Determination of rates of protein synthesis, gain and degradation in intact hind-limb muscle of lambs

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A model of leucine metabolism in the hind-limb muscles of the milk-fed lamb was developed which permitted simultaneous estimation of the rates of protein synthesis (K_s , days⁻¹), degradation (K_d) and therefore gain (K_g) of muscle *in vivo*. The conclusions drawn from the model were: the rate of protein synthesis in muscle was related to uptake of leucine; the rate of degradation of protein was related to leucine output, as leucine, or its corresponding oxo acid, 4-methyl-2-oxopentanoic acid, or CO₂. These findings support findings drawn from a wide range of studies *in vitro*. There was no correlation between rate of protein synthesis and rate of protein degradation, which suggests that the method can allow independent estimates of each. Estimates of protein synthesis obtained from the model (of leucine metabolism in muscle) were compared with those obtained simultaneously by constant infusion of radioisotope and analysis of incorporation into tissue. There were no significant differences between the mean values obtained for synthesis (K_s), gain (K_g) and degradation (K_d) by either method (K_s 0.051 ± 0.002, 0.046 ± 0.007; K_g 0.016 ± 0.002, 0.004 ± 0.008; K_d 0.035 ± 0.004, 0.041 ± 0.008 day⁻¹, respectively, for tissue analysis and the model). However, K_s obtained from the model was significantly and positively correlated with uptake of leucine from plasma, whereas K_s obtained from tissue analysis was not.

INTRODUCTION

The rate of protein deposition is determined by rates of both protein synthesis and degradation in a tissue. These rates can be obtained in the process of determining the rate of interconversion of leucine and its corresponding oxo acid, 4-methyl-2-oxopentanoic acid (KIC), within tissues, through the use of a kinetic model (Nissen & Haymond, 1981, 1983). These procedures are of particular value for studies of protein turnover in large animals, since they permit serial measurement of rates of protein synthesis and degradation in individual tissues of the same animal, which is not possible with the large-dose or continuous-infusion methods that have been used in smaller species (Garlick *et al.*, 1973; McNurlan & Garlick, 1980). An added advantage is that the rates of protein degradation and gain are obtained over the same measurement period without assumptions as to the change in composition of the tissue. Moreover, since leucine has been implicated in control of the rate of protein synthesis and degradation (see, e.g., Tischler *et al.*, 1982) information about its metabolism within tissues may provide further clues as to the mechanisms which control protein deposition *in vivo*.

We describe here a model of leucine metabolism which we have applied to the hind-limb muscles of milk-fed lambs. It has been used to explore the relationship between leucine metabolism and protein synthesis, degradation and gain. To show that it provides some results comparable with those by previous methods, we have compared the rates of protein synthesis and degradation obtained from the leucine model with those obtained by a procedure using tissue analysis.

MATERIALS AND METHODS

Chemicals and radioisotopes

The sodium salts of KIC and 2-oxohexanoic acid, and L-leucine, were obtained from Sigma (Poole, Dorset, U.K.). L-Amino-acid oxidase (EC 1.4.3.2) and catalase (EC 1.11.1.6) were purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). L-[1-¹⁴C]Leucine, L-[4,5-³H]leucine, [1(n)-¹⁴C]- and [1,2(n)-³H]-hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. L-[4,5-³H]KIC was prepared from L-[4,5-³H]leucine by the procedure of Rudiger *et al.* (1972). All other chemicals and reagents were of the highest purity available from standard suppliers.

Animals and surgical preparation

Five male Clun Forest lambs were removed from their mothers at 2 days of age and bottle-fed with an artificial milk (Lamlac; Volac, Orwell, Herts., U.K.) four times daily. Each litre of Lamlac contained 200 g of dry matter, 5 g of N and 4.5 MJ of gross energy. At 4–10 days of age the lambs were placed in individual cages and Lamlac was offered from a reservoir from which they could suck at will, but to which the rate of supply could be controlled. Milk intake was recorded daily, and the lambs were weighed three times a week. The ages of the lambs during the subsequent experiment, their weights, rates of live-weight gain, and mean milk, N, and gross energy intakes for the 4 days preceding and the day of the experiment are shown in Table 1.

At 3–4 weeks of age, polyethylene catheters (0.8 mm internal diam., 1.2 mm external diam.; Dural Plastics,

Table 1. Ages and weights of the lambs on the day of the experiment, and their mean weight gains and mean ($\pm$ S.E.M.) milk, nitrogen and gross energy intakes for the 4 days preceding and the day of the experiment

Lamb	Age (days)	Live weight (kg)	Weight gain (g/day)	Intake of		
				Milk (g/day)	N (g/day)	Energy (MJ/day)
X458	37	13.9	283	2314 $\pm$ 44.6	18.5	10.3
X479	38	16.3	353	2670 $\pm$ 109.8	21.4	11.9
X532	38	14.5	364	2678 $\pm$ 190.2	21.4	11.9
X692	35	10.1	50	1500 $\pm$ 10.0	12.0	6.7
X694	36	9.8	133	1260 $\pm$ 98.5	10.1	5.6

Dural, N.S.W., Australia) were inserted into the jugular vein under local anaesthesia. Carotid-arterial catheters (polyvinyl chloride; 0.8 mm internal diam., 1.2 mm external diam.) were implanted under halothane anaesthesia on the same day. At approx. 5 weeks of age and 2 days before experiments, catheters were placed via the recurrent tarsal veins, under local anaesthesia, in the deep femoral vein of both hind legs. All catheters were filled with sterile heparin/saline (0.9% NaCl) solution (100 units/ml) and flushed thrice weekly.

