Estimation of tissue protein synthesis in sheep during sustained elevation of plasma leucine concentration by intravenous infusion

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1. The fractional rate of protein synthesis (FSR) was determined in skeletal muscle, liver, rumen and cardiac muscle of wether sheep by continuous intravenous infusion of L-[4,5-^{3}H]leucine accompanied by infusion of 0, 7·6, 15·2 or 22·8 mmol leucine/h (three sheep per treatment). FSR was calculated assuming plasma (k_{sp}) or intracellular (k_{si}) leucine-specific radioactivity (SRA) was representative of the leucine precursor pool SRA for protein synthesis.

2. Plasma leucine concentration (plateau) was linearly related to leucine infusion rate, 22·8 mmol/h evoking a tenfold increase in plasma concentration.

3. Difference between plasma leucine SRA and intracellular leucine SRA in all tissues diminished as plasma leucine concentration increased.

4. There were significant differences between k_{si} and k_{sp} estimates for liver and rumen in control sheep.

5. As leucine infusion rate increased, differences between k_{si} and k_{sp} diminished in all tissues. With increasing leucine infusion, in liver k_{si} decreased and k_{sp} was increased, in rumen k_{si} decreased and k_{sp} was stable, while in cardiac and skeletal muscle k_{si} and k_{sp} both increased.

6. At a leucine infusion rate of 22·8 mmol/h, mean k_{sp} and k_{si} respectively were: rumen 11 (SE 2), 13 (SE 1); liver 19 (SE 2), 21 (SE 2); cardiac muscle 3·6 (SE 0·4), 3·8 (SE 0·3); skeletal muscle 4·1 (SE 0·2), 4·5 (SE 0·5) and did not differ significantly in any tissue.

Measurement of protein synthesis in animal tissues in vivo by continuous infusion of a radioactive tracer amino acid requires definition of amino acid specific radioactivity (SRA) in the precursor pool for protein synthesis (see Waterlow *et al.* 1978); ideally that of amino acyl-tRNA. Because of analytical difficulties most investigators have assumed that either intracellular (ICF) or extracellular (plasma) free amino acid SRA represented that of amino acyl-tRNA.

However, studies, notably in liver and heart, have demonstrated that amino acyl-tRNA SRA was intermediate to that found in ICF or plasma (Airhart *et al.* 1974; McKee *et al.* 1978), suggesting preferential channelling of amino acid from plasma into protein (Airhart *et al.* 1974). However, in cultured skeletal muscle cells, leucyl-tRNA SRA was lower than that of the culture medium or ICF suggesting preferential input of non-radioactive amino acid from protein degradation into the amino acid pool for protein synthesis (Schneible *et al.* 1981).

In perfused liver (Khairallah & Mortimore, 1976) and heart (McKee *et al.* 1978) it was shown that disparity between amino acid SRA in ICF, plasma and amino acyl-tRNA could be largely removed by raising the perfusate concentration of the amino acid to be traced. Using a similar principle, measurement of protein synthesis in rat tissues was achieved following injection of a large dose of the amino acid to be traced with the intention of equalizing SRA in all tissue pools. Thus uncertainty in calculation of tissue protein synthesis arising from assumptions as to the precursor pool was removed (McNurlan *et al.* 1979; Garlick *et al.* 1980).

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Studies on large domestic species present problems additional to those encountered in vitro or in rats. The 'flooding dose' procedure of McNurlan *et al.* (1979) is prohibitively expensive in large animals. Other factors such as physical volume of dose required and the probability of less accurate measurement of proteins with slow rates of turnover also militate against this procedure in large animals.

There remains a requirement for an improved method of measurement of protein turnover in large animals, unencumbered by the uncertainty in choosing ICF or plasma pools for calculating synthetic rates.

The experiment described in the present paper was designed to investigate variation in ICF and plasma SRA of leucine, in response to incremental increases in plasma leucine concentration in sheep. Further, it was envisaged that differences between ICF and plasma SRA would diminish as plasma leucine content increased. Thus the uncertainty in calculating protein synthesis because of differing SRA in ICF and plasma pools (see Davis *et al.* 1981) would be removed.

