The retention and metabolism of N^{τ}-methylhistidine by cockerels: implications for the measurement of muscle protein breakdown determined from the excretion of N^{τ}-methylhistidine in excreta

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1. Excreta were collected for four consecutive days from 4- to 18-week-old cockerels following subcutaneous injection of N^{τ}-[$^{14}CH_3$]methylhistidine.

2. The recoveries of radioactivity in excreta were incomplete and progressively decreased with increasing age.

3. Most of the radioactivity not recovered in excreta after 4 d was found in skeletal muscle where > 55% of the radioactivity present was in the N^{τ}-methylhistidine-containing dipeptide, balenine.

4. This peptide appeared to be relatively stable so that most of the labelled N^{τ}-methylhistidine incorporated was not released during the period of the recovery measurements.

5. The total pool of non-protein bound N^{τ}-methylhistidine (free N^{τ}-methylhistidine + balenine) in pectoral and mixed thigh muscles increased with age and relative to the daily excretion of N^{τ}-methylhistidine. At 18 weeks the pool was 3·3 times the daily excretion of N^{τ}-methylhistidine.

6. These observations account for the decreasing recoveries of radioactivity in excreta described previously, due to progressive dilution of labelled N^{τ}-methylhistidine in an expanding pool of non-protein-bound N^{τ}-methylhistidine, part of which was relatively stable.

7. It is concluded that excretion of N^{τ}-methylhistidine by 4- to 18-week-old cockerels cannot be used as a reliable index of muscle protein breakdown in vivo.

Poultry are efficient meat-producting animals and provide one of the highest contents of muscle in the carcass (Fowler, 1980), being only surpassed by deer and rabbits. Rapid deposition (growth) of lean tissue has been further accentuated in selected strains and the relative growth rates of individual tissues are well documented (Latimer, 1925; Wilson, 1953). However, protein turnover in the skeletal muscle of poultry has been little studied.

Protein synthesis in the skeletal muscle of chicken has been investigated by Maruyama *et al.* (1978), MacDonald & Swick (1981) and Muramatsu *et al.* (1985), but in all these examples degradation was assessed as the difference between growth and synthesis. In contrast, the degradation of myofibrillar proteins in vivo has been estimated from the excretion of the non-reutilizable amino acid, N^{τ}-methylhistidine (MH), in a comparison of broiler and layer chickens (Hayashi *et al.* 1985) and in normal and dystrophic strains as well as in perfused preparations (Hillgartner *et al.* 1981). However, neither of these reports considered the possibility that the excretion of MH by chickens may not have been complete although the presence of appreciable concentrations of balenine, a dipeptide containing MH and β-alanine, had been demonstrated in chicken pectoral muscle (Harris & Milne, 1981*a*). In spite of this, the excretion of MH by poultry was claimed to be quantitative (Saunderson & Leslie, 1983).

The present study of the metabolism of MH was to determine if the presence of balenine influenced the excretion of MH by cockerels and whether this excretion might be used to assess the breakdown of myofibrillar protein in vivo. Preliminary reports of some of these findings have appeared (Harris *et al.* 1983*a*, *b*).

MATERIALS AND METHODS

Birds and diet

Cockerels (Ross I broiler chickens, Ross Poultry (GB) Ltd, Inverurie, Aberdeenshire) were purchased as 1-d-old chicks and fed on a cereal-based diet devoid of fish meal and meat products (Table 1). Feed and water were offered *ad lib.*

^{14}C-labelled N^{τ}-methylhistidine

N^{τ}-[$^{14}CH_3$]-methylhistidine was obtained by custom synthesis from New England Nuclear, Boston, Mass., USA. The product has a specific activity of 46 mCi/mmol and purity was $> 97{\cdot}2\%$ by thin-layer chromatography. The labelled MH was injected subcutaneously over the left pectoral muscle as a solution in sterile saline (9 g sodium chloride/l) at a dose of 3–6 μCi/kg body-weight.

Collection and processing of excreta and tissue

Measurements were made on groups of three birds at 4, 6, 9, 12 and 18 weeks of age for the 4 d following injection of labelled MH. A fourth bird was included in the groups at 4, 12, and 18 weeks but was killed 16 h after injection. Tissues from these latter birds were included in some analyses and account for some groups containing four birds, e.g. Table 6.

Birds were housed individually in wire-mesh cages in a room maintained at 18–20° and with a 12 h light–12 h dark cycle. The cages were fitted with flexible plastic skirts that dipped into the collecting tray containing sufficient hydrochloric acid to cover its base. This arrangement ensured that all excreta were collected and the acid minimized, if not prevented, the bacterial breakdown of components in excreta, including MH. Faeces from 4 and 6-week-old birds were collected in 0·25 M-hydrochloric acid while 1·0 M acid was used in the collections from older birds in order to avoid excessively large volumes of faecal slurry. The large mesh size of the cage bases ensured that excreta, when voided, rapidly fell into the acid in the collecting tray and so prevented coprophagy. Cage bases and plastic skirts were washed before the daily collection of excreta. The excreta were homogenized to a slurry and centrifuged at 2000 ***g*** for 20 min at 4°. Precipitates were twice washed with water and centrifuged and the resulting supernatant fractions, after combining with the original, were used for the measurements of recovery and distribution of radioactivity.

