points and then multiplying this value by the duration between the two sample points (e.g. average the flux between 5 and 15 min and then multiply by 10). These values were summed to obtain an estimate of the total exchange (always termed total release or uptake). In some cases it was necessary to calculate the net production of a measured parameter. To obtain this estimate, the intramuscular concentrations were converted to wet weights using the wet/dry weight ratios. The overall shift in the intramuscular concentration (muscle accumulation) was then calculated and this was added to the total release/uptake to obtain the net production (always termed net production, release or uptake). The BCAAs were calculated by summing isoleucine, leucine and valine, the essential amino acids (EAAs) by summing the BCAAs, threonine, methionine, phenylalanine, tryptophan and lysine, and total amino acids (TAAs) by summing all amino acids except hydroxyproline and 3-methylhistidine.

Statistics

Since the BCAA trial always followed the control trial, any potential effects of the previous exercise bout and the effects of BCAA supplementation were analysed by a one-way repeated-measures analysis of variance (ANOVA) between time point −45 min (BCAA leg) and time point 0 (Control and BCAA legs). Each subject exercised at the same relative workload and therefore treatment effects were analysed with a Student's paired t test at each time point during exercise. To assess the effects of exercise, the data from time points 0 to 90 min were analysed by ANOVA. If significance was indicated a Tukey's (honestly significant difference) *post hoc* point-to-point comparison test was used to determine where the significance occurred. Significance was accepted at $P < 0·05$ and all values are given as means ± S.E.M.

RESULTS

Cardiorespiratory and blood flow data

Pulmonary and muscle oxygen consumption were not different between trials and did not vary during the exercise period. The respective means for these parameters were 15·6 ± 0·7 and 204·8 ± 11·8 ml min^{-1} kg^{-1} for the control leg and 16·2 ± 1·0 and 195·6 ± 14·2 ml min^{-1} kg^{-1} for the BCAA leg. Similarly, there were no differences between trials in blood or plasma flow, nor did these parameters vary after the first 10 min of exercise. The respective means for these variables were 1·68 ± 0·10 and 0·91 ± 0·08 ml min^{-1} kg^{-1} in the control trial and 1·68 ± 0·14 and 0·95 ± 0·08 ml min^{-1} kg^{-1} in the BCAA trial.

Blood, plasma and serum metabolites

There were no differences between trials in the arterial glucose concentrations and they did not change throughout the experiment. After the onset of exercise an uptake ($P < 0·05$) of glucose was demonstrated by 5 min in both trials and this uptake remained elevated ($P < 0·05$) throughout the experiment. The arterial lactate concentrations were elevated ($P < 0·05$) by 5 min in both trials and remained higher ($P < 0·05$) throughout the experiment. Similarly, lactate release demonstrated the same pattern in both trials but was higher ($P < 0·05$) than at rest at 5, 30, 60 and 75 min. Both the arterial lactate levels and the lactate release values were all consistently higher in the control than in the BCAA trial, but the difference was not found to be significant at any individual time point. There was also no significant difference in intramuscular lactate levels (Table 1) between trials. However, the net lactate production (total release + intramuscular shifts) for the 90 min exercise period was higher ($P < 0·05$) in the control trial (64·0 ± 11·7 mmol kg^{-1}) than in the BCAA trial (49·9 ± 11·4 mmol kg^{-1}).

The arterial FFA concentrations in both trials were elevated ($P < 0·05$) above the 0 min level at 60, 75 and 90 min, while the arterial glycerol concentrations in both trials were higher ($P < 0·05$) than at 0 min throughout the experiment. The arterial FFA and glycerol levels were higher ($P < 0·05$) in the BCAA trial than the control trial at 30 min and at 0, 5 and 30 min, respectively. Although the arterial FFA and glycerol concentrations had not completely returned to resting values by the time administration of the BCAA supplement took place, this was found to be significant only for glycerol. Despite consistently higher arterial FFA concentrations in the BCAA trial, there were no differences between trials in FFA uptake. The uptake of FFAs was increased ($P < 0·05$) after 5 min and remained elevated ($P < 0·05$) throughout the remainder of the experiment in both trials.

Table 1. Muscle metabolites

Metabolite	Trial	0 min	5 min	90 min
Glycogen (mmol kg^{-1})	C	365 ± 35	324 ± 43	101 ± 50†
	B	370 ± 31	299 ± 35	97 ± 46†
Lactate (mmol kg^{-1})	C	1·8 ± 0·3	5·7 ± 1·6†	1·9 ± 0·5
	B	2·0 ± 0·3	8·6 ± 2·6†	1·8 ± 0·4
NH_3 (μmol kg^{-1})	C	299 ± 30	537 ± 78†	459 ± 33†
	B	429 ± 95	652 ± 26†	567 ± 30*†
PCr (mmol kg^{-1})	C	80·7 ± 1·7	46·6 ± 9·6†	61·1 ± 5·8†
	B	83·2 ± 0·8	37·2 ± 6·0†	60·0 ± 6·5†
Cr (mmol kg^{-1})	C	37·1 ± 2·4	71·2 ± 11·0†	56·7 ± 4·3†
	B	34·6 ± 3·3	79·8 ± 8·7†	57·8 ± 5·9†
ATP (mmol kg^{-1})	C	25·1 ± 0·4	25·0 ± 0·5	25·0 ± 0·5
	B	24·9 ± 0·5	24·0 ± 1·1	25·1 ± 1·6
ADP (mmol kg^{-1})	C	4·0 ± 0·2	4·6 ± 0·2	4·6 ± 0·3
	B	4·3 ± 0·3	4·9 ± 0·3	4·6 ± 0·4
AMP (mmol kg^{-1})	C	0·16 ± 0·01	0·18 ± 0·02	0·18 ± 0·02
	B	0·18 ± 0·02	0·21 ± 0·03	0·18 ± 0·02
IMP (mmol kg^{-1})	C	0·43 ± 0·08	0·39 ± 0·09	0·71 ± 0·12†
	B	0·40 ± 0·04	0·52 ± 0·06	0·63 ± 0·11†
TAN (mmol kg^{-1})	C	29·3 ± 0·5	29·8 ± 0·6	29·8 ± 0·7
	B	29·4 ± 0·4	29·1 ± 1·0	29·9 ± 1·4

Values are means ± S.E.M.; $n = 5$; data from dry muscle samples, except for lactate and NH_3 values which were from wet muscle samples. C, control leg; B, BCAA leg; PCr, phosphocreatine; Cr, creatine; IMP, inosine monophosphate; TAN, total adenine nucleotide pool (ATP + ADP + AMP). * Significant difference from control leg; † significant difference from 0 min, $P < 0·05$.

