Interactions among the branched-chain amino acids and their effects on methionine utilization in growing pigs: effects on plasma amino- and keto-acid concentrations and branched-chain keto-acid dehydrogenase activity

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The present experiment was designed to elucidate the mechanism of the methionine-sparing effect of excess branched-chain amino acids (BCAA) reported in the previous paper (Langer & Fuller, 2000). Twelve growing gilts (30–35 kg) were prepared with arterial catheters. After recovery, they received for 7 d a semipurified diet with a balanced amino acid pattern. On the 7th day blood samples were taken before (16 h postabsorptive) and after the morning meal (4 h postprandial). The animals were then divided into three groups and received for a further 7 d a methionine-limiting diet (80 % of requirement) (1) without any amino acid excess; (2) with excess leucine (50 % over requirement); or (3) with excesses of all three BCAA (leucine, isoleucine, valine, each 50 % over the requirement). On the 7th day blood samples were taken as in the first period, after which the animals were killed and liver and muscle samples taken. Plasma amino acid and branched-chain keto acid (BCKA) concentrations in the blood and branched-chain keto-acid dehydrogenase (BCKDH; *EC* 1.2.4.4) activity in liver and muscle homogenates were determined. Compared with those on the balanced diet, pigs fed on methionine-limiting diets had significantly lower ($P < 0{\cdot}05$) plasma methionine concentrations in the postprandial but not in the post-absorptive state. There was no effect of either leucine or a mixture of all three BCAA fed in excess on plasma methionine concentrations. Excess dietary leucine reduced ($P < 0{\cdot}05$) the plasma concentrations of isoleucine and valine in both the postprandial and postabsorptive states. Plasma concentrations of the BCKA reflected the changes in the corresponding amino acids. Basal BCKDH activity in the liver and total BCKDH activity in the *biceps femoris* muscle were significantly ($P < 0{\cdot}05$) increased by excesses of leucine or all BCAA.

Branched-chain amino acids: Methionine: Amino acid utilization

Interactions between leucine, isoleucine and valine in growing pigs have been reported by numerous authors (Oestemer *et al*. 1973; Henry *et al*. 1976; Taylor *et al*. 1984) who showed that even moderate dietary excesses of leucine (i.e. >3 g/kg) reduced isoleucine and valine concentrations in plasma. Much larger quantities of leucine were required to reduce growth peformance (Taylor *et al.* 1984). Changes in plasma and tissue branched-chain amino acid (BCAA) concentrations of rats (Rogers *et al.* 1962; Tannous *et al.* 1966), poultry (D'Mello & Lewis, 1970; Smith & Austic, 1978), kittens (Hargrove *et al.* 1988), lambs (Papet *et al.* 1988) and human subjects (Synderman *et al.* 1959; Nair *et al.* 1992) receiving leucine in excess were similar to the changes reported in pigs (Oestemer *et al.* 1973; Henry *et al.* 1976; Taylor *et al.* 1984) indicating a common regulatory mechanism in these species. All three BCAA share a common enzyme complex, the branched-chain keto-acid dehydrogenase (*EC* 1.2.4.4; BCKDH) complex, in their degradative pathways. Activation of BCKDH by dietary leucine excess increased the oxidation of isoleucine and valine in rats and chicks (Calvert *et al.* 1982; Block & Harper, 1984) and this has been proposed as the reason for the altered BCAA concentrations (Harper *et al.* 1984; Block, 1989).

Methionine is catabolized not only via the trans-sulfuration pathway but also by a transamination pathway (Case & Benevenga, 1976; Benevenga, 1984), by which methionine is transaminated to α-keto-γ-methiolbutyric

Abbreviations: BCAA, branched-chain amino acids; BCKA, branched-chain keto acids; BCKDH, branched-chain keto-acid dehydrogenase; KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; KMTB, α-keto-γ-methiolbutyric acid; KMV, α-keto-β-methyl-n-valeric acid.

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acid (KMTB) followed by an irreversible decarboxylation to 3-methylthiopropionic acid and CO_2 (Mitchell & Benevenga, 1978). The decarboxylation reaction is also catalysed by BCKDH which raises the possibility that the interactions amongst the BCAA may extend to the metabolism of methionine.

Another connection between the BCAA and methionine is their transport via the L-system (Oxender & Christensen, 1963; Baumrucker, 1985; Guidotti & Gazzola, 1992) which may involve direct competition for uptake into tissues, especially the brain (Eriksson *et al.* 1981; Wahren *et al.* 1981). Changes in brain amino acid concentrations appear to regulate food intake (Peng *et al.* 1973; Harper & Peters, 1983; Tackman *et al.* 1990).

Furthermore, leucine has been proposed as a specific regulator of protein metabolism and effects on protein synthesis and degradation *in vitro* have been observed in a number of experiments (Buse & Reid, 1975; Li & Jefferson, 1978; Goldberg & Tischler, 1981; Mitch & Clark, 1984). This could affect amino acid utilization and consequently animal performance as well as amino- and keto-acid concentrations in plasma and tissues.