METHODS

Animals

Twelve 3-year-old Romney × Suffolk wether sheep (57·6 (SE 1·7) kg) were offered a pelleted barley–dried grass–pea (*Pisum sativum*) diet (122 g protein/kg: 11·0 MJ metabolizable energy/kg; NRM Feeds Ltd, Auckland) for 7–10 d before the experiments. The sheep were then housed individually in crates and fed at 2 h intervals to maintain intakes of 1500 g/d. After a further period of 7–10 d of continuous (2 h) feeding, infusions were carried out. Salt and water were available to the animals throughout. Room temperature was 17·0 (SE 0·9) and the animals were subjected to natural lighting.

Infusion procedure and treatments

At 16 h before infusion of leucine a polyethylene catheter was inserted into each jugular vein. The intravascular length of the sampling catheter was restricted to approximately 50 mm to avoid direct sampling of infusate.

L-Leucine dissolved in sterile physiological saline (9 g sodium chloride/l) was infused using a peristaltic pump (60 ml/h) at one of four levels (0, 7·6, 15·2 or 22·8 mmol/h, three sheep per level) for 8 h. In all animals, L-[4,5-^{3}H]leucine was infused via the same catheter at approximately 100 μCi/h beginning 2 h after the start of leucine infusion, and continuing for the remaining 6 h of leucine infusion.

Tissue and blood sampling

Blood samples were collected and prepared as described by Davis *et al.* (1981). Sheep were killed with a lethal dose of saturated magnesium sulphate (40 ml) injected via the sampling catheter after induction of light anaesthesia with 200 mg sodium pentobarbitone (Sagatal: May and Baker Ltd, Dagenham). Tissue samples of liver, heart, rumen and muscle (biceps femoris) were collected immediately, rinsed in saline and frozen in liquid nitrogen. All tissues were immersed in liquid N_2 within 3 min of $MgSO_4$ injection. Free and protein-bound leucine in tissues was separated following homogenization as described by Davis *et al.* (1981). The 'free' leucine released by homogenization is referred to as 'intracellular free' (ICF) in the following text. Differences between homogenate and ICF leucine SRA are likely to be small as discussed by Waterlow *et al.* (1978).

Analytical techniques

Amino acid analysis. Leucine was fractionated by ion-exchange chromatography as described by Davis *et al.* (1981) with the modification that fluorometric detection was

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employed using *o*-phthalaldehyde reagent (Benson & Hare, 1975) and an Aminco-SP-125 fluorimeter (American Instrument Co., Silver Spring, Maryland 20910, USA). This procedure increased throughput and allowed 75% of column effluent to be used for radioactivity determination.

All other analytical procedures were as described by Davis *et al.* (1981).

Calculations

Plasma leucine flux. The flux of leucine in plasma was calculated from the formula:

$$I = F.Sp_{\max}, \tag{1}$$

where I is the rate of infusion of isotope (μCi/h), F is the flux (mmol/h) and $Sp_{\max}$ is the plateau SRA of leucine in plasma (μCi/mmol).

The protein fractional synthetic rate (FSR) in the tissue was calculated as described by Waterlow *et al.* (1978) from the formula:

$$\frac{S_B}{S_i} = \left(\frac{\lambda_i}{\lambda_i - k_s}\right)\left(\frac{1-e^{-k_s t}}{1-e^{-\lambda_i t}}\right) - \left(\frac{k_s}{\lambda_i - k_s}\right), \tag{2}$$

where S_B is the SRA of protein-bound leucine and S_i the SRA of 'free' (homogenate) leucine in the tissues, k_s is the FSR (/d), t is the period of infusion (d) and λ_i is the rate-constant describing the rise to plateau of the SRA of free leucine in the tissue. In the following text k_s has been expressed $\times 100$.

Because λ_i was not determined directly the approximations of Garlick *et al.* (1973) were used such that for muscle, $\lambda_i = Rk_s$, where R is the concentration ratio, protein-bound leucine: free leucine in the tissue. For liver and rumen, $\lambda_i = \lambda_p$, where λ_p is the rate constant in the formula describing the rise to the plateau of leucine SRA in plasma (Sp) as:

$$Sp = Sp_{\max}\,(1 - e^{-\lambda_p t}), \tag{3}$$

where $Sp_{\max}$ is the plasma leucine plateau SRA and t is the period of infusion (d). Because the calculation of k_s is relatively insensitive to variation in λ_p the average λ_p was used, determined by non-linear regression using the values from two animals in each infusion group (Fig. 1).

Calculation of k_s was also made by substituting S_B: Sp in eqn (2). These estimates are distinguished using the notation k_{si} (S_B/S_i) and k_{sp} (S_B/Sp).

Statistical analysis

Statistical comparisons between treatment groups were made using Student's t test.

