The urinary excretion of N^{τ}-methyl histidine in sheep: an invalid index of muscle protein breakdown

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1. The validity of the urinary excretion of N^{τ}-methyl histidine (N^{τ}-MH) in sheep as a measure of the breakdown of muscle protein in vivo was assessed from the urinary recovery of radioactivity following the intravenous administration of N^{τ}-[$^{14}CH_3$]methyl histidine.

2. Recoveries of radioactivity in urine from animals of 4 weeks to 7 years of age were incomplete in 7 d but progressively increased with the age of the animal, becoming almost quantitative (90%) in older animals after recovery for 3 weeks.

3. The incomplete urinary recoveries were not due to partial excretion of N^{τ}-MH in faeces or its oxidation and elimination in expired gases but were related to the presence in muscle of a pool of non-protein-bound N^{τ}-MH which was several times larger than the expected daily urinary excretion.

4. This pool in newly accreted muscle tissue was maintained by retention of some of the N^{τ}-MH released by breakdown of muscle protein. Hence, only a proportion of the N^{τ}-MH released from protein breakdown was available for excretion. This proportion increased with the age of the animal and was probably the main determinant of the improved recoveries of radioactivity obtained in urine from older animals.

5. The non-protein-bound N^{τ}-MH in muscle consisted of free N^{τ}-MH and a dipeptide containing N^{τ}-MH, the latter comprising on average approximately 82% of the total non-protein-bound N^{τ}-MH in muscle. This proportion did not change appreciably with the age of the animal.

6. The dipeptide appeared to be synthesized in muscle from free N^{τ}-MH and was not a terminal product of protein breakdown.

7. The results show that urinary excretion of N^{τ}-MH is not a reliable index of muscle protein breakdown in sheep.

Skeletal muscle protein constitutes the major protein deposit of the body and as such functions as a protein store for whole-body protein metabolism (Daniel *et al.* 1977). The quantitative contribution of muscle protein to whole-body protein metabolism, however, is poorly characterized and its role in pregnancy and lactation is essentially unknown.

Methods of measuring muscle protein breakdown in vivo by the rate of loss of label from protein following administration of a radioactive amino acid are unsatisfactory in general due to the re-utilization of the labelled amino acid (Schimke, 1970). In addition, these methods do not allow an accurate assessment of the contribution of an individual tissue such as muscle to whole-body protein metabolism. However, two of the structural proteins of muscle, actin and myosin, contain an amino acid, 3-methyl histidine or N^{τ}-methyl histidine (N^{τ}-MH), which is not re-utilized in protein synthesis and which has been shown to be rapidly and quantitatively excreted in the urine of rats (Young *et al.* 1972), man (Long *et al.* 1975), rabbits (Harris *et al.* 1977) and cattle (Harris & Milne, 1978) as judged by the recovery in urine of labelled N^{τ}-MH after intravenous administration of the amino acid. Since N^{τ}-MH occurs almost exclusively in muscle protein (Asatoor & Armstrong, 1967; Haverberg *et al.* 1975), its excretion not only provides a measure of muscle protein breakdown in the previously-mentioned species but also allows the contribution of muscle protein to general protein metabolism to be assessed when whole-body protein breakdown is measured independently. This approach has been utilized in the rat and man (Young & Munro, 1978) where muscle protein contributes 8–9% and 20–26% respectively to whole protein breakdown.

The urinary excretion of N^{τ}-MH in man as a measure of muscle protein breakdown in

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vivo has been exploited in the human clinical field and it is tempting to apply the method to farm species where one of the main aims is the identification of animals with improved efficiency of protein accretion. However, caution is necessary in the indiscriminate application of the method to species in which it has not been validated. The variable excretion of N^{τ}-MH encountered in young lambs by Buttery *et al.* (1977) illustrates this point for these workers had not demonstrated the validity of the approach in their animals.

This paper describes the attempted validation of urinary excretion of N^{τ}-MH in sheep as a measure of muscle protein breakdown. It is the first instance reported in which the method was unsuccessful and provides an insight into reasons for the failure. A brief account of this work has been published (Harris & Milne, 1977) and later studies suggest that urinary N^{τ}-MH is also an invalid index of muscle protein breakdown in the pig (Milne & Harris, 1978). However, the method does appear to be valid in cattle (Harris & Milne, 1978).

MATERIALS AND METHODS

Animals

The sheep used were of various breeds: Suffolk × Suffolk Finn Dorset Poll (SSFDP), Suffolk × Finn Dorset Poll (SFDP), Suffolk × Blackface (SBF), Suffolk × Cheviot (SC), Blackface × Blackface (BF) and details are given in the Tables. Male animals were castrates except for a few intact males (IM). The animals were fed on a cereal-based diet containing (g/kg): rolled barley 828, soya-bean meal 146, steamed bone flour 24, and a trace mineral mixture, as defined by Ørskov *et al.* (1974), at 1 kg mix/ton diet.

