The inadequacy of urinary N^τ-methyl histidine excretion in the pig as a measure of muscle protein breakdown

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1. The validity of the urinary excretion of N^{τ}-methyl histidine (N^{τ}-MH) by pigs as an index of muscle protein breakdown in vivo was tested using the criterion of the rate of recovery of radioactivity in urine following an intravenous dose of N^{τ}-[$^{14}CH_3$]methyl histidine.

2. Urinary recoveries of radioactivity from five animals were less than 21% of dose in 7 d after which the daily recovery was less than 0·3% per day.

3. The incomplete recoveries of radioactivity were associated with the presence in muscle of a large pool of non-protein-bound N^{τ}-MH, the concentration of which increased with age.

4. The N^{τ}-MH in this pool was present as free N^{τ}-MH and in a dipeptide which constituted more than 90% of the total non-protein-bound N^{τ}-MH. The contribution of the peptide increased with age, reaching 99·8% in older animals.

5. The pool of non-protein-bound N^{τ}-MH was maintained and increased in both established and newly accreted tissue by retention of some of the N^{τ}-MH released by muscle protein breakdown, only a proportion of which was therefore available for excretion. Hence, the urinary excretion of N^{τ}-MH is not a valid index of muscle protein breakdown in pigs.

Interest in the contribution of individual tissues to general protein metabolism has been stimulated by the possibility of measuring non-destructively the rate of breakdown of muscle tissue in vivo from the excretion of N^{τ}-methyl histidine (N^{τ}-MH) or 3-methyl histidine in urine (Young *et al.* 1972; Long *et al.* 1975; Young & Munro, 1978).

Previous reports have shown the method to be valid in cattle (Harris & Milne, 1979, 1981) but not in sheep (Harris & Milne, 1977, 1980*a*). This paper describes the attempts to validate the urinary excretion of N^{τ}-MH as a measure of muscle protein breakdown in pigs using the criterion of recovery of radioactivity in urine following an intravenous dose of N^{τ}-[$^{14}CH_3$]methyl histidine. A preliminary report of this work (Milne & Harris, 1978) has been published.

EXPERIMENTAL

Animals

Male castrates and female pigs from Large White × (Landrace × Large White) crosses were used. Urine samples from male animals in metabolic cages were collected under toluene. Samples from female animals were collected under acid using urethral bladder catheters (Foley type, 2 way catheters, FG14 and 18 with 5–15 ml and 30 ml balloons respectively; Warne Surgical Products Ltd, Andover, Hants). Animals were fed on a cereal-based diet devoid of fish meal and other animal products but supplemented with soya-bean meal. Four animals over 300 kg in weight were given a similar diet but which contained 50 g white fish meal/kg.

Materials and methods

The sample of N^{τ}-[$^{14}CH_3$]methyl histidine has been described (Harris & Milne, 1980*a*) and was injected as a solution in sterile saline (9 g sodium chloride/l) into an ear vein while the pig was lightly anaesthetized with Trilene (ICI Pharmaceuticals Ltd, Macclesfield, Cheshire). The preparation and analysis of N^{τ}-MH in blood and muscle samples has been detailed (Harris & Milne, 1981) together with the method of isolation and analysis of the dipeptide

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Table 1. *The recovery of radioactivity in urine after intravenous injection of N^{τ}-[$^{14}CH_3$]methyl histidine*

Pig wt (kg) ...	26·5	52·7	53·7	58·0	82·0
Sex ...	♂	♂	♂	♀	♀
Period after injection (d)	Recovery of radioactivity in urine (% dose)				
1	15·68	No urine	15·95	11·42	17·66
2	1·08	1·84	1·09	1·30	1·42
3	0·35	0·43	0·34	0·69	0·53
4	0·15	0·34	0·21	0·61	0·42
5	0·12 }	0·19	0·09	0·26	0·22
6	0·09 }		0·07	0·24	0·26
7	0·09	0·08	0·08	—	0·23
Total	17·56	2·88	17·83	14·52*	20·74

* 6 d recovery.

balenine (β-ananyl-N^{τ}-methyl histidine) from extracts of muscle. The distribution of radioactivity in muscle extracts and the detection of radioactivity in expired gases were carried out as described by Harris & Milne (1980*a*).