RESULTS

Plasma and tissue leucine concentrations and plasma leucine entry rate

Concentrations of leucine in plasma and ICF pools are given in Table 1. Leucine concentration in plasma rose to a plateau within 2 h of the start of infusion and plateau concentration increased linearly with infusion rate, 22·8 mmol/h evoking a ten-fold increase in plasma concentration (Table 1). Tissue leucine concentrations were lower than those in plasma, muscle and heart but slightly higher in rumen and liver tissue in control sheep. The ratio, ICF: plasma concentration remained approximately constant for heart and muscle with increasing infusion rate, while the corresponding value declined in liver and rumen (Table 1).

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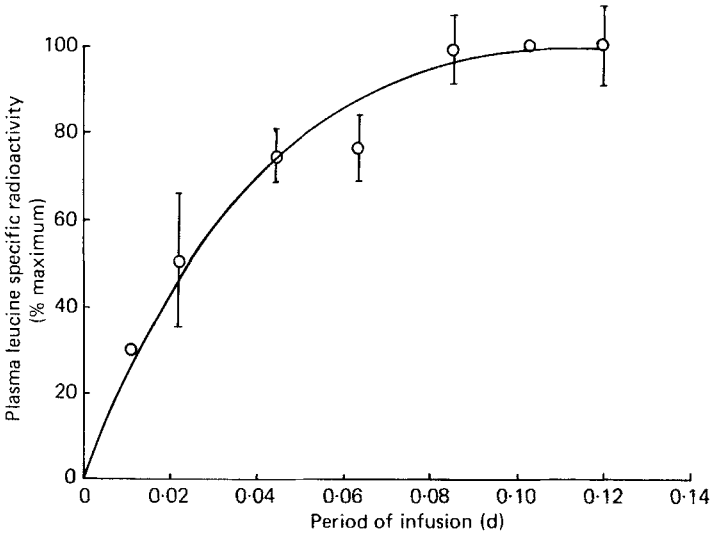

Fig. 1. The rise of plasma leucine-specific radioactivity (expressed as a percentage of plateau value) during the first 3 h of L-[4,5-^{3}H]leucine infusion. λ_p, the rate-constant in the formula describing the rise to the plateau of leucine-specific radioactivity in plasma (eqn (3)), was 39/d. The values from two animals in each infusion group were used to derive λ_p using non-linear regression. Vertical bars represent standard errors.

Table 1. *Change in plasma and intracellular (ICF) leucine concentration (μmol/g tissue) with intravenous leucine infusion rate*

(Mean values with their standard errors for three sheep per infusion level)

Leucine infusion rate (mmol/h)...	0		7·6		15·2		22·8	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Plasma (plateau) (μmol/ml)	0·13	0·03	0·48	0·07	0·82	0·11	1·32	0·10
Cardiac muscle (μmol/g)	0·09	0·01	0·23	0·02	0·49	0·04	0·80	0·07
Skeletal muscle (μmol/g)	0·08	0·02	0·27	0·02	0·54	0·04	0·88	0·08
Liver (μmol/g)	0·16	0·03	0·29	0·08	0·76	0·10	1·17	0·17
Rumen (μmol/g)	0·13	0·02	0·27	0·02	0·58	0·02	0·91	0·09

Plasma leucine entry rate increased linearly and was highly correlated ($P < 0{\cdot}001$) with leucine infusion rate, the slope of the regression being close to unity (Fig. 2).

As the pool size of free leucine increased from eight- to tenfold (Table 1) and plasma entry rate increased three- to fourfold between control and the highest level of leucine infusion, it is likely that λ_p (eqn (3)) was reduced from 39/d in control sheep to < 20/d in leucine-infused animals. However, insufficient samples were assayed to define this reduction accurately.

Failure to account for a reduction in λ_p has only a small effect on the calculation of k_s in liver and rumen. For example, rumen k_{si} at the highest level of leucine infusion is increased from 12·7 to 14·1%/d when λ_p was assumed to be 20/d rather than 39/d. In cardiac and skeletal muscle the increased pool size of free leucine in the tissue is accounted for in the estimation of λ_i from Rk_s.