Materials

N^{τ}-[$^{14}CH_3$]methyl histidine, obtained from New England Nuclear, Boston, Mass., USA, was supplied at a specific activity of 46 mCi/mmol. The purity of the preparation was between 85 and 95% during the period of the investigation.

Standard amino acid mixtures, type PB, for calibration of amino acid analyses were prepared by the Hamilton Company, Reno, Nevada, USA, and supplied by V. A. Howe & Co. Ltd, London. Individual amino acids were purchased from Calbiochem Ltd, Bishops Stortford, Herts. All other chemicals were of analytical grade, or the highest quality available.

Route of administration of labelled N^{τ}-MH

Previous studies with rats (Young *et al.* 1972) showed that quantitative recoveries of radioactive N^{τ}-MH and its metabolites in urine were obtained after either oral or intravenous administration. Labelled N^{τ}-MH was therefore administered by direct intravenous (jugular) injection or by jugular catheter. The possibility of degradation of N^{τ}-MH in the rumen was thus avoided (Annison & Lewis, 1959; Barnett & Reid, 1961; Mangen, 1972).

Collection of excreta

Animals were kept in metabolic cages fitted with urine-faeces separators for collection of excreta. Separators were washed with water each day before collection of urine samples, the washings being mixed with the respective urine samples. Urine was collected under toluene to minimize bacterial contamination and to facilitate the subsequent identification of any acid-labile metabolites of N^{τ}-MH. Expired gases were monitored for radioactivity by passing them through a Cary-Tolbert ionization chamber connected to a Cary model 401 Vibrating Reed Electrometer (Varian Associates, Palo Alto, California, USA).

Sample preparation and analysis

Blood and muscle samples were deproteinized with perchloric acid (PCA) and treated as described by Nicholas *et al.* (1977). PCA-soluble extracts were hydrolysed in 6 M-HCl at 110° for 16 h and the acid subsequently removed in vacuo.

Chromatographic analyses were carried out on an amino acid analyser (The Locarte Co., Wendell Road, London) using the system of Spackman *et al.* (1958) for the basic components of physiological fluids. The distribution of radioactivity in PCA-soluble extracts of muscle was determined on a 6 × 240 mm bed of M72 resin (Beckman Instruments Ltd, Glenrothes, Fife) thermostated at 28° and operated at 30 ml/h in sodium citrate buffer, 0·38 N in Na^+, pH 4·18. The column effluent was collected in 3 ml fractions.

The concentration of N^{τ}-MH in extracts, equivalent to 0·5–1·0 ml blood or 0·5–1·0 g muscle, were measured analytically using the same system except that a longer column (6 × 400 mm resin bed) was used. The buffer pH was 4·18 for hydrolysed extracts and 4·12 for unhydrolysed samples.

Liquid-scintillation counting

Radioactive samples were counted in 'mini' tubes or standard vials containing 3 and 10 ml respectively of liquid scintillator (NE 260; Nuclear Enterprises (GB) Ltd, Edinburgh) in a Packard Tri-carb Model 3315 (Packard Instrument Co., Illinois, USA) or Beckman LS 345 liquid-scintillation spectrometer (Beckman Instruments Ltd, Glenrothes, Fife). Samples were corrected for quench by the use of an internal standard.

RESULTS

Recovery of radioactivity in excreta

A solution of N^{τ}-[$^{14}CH_3$]methyl histidine in sterile saline (9 g sodium chloride/l) was injected into the jugular vein of two young lambs and repeated at intervals of 5–16 weeks. Urine was collected daily and the cumulative recoveries of radioactivity from the lambs (nos. 740, 764) are shown in Table 1. The results show that, at all ages, the recovery of radioactivity in urine in 7 d was less than quantitative and that the percentage of the administered dose recovered in 7 d increased with the age of the animals.

Because of the low recoveries of radioactivity in urine and also to determine whether the gut might be an alternative pathway for excretion of N^{τ}-MH in sheep, aqueous extracts of faeces from the animals noted in Table 1 were monitored for radioactivity. The total radioactivity in faeces did not account for more than 3% of the injected dose and this was attributed to contamination of the faeces with urine in the separator. Faeces were not monitored in subsequent recovery measurements.

The incomplete urinary recoveries of radioactivity in the 7 d following a dose of labelled N^{τ}-MH was confirmed in additional animals (Table 1), each of which was used for a single recovery experiment. The results given in Table 1 were obtained from animals covering a range from weaning at 4 weeks to 7 years of age and also demonstrated the age-related increase in urinary recovery of radioactivity previously identified in the two lambs. The increasing recoveries with age from these animals eliminated the possibility that the changing recoveries found for lambs 740 and 764 (Table 1) were due to the progressive carry-over of radioactivity from one injection to the next. In five studies of recoveries of radioactivity in urine over a longer time-scale, it was found that significant levels of radioactivity were still being excreted during the third week after administration (Table 1) and that the cumulative recoveries in the oldest animals then became almost quantitative (90%).