There were no differences between trials in arterial plasma NH_3 concentrations (Fig. 1) from −45 to 0 min. At the onset of exercise, both the arterial and NH_3 flux (Fig. 1) levels were elevated and remained elevated ($P < 0·05$) throughout the experiment in both trials. The arterial NH_3 concentrations were higher ($P < 0·05$) in the BCAA trial than in the control trial after 5, 30 and 75 min of exercise. The release of NH_3 was higher ($P < 0·05$) in the BCAA trial than in the control trial at each time point except at 75 min. There were no significant differences between trials in the intramuscular NH_3 levels (Table 1) at 0 and 5 min, despite consistently higher values being observed in the BCAA trial. However, following 90 min of exercise the intramuscular NH_3 concentration was significantly higher ($P < 0·05$) in the BCAA trial than in the control trial.

Muscle metabolites

There were no differences between trials in glycogen, PCr, Cr, ATP, ADP, AMP, IMP or TAN concentrations (Table 1). Muscle glycogen was decreased ($P < 0·05$) following 90 min of exercise in both trials. Exercise resulted in a decrease ($P < 0·05$) in PCr and a reciprocal increase in Cr by 5 min in both trials. Although these variables remained different ($P < 0·05$) from resting values, both PCr and Cr had recovered modestly by the end of the exercise bout. There were no measurable shifts in ATP, ADP, AMP or TAN during exercise in both trials, but there was an increase ($P < 0·05$) in IMP after 90 min of exercise in both trials. The increase in IMP was very modest and corresponded to only a 0·2–0·3 mmol (kg dry weight)$^{-1}$ increase at 90 min.

It should be noted that due to the invasiveness of the study it was necessary to perform both trials on each subject in the same day. Therefore, it was necessary to perform the control trial first. In an effort to minimize the effects of the previous exercise bout, 90 min of rest and 45 min of BCAA supplementation were allowed between the end of the control exercise bout and the onset of the BCAA exercise bout. It should be remembered that the second trial was carried out on the contralateral unexercised leg. Several studies have demonstrated no effect of a previous exercise bout on such parameters as heart rate, oxygen consumption, blood flow or fatigue when exercising the contralateral leg (Savard, Kiens & Saltin, 1987; Bangsbo, Graham, Kiens & Saltin, 1992). In the present study there were no differences between trials in glucose or FFA uptake, oxygen consumption, or glycogen utilization. Therefore, we feel that the effects of the previous exercise bout were minimal and had no significant impact on the major focus and findings of the present study.

Arterial amino acids

BCAA supplementation resulted in an increase ($P < 0·05$) in the arterial plasma BCAA concentrations (Table 2). There were no shifts in any of the other individual amino acids as a result of BCAA supplementation. At the onset of

exercise the arterial BCAA levels had increased more than 4-fold and by 75 min they had plateaued at more than six times ($P < 0{\cdot}05$) the pre-supplementation value. In contrast, the arterial BCAA concentration in the control trial remained unchanged during the exercise bout. Since the BCAA levels were artificially elevated by supplementation, they were subtracted from the essential amino acid (EAA − BCAA) and total amino acid (TAA − BCAA) concentrations. Lower ($P < 0{\cdot}05$) EAA − BCAA levels were observed at 0, 30, 75 and 90 min in the BCAA trial than in the control trial, while no differences between trials were observed in the TAA − BCAA levels (Table 2). The BCAA trial was also characterized by consistently higher arterial glutamine levels than were observed in the control trial, the differences being significant at 5, 30 and 60 min (Table 2). The arterial alanine and glutamine levels were higher ($P < 0{\cdot}05$) than at 0 min at 5, 30, 60, 75 and 90 min and 75 and 90 min, respectively. There were no other relevant differences between trials for any of the other amino acids.

Intramuscular amino acids

The administration of BCAA resulted in higher ($P < 0{\cdot}05$) intramuscular BCAA levels by the onset of exercise, and these levels remained higher ($P < 0{\cdot}05$) throughout the experiment than in the control trial (Fig. 2). The intramuscular BCAA levels remained constant in the control trial, but they continued to increase as exercise progressed in the BCAA trial. By the end of the 90 min exercise period, the intramuscular BCAA levels had approximately doubled in the BCAA trial. At rest there were no differences in EAA − BCAA levels between trials ($4{\cdot}96 \pm 0{\cdot}28$ *vs.* $4{\cdot}60 \pm 0{\cdot}11$ mmol (kg dry weight)$^{-1}$). However, after exercise the EAA − BCAA levels were elevated ($P < 0{\cdot}05$) in both trials, and this increase was greater ($P < 0{\cdot}05$) in the control trial ($6{\cdot}59 \pm 0{\cdot}63$ mmol (kg dry weight)$^{-1}$) than in the BCAA trial ($5{\cdot}61 \pm 0{\cdot}49$ mmol (kg dry weight)$^{-1}$). Despite these shifts in the intramuscular EAA − BCAA concentrations, there were no differences or shifts in TAA − BCAA pools in both trials.

Exercise resulted in an elevation ($P < 0{\cdot}05$) in the intramuscular aspartate, serine, asparagine, glycine, histidine, threonine, methionine, phenylalanine and lysine concentrations by 90 min, compared with their respective concentrations at 0 min, in both trials. In contrast, intramuscular glutamate was depressed ($P < 0{\cdot}05$) by 5 min and remained depressed ($P < 0{\cdot}05$) in both trials for the

