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Splanchnic-bed transfers of amino acids in sheep blood and plasma, as monitored through use of a multiple U-^{13}C-labelled amino acid mixture

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The response in whole-body and splanchnic tissue mass and isotope amino acid transfers in both plasma and blood has been studied in sheep offered 800 g lucerne (*Medicago sativa*) pellets/d. Amino acid mass transfers were quantified over a 4 h period, by arterio–venous procedures, across the portal-drained viscera (PDV) and liver on day 5 of an intravenous infusion of either vehicle or the methylated products, choline (0·5 g/d) plus creatine (1·0 g/d). Isotopic movements were monitored over the same period during a 10 h infusion of a mixture of U-^{13}C-labelled amino acids obtained from hydrolysis of labelled algal cells. Sixteen amino acids were monitored by gas chromatography–mass spectrometry, with thirteen of these analysed within a single chromatographic analysis. Except for methionine, which is discussed in a previous paper, no significant effects of choline plus creatine infusion were observed on any of the variables reported. Whole-body protein irreversible-loss rates ranged from 158 to 245 g/d for the essential amino acids, based on the relative enrichments (dilution of the U-^{13}C molecules by those unlabelled) of free amino acids in arterial plasma, and 206–519 g/d, when blood free amino acid relative enrichments were used for the calculations. Closer agreement was obtained between lysine, threonine, phenylalanine and the branched-chain amino acids. Plasma relative enrichments always exceeded those in blood ($P < 0·001$), possibly due to hydrolysis of peptides or degradation of protein within the erythrocyte or slow equilibration between plasma and the erythrocyte. Net absorbed amino acids across the PDV were carried predominantly in the plasma. Little evidence was obtained of any major and general involvement of the erythrocytes in the transport of free amino acids from the liver. Net isotope movements also supported these findings. Estimates of protein synthesis rates across the PDV tissues from [U-^{13}C] leucine kinetics showed good agreement with previous values obtained with single-labelled leucine. Variable rates were obtained between the essential amino acids, probably due to different intracellular dilutions. Isotope dilution across the liver was small and could be attributed predominantly to uni-directional transfer from extracellular sources into the hepatocytes and this probably dominates the turnover of the intracellular hepatic amino acid pools.

Stable isotopes: Amino acids: Protein metabolism: Liver

Inter-organ transport of free amino acids (AA) by the blood involves different distributions between the erythrocytes and plasma (e.g. Elwyn *et al.* 1972; Heitmann & Bergman, 1980; Houlier *et al.* 1991). Some AA (e.g. glutamate, glycine, lysine, histidine) are present at greater concentrations in erythrocytes than in plasma which, in the absence of Na^+-dependent concentrative uptake (Young & Ellory, 1977), indicates production or synthesis *in situ*; for others (e.g. valine, isoleucine, phenylalanine) there is no concentration gradient, while some are more concentrated in the plasma (e.g. arginine, glutamine), which suggests

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either a restriction on uptake or catabolism within the erythrocyte. It has been proposed that, in non-ruminants, AA transport to the liver involves predominantly plasma, but for movements from the hepatic-bed erythrocyte involvement becomes more important (Elwyn *et al.* 1972). Whether this is true for ruminants is controversial (cf. Heitmann & Bergman, 1980; Houlier *et al.* 1991) and, furthermore, it is not resolved whether the transfers of AA from both blood and plasma across specific tissue beds occur in the same direction (cf. Aoki *et al.* 1976; Heitmann & Bergman, 1980).

For various reasons, net AA exchanges across tissues should be measured in blood but in isotopic studies plasma is often used (e.g. Bennet *et al.* 1990; Tessari *et al.* 1990). One reason for this decision relates to the different isotope activities observed for blood and plasma free AA following infusion of a labelled form (Barrett *et al.* 1987). Although probably neither blood nor plasma isotope activities define accurately that of the true precursor(s) for tissue protein synthesis, comparisons of both whole-body and tissue protein fluxes based on continuous infusion and arterio–venous (A–V) procedures provide important semi-quantitative data and can identify mechanisms which underlie metabolic responses. Both the magnitude and direction of AA movements can be affected by the choice of either the blood or plasma free amino acid pools but for isotope movements, at least, the data available are limited. Consequently, the extent to which errors can arise due to selection of either plasma or blood is unclear.

As part of a study to monitor changes in ovine methionine metabolism in response to altered provision of methylated products (choline plus creatine), the use of U-^{13}C-labelled algal-protein hydrolysates provided additional kinetic information for other AA besides methionine. The protocol required A–V measurements, hence data on mass and isotope transfers for both blood and plasma across the portal-drained viscera (PDV) and liver were obtained. The specific effects on methionine metabolism of methyl group provision (Lobley *et al.* 1996) are reported elsewhere.

MATERIALS AND METHODS

Animals and surgical preparations

Six Suffolk cross wether lambs (age 9–15 months, 28–45 kg live weight) were surgically prepared with indwelling catheters in the aorta, mesenteric, portal and hepatic veins plus two temporary catheters in the external jugular veins, as described by Lobley *et al.* (1995). Animals were offered 800 g lucerne (*Medicago sativa*) pellets/d (Lobley *et al.* 1995) as twenty-four hourly portions by means of automated feeders. In an attempt to ensure that methionine was the limiting amino acid, the sheep were continuously infused throughout with lysine (1·5 g/d) and histidine (0·5 g/d) into the mesenteric vein. The two treatments consisted of continuous infusion (20 g/h) either of (1) sterile saline (8·9 g NaCl/l) into the jugular and mesenteric veins or (2) choline (0·5 g/d), intra-jugular, and creatine (1·0 g/d), intra-mesenteric. Choline and creatine were dissolved in sterile saline and each solution adjusted to pH 7·4. Treatments (1) and (2) were each continued for 14 d and, on day 5 of each treatment, infusions of U-^{13}C-labelled algal hydrolysate were administered. Other measurements of methionine kinetics and wool growth which were made during the 14 d infusion period have been reported separately (Lobley *et al.* 1996). Treatments were successive and three sheep received the 14 d saline infusion first while the other three started with the choline and creatine infusions. In two sheep, already used in a previous study (Lobley *et al.* 1995), catheter failures meant that in one case only arterial blood samples were obtained, under both control and treatment conditions, while for the other sheep complete trans-splanchnic samples were obtained only during the control infusion. Data from all sheep are included in the calculations of whole-body AA fluxes but tissue exchange values are restricted to the four animals which successfully completed the experiment.

Preparations of ^{13}C-labelled amino acids

Lipid- and starch-extracted freeze-dried phototrophic microalgae, which had been grown in an atmosphere of $^{13}CO_2$, was obtained from Martek Corporation (Columbia, MD, USA). This was then hydrolysed to predominantly constituent free AA in redistilled 5·8 M-HCl (200 ml/g algal powder) in the presence of dithiothreitol (DTT, 100 mg) and phenol crystals (5–10 mg) under reflux in an atmosphere of pure N_2 for 18 h. The hydrolysate was taken to dryness under reduced pressure, dissolved in 0·1 M-sodium phosphate buffer, pH 7·4 (100 g/g algal powder) containing DTT (0·5 g/kg), centrifuged at 1000 *g* to remove debris and then filtered under aseptic conditions through a 0·2 μm filter. If it was to be used directly the solution was diluted with an equal weight of sterile saline and sodium heparin (final concentration 400 i.u./g solution) was added. If storage was likely to exceed 7 d, 100 g portions of the alga–sodium phosphate solution were freeze-dried and kept at −20° until required for use, then 200 g saline was added, followed by refiltration under aseptic conditions before addition of sodium heparin as described previously.