Since the recoveries of radioactivity in the urine from young animals were less than 50%

Table 1. *Variation in the urinary excretion of radioactivity with age in sheep after single or repeated dosing with N^{τ}-[$^{14}CH_3$]methyl histidine*

	Age (weeks)	Sex	Breed	Wt (kg) at injection	Cumulative recoveries (% injected dose) in: 7 d	14 d	21 d
(no. 740)	8	♂	SFDP	17·5	27·0	—	—
	13			20·0	31·6	—	—
	23			47·0	45·3	—	—
	36			66·0	70·5	—	—
(no. 764)	8	♂	SFDP	15·5	36·6	—	—
	13			22·5	*41·8	—	—
	23			45·0	49·4	—	—
	52			65·0	62·0	—	—
	4	♂	SFDP	9·75	41·8	—	—
	9	IM		10·0	34·4	47·0	51·3
	10	♂	SSFDP	12·3	26·8	—	—
	15	♂		22·75	34·7	—	—
	16	♀		16·0	47·2	—	—
	20	IM	SFDP	24·75	47·7	62·6	68·6
†	27	IM		44·9	33·8	45·1	54·9
†	39			64·25	54·3	69·3	77·7
	15 months	♀	SC	49·0	74·6	86·8	90·2
	7 years	♀	BF	39·0	78·0	—	—

IM, intact male.
SFDP, Suffolk × Finn Dorset Poll; SSFDP, Suffolk × Suffolk Finn Dorset Poll; SC, Suffolk × Cheviot; BF, Blackface × Blackface.
* 6 d recovery. † Values from the same animal.

of the dose after 1 week, the possibility of oxidation of N^{τ}-MH and subsequent elimination as $^{14}CO_2$ was considered. Lambs were injected intravenously as before and placed in metabolic cages fitted with Perspex chambers for the head and made leak-proof with flexible neck-cuffs. Expired air from the head chamber was passed through an ionization chamber. In two separate experiments less than 5% of the dose of ^{14}C was extracted in 3 d in expired gases. This level of radioactivity was at the limit of detection of the apparatus and it was concluded that oxidation of N^{τ}-MH did not form an important route of elimination from the body. The failure to detect pathways of elimination of radioactivity other than urine indicated that the fraction of the dose not recovered in urine remained in the animal body.

The site of retention of radioactivity

Amino acid analyses of deproteinized extracts of blood and muscle (longissimus dorsi) showed that both tissues had relatively low concentrations of free N^{τ}-MH (Tables 2 and 3). Acid-hydrolysis of muscle extracts resulted in increases in N^{τ}-MH by up to a factor of fifteen times (Table 2). The mean concentration (nmol/g muscle) of total non-protein-bound N^{τ}-MH in the muscle of 9–18-week-old lambs (n 12) was 294·0 (Table 4) compared with mean values of 287·6 at 15 months (n 2), 196·3 at 33 months (n 3) and 138·0 at 84 months (n 2). The mean concentration of N^{τ}-MH from all 15–84-month-old sheep (n 7) was 205·7 nmol/g muscle. The mean percentage of N^{τ}-MH in the acid labile form (Table 4) from 9–18-week-old lambs was 82·4 compared with values of 85·9, 87·1 and 72·6 from 15, 33 and 84-month-old sheep respectively. The mean percentage for all 15–84-month-old animals was 82·6. The apparent decrease in the total concentration of non-protein-bound N^{τ}-MH in

Table 2. *Concentration (nmol/g muscle) of non-protein bound N^{τ}-methyl histidine in m. longissimus dorsi of sheep*

Sheep				Non-protein bound N^{τ}-MH		N^{τ}-MH in acid-labile form (%)
Age (weeks)	Sex	Breed	Wt (kg)	Free	Total	
9	♀	SFDP	12·25	35·4	270·0	86·9
10	♂	SFDP	13·0	17·9	201·3	91·1
10	♂	SFDP	14·0	30·6	202·5	84·9
10	♂	SFDP	14·0	44·4	339·6	86·9
10	♂	SFDP	15·75	54·5	335·3	83·8
10	♂	SFDP	12·5	24·4	132·7	81·6
12	♂	SFDP	15·0	61·6	248·9	75·3
12	♂	SFDP	23·4	50·4	331·9	84·8
12	♂	SSFDP	14·25	116·2	597·6	80·6
13	♂	SFDP	15·7	72·2	203·2	64·5
13	♂	SFDP	15·6	60·9	244·3	75·1
18	♀	SSFDP	16·75	27·0	420·6	93·6
Age (months)						
15	♂	SFDP	71·5	34·5	222·3	84·5
15	♀	SC	49·0	44·6	352·9	87·4
33	♂	SFDP	96·0	36·3	264·6	86·3
33	♂	SFDP	105·0	30·5	164·5	81·5
33	♂	SFDP	104·0	10·6	159·9	93·4
84	♀	BF	39·0	16·7	81·6	79·5
84	♀	SC	—	66·8	194·4	65·5

muscle with age suggested by the mean values for the 4 groups and the difference between the mean values of the proportion of N^{τ}-MH in the acid-labile component were not significant.