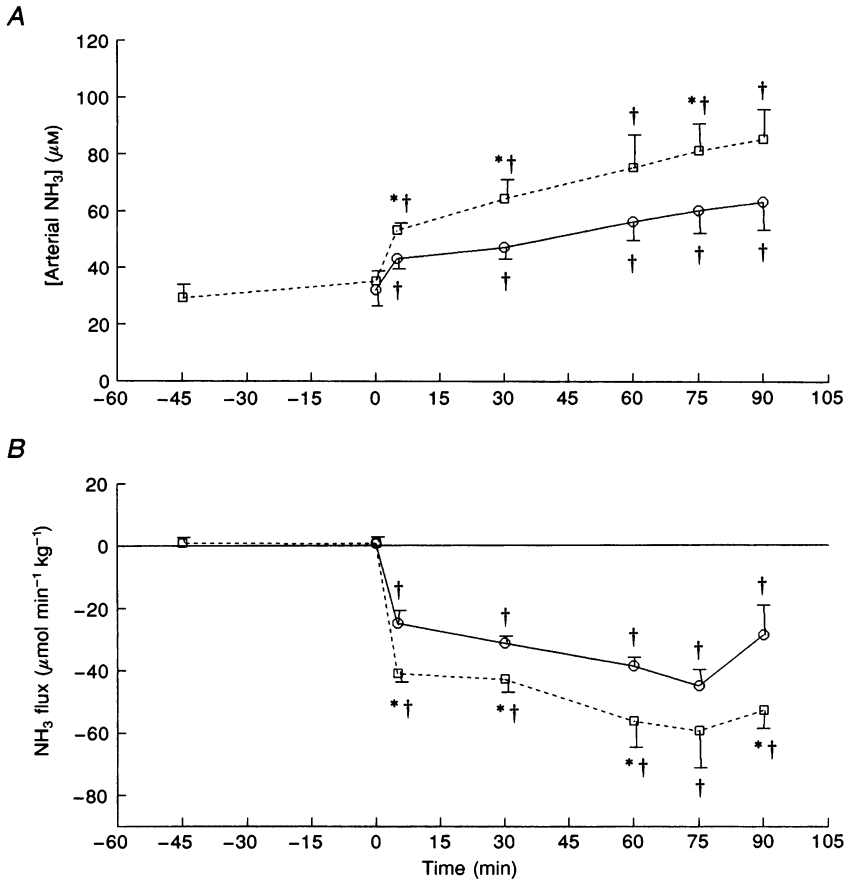

Figure 1. Summary of plasma NH_3 metabolism

○, control trial; □, BCAA trial. *A*, arterial NH_3. *B*, muscle NH_3 flux; a negative value indicates a release. * Significant difference from control leg; † significant difference from 0 min, $P < 0{\cdot}05$.

Table 2. Some arterial plasma and flux amino acid data

Amino acid	Trial	−45 min	0 min	5 min	30 min	60 min	75 min	90 min
				Arterial (μM)				
Glutamate	C		48 ± 5	42 ± 5	44 ± 4	41 ± 4	44 ± 4	45 ± 4
	B	58 ± 4	56 ± 7	40 ± 2	45 ± 3	42 ± 2	40 ± 4	41 ± 1
Glutamine	C		503 ± 23	467 ± 14	504 ± 15	493 ± 19	535 ± 20†	541 ± 40†
	B	517 ± 12	511 ± 14	547 ± 22*	578 ± 29*	589 ± 26*	601 ± 34†	607 ± 42†
Alanine	C		278 ± 20	314 ± 14†	406 ± 21†	342 ± 18†	341 ± 25†	325 ± 25†
	B	246 ± 17	227 ± 13	331 ± 16†	380 ± 13†	377 ± 21†	364 ± 24†	346 ± 29†
BCAA	C		417 ± 18	396 ± 17	383 ± 19	356 ± 8	352 ± 12	366 ± 9
	B	373 ± 15	1537 ± 162*‡	1662 ± 119*	1731 ± 109*	2163 ± 229*	2285 ± 243*	2062 ± 226*
EAA − BCAA	C		362 ± 31	337 ± 34	351 ± 36	317 ± 26	341 ± 37	353 ± 36
	B	321 ± 31	313 ± 28*	333 ± 28	301 ± 21*	299 ± 26	289 ± 20*	293 ± 19*
TAA − BCAA	C		2104 ± 117	2080 ± 103	2236 ± 145	2100 ± 98	2144 ± 126	2216 ± 190
	B	2046 ± 105	1952 ± 75	2144 ± 68	2198 ± 56	2252 ± 69	2217 ± 98	2174 ± 131
				Flux (μmol min^{-1} kg^{-1})				
Glutamate	C		2·7 ± 0·7	7·4 ± 1·2†	9·0 ± 0·8†	5·9 ± 1·1†	4·3 ± 1·7†	5·4 ± 2·4†
	B	3·4 ± 0·7	3·3 ± 0·7	7·6 ± 1·6†	7·5 ± 2·6†	8·0 ± 2·1†	7·0 ± 1·2†	6·6 ± 2·0†
EAA − BCAA	C		−2 ± 1	−5 ± 4	−10 ± 3†	−21 ± 8†	−23 ± 12†	−24 ± 4†
	B	−4 ± 1	−6 ± 2	6 ± 15	−15 ± 7	−7 ± 11	−11 ± 7	−16 ± 7
TAA − BCAA	C		−19 ± 3	−41 ± 24	−72 ± 12†	−129 ± 23†	−140 ± 37†	−119 ± 14†
	B	−38 ± 14	−29 ± 13	−64 ± 25	−169 ± 37†	−83 ± 50	−84 ± 33	−111 ± 55

Values are means ± S.E.M., $n = 5$. C, control trial; B, BCAA trial; EAA − BCAA, EAA data minus BCAA data; TAA − BCAA, TAA data minus BCAA data. * Significant difference from control leg; † significant difference from 0 min; ‡ significant difference from −45 min, $P < 0·05$.

remainder of the experiment. Intramuscular alanine (Fig. 3) was elevated ($P < 0·05$) in both trials by 5 min but was not different from the concentration at 0 min by the end of exercise. There were no differences or shifts in intramuscular taurine, glutamine (Fig. 4), proline, tyrosine and ornithine concentrations in both trials.

Amino acid flux data

Following BCAA supplementation there was an uptake ($P < 0·05$) of BCAAs by muscle, which was not observed in the control trial (Fig. 2). After the start of exercise there was a 6-fold increase in the uptake of BCAAs by 5 min in the BCAA trial. The BCAA trial was further characterized by a consistently greater BCAA uptake throughout the experiment and this was greater ($P < 0·05$) than the control trial at 5, 30 and 60 min. The BCAA flux was greater ($P < 0·05$) than at 0 min at 5 and 60 min in the BCAA trial and at 60 and 75 min in the control trial. Despite a consistently lower release of EAA − BCAA in the BCAA trial, there were no significant differences between trials in EAA − BCAA flux (Table 2). However, the control trial did demonstrate greater ($P < 0·05$) EAA − BCAA flux than at 0 min at 30, 60, 75 and 90 min. There were no differences between trials in the TAA − BCAA flux, but the TAA − BCAA flux was greater ($P < 0·05$) than at 0 min at 30 min in the BCAA trial and at 30, 60, 75 and 90 min in the control trial (Table 2).

Exercise resulted in an increase ($P < 0·05$) in flux from the 0 min level for all amino acids except histidine, threonine, arginine, proline, methionine, tryptophan, ornithine, lysine and 3-methylhistidine. Glutamate was the only amino acid that was consistently taken up by muscle throughout the experiment in both trials (Table 2). With the onset of exercise there was an increase in the efflux of alanine (Fig. 3) and glutamine (Fig. 4) in both trials. The efflux of alanine and glutamine at 30 min was greater ($P < 0·05$) in the BCAA trial than in the control trial. The flux of alanine and glutamine was greater ($P < 0·05$) than at 0 min throughout the experiment in both trials. There were no other relevant differences between trials for any of the other amino acids.