The free AA composition of each hydrolysed preparation was quantified by ion-exchange chromatography with lithium citrate buffers, using an Alpha Plus Amino Acid Analyzer (Pharmacia-LKB Biochrom Ltd, Cambridge), with L-norleucine as the internal standard and a ninhydrin detection system. The average recovery of AA was 450 g/kg original algal powder.

Infusion protocol and blood sampling

The equivalent of 1 g original algal powder was continuously infused (20 g infusate/h) into a jugular vein over a 10 h period. Over the last 6 h of infusion 0·18 M-sodium *p*-aminohippurate (pH 7·4; 20 g/h) was infused into the mesenteric vein to allow determination of plasma flow. Blood samples were withdrawn continuously by peristaltic pump from the aorta, hepatic portal and hepatic veins (10 ml/h per catheter) over hourly intervals during the last 4 h of infusion (Lobley *et al.* 1995). The nature of the preparation meant that other U-^{13}C-labelled cell products were also present and, therefore, estimates of total AA oxidation through $^{13}CO_2$ kinetics were not attempted. Packed cell volume (PCV) was determined by haematocrit.

Approximately 7 ml blood was centrifuged at 1000 *g* for 10 min and 1 g of the plasma taken for *p*-aminohippurate analysis. To a known weight (about 3 g) of the remaining plasma was added 0·2 parts by weight of a solution containing 1 mmol L-norleucine/kg and 5 mmol DTT/kg. The remainder of the blood (approximately 3 g) was haemolysed with an equal weight of an aqueous solution containing 0·15 mmol L-norleucine/kg and 1 mmol DTT/kg. These samples were stored as approximately 1–1·2 ml portions in micro-centrifuge tubes at −20° until required for further analysis. Samples of blood and plasma were obtained on the day before infusion and prepared similarly to provide natural abundance background samples for gas chromatography–mass spectrometry (GCMS) analyses.

Chemical analyses

Plasma flow (PF; g/min) was determined as described previously (Lobley *et al.* 1995); blood flow was then derived from PF/(1-PCV). Samples of the stored plasma and haemolysed blood were thawed to 4°, an important consideration to inhibit peptide and protein hydrolysis, and deproteinized by addition of 0·15 vol. sulphosalicylic acid (480 g/l) and centrifuged at 13000 *g* for 5 min. The supernatant fractions of those destined for free AA analysis were clarified through 0·22 μm micro-centrifuge filters (Sigma Chemical Co., Poole, Dorset) and the filtrates adjusted to approximately pH 2 with NaOH (100 g/l) before immediate analysis using the Alpha Plus Amino Acid Analyzer. For blood free AA, proline quantification was hampered by contamination from erythrocyte glutathione, while

in both plasma and blood a small overlapping peak created difficulties with threonine estimation. For these reasons caution should be exercised in interpretation of data for these AA. The concentrations of glycine, and occasionally valine, in the fluids were so large that a second run at a lower injection volume was necessary to remain within the linear range of the ninhydrin reaction.

Those supernatant fractions destined for GCMS analyses were desalted by application to 0·6 ml Dowex-50 (H^+, 100–200 mesh, $\times 8$), washed twice with 1 ml water and eluted with 3 ml 4 M-NH_4OH; the eluate was freeze-dried and stored at $-20°$.

GCMS analyses

AA from the freeze-dried eluate were prepared as the *n*-butyl heptafluorylbutyryl (HFB) derivatives, based on the methods of McKenzie & Tenaschuk (1979*a*, *b*); during this derivatization glutamine and asparagine are hydrolysed to the dibasic acids, which then appear with lower enrichments. GCMS analyses were performed using an HP5889A MS Engine (Hewlett Packard, Avondale, PA., USA). Most of the HFB-AA were then examined in a single chromatographic step under negative chemical ionization (CI) conditions with separations performed, under split (1:40) conditions, on a 30 m × 0·25 mm × 25 μm SE-30 capillary column at 118° for 4 min followed by a 10°/min temperature change to 230° which was maintained for 5 min. Thus, fourteen AA could be analysed with a 15 min run. The source temperature was 200°, electron energy 230 eV with the reagent gas (methane) pressure at 1·3 Torr. Because of formation of multi-derivatives of HFB-arginine this was separated, at a greater concentration, under the conditions: 207° for 5·5 min, then a temperature rise of 30°/min to 220° which was maintained for 3 min. Either as a natural product or a consequence of the acid-hydrolysis conditions extensive quantities of D-lysine (22–43%) were present in the algal infusate and the two isomers were separated on a 25 m × 0·25 mm × 0·08 μm Chiral-L-VAL capillary column in the split mode under the conditions: 170° for 8 min followed by a temperature rise at 30°/min to 200° which was maintained for 3 min. All other AA had less than 2% of the D-isomer. HFB-methionine was separated under splitless conditions starting at 60° for 0·75 min then increased to 210° at 30°/min and after being held for 5 min was further elevated at 30°/min to 250° which was maintained for 4 min. Small amounts of impurities occasionally co-chromatographed with methionine and phenylalanine so these AA were also examined as the tertiary butyldimethylsilyl derivative (Calder & Smith, 1988), using electron impact (EI) ionization conditions with a source temperature of 200°, electron energy 70 eV and emission current 300 μA.

For both CI and EI determinations only fragment ions which contained all the original carbon atoms of the AA were monitored (Table 1), except for methionine for which molecules where all the non-methyl-carbon atoms were labelled were also included. Selective ion recording conditions for the m/z ions were used throughout. Since only the m+0 and (m+*n*) ions (where *n* is the number of carbon atoms in the molecule) were monitored, neither true molar % excess nor tracer:tracee can be calculated. Instead the values represent the relative enrichments (re) of $(m+n)/[(m+0)+(m+n)]$ or in the case of methionine $[(m+5)+(m+4)]/[(m+0)(m+5)+(m+4)]$ with the calculations based on

$$(R_s - R_0)/(1 + R_s - R_0) \text{ (Campbell, 1974),}$$

where R_s and R_0 are $(m+n):(m+0)$ ratios in an enriched and natural abundance sample respectively. For the individual amino acids in the infusate of algal hydrolysate, > 99% of the molecules were in the (m+*n*) form, i.e. with all carbon atoms fully labelled. Under these circumstances the re for the essential AA will approximate to mpe. For non-essential amino acids the (m+*n*) species may also be formed from other AA (or metabolites present in the

algae) and of course molecules with fewer than n labelled carbon atoms may also arise (Berthold *et al.* 1991).

