Muscle protein synthesis in response to testosterone administration in wether lambs

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A method has been developed based on stable isotopes and biopsy procedures which allows the large-dose procedure for measurement of protein synthesis to be applied in serial studies to farm species. Measurements of total nitrogen retention and protein synthesis in m. longissimus dorsi and m. vastus lateralis were made in five wether lambs (40–44 kg) infused intravenously, successively, with vehicle (10 d); testosterone (15 d; 9 mg/d); vehicle (15 d). N retention was improved by testosterone infusion (+2·9 g N/d; a 96% improvement total over control periods). Muscle protein synthesis was not significantly altered by exogenous hormone administration, nor were RNA:protein, RNA:DNA or protein:DNA. The implication of the developed procedure for dynamic studies in accessible tissues of large animals is discussed.

Protein synthesis: Testosterone: Lambs: Muscle

For most species entire males have faster rates of growth, increased muscle mass and lower body fat than castrated males. Most of these attributes are related to the presence of the androgen, testosterone, as shown by exogenous administration of the hormone to female and castrated animals (e.g. O'Mary *et al.* 1952; Burgess & Lamming, 1960; Scow & Hagan, 1965; Kochakian, 1975; Schanbacher *et al.* 1980; Singh *et al.* 1985). While some of the improvement in growth rate and muscle mass can be attributed to stimulation of voluntary food intake (Schanbacher *et al.* 1980; Singh *et al.* 1985) even at controlled intake the entire male has a greater rate of protein accretion than the castrate (Prescott & Lamming, 1964; Fletcher *et al.* 1986).

The mechanism by which the myotrophic action of testosterone is achieved remains controversial; direct measurement by isotope incorporation in either males of the laboratory species or in muscle cells in culture have given inconsistent results for changes in either protein synthesis or degradation (e.g. de Loecker, 1965; Powers & Florini, 1975; Grigsby *et al.* 1976; Stratman, 1978; Dohm *et al.* 1979; Ballard & Francis, 1983). Only in the young female rat have pronounced effects on muscle protein synthesis been reported (Martinez *et al.* 1984), although with older animals the muscle anabolism was not associated with changes in protein synthesis and modification of protein degradation has been advanced as the cause (J. J. Choo and P. W. Emery, personal communication). In wether lambs, episodically infused with testosterone, Lobley *et al.* (1987) observed that whole-body protein synthesis (estimated from leucine irreversible loss rate) declined, although because skeletal muscle makes a small contribution to total synthesis (20–25%; see Lobley *et al.* 1980; Attaix *et al.* 1988), stimulation of muscle metabolism in muscle may have been masked by decreases in the rates for other tissues.

In the current study muscle protein synthesis was measured by an extension of the large-dose ('flood') procedure, which was developed originally by Garlick *et al.* (1980) and has become widely adopted for studies with laboratory animals. Usually the technique involves

radioisotopes and terminal procedures and, in this form, has been applied on a few instances to farm species (young lambs, Attaix *et al.* 1986, 1988; sheep, Pell & Bates, 1987; piglets, Seve *et al.* 1986). The alternative methodology described in the current study is based on stable isotopes and biopsy procedures; this allows repeat measurements on the same animals and should reduce the variability observed with large-animal experiments. A preliminary report on some of the technical aspects of the present study has been published (Lobley *et al.* 1990).

MATERIALS AND METHODS

Animals and diet

Five Suffolk × wether lambs (12–15 months, 40–44 kg) were used; each had been castrated within 6 weeks of birth. Each was offered 1·2 kg (as fed) grass pellets (10·3–10·9 MJ metabolizable energy (ME)/kg dry matter (DM); 21–23 g nitrogen/kg DM); for each animal sufficient diet was mixed and weighed to encompass the total experimental period (approximately 60 d). The amounts of ration supplied approximated to 1·7 × energy maintenance (i.e. 630 kJ ME/kg live weight$^{0.75}$) and was delivered in equal portions at hourly intervals by means of automated feeders. Animals were allowed at least 3 weeks of adjustment to the quantity of food and method of supply before the start of the experiment. Average live-weight gain over the experimental period was 6·4 kg.

Catheterization and balance procedures

Each sheep was prepared with a Silastic sample catheter (1·02 mm i.d., 2·16 mm o.d.; Dow Corning, Health Care Group, Reading) introduced, through a size 10 needle, 250 mm into the right external jugular vein. A double-bore, polyvinyl infusion catheter (0·8 mm i.d., 1·2 mm o.d.; Dural Plastics Ltd, Dural, NSW, Australia) was introduced 300 mm into the left external jugular vein.