Total amino acid release/uptake data

Summed over the 90 min exercise period, the BCAA trial demonstrated a total uptake ($P < 0·05$) of 7104 ± 2572 μmol kg^{-1} of BCAAs, whereas a total release of 588 ± 86 μmol kg^{-1} of BCAAs took place in the control trial (Fig. 2). The total release of EAA − BCAA was consistently lower in the BCAA trial than in the control trial and, summed over the 90 min exercise period, was 1318 ± 350 μmol kg^{-1} in the control trial and 736 ± 405 μmol kg^{-1} in the BCAA trial. However, although the difference between trials appears to be large it was not found to be significant. The total release of TAA − BCAA over the 90 min exercise period was 8240 ± 1104 μmol kg^{-1}

in the control trial and 9417 ± 1426 μmol kg^{-1} in the BCAA trial, and was not significantly different between trials. The release of alanine between 0 and 5, between 5 and 30 and between 30 and 60 min, as well as the total release of alanine, were all higher ($P < 0{\cdot}05$) in the BCAA trial than in the control trial (Fig. 3). Similarly, the release of glutamine between 5 and 30 min and the total release of glutamine were greater ($P < 0{\cdot}05$) in the BCAA trial than in the control trial (Fig. 4). There were no other relevant differences between trials in the total release or uptake of any other amino acid.

NH_3, alanine and glutamine balance

The NH_3 balance calculations are summarized in Fig. 5. There was no difference in the increase in intramuscular NH_3 over the 90 min exercise period (muscle accumulation) between trials. The total release of NH_3 over 90 min (muscle flux) was higher ($P < 0{\cdot}05$) in the BCAA trial than

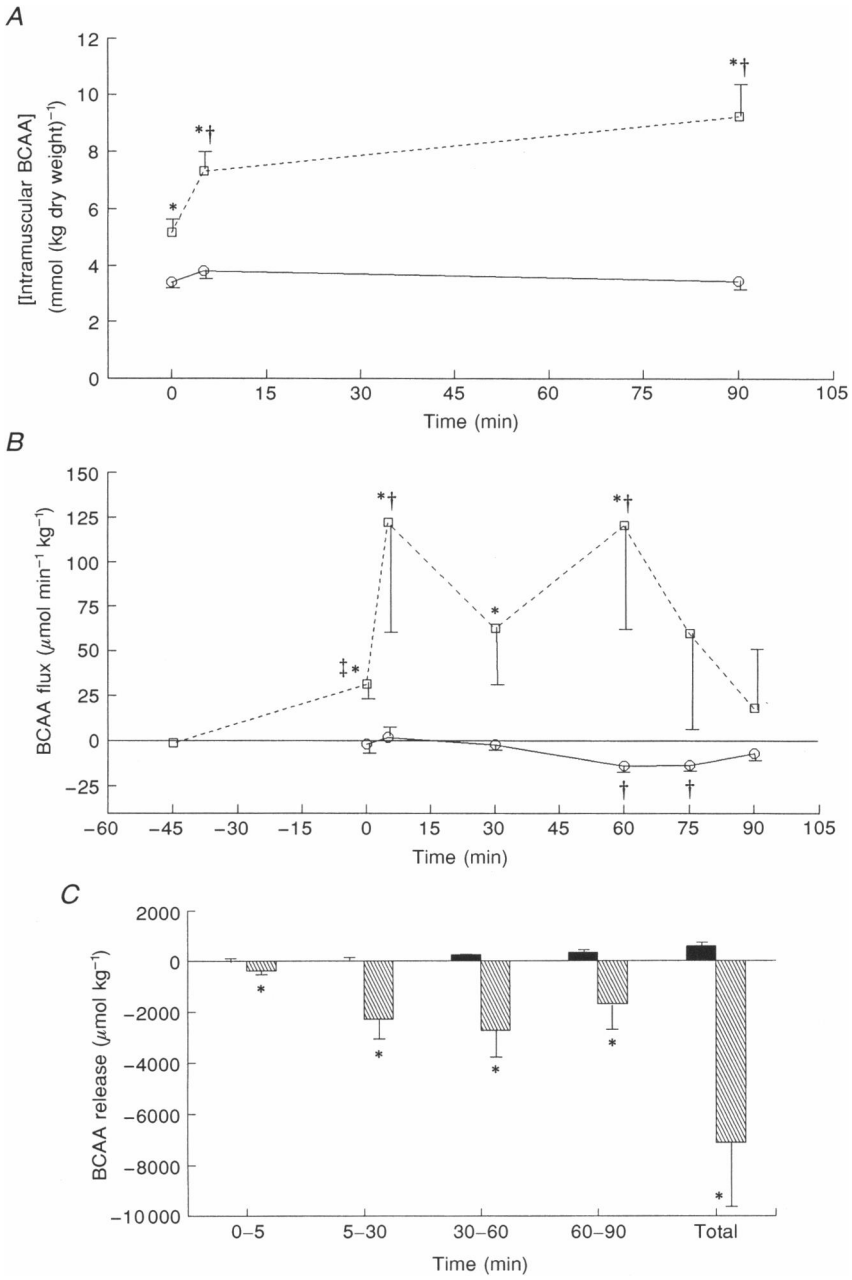

Figure 2. Summary of BCAA metabolism

A, intramuscular BCAA levels in the control (○) and BCAA (□) trials. *B*, BCAA flux during exercise in the control (○) and BCAA (□) trials; a positive value indicates an uptake. *C*, BCAA release over various time segments during exercise and then summed for the entire experiment (Total). Negative value indicates an uptake. ■, control trial; ▨, BCAA trial. * Significant difference from control leg; † significant difference from −45 min; ‡ significant difference from 0 min, $P < 0{\cdot}05$.

in the control trial. When muscle accumulation and muscle flux were summed, the total muscle production was also higher ($P < 0{\cdot}05$) in the BCAA trial than in the control trial. The deamination of one molecule of AMP to IMP produces one molecule of NH_3; thus the intramuscular increase in IMP shares a 1:1 stoichiometric relationship with NH_3. The increase in intramuscular IMP (NH_3 production from AMP) was very small and was not different between trials. When the NH_3 production from AMP was subtracted from the total muscle NH_3 production, the net muscle NH_3 production was 4401 ± 589 μmol kg^{-1} in the BCAA trial and 2993 ± 316 μmol kg^{-1} ($P < 0{\cdot}05$) in the control trial.