Calculations

Whole-body irreversible-loss rate (ILR; mmol/h) for each AA was calculated from

$$\text{ILR} = ((0{\cdot}99/\text{re}_{b(\text{or } p)}) - 1) \times \text{I},$$

where b or p represent the relative enrichments of each free AA in either blood or plasma respectively and I is the rate of infusion (mmol/h) of the U-^{13}C labelled L-AA in the algal hydrolysate. The ILR values were converted to equivalent daily protein fluxes (PrF; g/d) by

$$\text{PrF} = \text{ILR} \times 2{\cdot}4 \times \text{MW}_{aa}/(\text{protein content}_{aa}),$$

where MW_{aa} is the molecular weight of the AA, and protein content_{aa} is the weight of the AA per 100 g ovine mixed body protein deposited during growth (values from MacRae *et al.* 1993).

Erythrocyte AA concentration, $[\text{AA}]_e$, was calculated from

$$([\text{AA}]_b - ([\text{AA}]_p \times (1 - \text{PCV})))/\text{PCV},$$

where the subscripts b and p refer to blood and plasma (concentration) respectively. Similarly, erythrocyte AA relative enrichment was derived from

$$(([\text{AA}]_b \times \text{re}_b) - ([\text{AA}]_p \times \text{re}_p \times (1 - \text{PCV})))/(\text{PCV} \times [\text{AA}]_e).$$

Both net mass and isotope transfers of AA between either blood or plasma and the PDV and hepatic tissues were calculated based on the principles described previously (Lobley *et al.* 1995). Thus, for isotope transfers of the uniformly-labelled AA molecules:

$$\text{PDV } (\mu\text{mol/min}) = \text{PVF} \times (([\text{AA}]_{pv} \times \text{re}_{pv}) - ([\text{AA}]_a \times \text{re}_a)),$$

$$\text{liver } (\mu\text{mol/min}) = (\text{HVF} \times [\text{AA}]_{hv} \times \text{re}_{hv}) - (\text{PVF} \times [\text{AA}]_{pv} \times \text{re}_{pv}) - (\text{HA} \times [\text{AA}]_a \times \text{re}_a),$$

where PVF, HVF and HA refer to blood (or plasma) flows (kg/min) in the hepatic portal vein, hepatic vein and hepatic artery, and the subscripts hv, pv and a refer to concentrations and re determined in blood (or plasma) from the hepatic portal vein, hepatic vein and artery respectively. Corresponding mass transfers were calculated after removing re from the equations.

Conversion of isotope transfers to a corresponding AA flux involves division by an reconsidered representative of the precursor pool for protein synthesis within the tissue. In reality, probably none of the vascular pools are strictly appropriate but to provide a comparison with conventional whole-body protein flux measurements (usually based on arterial(ized) blood enrichments) the re of the vascular pool(s) supplying the tissue have been used. Thus, for the PDV this is the arterial supply but for liver which receives an unbalanced mixture from the portal vein and the hepatic artery the mean re was calculated from AA concentration, re and blood (plasma) flow in each vessel.

For all the previous calculations values were determined for each time-point and then averaged for each treatment within an animal.

Statistics

Whole-body ILR, of AA based on both arterial blood and plasma re, were tested for effect of treatment in the four animals which completed the full study and, because there were no significant responses, these data were combined with those from the incomplete studies and analysed for differences between blood and plasma by ANOVA. All other data were tested using ANOVA for the four animals that completed the experiments. Animals were considered as blocks, with treatment and either fluid type (blood or plasma) or fluid site (arterial, portal or hepatic vein) as sub-blocks. Analyses were examined also for interactions

between treatment and fluid type or treatment and fluid site. For data from different sites the analysis was performed for four animals and two periods, i.e. in effect eight replications per site. The analysis of variance applied was essentially equivalent to that for a split-plot design where animal × period combinations formed the main plots and sites the sub-plots. Because of the small number of animals certain biological trends are considered worthy of discussion when probabilities attain 0·1 or less.

RESULTS

Gas-chromatography–mass-spectrometry analyses

The amount of AA in the mixed algal protein varied from 16 to 127 mg/g with histidine and lysine representing the respective extremes. This pattern differed from the relative proportions of AA ILR and, thus, the re (× 100) of the individual L-AA in blood varied from 0·17–1·20 (excluding glutamate which was substantially lower, probably due to a combination of high ILR values and dilution from hydrolysis of glutamine during the derivatization procedure). The precision expected depended on the relative proportions of the m and m+*n* peak areas occurring between the natural abundance and enriched samples. For AA containing only a few carbon atoms (e.g. glycine, alanine) the probability of reasonable quantities of the m+*n* species occurring naturally is greater than for leucine or phenylalanine, for example. Fortunately, the shorter-chain-length AA are present in greater proportions in the algal protein which mitigates the problem and, in consequence, the mean precision (SD) for the re (× 100) of most AA was 0·02. This can be compared with errors of 0·05 atom % excess for singly-labelled AA, e.g. [1-^{13}C]leucine, which are infused at amounts sufficient to raise vascular enrichments to five to ten times those achieved here; hence the precisions are of similar proportion.

Whole-body amino acid ILR

Significant differences ($P < 0·02$) existed for all AA between the re in arterial blood and plasma, with the latter always higher. The blood : plasma re varied between AA, with valine and isoleucine at 0·93–0·95; leucine and phenylalanine 0·81–0·86; threonine, serine, alanine, methionine, tyrosine and lysine 0·70–0·78; glycine 0·66, proline 0·60 and histidine 0·44. Obviously ILR values based on plasma or blood free AA enrichments also differed by similar proportions (Table 1). Data for glutamate and aspartate should not be considered because of amide hydrolysis during derivatization while the presence of arginase (*EC* 3.5.3.1) within the erythrocyte means that most of whole-blood arginine is present in the plasma (Covolo & West, 1947). The lowest calculated whole-body PrF was based on plasma proline enrichments (Table 1); similar values were obtained for lysine, tyrosine and threonine (158–165 g/d); aspartate, valine, glycine, leucine, arginine and alanine (193–211 g/d); histidine, phenylalanine, isoleucine and methionine (230–245 g/d); with the highest values recorded for serine and glutamate (> 262 g/d).