Lambs were harnessed to collect faeces daily by bag while urine was continuously collected by suction into 2 M-sulphuric acid; excreta were pooled for analysis as 5 d batches. Analysis of feed and excreta for N (Kjeldahl) was by an approved procedure of the Association of Official Agricultural Chemists (1980).

Experimental design and testosterone administration

Each animal was measured for a 10 d (i.e. two 5 d balance) control period, followed by a 15 d (three 5 d) period of episodic testosterone infusion, then a 10 d post-treatment period was allowed to elapse before a final 5 d control measurement was made. Through one bore of the left catheter was infused, by means of a syringe pump, either water–ethanol (50:50, v/v) during control periods or a similar solution containing testosterone (1 mg/ml) at a rate of 9 ml/d. A time clock switched the infusion pump on and off during consecutive hourly periods. Episodic, rather than continuous, infusion of testosterone would better simulate the condition in the intact male where androgen release from the testes is regulated through hypothalamic negative feedback on luteinizing hormone release and results in considerable diurnal fluctuations in plasma testosterone concentration (e.g. D'Occhio *et al.* 1982; Lobley *et al.* 1987). The Silastic catheter was maintained patent by flushing, three times a week, with heparin solution (25 i.u. heparin/ml saline (9 g sodium chloride/l)).

Measurement of muscle protein synthesis

A preliminary trial was conducted with two 40 kg lambs, offered a similar intake to the main study, to ensure that appropriate flooding of plasma and muscle free-leucine pools could be achieved and maintained. Each sheep was injected intravenously with 5 g [^{13}C]leucine (a mixture of 0·25 g [1-^{13}C]leucine, 99 atoms %; Tracer Technologies,

Somerville, MA, USA; plus 4·75 g L-leucine, hospital grade; Ajinomoto Co. Inc., Japan) dissolved in 250 ml saline. All solutions were passed through a 0·2 μm filter and autoclaved. Solutions were warmed to 39 ° and injected continuously over an 8–10 min period. Blood samples (5 ml) were taken into heparinized syringes before and at 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105 and 120 min after the start of injection. Biopsies were taken from m. longissimus dorsi at −5, 20, 45, 60 and 120 min. Skin incisions were made under local anaesthesia and muscle samples (50–100 mg) taken under suction with a 6 mm Allendale biopsy needle; incision sites were closed immediately. The muscle sample was quickly washed with ice-cold saline, blotted dry and frozen in liquid N_2. Long-term storage of plasma and muscle samples was at −20°.

For the main study the sample collection and storage was as for the preliminary investigation. The leucine injectates comprised 0·8 g [1-^{13}C]leucine (99 atoms %) plus 4·2 g L-leucine (hospital grade) prepared and injected as described previously. Blood samples (5 ml) were taken into heparinized syringes before and at 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105 and 120 min after the start of injection and biopsies (200 mg) were taken from m. longissimus dorsi and m. vastus lateralis at −5, 60 and 120 min. These two muscles, easily accessible to biopsy techniques, were selected as they contain mixed fibre populations, representative of the majority of the ovine musculature (e.g. Suzuki, 1971). Protein synthesis was measured twice during the initial control periods (at days −12 and −5, C_1 and C_2 respectively), at days 2, 9 and 15 of testosterone infusion (T_1, T_2 and T_3 respectively) and then 16 d after cessation of hormone administration (post-treatment control period C_3).

Preparation of plasma for measurement of isotopic enrichment of free leucine and 4-methyl-2-oxo-pentanoate (MOP; 2-ketoisocaproate) was as described by Calder & Smith (1988). The free and protein-bound leucine from muscle were separated by maceration, with a ground-glass homogenizer, of the tissue in 3 ml ice-cold ethanol containing 1 mM-hydrochloric acid, followed by centrifugation. The supernatant fraction was stored at −20° for not more than 7 d before analysis. The wet protein precipitate (of known weight) was washed twice with 5 ml 0·23 M-perchloric acid (PCA), then suspended in a solution of water (5 ml) and 0·6 M-sodium hydroxide (5 ml) for 1 h at 37° and the mixture reweighed; 0·5 g solution was removed for protein analysis. To the remainder, 2 ml 2·3 M-PCA was added, the mixture stored on ice for 10 min and then centrifuged. The supernatant fraction was retained for RNA analysis. The pellet was washed with 5 ml 0·23 M-PCA and then 0·6 M-PCA (2·5 ml) added, the mixture reweighed and the suspension incubated, with occasional mixing, at 70° for 30 min. The mixture was then placed on ice for 10 min, centrifuged, and the supernatant fraction retained for DNA analysis. The pellet was washed first with 5 ml 0·6 M-PCA, then twice with 5 ml chloroform–methanol (2:1, v/v) and finally twice with at least 5 ml diethyl ether before allowing to air-dry. Plasma protein precipitates from the pre-injection samples were also washed with 0·23 M-PCA and then treated similarly. Samples of dried protein (20–30 mg) were dissolved for 1 h in 1 ml 0·3 M-NaOH and then 7 ml concentrated HCl added before hydrolysis under sealed tube conditions *in vacuo* at 110° for 18 h. The HCl was then removed by evaporation under reduced pressure.