The NH_3 produced during an exercise bout leaves the muscle in predominantly three forms: NH_3, alanine and glutamine (Fig. 5). When the total glutamine release was added to the net NH_3 release, the total release of

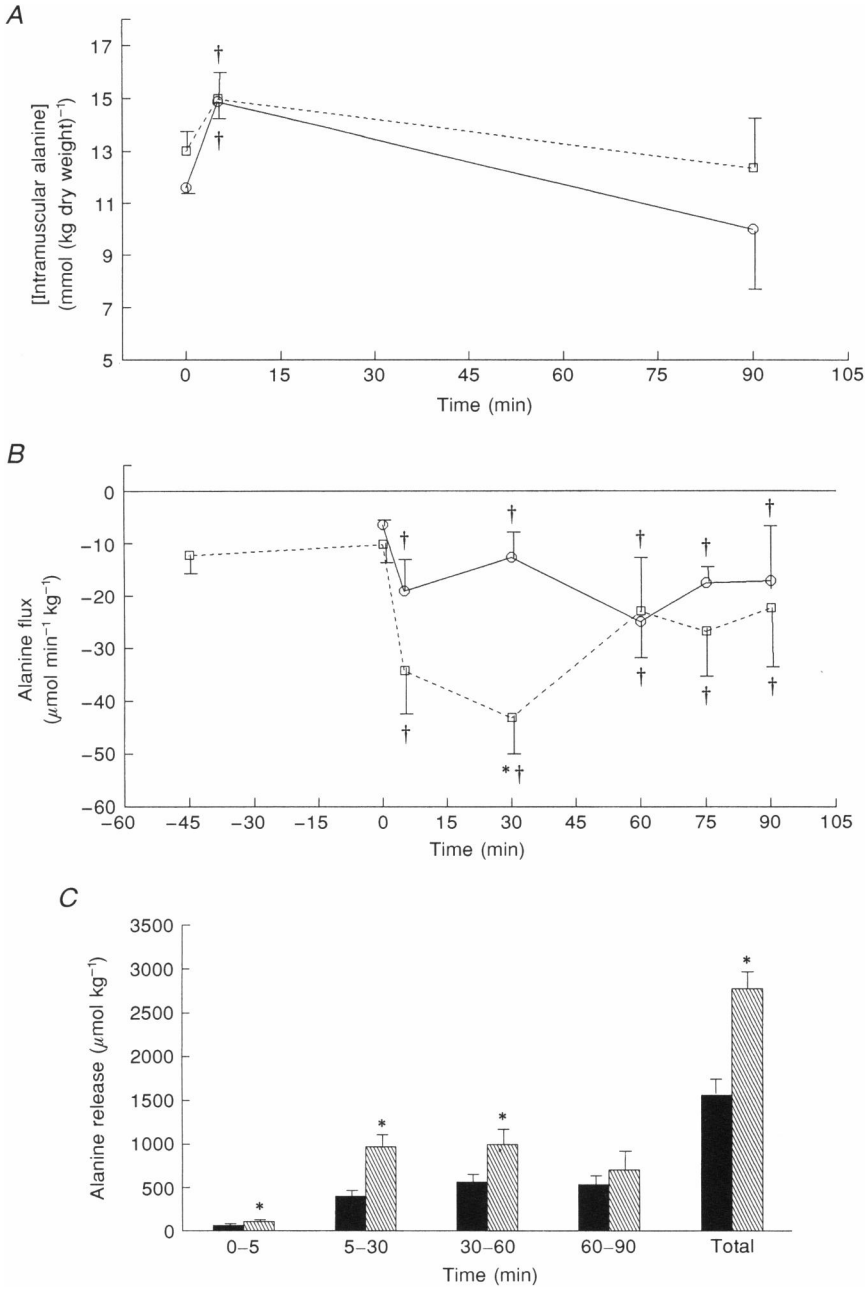

Figure 3. Summary of alanine metabolism

A, intramuscular alanine levels in the control (○) and BCAA (□) trials. *B*, alanine flux during exercise in the control (○) and BCAA (□) trials; a negative value indicates a release. *C*, alanine release over various time segments during exercise and then summed over the entire experiment (Total). ■, control trial; ▨, BCAA trial. * Significant difference from control leg; † significant difference from 0 min, $P < 0{\cdot}05$.

NH_3 + glutamine was 7877 ± 659 μmol kg^{-1} in the BCAA trial and 5206 ± 491 μmol kg^{-1} ($P < 0.05$) in the control trial (Fig. 5). When all three of these parameters were summed, the total release of NH_3 + glutamine + alanine was 10648 ± 702 μmol kg^{-1} in the BCAA trial and 6762 ± 637 μmol kg^{-1} ($P < 0.05$) in the control trial. It is evident that administration of a BCAA dose results in a significantly larger NH_3 load than is indicated by the free NH_3 concentration alone.

DISCUSSION

In the present study a large dose of BCAAs was administered to subjects, which resulted in high (non-physiological) circulating levels of these amino acids. During subsequent exercise, a major finding was that muscle released larger amounts of NH_3 (1·5-fold), alanine (1·8-fold) and glutamine (1·6-fold) than were produced during control exercise. The two potential sources of NH_3 in skeletal muscle during exercise are: (1) the deamination