However, the response of growing pigs to leucine or BCAA appears to depend on the limiting amino acid. Langer & Fuller (2000) reported a reduced protein utilization (measured as N retention) in growing pigs fed on isoleucine-limiting diets after increasing the leucine concentration from 7·2 g/kg to 10·8 g/kg. In contrast, protein utilization was improved rather than reduced by additions of leucine or a mixture of BCAA to a methionine-limiting diet. In order to explore the mechanism underlying these observations the following experiment was carried out.

Materials and methods

Animals

Twelve growing gilts (Cotswold crossbred) with an average initial weight of 30–35 kg were used in the experiment. About 2 weeks before the experiment all animals were prepared with two blood sampling catheters (PVC – NT2; 0·99 mm internal and 1·60 mm external diameter, inserted length 275 mm; Portex Ltd, Hyde, Kent, UK) which were inserted into the lumbar aorta, via the medial saphenous arteries of the hindlegs, as described by Fuller *et al.* (1977), except that Fluothane® (Pitman-Moore, Crewe, Cheshire, UK) anaesthesia was used. Animals were housed in open pens for about 1 week after surgery; after that they were kept in individual metabolism cages in a temperature-controlled room (22–24°) where they were allowed a second week of recovery, and where they remained for the duration of the experiment. All management and experimental procedures in this study were carried out in strict accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986 by staff licensed under this Act to carry out such procedures.

Diets and experimental design

The isoenergetic and isonitrogenous diets based on casein and synthetic amino acids were identical to the diets described in the previous paper (Langer & Fuller, 2000): there was one diet with an 'ideally' balanced amino acid pattern according to Wang & Fuller (1989) (control; no limiting amino acid, no excess) and three methionine-deficient diets (80 % of requirement). The methionine-deficient diets contained either no excess, excess leucine (50 % over requirement) or excesses of all three BCAA (each 50 % over requirement). Leucine, isoleucine, valine and methionine were added or removed isonitrogenously at the expense of aspartic acid and monosodium glutamate. Before the start of the experiment the animals were adapted to a semipurified low-protein (100 g crude protein/kg) diet for 7 d. During the experiment feed was given twice daily (at 07.00 and 15.00 hours) at a rate of 80 g/kg body weight$^{0·75}$. The animals consumed all the feed given, usually within 15 min. The planned daily N intake on all diets was 1·25 g/kg body weight$^{0·75}$. Water was available *ad libitum* from nipple drinkers and also added to the diets during feeding to reduce spillage.

The experiment consisted of two 7 d periods. In the first period the animals received a diet with the 'ideally' balanced amino acid pattern. On day 7 blood samples were taken before (16 h postabsorptive, before morning feeding) and after the morning feeding (4 h postprandial). In the second period the twelve pigs were randomly divided into three groups and received either a methionine-limiting diet without any amino acid excess, or the same diet with excess leucine or with excesses of all three BCAA. Blood samples were taken as in the first period. After the postprandial blood samples had been taken a lethal dose of Euthesate® (pentobarbitone sodium Ph Eur 200 mg/ml; Willows Francis Veterinary, Crawley, Sussex, UK) was given and samples of liver, biceps femoris and adductor muscle were taken immediately.

Sample collection, storage and chemical analysis

Diets. Diets were analysed for crude protein (N × 6·25) by the macro Kjeldahl procedure (Davidson *et al.* 1970) and for amino acid concentrations (except methionine, cyst(e)ine and tryptophan) by ion-exchange chromatography. Methionine and cyst(e)ine concentrations were determined after oxidation with performic acid as described by Bech-Andersen *et al.* (1990) and tryptophan was measured according to Naumann & Bassler (1993).

Plasma. After collection in prechilled heparinized tubes the blood samples (10 ml) were kept on ice for not more than 2 h before separating the plasma by centrifugation (2800 ***g***, 15 min, 4°). The plasma was divided into portions of 0·8 ml, to which 0·1 ml 200 μM-norleucine standard with 1·0 mM-dithiothreitol and 0·1 ml α-ketocaproic acid standard were added. Samples and standards were mixed using a vortex mixer and stored in Eppendorf tubes at −70° until required for analysis. For the determination of amino and keto acids plasma was deproteinized with 0·2 ml ice-cold 5-sulfosalicyclic acid (1·9 M), vortex mixed and centrifuged at 15 000 ***g*** for 15 min at 4°. An ion-exchange column (Dowex 50W (X8), H^+ form) was used to separate amino and keto acids. Keto acids were eluted with 2 ml distilled water added slowly to the column. The column was then washed twice with 2 ml distilled water before eluting the amino acids

twice with 2 ml 4 M-NH_4OH and 1 ml water. The eluate was freeze-dried twice to remove the NH_3 and redissolved in citrate buffer pH 2·2 before amino acid analysis by ion-exchange chromatography using a Pharmacia Alpha-Plus analyser with a Li-form high performance 202 × 4·6 mm column (Pharmacia, Cambridge, Cambs., UK). Keto acids were measured as their dimethylsilyated quinoxalinol derivatives by GC with *o*-phenylenediamine and *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluroacetamide as derivatives using a Hewlett-Packard model 5890A analyser with flame-ionization detector and a Chrompack SIL-5 column (25 m × 0·25 mm) (Packard Instruments, Downers Grove, IL, USA).