Experimental

Three lambs (X458, X479, X532) were allowed unlimited access to milk throughout the experimental period, whereas the intakes of the remaining two lambs (X692, X694) were restricted to a constant (but lower) rate over a 24 h period.

A mixture of [1^{14}C]leucine and [$4,5\text{-}^3\text{H}$]KIC ($\sim 0.1 \mu\text{Ci}$ of ^{14}C plus $\sim 0.23 \mu\text{Ci}$ of ^3H /min) in saline was infused into a jugular vein at a constant rate for 6 h. This was carried out in the same cage in which the lamb had previously been maintained. To facilitate sampling of blood, each lamb was injected intravenously with 10000 units of heparin (Multiparin; 5000 units/ml; Weddel Pharmaceuticals, Wrexham, Clwyd, U.K.) before the first samples were taken, and heparin content was maintained by infusing 30 units/min between sampling periods. Blood samples (10 ml) were taken for determination of the packed cell volume (PCV), the concentrations of O_2 , CO_2 , leucine and KIC, and the specific radioactivities (SRA) of CO_2 , leucine and KIC. Blood was taken from the carotid artery and left and right deep femoral veins before, and at hourly intervals during, infusion.

Blood flow to both hind legs was determined by diffusion of ^3HOH (Oddy *et al.*, 1981). This measurement was made between 5 and 6 h of infusion. In two experiments blood flow was not determined and was estimated from the empirical relationship between arteriovenous difference for oxygen (X , mm) and blood flow (Y , $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$) that has been obtained in this laboratory;

$$Y = 0.173 - 0.0273X; r = 0.66, P < 0.05, n = 47$$

Immediately after the sample taken after 6 h, the infusion was stopped and the lambs were killed by rapid intravenous injection of a lethal dose of barbiturate (Sagatal; May and Baker, Dagenham, U.K.). Samples (approx. 10 g) of vastus lateralis, biceps femoris and gastrocnemius muscles were removed from each leg within 5 min of the end of the infusion, and they were

immediately frozen in liquid N_2 . These three muscles were chosen because together they represent 40% of hind-limb muscle mass, and receive 40% of hind-limb muscle capillary blood flow (Oddy *et al.*, 1981). All the muscles of the hind limb were then removed and weighed, and combined with those from other lambs used to predict rate of hind-limb muscle gain from live-weight gain (see below).

Analyses for PCV, O_2 and CO_2 concentrations and SRA of CO_2 were begun on the day of experiment. Plasma and tissue samples were stored at -20°C until analysis was practicable.

Analytical

Blood. CO_2 concentration and specific radioactivity were determined as described by Hinks *et al.* (1966). O_2 concentration was determined with a model 182 Co-oximeter (Instrumentation Laboratory Inc., Lexington, MA, U.S.A.) calibrated for sheep blood. PCV was determined with a Hawkesly micro-haematocrit centrifuge (Hawkesly, Lancing, Sussex, U.K.).

Plasma. Plasma was chosen for analysis of leucine and KIC, because preliminary studies with sheep blood showed there was little KIC in red cells (concentration in lysed cells was $2.27 \mu\text{M}$, compared with $12.8 \mu\text{M}$ in plasma). Plasma was also satisfactory for leucine analysis, since the arteriovenous difference was $22 \pm 3 \mu\text{M}$ ($n = 13$ samples), and the comparable whole-blood value ($18 \pm 4 \mu\text{M}$) was not significantly different.

Leucine and KIC concentrations and SRA were determined by a modification of the method described by Nissen *et al.* (1982). Plasma (2 ml), to which 0.4 ml of 1 M-HCl, 220 nmol of norleucine and 19.6 nmol of 2-oxohexanoic acid were added as internal standards for leucine and KIC determinations respectively, was passed through Dowex 50 W ion-exchange resin (H^+ form; 200–400 mesh; 1 ml bed volume). The acid fraction, containing KIC, was eluted with 4×1 ml of 0.01 M-HCl into 35 ml stoppered tubes. The amino acid fraction (containing leucine) was eluted with 4×1 ml of 4 M- NH_3 into a 20 ml scintillation vial. The acid fraction was then extracted as described by Nissen *et al.* (1982). The eluate containing the amino acids was freeze-dried and the dry material taken up in 1 ml of water. A 0.2 ml sample was taken for analysis of leucine, the remainder being used for measurement of radioactivity after addition of 10 ml of scintillation fluid. It was assumed that all the radioactivity in the amino acid fraction was in leucine, for preliminary studies with plasma from a goat infused with [^3H]leucine

indicated that there was no detectable radioactivity in any other amino acid.