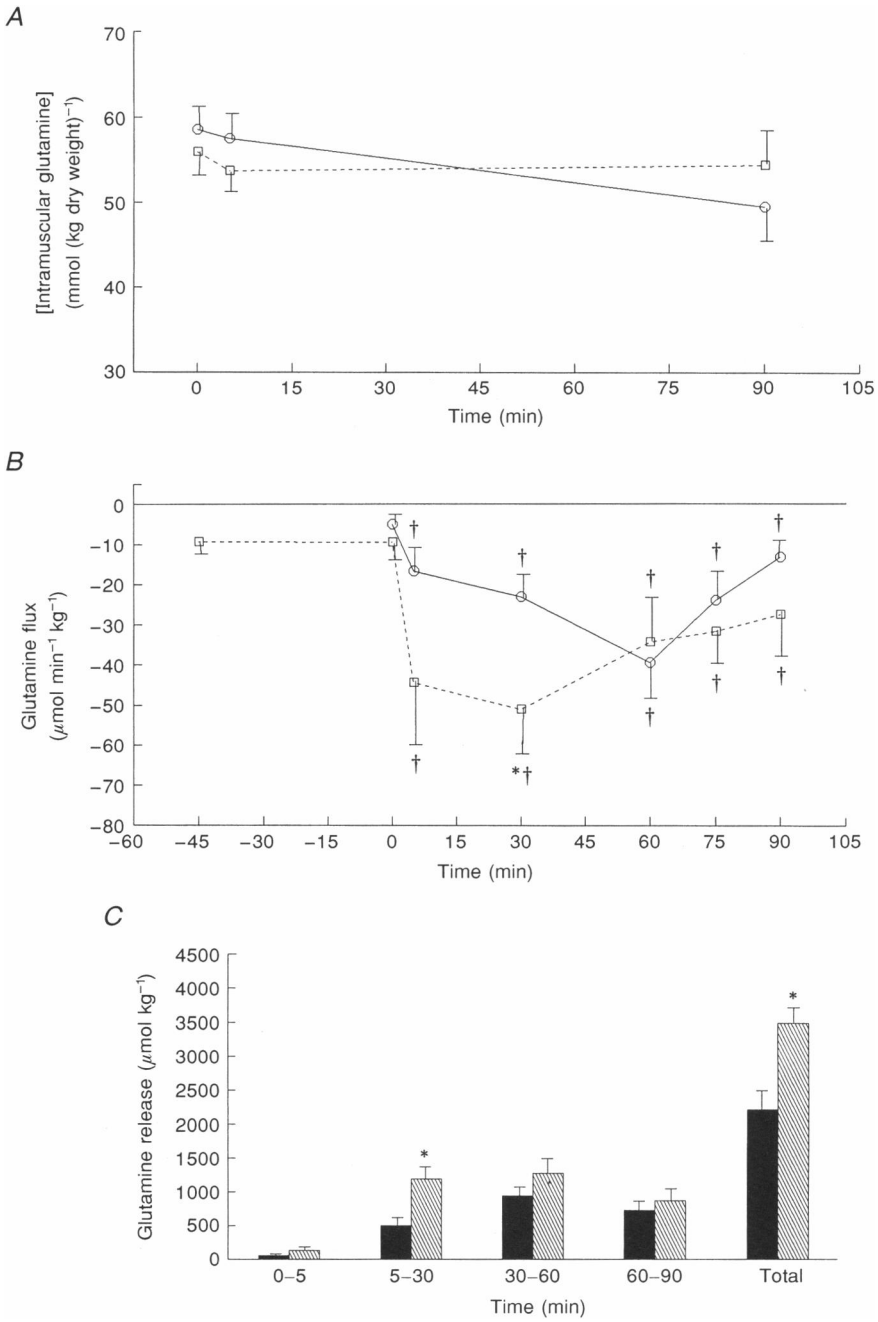

Figure 4. Summary of glutamine metabolism for the experiment

A, intramuscular glutamine levels in the control (○) and BCAA (□) trials. *B*, glutamine flux during exercise in the control (○) and BCAA (□) trials; a negative value indicates a release. *C*, glutamine release over various time segments during exercise and then summed for the entire experiment (Total). ■, control trial; ▨, BCAA trial. * Significant difference from control leg; † significant difference from 0 min, $P < 0.05$.

of AMP to IMP and NH_3 as one of the steps of the PNC; and (2) the deamination of BCAAs. In recent years a number of studies have proposed that a substantial portion of the NH_3 produced during a submaximal exercise bout comes from the deamination of amino acids (MacLean *et al.* 1991, 1994; Wagenmakers *et al.* 1991). Much of the reasoning for this proposal is based on an examination of the reactions of the PNC. The first step in the PNC involves the formation of IMP and NH_3 from AMP. The primary function of this reaction is to maintain the energy state of the cell (Lowenstein, 1990). During high intensity exercise, when the ATP/ADP ratio becomes compromised, the deamination of AMP to IMP and NH_3 allows the near-equilibrium adenylate kinase reaction to move in the direction of ATP production ($2ADP \rightleftharpoons ATP + AMP$). The function of the last two reactions involves the salvaging of adenine nucleotides by reaminating IMP back to AMP. Based on this, it has been argued that the reactions of the PNC may not play a major role during a prolonged submaximal exercise bout. Briefly, the ATP/ADP ratio during an exercise bout of the intensity and duration used in the present study is easily maintained, as is reflected by the lack of significant shifts in the intramuscular concentrations of ATP, ADP, AMP or TAN. Similarly, it is very unlikely that the PNC would be acting in both directions simultaneously, such that AMP deamination is followed by immediate IMP reamination resulting in NH_3 production with no measurable changes in AMP. The main support for this contention is the finding in rodents that the PNC does not act as a cycle in metabolically active fibres (Meyer & Terjung, 1980). Furthermore, for each IMP reaminated, one aspartate is needed as an NH_3 donor. In the present study the amount of aspartate required to produce all the NH_3 (not including alanine and glutamine) in the control and BCAA trials is severalfold higher than the amount available from intramuscular sources. Lastly, in the present study the rate of NH_3 production steadily increased in both trials for the first 75 min of the experiment and this production was significantly higher in the BCAA trial. Therefore, for PNC cycling to have produced all the NH_3 during the exercise trials, the rate of cycling must have steadily increased and must have increased at a greater rate in the BCAA trial. It is difficult to suggest what could be activating the cycle to a greater extent as exercise progresses and what could be triggering a greater degree of cycling following BCAA administration. Based on the above discussion it is apparent that the activity of the PNC should be minimal during an exercise bout of the intensity and duration used in the present study.

The first step in the degradation of BCAAs in skeletal muscle involves the removal of the NH_3 group by transamination with 2-oxoglutarate (2-OG) to form glutamate

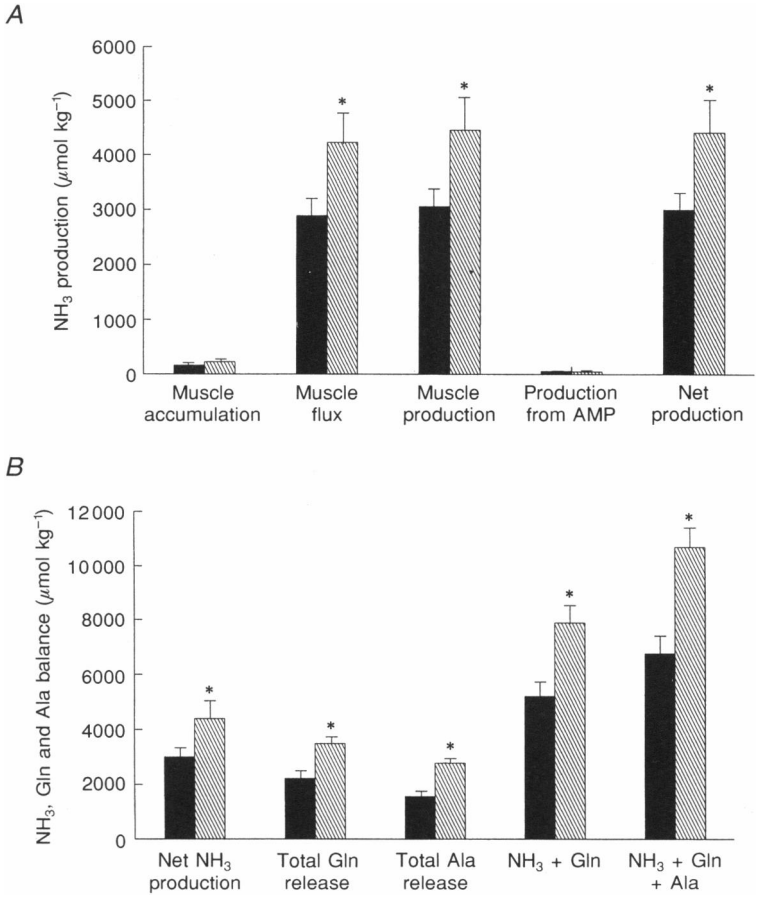

Figure 5. Summary of the estimates for net muscle NH_3 production (*A*) and overall NH_3, glutamine and alanine balance (*B*)