RESULTS

Recoveries of radioactivity in urine following intravenous administration of N^{τ}-[$^{14}CH_3$]methyl histidine

The daily recovery of radioactivity in urine from five animals over 7 d (Table 1) shows that only a small proportion of the injected dose was excreted and, with the exception of the first day, that less than 2% of dose was eliminated in urine each day. The failure to recover most of the radioactivity of the dose in urine might suggest that the labelled N^{τ}-MH was being oxidized to $^{14}CO_2$, excreted in faeces or retained in the body. To test the first possibility, the recovery measurements on the 52·7 and 53·7 kg animals in Table 1 were conducted in a calorimeter connected to an ionization chamber for the detection of radioactivity in expired gases. No detectable radioactivity was found after 5 d in spite of the fact that between 82 and 97% of the dose presumably remained in the body at that time (Table 1). Although faeces were not monitored routinely, the level of radioactivity in aqueous extracts of faeces following a spillage of urine were those expected from such contamination and were not elevated as might be expected if the faeces were a major route of excretion of N^{τ}-MH (or its breakdown products) in the pig.

The concentration of non-protein-bound N^{τ}-MH in muscle and blood

To determine whether the low recoveries of radioactivity in urine resulted from a large body pool of N^{τ}-MH, the concentration of non-protein-bound N^{τ}-MH was measured in deproteinized extracts of muscle (m. longissimus dorsi) and whole blood. The concentrations of free N^{τ}-MH were low in both muscle and blood (Tables 2 and 3) and did not change appreciably in blood after acid-hydrolysis (Table 3). In contrast, extracts of muscle showed marked increases in the concentration of N^{τ}-MH after hydrolysis, thus demonstrating that almost all (90·0–99·8%) of the non-protein-bound N^{τ}-MH in muscle was present in an acid-labile form(s) distinct from the free N^{τ}-MH (Table 2). Furthermore, the concentration of the acid-labile form appeared to increase progressively with age, reaching concentrations of 2 μmol total non-protein-bound N^{τ}-MH/g muscle tissue at approximately 9 months of age and body-weights of 160–170 kg (Table 2). The total concentration of N^{τ}-MH in the

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Table 2. *Concentrations of non-protein-bound N^{τ}-methyl histidine (N^{τ}-MH) in pig muscle (m. longissimus dorsi)*

Wt (kg)	Age (months)	Sex	Concentration of non-protein-bound N^{τ}-MH (μmol/g muscle) Free	Total	Percentage of N^{τ}-MH in acid-labile form
0·8	Birth	♀	0·017	0·19	91·1
1·3	Birth	♀	0·008	0·08	90·0
33·0	3·0	♂	0·004	0·28	98·6
62·8	4·5	♂	0·005	0·75	99·3
158·0	9·5	♀	—	1·97	—
159·0	9·5	♀	0·007	2·06	99·7
171·0	9·5	♀	0·005	1·98	99·7
174·0	9·0	♀	0·004	2·27	99·8
309·0*	49·0	♂	0·039	10·20	99·6
309·0*	50·0	♀	0·021	6·9	99·7
342·0*	50·0	SF	0·034	14·00	99·8

SF, spayed female.
* Diet contained 50 g white fish meal/kg.

Table 3. *Concentration of non-protein-bound N^{τ}-methyl histidine (N^{τ}-MH) in whole blood*

Wt (kg)	Age (months)	Concentration of non-protein-bound N^{τ}-MH (μmol/ml) Free	Total	Percentage of N^{τ}-MH in acid-labile form
0·8	Birth	0·010	0·007	—
1·3	Birth	0·009	—	—
33·0	3·0	0·008	0·029	72
62·8	4·5	—	0·018	—
97·7	6·5	0·006	0·013	54
158·0	9·5	0·019	0·025	24
309·0*	49·0	0·060	0·073	18
342·0*	50·0	—	0·052	—
350·0*	49·0	0·027	0·043	37

* Diet contained 50 g white fish meal/kg.

muscle of animals over 300 kg (Table 2) reached 14 μmol/g muscle but may be unusually elevated since these animals were fed on a diet containing 50 g white fish meal/kg, a potential source of N^{τ}-MH. It is relevant to note that the levels of both free and total N^{τ}-MH in blood (Table 3) and the concentration of free N^{τ}-MH in muscle (Table 2) appeared elevated in these animals.