In contrast to the findings in muscle, extracts of blood showed only small increases in the amount of N^{τ}-MH after acid-hydrolysis (Table 3). The mean concentration of total non-protein-bound N^{τ}-MH/ml blood from the five youngest animals (9–30 weeks) was 63·8 nmol with a mean percentage of 30·9 of the N^{τ}-MH being in the acid-labile form. Comparable values for the five oldest animals (15–84 months) were a mean of 36·3 nmol N^{τ}-MH/ml blood, the mean percentage in the acid-labile form being 13·2. The apparent decrease in both the total concentrations of non-protein-bound N^{τ}-MH in blood and the proportion of the N^{τ}-MH in the acid-labile form with age were not significant between the two groups.

Chromatographic analysis of extracts prepared from serial muscle biopsies taken from a sheep following a pulse injection of labelled N^{τ}-MH showed two radioactive components which together accounted for all the radioactivity (Table 4). The first component co-chromatographed with N^{τ}-MH while the second component eluted later close to the position of carnosine (Fig. 1). Radioactive N^{τ}-MH was rapidly incorporated into component no. 2 (Table 4) which was labelled to the same extent as component no. 1 after approximately 2·5 h. Analysis of the last biopsy sample after acid-hydrolysis showed that all the radioactivity of the sample was then in the position of the first component (Table 4).

The acid-labile component containing N^{τ}-MH has been identified as the dipeptide β-alanyl-N^{τ}-methyl histidine (unpublished observations). The identification of the perchloric acid-soluble, acid-labile compound containing N^{τ}-MH in blood (Table 3) has not been pursued. It is assumed that this compound is identical to the dipeptide identified in muscle.

Table 3. *Concentration (nmol/ml blood) of non-protein bound N^{τ}-methyl histidine in blood of sheep*

Sheep			Non-protein bound N^{τ}-MH		
Age (weeks)	Sex	Breed	Free	Total	N^{τ}-MH in acid-labile form (%)
9	♀	SFDP	32·6	55·9	41·7
12	♂		49·1	59·9	18·0
12	♂		37·6	89·8	58·1
15	♂	SBF	39·9	44·9	11·1
21	♂	SFDP	25·3	34·1	25·8
30	♂		24·6	34·4	28·5
52	♂		39·6	46·6	14·7
Age (months)					
15	♂	SFDP	43·4	55·3	21·5
33	♂		33·1	36·4	9·1
33	♂		40·0	43·2	7·4
33	♂		23·7	25·8	8·1
84	♀	BF	16·7	20·9	20·1

The rate of urinary excretion of radioactivity

The recovery of radioactivity in urine 24 h after injection of labelled N^{τ}-MH always appeared anomalously high compared with subsequent 24 h collections from animals aged between 4 weeks and 15 months. These results are presented in Fig. 2 as a semilog plot of dose remaining in the body (100 minus percentage excreted) *v.* time and show in all instances an apparent first-order clearance of N^{τ}-MH from the body pool of non-protein-bound N^{τ}-MH. For each animal the clearance extrapolated back to less than 90% of the dose at zero time and showed that the expected clearances in the first 24 h ranged from 2–3% of the dose at 10 weeks of age to approximately 12% of the dose at 15 months, values which were considerably less than the observed recoveries which varied between 15% of dose at 10 weeks of age and 32·5% at 15 months.

DISCUSSION

The first serious attempt to determine the metabolic fate of administered N^{τ}-MH in a number of species (Cowgill & Freeberg, 1957) showed that in all instances most of the administered N^{τ}-MH was rapidly eliminated from the body in excreta but that the chemical identity of the labelled compounds differed between species as judged by comparative chromatography and acid lability. Although thought to be of endogenous origin (Tallan *et al.* 1954; Block *et al.* 1965), N^{τ}-MH was only later found to occur in actin and myosin of skeletal muscle (Asatoor & Armstrong, 1967; Johnson *et al.* 1967). Asatoor & Armstrong (1967) realized that the excretion of N^{τ}-MH might be used as an index of muscle protein breakdown in vivo and this has been established in a number of species (see p. 129), the criterion of validity in all instances being the rapid and quantitative recovery in urine of an intravenous dose of labelled N^{τ}-MH.