Amino acid mass and isotope distribution between plasma and blood

PCV values ranged from 0·35 to 0·26 between sheep but within animal were not significantly different between arterial, hepatic portal and hepatic venous blood nor between treatments (Table 2). For most AA the concentration in the plasma was significantly greater for the hepatic portal vein than arterial or hepatic vein samples, aspartate and glutamate being the exceptions (Table 2). Plasma volume averaged 0·71 of blood and for arterial samples only the concentrations of valine, isoleucine and phenylalanine matched this, i.e. plasma and erythrocyte concentrations were similar (Table 2). For threonine, alanine and tyrosine erythrocyte concentrations were apparently slightly greater than those in plasma, more so for serine, glycine, proline and leucine and considerably in excess for aspartate, glutamate,

Table 1. *Blood and plasma amino acid irreversible-loss rates (ILR) and equivalent whole-body protein flux (PrF) for young sheep offered 800 g lucerne* (Medicago sativa) *pellets/d**

			ILR (mmol/h)							
	Ions monitored		Blood		Plasma			Statistical significance of difference: P†	PrF‡ (g/d)	
Amino acid	m/z	m/z	Mean	SE	Mean	SE	SED		Blood	Plasma
Histidine	406	412	3·45	0·28	1·53	0·13	0·200	< 0·001	519	230
Isoleucine	363	369	2·91	0·21	2·72	0·21	0·037	< 0·001	259	242
Leucine	363	369	6·01	0·49	5·09	0·44	0·174	< 0·001	240	203
Lysine	574	580	4·43	0·28	3·34	0·23	0·116	< 0·001	209	158
Methionine§	320	325	1·11	0·11	0·85	0·10	0·049	< 0·001	323	245
Phenylalanine§	204	213	2·68	0·25	2·31	0·21	0·063	< 0·001	269	231
Threonine	351	355	4·18	0·33	3·19	0·25	0·132	< 0·001	217	165
Valine	349	354	3·90	0·29	3·72	0·28	0·024	< 0·001	206	196
Alanine	321	324	11·12	0·50	8·53	0·40	0·283	< 0·001	276	211
Arginine‖	778	784	4·94	0·16	4·05	0·26	0·075	0·021	256	210
Aspartate¶	421	425	13·49	1·36	5·43	0·66	1·051	< 0·001	480	193
Glutamate¶	435	440	34·56	3·50	17·12	2·39	2·801	< 0·001	878	435
Glycine	307	309	15·62	1·01	10·34	0·79	0·346	< 0·001	297	197
Proline	347	352	3·94	0·38	2·40	0·27	0·154	< 0·001	190	116
Serine	533	536	7·47	0·65	5·44	0·58	0·107	< 0·001	360	262
Tyrosine	431	440	2·12	0·19	1·60	0·15	0·070	< 0·001	210	159

* For details of animals and procedures, see pp. 218–221.

† Data are for six sheep, twelve observations; comparison of differences between blood and plasma by analysis of variance with animals treated as blocks, 11 residual df.

‡ Calculated from ILR adjusted to the mean composition of ovine body protein (MacRae *et al.* 1993).

§ Monitored under electron impact conditions; all other amino acids monitored under chemical ionization conditions.

‖ Arginine concentration in whole blood was very low on several occasions and only five blood values are reported; residual df 4.

¶ Values for aspartate and glutamate are compromised due to cleavage to the dibasic acid of asparagine and glutamine during derivatization prior to GCMS separation.

Table 2. *Plasma free amino acid concentrations and the distribution between erythrocytes and plasma for blood samples from the aorta (A), hepatic portal (P) and hepatic (H) veins of young sheep offered 800 g lucerne* (Medicago sativa) *pellets/d**

(Mean values for eight observations†)

	Plasma concentrations (μM)					Erythrocyte‡: plasma concentrations				
	A	P	H	SED	Statistical significance of difference†: *P*	A	P	H	SED	Statistical significance of difference†: *P*
Histidine	64	70	63	1·1	< 0·001	2·55	2·34	2·53	0·151	NS
Isoleucine	90	107	98	2·3	< 0·001	0·95	0·72	0·93	0·050	< 0·001
Leucine	124	141	134	4·6	0·008	1·48	1·39	1·60	0·172	NS
Lysine	178	197	184	2·9	< 0·001	2·75	2·51	2·50	0·184	NS
Methionine	15	20	17	1·0	< 0·001	0·34	0·12	0·24	0·134	NS
Phenylalanine	50	62	53	1·5	< 0·001	1·03	0·77	0·93	0·095	0·046
Threonine	129	142	131	2·1	< 0·001	1·16	1·41	1·36	0·193	NS
Valine	239	261	251	3·5	< 0·001	1·02	0·90	0·98	0·056	NS
Alanine	141	171	138	3·7	< 0·001	1·26	1·09	1·43	0·116	0·032
Arginine	142	152	140	3·0	0·004	§	§	§	—	—
Aspartate	24	29	33	3·0	0·029	4·24	4·11	5·44	0·821	NS
Glutamate	114	112	144	8·7	0·004	3·77	4·21	3·33	0·288	NS
Glycine	463	489	456	13·0	0·059	1·63	1·44	1·72	0·123	NS
Proline	110	129	113	5·7	0·011	1·41	1·28	1·54	0·285	NS
Serine	66	89	71	1·7	< 0·001	1·53	1·18	1·49	0·144	0·056
Tyrosine	51	62	54	1·6	< 0·001	1·17	0·88	1·12	0·111	0·048
Packed cell volume (PCV)	0·289	0·289	0·290	0·0004						

* For details of animals and procedures, see pp. 218–221.

† Four animals each with two treatments (plus and minus choline and creatine) by ANOVA with 12 residual df. There was no effect of methyl acceptor provision and probabilities refer to differences between sample sites. No significant treatment × sample site differences were observed.

‡ Calculated from (blood concentration − ((1 − PCV) × plasma concentration)) ÷ PCV, details on p. 221.

§ Blood arginine concentrations low due to action of arginase (*EC* 3.5.3.1) released from erythrocytes on haemolysis.

lysine and histidine. In contrast, methionine was present predominantly in the plasma. There were no significant differences in the erythrocyte AA concentrations between sample sites (data not shown) and, therefore, the erythrocyte:plasma values were lower in the hepatic portal vein (except for threonine and glutamate) than the aorta or hepatic vein (Table 2). These latter two values were statistically similar.

The re of all free AA in arterial plasma exceeded significantly those in hepatic portal and hepatic venous plasma, which themselves were not significantly different (Table 3). The calculated re in the erythrocyte were always significantly lower ($P < 0{\cdot}001$) than those in the respective plasma sample (Table 3); therefore, the proportion of labelled free AA in the plasma exceeded the mass distribution. The extent to which erythrocyte re were lowered varied markedly between AA. Valine showed the closest agreement between plasma and erythrocytes followed closely by isoleucine and phenylalanine, i.e. in line with the mass equilibrations. Poorer isotope exchange occurred for lysine, leucine, threonine and methionine. The greatest disparity between mass and isotope distributions was observed for histidine for which mass was concentrated in the erythrocyte (Table 2) but little of the label entered from plasma (from Table 3). Isotope exchange was limited for most of the non-essential AA, in particular glycine and glutamate, while tyrosine was also excluded from the erythrocyte cell to a much greater extent than that observed for the other aromatic AA, phenylalanine.

For all AA the re in the erythrocytes showed lower proportional changes between sample sites than did plasma and in no case were the values significantly different (Table 3).

Mass and isotope transfers across the portal-drained viscera and liver

Plasma flow across the splanchnic tissues was not altered by choline plus creatine infusion and averaged (*n* 8) 1286 (SE 61), 1355 (SE 75) and 69 (SE 22) g/min for portal vein, hepatic vein and artery respectively. Mean blood flow was approximately $1{\cdot}38 \times$ PF.