The limited values in Table 3 suggest that more than 50% of non-protein-bound N^{τ}-MH in blood was present as free N^{τ}-MH since the mean content of N^{τ}-MH in the acid-labile form was 41% of the total (Table 3) although the values ranged from 18–72%.

The distribution of radioactivity and identification of the labelled compound in muscle extracts

Deproteinized muscle extracts from pigs used in recovery experiments (Table 1) were prepared from samples taken at slaughter 10–16 d after injection with labelled N^{τ}-MH and analysed for the distribution of radioactivity. In each instance a single radioactive

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Table 4. *Distribution of radioactivity in deproteinized extracts of muscle (m. longissimus dorsi) following an intravenous dose of N^{τ}-[$^{14}CH_3$]methyl histidine*

Wt (kg)	Period after injection (d)	Percentage of applied radioactivity in acid labile component
33	10	99·4
60	11	94·9*
63	16	93·5
84	10	(1) 88·2
		(2) 89·3

* Distribution in mixed thigh muscle.

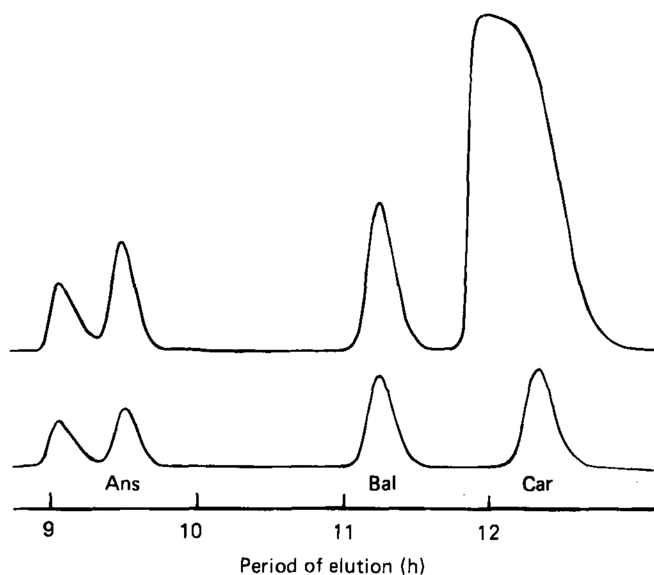

Fig. 1. Demonstration of the occurrence of balenine in extracts of muscle (m. longissimus dorsi) of the pig. Upper trace, extract equivalent to 50 mg muscle tissue; lower trace, separation of standards. Ans, anserine (25 nmol); Bal, balenine (50 nmol); Car, carnosine (50 nmol).

component was found which accounted for essentially all the radioactivity in the sample (Table 4). This labelled component was eluted after free N^{τ}-MH and slightly before carnosine, a position approximating to the elution volume of the dipeptide balenine (Harris & Milne, 1980*a*). A more extended analysis of such muscle extracts for ninhydrin-positive material showed that a component was present which eluted at the same position as standard balenine and was separated from carnosine (Fig. 1).

The identity of the component was confirmed as the dipeptide, balenine, by amino acid composition and determination of the N-terminal residue (Harris & Milne, 1980*b*).

DISCUSSION

Recoveries of radioactivity in urine

The low recoveries of radioactivity in urine (Table 1) are similar to those reported in young lambs (Harris & Milne, 1980*a*) in that only a small proportion of the dose was excreted in 7 d but the recovery from pigs was a smaller proportion of dose than was excreted by lambs. A relatively high excretion of radioactivity on day 1 (Table 1) has also been observed

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in rats (Young *et al.* 1972), man (Long *et al.* 1975), rabbits (Harris *et al.* 1977), sheep and cattle (Harris & Milne, 1981) and in the two latter species was attributed to a proportion of the dose being excreted before the intravascularly administered labelled N^{τ}-MH had equilibrated with the extravascular fluid in the body. In young sheep approximately 21% of the 26% of dose recovered in urine in the first 24 h was attributed to excretion before equilibration (Harris & Milne, 1980*a*). A closer approximation to the probably true rate of excretion of radioactivity by pigs is illustrated by the results for the 52·7 kg animal which produced no urine on day 1 and for which the cumulative excretion during days 2–7 was 2·9% of dose (Table 1).