Leucine from the protein hydrolysates was separated by ion-exchange chromatography (600 × 9 mm column, 10 μm diameter resin; Locarte Co. Ltd, London) using 0·2 M-sodium citrate, pH 3·7, at a flow-rate of 30 ml/h. Approximately 5% of the eluate was diverted for peak identification by ninhydrin while the remainder flowed either to waste or a fraction collector through a valve controlled by a time clock. Fractions, each of 2·5 min duration (i.e. 1·25 ml), were collected between 260 and 380 min from the start of chromatography; the appropriate tubes containing leucine were identified from the ninhydrin trace. The fractions containing the leucine peak were combined (total volume 40–45 ml), acidified and

Table 1. *Nitrogen retention (g N/d) before, during and after treatment with 9mg testosterone/d supplied by episodic, intravenous infusion*

(Each value represents the mean of a 5 d measurement for five animals)

	Pre-controls		For treatment days*			Post-control
	C_1	C_2	T_1	T_2	T_3	C_3
N retention (g N/d)	2·18	2·32	6·22	6·05	5·62	3·79

C_1, C_2, C_3, are each 5 d control periods either immediately before (C_1, C_2) or between 10–15 d after treatment (C_3); T_1, T_2, T_3, are 5 d treatment periods between 0–15 d of testosterone infusion.

* Mean increase in N retention over treatment period compared with pre- and post-treatment controls 2·9 g N/d ($P < 0.001$; standard error of difference 0·79, residual df 16).

desalted on 3 ml Biorad AG50 (H^+; × 8, 100–200 mesh). The leucine was eluted with 30 ml 4 M-ammonium hydroxide and the sample evaporated to dryness under reduced pressure, redissolved in 2 ml 0·1 M-HCl and the leucine concentration determined on a Rank-Hilger Chromaspek (Hilger Analytical, Margate). Subsamples, containing 3 μmol leucine, were evaporated to dryness at 100° under a N_2 stream in 10 ml. Vacutainer tubes (red cap, Silicone-treated, sterile, no additive; Beckton Dickinson, Rutherford, N.J., USA) were used from batch numbers determined to have minimal interference of mass contaminant 46 (see Milne & McGaw, 1987). Measurement of leucine enrichment followed the procedure of Read *et al.* (1984), with minor modifications. The tubes were placed in ice and 20 mg ninhydrin and 1 ml cold, degassed lithium citrate buffer (0·1 M, pH 2·2) added. Septa (which had just previously been degassed at 90° under 10 Pa for 1 h) were reinserted and the tubes evacuated (0°, < 1 Pa, 5 min). Carbon dioxide was liberated from the carboxyl group of leucine by reaction at 100° for 1 h, followed by standing at room temperature for a further 1 h. Samples were then analysed within 18 h on a gas isotope-ratio mass spectrometer (GIRMS; SIRA 12, VG Isogas, Middlewich, Cheshire) fitted with an automatic cryogenic purification system and samples expressed either as atoms % excess by reference to a standard PeeDee Belemnite (1·1112328 atoms %) or as ‰ calculated as

$$\delta\,^{13}\mathrm{C}\ ‰ = \frac{\text{R sample} - \text{R standard}}{\text{R standard}} \times 1000,$$

where R is the $^{13}C:^{12}C$ ratio and standard is PeeDee Belemnite where R = 0·0112372.

The enrichment of free leucine and MOP from plasma was measured by gas–liquid chromatography–mass spectrometry (GCMS) on a VG 12–250 quadrupole mass spectrometer (VG MassLab, Manchester). Leucine was measured as the tertiary butyldimethylsilyl derivative and MOP as the quinoxalinol tertiary butyldimethylsilyl derivative under electron-impact ionization with selective ion monitoring (Calder & Smith, 1988). The enrichment of leucine from the muscle biopsies was measured by GCMS as the N-heptafluorobutyryl *n*-butyl ester derivative under negative chemical ionization with methane as the reagent gas, by a modification of a previously described method (MacKenzie & Tenaschuk, 1974; Ford *et al.* 1985). The enrichments of free leucine and MOP and of the injected leucine, were expressed as atoms % excess over the zero-time value.