Across the PDV, mass transfers of most AA were similar between blood and plasma (Table 4), indicative that the majority of the appearance was in the latter. Significant differences existed only for threonine and glycine, both of which favoured blood. There was a negative uptake (i.e. removal from the systemic circulation) of glutamate from plasma. Daily free AA-N uptake (excluding glutamine, asparagine, ornithine, citrulline and tryptophan transfers) was 8·5 and 10·4 g N/d for plasma and blood respectively, equivalent to 47 and 57% of N intake.

Except for isoleucine and proline, hepatic AA transfers were similar between plasma and blood (Table 4). The substantial quantity of glutathione in the erythrocyte interfered with proline determination but no technical problems were apparent with isoleucine quantification. The proportion of AA apparently absorbed across the PDV which were extracted by the liver ranged, for plasma transfers, from 0·34 to 1·14; the branched-chain AA gave the lowest proportional extractions while absorbed glycine, alanine and histidine were removed completely. Similar proportional extractions were observed for blood, except for glycine (0·51 *v.* 1·08) and isoleucine (0·26 *v.* 0·50) where plasma removal was greater.

Transfers of ^{13}C-labelled AA across the PDV were not significantly different between blood and plasma (Table 5) and were all negative, except for arginine, indicative of removal for tissue protein synthesis and other metabolic purposes. These values can be converted to equivalent PrF, based on the AA composition of total body protein and selection of free AA enrichment from either arterial or portal vein blood (plasma) as most representative of the precursor pool for protein synthesis. For the essential AA, and based on plasma enrichments, arterial PrF ranged from 32 to 62 g/d with the lowest values for valine, phenylalanine and histidine (Table 6). Over all, these represented 15–39% of the whole-body PrF (plasma) determined using the equivalent AA (Table 6). Extremes were recorded for histidine (low) and lysine (high), but the average contribution was 23 (SE 2)%. For

Table 3. *Relative enrichments* ($\times 100$) *of* (m+n) *ions*† *in plasma and erythrocytes from the aorta* (*A*), *hepatic portal* (*P*) *and hepatic* (*H*) *veins of young sheep offered 800 g lucerne* (Medicago sativa) *pellets/d**†

(Mean values for eight observations‡)

	Plasma					Erythrocyte§			
	A	P	H	SED	Statistical significance of difference‡: *P*	A	P	H	SED
Histidine	0·375	0·315	0·308	0·011	< 0·001	−0·002	0·000	0·001	0·004
Isoleucine	0·578	0·440	0·425	0·010	< 0·001	0·425	0·419	0·423	0·024
Leucine	0·735	0·546	0·514	0·015	< 0·001	0·343	0·365	0·359	0·023
Lysine	1·068	0·828	0·820	0·019	< 0·001	0·539	0·511	0·506	0·023
Methionine	0·788	0·516	0·519	0·026	< 0·001	0·081	0·189	0·173	0·078
Phenylalanine	0·723	0·510	0·503	0·021	< 0·001	0·456	0·389	0·410	0·079
Threonine	0·706	0·551	0·538	0·017	< 0·001	0·296	0·273	0·261	0·038
Valine	0·661	0·578	0·564	0·007	< 0·001	0·542	0·567	0·511	0·035
Alanine	0·567	0·387	0·361	0·026	< 0·001	0·124	0·133	0·159	0·027
Arginine	0·493	0·440	0·425	0·008	< 0·001	‖	‖	‖	—
Aspartate	0·714	0·441	0·415	0·048	< 0·001	0·131	0·115	0·082	0·023
Glutamate	0·224	0·194	0·174	0·008	< 0·001	0·041	0·038	0·009	0·014
Glycine	0·406	0·317	0·343	0·005	< 0·001	0·091	0·015	0·002	0·096
Proline	1·050	0·822	0·805	0·026	< 0·001	0·185	0·183	0·110	0·090
Serine	0·417	0·234	0·205	0·025	< 0·001	0·108	0·104	0·109	0·024
Tyrosine	0·821	0·606	0·590	0·017	< 0·001	0·111	0·118	0·140	0·017

* For details of animals and procedures, see pp. 218–221.

† Calculations based on ratios of $(m+n)/[(m+n)+(m+0)]$ ions where *n* is the number of C in the amino acid except for methionine where the m+5 and m+4 ions are summated, see p. 220.

‡ Four animals each with two treatments (plus and minus choline and creatine) by analysis of variance with 12 residual df. There was no effect of methyl acceptor provision and probabilities refer to differences between sample sites. No significant treatment × sample site differences were observed.

§ Calculated from [(blood concentration × relative enrichment) − ((1 − PCV) × plasma concentration × relative enrichment)] ÷ PCV, where PCV is packed cell volume; for details see p. 221.

‖ Not calculated.

Table 4. *Portal-drained viscera appearance and hepatic removal of free amino acids from plasma and blood (μmol/min) for young sheep offered 800 g lucerne* (Medicago sativa) *pellets/d**

	PDV appearance†				Hepatic removal†			
	Plasma	Blood	SED	Statistical significance of difference‡: *P*	Plasma	Blood	SED	Statistical significance of difference‡: *P*
Histidine	8·0	8·6	4·36	NS§	−8·7	−11·2	5·10	NS
Isoleucine	22·0	17·6	3·51	NS	−11·1	−4·6	3·06	0·08
Leucine	26·4	22·9	3·36	NS	−9·1	−4·5	3·99	NS
Lysine	24·2	33·2	6·54	NS	−13·7	−20·2	5·92	NS
Methionine	7·7	5·6	1·57	NS	−4·1	−3·1	1·25	NS
Phenylalanine	16·6	15·0	2·55	NS	−12·8	−13·7	1·70	NS
Threonine	15·4	31·0	5·64	0·032	−13·1	−22·2	7·54	NS
Valine	28·5	22·9	6·91	NS	−13·3	−10·0	7·68	NS
Alanine	38·4	43·3	4·70	NS§	−43·8	−45·2	7·36	NS
Arginine	20·5	17·6	13·24	NS‖	−14·8	−12·7	8·40	NS‖
Aspartate	7·4	10·7	7·42	NS	7·2	28·4	12·33	NS
Glutamate	−11·0	3·2	10·48	NS	48·0	50·9	9·25	NS
Glycine	45·3	112·6	15·60	0·023	−48·6	−56·7	16·68	NS
Proline	24·5	27·6	16·55	NS	−23·5	1·1	9·32	0·039
Serine	29·7	29·4	3·75	NS	−22·4	−21·3	2·52	NS
Tyrosine	14·9	12·8	1·35	NS	−12·4	−11·7	3·36	NS

* For details of animals and procedures, see pp. 218–221.

† Positive values indicate net appearance across tissue bed, negative values net removal. Based on net movements from blood (plasma) flow × AA concentrations as described on p. 221.

‡ By analysis of variance for four sheep each on two treatments as described in Table 2. Differences between plasma and blood net transfers have six residual df except for glycine (3 residual df) and arginine (5 residual df) across the PDV and arginine (5 residual df) across the liver.

