Effect of feed intake on amino acid transfers across the ovine hindquarters

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Responses in variables of amino acid (AA) metabolism across peripheral tissues to feed intake were studied in six sheep (mean live weight 32 kg) prepared with arterio–venous catheters across the hindquarters. Four intakes (0·5, 1·0, 1·5 and 2·5 × maintenance energy) were offered over 2-week periods to each sheep in a Latin square design with two animals replicated. Animals were infused intravenously with a mixture of U-^{13}C-labelled AA for 10 h and integrated blood samples withdrawn from the aorta and vena cava hourly between 5 and 9 h of infusion. Biopsy samples were also taken from skin and *m. vastus lateralis*. Data from both essential (histidine, isoleucine, leucine, lysine, phenylalanine, threonine) and nonessential (glycine, proline, serine, tyrosine) AA were modelled to give rates of inward and outward transport, protein synthesis and degradation, plus the fraction of total vascular inflow that exchanged with the hindquarter tissues. Rates of inward transport varied more than 10-fold between AA. For all essential AA (plus serine), inward transport increased with food intake ($P<0.04$). There were corresponding increases in AA efflux ($P<0.05$) from the tissues for threonine and the branched-chain AA. Protein synthesis rates estimated from the kinetics of these AA also increased with intake ($P<0.02$). Rates of inward transport greatly exceeded the amount of AA necessary to support protein retention, but were more similar to rates of protein synthesis. Nutritional or other strategies to enhance AA transport into peripheral tissues are unlikely to increase anabolic responses.

Amino acid transport: Protein metabolism: Feed intake: Hindlimb: Muscle: Skin: Sheep

Flexibility in partition of nutrients between tissues permits both short- and long-term adaptation to environmental and physiological stimuli. Intake can also regulate metabolic responsiveness, with the priority of nutrient use altered between 'visceral' and 'productive' tissues during under- and overnutrition (Lobley, 1993). This alters the efficiency by which farmed species divert feed eaten to tissues such as muscle, skin and the mammary gland. While in such tissues certain aspects of protein metabolism, including synthesis and degradation, have been shown to alter with feed intake (e.g. Seve *et al.* 1986; Oddy *et al.* 1987; Dawson *et al.* 1991; Boisclair *et al.* 1993; Ponter *et al.* 1994; Liu *et al.* 1998; Hoskin *et al.* 2001) the involvement of other processes is less clear. For example, increases in feed intake are usually accompanied by elevated blood flow to peripheral tissues (e.g. Harris *et al.* 1994; Hoskin

et al. 2001) and, in some cases, arterial concentrations of certain amino acids (AA) (Hoskin *et al.* 2001). These two factors, acting either in isolation or together, provide a greater vascular supply of most AA, but how this relates to transport into cells and subsequent use for protein metabolism is unclear. In other tissues, such as the mammary gland, transport of specific nutrients (glucose) is thought to limit metabolic activity (milk production; Cant *et al.* 1999) and here, nutritional, endocrinological or genetic approaches to enhance rates of transport appear attractive.

Whether similar strategies should be extended to increase AA transport in other peripheral tissues depends on whether transport of AA is a limitation to increasing protein synthesis and ultimately net protein accretion. Unfortunately, the techniques necessary to obtain such

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knowledge are limited for situations *in vivo*, requiring either multiple sampling (Banos *et al.* 1973) or perfused preparations (Hundal *et al.* 1989). An alternative indirect approach was developed from traditional, trans-tissue, arterio–venous methods by Biolo *et al.* (1992, 1995) and involved a mathematical, three-compartment model that allowed both inward and outward transport of AA to be estimated. Based on this model, the relationships between transmembrane AA movements and rates of intracellular (tissue homogenate) protein metabolism were explored in a number of species, including human subjects, under various physiological and nutritional conditions (e.g. Biolo *et al.* 1992, 1994, 1995).

This model has been used in the current study to examine the effect of increasing feed intake, from less than to greater than maintenance energy (M), on the dynamics of a number of AA across the tissues of the ovine hindquarters. In particular, the sensitivity of inward transport of AA and the potential limitation for either protein synthesis or protein retention was examined. Possible alternative constraints on protein metabolism, for example intracellular AA concentrations, were also investigated. Other components of the present study have been reported previously (Hoskin *et al.* 2001; Savary *et al.* 2001).

Materials and methods

Animals and treatments

Six Suffolk-cross wether lambs (aged 6–8 months, live weight 30·5–34·5 kg) were surgically prepared with hind-limb arterio–venous catheters according to the methods described by Savary *et al.* (2001). Permanent indwelling polyvinyl or silicone rubber catheters were inserted into the aorta (two, 30 mm apart; caudal for infusion of plasma flow marker *p*-aminohippurate and cranial for arterial blood sampling) and posterior vena cava (venous blood sampling), caudal to entry of the renal veins. As required, temporary polyvinyl catheters were inserted into the jugular vein (for labelled algal hydrolysate infusion), posterior vena cava via tarsal vein (550–600 mm; if permanent vena cava catheter lost patency) and auricular artery (arterial blood sample), or occasionally into the heart via jugular vein (mixed venous blood sample). The latter two options were used only if: (1) one of the aortal catheters lost patency; (2) if the auricular artery catheter failed.

The sheep were fed a pelleted dried grass diet (estimated metabolisable energy 10 MJ/kg DM, 26 g N/kg DM) from automatic feeders, supplied daily as twenty-four equal portions per h, whilst housed in metabolism cages. In a Latin-square design with two sequences repeated, the animals were offered each of four levels of feed intake corresponding to 0·5, 1·0, 1·5 and 2·5 × M (based on daily M requirements of 400 kJ/kg body weight$^{0.75}$). Each level of intake was fed for 2 weeks prior to measurement, except for 0·5 × M, which was only fed during the week of measurement, with animals fed at 1·0 × M the week before measurement at 0·5 × M. All animals consumed all the ration offered, and the 24 h portions were consumed immediately, except for two animals at the 2·5 × M intake level who

were sometimes delayed by up to 1 h in consuming portions delivered between 02.00 and 06.00 hours.

During the week before measurement, animals were housed in metabolism cages. The rest of the time they were individually housed in floor pens with straw bedding and fed two times per d. Animals were kept under constant light cycle of 10 h light–14 h dark, with free access to water and a multi-mineral salt block. All procedures involving animals were conducted according to Home Office (UK) requirements for animal ethics and welfare.