Protein, DNA and RNA measurements

The alkali-soluble supernatant fraction was diluted, by weight, with 0·3 M-NaOH (1:50) and protein determined by an autoanalyser procedure (Gaunce & D'Iorio, 1970). RNA was measured in the 2·3 M-PCA supernatant fraction by a modified orcinol procedure

(Kerr & Seraidarian, 1945), using weighed rather than volumetric additions. DNA was quantified in the 0·6 M-PCA supernatant fraction by the method of Burton (1956), again all additions were weighed. Supernatant fractions were in general analysed immediately; repeat measurements, where necessary, were conducted on samples of frozen (−20°) supernatant fraction.

Other analytical techniques

Plasma immunoreactive insulin was assayed based on the method of Midgley *et al.* (1969), using anti-porcine antibody (ICN Biomedicals Ltd, High Wycombe, Bucks). Inter- and intra-assay coefficients of variation were 12 and 8% respectively.

Calculations and statistics

The fractional rate of muscle protein synthesis (k_s; /d) was calculated from the formula described by Garlick *et al.* (1980)

$$k_s = (P_t - P_0)/A,$$

where P_0 and P_t are enrichments (atoms % excess) of leucine in muscle protein at times 0 and t respectively and A is the area under the curve for precursor enrichment (i.e. atoms % excess × time (d)), calculated by trapezium-based analysis.

Six periods × five animals formed a randomized block design. The interactions between animals and periods could not be used, however, to provide an estimate of error as the animals varied in their change-over time. The main effect of period was, therefore, divided into three components and the interaction into three associated components, i.e. (1) [control 1 and 2 *v.* control 3] × animal, 4 df; (2) (treatment *v.* mean of controls) × animal, 4 df; (3) (within control plus within treatment) × animal, 12 df. Assuming any time-related changes to be largely linear the first component would be expected to contain the largest error. The second component which provides a genuine estimate of error (but with only 4 df) was expected to be of the same magnitude or slightly larger than the third. The analysis indicated, however, that there was no difference in components 2 and 3 and they were combined to provide a measure of error (with 16 df).

RESULTS

N retention studies

Testosterone administration induced a significant improvement in N retention (average of 3·7 g N/d, $P < 0{\cdot}001$, +265%; Table 1) compared with pretreatment controls. For three of the sheep the improvement was due exclusively to a decrease in urinary N elimination; for the other two a proportion of the effect was in N digestibility. The post-treatment control was also higher than pretreatment controls which indicates that the testosterone effect, or the androgen itself, was not totally cleared 2 weeks after cessation of infusion. This pattern in the partition of N excretion observed during testosterone infusion was maintained for the post-treatment control although the net N retention had declined (Table 1).

Muscle composition

The chemical analyses for the two muscles were similar and near constant across the control and treatment periods (Tables 2 and 3). The use of gravimetric rather than volumetric procedures improved precision considerably and reduced variation, e.g. the mean RNA:protein of three biopsy samples for each of six different treatment periods gave a coefficient of variation of 3·05% by volumetric procedures but only 0·17% when calculated gravimetrically. Despite the increase in N retention no change in the protein:DNA, an indicator of hypertrophic growth, was observed for either muscle.

Table 2. *M. longissimus dorsi RNA:protein, protein:DNA, RNA:DNA and fractional protein synthesis rates (× 100; $k_{s,1}$; $k_{s,m}$) before, during and after treatment with 9 mg testosterone/d supplied by episodic, intravenous infusion*

(Means of five animals)

	Period							Statistical significance of difference:	
	Pre controls		Treatment			Post-control			
	C_1	C_2	T_1	T_2	T_3	C_3	SED	*P*	Residual df*
RNA:protein (μg/mg)	3·57	3·56	3·56	3·57	3·57	3·56	0·005	NS	16
Protein:DNA (μg/μg)	341	342	340	340	341	341	0·7	< 0·1	16
RNA:DNA (μg/μg)	1·217	1·218	1·212	1·212	1·218	1·214	0·003	NS	16
$k_{s,l}$†	2·99	2·33	2·56	2·31	2·69	3·16	0·34	NS	14
$k_{s,m}$†	2·93	2·24	2·49	2·34	2·59	3·06	0·33	NS	14

C_1, C_2, C_3, pre-, −12 and −5 d (C_1, C_2) and post, +16 d (C_3) control periods; T_1, T_2 and T_3, times (2, 9 and 15 d respectively) after commencement of testosterone infusion; SED, standard error of difference; NS, not significant.

* For complete data set then residual df 16; mean values include estimated missing values.

† Fractional synthesis rates (× 100) based on either plasma free leucine ($k_{s,l}$) or 4-methyl-2-oxo-pentanoate ($k_{s,m}$) enrichment areas.