This work shows that, following an intravenous dose of N^{τ}-[$^{14}CH_3$]methyl histidine, the recovery of radioactivity in sheep urine is incomplete in 7 d and in young lambs is substantially less than 50% of the dose (Table 1). The results also demonstrate that urinary recovery of radioactivity in 7 d increases with the age of the animal, a point further enforced in Table 1 where the weekly and cumulative urinary recoveries in 3 weeks are shown for

Table 4. *Distribution of radioactivity in extracts of m. longissimus dorsi of sheep after injection of N^{τ}-[$^{14}CH_3$]methyl histidine*

Time of biopsy sample (min)	Total radio-activity applied (counts/min)	Total radioactivity (counts/min) in: Component 1	Component 2	Component 1 / Component 2
14	590	402	210	1·9
92	702	596	252	1·6
164	632	360	330	1·1
164 (after acid-hydrolysis)	632	530	—	—

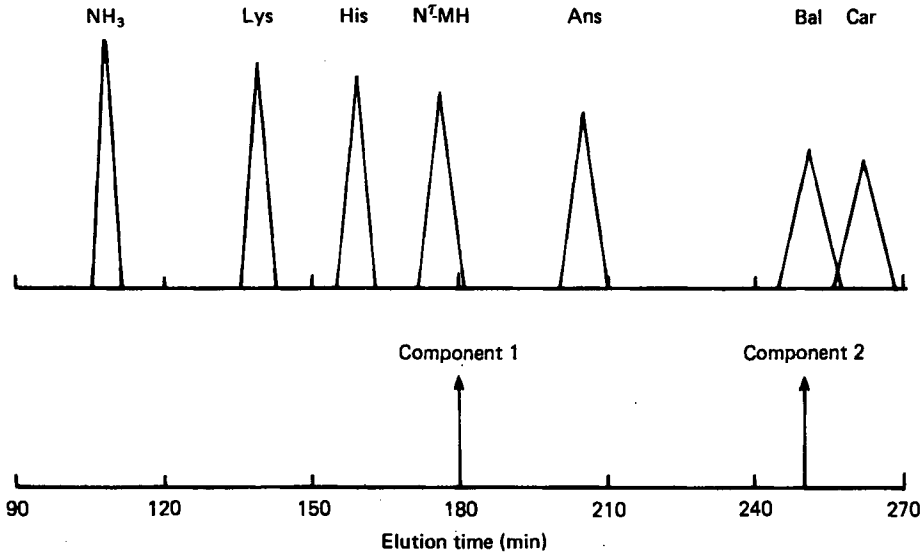

Fig. 1. Elution position of radioactive components from sheep muscle relative to other basic amino acids and peptides after injection of N^{τ}-[$^{14}CH_3$]methyl histidine. NH_3, ammonia; Lys, lysine; His, histidine; N^{τ}-MH, N^{τ}-methyl histidine; Ans, anserine; Bal, balenine; Car, carnosine.

animals ranging from 9 weeks to 15 months of age. These recoveries should be contrasted with those obtained in other species. Thus rats gave 100% recoveries in 3 d (Young *et al.* 1972), adult humans 95% in 2 d (Long *et al.* 1975), adult rabbits 90–97% in 7 d (Harris *et al.* 1977) and cattle 86–99% in 6 d (Harris & Milne, 1978).

Attempts to account for the low urinary recoveries of a dose in sheep due to partial excretion of N^{τ}-MH through other routes were unsuccessful. Thus the level of radioactivity found in faeces could be accounted for by contamination with urine. A similar finding was reported for rats (Young *et al.* 1972). That the N^{τ}-MH was not extensively degraded in vivo and oxidized to CO_2 was demonstrated by the failure to detect $^{14}CO_2$ in expired gases in two experiments each continued for 3 d. During this time not more than 40% of the dose was excreted in urine and hence 60% or more of the dose presumably remained in the body. That this interpretation is probably correct is supported by the longer-term urinary recoveries (Table 1) where appreciable radioactivity was excreted during the third week following a dose of labelled N^{τ}-MH and the fact that the cumulative urinary recovery approached quantitation (90%) in a 15-month-old animal (Table 1).

For those species, mentioned previously, in which a dose of labelled N^{τ}-MH is rapidly