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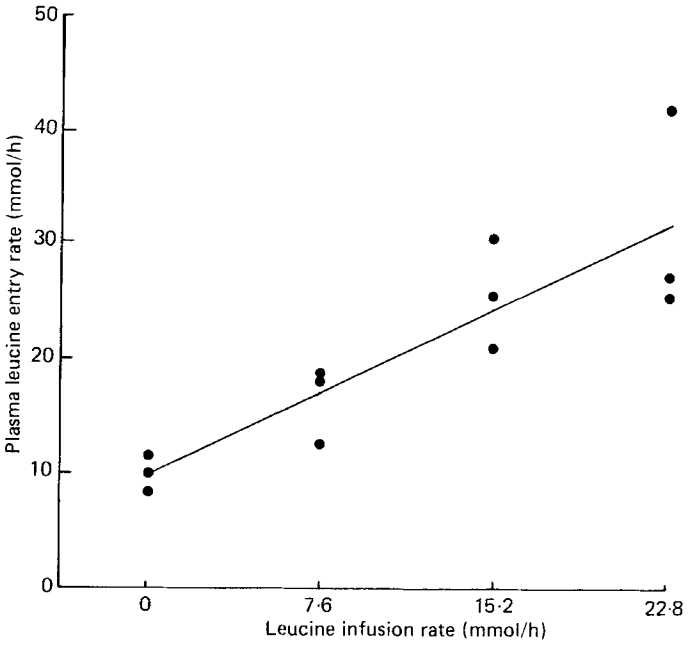

Fig. 2. Change in plasma leucine entry rate (mmol/h) with increasing leucine infusion rate (mmol/h). Mean values for individual sheep are shown. The line was fitted by weighted regression using weights inversely proportional to variance of entry rates for each infusion rate. The equation describing the relation was:

$$Y = 0{\cdot}96X + 1{\cdot}28.$$

Standard errors for slope and intercept were 0·13 and 0·10 respectively.

Free leucine SRA in tissue and plasma

The trend in all tissues was for ICF leucine SRA to approach that of plasma as leucine infusion rate increased. At 22·8 mmol/h infusion rate, leucine SRA in ICF in all tissues was at least 85% of that in plasma (Fig. 3). In control sheep, ICF leucine SRA (expressed as a percentage of plasma leucine SRA) in heart, rumen, liver and muscle was 82, 50, 36 and 66 respectively and was 99, 85, 93 and 94 respectively in animals infused with 22·8 mmol leucine/h.

Tissue protein FSR

Tissue protein synthetic rates calculated using ICF (k_{si}) or plasma leucine SRA (k_{sp}) as the assumed precursor pool estimates are shown in Fig. 4. In both heart and skeletal muscle k_{si} and k_{sp} increased with increasing leucine infusion rate. In skeletal muscle the increase in k_{si} was from 2·1%/d (control) to 4·5%/d (22·8 mmol/h) and the increase was evident, although not statistically significant, in the 7·6 mmol/h infusion group. Of the four tissues, the difference between k_{si} and k_{sp} in control animals was least in skeletal muscle and heart tissue, tending to reduce as infusion rate increased in the experimental animals.

In liver, k_{si} decreased from 35%/d (control) to 22%/d (22·8 mmol/h) with increasing leucine infusion rate. While the difference between k_{si} and k_{sp} was significant in control animals ($P < 0{\cdot}001$) it was not statistically significant at any other infusion level and diminished as the infusion rate was increased. In control sheep, liver k_{sp} was significantly lower than that found in infused sheep ($P < 0{\cdot}05$).

In rumen there was no significant change of k_{sp} with infusion rate but k_{si} declined from

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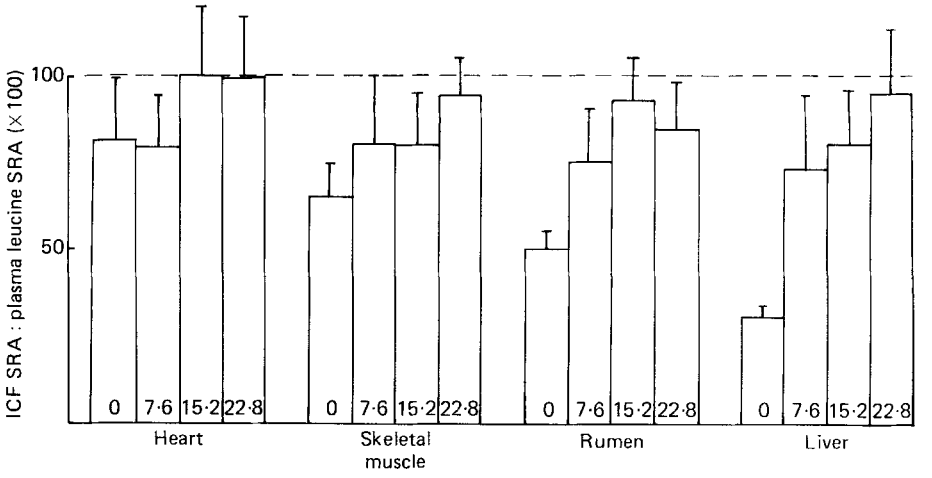