Table 3. *M. vastus lateralis RNA:protein, protein:DNA, RNA:DNA and fractional protein synthesis rates (× 100; $k_{s,1}$; $k_{s,m}$) before, during and after treatment with 9 mg testosterone/d supplied by episodic, intravenous infusion*

(Means of five animals)

	Period							Statistical significance of difference:	
	Pre controls		Treatment			Post-control			
	C_1	C_2	T_1	T_2	T_3	C_3	SED	*P*	Residual df*
RNA:protein (μg/mg)	3·58	3·57	3·57	3·56	3·58	3·57	0·01	NS	16
Protein:DNA (μg/μg)	341	340	341	341	341	341	0·8	NS	16
RNA:DNA (μg/μg)	1·220	1·213	1·219	1·214	1·220	1·218	0·005	NS	16
$k_{s,l}$†	2·43	2·39	2·93	2·59	2·69	2·62	0·49	NS	11
$k_{s,m}$†	2·13	2·26	2·86	2·60	2·59	2·44	0·45	NS	11

C_1, C_2, C_3, pre-, −12 and −5 d (C_1, C_2) and post-, +16 d (C_3) control periods; T_1, T_2 and T_3, times (2, 9 and 15 d respectively) after commencement of testosterone infusion; SED, standard error of difference; NS, not significant.

* For complete data set then residual df 16; mean values include estimated missing values.

† Fractional synthesis rates (× 100) based on either plasma free leucine ($k_{s,l}$) or 4-methyl-2-oxo-pentanoate ($k_{s,m}$) enrichment areas.

Validation of isotope procedure

In the preliminary studies plasma leucine concentrations were elevated 15-fold immediately post-injection compared with pre-injection values and at the end of 2 h were still 2–4-fold greater (Fig. 1(*a*)). Plasma free leucine enrichment was increased to 4 atoms % excess and then declined in a near linear fashion to 2·2–2·4 atoms % excess over 2 h (Fig. 1(*b*)). Plasma MOP showed similar kinetics but took longer to reach maximum enrichment (delay approximately 5 min) and, thereafter, remained slightly higher than plasma free leucine; this is compatible with entry of leucine into cells, production of MOP therein, followed by transport into the blood. Muscle free leucine enrichment also exhibited an initial lag, as

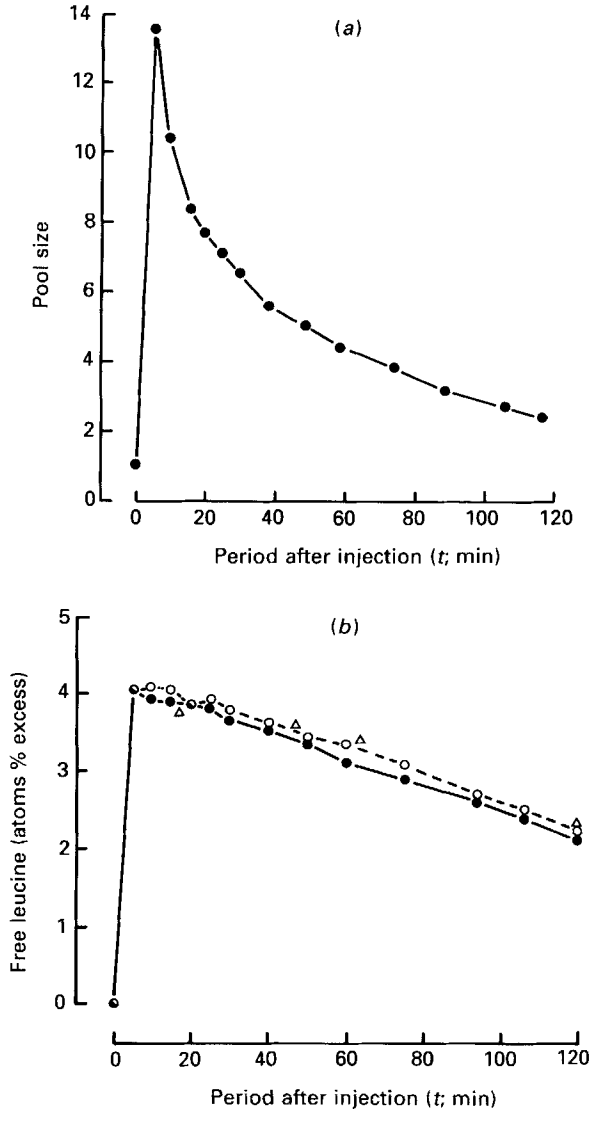

Fig. 1. Changes in (*a*) plasma free leucine concentration and (*b*) enrichment of plasma free leucine (●), plasma 4-methyl-2-oxopentanoate (○) and muscle homogenate free leucine (Δ), after intravenous injection of a mixture of 0·25 g [1-^{13}C]leucine plus 4·75 g L-leucine into 40-kg lambs (values are means of two animals). For details of procedures, see p. 692.

would be expected for amino acid entry, but at 60 and 120 min tissue free amino acid enrichment was equal to or slightly greater than that for the plasma leucine or MOP (see Fig. 1(*b*)).