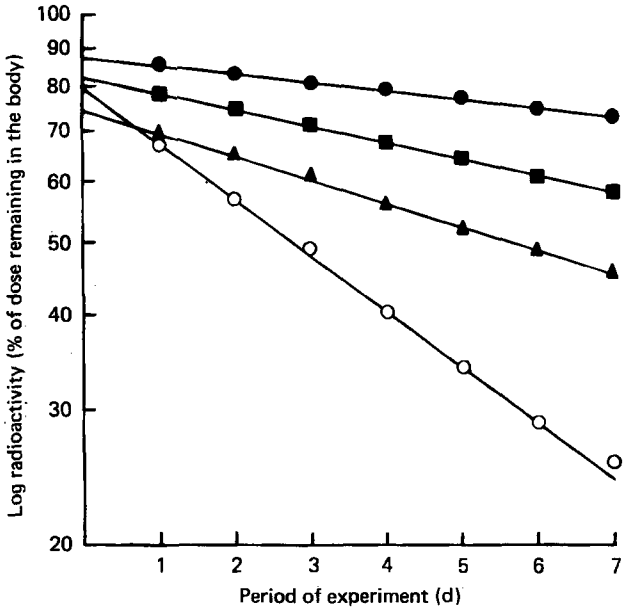

Fig. 2. Clearance of radioactivity (% of dose remaining in body; log form) from the body pool of non-protein-bound N^{τ}-methyl histidine in sheep of different ages after adminstration of N^{τ}-[$^{14}CH_3$]-methyl histidine: (●), 10 weeks; (■), 4 weeks; (▲), 39 weeks; (○), 15 months. For details of animals, see Table 2.

and quantitatively eliminated from the body, one of the underlying assumptions (Harris & Milne, 1977) is that N^{τ}-MH released from muscle protein breakdown is rapidly excreted in urine. This obtains only if the body pool of non-protein-bound N^{τ}-MH is not appreciably larger than the daily urinary excretion. By this criterion and in the absence of other routes of elimination, the incomplete recoveries of radioactivity in urine after 7 d (Table 1) indicate the presence in sheep of a body pool of non-protein-bound N^{τ}-MH which is large relative to the daily urinary excretion of N^{τ}-MH.

Analysis of deproteinized extracts of muscle (Table 2) and blood (Table 3) showed that the levels of free N^{τ}-MH in these tissues were not particularly elevated. However, acid-hydrolysis of muscle extracts resulted in increases in free N^{τ}-MH by up to fifteen-fold (Table 2). Although a change occurred in the levels of N^{τ}-MH in blood extracts on hydrolysis, the magnitude of the increase was usually less than two-fold (Table 3).

The total concentration of non-protein-bound N^{τ}-MH in both muscle and blood and the percentage of non-protein-bound N^{τ}-MH present in blood in the acid labile form all appeared to decrease with age although such changes were not statistically significant. However, the two tissues showed marked differences in the distribution of non-protein-bound N^{τ}-MH between the free and peptide-bound forms, most of the N^{τ}-MH being present in muscle in the dipeptide form at all ages (Table 2) while the free form of N^{τ}-MH predominated in blood (Table 3).

The previously-mentioned findings suggest that the muscle pool of non-protein-bound N^{τ}-MH in young sheep is large, assuming m. longissimus dorsi to be representative of the skeletal musculature. If muscle tissue is 35% of the body mass at a live weight of 15·2 kg (mean weight of 9–18-week-old lambs, Table 2), the total muscle pool of non-protein-bound N^{τ}-MH is approximately 1·6 mmol (using the mean concentration of 294·0 nmol/g muscle

for 9–18-week-old-lambs) and is approximately twenty-five times the expected daily urinary excretion of N^{τ}-MH in lambs at 15 kg as calculated in Table 5. The incomplete urinary recoveries of radioactivity in 7 d from young lambs (Table 1) can thus be explained in terms of a large muscle pool of non-protein-bound N^{τ}-MH, most of which (> 80%) is present as a dipeptide (Table 2) which is in equlibrium with the pool of free N^{τ}-MH.

Two groups of factors can be identified which may influence the progressively increasing recovery of radioactivity in urine with age: (*a*) those determining the amount of N^{τ}-MH released from muscle protein breakdown and (*b*) those controlling the amount of N^{τ}-MH retained in muscle tissue and hence the proportion of the N^{τ}-MH from protein breakdown that is excreted in urine.

(*a*) Three factors appear to regulate the amount of N^{τ}-MH released from muscle protein (Table 5); (1) the muscle mass, (2) the proportion of the muscle mass broken down each day, that is, the fractional breakdown rate (FBR) and (3) the protein-bound content of N^{τ}-MH. All three components change with age, the muscle mass first increasing at a faster rate than the body mass and then remaining as a fairly constant proportion of body-weight in the young adult animal (Young, 1970).

The FBR decreased with age in general and has been fully described in the rat (Millward *et al.* 1975). The information for sheep is limited but an FBR of approximately 2%/d for muscle can be inferred from the results of Arnal (1977) for lambs at approximately 15 kg weight. Similarly, an FBR of 1·7–1·8%/d may be assumed from the results of Buttery *et al.* (1975) for non-growing, mature wethers of 40–50 kg weight.