Infusion, blood sampling and tissue biopsy

Measurements of AA kinetics were made on the last day of each 2-week feeding period and involved infusion of a U-^{13}C-labelled algal hydrolysate, prepared as described by Savary *et al.* (2001), based on Lobley *et al.* (1996). Samples of plasma were obtained on the day prior to infusion to provide background (natural abundance) samples for GC–MS analyses. The same amount of algal hydrolysate was continuously infused (20 g infusate/h; 22–198 μmol AA/h) into the jugular vein over a 10 h period, regardless of level of feed intake. Between 3 and 9 h a solution of 0·15 M-sodium *p*-aminohippurate, pH 7·4, 20 g/h) containing sodium heparin (400 IU/g) was infused into the more caudal aortal catheter to allow the determination of plasma flow. Integrated blood samples were withdrawn continuously by peristaltic pump from both the cranial aortal catheter (or auricular artery or heart) and the vena cava (10 ml/h per catheter) over 30 min intervals during the last 4 h of *p*-aminohippurate infusion. The infusion of heparinised *p*-aminohippurate was discontinued after 9 h, but the infusion of ^{13}C-labelled AA was maintained. A small biopsy (about 200–400 mg) was taken 1 h later under local anaesthesia (Lignavet; C-Vet Veterinary Products, Leyland, Lancs., UK) from the skin and the underlying *m. vastus lateralis* of one leg. These tissue samples were rapidly cleaned of any fascia, fat and blood contamination, washed in ice-cold physiological saline (9 g NaCl/kg), swabbed dry and then frozen in liquid N$_2$, subsequent to storage at −80°C. Biopsies were taken from alternate legs for consecutive infusions. The two biopsies from each leg (4 weeks apart) were taken from sites approximately 100 mm apart. Spot arterial and venous blood samples were taken at approximately 30 min intervals from the end of *p*-aminohippurate infusion to immediately following the biopsy.

One concern included the possible effect of the local anaesthetic on AA transfers. This was tested in a companion study, where sheep infused with [ring-^{2}H$_5$]-phenylalanine were injected with the same local anaesthetic into a marked area of one leg, 15 min prior to terminal anaesthesia. Tissue-free phenylalanine enrichments were similar for tissue samples from either the site of local anaesthesia or a similar area of the other leg (SO Hoskin, IC Savary-Auzeloux and GE Lobley, unpublished results).

Laboratory analyses

Blood (5 g) was centrifuged for 10 min at 1000 ***g***, to obtain

3 g plasma. Plasma flow (kg/min) was determined from 1 g plasma according to the gravimetric method described by Lobley *et al.* (1996). To another 1 g plasma was added 0·3 g algal infusate solution (diluted × 2·5) for measurement of AA concentration by the isotope-dilution technique using the *t*-butyldimethylsilyl derivatives (Calder *et al.* 1999). The remainder of plasma was stored in a microcentrifuge tube for later preparation of freeze-dried eluates (Savary *et al.* 2001). Enrichments of AA (as molar % excess) were determined from freeze-dried eluates as *n*-butylheptafluorylbutryl derivatives, with the D and L proportions of lysine resolved on a chiral column (Lobley *et al.* 1996). Tissue biopsy samples were freezer-milled (Spex 6700; Glen Creston, Stanmore, Middlesex, UK) to a fine powder under liquid N_2. These samples were then divided and prepared either for tissue enrichments (Connell *et al.* 1997) or for free AA concentrations (Rocha *et al.* 1993) measured on an AA analyser (Pharmacia-LKB Alpha Plus, Uppsala, Sweden), with post-column derivatisation with *o*-phthalylaldehyde and fluorescent detection.

A number of AA were not determined in either plasma and/or tissue for the following reasons. The derivitisation protocol adopted (Lobley *et al.* 1996) caused hydrolysis of glutamine and asparagine with consequent production of glutamate and aspartate and thus not all of these were analysed. During preparation of the algal hydrolysate, both cysteine and tryptophan are degraded and thus no flux values could be obtained. Methionine and alanine enrichments in the tissue samples were found to be unreliable due to the presence of unidentified interfering compounds.

Calculations

The model shown in Fig. 1 is based on Biolo *et al.* (1992, 1995). Let F be the metabolite flow (concentration ×

plasma flow) and E be metabolite enrichment. The term F_{yx} represents the flow to pool y from pool x. The subscripts m, a, and v refer to the tissue homogenate, arterial and venous pools of free AA respectively, while o refers to the protein-bound AA. F_{in} and F_{out} are the arterial inflows and venous outflows of AA respectively, and F_{ma} and F_{vm} reflect inward and outward transmembrane transport respectively. F_{mo} represents appearance of AA from protein breakdown and *de novo* synthesis, whereas F_{om} represents use of AA for protein synthesis, oxidation and other fates.

Assuming metabolic steady state, the steady-state equations of free AA for the arterial pool, venous pool and mixed tissue homogenate pool are given by:

$$F_{in} - F_{va} - F_{ma} = 0, \qquad (1)$$

$$F_{va} + F_{vm} - F_{out} = 0, \qquad (2)$$

and

$$F_{ma} + F_{mo} - F_{om} - F_{vm} = 0. \qquad (3)$$

It is assumed that steady state has also been achieved for the labelled AA and, furthermore, that no labelled AA is returned from protein breakdown. Then, the steady-state equations for the labelled AA for the venous pool and tissue homogenate pool are given by:

$$E_a \ F_{va} + E_m \times F_{vm} - E_v \times F_{out} = 0 \qquad (4)$$

and

$$E_a \ F_{ma} - E_m \times F_{om} - E_m \times F_{vm} = 0, \qquad (5)$$

where E_a, E_m and E_v are the arterial, tissue homogenate and venous enrichments of labelled AA respectively.
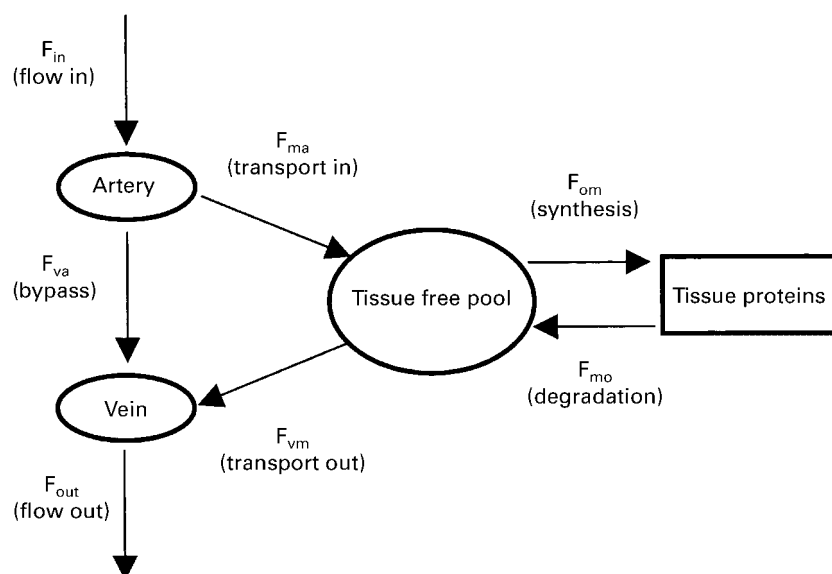

Fig. 1. The hindlimb kinetic model (based on Biolo *et al.* 1992, 1995).

Combining (2) with (4) gives:

$$F_{vm} = \frac{E_a - E_v}{E_a - E_m} \times F_{out}$$

The remaining flows are obtained from rearranging (2), (1), (5) and (3) respectively:

$$F_{va} = F_{out} - F_{vm},$$

$$F_{ma} = F_{in} - F_{va},$$

$$F_{om} = \frac{E_a \times F_{ma} - E_m \times F_{vm}}{E_m}$$

and

$$F_{mo} = F_{om} + F_{vm} - F_{ma}.$$

Assumptions and limitations of the model

The various forms of the model developed by Biolo *et al.* (1992, 1995) all have the inherent assumption that the tissue homogenate pool represents a common precursor through which pass all inward (from AA influx and protein degradation) and outward transfers (AA efflux, protein synthesis and, where appropriate, AA synthesis *de novo*). While this assumption is difficult to test directly, nonetheless, the homogenate pool is probably the precursor for protein synthesis and AA oxidation in muscle for both pigs (Watt *et al.* 1992) and sheep (Lobley *et al.* 1992). In contrast, other tissues, such as skin, may rely more on direct use of extracellular AA for protein anabolism (Lobley *et al.* 1992). This complicates matters when the vascular drainage encompasses a mixture of tissues, as is the case for the hindquarter preparation, where muscle, skin, bone and fat will all contribute to the venous outflow. For such preparations, the correct approach would necessitate determination of both the blood flows and the respective metabolic activities for each tissue (Biolo *et al.* 1994) and then application of weighted average values to encompass the composite hindquarters. Another option is to measure the aminoacyl–tRNA for all AA within the various tissue biopsies. This would address possible differences between homogenate free AA enrichments and that of the precursor pool for protein synthesis (Caso *et al.* 2001); however, the half–lives of the aminoacyl–RNA are only a few seconds and values may alter during sampling and extraction. This may account for differences observed between various techniques (Lobley *et al.* 1992; Watt *et al.* 1992; Davis *et al.* 1999; Caso *et al.* 2001). In practice, none of these alternative approaches was feasible within the constraints of the present study and, therefore, it was assumed that the muscle biopsy values reflect the mean for the composite tissues.

Although the protein metabolic activity of skin, muscle and bone differ (e.g. Seve *et al.* 1986; Lobley *et al.* 1992; Ponter *et al.* 1994; Liu *et al.* 1998), the homogenate enrichments of skin and muscle were similar in the current study (see later), except for serine (skin > muscle) and histidine (muscle > skin). This approach has also been shown to be robust in other species, with similar trends between arterio–venous and direct incorporation techniques (Harris *et al.* 1992; Lobley *et al.* 1992; Zhang *et al.* 1998). No allowance was included for the contribution of extracellular fluid to the biopsy sample, but this is usually minor (Biolo *et al.* 1992).

Statistics

Comparisons were by ANOVA using Genstat 4.2 for Windows (Lawes Agricultural Trust, Rothamsted, Harpenden, Herts., UK). For main effects, animals were treated as blocks with period × intake as treatment. In all cases there was no effect of period and this was excluded. In a number of cases it was obvious that, at the three lower intakes, some kinetic variables were similar but changed markedly at the highest intake. For this reason, data were also analysed for both linear and quadratic effects, but were found to be predominantly linear, as found by Hoskin *et al.* (2001) with associated data.

Results

Results for blood flow, arterial concentrations and mass transfers across the hindlimb have been presented elsewhere (Hoskin *et al.* 2001; Savary *et al.* 2001). For ease of comparison, results are separated into three sections: the measured essential AA (histidine, lysine, phenylalanine and threonine, but excluding the branched-chain AA isoleucine, leucine and valine) and the measured nonessential AA (glycine, proline, serine and tyrosine).

Amino acid supply and transport

Absolute arterial plasma inflows (F_{in}) between AA varied 10-fold (glycine and serine represented the extremes) in proportion to arterial concentrations. For most AA, F_{in} increased with intake ($P<0.01$) and with strong linear responses ($P<0.01$; Tables 1, 2 and 3). The exception was glycine, where only the linear response was significant ($P=0.028$). This was due to the large increase for $2.5 \times M$ compared with the other three intakes (Table 3). For multi-treatment comparisons, ANOVA is not efficient at separating relatively small changes in one group when the remaining groups are similar. The linear regression analysis compares weighted means of the lower two and upper two intakes and thus includes a greater emphasis on comparison between the extreme intakes. The observed changes in F_{in} at the lower intakes were mainly related to increased AA plasma concentrations, but at the highest intake a combination of increased concentration (Savary-Auzeloux *et al.* 2002) and greater blood flow (Hoskin *et al.* 2001) occurred. For most AA, the larger inflow to the hindquarters was accompanied by a greater outflow in the venous drainage (F_{out}, $P<0.05$ or better). The exceptions were histidine and glycine, but even for these the linear responses remained significant ($P<0.05$).