See Results section for explanation of the calculation for net muscle NH_3 production. Overall NH_3, NH_3 + glutamine and NH_3 + glutamine + alanine balance values were estimated by summing the net NH_3 production (*A*) with the total release of these other amino acids. ■, control trial; ▨, BCAA trial. * Significant difference from control leg, $P < 0{\cdot}05$.

and branched-chain oxo acids (BCOAs), catalysed by BCAA aminotransferase. Glutamate can then be oxidatively deaminated by glutamate dehydrogenase (GDH), releasing the NH_3 and reforming 2-OG. Both of these reactions are near equilibrium and, when coupled, form a trans-deamination reaction which has been suggested to be the primary pathway for BCAA deamination in skeletal muscle (Newsholme & Leech, 1983). Since these reactions are near equilibrium any large increase in the concentration of substrates for these reactions should result in a subsequent increase in the concentration of products. In the present study the BCAA trial removed over 7000 μmol kg^{-1} of BCAAs from the circulation during the 90 min exercise period, which resulted in a more than doubling of the intramuscular BCAA levels. Therefore, it is reasonable to suggest that during the BCAA trial more BCAAs were deaminated and thus more NH_3 and BCOAs were produced. The release of NH_3 during exercise was significantly higher in the BCAA trial than in the control for every time point except for 75 min. Summed over the entire experiment approximately 1300 μmol kg^{-1} more NH_3 was released in the BCAA trial than in the control trial. Furthermore, despite the large NH_3 production in both trials there were only very small changes in the intramuscular NH_3 levels, indicating that the NH_3 was rapidly diffusing from the muscle and not being trapped. These data clearly show that BCAAs can be a considerable source of NH_3 in exercising skeletal muscle.

The production of alanine involves the addition of an NH_3 group to pyruvate. This is accomplished by a transamination reaction involving glutamate and pyruvate forming alanine and 2-OG, catalysed by alanine aminotransferase. It is well established that alanine release increases during exercise (Felig & Wahren, 1971; Ahlborg, Felig, Hagenfeldt, Hendler & Wahren, 1974). However, the NH_3 (in the form of glutamate) for alanine production can come from many sources. For example, the glutamate can arise from intramuscular protein breakdown, the intramuscular free glutamate pool or be produced by the transamination of NH_3 from BCAA to 2-OG. Therefore, under normal exercise conditions it is difficult to determine how much of the NH_3 leaving the muscle in the form of alanine is produced from the latter source. In the present study, however, a significantly greater amount of alanine was released during exercise in the BCAA trial than in the control trial. There were no significant differences between trials in glutamate uptake or intramuscular glutamate. Similarly, there were no significant differences between trials in glycogen breakdown, suggesting that the same quantity of pyruvate was available for alanine formation. There was also no evidence to suggest that the amount of glutamate produced from net protein degradation was significantly different between trials. For the above reasons and since the BCAA trial was characterized by a greater abundance of BCAAs, it is reasonable to suggest that the difference between the amount of alanine produced in the BCAA trial and that produced in the control trial represents NH_3 from BCAAs.

On the other hand, glutamine is formed in muscle by the addition of a free NH_3 group to glutamate catalysed by glutamine synthetase. The source of the glutamate is the same as above, but there are only two sources of the NH_3. It can either come from the PNC, but this has been argued to be most unlikely during this type of exercise, or from the deamination of glutamate by GDH. It is evident that glutamine represents the release of two NH_3 groups. Since one of the NH_3 groups must be derived from the intramuscular NH_3 pool, the release of glutamine represents additional NH_3 production, a fact very often overlooked. In the present study the total release of glutamine was higher ($P < 0{\cdot}05$) in the BCAA than in the control trial. Given the elevated BCAA concentrations in the BCAA trial and the central role glutamate (and BCAA transamination) plays in NH_3 production, it is reasonable to suggest that the NH_3 released in the form of glutamine in the BCAA trial in excess of that produced in the control trial, originated from BCAAs.

This study clearly shows that when circulating BCAA levels are elevated (non-physiologically) by supplementation prior to exercise, significantly greater muscle NH_3 production occurs during exercise than is obtained without supplementation. As discussed above, the NH_3 released in the form of alanine and glutamine can represent a substantial amount of NH_3, which is not usually included when total NH_3 production is discussed. In our experiments, when these amino acids were included, 3886 $\pm$ 651 μmol kg^{-1} more NH_3 was released in the form of alanine + glutamine + NH_3 in the BCAA trial than in the control trial. Thus, BCAA supplementation put a substantially higher NH_3 load on the muscle than was indicated by the free NH_3 data alone.

As outlined above, the production of glutamine requires free NH_3 and its release represents the efflux of at least one NH_3 group. Furthermore, the production of NH_3 (NH_3 + glutamine) can originate from only two sources, the PNC or the metabolism of BCAA. On this basis it is possible to make an estimate of the contribution to NH_3 production from the two sources in the BCAA trial, and this estimate is summarized in Table 3. To make this calculation the net metabolism of BCAA (excluding any other fates such as incorporation into protein) must be determined. This is not possible in the control trial, but an estimate in the BCAA trial can be made. Firstly, the net BCAA production was determined by summing the total BCAA release and the intramuscular BCAA accumulation in the control and BCAA trials (calculation 1 in Table 3). This represents the net quantity of BCAAs produced (in the control trial) and consumed (in the BCAA trial) over the exercise period. The difference between the net BCAA production in the control trial and the net BCAA consumption in the BCAA trial represents the amount of

Table 3. Estimation of the contribution to NH_3 + glutamine production from BCAA metabolism and the purine nucleotide cycle in the BCAA trials