Tissue samples. Liver and muscle samples were removed as quickly as possible, frozen with Wollenberg tongs cooled in liquid N_2, immersed in liquid N_2 for 2–3 h and then stored at −70° until further analysis. BCKDH activity in sample homogenates was measured as $^{14}CO_2$ released from α-keto[carboxyl-^{14}C]isovaleric acid ([^{14}C]KIV) or L-[carboxyl-^{14}C]valine (L-[^{14}C]valine). L-[^{14}C]valine was purchased from American Radiolabeled Chemicals Ltd (Tocris-Cookson, Bristol, UK). [^{14}C]KIV was produced from the radiolabelled amino acid according to the method described by Rüdiger *et al.* (1972). The yield was approximately 84 % keto acid with a purity of about 97·8 % (checked by TLC). The activation state of the enzyme was measured as the ratio basal activity : total activity. The basal activity was determined in the presence of phosphatase and kinase inhibitors, 100 mM-KFl and 10 mM-dichloroacetic acid respectively. Total activity was estimated after preincubation of the enzyme with Mg^{2+}. The homogenization buffer (pH 7·4 at 4°) contained: 2 ml/l Triton X-100, 70 mM-sucrose, 200 mM-D-mannitol, 10 mM-HEPES and 0·4 mM-ethylene glycol bis(β-aminoethyl ether)*N*,*N*′-tetraacetic acid (EGTA). The radioactive cofactor solution (pH 7·4 at 37°) contained: 4 mM-NAD^+, 0·8 mM-CoA-lithium salt), 0.8 mM-thiamine pyrophosphate, 4 mM-dithiothreitol, 10 mM-HEPES, 4 mM-EGTA, 10 mM-sodium carbonate, KIV and [^{14}C]KIV. Frozen liver samples were quickly weighed and a 75 g/l homogenate was prepared using a Polytron homogenizer (15 s at setting 3; Kinematica AG, Lucerne, Switzerland). Muscle samples were crushed and weighed before preparing a 160 g/l homogenate with a Polytron homogenizer (30 s at setting 3). Homogenates were centrifuged for 5 min at 600 ***g*** for liver and 800 ***g*** for muscle. To remove larger particles the supernatant fraction was filtered through gauze. BCKDH assays were optimized for each tissue. Until incubation the homogenates were stored and processed on ice. Total enzyme activity was measured after preincubation at 37° with 5 mM-$MgSO_4$ for 20 min (liver) or 75 min (muscle). The incubations were carried out in sealed 25 ml Pyrex vials with a hanging centre well containing 0·3 ml phenylethylamine–methanol (1 : 1, v/v) to trap the released $^{14}CO_2$. The reaction was started by adding 0·6 ml homogenate to 0·6 ml cofactor solution. For liver BCKDH activity about 60 000 disintegrations/min (dpm) and 1 mM-KIV and for muscle BCKDH activity about 50 000 dpm and 2 mM-KIV were used per incubation. For basal and total activity liver was incubated for 4 min and muscle for 10 min. The reaction was stopped with 0·3 ml 1 M-$HClO_4$. Tissue homogenates treated with 1 M-$HClO_4$ before the incubation served as blanks for background. CO_2 was trapped for 2 h. Centre wells were placed in scintillation vials with 5 ml Ultima Gold® scintillation fluid (Hewlett-Packard) and the radioactivity was counted using a Hewlett-Packard Tri-carb 1900CA liquid scintillation analyser.

For the indirect determination of basal BCKDH activity in adductor muscle samples the method used for muscle samples was modified. The cofactor solution contained instead of [^{14}C]KIV, L-[^{14}C]valine (150 000 dpm/incubation), 0·2 mM-unlabelled KIV, 1 mM-L-valine and 100 μM-pyridoxal 5′ phosphate. L-[^{14}C]valine was more resistant to chemical decarboxylation than [^{14}C]KIV which led to a reduced background and improved the sensitivity. The method is based on the assumption that in muscle, transamination is not a limiting factor in BCAA catabolism. However, the method only gives information about the valine oxidative flux and the percentage of enzyme that is active and does not allow conclusions to be drawn about absolute activities. Nevertheless, knowing the total activity measured with [^{14}C]KIV and the percentage of active enzyme it is possible to predict the basal activity indirectly.

Protein in tissue samples was assayed by the biuret method using the Total Protein Reagent (Sigma Chemical Company Ltd, Poole, Dorset, UK).

Statistical analysis

Data were analysed using GENSTAT 5.2 (Rothamsted Experimental Station, Harpenden, Herts., UK). Plasma amino- and keto-acid concentrations were analysed by ANOVA using a split-plot analysis for a 3 × 2 factorial design. The three-level factor defining treatment group determined main plots and the two level factor (control, treatment) the sub-plot factor. Each treatment mean was compared against its own control and treatment differences were assessed by (t1 − c1) − (t2 − c2), i.e. by difference between changes. From the analysis of previous experiments of similar design (e.g. Wang & Fuller, 1989) the effect of time (i.e. period) was considered negligible. BCKDH activities in liver and muscle samples, as well as flux rates in adductor muscle, were analysed by ANOVA using a simple block design.