The protein-bound content of N^{τ}-MH in muscle increases in the young animal, reaching the adult level in lambs at approximately 20 kg (Buttery *et al.* 1977). A similar developmental change also occurs in rabbits (Trayer *et al.* 1968) and in children (Bilmazes *et al.* 1978). The cumulative effect of these age-related changes is to increase the amount of N^{τ}-MH released from muscle protein breakdown each day relative to the muscle pool of non-protein-bound N^{τ}-MH, resulting in a greater flux of N^{τ}-MH through the muscle pool and hence an increased recovery of radioactivity in urine from older animals. This is illustrated by the results in Table 5, which show that the N^{τ}-MH released from muscle protein breakdown increased six-fold between the 15 kg and 60 kg animal while the muscle mass increased four times and the total pool of N^{τ}-MH only 2·7 times. Even if the concentration of non-protein-bound N^{τ}-MH in muscle remained constant between the 15 and 60 kg animals (Table 5) so that the total pool of non-protein-bound N^{τ}-MH increased by the same factor as the muscle mass, the flux of N^{τ}-MH through the pool would still be higher in the older animal.

(*b*) A further consequence of the presence of an appreciable pool of non-protein-bound N^{τ}-MH in sheep muscle is that the amount of N^{τ}-MH excreted daily in urine is less than that released from muscle protein breakdown per d. This is because the only known source of N^{τ}-MH in the animal body is that released from breakdown of the muscle proteins actin and myosin and, to a much smaller extent, similar proteins in non-muscle tissues. It follows, therefore, that a growing animal can only maintain the tissue concentrations of non-protein-bound N^{τ}-MH (both free and peptide bound) in newly-accreted muscle tissue by retention of some of the N^{τ}-MH released from protein breakdown. Hence, only a proportion of the N^{τ}-MH released from protein breakdown is available for excretion. This proportion also varies with age for two reasons: (1) the concentration of non-protein-bound N^{τ}-MH in muscle may decrease with age (Table 2) and (2) the growth in muscle tissue, expressed as a proportion of muscle already present (i.e. the fractional growth rate) is larger in the young animal than in the 60 kg adult. Each factor alone would result in the retention of a larger proportion of N^{τ}-MH in the young animal and the combined effect of both factors is illustrated in Table 5, when both animals are growing at 300 g/d, the growth increment being of the same composition as the animal body.

Table 5. *The effect of various factors on urinary excretion of N^{τ}-methyl histidine in young and adult sheep*

	Young lamb	Adult sheep	Factor from: value at 60 kg / value at 15 kg
Body-wt (kg)	15	60	4·0
Muscle mass (kg)	5·25	21·00	4·0
Protein bound N^{τ}-MH (μmol/g muscle)	0·6*	1·0*	1·6
Fractional breakdown rate (%/d)‖	2·0†	1·8‡	0·9
N^{τ}-MH released from muscle protein breakdown/d (μmol)	63	378	6·0
Non-protein-bound N^{τ}-MH in muscle (μmol/g muscle)	0·300§	0·200§	0·7
Total pool of non-protein bound N^{τ}-MH in muscle (μmol)	1575	4200	2·7
Growth increment (g/d)	300	300	
Muscle increment (g/d)	105	105	
FGR (%/d)	2·0	0·5	
N^{τ}-MH retained in new muscle tissue (μmol/d)	31·5	21·0	
Percentage of N^{τ}-MH from protein breakdown retained (%/d)	50·0	5·6	
Percentage of N^{τ}-MH from protein breakdown excreted (%/d)	50·0	94·4	

FGR, fractional growth rate.

* From Buttery *et al.* (1977).

† Deduced from synthesis and growth values of Arnal (1977).

‡ From Buttery *et al.* (1975).

§ Approximations to the mean values calculated previously from Table 2.

‖ The fractional breakdown rates are for total muscle proteins since the fractional rates for myofibrillar and sarcoplasmic fractions have been assumed to be very similar. Hence, N^{τ}-MH (μmol) released from protein breakdown/d is given by muscle mass (g) × fractional rate × protein bound content of N^{τ}-HM (μmol/g).

The model clearly demonstrates that a much larger proportion of the N^{τ}-MH from muscle protein breakdown is retained in the young animal compared with the 60 kg adult and correspondingly, a smaller proportion of the N^{τ}-MH is available for excretion in the young lamb. The larger proportion of the N^{τ}-MH from protein breakdown excreted in the adult animal is probably the main determinant of the improved urinary recoveries of radioactivity observed in older animals (Table 1). The fact that only a proportion of the N^{τ}-MH released from protein breakdown is excreted in urine in young lambs and that this proportion is not constant with age or weight as shown by the recovery values for young animals in Table 1, and the muscle concentrations of non-protein-bound N^{τ}-MH in Table 2, provides a logical explanation of the variability in urinary excretion of N^{τ}-MH reported in young lambs by Buttery *et al.* (1977).

Kidney function might also influence the proportion of N^{τ}-MH from protein breakdown that is retained in body pools by altering in turn the concentration of N^{τ}-MH in blood and muscle. However, there is no significant difference between the mean values of the concentration of either free or total non-protein-bound N^{τ}-MH in blood (Table 3) or muscle (Table 2) of the age groups and it is concluded that the kidney probably does not contribute to changing recoveries of radioactivity in sheep urine.