Similar observations were made during the main study (values not shown) except that the plasma free leucine enrichment attained a maximum of 14 atoms % excess followed by a linear decline to 6–7 atoms % excess. Areas under the plasma free leucine or MOP curves were nearly identical, e.g. MOP:leucine area was 1·011 (SD 0·017; *n* 30) for 1 h and 1·028 (SD 0·015; *n* 30) over the 2 h.

Protein-bound fractions of leucine required to be separated from other carboxyl-containing compounds, notably isoleucine; this necessitated chromatographic conditions which ensured at least 5 min baseline between the two amino acids. Analysis of leucine, mixed with isoleucine in proportions similar to muscle protein (1:0·6, molar basis), separated through the procedure produced analyses of 25·23 (SD 0·11; *n* 6) ‰ for standard and 25·29 (SD 0·17; *n* 6) ‰ for separated standard. Hydrolysed protein, from slightly enriched sheep muscle, gave on repeat analyses 23·11 (SD 0·38; *n* 7) ‰.

Protein synthesis

The similarity in areas under the plasma enrichment *v.* time curves for free leucine and MOP meant that synthesis rates calculated assuming either as the precursor were very close (Tables 2 and 3). The leucine:MOP areas ratio did not alter throughout the course of the experiment, nor with treatment (values not shown).

Muscle protein synthesis (k_s) did not differ significantly between control and hormone-treatment periods for either m. longissimus dorsi or m. vastus lateralis (Tables 2 and 3). This was valid no matter whether plasma free leucine or MOP or tissue homogenate free leucine enrichment was chosen as appropriate precursor. Similarly no change in protein:RNA was observed; this index sometimes indicates whether the synthetic capacity of tissues is altered.

In the present study initial [1–^{13}C]leucine enrichment from plasma protein was 1·07186 (SD 0·0017; *n* 5) atoms % compared with 1·07347 (SD 0·0004; *n* 8) atoms % from muscle protein. These differences should be compared with the usual increase in enrichments, obtained 2 h after isotope injection, of approximately 0·02 atoms % (i.e. approximately 18‰). As would be expected muscle protein enrichment increased throughout the period of study; under the conditions employed each week muscle presamples were approximately 20 ‰ more enriched than the previous pre-injection biopsy. Thus, by the end of the study the presample had an enrichment of +100 ‰ (i.e. +0·11 atoms %) compared with the first biopsy and the standard deviation of replicate sample analyses was greater at 0·001 ‰ (*n* 15). Part of the increased variance could be ascribed to technical (GIRMS) factors, but part may have been due to slightly different protein enrichments within the biopsy samples. The close relationship between initial muscle and plasma protein leucine enrichment only held for the first control period.

Leucine injections altered plasma concentrations of other amino acids. The extent of these changes varied from animal to animal but, under worst conditions, resulted in decreases in plasma free amino acid concentrations of 40–50% for valine, isoleucine, phenylalanine and tyrosine (Lobley *et al.* 1990).

Effect of insulin

In response to leucine (but not a similar volume of physiological saline), insulin plasma concentrations increased maximally by 20–40 mU/l from a baseline of 31 (SE 4) mU/l; within 40 min from the start of injection, hormone concentration was restored to pre-injection values. The insulin secretagogue activity of large-dose injections of other amino acids has been reported elsewhere (Lobley *et al.* 1990).

DISCUSSION

Large-dose procedure

General considerations. The popularity of the large-dose procedure rests on the equalization of the free amino acid isotopic activity between the tissue and plasma pools, with the consequent removal of uncertainty as to which best represents the precursor for protein

synthesis (Garlick *et al.* 1980). This provides a considerable advantage, but there are drawbacks to the technique which need also to be considered. First, the period of measurement is short, usually minutes or a few hours and, therefore, maintenance of an acceptable metabolic steady-state is needed if results are to be extrapolated to a daily basis. This was the reason for the adoption of the frequent-feeding pattern in the current study. Second, perturbations in either hormone or substrate concentrations may occur as a consequence of the amino acid injection and these may alter rates of protein synthesis.

Technical developments. The new method was developed to allow repeat measurement of muscle protein synthesis (or other accessible tissues) in farm livestock and to achieve this use was made of the sensitivity of gas isotope ratio mass spectrometry. Approximately 50 mg muscle tissue provided adequate protein for isolation of 3 μmol leucine, enough for one analysis by the ninhydrin decarboxylation procedure (Read *et al.* 1984). Good repeatability was achieved provided considerable care was taken to ensure that the complete leucine peak was collected and was free of any other amino acid contamination. This was important because of the isotopic fractionation which occurs during separation of both stable and radio-labelled materials on ion-exchange (e.g. Gaitonde & Nixey, 1972; Nicholas *et al.* 1977), gas (e.g. Millard, 1979) and reverse-phase high-performance liquid chromatography (e.g. Brown *et al.* 1986).