The concentration of leucine was determined by automated amino acid analysis (Locarte Co., London W14, U.K.), by using Durrum DC6a resin in a 30 cm × 9 mm column maintained at 38 °C, and the first two lithium citrate buffers described by Lepoire & Pearson (1977). This resulted in separation of amino acids up to and including phenylalanine.

The concentration and specific radioactivity of KIC were determined by h.p.l.c. (Gilson Medical Electronics, Middleton, WI, U.S.A.) on a 25 cm × 4.5 mm Zorbax-ODS column by using 0.05 M-sodium phosphate buffer (pH 4.0)/acetonitrile (9:1, v/v) at 1 ml/min. KIC and 2-oxohexanoic acid were detected at 210 nm; 1 min fractions were collected before, during and after elution of the KIC peak and radioactivity was measured after addition of 10 ml of scintillator.

Tissue. Portions (~ 5 g) of tissue frozen in liquid N₂ were crushed in a percussion mortar cooled in liquid N₂, and then transferred to a pre-weighed 50 ml plastic centrifuge tube containing 3.0 ml of HClO₄ [30 ml of 72% (v/v) HClO₄ made up to 100 ml with water] plus the internal standards (550 nmol of norleucine and 49 nmol of 2-oxohexanoic acid), frozen in liquid N₂ and re-weighed. Ice-cold water (12 ml) was added and the tissue was homogenized in an Ultra-Turrax homogenizer (Camlab, Cambridge, U.K.). The probe was washed with 5 ml of HClO₄ (5 ml of 72% made up to 100 ml with water) and the washings plus original homogenate were centrifuged for 15 min at 1000 g. The precipitate was washed and re-centrifuged twice with 10 ml of HClO₄ (3%), and the washings plus original supernatant constituted the intracellular (protein-free) fraction. This was neutralized with 5 M-KOH, cooled in ice for 20 min, and centrifuged for 10 min at 1000 g. The supernatant was freeze-dried, resuspended in 8 ml of distilled water, filtered, adjusted to 10 ml and stored at -20 °C until analysis.

The HClO₄ precipitate was treated essentially as described by Nicholas *et al.* (1977). It was washed with the following solutions, centrifuged (10 min at 1000 g), and the supernatants were discarded. Two washings were made with 20 ml of 10% trichloroacetic acid, then one with 20 ml of ethanol/water (19:1, v/v) containing 100 g of potassium acetate/l, followed by 20 ml of warm (40 °C) chloroform/ethanol/diethyl ether (1:2:2, by vol.). The suspension was left for 15–20 min before centrifugation, and the precipitate finally was washed twice with 20 ml of ether.

The tubes and precipitates were dried at 50 °C overnight, weighed, and the precipitate was ground in a pestle and mortar and stored in sealed vials at room temperature. The precipitate was taken to be the bound protein fraction of the tissue (mean ± S.E.M. for five lambs was 17.7 ± 0.4% of wet weight of tissue). The N content of precipitated protein, as determined by a micro-Kjeldahl technique (Mangan & West, 1977), was 128.3 ± 0.7 mg of N/g.

A weighed amount (~ 100 mg) of protein was digested with 3 ml of NCS (Amersham, Arlington Heights, IL, U.S.A.) at 50 °C overnight, and 10 ml of scintillation fluid was added for determination of the total radioactivity in the leucine. It was assumed that all the radioactivity in the protein was in leucine. The leucine content of protein

Fig. 1. The model for leucine and 4-methyl-2-oxopentanoic acid (KIC) metabolism in hind-limb muscle used to determine rates of protein synthesis (R_{41}) and degradation (R_{14})

Rates R_{10} and R_{20} represent gross transfer of leucine and KIC respectively from arterial plasma. Outputs R_{01} and R_{02} are corresponding flows into deep-femoral-venous plasma. R_{32} is the output of leucine-derived CO₂ from muscle. R_{21} and R_{12} represent rate constants for the transamination reaction between leucine and KIC. Values for the rate constants (nmol · g⁻¹ · min⁻¹) are: R_{01} , 2.02 ± 0.22; R_{02} , 0.55 ± 0.12; R_{10} , 4.25 ± 0.40; R_{12} , 0.35 ± 0.13; R_{14} , 2.64 ± 0.50; R_{20} , 0.40 ± 0.09; R_{21} , 2.38 ± 0.53; R_{32} , 1.87 ± 0.12; R_{41} , 2.84 ± 0.36. Values are means ± S.E.M. for ten hind-limbs.

was determined as described for plasma, after hydrolysis in a sealed tube of approx. 100 mg of protein in 10 ml of 6 M-HCl at 110 °C for 18 h. The mean leucine content was 7.014 ± 0.064 g/100 g precipitated protein, or 8.75 g of leucine/16 g of N.