Results

Postprandial plasma amino acid concentrations

Reducing dietary methionine by 20 % caused a 55 % reduction in plasma methionine concentration (Table 1). Plasma concentrations of both threonine and valine increased significantly in animals given the methionine-deficient diet, compared with those given the balanced control diet.

The greatest changes in plasma amino acid concentrations were caused by excesses of leucine or BCAA in the methionine-deficient diets. A 50 % excess of leucine increased plasma leucine concentration by about 60 %; at the same time isoleucine and valine concentrations both dropped by more than 35 % when compared with the

Table 1. Postprandial and postabsorptive plasma amino acid concentrations in pigs fed on a control diet with a balanced amino acid pattern and on a methionine-deficient diet with no excess amino acids, excess leucine or an excess of the branched-chain amino acids (BCAA) for 7 d*

(Values are means for four pigs)

	Amino acid concentration (μmol/l)							
	No excess		Leucine excess		BCAA excess		SED	
	Control	Treatment	Control	Treatment	Control	Treatment	Control†	Treatment‡
Postprandial (4 h)								
Valine	278[a]	355[b]	289[a]	227[c]	286[a]	530[d]	31·0	43·8
Cyst(e)ine	84	91	88	79	85	77	9·6	13·5
Methionine	41[a]	18[b]	45[a]	13[b]	42[a]	23[b]	5·9	8·3
Isoleucine	94[a]	120[a]	82[ab]	73[b]	97[a]	222[c]	13·3	18·8
Leucine	213[a]	216[a]	188[a]	345[b]	207[a]	369[b]	27·3	38·6
Threonine	182[a]	260[b]	175[a]	241[b]	176[a]	257[b]	21·9	31·0
Postabsorptive (16 h)								
Valine	249[a]	307[b]	230[a]	161[c]	244[a]	368[d]	14·0	19·8
Cyst(e)ine	86	76	78	75	93	69	20·4	28·8
Methionine	19	19	16	19	20	22	4·4	6·2
Isoleucine	79[a]	104[b]	62[a]	67[a]	74[a]	128[b]	9·9	14·0
Leucine	154[a]	168[ab]	141[a]	180[b]	151[a]	185[b]	7·3	10·3
Threonine	99[a]	165[b]	85[a]	149[b]	88[a]	147[b]	17·7	25·1

[a,b,c,d] Mean values within a row not sharing a common superscript letter indicate significant ($P < 0·05$) differences between a treatment and its own control, or between two treatments.
* For details of diets and procedures, see pp. 50–51.
† For comparison of group mean against its own control.
‡ For comparison of treatment means against each other.

methionine-deficient diet without excess. Plasma concentrations of valine and isoleucine were significantly higher on the BCAA-supplemented diet. Neither methionine nor cyst(e)ine concentrations in the plasma of pigs given the methionine-deficient diets were altered by excesses of leucine or of all BCAA.

Postabsorptive plasma amino acid concentrations

In general plasma amino acid concentrations were lower in the postabsorptive than in the postprandial state (Table 1). Threonine, isoleucine and valine concentrations were significantly increased by methionine deficiency compared with the control diet. Although plasma leucine concentrations were similar in all three methionine-deficient treatment groups, isoleucine and valine concentrations were significantly reduced by leucine excess. Whereas plasma isoleucine was reduced by approximately 35 %, as it was in the postabsorptive state, valine decreased rather more, by nearly 50 %. Addition of BCAA significantly increased the plasma concentration of valine but the increase in isoleucine was not significant.

Table 2. Postprandial and postabsorptive plasma keto acid concentrations in pigs fed on a control diet with a balanced amino acid pattern and on a methionine-deficient diet with no excess amino acids, excess leucine, or an excess of the branched-chain amino acids (BCAA) for 7 d*

(Values are means for four pigs)

	Keto acid concentration (μmol/l)							
	No excess		Leucine excess		BCAA excess		SED	
	Control	Treatment	Control	Treatment	Control	Treatment	Control†	Treatment‡
Postprandial (4 h)								
KIV	21[a]	19[a]	16[a]	6[b]	23[a]	19[a]	3·2	4·5
KMV	55[a]	49[a]	40[ab]	28[b]	58[a]	56[a]	6·6	9·3
KIC	80[a]	77[ab]	67[a]	100[b]	80[a]	90[ab]	11·9	16·8
Postabsorptive (16 h)								
KIV	11[a]	18[bc]	14[a]	9[ab]	10[a]	20[c]	3·5	4·9
KMV	29[a]	46[ab]	32[a]	31[ab]	28[a]	54[b]	8·9	12·5
KIC	53[a]	56[ab]	49[a]	62[b]	57[a]	56[ab]	5·8	8·2

KIV, α-ketoisovaleric acid; KMV, α-keto-β-methyl-n-valeric acid; KIC, α-ketoisocaproic acid.
[a,b,c] Mean values within a row not sharing a common superscript letter indicate significant ($P < 0·05$) differences between a treatment and its own control, or between two treatments.
* For details of diets and procedures, see pp. 50–51.
† For comparison of group mean against its own control.
‡ For comparison of treatment means against each other.

Postabsorptive methionine and cyst(e)ine concentrations were similar for all treatments, regardless of the deficiency of methionine or the excess of leucine or the BCAA.