With animals not previously injected or infused with enriched ^{13}C-labelled compounds it may be possible to use either plasma or blood protein as the baseline sample rather than use a biopsy presample (see also Heys *et al.* 1990*a*, *b*). In the current study adoption of this procedure would have resulted in a 7·5% overestimate of muscle protein synthesis, while for more rapidly turning-over tissues (e.g. liver, gastrointestinal tract, skin) the error would have been proportionally less. Such an application would also not be appropriate if dietary conditions (and, therefore, possibly the natural enrichment of amino acids) had been switched in the recent past, since the turnover of the blood proteins differs from that of the tissues.

Biological considerations. The amount of leucine injected approximated to 0·8 mmol/kg body-weight or four to eight times body free leucine; this is more than in human measurements (0·4 mmol/kg; Garlick *et al.* 1989) but less than the phenylalanine injected in rats (1·5 mmol/kg; Garlick *et al.* 1980), the valine used for growing lambs (3·5 mmol/kg; Attaix *et al.* 1986) and the proline administered to growing lambs (6 mmol/kg; Pell & Bates, 1987). Rapid injection of leucine (< 3 min) induced pyrexia, muscle tremor, and on occasions, inappetance; none of these symptoms was observed with the slower injection rate adopted.

The stimulation of insulin secretion by large amounts of leucine (and other amino acids) has been shown for many species (e.g. Fajans *et al.* 1967; Rocha *et al.* 1972; Spangler & Phillips, 1982; Mazzaferri *et al.* 1983). The question is whether protein metabolism will be altered by changes in insulin status? Certainly, lambs offered grass pellets did not alter N balance when glucose plus insulin were infused over several days (Sumner & Weekes, 1983) even though a similar protocol increased protein gain in pigs (Fuller *et al.* 1977). Infusion of the hormone increased plasma immunoreactive insulin from 14 to 35 mU/l and this can be compared with the average peak increase in immunoreactive insulin of 34 mU/l induced by the leucine injection in the present investigation, albeit the response was transient with pre-injection values re-established within 40 min. Lack of response in N retention studies to chronic hormone administration does not mean necessarily that changes in protein synthesis within specific tissues may not occur during acute changes in endocrine status but, in ruminants, the involvement of insulin in control of protein synthesis is controversial. Thus, Early *et al.* (1988*a*, *b*) observed a non-significant increase in fractional synthesis rate of sheep muscle with infusion of a mixture of glucose plus insulin while, in contrast, Oddy

et al. (1987) observed no increase in hind-leg protein synthesis, degradation or retention in milk-fed lambs in response to insulin infusion, although the hormone decreased synthesis and, to an even greater extent, degradation in the fasted animal. This contrasts with the stimulation of muscle protein synthesis in simple-stomached animals (e.g. Garlick *et al.* 1983; Palmer *et al.* 1985; Jepson *et al.* 1988), although the sensitivity may decline with age (cf. Baillie *et al.* 1988; Garlick & Grant, 1988). In the current study pre-injection plasma insulin concentrations were 15–30 mU/l for four of the animals and 60–90 mU/l for the other but, despite this wide range in systemic hormone concentrations, k_s measurements were similar. These concentrations, achieved in fed animals, may be above the response limits for muscle protein synthesis (reported to be 20 mU/l for the rat; Jepson *et al.* 1988) and, thus, further increases may produce no effect.

Leucine *per se* has been reported to stimulate muscle protein synthesis, mainly in 'stepped down' or in vitro conditions (e.g. Buse & Reid 1975; Fulks *et al.* 1975; Buse & Weigand, 1977; Li & Jefferson, 1978; Buse *et al.* 1979; Smith, 1985). This has been difficult to confirm in rodents in vivo (McNurlan *et al.* 1982). In sheep, however, Schaefer *et al.* (1986) observed a doubling of muscle k_s when infusion of leucine for 8 h was increased from 0 to 22·8 mmol/h (compared with our injection of 38 mmol/8–10 min). In contrast, neither in ruminant (Matras & Preston, 1985) nor preruminant lambs (Papet *et al.* 1988) did exogenous leucine administration alter N retention and there is evidence from studies on human muscle that apparent effects of amino acids on protein synthesis depend on which precursor pool is selected (Bennet *et al.* 1989).