The concentration and specific radioactivity of intracellular leucine were determined by the procedures described for plasma. KIC was detected in intracellular (muscle) water, but in amounts so small as to make impracticable any attempt to determine its SRA.

Liquid-scintillation counting. All radioactivity measurements were made in Scintillator 299 (Packard Instrument Co., Davners Grove, IL, U.S.A.) in an Analytic 92 liquid-scintillation counter (G. D. Searle, Chicago, IL, U.S.A.). External-standard channel ratios were used to correct for quenching, by using curves obtained from addition of known amounts of [¹⁴C]- and [³H]-hexadecane to a range of samples obtained from each analysis.

CALCULATIONS

Measurements of rates of synthesis and degradation in the muscle of the hindlimb were derived by two distinct methods.

(i) By solving the model shown in Fig. 1. R_{41} is the rate of protein synthesis and R_{14} the rate of protein degradation. Rate of gain is the difference between the two rates.

To solve the model, the following rates are needed:

$$\begin{aligned} R_{10} &= \frac{(L_{sa} \cdot L_a - L_{sv} \cdot L_v)}{L_{sa}} \cdot PF \\ R_{01} &= R_{10} - (L_a - L_v) \cdot PF \\ R_{20} - R_{02} &= (K_a - K_v) \cdot PF \\ R_{32} &= \frac{(C_{sv} \cdot C_v - C_{sa} \cdot C_a)}{K_{sv}} \cdot BF \end{aligned}$$

R_{12} , R_{21} , R_{14} , R_{41} are then obtained by solving simultaneous equations by using the following matrix (eqns. 1 and 2 are obtained from consideration of isotope balance and eqns. 3 and 4 from consideration of carbon balance):

$$\frac{K_{sv}}{L_{sv}} \cdot R_{12} - R_{21} + \frac{L_{sb}}{L_{sv}} \cdot R_{14} - R_{41} = \frac{(L_{sv} \cdot L_v - L_{sa} \cdot L_a)}{L_{sv}} \cdot PF \quad (1)$$

$$-R_{12} + \frac{L_{sv}}{K_{sv}} \cdot R_{21} - 0 + 0 = \frac{(K_{sv} \cdot K_v - K_{sa} \cdot K_a)}{K_{sv}} \cdot PF + R_{32} \quad (2)$$

$$R_{12} - R_{21} + R_{14} - R_{41} = R_{01} - R_{10} \quad (3)$$

$$-R_{12} + R_{21} - 0 + 0 = (R_{02} - R_{20}) + R_{32} \quad (4)$$

Separate values for R_{20} and R_{02} are obtained by infusing [4,5- ^{3} H]KIC:

$$\begin{aligned} R_{20} &= \frac{(K_{sa} \cdot K_a - K_{sv} \cdot K_v)}{K_{sa}} \cdot PF \\ R_{02} &= R_{20} - (K_a - K_v) \cdot PF \end{aligned}$$

L_{sa} , L_{sv} , L_{sb} represent SRA of leucine in arterial or venous plasma or in tissue protein, respectively.

K_{sa} , K_{sv} represent corresponding values for SRA of KIC in arterial and venous plasma.

C_{sa} , C_{sv} represent SRA of CO_2 in arterial or venous blood.

L_a , L_v , K_a , K_v , C_a , C_v are the corresponding arterial and venous concentrations of leucine or KIC in plasma or of CO_2 in blood.

PF is the plasma flow = $(1 - \text{fractional PCV}) \cdot BF$, where BF is the blood flow.

L_{sb} , the SRA of protein-bound leucine, was obtained from tissue analysis; in practice the model is not sensitive to values of L_{sb}/L_{sv} varying from 0.001 to 0.05. Actual values were less than 0.01.

When SRA is expressed as nCi/ μ mol, concentration as μ mol/litre and blood flow as ml/min per g of tissue, R values are obtained as μ mol/min per kg. Then:

$$K_{sm} = \frac{R_{41} \times 1.44}{\text{leucine content of hind-limb muscle}}$$

$$K_{dm} = \frac{R_{14} \times 1.44}{\text{leucine content of hind-limb muscle}}$$

$$K_{gm} = K_{sm} - K_{dm}$$

where K values (K_{sm} , K_{dm} , K_{gm} indicate respectively fractional rates of synthesis, degradation or gain as obtained from the model) are expressed as day $^{-1}$, and leucine content was 93.61 mmol/kg wet wt.

The mean concentrations of leucine, KIC and CO_2 for the entire sampling period, and the mean specific radioactivity from 2 to 6 h of infusion, were used for the calculation of rates of flux in each hind limb. To assess the effect of the considerable variation in leucine concentration and SRA during the course of the experiment, flux rates were also calculated from individual values of concentration and SRA in one lamb (X458). The mean flux rate calculated from the samples obtained each hour did not differ substantially from that calculated by using the mean of all values of concentration and SRA over the period 2–6 h after commencement of infusion. Where calculated from individual hourly samples R_{01} and R_{10} were respectively 1.589 ± 0.3176 and 4.223 ± 1.069 (S.E.M.) μ mol/min per kg, and when calculated from the mean values of concentration and SRA over the same period were 2.02 and 4.97 μ mol/min per kg.