Fig. 3. The ratio, intracellular free (ICF) leucine-specific radioactivity (SRA): plasma leucine SRA (× 100) in heart, muscle, rumen and liver at four levels (0, 7·6, 15·2, 22·8 mmol/h) of intravenous leucine infusion. Infusion rates are shown within each histogram. Vertical bars represent standard errors.

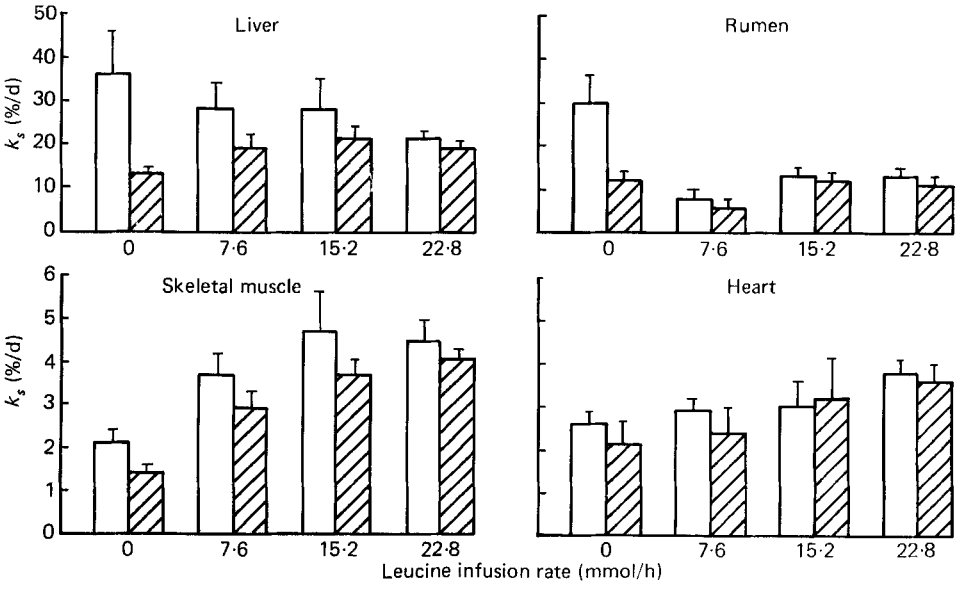

Fig. 4. Tissue protein fractional synthethic rate (k_s) calculated assuming intracellular (k_{si}, □) or plasma (k_{sp}, ▨) leucine specific radioactivity as precursor specific radioactivity. Values for k_{si} and k_{sp} are shown for liver, rumen, muscle and heart at each of four levels of leucine infusion (0, 7·6, 15·2, 22·8 mmol/h). Vertical bars represent standard errors. k_{si} and k_{sp} differed significantly (paired t test; $P < 0.01$) in liver and rumen in control sheep. k_{si} and k_{sp} were significantly (t test; $P < 0.05$) greater than controls at 15·2 and 22·8 mmol/h leucine infusion rate for muscle and heart respectively. In control sheep, liver k_{sp} was significantly lower than that found in infused sheep (t test; $P < 0.05$).

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a control value of 30% to approximately 10% at all levels of leucine infusion. In conjunction with this decline the significant difference between k_{si} and k_{sp} in control sheep ($P < 0.01$) was removed.

DISCUSSION

Leucine infusion and measurement of protein synthesis

The strategy of raising plasma leucine concentration was successful in removing the disparity between plasma and ICF leucine SRA. In the tissues examined the uncertainty as to the validity of k_{si} or k_{sp} was substantially removed.

The response of individual tissues to leucine infusion differed. In liver, convergence of k_{si} and k_{sp} was achieved by a decline in k_{si} and a small rise in k_{sp}. Airhart *et al.* (1974) and Khairallah & Mortimore (1976) showed that valyl-tRNA in perfused rat liver was intermediate in SRA to that of plasma and ICF implying that valyl-tRNA was charged by amino acid derived directly from plasma as well as ICF. The results in Fig. 4 support the rat studies indicating that leucyl-tRNA SRA was intermediate to that of plasma and ICF leucine SRA in sheep liver in vivo as k_{sp} increased and k_{si} decreased as plasma leucine concentration increased. However, this point assumes that liver protein synthesis was unchanged in animals receiving leucine infusions.