This argument presupposes that the poor recovery of radioactivity in urine is due entirely to the presence of a large pool of non-protein-bound N^{τ}-MH in muscle and is not an indication of loss of radioactivity by routes of excretion other than urine. That this deduction is correct is supported by the failure to detect radioactivity in expired gases or in faeces other than could be ascribed to contamination with urine after spillage. Furthermore, it was demonstrated that the radioactivity in extracts of muscle was present in an N^{τ}-MH-containing dipeptide (Table 4, Fig. 1) which also suggested that the labelled N^{τ}-MH not recovered in urine was retained in the body in an undegraded form (see later).

Although no trend is apparent in the urinary recovery of radioactivity with age from the limited results in Table 1, it is anticipated that the small recoveries measured in 7 d would decrease further in larger animals in view of the increasing pool of non-protein-bound N^{τ}-MH in muscle suggested by the results in Table 2. It is also possible that such an anticipated trend in recovery of dose with age would be concealed by the proportion of dose excreted before equilibration on the first day.

Tissue concentrations of non-protein-bound N^{τ}-MH

The low concentrations of free N^{τ}-MH in deproteinized extracts of muscle (m. longissimus dorsi) (Table 2) are similar to those reported in cattle (Harris & Milne, 1981) and man (Delaporte *et al.* 1978) but are lower than found in sheep (Harris & Milne, 1980*a*). After acid-hydrolysis, the concentration of free N^{τ}-MH increased dramatically, demonstrating that more than 90% of the total non-protein-bound N^{τ}-MH was present in the acid-labile form (Table 2). This proportion increased with age (Table 2) and contrasts with the value of approximately 80% of non-protein-bound N^{τ}-MH found in the acid-labile form in the muscle of sheep and cattle and which did not change appreciably with age (Harris & Milne, 1981). Rangley & Lawrie (1976) had previously reported the presence of balenine in pork and although their limited evidence from four animals suggested that the concentration of balenine might increase with age, the implications for the excretion of N^{τ}-MH in urine were not appreciated. The units used in Rangley & Lawrie's (1976) paper (mg N^{τ}-MH/g N) do not facilitate comparison with the present values but assuming the nitrogen arises largely from protein which has 16% N and constitutes 20% of tissue weight, it can be deduced that the total concentration of N^{τ}-MH in m. longissimus dorsi was 2·24, 3·15, 3·78 and 10·6 μmol N^{τ}-MH/g tissue from animals at 3, 6, 6 and 36 months respectively. These values include the contribution of protein-bound N^{τ}-MH which, when calculated in the same way, amounts to 0·99 μmol/g muscle. The corrected values of non-protein-bound N^{τ}-MH are thus 1·25, 2·16, 2·79 and 9·61 μmol N^{τ}-MH/g muscle at 3, 6, 6 and 36 months respectively; values which are somewhat higher than those reported in the present paper (Table 2) but which are probably acceptable in view of the approximations involved in their calculation.

These concentrations of total non-protein-bound N^{τ}-MH (as balenine) in pig muscle, especially in older animals, far exceed the levels of non-protein-bound N^{τ}-MH reported in any other species except the Fin and Sperm whales where balenine occurs at concentrations of 45 μmol/g m. longissimus dorsi (reviewed in Crush, 1970), although higher values of

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approximately 68 μmol/g muscle (unspecified) were found by Rangley & Lawrie (1976). For comparison, the highest concentrations of balenine found in m. longissimus dorsi of young lambs at 12 weeks of age was 0·48 μmol/g muscle (Harris & Milne, 1980*a*) and was 0·2–0·3 μmol/g in young calves at 5 weeks of age (Harris & Milne, 1981) and below 0·1 μmol/g muscle in rats, rabbits and the psoas muscl of man (unpublished observations). The high concentration of total non-protein-bound N^{τ}-MH in pig muscle as represented by analyses of m. longissimus dorsi (Table 2) thus substantiates the prediction of a large body pool of N^{τ}-MH as judged by the very low recoveries of radioactivity in urine after a dose of labelled N^{τ}-MH (Table 1) and by the absence of evidence of alternative routes of excretion of radioactivity.