Calculation	Parameter	Trial C	Trial B
1a	Net BCAA production (μmol kg^{-1})	596	—
1b	Net BCAA consumption (μmol kg^{-1})	—	6405
2	Net BCAA metabolism (μmol kg^{-1})	—	7001
3	Alanine difference (μmol kg^{-1})	—	1214
4	Net BCAA − alanine (2 − 3) (μmol kg^{-1})	—	5787
5	NH_3 from AMP deamination (μmol kg^{-1})	—	47
6	NH_3 + glutamine (μmol kg^{-1})	—	7924
7	Percentage of NH_3 + glutamine from BCAA metabolism (4/6)	—	73·0
8	Percentage of NH_3 + glutamine from AMP deamination (5/6)	—	0·6
9	Percentage of NH_3 + glutamine from BCAA degradation and PNC cycling	—	26·4

C, control trial; B, BCAA trial; PNC, purine nucleotide cycle. Calculations 1a and 1b, total BCAA release + muscle accumulation, with a net production for the C trial and a net consumption for the B trial. Calculation 2, the difference between trials in BCAA production. Calculation 3, the difference in the amount of alanine produced in the B trial from that produced in the C trial. Calculation 4, net BCAA metabolism minus the alanine difference. Calculation 5, the NH_3 represented by IMP accumulation. Calculation 6, the sum of the total productions of NH_3 and glutamine. Calculations 7 and 8, percentage of NH_3 + glutamine (calculation 6) derived from calculations 4 and 5, respectively. Calculation 9, the remainder of the NH_3 produced from unmeasurable sources, i.e. BCAA metabolized as a result of protein degradation and PNC cycling.

BCAAs that were metabolized during the BCAA trial instead of being released. Therefore, to estimate net BCAA metabolism (calculation 2), the net BCAA production value in the control trial must be added to the net consumption value in the BCAA trial. This calculation only roughly estimates those BCAAs that would normally have been released and does not determine the quantity of BCAAs that were metabolized (i.e. deaminated) during the experiment in the control trial.

To estimate NH_3 obtained from BCAAs, the amount of alanine produced in excess of that produced in the control trial (calculation 3) was subtracted from the net BCAA metabolism value. Although this most probably represents NH_3 from BCAAs, it is not free NH_3. Therefore, the PNC could not have contributed to its formation and it must be subtracted from the calculation. To determine the NH_3 produced from AMP deamination the accumulation of IMP was used since it shares a 1:1 stoichiometric relationship with NH_3 (calculation 5). Lastly, the total production of NH_3 as NH_3 + glutamine was determined (calculation 6). The percentage contribution to NH_3 + glutamine production from BCAA metabolism (calculation 4/calculation 6 × 100) and from AMP deamination (calculation 5/calculation 6 × 100) was determined. The remainder represents the NH_3 produced from PNC cycling and/or BCAAs made available from endogenous protein breakdown which would normally be metabolized during exercise (which we cannot estimate in this study). It should be noted that these calculations are used in an effort to compare qualitatively the contribution to NH_3 production from the two potential sources. It is realized that there may be a substantial difference in the amount of BCAAs potentially available between the control and BCAA trials. However, from these calculations it is evident that the contribution to NH_3 production from AMP deaminase is very small. In contrast, 73·0% of the free NH_3 produced (in the form of NH_3 + glutamine) could be accounted for by the disappearance of BCAAs. Thus, these data show that the contribution to NH_3 production from BCAAs can be significant during exercise.

The last step in the catabolism of BCAAs is rate limiting and involves the non-reversible decarboxylation of the BCOAs by branched-chain oxo acid dehydrogenase (BCOADH). The BCOADH complex is almost totally inactive in skeletal muscle at rest but is significantly activated during exercise (Kasperek, Dohm & Snider, 1985; Wagenmakers, Brookes, Coakley, Reilly & Edwards, 1989). This enzyme is also a classic example of a multi-complex enzyme and is activated and deactivated by dephosphorylation and phosphorylation, respectively. The BCOADH kinase responsible for the ATP-mediated inactivation of the complex has been shown to be inhibited by BCOAs as well as by ADP (Lau, Fatania & Randle, 1982; Paxton & Harris, 1984; Aftering, Block & Buse, 1986). Similarly, it has been shown that elevated intramuscular BCAA levels result in significant activation of the BCOADH complex both before and during exercise (MacLean, Saltin, Wagenmakers & Graham, 1993*b*). With such a low initial activity state and the potential for activation from both BCOAs and exercise, the potential increase in BCAA oxidation could be great. In the present study, the intramuscular BCAA concentrations were significantly elevated prior to the onset of exercise and

continued to increase throughout the experiment. Since the two reactions involved in the formation of the BCOAs are near equilibrium, any increase in the intramuscular BCAA concentrations would result in an increase in the intramuscular BCOA concentrations. The overall effect would be an increased activation of the BCOADH complex. Therefore, it is reasonable to suggest that in the present study, BCAA supplementation resulted in an increased activation of the rate-limiting enzyme in BCAA oxidation.

An interesting finding in this study was the lower quantity of lactate produced in the BCAA trial than in the control trial. From the glucose uptake and glycogen measurements it is possible to determine the total amount of pyruvate that was available in both trials. The total amounts of pyruvate produced in the control and BCAA trials were 233 ± 18 and 226 ± 26 mmol kg^{-1}, respectively, and there was no significant difference between the values. On the other hand, the total amount of alanine that was produced was significantly higher ($P < 0.05$) in the BCAA trial than in the control trial. However, approximately 14 mmol kg^{-1} less lactate, but only 1·2 mmol kg^{-1} more alanine, was produced in the BCAA trial than in the control trial. Thus, approximately 12·8 mmol kg^{-1} of pyruvate had to be utilized by the tricarboxylic acid (TCA) cycle. Although this may appear to be a relatively large amount, it should be noted that compared with the total amount of pyruvate available, this represents only approximately 5·7% more pyruvate entering the TCA cycle. It is difficult to explain this finding, but, since more alanine was formed, it may be that this change in the pyruvate/lactate ratio may have resulted in the shunting of more pyruvate into the TCA cycle.

In summary, this study clearly shows that muscle BCAA metabolism is influenced by the magnitude of the dose of BCAAs administered prior to exercise. The larger dose of BCAAs used in this study resulted in higher circulating levels (non-physiological) of BCAAs and a larger uptake and metabolism of these amino acids during exercise than were observed in a previous study (MacLean *et al.* 1994). The increased BCAA metabolism by muscle was further characterized by significantly greater NH_3, alanine and glutamine production, as well as a significantly lower lactate production. Lastly, it was estimated that approximately 73% of the NH_3 (NH_3 + glutamine) produced during exercise following BCAA supplementation, could be accounted for by the disappearance of the ingested BCAAs.

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