F_{in} is distributed between entry to the tissues and vascular bypass. The proportion of F_{in} transported into the tissues varied: $0.4–0.5$ for isoleucine, leucine, phenylalanine and serine; $0.3–0.4$ for lysine and threonine;

Table 1. Effect of feed intake on hindlimb histidine, lysine, phenylalanine and threonine arterial (F_{in}), venous (F_{out}) and bypass (F_{va}) flows, transport from the artery to the 'muscle' free pool (F_{ma}) and from the 'muscle' free pool to the vein (F_{vm}), protein synthesis (F_{om}) and degradation (F_{mo})*

(Mean values for six sheep at each feed intake)

| | Feed intake | | | | Statistical significance of effect (ANOVA): P† | | |
Amino acid (μmol/min)	$0.5 \times M$	$1.0 \times M$	$1.5 \times M$	$2.5 \times M$	Intake	Linear	SED
Histidine							
F_{in}	26·9	28·7	31·7	42·1	0·029	0·004	4·78
F_{out}	27·7	28·3	31·1	39·7	NS	0·019	4·94
F_{ma}	3·5	3·6	4·0	6·1	0·017	0·003	0·79
F_{vm}	4·3	3·3	3·4	3·7	NS		0·85
F_{va}	23·4	25·1	27·7	36·0	0·047	0·007	4·28
F_{om}	63·2	50·3	56·6	70·8	NS		14·43
F_{mo}	64·0	50·0	56·0	68·4	NS		14·40
Lysine							
F_{in}	36·8	47·5	56·1	74·4	0·011	0·001	9·45
F_{out}	37·6	45·7	52·8	66·6	0·048	0·007	9·34
F_{ma}	13·0	14·2	16·5	28·9	<0·001	<0·001	3·11
F_{vm}	13·8	12·4	13·3	21·1	0·056	0·020	3·13
F_{va}	23·8	33·3	39·6	45·5	0·079	0·015	7·79
F_{om}	28·8	25·0	35·6	30·6	NS		8·15
F_{mo}	29·6	23·2	32·4	22·8	NS		8·38
Phenylalanine							
F_{in}	22·8	25·3	31·4	60·2	0·002	<0·001	7·80
F_{out}	23·9	25·2	31·5	58·3	0·003	<0·001	7·91
F_{ma}	10·9	10·9	12·0	22·1	0·037	0·009	4·56
F_{vm}	12·1	10·8	12·0	22·1	0·096	0·030	4·57
F_{va}	11·9	14·3	19·5	36·2	0·002	<0·001	4·96
F_{om}	10·5	9·2	16·4	10·1	NS		3·58
F_{mo}	11·6	9·1	16·4	8·1	NS		3·76
Threonine							
F_{in}	33·4	51·8	62·3	99·8	<0·001	<0·001	12·06
F_{out}	34·9	51·2	60·6	95·6	0·001	<0·001	11·86
F_{ma}	12·9	15·5	18·5	32·8	<0·001	<0·001	3·98
F_{vm}	14·4	14·9	16·9	28·6	0·011	0·002	4·03
F_{va}	20·5	36·3	43·7	67·1	0·003	<0·001	9·89
F_{om}	18·4	19·2	26·7	32·8	0·005	<0·001	3·71
F_{mo}	19·8	18·6	25·0	28·7	NS	0·033	4·45

M, maintenance energy.

* For details of diets and procedures, see p. 168.

† The expected d.f. were 15, but there were some missing values: histidine and theonine d.f. 14, lysine d.f. 13, phenylalanine d.f. 12.

0·2–0·3 for glycine, proline, tyrosine and valine; 0·13 for histidine. This partition of F_{in} was not altered by intake for most AA, although for tyrosine ($P<0.02$) a lesser fraction (0·22 compared with 0·40) was transported into tissues as intake increased.

Absolute rates of tissue entry (F_{ma}, mass/time) again varied more than 10-fold between AA, with the lowest values for histidine and the highest for glycine. For the essential AA, branched-chain AA and serine, F_{ma} increased with intake ($P<0.05$ or better) and with linear effects ($P<0.01$ or better, except for isoleucine $P=0.022$). Inward transport of proline showed a linear trend ($P=0.013$), but neither glycine nor tyrosine were altered by intake. The linear contrasts of the ANOVA involve comparison between the two lower and the two higher intakes. Where linear effects were observed, but in the absence of significant responses from conventional ANOVA, these were invariably due to the large changes observed at the highest ($2.5 \times M$) intake. Apparent net retention of individual AA across the hindquarters has been

reported elsewhere (Hoskin *et al.* 2001), but can be obtained as the difference between F_{in} and F_{out}.

Protein synthesis and retention

If there is no oxidation of the AA, or transformation to other metabolites, then apparent net retention should truly represent protein gain. This will be the case for histidine, lysine, phenylalanine, threonine and tyrosine. The proportion of inward flow (F_{ma}) used to support apparent AA retention by the tissues varied with intake for all AA ($P<0.02$ or better), except for glycine and serine. These comparisons included the negative values obtained at $0.5 \times M$, when the hindquarter tissues underwent net protein mobilisation and the sheep were losing body weight (Hoskin *et al.* 2001). At the highest intake, the proportion of inward transport retained on a net basis varied between 0·1–0·2 for phenylalanine, threonine and tyrosine; 0·2–0·3 for isoleucine, leucine, lysine and valine; 0·32 for proline; 0·43 for histidine. AA effluxes (F_{vm}) from the hindlimb

Table 2. Effect of feed intake on hindlimb branched-chain amino acid arterial (F_{in}) venous (F_{out}), and bypass (F_{va}) flows, transport from the artery to the 'muscle' free pool (F_{ma}) and from the 'muscle' free pool to the vein (F_{vm}), protein synthesis (F_{om}) and degradation (F_{mo})*

(Mean values for six sheep at each feed intake)