Branched-chain keto acid concentrations

Changes in plasma keto acid concentrations caused by diet and time after feeding are shown in Table 2. The concentrations of α-keto-β-methyl-n-valeric acid (KMV) and KIV, the keto acids of isoleucine and valine, were altered in the same direction as the corresponding amino acid concentrations. Postprandial plasma concentrations of KIV and KMV were significantly ($P < 0{\cdot}05$) reduced by dietary leucine excess whereas in the postabsorptive state the effect was not significant. Additions of isoleucine and valine together with leucine (BCAA excess treatment) reversed the effects of leucine on KIV and KMV. It was not possible to determine the concentration of KMTB, the keto acid of methionine as this keto acid appears to be unstable in the conditions used for analysis of the BCAA.

The relationship between branched-chain amino acid and keto acid concentrations

In the postprandial state (Fig. 1) there was not a strong relationship between the plasma concentrations of valine or isoleucine and their corresponding keto acid concentrations. However, plasma α-ketoisocaproic acid (KIC) increased with plasma leucine concentration and both were increased with dietary leucine excess. The relationship between the plasma concentrations of KIC (y) and leucine (x) can be described by the equation:

$$y = 50{\cdot}2 + 0{\cdot}125x;\ r^2\ 0{\cdot}760.$$

In the postabsorptive state, there was a linear relationship between isoleucine and KMV as well as between valine and KIV (Fig. 2). The relationship between KMV (y) and isoleucine (x) can be described by the equation:

$$y = 2{\cdot}76 + 0{\cdot}396x;\ r^2\ 0{\cdot}845,$$

and that between KIV and valine by the equation:

$$y = -1{\cdot}20 + 0{\cdot}057x;\ r^2\ 0{\cdot}758.$$

Changes in branched-chain keto-acid dehydrogenase activity in liver

Measurements with [^{14}C]α-ketoisovaleric acid. Enzyme activities are expressed per g tissue and per mg protein (Table 3). Pig liver contained approximately 170 mg extractable protein per g tissue. Whether expressed per g tissue or per mg protein, basal BCKDH activity in the liver was significantly increased ($P < 0{\cdot}05$) by leucine or BCAA excess. The percentage enzyme active was significantly increased ($P < 0{\cdot}05$) by leucine excess and even more by BCAA excesses.

Changes in branched-chain keto-acid dehydrogenase activity in muscle

Measurements with [^{14}C]α-ketoisovaleric acid. Using the radioactive keto acid as substrate the basal activities were below the detection limit of the assay. The total BCKDH activities of adductor and biceps femoris muscles, measured with [^{14}C]KIV, are shown in Table 4. Pig muscle contained approximately 60 mg extractable protein per g tissue. Both muscles had similar total activities but the activity in adductor muscle was not affected by the dietary treatments. Total BCKDH activity in biceps femoris muscle was significantly ($P < 0{\cdot}05$) increased by excess leucine and excess BCAA.

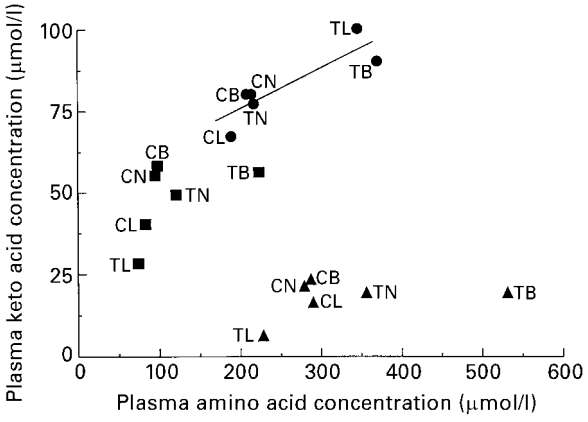

Fig. 1. Relationships between 4 h postprandial plasma concentrations of branched-chain amino acids and branched-chain keto acids in pigs fed on diets containing different proportions of amino acids. C, control treatment (balanced amino acid pattern); T, treatment; N, methionine-deficient diet with no excess amino acids; L, methionine-deficient diet with excess leucine; B, methionine-deficient diet with excess branched-chain amino acids. The relationships shown are: (■), isoleucine : α-keto-β-methyl-n-valeric acid; (●), leucine : α-ketoisocaproic acid ($y = 0{\cdot}125x + 50{\cdot}2$, r^2 0·760); (▲), valine : α-ketoisovaleric acid.