§ Significant effects between treatments for alanine ($P = 0{\cdot}031$) and histidine ($P = 0{\cdot}051$) with lower appearances during infusion of choline plus creatine across the PDV and for valine ($P = 0{\cdot}044$) and methionine ($P = 0{\cdot}033$) across the liver with again lower removal values in the presence of the two methyl group acceptors; 3 residual df.

‖ Values for blood arginine transfers compromised by presence of arginase (*EC* 3.5.3.1) in the erythrocyte.

Table 5. *Blood and plasma ^{13}C-labelled amino acid transfers (nmol/min) across the portal drained viscera and liver of young sheep offered 800 g lucerne* (Medicago sativa) *pellets/d**

	PDV transfer†				Hepatic transfer†			
	Plasma	Blood	SED	Statistical significance of difference‡: *P*	Plasma	Blood	SED	Statistical significance of difference‡: *P*
Histidine	−19	−11	14·7	NS	−34	−5	18·5	NS
Isoleucine	−57	−80	19·8	NS	−74	−53	13·8	NS
Leucine	−166	−172	17·4	NS	−113	−98	19·6	NS
Lysine	−269	−313	37·3	NS	−156	−205	40·3	NS
Methionine	−25	−20	8·2	NS	−20	−11	8·6	NS
Phenylalanine	−48	−35	19·7	NS	−78	−84	10·2	NS
Threonine	−142	−92	40·7	NS§	−104	−146	42·2	NS
Valine	−83	−123	41·1	NS	−135	−147	61·9	NS
Alanine	−146	−153	37·4	NS	−215	−199	28·6	NS
Arginine	1	‖			−98	‖		
Aspartate	−50	−43	16·2	NS	21	38	36·9	NS
Glutamate	−68	−62	26·6	NS	56	−5	17·7	0·013
Glycine	−94	−18	111·4	NS	−363	−370	71·5	NS
Proline	−136	−28	109·3	NS	−182	−93	55·3	NS
Serine	−73	−64	20·4	NS	−87	−86	14·9	NS
Tyrosine	−56	−47	15·7	NS§	−89	−77	14·8	NS

* For details of animals and procedures, see pp. 218–221.

† Positive values indicate net appearance across tissue bed, negative values net removal. Based on tissue inflows and outflows of [m+*n* ^{13}C] amino acids from blood (plasma) flow × amino acid concentration × relative enrichment; for details, see p. 221.

‡ By analysis of variance for four sheep each on two treatments as described in Table 2. Differences between blood and plasma transfers have six residual df except for glycine (four residual df).

§ Significant interaction between treatment and fluid.

‖ Not calculated due to loss of arginine through erythrocyte arginase (*EC* 3.5.3.1) activity.

Table 6. *Protein fluxes (PrF, g/d) across the portal-drained viscera (PDV), the contribution to whole-body flux (WBF) and isotope dilutions across splanchnic bed tissues of young sheep offered 800 g lucerne* (Medicago sativa) *pellets/d**

	PDV PrF (g/d)†		% WBF‡		Isotope dilution§ PV:A		Isotope dilution§ HV:PV	
	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood
Histidine	34·8	54·5	15·2	10·5	0·65	0·88	0·80	0·93
Isoleucine	48·7	78·6	20·1	30·3	0·68	0·80	0·92	0·96
Leucine	48·4	71·1	23·9	29·6	0·67	0·80	1·01	0·95
Lysine	61·9	106·2	39·2	50·8	0·72	0·83	1·00	0·97
Methionine	56·0	58·6	22·9	18·1	0·55	0·65	1·02	1·03
Phenylalanine	32·4	31·6	14·0	11·8	0·62	0·77	0·99	0·96
Threonine	61·2	51·1	37·0	23·5	0·68	0·82	1·28	1·19
Valine	39·5	62·7	20·1	30·5	0·78	0·90	0·98	0·94
Alanine	34·3	53·8	16·2	19·5	0·60	0·73	0·94	0·95
Arginine	8·8	—	4·2	—	0·56	—	0·87	—
Aspartate	9·8	20·0	5·1	4·2	0·51	0·51	1·03	1·53
Glutamate	38·7	81·4	8·9	9·3	0·78	0·83	0·97	0·60
Glycine	12·7	46·5	6·5	15·7	0·75	0·93	0·77	0·93
Proline	19·0	9·4	16·4	4·9	0·73	0·68	1·03	0·88
Serine	41·8	60·3	15·9	16·7	0·48	0·63	0·86	0·83
Tyrosine	36·0	48·9	22·7	23·3	0·67	0·80	0·99	0·97

PV, portal vein; A, artery, HV, hepatic vein.

* For animals and procedures, see pp. 218–221.

† Calculated from isotope transfers (Table 5) corrected for time and concentration of amino acid in protein gain (MacRae *et al.* 1993) and based on arterial free amino acid enrichment as precursor. Conversion to values based on PV enrichment as precursor can be obtained by dividing by the PV:A isotope dilutions.

‡ Values are percentage contribution to protein WBF (from arterial relative enrichments) based on calculations for the corresponding individual amino acids (see Table 1).

§ Free amino acid relative enrichment ratios.

blood transfers, arterial PrF ranged from 31 to 106 g/d (phenylalanine and lysine were the extremes) or from 11 to 51% of PrF (blood), with a mean of 26 (SE 5)%, or 22 (SE 3)% if lysine is excluded. Enrichment dilutions (hepatic portal vein:artery) across the PDV, with aspartate excluded, were always greater for plasma than for blood and ranged from 0·48 to 0·78 and from 0·63 to 0·93 respectively (Table 6).

The corresponding isotope dilutions across the liver were less than those for the PDV and ranged from 0·77 to 1·28 for plasma and from 0·60 to 1·19 for blood (Table 6). With the exception of glutamate and aspartate there was net hepatic removal of label for the AA (Table 5). Direct conversion of these values to protein equivalents would be misleading as the metabolic fates of the removed AA involve substantial oxidation and transformation into other substrates in addition to protein synthesis. If the amounts of ^{13}C removed from plasma across the liver are compared with those extracted by the PDV then values of 0·65–0·70 are observed for threonine, leucine and lysine while, for the remainder of essential AA, hepatic extraction exceeded that from the PDV by 1·28–1·95 (from Table 5).

DISCUSSION

Whole-body amino acid and protein flux

Whole-body flux measurements have become a common feature of many protein metabolism studies involving either large animals or man. The method is simple to apply, requires a minimum of vascular catheterization, but is subject to a series of limitations.

First, the method, at least when based on the isotope activities of plasma free AA, provides only minimum values for whole-body turnover since the isotope dilutions are based on changes in the infused (primary) pool. Second, it is not known how much of the transfer into the tissues is from the erythrocytes as opposed to plasma, a situation complicated if the isotope activity of the AA in these pools differ. Third, only a single AA is normally used as tracer (e.g. leucine, phenylalanine, lysine), with assumptions made as to the partition of the infused isotope between protein synthesis, AA oxidation and, if appropriate, conversion to other metabolites. Extrapolations are often then made to whole-body protein kinetics, but imbalances in the amount of a specific AA available from dietary sources, or released from endogenous protein degradation, can result in misleading estimates. Elements of the latter two limitations are addressed within the current study.