| Amino acid (μmol/min) | Feed intake | | | | Statistical significance of effect (ANOVA): P† | | |
	$0.5 \times M$	$1.0 \times M$	$1.5 \times M$	$2.5 \times M$	Intake	Linear	SED
Isoleucine							
F_{in}	30·5	40·9	46·8	106·6	<0·001	<0·001	7·82
F_{out}	30·8	38·5	44·1	97·8	<0·001	<0·001	7·58
F_{ma}	12·6	34·8	12·4	54·5	0·032	0·022	13·92
F_{vm}	12·9	32·4	9·8	45·7	0·071	0·064	13·60
F_{va}	17·9	6·1	34·3	52·1	0·024	0·007	13·07
F_{om}	13·2	11·3	16·0	34·7	0·030	0·007	7·28
F_{mo}	13·5	8·9	13·4	25·9	NS	0·059	7·13
Leucine							
F_{in}	50·9	59·9	81·1	158·9	<0·001	<0·001	15·62
F_{out}	51·5	56·5	75·8	145·4	<0·001	<0·001	15·13
F_{ma}	25·3	35·0	37·8	63·8	0·013	0·002	10·08
F_{vm}	25·9	31·6	32·5	50·3	NS	0·020	9·45
F_{va}	25·6	25·0	43·3	95·1	<0·001	<0·001	10·56
F_{om}	26·0	25·3	31·1	60·6	<0·001	<0·001	4·82
F_{mo}	26·6	21·9	25·8	47·1	<0·001	<0·001	4·96
Valine							
F_{in}	68·5	102·1	150·4	280·9	<0·001	<0·001	26·11
F_{out}	69·1	99·9	146·5	270·2	<0·001	<0·001	25·77
F_{ma}	23·0	23·8	29·3	53·6	0·015	0·003	9·01
F_{vm}	23·6	21·6	25·3	42·9	NS	0·032	9·07
F_{va}	45·5	78·3	121·2	227·3	<0·001	<0·001	23·25
F_{om}	17·1	16·7	25·5	54·8	0·013	0·02	11·03
F_{mo}	17·7	14·4	21·6	44·1	0·068	0·017	10·95

M, maintenance energy.

* For details of diets and procedures, see p. 168.

† The expected d.f. were 15, but there were some missing values: isoleucine d.f. 11, leucine and valine d.f. 13.

tissues showed weaker trends in response to intake and were only significant for threonine and serine. In addition, linear responses were also observed for leucine, lysine, phenylalanine and valine.

Isoleucine, leucine, threonine and valine all showed increases in F_{om} (protein synthesis plus oxidation; Tables 1 and 2) in response to higher intakes ($P=0.03$ or better). Protein degradation (F_{mo}) increased with intake only for leucine ($P<0.01$), but with a linear response also for threonine and valine ($P<0.05$). The combination of F_{ma} (inward transport) and F_{mo} (proteolysis and any AA synthesis *de novo*) gives the total flow through the homogenate pool (as does F_{vm} plus F_{om}). This total flow was sensitive to intake for the branched-chain AA and threonine ($P<0.03$ or better), with additional linear trends for phenylalanine, serine and tyrosine. There were marked differences between the AA in the relative inflows from either F_{ma} or F_{mo}. The contributions from inward transport (F_{ma}) were: branched-chain AA plus glycine and phenylalanine 0·5–0·6; lysine, threonine and tyrosine 0·4–0·5; proline and serine 0·2–0·3; histidine 0·07. This proportional contribution was sensitive to intake only for histidine ($P<0.03$), but with linear trends for lysine, phenylalanine, serine and threonine ($P<0.05$ or better). The proportion of inward transport used for F_{om} (synthesis and oxidation) was insensitive to intake (except for a linear trend for histidine, $P<0.02$). For several AA the use

(apparent disposal) of inward transport (F_{ma}) for synthetic and oxidative purposes approached unity (glycine, isoleucine, leucine, phenylalanine, threonine and valine). For the other AA, apparent disposal exceeded inward transport by 2–4-fold, except for histidine, where inward transport from plasma only accounted for 0·1–0·2 of the needs for protein synthesis. In terms of total inflow to the homogenate pool (i.e. $F_{ma} + F_{mo}$), disposal (F_{om}) accounted for 0·39–0·66 for most AA, except for histidine, proline and serine where values ranged from 0·79 to 0·94.

Tissue amino acid concentrations and enrichments

Responses to intake in muscle biopsy AA concentrations were of three types. Some AA (glycine, isoleucine, leucine, phenylalanine and serine) remained unaltered (Table 4). Of these, the essential and branched-chain AA forms had concentrations (mM) lower than for arterial plasma, while glycine and serine were concentrated approximately 3·0-fold within the muscle. Concentrations of some AA declined as intake increased (histidine and lysine; Table 4). Across the intake range, histidine remained concentrated in muscle by approximately 1·5–2·5-fold, while lysine decreased from double that of arterial plasma at the lowest intake to similar values at the higher intakes. The remainder of the AA (threonine, tyrosine and valine) increased tissue concentrations as intake was raised.

Table 3. Effect of feed intake on hindlimb nonessential amino acid arterial (F_{in}), venous (F_{out}), and bypass (F_{va}) flows, transport from the artery to the 'muscle' free pool (F_{ma}) and from the 'muscle' free pool to the vein (F_{vm}), protein synthesis (F_{om}) and degradation (F_{mo})*

(Mean values for six sheep at each feed intake)

Amino acid (μmol/min)	Feed intake				Statistical significance of effect (ANOVA): P†		
	$0.5 \times M$	$1.0 \times M$	$1.5 \times M$	$2.5 \times M$	Intake	Linear	SED
Glycine							
F_{in}	216	216	217	302	NS	0·028	37·5
F_{out}	219	219	221	306	0·087	0·025	37·5
F_{ma}	59·1	52·0	53·8	80·0	NS		14·02
F_{vm}	61·8	54·5	57·8	84·6	NS	0·063	13·17
F_{va}	157	164	163	222	NS	0·037	29·5
F_{om}	45·5	35·2	42·7	52·6	NS		12·44
F_{mo}	48·2	37·1	46·7	57·0	NS		10·84
Proline							
F_{in}	28·5	33·0	41·3	60·5	0·002	<0·001	6·97
F_{out}	29·2	32·3	39·8	57·0	0·005	<0·001	6·85
F_{ma}	6·1	7·6	8·3	11·8	0·081	0·013	2·06
F_{vm}	6·7	6·8	6·8	8·4	NS		1·93
F_{va}	22·5	25·5	33·0	48·7	0·003	<0·001	5·98
F_{om}	30·0	25·3	28·9	38·3	NS		7·29
F_{mo}	30·6	24·6	27·5	34·9	NS		7·18
Serine							
F_{in}	15·2	21·4	25·9	42·8	<0·001	<0·001	4·97
F_{out}	15·1	19·2	23·9	36·6	0·001	<0·001	4·41
F_{ma}	7·4	9·9	11·8	20·4	0·001	<0·001	2·62
F_{vm}	7·3	7·7	9·8	14·3	0·022	0·003	2·15
F_{va}	7·8	11·5	14·1	22·4	0·004	<0·001	3·30
F_{om}	40·7	34·4	42·4	54·6	NS		10·36
F_{mo}	40·6	32·2	40·4	48·5	NS		10·50
Tyrosine							
F_{in}	17·1	23·8	30·4	60·2	<0·001	<0·001	5·90
F_{out}	17·9	24·0	30·8	60·1	<0·001	<0·001	5·99
F_{ma}	6·8	8·3	8·2	10·2	NS		2·04
F_{vm}	7·6	8·5	8·5	10·0	NS		2·18
F_{va}	10·3	15·5	22·3	50·0	<0·001	<0·001	5·14
F_{om}	9·8	9·5	19·1	21·5	NS	0·062	6·74
F_{mo}	10·6	9·7	19·4	21·3	NS	0·075	6·67

M, maintenance energy.