(ii) K_{si} was obtained from measurement of SRA of

intracellular and protein-bound leucine as described for muscle by Garlick *et al.* (1973). The ratio of bound to free leucine in muscle (R) was 1102 ± 120 , with no significant difference between the muscles sampled. An estimate of the rate of hind-limb muscle gain (K_{gi}) was derived from the relationship between live weight (X , kg) and hind-limb muscle weight (Y , g) obtained by dissection from male Clun Forest lambs 1–6 weeks of age:

$$Y = 28.031 + 46.386X; r = 0.97, P < 0.01, n = 11$$

and measured rate of gain.

Thus:

$$K_{gi} = (\text{muscle gain/day})/\text{muscle mass.}$$

K_{di} , the fractional rate of protein degradation, was derived from:

$$K_{di} = K_{si} - K_{gi}$$

RESULTS

Leucine metabolism in hind-limb muscle

The measurements from which the rate constants for leucine metabolism in hind-limb muscle were derived are shown in Table 2. A typical time course of radioactivity (d.p.m./ml) in arterial and deep-femoral venous-blood leucine, CO_2 and KIC is shown in Fig. 2 (data for lamb X458). There was substantial variation in arterial plasma leucine concentration throughout each experiment (average coefficient of variation 32%), which was responsible for equally large fluctuations in plasma leucine SRA (Fig. 3). The arterio-venous difference of hind-limb leucine was significantly related to arterial leucine concentration, despite the variation in the latter (Fig. 4).

Table 2. Hind-limb blood flow, PCV and plasma leucine and KIC and blood CO₂ concentrations (μM) and SRA (d.p.m./nmol) in lambs used in this study

All values are means over the 6 h experimental period. Blood flow was determined once, between 5 and 6 h of infusion of [$1\text{-}^{14}\text{C}$]leucine and [$4, 5\text{-}^3\text{H}$]KIC. Abbreviations: A, artery; LV and RV, left- and right-leg deep femoral veins.

Lamb	Blood flow (ml·min ⁻¹ ·g ⁻¹)	PCV (%)	Plasma leucine		Plasma KIC			Blood CO ₂	
			Concn.	¹⁴ C SRA	Concn.	¹⁴ C SRA	³ H SRA	Concn.	¹⁴ C SRA
X458	A	25.7	189.2	3.01	13.05	2.14	35.4	24986	0.00486
	LV 0.132*		159.1	2.62	14.52	2.04	18.3	25695	0.00518
	RV 0.130*		157.8	2.57	14.36	2.57	21.8	26125	0.00546
X479	A	27.8	174.8	2.00	14.76	1.23	11.1	25323	0.00394
	LV 0.115*		153.4	1.67	16.78	1.45	10.9	27133	0.00468
	RV 0.108*		155.2	1.75	15.86	1.19	6.2	26638	0.00498
X532	A	23.8	154.0	2.51	11.01	1.68	63.0	33662	0.00383
	LV 0.128		123.8	2.20	11.86	1.55	38.8	34881	0.00393
	RV 0.120		124.1	2.23	10.7	1.61	45.5	34698	0.00377
X692	A	29.2	145.2	3.16	15.23	1.82	53.5	23390	0.00356
	LV 0.185		125.4	2.55	18.22	1.81	28.0	26745	0.00418
	RV 0.200		126.0	2.59	18.14	1.40	28.0	25388	0.00439
X694	A	25.5	102.0	4.24	15.50	2.27	50.0	30030	0.00359
	LV 0.114		87.1	3.12	16.34	1.86	38.2	31633	0.00425
	RV 0.079		89.2	3.21	16.98	2.14	34.7	30440	0.00463
Average S.E.M.			18.6	0.38	1.09	0.23	6.18	752	0.00038

* Blood flow estimated from oxygen arterio-venous difference, according to the equation described in the text.

* Blood flow estimated from oxygen arterio-venous difference, according to the equation described in the text.

There was no evidence that the fluctuations in arterial leucine concentration were linked to feeding behaviour, for the lambs had access to food at all times, and drank at frequent, but irregular, intervals. The possibility that blood sampling, or handling during the course of the experiment, contributed to the variation was investigated separately in two additional lambs. A similar pattern of variation in plasma leucine concentration was seen throughout the day when just five 2 ml blood samples were removed at 2 h intervals, or larger amounts (40 ml) at 1 h intervals.