Obled *et al.* (1989) compared the disposal rates from plasma of four AA, infused simultaneously into rats, and reported close agreement for oxidation-corrected protein flux between leucine, lysine and tyrosine but with somewhat greater values for threonine. Other studies which have compared phenylalanine and leucine have, for man and dogs, yielded data in which calculated protein flux is higher with the former (Barrett *et al.* 1987; Bennet *et al.* 1990; Biolo *et al.* 1992), which contrasts with observations in fed sheep where whole-body protein synthesis rates were slightly greater for leucine (Harris *et al.* 1992). The current data are uncorrected for oxidation of the individual AA, but for the essential AA good agreement for estimated PrF was obtained between lysine, threonine (and tyrosine). Somewhat greater (22–26%) values were observed with leucine and valine. Histidine, phenylalanine, isoleucine and methionine were higher still (43–52%), while the largest flux was recorded for histidine based on blood enrichment. Based on plasma AA fluxes, Cronjé *et al.* (1992) observed good agreement between lysine, leucine and methionine for sheep fed on a roughage diet low in protein, but at high protein intakes lysine ILR increased by 50% while the other two were more than doubled, thus creating a considerable disparity.

Such differences between the AA can be based on four possibilities. First, the AA composition of protein degraded within the body may differ from that for retained protein. The half-life of specific proteins can vary from minutes to weeks, although current evidence does not suggest a sufficiently great disparity in AA composition to account for the calculated differences. Second, the pattern of absorbed AA favours those with the greater flux and differences in the relative proportions of absorbed essential AA compared with those in body protein are known to exist for ruminants (see MacRae & Reeds, 1980). Third, and linked to the previous comment, the amount of absorbed AA removed by the liver varies considerably (e.g. Wolff *et al.* 1972; Biolo *et al.* 1992; Lobley *et al.* 1995 and present study). Thus, the isotope dilution occurring in the peripheral blood or plasma will be less for those with larger hepatic extractions and lower apparent fluxes will result. Fourth, the extent of interchange between the tissues and either plasma or erythrocyte fluids may vary and, because intracellular isotopic activities will be lower due to entry of unlabelled AA from protein breakdown, this will alter the apparent flux.

Plasma and erythrocyte amino acid mass and isotope distributions

For some AA, concentrations were similar between plasma and blood as reported by others (e.g. Heitmann & Bergman, 1980) and in accordance with non-concentrative, Na^+-independent transport across the erythrocyte membrane (see Young & Ellory, 1977). For others, the concentration within the erythrocytes was greater than that in plasma and, of the essential AA, this was particularly noticeable for histidine and lysine, as observed in other species (Dewes *et al.* 1977). Despite this, [^{13}C]histidine was almost completely excluded from the erythrocyte. Several explanations may account for this observation.

First, conformational changes may occur with histidine when all the ^{12}C atoms are replaced with ^{13}C such that the transporter is either unable to recognize the AA and/or effect transfer. Second, histidine cannot enter against the concentration gradient and, indeed, the rate of entry for AA into the erythrocyte is generally lower for ruminants than for non-ruminants, including man (Young & Ellory, 1977), with histidine amongst the slowest (Young *et al.* 1976; Vadgama & Christensen, 1985).

This latter point raises the specific question as to how such gradients are established and does this relate to the general observation that enrichments for free AA within the erythrocyte are lower than those in plasma? Again several possibilities exist. First, the rate of equilibration between plasma and the erythrocyte might be slow and, because infusion of the hydrolysate is into the plasma, a permanent differential would be established. A pseudo-plateau enrichment was established, however, for plasma AA for the last 6–7 h of the infusion and, within this period, there was no indication of blood enrichments continuing to rise (data not shown), as would be expected if a slow, but finite, rate of transport was occurring (Young *et al.* 1976; Vadgama & Christensen, 1985). The second option involves synthesis, or production, of free AA from low- or unlabelled sources within the erythrocyte. For certain of the non-essential AA, biosynthesis of the carbon skeleton or transamination of the appropriate oxoacid (Dauman *et al.* 1989) provide potential routes of production, but this is not generally applicable to the essential AA. Erythrocytes, however, do contain considerable oligopeptide hydrolase activity (Adams *et al.* 1952) and the uptake and hydrolysis of exogenous or synthetic peptides has been demonstrated in both man *in vitro* and goats *in vivo* (e.g. King & Kuchel, 1985; Odoom *et al.* 1990; Lochs *et al.* 1990; Backwell *et al.* 1994), as has protein degradation within the reticulocyte (Rapoport *et al.* 1985). Furthermore, it has been proposed that erythrocyte concentrations of glycine are maintained by hydrolysis of glutathione (Dauman *et al.* 1989). Although erythrocyte involvement is not considered a major contributor to whole-body peptide clearance, rates of hydrolysis may be sufficient to cause the dilutions in enrichment observed during the current study. The presence of histidyl-peptides, notably carnosine, in plasma may provide sufficient substrate to generate *in situ* erythrocyte free histidine to maintain the observed concentration gradient and lead to the exclusion of the labelled AA. AA may arise also from intracellular proteolysis, as has been shown for reticulocytes (e.g. Müller *et al* 1980; Rapoport *et al.* 1985) and this might provide a general mechanism for the isotope dilutions observed.

Inter-organ amino acid transport

An alternative explanation for the plasma:erythrocyte differences might relate to inter-organ transport. Early studies on net AA movements between body tissues led to the hypothesis that transport from the gastrointestinal tract (GIT) to the liver and from peripheral tissues to the GIT was predominantly via the plasma, but that a substantial proportion of AA transported from the liver to the periphery was in the erythrocytes (Elwyn *et al.* 1972). Because free AA isotope activities are lower in the liver cytosol of non-ruminants and ruminants compared with plasma (Garlick *et al.* 1973; Eisemann *et al.* 1989), due to dilution from protein degradation, then if the intracellular pool exchanged directly with the erythrocyte this would lower isotope activity within the erythrocyte.

Support for the concept advanced by Elwyn *et al.* (1972) has been obtained from cattle data based on the shifts in plasma:blood free AA concentration between arterial and hepatic portal blood (Houlier *et al.* 1991). Hepatic portal venous blood represents mixed drainage from the small intestine which adds AA to the bloodstream during the absorptive phase of digestion, and from the forestomachs (including the pancreas) and caecum, which are net removers so that mesenteric-drained viscera net uptake exceeds that of the PDV

(Reynolds & Huntington, 1988). Hepatic portal venous blood composition is, therefore, a result of both entry and removal of AA and simple comparisons may not accurately reflect the site of transport for those newly absorbed. With this reservation in mind, the current study supports the concept that the majority of AA absorbed across the PDV are carried predominantly in plasma with threonine and glycine as possible exceptions.