* For details of diets and procedures, see p. 168.

† The expected d.f. were 15, but there were some missing values: glycine, proline and serine d.f. 14, tyrosine d.f. 13.

Threonine was concentrated 2·0-fold with regard to arterial concentrations, whilst tyrosine and valine were either lower or similar to plasma. Across increasing intakes, the muscle:arterial plasma concentrations did not vary significantly except for lysine, serine and valine where the value of the ratio declined, particularly between $0.5 \times M$ and $1.0 \times M$ (Table 5). In skin, the concentrations of branched-chain AA, phenylalanine and glycine were all raised by increasing intake (Table 4), whilst the remaining AA were unchanged. There were no intake-related effects on the skin:arterial plasma concentration ratios. Similarly, the muscle:skin AA concentrations were not altered significantly by level of intake, except for lysine ($P<0.02$) where the value of the ratio declined. Overall, the enrichments of most AA in muscle were equal to or exceeded those in skin, with the value of the ratio between 1·0 and 1·5, except for histidine (0·14), proline (0·51) and serine (3·54). The value of the ratio of enrichments between the tissues was unaffected by intake.

Discussion

This present study was based on two questions, which are not considered to be mutually exclusive: 'Is transport of AA into cells sensitive to food intake?'; 'Does transport of AA into cells of hindquarter tissues limit their ability to either synthesise or accrete protein?'. If the answer to the second question was 'yes', then approaches to enhance either transporter number or activity become attractive.

Intake, tissue amino acid supply and uptake

While intake can influence both arterial concentrations of AA and total blood flow to muscle, it has been difficult to explain responses (protein synthesis or accretion) in terms of these two variables alone (Lescoat *et al.* 1996). This is also shown in Fig. 2(a), in which regression analysis of the combined results for three AA are shown. The relationship between F_{in} and net retention is low (<0.18)

Table 4. Effect of feed intake (× maintenance energy) on homogenate muscle (*m. vastis lateralis*) and skin amino acid concentrations*

(Mean values for six sheep at each feed intake)

Amino acid (μmol/kg)	Feed intake				Statistical significance of effect (ANOVA): P†		SED
	0·5 × M	1·0 × M	1·5 × M	2·5 × M	Intake	Linear	
Muscle							
Glycine	1268	1045	1220	1162	NS		253·3
Histidine	122	95	107	88	0·065	0·036	12·2
Isoleucine	39	34	34	45	NS		5·8
Leucine	77	64	61	83	NS		9·9
Lysine	127	74	82	52	0·057	0·017	25·3
Phenylalanine	34	31	29	38	NS		5·4
Serine	193	131	135	127	NS		32·2
Threonine	175	192	209	279	0·035	0·005	33·5
Tyrosine	33	34	34	47	0·007	0·002	3·9
Valine	107	102	103	158	0·003	0·001	14·0
Skin							
Glycine	861	1262	1028	1401	0·035	0·020	174·0
Histidine	70	79	66	83	NS		11·8
Isoleucine	40	45	44	79	0·001	<0·001	8·4
Leucine	88	85	76	135	0·020	0·012	17·6
Lysine	65	64	58	80	NS		9·6
Phenylalanine	48	59	49	81	0·089	0·031	13·5
Serine	312	394	389	748	NS		178·2
Threonine	129	157	146	211	NS		33·6
Tyrosine	50·8	69·4	59·3	68·0	NS		9·31
Valine	138	154	150	264	0·001	<0·001	27·6

M, maintenance energy.
* For details of diets and procedures, see p. 168.
† d.f. 15.

and with only a moderate correlation (R^2 0·73). Such poor correlations may be due, in part, to the fact that not all inflow to an organ is either available to, or exchanges with, the cells. In the mammary gland, for example, flow through individual capillaries is not constant and changes with time (Prosser *et al.* 1996). Thus, the temporal pattern of nutrient supply to different parts of the udder varies and

the concept of 'nutritive' flow was introduced to include only the quantity that perfuses 'active' parts of the tissue and exchanges with the cells (Prosser *et al.* 1996).

This regionalisation of blood flow would, of necessity, also involve 'non-nutritive', or 'bypass', flow via anastomose or 'shunt' vessels, as measured by F_{va} in the current study. At its simplest, the theory would propose that blood

Table 5. Free amino acids in muscle (*m. vastis lateralis*) water:free amino acids in arterial plasma water ratios*†

(Mean values for six sheep at each feed intake)

Amino acid	Feed intake				Statistical significance of effect (ANOVA): P‡		SED
	0·5 × M	1·0 × M	1·5 × M	2·5 × M	Intake	Linear	
Glycine	4·03	3·40	3·73	4·01	NS		1·347
Histidine	2·65	2·10	2·31	1·81	NS	0·058	0·362
Isoleucine	0·78	0·62	0·48	0·38	NS	0·041	0·177
Leucine	0·84	0·75	0·52	0·49	NS	0·060	0·185
Lysine	2·10	1·18	0·96	0·91	0·036	0·017	0·367
Phenylalanine	0·95	0·90	0·68	0·63	NS	0·045	0·159
Serine	8·68	4·42	3·35	2·82	0·026	0·011	1·818
Threonine	3·31	2·82	2·07	2·57	NS		1·018
Tyrosine	1·111	0·91	0·77	0·78	NS	0·040	0·143
Valine	0·79	0·65	0·48	0·54	0·058	0·033	0·106