Total flux through the leucine and KIC pools, CO₂ output and values of K_s , K_g and K_d derived from the model are shown in Table 3. A number of significant correlations are seen in the correlation matrix (Table 4). Some of these may be fortuitous (with 35 comparisons, two are expected by chance at the $P < 0.05$ level), and some may be inevitable [e.g. leucine influx and efflux (R_{10} and R_{01})]. It is biologically significant that the rate of protein synthesis (R_{41}) was correlated only with the rate of leucine uptake (R_{10}), whereas the rate of protein degradation was correlated with leucine efflux (R_{01}), KIC efflux (R_{02}) and oxidation (R_{32}). It is striking that the rate of protein synthesis (R_{41}) was not significantly related to the rate of protein degradation (R_{14}). Significant correlations between rate of leucine uptake and protein synthesis, and rate of leucine efflux and protein degradation, were also observed within experiments.

The rate of protein gain, K_{gm} , was significantly and negatively correlated with the proportion of leucine catabolized [that is, the ratio of KIC/leucine fluxes in muscle (Y_1)]:

$$Y_1 = 0.387 - 5.365 K_{gm}; r = 0.94, n = 10 \text{ hind limbs, } P < 0.01$$

and also by the percentage of leucine flux oxidized to CO₂ in hind-limb muscle (Y_2):

$$Y_2 = 26.97 - 467.8 K_{gm}; r = 0.91, n = 10, P < 0.01.$$

The rate of protein gain was also found to be negatively correlated with rate of protein degradation (K_{dm}):

$$K_{gm} = 0.033 - 0.71 K_{dm}; r = 0.71, n = 10, P < 0.05$$

but was not significantly correlated with the rate of protein synthesis ($P > 0.2$).

Hind-limb muscle protein synthesis obtained from tissue analysis

The ratio SRA of protein-bound leucine/SRA of intracellular leucine was similar in the three muscles sampled from each leg (means $\pm$ S.E.M. for biceps femoris, vastus lateralis and gastrocnemius were 0.01161 ± 0.0010 , 0.01133 ± 0.0009 , 0.01281 ± 0.0006 respectively). The values were therefore pooled to derive a mean rate of protein synthesis, K_{si} , in each leg. Table 5 shows these values, together with K_{di} derived from K_{si} and the estimated rate of hind-limb muscle gain.

Comparison of estimates of protein synthesis in muscle derived by leucine metabolism or tissue analysis

Individual estimates of K_s , K_g and K_d obtained by the two methods (Tables 3 and 5) were not correlated, although for K_g and K_d there was a weak trend ($P < 0.1$). However, the mean values for K_{si} and K_{sm} (0.051 ± 0.002 and 0.046 ± 0.007), for K_{gi} and K_{gm} (0.016 ± 0.002 and 0.004 ± 0.008) and for K_{di} and K_{dm} (0.035 ± 0.004 and 0.041 ± 0.008) were not significantly different.

Comparison of rates of protein synthesis and net leucine uptake by the hind limb reveal contrasting methodological differences. There was a significant

Fig. 2. Change of radioactivity with time in leucine and 4-methyl-2-oxopentanoic acid (KIC) of blood plasma and blood CO_2 during a constant intravenous infusion of $[1-^{14}\text{C}]$ leucine for 6 h into a lamb (X458)

□, ■, Leucine, ○, ●, KIC and △, ▲, CO_2 from arterial and deep-femoral-venous blood respectively.

negative correlation between net rate of leucine uptake (Y_2 , nmol/min per g) and K_{si} (days^{-1});

$$Y_2 = 8.27 - 102.45 K_{si}; r = -0.67, n = 10, P < 0.05$$

and a significant positive correlation between Y_2 and K_{sm} ;

$$Y_2 = 1.22 + 39.19 K_{sm}; r = 0.80, n = 10, P < 0.01$$

The former suggests that increased protein-synthesis rate (as determined by tissue analysis) is associated with decreased leucine uptake, whereas the latter indicates that, as the rate of protein synthesis (measured in conjunction with muscle leucine metabolism) increases, so also does net leucine uptake.

DISCUSSION

The measurement of leucine metabolism in muscle appears to provide a sensitive measure of protein synthesis, gain and degradation. The mean results obtained by this new procedure were not significantly different from those obtained with a conventional tissue-analysis procedure, but over the (rather small) range of values observed there was no significant correlation between the two methods.

The potential sources of error peculiar to this new procedure are due particularly to measurement of arteriovenous difference, and blood flow (and the possibility that the rate of blood flow may change during the course of an experiment), and to the variability of leucine concentration during the experiment (and the associated risk of non-steady-state conditions). Furthermore, the assumption that net leucine uptake (i.e. after

Fig. 3. Mean ($\pm$ S.E.M.) arterial plasma concentration of leucine (●), specific radioactivity of leucine (▲) and the amount of radioactivity in plasma leucine (■) of the lambs used in this study

Fig. 4. Relationship between arterial plasma leucine concentration and the arterio-venous difference of leucine across the hind limb

correction for transamination and oxidation) is solely incorporated into muscle protein, although quite plausible, depends on there being no significant export of peptide leucine from muscle. This has yet to be verified.