For hepatic transfers the situation is less clear, as the extensive data of Heitmann & Bergman (1980) failed to observe a preferential transport of amino acids from the liver in erythrocytes in contrast to the findings of Elwyn *et al.* (1972) and Houlier *et al.* (1991). If AA movements across the liver involved removal from the plasma and/or addition to the erythrocytes then disappearance from plasma should exceed that from blood. In the present study, only for isoleucine and proline was this shown to occur at a statistically significant level, although the two other branched-chain amino acids showed a similar trend. Trans-hepatic erythrocyte exchanges would, if a general phenomenon, also result in labelling of erythrocyte histidine and, possibly, more marked changes than those actually observed in re between hepatic portal and hepatic venous erythrocytes. It is unlikely, therefore, that substantial transport of AA from the liver has occurred under the current experimental situations.

Splanchnic tissue amino acid and protein metabolism

Leucine isotope transfers across the splanchnic tissues within this multi-AA approach can be compared with data obtained for sheep of similar weight and intake but infused, more conventionally, with single-labelled [1-^{13}C]leucine (Lobley *et al.* 1995) and based on a similar choice of precursor pool enrichment. Rates were slightly greater in the present study across the PDV (-37 *v.* -30 μmol/min) and liver (-22 *v.* -13 μmol/min) but are uncorrected for oxo-acid conversions and oxidation to CO_2 which, if similar conditions to the previous conditions prevail, would reduce the flux for protein synthesis by 2 and 5 μmol/min respectively. This indicates that, although measured at lower enrichments, values for the [U-^{13}C]leucine are similar to accepted procedures and, furthermore, isotope discrimination does not appear to be a serious problem. Despite the reasonable similarity in whole-body ILR between most of the essential AA there was a greater disparity for values across both the PDV and liver. Thus, both the proportions of AA flux and equivalent PrF values varied over a 2–3-fold range, with precursor pool selection influencing the absolute magnitude of the values. The latter problem is exaggerated in the fed condition, when substantial isotope dilution occurs within the enterocytes (Lobley *et al.* 1980) and probably to different extents for each AA.

The current isotope method has certain limitations, one being that the various metabolic fates of the AA are not monitored and, thus, corrections cannot be applied to the gross ILR or PrF estimations. This problem is best exemplified for the liver where the extracted AA can be used for protein synthesis, metabolite formation (e.g. hippurate, glutathione) or be oxidized. Catabolism of AA is a major function of the liver but currently few data are available for ruminants to resolve hepatic partition of their fates and, for essential AA, these are restricted to leucine (Pell *et al.* 1986; Lobley *et al.* 1995). The branched-chain AA are interesting in that a relatively small proportion of the amount absorbed is removed by the liver and oxidation is divided between hepatic and peripheral tissues (Pell *et al.* 1986). What is required is information on the fates of, for example, phenylalanine or methionine, for which most of the absorbed fraction is removed by the liver but where sufficient quantities must still be available to support peripheral tissue gain. In particular, it is necessary to determine how much of the AA leave the liver in non-free form, i.e. as small proteins or peptides which then provide substrates for peripheral tissue protein synthesis.

A second limitation to the kinetic approach involves interconversions between AA. To

use conventional mpe or tracer:tracee requires quantification of all the ions from m+0 to m+*n*. For most essential AA this is not necessary as the purity of the initial tracer means that new labelled intermediates will not arise. The exception is methionine because the labelled methyl group can be replaced during the methionine↔homocysteine cycle (see Lobley *et al.* 1996). Such replacement will probably involve a non-labelled moiety (thus creating an m+4 ion from the original m+5 methionine ion), but if the methyl group arises from, for example, serine metabolism then the m+5 species would be reformed, and as this could not be distinguished from the original methionine molecule, then the rate of transmethylation but not the net flux of methionine would be underestimated. The dilution of labelled serine which occurs in the body is considerable (Table 3), however, and the probability of label return is correspondingly small. Similar considerations arise for tyrosine derived from phenylalanine. Potentially more serious errors arise with other non-essential AA, for example glycine–serine and proline–arginine–glutamate interconversions. Indeed glutamate metabolism using this approach was studied in detail in the laying hen (Berthold *et al.* 1991) and two main labelled species occurred, m+5 and m+2. The latter would arise from glutamate derived from tricarboxylic acid cycle reactions, while the m+5 species represents either the original labelled infused AA or that arising from for example arginine or proline metabolism. Again, however, the respective dilutions reduce the probability of m+5 glutamate arising from intermediary metabolism and, anyway, as indicated earlier both glutamate and aspartate kinetics are compromised by hydrolysis of their respective amides during the derivatization procedure. Nonetheless, the use of isotopomer analysis (the identification of the extent of labelling for each atom of a metabolite) is a very powerful resource of GCMS techniques and offers a simple and rapid approach to quantifying metabolic rates and identifying the source and fate of individual moieties within a molecule.

Despite the fact that the liver of the ruminant lamb has a considerable fractional synthesis rate (20%/d; Lobley *et al.* 1994), isotope dilution across the hepatic bed was much lower than that observed for less-active tissues e.g. muscle and skin (Harris *et al.* 1992, 1994). Thus, either the isotope flux is primarily uni-directional (from plasma into the hepatocyte) or the free AA enrichments of the blood supply (predominantly the hepatic portal vein) and the intracellular pool are more similar than those for the corresponding comparisons across peripheral tissues. Both possibilities may be correct. The absolute and fractional removal of blood (plasma) AA is much greater across the liver than the hind leg (cf. Harris *et al.* (1992) *v.* present study). The uptake of AA from the blood, therefore, will make the greatest contribution to the fractional turnover of the intracellular pool, e.g. for phenylalanine the relative inflows from uptake and protein degradation (Lobley *et al.* 1994) can be calculated at 4:1. In consequence, intracellular:extracellular enrichments will be $> 0{\cdot}8$ and, indeed, the mean enrichment of protein-bound lysine in human VLDL-apolipoprotein B-100, synthesized in the liver, attained 0·68 that of free lysine in peripheral venous plasma (Motil *et al.* 1994), a value similar to the PDV:arterial free lysine dilution observed in the current study.

Summary

Overall, the use of the uniformly-labelled ^{13}C-labelled algal protein hydrolysate provides a rapid means for identification of changes or abnormalities in either whole-body or trans-tissue fluxes of amino acids. In comparison with the conventional use of single-labelled tracer amino acids the method has the advantage of scanning up to fifteen amino acids simultaneously, although the costs are greater than when a single tracer amino acid is infused (commercially 1 g algal powder costs approximately £150). Such information not only increases general understanding but avoids distortions which may occur through use

of a single amino acid, which may not necessarily be representative of amino acid metabolism or protein flux. In combination with mass transfers the current data support the concept that absorbed amino acids are carried to and from the liver predominantly in the plasma. With regard to choice between blood or plasma pools for 'precursor' activity the branch chain amino acids and lysine offer the least uncertainty and methionine and histidine the greatest. The best 'precursor' selection for tissue protein studies may be resolved if the multi-amino acid infusion is used in combination with determination of enrichments of rapid turnover export proteins (e.g. Motil *et al.* 1994), although this approach may not necessarily be definitive for synthesis of constitutive proteins.

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