M, maintenance energy.
* For details of diets and procedures, see p. 168.
† Mean values for water contents of plasma and muscle were 0·931 and 0·836 respectively.
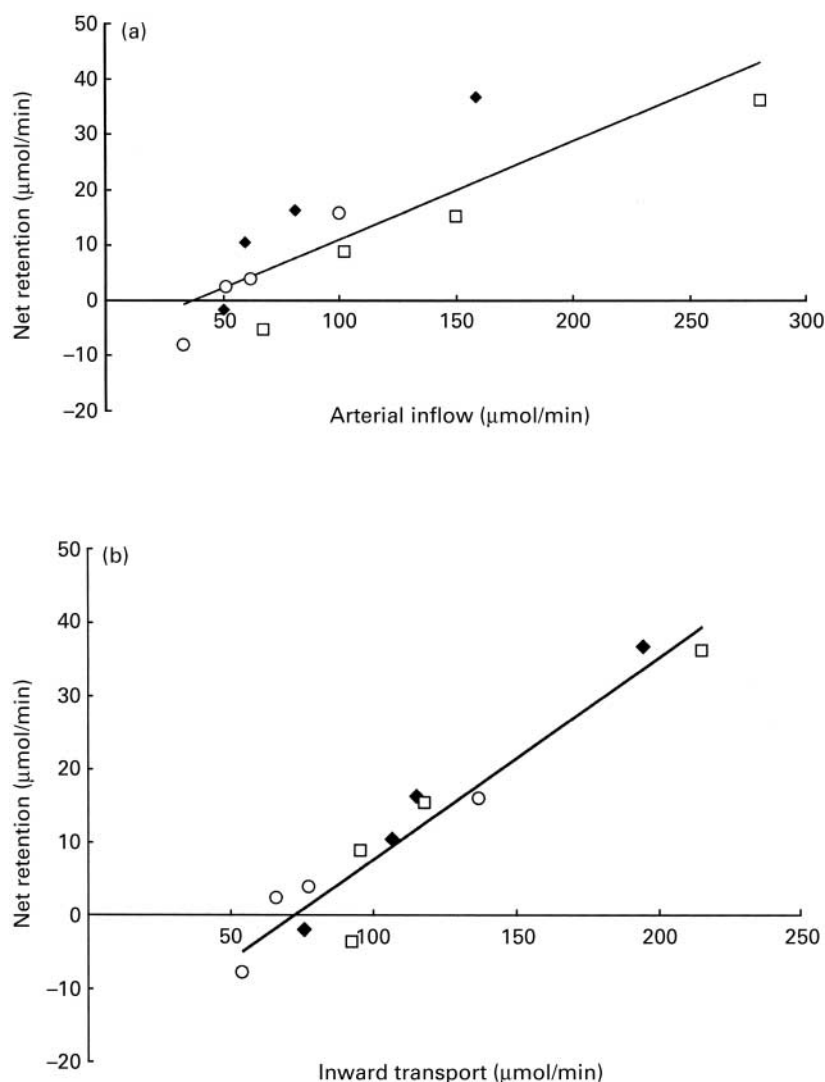‡ d.f. 13 (two missing values).

Fig. 2. Amino acid ($\blacklozenge$, leucine; $\bigcirc$, threonine; $\square$, valine) kinetics in sheep in response to alteration of intake from 0.5 to $2.5 \times$ maintenance energy. Response in net retention to either (a) altered amino acid supply via arterial inflow ($y = 0.177x - 6.64$, R^2 0.73) or (b) transport of amino acid into cells of hindquarter tissues ($y = 0.276x - 19.97$, R^2 0.92). For details of diets and procedures, see p. 168.

passing through nutritive capillaries exchanges fully with adjacent cells and that metabolic extraction depends on the 'nutritive' flow: 'non nutritive' flow value. An alternative hypothesis would stress the importance of differences between individual metabolites and cells. The latter would account for scenarios where some metabolites are completely extracted across a tissue while others are not, e.g. nearly 100% propionate and NH_3 total inflows to the liver are removed (e.g. Eisemann *et al.* 1996; Nieto *et al.* 1996), while AA extractions can be $<10\%$ (e.g. Wray-Cahen *et al.* 1997; Lobley *et al.* 2001). In the current study, the second hypothesis also appears more probable, as the 4-fold differences in the $F_{ma}:F_{in}$ ratio ($0.13-0.50$) for the various AA clearly indicate differential extraction efficiencies, even though vascular distribution would be similar.

With regard to the first question posed, intake did influence the absolute amounts of individual AA transported into muscle, but this was a function of increased supply (F_{in}) to the tissues rather than a change in the 'transport efficiency' (i.e. $F_{ma}:F_{in}$). Furthermore, the differential in $F_{ma}:F_{in}$ ratio observed between individual AA was maintained across a wide intake range. Thus, an important feature of AA transport appeared to be total supply (i.e. the combination of blood flow and arterial concentrations) to the tissues. The constancy of 'transport efficiency' may be a feature of changing total intake and thus maintaining similar relative proportions of the individual AA. If one AA becomes limited then its extraction can reach nearly 100%, as observed across the mammary gland of lactating goats during histidine deficiency (Bequette *et al.* 2000).

Transport, protein synthesis and retention

Feed intake altered the amount of AA transported into the tissues, but did this limit either protein retention or

synthesis? Less than 50 % arterial AA flow exchanged with the hindlimb tissues, yet this still provided a large excess of AA compared with the needs for net retention. Indeed, at the highest intakes and rates of protein accretion (Hoskin *et al.* 2001), the proportion of inward transport retained did not exceed 40 %. In addition, even when there was net mobilisation of protein by the hindquarter tissues (i.e. net outward transfer), inward transport was still considerable. Thus, absolute rates of inward transport did not appear to limit protein accretion. In practice, net gain may be limited by the inward transport of just one AA, with transfers of the rest being in 'excess' of metabolic needs. Due to technical problems and limitations not all AA were evaluated: notable omissions included methionine and tryptophan and it must be assumed that neither of these was limiting. One alternative possibility is that retention is a function of transport greater than some 'basal' value, but even when differences between the highest and lowest intake were considered, < 30 % of the incremental inward transport was retained for most of the essential AA and branched-chain AA. Again, this can be seen graphically for the three AA (Fig. 2(b)). The exception was histidine, where the increased retention exceeded the extra transfer into the cell (123 %). Furthermore, because increased transport of one AA can stimulate the uptake of others (Oxender & Christensen, 1963; Jacquez, 1967; Shennan *et al.* 1994), altered metabolism of just a few AA might provoke the universal responses observed here. This needs to be examined further.