An accurate determination of total (i.e. capillary plus arterio-venous anastomoses) blood flow is crucial to obtain absolute values of the rates in the model, and in turn K_{sm} , K_{gm} and K_{dm} , although blood flow is not of importance if only the relative rates of synthesis and degradation are required. The suitability of $^3\text{H}_2\text{O}$ as a marker for measurement of blood flow in this hind-limb preparation has been previously determined (Oddy *et al.*, 1981). We have assumed that blood flow measured over a 1 h period at the end of the experiment is representative of that over the entire infusion period, without direct evidence that it is. Nonetheless we did take particular care that the lambs did not become excited and vary their level of activity during any part of the experiment, for perhaps the major source of variation in blood flow in the hind limb is the level of activity of the animal (see Bird *et al.*, 1981; Oddy *et al.*, 1985). Further limitations (particularly

Table 3. Flux through leucine and KIC pools and leucine-derived CO₂ output in hind-limb muscle

Fluxes are defined as the net movements through leucine and KIC pools as shown in Fig. 1. All values are expressed as nmol·min⁻¹·g⁻¹. Values of K_{sm} , K_{dm} and K_{gm} (day⁻¹) are derived from the model as described in the text.

Lamb	Leg	Leucine flux	KIC flux	CO ₂ output	K_{sm}	K_{gm}	K_{dm}
X458	L	6.60	1.77	0.86	0.055	0.032	0.023
	R	7.15	1.77	1.21	0.042	0.028	0.015
X479	L	7.91	3.70	2.46	0.030	-0.012	0.042
	R	6.02	3.63	3.06	0.018	-0.023	0.042
X532	L	6.86	1.71	1.05	0.063	0.029	0.035
	R	5.38	0.71	0.33	0.058	0.038	0.020
X692	L	10.04	4.05	2.88	0.058	-0.011	0.069
	R	12.13	6.02	4.03	0.088	-0.027	0.094
X694	L	6.59	2.45	1.69	0.035	-0.007	0.043
	R	3.64	1.53	1.11	0.015	-0.006	0.022

Table 4. Correlation matrix of rates determined for the metabolism of leucine in muscle of 5–6-week-old milk-fed lambs

The rates (R) refer to the model shown in Fig. 1. Levels of significance: $P < 0.01$, $r = 0.77$, $P < 0.05$, $r = 0.63$.

	R_{01}	R_{10}	R_{02}	R_{20}	R_{32}	R_{12}	R_{21}	R_{14}	R_{41}
R_{01}	1								
R_{10}	0.796	1							
R_{02}	0.792	0.726	1						
R_{20}	0.611	0.651	0.958	1					
R_{32}	0.698	0.314	-0.585	0.412	1				
R_{12}	-0.618	-0.525	-0.551	-0.493	-0.519	1			
R_{21}	-0.146	-0.331	-0.157	-0.228	0.169	0.753	1		
R_{14}	0.755	0.426	0.673	0.510	0.901	-0.355	0.295	1	
R_{41}	0.476	0.797	0.545	0.549	0.044	-0.093	-0.043	0.367	1

Table 5. Characteristics of protein turnover in hind-limb muscle of lambs, measured by tissue analysis

Muscle weight gain (g/day) was estimated from body weight and rate of body-weight gain, and fractional rate of protein synthesis (K_{si}) was derived from tissue analysis (Garlick *et al.*, 1973). Fractional rate of muscle protein gain (K_{gi}) was calculated as muscle gain (g/day)/muscle wt. (g), and rate of degradation (K_{di}) was calculated as $K_{di} = K_{si} - K_{gi}$. K_{si} , K_{gi} and K_{di} are expressed as day⁻¹.

Lamb	Leg	Muscle gain	K_{si}	K_{gi}	K_{di}
X458	L	13.13	0.047	0.0195	0.0295
	R	13.13	0.049	0.0195	0.0275
X479	L	16.37	0.050	0.02038	0.0291
	R	16.37	0.0515	0.02038	0.0306
X532	L	16.89	0.0385	0.0241	0.0144
	R	16.89	0.0435	0.0241	0.0194
X692	L	2.32	0.0535	0.0047	0.0488
	R	2.32	0.0565	0.0047	0.0518
X694	L	6.17	0.0605	0.0128	0.0477
	R	6.17	0.0615	0.0128	0.0487

with regard to comparison of biceps femoris, vastus lateralis and gastrocnemius muscle with all the muscles of the hind limb) may be that muscles differ with respect to leucine metabolism. Our data suggested that variation in K_{si} between the three muscles sampled was small. Another source of error could be introduced if the catheter in the deep femoral vein sampled blood arising from non-muscle sources. In the preparation used here, non-muscle tissues (excluding arteriovenous anastomoses) contribute to less than 10% of blood sampled from this site in adult sheep (see Domanski *et al.*, 1974; Oddy *et al.*, 1981; Teleni, 1984). It should be made clear here that this preparation is better suited to animals which can be trained to stand quietly during the experiment. Widely differing postures and level of activity, such as occur, for example, with young pigs, would alter both rate of blood flow and the proportion of sampled blood derived from muscle.