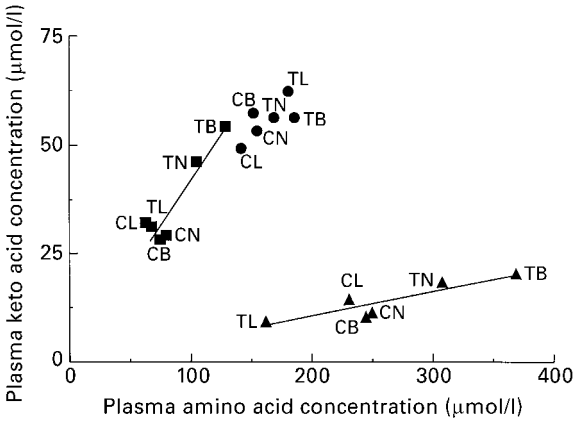

Fig. 2. Relationships between 16 h postabsorptive plasma concentrations of branched-chain amino acids and branched-chain keto acids in pigs fed on diets containing different proportions of amino acids. C, control treatment (balanced amino acid pattern); T, treatment; N, methionine-deficient diet with no excess amino acids; L, methionine-deficient diet with excess leucine; B, methionine-deficient diet with excess branched-chain amino acids. The relationships shown are: (■), isoleucine : α-keto-β-methyl-n-valeric acid ($y = 0{\cdot}396x + 2{\cdot}76$, r^2 0·876); (●), leucine : α-ketoisocaproic acid; (▲), valine : α-ketoisovaleric acid ($y = 0{\cdot}057x - 1{\cdot}20$, r^2 0·806).

Table 3. Hepatic branched-chain keto-acid dehydrogenase activities measured with α-keto[carboxyl-^{14}C]isovaleric acid in pigs fed on methionine-deficient diets with either no excess, excess leucine or excess branched-chain amino acids (BCAA)*

(Values are means for four pigs)

	Branched-chain keto-acid dehydrogenase activity			
	No excess	Leucine excess	BCAA excess	Pooled SED
Activity (nmol/min per g tissue)				
Basal	18[a]	38[b]	54[b]	8·1
Total	224	250	272	26·0
Active (%)†	7·8[a]	15·2[b]	19·7[b]	2·32
Activity (nmol/min per mg protein)				
Basal	0·11[a]	0·22[b]	0·34[c]	0·052
Total	1·32	1·44	1·71	0·205
Active (%)	7·8[a]	15·2[b]	19·7[b]	2·32

[a,b,c] Mean values within a row not sharing a common superscript letter were significantly different: $P < 0{\cdot}05$.
* For details of diets and procedures, see pp. 50–51.
† Active (%) is the basal activity/total activity × 100.

Table 5. Branched-chain keto-acid dehydrogenase activities measured with L-[^{14}C] valine in the adductor muscle of pigs fed on methionine-deficient diets with either no excess, excess leucine or excess branched-chain amino acids (BCAA)*

(Values are means for four pigs)

	Branched-chain keto-acid dehydrogenase activity			
	No excess	Leucine excess	BCAA excess	Pooled SED
Activity (nmol/min per g tissue)				
Basal	0·041	0·060	0·054	0·0089
Total	0·55	0·42	0·57	0·196
Active (%)†	9·9[a]	14·3[b]	9·6[a]	2·13
Activity (nmol/min per g protein)				
Basal	0·0008	0·0011	0·0010	0·00018
Total	0·0104	0·0078	0·0101	0·00385
Active (%)	9·5[a]	14·0[b]	9·6[a]	2·01

[a,b] Mean values within a row not sharing a common superscript letter were significantly different: $P < 0{\cdot}05$.
* For details of diets and procedures, see pp. 50–51.
† Active (%) is the basal activity/total activity × 100.

Measurements with L-[^{14}C]valine. Basal BCKDH activity in adductor muscle was not significantly increased by excesses of leucine or BCAA, whether expressed per g tissue or per mg protein.

When L-[^{14}C]valine was used as substrate, there was no difference in total enzyme activity amongst the treatments (Table 5). The percentage of active enzyme rose significantly from about 10 % on the treatment without excess to 14 % with leucine excess. Using the ratio basal : total activity determined with L-[^{14}C]valine the indirect calculation of absolute basal activity was possible using the results for total activity obtained with [^{14}C]KIV. Assuming that adductor muscle BCKDH was about 10 % active the basal activity would be approximately 0·50 nmol KIV/min per g tissue.

Discussion

This work was undertaken to elucidate the mechanisms by which dietary branched-chain amino acids affect the utilization of a methionine-deficient diet, as reported in the previous paper (Langer & Fuller, 2000). To try to understand how the alterations in amino acid utilization were brought about we examined changes in plasma concentrations of amino acids and keto acids and changes in the activity of BCKDH in muscle and liver.

Table 4. Total branched-chain keto-acid dehydrogenase activities in adductor and biceps femoris muscles of pigs fed on methionine-deficient diets with either no excess, excess leucine or excess branched-chain amino acids (BCAA)*

(Values are means for four pigs)

	Branched-chain keto-acid dehydrogenase activity			
	No excess	Leucine excess	BCAA excess	Pooled SED
Activity (nmol/min per g tissue)				
Adductor	4·8	5·3	3·9	0·74
Biceps femoris	4·2[a]	6·5[b]	7·0[b]	1·07
Activity (nmol/min per mg protein)				
Adductor	0·079[ab]	0·091[a]	0·064[b]	0·0119
Biceps femoris	0·072[a]	0·114[b]	0·115[b]	0·0147

[a,b] Mean values within a row not sharing a common superscript letter were significantly different: $P < 0{\cdot}05$.
* For details of diets and procedures, see pp. 50–51.