Why does AA transport increase with intake when sufficient material is apparently already available to the cell? One possibility relates to preferential use of specific AA pools for metabolic purposes, i.e. 'compartmentalisation'. For example, the rates of inward transport and protein synthesis were similar for many of the essential AA. In theory, protein synthesis can arise from AA that have just entered the cell or those released from protein degradation (plus any synthesised *de novo* in the case of the nonessential AA). If protein synthesis occurred mainly from AA that entered the cell, with conversely those liberated from proteolysis directed towards efflux, then rates of inward transport would need to be both high and sensitive to intake. Unfortunately, evidence for such cellular compartmentalisation appears weak. Although in some tissues, such as the liver, polypeptide synthesis can involve AA newly-transported into the cell, this appears to be confined mainly to export proteins (Fern & Garlick, 1976; Connell *et al.* 1997), with constitutive proteins synthesised from intracellular pools (Fern & Garlick, 1976; Smith & Sun, 1995). In muscle, proteins appear to be synthesised from AA in the intracellular compartment, as shown in a variety of studies involving either reciprocal pool or large dose approaches (e.g. Horber *et al.* 1989; Lobley *et al.* 1992; Watt *et al.* 1992; Ljungqvist *et al.* 1997). Thus, the large inward transport rates do not appear to be a feature of compartmentalisation but, instead, may confer metabolic flexibility and the ability to respond rapidly to changes in substrate supply or hormonal changes without the delay incurred if synthesis or recruitment of transporters were needed. This would be in line with metabolic control logic (Newsholme & Board, 1991).

Intake effects on tissue homogenate concentrations of amino acids

Another putative regulator of protein synthesis and accretion could be intracellular concentrations of AA. These are a function of the inflows (inward transport, protein degradation, synthesis *de novo*) and outflows (efflux, protein synthesis and AA catabolism) and clearly would be influenced by changes in rates of transport. Despite the differences in AA kinetics in response to intake, however, this did not result in consistent changes of the free essential AA concentrations in the homogenate, either within or between muscle and skin. This may be due to the links between counter-regulatory transport mechanisms (Christensen, 1990) with influx-driven increases in rate of efflux (Shennan *et al.* 1994; Ferruzza *et al.* 1997). Indeed, the current results do indicate that increased AA inward transport is associated with elevated efflux, but such responses may differ between tissues. For example, when a large dose of an AA transported mainly by the L-system is injected into sheep then the concentrations of other AA co-transported by the L-system increase in skin, but either decrease, or remain unaltered, in muscle (Rocha *et al.* 1993). Nonetheless, any changes in homogenate AA concentrations may have limited effect, because in the process of protein synthesis the K_m for aminoacyl tRNA transferases rarely exceeds $1–2\,\mu M$ (e.g. Nazarenko *et al.* 1992). Therefore, any increase of homogenate concentrations beyond the $50–250\,\mu M$ already found at low intakes will not exert a mass action effect on protein synthesis.

Inter-species comparisons

There are only a limited number of studies that have measured, or estimated, AA transport into tissues. Direct estimates for muscle have been reported for rodents, both *in vivo* (Banos *et al.* 1973) and for the perfused hindlimb (Hundal *et al.* 1989), while values for dogs (Biolo *et al.* 1992), rabbits (Zhang *et al.* 1998) and human subjects (Biolo *et al.* 1995) have been obtained by the procedure adopted in the current study. Based on the assumption that sheep hindquarters represent 18 % body weight (Harris *et al.* 1992), then inter-species comparisons of normalised results reveal that rates of inward AA transport were rat $>$ dog $=$ sheep $>$ rabbit $=$ human subjects. For example, inward transport rates for phenylalanine were (nmol/min per g tissue): rat 7.7, dog 4.2, sheep (low to high intake) 1·9–4·0, human subjects 1·9, rabbit (fasting and plus AA) 1·1–1·9. The same order is found when rates of fractional protein synthesis in muscle between these species are compared (e.g. Lewis *et al.* 1984; Garlick *et al.* 1989; Lobley *et al.* 1992; Caso *et al.* 2001). Thus, the various experimental approaches all provide indirect support for a relationship between inward AA transport and tissue protein turnover.

While for most AA, enrichments in both muscle and skin were similar, suggesting that the proportional inflows into the homogenate pool from inward transport and protein degradation were similar between the two tissues, this was not the case for histidine. For this AA, intra-muscular

enrichments (but not those in skin) were low compared with other AA. The histidine content of ovine muscle protein in the current study was not unusual (Macrae *et al.* 1993), so homogenate release from protein degradation should be in reasonable proportion relative to other AA. Thus, the results suggest that either inward transport into muscle is low or that other, unlabelled, sources of histidine are available to the tissue. Low rates of histidine transport into muscle are not a feature of other species (e.g. rat; Banos *et al.* 1973) and thus this explanation would require a specific feature of ovine (or ruminant animal) metabolism. Histidine may also be liberated from carnosine (β-alanylhistidine), present in large concentrations in muscle (Maynard *et al.* 2001), and can provide the AA in times of histidine deficiency (Tamaki *et al.* 1977). This would act as a source of unlabelled histidine (in the short-term). This may be similar to the situation in ovine erythrocytes, where histidine concentration exceeds that of plasma, but has much lower enrichment (Lobley *et al.* 1996). Histidine in muscle, therefore, may be either sourced from small peptides or exchanged directly with the erythrocyte.

In summary, most of the AA examined showed similar generalised responses to increased food intake. Thus, both cellular influx and efflux of AA were elevated, as was protein turnover. Despite this, absolute rates of AA transport did not appear to limit protein anabolism and strategies to enhance AA transport in order to increase anabolic responses to food intake are likely to have limited benefit. As supply to the cell of precursor AA appeared to be always in considerable excess to that necessary for gain, then other factors, such as hormonal action, must be the key regulators. The scope of the present study was limited to effects of feed intake, with the sole focus on transport of AA from arterial supply into tissues of the ovine hindquarters. It is almost certain that hormonal and endocrine factors such as insulin and insulin-like growth factor 1 (Lobley, 1998), the plasma concentrations of which alter with feed intake, were involved in the changes in protein metabolism observed in the present study. However, investigation of these factors and their interactions with AA transport requires alternative approaches (Davis *et al.* 2002) that were beyond the scope of the present study. Nonetheless, as neither total AA supply nor inward transport appear to limit protein gain or metabolism within muscle, then such areas do need to be investigated as a matter of urgency.

Acknowledgements

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