In rumen, k_{sp} did not change with leucine infusion but k_{si} was markedly reduced even at the 7·6 mmol/h infusion level. These values indicate that k_{si} was a substantial overestimate of protein synthetic rate in rumen tissue of control sheep. However, the assessment of protein synthesis in rumen tissue is complicated by the heterogeneity of tissue types (smooth muscle, epithelial) in the sample.

In skeletal muscle and heart the relatively small difference in leucine SRA between plasma and ICF was diminished by leucine infusion. Also, estimates of k_{si} and k_{sp} both increased, implying a stimulation of protein synthesis in these tissues in association with leucine infusion.

Several studies have indicated that leucine can increase protein synthesis in diaphragm (Buse & Weigand, 1977), perfused rat heart (Rannels *et al.* 1974; Chua *et al.* 1979), perfused rat hind-limbs (Buse *et al.* 1984) and isolated skeletal muscles (Chang Hong & Layman, 1984). However, such responses have not always been apparent in vivo (McNurlan *et al.* 1982) or in vitro (Wijayasinghe *et al.* 1984). Precisely which circumstances permit the expression of a response of tissue protein synthesis to leucine requires clarification but the effect is probably not simply a removal of substrate limitation.

For accuracy of determination of tissue protein synthesis it is required that SRA of leucyl-tRNA be known. In vitro (perfused rat heart) it has been shown that amino acyl-tRNA SRA converged with that of plasma and ICF as perfusate concentration of the traced amino acid increased (McKee *et al.* 1978) while in rat liver, ICF and perfusate valine SRA converged as perfusate valine concentration increased (Mortimore *et al.* 1972). In perfused heart, parity between plasma, phenylalanyl-tRNA and ICF phenylalanine SRA was achieved when perfusate phenylalanine concentration was as low as 0·4 mM.

However, Schneible *et al.* (1981) showed with cultured skeletal muscle that even when plasma and ICF SRA were equal, amino acyl-tRNA SRA was lower and relatively unresponsive to increasing amino acid concentration in the medium. Whether such results are a feature entirely of this experimental model is unknown but they emphasize the requirement for estimation of leucyl-tRNA SRA in tissues of sheep with high plasma leucine concentrations before deriving conclusions to the accuracy of k_{si} or k_{sp} estimations.

Measurements of k_{si} and k_{sp} in tissues of growing lambs (20 kg live weight) by L-[4,5-^{3}H]leucine infusion gave k_{sp} values slightly higher than those determined here (Davis

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et al. 1981). Differences were greatest for liver and rumen where in the younger lambs k_{si} was 54%/d and 79%/d respectively compared with 40%/d and 30%/d (control animals) in the present study. Cardiac muscle showed a similar discrepancy in k_{si} between these studies.

Metabolic effects of leucine infusion

Intravenous infusion of leucine into sheep did not cause any deviation in measurements of heart rate (90 (SE 4)/min), respiration rate (22 (SE 1)/min), packed cell volume (0·32 (SE 0·1)), rectal temperature (38·9 (SE 0·2)°) or plasma glucose (693 (SE 2) mg/l). The presence of high concentrations of leucine in the diet of pigs (Taylor *et al.* 1984) and rats (Block & Harper, 1984; Harper & Benjamin, 1984) as well as intravenous infusion of leucine in man (Sherwin, 1978) and rats (Zapalowski *et al.* 1984) has been reported to depress plasma concentrations of valine and isoleucine. Quantitative measurements of valine and isoleucine were not made in the present experiments but the limited aminograms did not show a decline in free isoleucine content of plasma and tissues.

Conclusions

Elevation of plasma leucine concentration by intravenous infusion of leucine reduced the disparity between plasma and ICF leucine SRA during continuous infusion of L-[4,5-^{3}H]leucine. While this procedure increased the precision of k_s determination, increased accuracy has not been shown, but must await the determination of leucyl-tRNA SRA when plasma leucine concentration is elevated.

The differing pattern of 'response' of k_{si} and k_{sp} suggests the relative contribution of intracellular and plasma amino acids to the protein precursor pool varies between tissues.

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