Plasma amino acid concentrations

The results demonstrate that BCAA interactions occur in the growing pig even at moderate levels of excess, and even when methionine, rather than one of the BCAA, is the limiting amino acid in the diet. The consumption of a moderate excess of leucine resulted in significant ($P < 0{\cdot}05$) depressions in the plasma concentrations of the other two BCAA as previously reported in pigs (Oestemer *et al.* 1973; Henry *et al.* 1976; Taylor *et al.* 1984), other animal species (D'Mello & Lewis, 1970; Block, 1989) and human subjects (Abumrad *et al.* 1984). These observed changes could be caused by various metabolic reactions: (1) effects on intestinal absorption or transport between tissues and blood; (2) effects on protein accretion due to (a) changes in protein synthesis, (b) changes in protein degradation; (3) effects on transamination to branched-chain keto acids (BCKA) and further decarboxylation.

The observed depressions of isoleucine and valine concentrations in plasma are probably not due to the inhibition by leucine of isoleucine or valine absorption or transport because leucine excess appears to have negligible effects on isoleucine and valine absorption (Rogers *et al.* 1962; Smith & Austic, 1978; Calvert *et al.* 1982; Block & Harper, 1984) and because intravenous infusion of leucine or KIC into human forearm resulted in a similar decrease in isoleucine and valine (Abumrad *et al.* 1984). Inhibition of isoleucine and valine uptake into tissues by leucine excess should increase their plasma concentrations rather than reduce them.

Increased accretion of protein due to leucine excess as observed by Langer & Fuller (2000) using exactly the same diets cannot be completely excluded as part of the reason for the changes in plasma isoleucine and valine concentrations. However, enhanced protein accretion should also affect the plasma concentrations of other amino acids, which was not observed. A selective accretion of protein with higher isoleucine and valine concentrations seems very unlikely and the changes in their plasma concentrations seem to be the result of a selective mechanism other than incorporation into protein (Harper *et al.* 1984).

Several authors (e.g. Harper *et al.* 1984; Block, 1989) have suggested that an excess of leucine or KIC can increase both the transamination of isoleucine and valine to KMV and KIV and their further decarboxylation and this could account for the changes observed here. Various experiments in other animal species showed that leucine excess supplied via the feed or by intravenous infusion can activate the catabolic pathway of BCAA causing a reduction in the plasma concentrations of isoleucine and valine (Calvert *et al.* 1982; Block & Harper, 1984).

Branched-chain keto acid concentrations

It seems that this is the first report of plasma keto acid concentrations and the effect of leucine or its metabolites on the keto acids of isoleucine and valine in pigs. Similar experiments investigating the effects of leucine or BCAA on their metabolism in other animals have usually been carried out using greater excesses. In rats, addition of large amounts of leucine (i.e. 15 g/kg diet) to a low-protein diet reduced the plasma concentrations of KMV and KIV but increased KIC significantly (Block & Harper, 1991). Similarly, the addition of KIC to a low-protein diet increased the plasma concentration of KIC and reduced the concentrations of KMV and KIV in rats (Crowell *et al.* 1990). This suggests that leucine and KIC cause the same response.

The present results show that similar changes in KMV and KIV occur in the pig even at moderate levels of excess. These could be explained by an activation of BCKDH due to leucine or KIC excess and an increase in the decarboxylation of KMV and KIV.

The relationship between branched-chain amino and keto acids

There was in general a close relationship between the plasma concentration of a BCAA and its corresponding keto acid. Similar observations were made by Matthews *et al.* (1982) in man. This relationship is presumably due to the presence of a near equilibrium transaminase in most tissues (Krebs & Lund, 1977). Transamination of BCAA to BCKA occurs very rapidly (Matthews *et al.* 1982) and therefore it is not surprising that plasma BCAA and BCKA concentrations were closely related. However, these relationships were closer in the postabsorptive than in the postprandial state. This suggests that the absorption and the transport of BCAA might play an important role. Transamination of the BCAA appears to occur mainly in skeletal muscle (Shinnick & Harper, 1977); therefore it is necessary to transport BCAA to the muscle before the BCKA can be released (Harper & Zapalowski, 1981). This might explain the weaker relationship during the postprandial state when the absorption is much higher than in the postabsorptive state. Furthermore, in both postprandial and postabsorptive states, the ratios BCAA : BCKA were always higher when all three BCAA were supplied together which indicates that all three BCAA or BCKA might compete for transamination or transport. Although there is no *in vivo* evidence, this is consistent with the different K_m values for the transaminases and the fact that all three BCAA are transported by the L-system.

Branched-chain keto-acid dehydrogenase activity in liver and muscle

In the pig, total liver BCKDH activity was much higher than total skeletal muscle activity. Expressed per g tissue, liver had about forty times higher total activity than muscle. Expressed per mg protein, the total activity in liver was still eighteen times higher than in muscle. This may be related to the higher concentration of mitochondria in liver.

Excesses of both leucine and BCAA enhanced the activity of BCKDH in liver. This effect could be explained by the larger amounts of KIC produced. It has been shown that enhanced levels of BCKA, especially KIC, inhibit the BCKDH kinase (*EC* 2.7.1.115), which results in a less phosphorylated BCKDH complex and therefore a higher BCKDH activity (Paxton & Harris, 1984). This response of BCKDH to KIC would explain why leucine excess reduced the concentrations of isoleucine and valine and their keto acids in plasma: the increased BCKDH activity would result in greater oxidation of isoleucine and valine.