These and other factors which contribute to variation of blood flow and arterio-venous difference account for the greater variability in values obtained from the measurement of leucine metabolism compared with those obtained by using tissue analysis. In particular this applies to lambs X694 and X479, for differences in blood

flow and arterio-venous difference of leucine, CO_2 and/or KIC between legs combined to produce quite different values of K_{si} . Nonetheless, where such changes occur they require the combined effect of blood flow and arterio-venous difference in concentration and SRA of three metabolites, which are probably real. Furthermore, this is not clarified by comparison with results obtained by a conventional tissue analysis procedure, for that also suffers from particular difficulties when used with muscle. Apart from the rather low rates of incorporation of label into tissues such as muscle necessitated by the use of appropriate whole-body doses in large animals, it is also doubtful whether the rate of change in the ratio of protein-bound label to that of free intracellular leucine was linear throughout the infusion period, not least because of the extent of the changes in leucine concentration seen in the plasma pool in this study. Consequently, in conditions as used here this technique may be relatively insensitive for measuring small changes in synthesis of muscle protein, which may account for the (physiologically improbable) negative correlation observed between leucine uptake and K_{si} .

The calculation of K_{di} involves additional errors, since this also requires an estimate of rate of protein gain, which cannot be determined in the period over which protein synthesis is estimated. Accordingly the procedure commonly used is to extrapolate from growth determined before the experiment to the period over which protein synthesis is measured, as we have done here (Waterlow *et al.*, 1978). Apart from the altered patterns of activity owing to an inevitable increase in handling of animals, sampling of blood must alter N and energy balance compared with pre-experimental days. However, variations in rate of protein gain caused by these factors (although induced by measurements across the hind leg) are accounted for in the measurement of leucine metabolism, whereas they are not by the tissue-analysis procedure. Moreover, it is not always clearly recognized that it is inevitable that there will be a calculated increase in the rate of degradation of muscle protein at the same time as there is an increase in rate of synthesis of protein when this method (tissue analysis) is used, for in muscle K_s is often 4-fold or more greater than K_g . Accordingly the estimates of K_{si} and K_{di} are not necessarily independent, because 80% or more of the value of K_{di} can be determined by K_{si} . However, this extreme case was not reflected in the distribution of error, for here K_{si} and K_{gi} each accounted for 30% of the variance of K_{di} .

One must always be cautious in supposing that a correlation implies any causal relationship. Nevertheless the results obtained from the model of leucine metabolism in muscle show that rate of protein synthesis (K_{sm}) was correlated with both net and gross leucine uptake, as has been observed with studies *in vitro* and perfusion studies. They also suggest a close association between muscle protein degradation and the transamination and oxidation of leucine, a finding previously demonstrated only during studies *in vitro* (Paul & Adibi, 1976; Zapalowski *et al.*, 1981; Tischler *et al.*, 1982). The observations that in muscle the net gain of protein (K_{gm}) is negatively correlated with rate of protein degradation, with leucine oxidation and with the proportion of leucine catabolized are all consistent with the notion that in these circumstances the rate of protein degradation may have more influence on the rate of protein deposition than has the rate of protein synthesis. Findings made with the

conventional technique (incorporation of label into muscle protein and changes in body weight) are consistent with this idea, for partition of variance of the estimate of K_{gi} revealed that K_{di} had a greater influence than K_{si} (44% of variance was due to K_{di} , compared with 12% due to K_{si}). Maruyama *et al.* (1978) and Saunderson & Bryant (1985) reached similar conclusions in studies with growing chickens, the latter workers using a completely different method (3-methylhistidine excretion). Li (1980) concluded that protein degradation was an important determinant of muscle protein deposition in hamsters. The findings are not, however, in agreement with those of Millward *et al.* (1976), that rate of synthesis of muscle protein was the factor of most importance in control of rate of growth of rat muscle. Nor are they compatible with the view summarized by Reeds & Fuller (1983) that whole-body protein synthesis is more important than degradation in controlling N balance over a range of energy intakes from starvation to 3 times maintenance of energy balance. However, in the present study muscle protein synthesis contributed to less than 15% of whole-body protein synthesis. It may also be pertinent that, in the study of Millward *et al.* (1976), wasting of muscle was induced in various ways, whereas in the present work and that of Saunderson & Bryant (1985) normal growing muscle was studied.

Disadvantages with the technique that we have proposed are that it is technically more complicated than methods based on tissue incorporation and that it does not permit study of differences in individual muscles, and it cannot necessarily be applied to other species. Nevertheless, the method that we have developed seems to be a valuable tool for the study of factors that may affect the rates of protein synthesis and degradation in muscle *in vivo*. Estimated rates are not significantly different from those obtained by more conventional techniques, and for a number of relationships we are able to support conclusions reached from studies of muscle *in vitro*. It offers the advantage of allowing repeated measures of muscle protein synthesis and degradation in the same animal, and in particular allows the study of factors that may affect the rate of muscle protein degradation *in vivo*.

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