A consequence of the large and increasing pool of non-protein-bound N^{τ}-MH in muscle (Table 2) is that a considerable proportion of the N^{τ}-MH released from muscle protein breakdown must be retained in the body to maintain and increase the tissue concentrations of N^{τ}-MH in newly-accreted muscle. Hence, the urinary excretions of N^{τ}-MH in pigs grossly underestimate protein breakdown and cannot be used as an index of muscle protein degradation. A similar situation is found in young sheep (Harris & Milne, 1980*a*). As an illustration, comparison of the values for 33 kg and 62 kg pigs (Table 2) shows that the pool of non-protein-bound N^{τ}-MH in muscle increased by 14·9 mmol in 6 weeks, equivalent to an increase in non-protein-bound N^{τ}-MH of 355 μmol/d and representing approximately 64% of the N^{τ}-MH released daily by protein breakdown in the 62 kg animal (calculated assuming muscle = 0·4 × body-weight, protein-bound N^{τ}-MH = 750 μmol/kg muscle tissue and the fractional breakdown rate for muscle protein 3%/d, derived from the fractional synthesis and growth rates in muscle of 4 and 1%/d respectively (Garlick *et al.* 1976)). Such calculations assume that the concentrations of non-protein-bound N^{τ}-MH in m. longissimus dorsi are representative of the whole musculature and also ignore possible variations in pool size between animals but serve to show that a large proportion of N^{τ}-MH released from muscle protein is retained in the body.

The concentrations of free N^{τ}-MH in whole blood (Table 3) were in the same range as found in muscle (Table 2), but, although an increase in free N^{τ}-MH occurred after hydrolysis of extracts of blood, a much smaller proportion of the total non-protein-bound N^{τ}-MH was present in the acid-labile peptide form (Table 3) than found in muscle (Table 2). A difference in the proportion of N^{τ}-MH as balenine in muscle and blood was also observed in sheep and cattle (Harris & Milne, 1981). The levels of free N^{τ}-MH in blood samples from animals less than 300 kg (Table 3) were lower than those reported by Badger & Tumbleson (1974) in serum from animals given a diet containing fish meal. It is interesting to note the similarity between concentrations of free N^{τ}-MH in the pig serum of Badger & Tumbleson (1974) and in blood from animals receiving a fish meal supplemented diet in the present study (Table 3), suggesting that a dietary source of N^{τ}-MH may influence blood or serum levels of free N^{τ}-MH.

The identity of the N^{τ}-MH-containing component in extracts of muscle

The identification of the radioactive component in deproteinized extracts of muscle as balenine was based on its chromatographic elution position relative to standard balenine (Fig. 1), the presumption that the radioactivity in the component was present in N^{τ}-MH since no evidence was found for degradation of N^{τ}-MH in vivo and the identification, isolation and analysis of unlabelled balenine in muscle extracts, the amount of which varied directly with the concentration of N^{τ}-MH determined subtractively in the acid-labile component (Table 2).

The labelled compound in pig muscle extracts (Table 4) was identical in chromatographic properties to the acid-labile component found in sheep muscle where it was formed from

the pool of free N^{τ}-MH and where the radioactivity in the component was associated with free N^{τ}-MH after acid hydrolysis (Harris & Milne, 1980*a*). The identification of the component in pig muscle as balenine was further strengthened by the confirmation of its presence in muscle extracts analysed for ninhydrin-positive material, where its elution position in a very discriminating system coincided with standard balenine (Fig. 1). The presence of the unusual amino acid, N^{τ}-MH, was confirmed by determination of the amino acid composition and sequence following the preparative isolation of the component (Harris & Milne, 1980*b*).

The physiological factors which control the accumulation of N^{τ}-MH in pig muscle are unknown and contrast with the situation in the muscle of sheep and cattle where the concentration of non-protein-bound N^{τ}-MH did not change significantly with age (Harris & Milne, 1981). However, this report has demonstrated that an intravenous dose of labelled N^{τ}-MH is largely retained in the body of pigs, mainly, if not exclusively, in muscle where considerable amounts of the N^{τ}-MH released from protein breakdown are retained in a large and increasing pool of non-protein-bound N^{τ}-MH. Since only the N^{τ}-MH which is not retained is available for excretion, the N^{τ}-MH measured in urine is an inadequate index of muscle protein breakdown in the pig.

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