Branched-chain amino acid metabolism in the whole animal

Comparing the values for liver and muscle basal BCKDH capacity (Table 6), the 40 kg pig fed on a methionine-deficient diet without excess amino acids was able to decarboxylate about 25·9 mmol KIV/d in liver and about 11·5 mmol in muscle. In the rat, the contribution of other

Table 6. Basal activities of branched-chain keto-acid dehydrogenase (BCKDH) in a 40 kg pig

Organ	Organ weight (g)	Organ : body (%)	BCKDH activity (nmol/min per g)	Organ BCKDH capacity (nmol/min)	Organ BCKDH capacity (nmol/d)	Liver : muscle ratio
Liver	1000	2·5	18·0	18 000	25·9	2·3 : 1
Muscle	16 000	40·0	0·5*	8000	11·5	

* Adductor muscle.

Table 7. Branched-chain amino acid intake, excretion in ileal digesta (i.e. passing into caecum), body concentrations, retention and oxidation in a 40 kg pig

Amino acids	Intake (g/d)	Passing into caecum* (g/d)	Concentration in body protein† (g/kg)	Retention (g/d)	Oxidation‡ (g/d)	Oxidation‡ (mmol/d)
Leucine	9·3	0·98	74	6·3	2·0	15·2
Valine	6·4	0·92	47	4·0	1·5	12·8
Isoleucine	5·1	0·74	35	3·0	1·4	10·7

* Wang & Fuller (1989).
† Kyriazakis & Emmans (1993).
‡ Amino acid oxidation is calculated as the difference between the amino acid digested (intake – that passing into the caecum) and that retained (calculated as N retention × 6·25 × amino acid concentration in body protein).

organs was relatively low compared with liver and muscle, except kidney with 6·5 % of the total decarboxylation capacity (Shinnick & Harper, 1977). Liver and muscle seem to be the major tissues degrading BCAA. The diet supplied about 70·9 mmol (9·3 g) leucine, 54·7 mmol (6·4 g) valine and 38·9 mmol (5·1 g) isoleucine per day. Knowing the daily BCAA intake, the N retention (taken from Langer & Fuller, 2000), the whole-body BCAA concentrations (Kyriazakis & Emmans, 1993) and the daily flow of BCAA in ileal digesta passing into the caecum (Wang & Fuller, 1989) it is possible to calculate how much BCAA (via BCKA) had to be degraded (Table 7) by animals given the methionine-deficient diet. These are simplified and approximate calculations in which it is assumed that the enzyme was completely extracted, that its activity was measured under nearly optimal conditions and that all three BCKA are equally good substrates for BCKDH. Nevertheless, the degradation of further excess of leucine or BCAA would require the activation of BCKDH or an increase in the amount of BCKDH. Both were observed in the present experiment. Total activation of the BCKDH after feeding BCAA in excess would allow for a daily degradation of up to 392 mmol KIV in liver and 161 mmol in muscle.

Possible effects of branched-chain keto-acid dehydrogenase activity on methionine metabolism

In the previous paper (Langer & Fuller, 2000) we reported that the addition of excess leucine or BCAA to a methionine-deficient diet enhanced N retention. This suggests that these amino acids reduced the oxidative loss of methionine, making more available for body protein accretion. We were unable to measure directly either the concentrations of KMTB or its oxidation so our discussion of this issue must be incomplete. The low K_m and non-inducibility of S-adenosyl methionine synthase (*EC* 2.5.1.6) probably limits the degradation of methionine by the trans-sulfuration pathway, whereas the K_m for transamination of methionine is high relative to plasma and tissue concentrations of this amino acid (Harper, 1983; Livesey, 1984); therefore regulation of the flux through the transamination pathway by supply of other substrates (i.e. BCAA and BCKA) might regulate methionine catabolism (Livesey, 1984). Besides activating a degradative enzyme for methionine, excessive amounts of leucine, isoleucine or valine, or of their keto acids, could affect methionine transamination as well as decarboxylation by supplying competitive substrates. Depression of methionine transamination and of methionine oxidation by addition of BCAA during incubation of rat skeletal muscle homogenates has been reported by Wu & Thompson (1989). However, BCAA had no effect on the transamination of methionine in rat liver (Livesey & Lund, 1980). Because BCKA, and particularly KIC, activate BCKDH this would be expected to increase rather than reduce the oxidation of KMTB. However, whereas the K_m values for the BCKA are in the range 10–20 μM, that for KMTB is at least three times higher, 67 μM (Jones & Yeaman, 1986). Thus the more important effect of additional BCAA might be the provision of alternative substrate. Dixon & Benevenga (1980) reported that addition of 1 mM-KIC depressed the decarboxylation of KMTB in rat liver mitochondria by 78 %. Studies with rat hepatocytes (Livesey, 1981) showed inhibition of the decarboxylation of KMTB by KIV, KMV and KIC.

It seems likely that the dominant effect of excess leucine or BCAA on methionine was to reduce the oxidation of KMTB by competitive inhibition, leading to increased availability of methionine for body protein synthesis.

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