Future improvements. The method developed is a useful addition to techniques already available for the measurement of tissue protein synthesis in farm species (Lobley, 1988); the certainty of pool isotopic activity offers advantages over tracer infusion techniques; the repeat biopsy technique allows intra-animal response analysis, with consequent reduction in biological variability; the cost of isotope use is also moderate and this would be reduced if more metabolically active tissues or animals were studied. Two improvements are recommended: first, an amino acid with less (zero) insulin secretagogue activity should be chosen as a marker (e.g. valine; see Lobley *et al.* 1990); second, for extensive repeat studies different amino acids (or isotopes of the same amino acid) should be considered to reduce the increased 'background' variability which occurs with frequent isotope administration.

Effects of testosterone infusion

In the current study the improvement in total N retention (mean + 2·9 g N/d, when compared with the average of the pre- and post-treatment periods) produced by intravascular testosterone infusion was even greater than that observed in an earlier, similar study (mean + 1·2 g N/d; Lobley *et al.* 1987). Despite this large change in rate of protein accretion, muscle k_s was unaffected by hormone treatment, a finding in agreement with some studies in laboratory species (e.g. Grigsby *et al.* 1976; Stratman, 1978; Dohm *et al.* 1979) and muscle cells in culture (Ballard & Francis, 1983). It must also be considered, however, whether large changes in k_s would be expected for fattening sheep for, despite the large effects on N balance, the response in body N fractional accretion rate, (k_g), is small in absolute terms. Based on a body N content of 25 g N/kg live weight then the sheep k_g ($\times 100$) during control periods would be 0·27 and 0·53 %/d during testosterone administration. It is not known if this anabolic response is distributed across all tissues but body composition findings suggests that effects may be concentrated in the carcass (muscle plus bone) components (Singh *et al.* 1985). If the anabolism were exclusively in the carcass (50·1 % of body N, J. C. MacRae, personal communication) then muscle k_g would be increased by 0·51 %/d. These increments should be compared with fractional synthesis rates ($\times 100$) of approximately 2·5 %/d such that changes of 10–20 % would be sufficient

to provide the extra protein accreted. Of course if the efficiency of deposition:synthesis were only approximately 50% (or less), as observed in a range of nutritional and hormonal studies on whole-body and muscle protein metabolism (see Lobley, 1988), then the synthesis changes would require to be doubled. In the latter circumstance they would certainly be detected by the method employed, while changes of only 10% would be beyond the limits of confidence.

Similarly it is not possible to decide if additional muscle growth had occurred during testosterone administration. Hypertrophic growth should increase protein:DNA unless proliferation of satellite cells or recruitment into fibres occurred. The protein:DNA ratio was unchanged by treatment and, over a 15 d period, if the extra protein gain was exclusively in muscle, but not accompanied by additional DNA synthesis, then the ratio would change by less than 4%. In fact, the repeatability of the method was such that this would have been detected. The RNA:protein was also unchanged and, if the concept of maximum synthesis at supramaintenance intakes is related to ribosome number, i.e. 'translation capacity', rather than changes in rates of protein synthesis per unit (ribosomal) RNA, i.e. 'translational activity', is correct, this would support the observation that muscle k_s is unaltered by testosterone.

Protein anabolism may also occur through reduction in protein breakdown and decreased proteolysis of myofibrillar proteins has been reported for the 'synthetic androgen', trenbolone acetate (Vernon & Buttery, 1978; van Eenaeme *et al.* 1983). Indeed, the activity of the protein-catabolic glucocorticoids (Millward *et al.* 1983; Garlick *et al.* 1987) can be modulated by testosterone (e.g. Mayer & Rosen, 1977; Thomas & Rodway, 1982). An alternative indirect action has been proposed involving growth hormone (GH) (Scow & Hagan, 1965; Jansson *et al.* 1983; Ohlsson *et al.* 1987) and differences in GH pulsatile secretion between rams, wethers and ewes can be altered by pharmacological, but not physiological, doses of testosterone (cf. Davis *et al.* 1977; Lobley *et al.* 1987). Similarly either testosterone or GH restores skeletal growth in hypophysectomized, prepubertal lambs (Young *et al.* 1989). To date, actions of GH, or more probably insulin-like growth factor-1, the production of which is GH-regulated at both the hepatic (e.g. Schimpff *et al.* 1976; Coxam *et al.* 1989) and muscle level (Isgaard *et al.* 1989), have involved alteration in protein synthesis (e.g. Pell *et al.* 1987; Pell & Bates, 1987; Eisemann *et al.* 1989). It is possible that testosterone interacts with both GH and glucocorticoids but that in any specific physiological condition one interaction predominates; this would explain the contradictory nature of the evidence available.

Under the conditions of the present study, however, the protein anabolic effect of testosterone is probably expressed through reduction of muscle protein degradation.

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