From the previously-mentioned discussion it might appear that information of limited value may be obtained from the urinary excretion of N^{τ}-MH in adult sheep, where the influence of body pools of non-protein-bound N^{τ}-MH will be least (Tables 2 and 3) and

held at constant body-weight to avoid retention of N^{τ}-MH released from muscle protein breakdown. The fractional breakdown rates calculated in this situation would be average values since the assumption implicit here is that the body pool of non-protein-bound N^{τ}-MH remains constant with time. If this is not so, small changes in the muscle pool of N^{τ}-MH would produce measurable changes in the daily excretion of N^{τ}-MH. As an example, if the 60 kg animal in Table 5 excretes in urine 95% of the N^{τ}-MH from protein breakdown (359 μmol/d), a decrease of 1% in the total, non-protein-bound N^{τ}-MH would result in a 12% increase in urinary excretion of N^{τ}-MH. Since the precision of the usual methods of analysis of N^{τ}-MH are at best 2–3%, a 1% change in the concentration of N^{τ}-MH in the muscle pool would be within the experimental error of the analysis, even assuming the muscle pool could be sampled in a representative manner. Hence, fluctuations in urinary output of N^{τ}-MH could not be ascribed with confidence to changes in the breakdown rate of muscle protein and the practical limitations, detailed previously, effectively preclude the use of urinary excretions of N^{τ}-MH for any useful purpose in sheep.

The extrapolation of the first-order clearance of radioactivity in urine to values less than 100% of dose at zero time (Fig. 2) is consistent with the anomalously high recoveries of radioactivity during the first 24 h periods being due to the rapid excretion of a proportion of the dose before it equlibrated in the body. The clearance rates determined from Fig. 2 during the first 24 h are then those rates which would have been found if the dose had equilibrated quickly or instantaneously in the body.

Since the observed clearances in urine from young lambs were only 2–5%/d of the dose remaining in the body (Fig. 2), suggesting a large body pool of N^{τ}-MH, it appeared likely that the labelled N^{τ}-MH in the extravascular pool was equilibrating with both the free N^{τ}-MH and its dipeptide form in muscle because the dipeptide constituted on average over 80% of the total non-protein-bound pool in that tissue (Table 2). Table 4 shows that such an equilibration occurred and that labelled N^{τ}-MH not only entered the pool of free N^{τ}-MH in muscle but also was rapidly incorporated into the dipeptide. Assuming the distribution of labelled N^{τ}-MH in muscle after equilibration was similar to N^{τ}-MH in the non-protein-bound N^{τ}-MH (Table 2), much of the labelled N^{τ}-MH incorporated into the peptide was subsequently released and excreted in urine mainly as free N^{τ}-MH (C. I. Harris, unpublished results) over a period of several weeks (Table 1). Thus, it is suggested that the second, slower phase of excretion of labelled N^{τ}-MH (Fig. 2) resulted from the turnover of the labelled N^{τ}-MH-containing dipeptide in muscle.

The relationship between the rate of urinary recovery of radioactivity and the presence of the dipeptide in muscle suggested in this work might imply that the dipeptide does not occur in those species which show rapid urinary recoveries of labelled N^{τ}-MH. However, the tentative identification of the same dipeptide in the muscle of rabbits and cattle (C. I. Harris and G. Milne, unpublished results) suggest that such a relationship may not be simple and may indicate that factors additional to the muscle pool of non-protein-bound N^{τ}-MH can influence the urinary recovery of labelled N^{τ}-MH.

In retrospect, the occurrence in muscle of the N^{τ}-MH-containing dipeptide, balenine, is not surprising since the enzyme systems responsible for the synthesis of the analogous peptides carnosine and anserine show a broad specificity for histidine and histidine derivatives, including N^{τ}-MH (Winnick & Winnick, 1958; Kalyankar & Meister, 1959). Since N^{τ}-MH occurs ubiquitously in muscle proteins and because the muscle of at least mammalian species contains the enzyme systems necessary for the synthesis of carnosine and anserine, the occurrence of balenine in muscle should perhaps be regarded as the norm rather than the biochemical rarity suggested by its original discovery in whale meat (Cocks *et al.* 1964). Those physiological factors which influence the concentration of balenine in muscle have not been defined but merit further study in the general context of this investigation. The

potential role of balenine in modulating the excretion of N^{τ}-MH must always be considered in any new species in which the validity of urinary excretion of N^{τ}-MH is being investigated and in which the possible influence of a body pool of balenine on urinary excretion of N^{τ}-MH can be demonstrated only by the use of labelled N^{τ}-MH. Such an application has shown that the urinary excretion of N^{τ}-MH does not provide a reliable measure of muscle protein breakdown in sheep.

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