Blood samples were taken from a pectoral vein and the birds killed by cervical dislocation and plucked. Mixed pectoral and thigh muscles were quickly removed and frozen in liquid nitrogen before being freeze-dried. The right and left pectoral muscles were processed separately. Extracts of the right muscle only were used for residual radioactivity (Table 4) and its distribution (Table 5), thus avoiding any complications due to residual radioactivity remaining at the site of injection over the left muscle. Results from the right muscle were doubled to obtain a value for total pectoral muscle. The dried tissues were dispersed to a fine powder in a grinder. The residual carcass excluding viscera was treated similarly. Tissue samples were extracted for low molecular weight, non-protein-bound materials in the proportion: 1·0 g dried tissue + 4·0 ml cold water + 1·0 ml internal standard (ϵ-amino caproic acid, 1·0 μmol/ml) + 3·0 ml chilled 1 M-perchloric acid (PCA). The mixture was homogenized (I.L.A. homogenizer, model X10, Scientific Instrument Centre Ltd, London) and then stood in ice for 30 min before centrifuging at 3600 ***g*** for 15 min at 5°. The precipitates were twice resuspended in 1·25 ml 0·5 M-PCA and centrifuged. The supernatant fractions and the respective washings were combined, freeze-dried and finally dissolved in 5·0 ml 0·1 M-HCl before analysis.

Blood samples (5 ml), freshly drawn, were added to 12·5 ml cold 0·5 M-PCA plus 0·125 ml ϵ-amino caproic acid standard (1·0 μmol/ml) while extracts of excreta were mixed with an

Table 1. *Composition of diet (g/kg) fed to cockerels*

Calculated protein content	177
Ground barley	250
Ground wheat	175
Ground maize	133
Wheatings	98
Soya-bean meal*	275
Grass meal	39
Limestone	20
Sodium chloride	5
Vitamin–mineral supplement†	5

* Soya-bean meal, 430 g protein/kg, purchased as Soya 44 from Harbro Farm Sales Ltd, Aberdeenshire.

† Composition of vitamin–mineral supplement (mg/kg) of final diet when added at 5 kg/tonne diet: retinol 60, cholecalciferol 15, tocopherol acid succinate 33, menadione 6, thiamin 2, riboflavin 20, pyridoxine 10, cyanocobalamin 60 μg, nicotinic acid 100, pantothenic acid 30, folic acid 3, biotin 200 μg, choline 500, copper 24, cobalt 2, manganese 200, zinc 120, magnesium 100, iodine 4, selenium 0·3.

equal volume of 1 M-PCA. Subsequent processing was as described previously for tissue extracts.

Samples for acid hydrolysis were mixed with an equal volume of concentrated HCl and incubated in a sealed tube at 110° for 18 h. The acid was removed in vacuo.

Chromatographic methods

MH was quantified using a Locarte amino acid analyser (The Locarte Co., London) using a 6 × 230 mm bed of Aminex A-5 resin (Biorad Laboratories Ltd, Watford, Herts) at 28° and eluted at 30 ml/h with 0·38 M-sodium citrate, pH 4·18, followed by 0·35 M-sodium citrate, pH 4·90.

Balenine was resolved from carnosine on a 6 × 450 mm bed of Locarte L-16 resin at 55° with 0·38 M-sodium citrate buffer, pH 4·06, at a flow rate of 15 ml/h. Under these conditions, anserine eluted at 9·5 h, balenine at 11 h and carnosine at 12·5 h.

Extracts from tissues and excreta to be analysed for MH were first purified by a modification of the pyridine fractionation system (Haverberg *et al.* 1974). The clarified sample containing internal standard was loaded on to 7 × 120 mm beds of Dowex 50 × 8, 200–400 mesh, H^+ form. Each column was eluted sequentially with 27 ml 1 M-HCl, 42 ml 0·2 M-aqueous pyridine and finally 27 ml 1·0 M-pyridine solution which eluted both the MH and internal standard. The important feature of the present method is that the colour change in the resin caused by the 0·2 M-pyridine solution should not be allowed to reach the bottom of the resin bed because the internal standard is eluted at the solvent front, i.e. where the colour change occurs on the resin. This front should be eluted by and collected with the 1·0 M-pyridine fraction which then contains both the standard and the MH. This modification does not result in such a high purification of MH as the original method but it does permit the use and recovery of an internal standard in the same fraction as the MH, a feature that was not possible in the method of Haverberg *et al.* (1974). After freeze-drying the 1·0 M-pyridine fraction, the standard and MH were analysed as described previously.

An estimate of the rate of incorporation of MH into balenine over the 4 d period may be obtained from the relation:

$$\frac{\text{MH incorporated into balenine in a given muscle}}{\text{MH excreted in total}} = \frac{\text{label incorporated into balenine in a given muscle}}{\text{label excreted in total}}.$$

The fractional synthesis rate (k_s) in a given muscle was obtained by dividing the daily incorporation of MH into balenine by the pool size of balenine in that muscle. The half-life ($t_{\frac{1}{2}}$) of balenine was then determined from the relation:

$$t_{\frac{1}{2}} = 0{\cdot}693/k_s.$$

Other measurements and statistics

Radioactivity was measured using an NE 260 scintillator (Nuclear Enterprises Ltd, Edinburgh) in a Beckman model 345 spectrometer (Beckman Ltd, High Wycombe, Bucks). Results are presented as means with their standard errors for the given number of observations. The significance of differences between groups was assessed by Student's *t* test and by analysis of variance.

RESULTS

The mean cumulative recoveries of radioactivity in excreta collected in 4 d from groups of three cockerels progressively decreased with increasing age (Table 2). Analysis of variance of the mean daily excretion of radioactivity showed that the proportion of dose excreted each day changed in a statistically significant way with age (Table 2). There was also a significant effect on excretion caused by the interaction of the age of the bird and the day of collection. Thus, the percentage of dose collected on day 1 at 18 weeks of age was half that found with 4-week-old birds while the reverse was found on day 4. Profiles of these excretion values (not shown) demonstrated a marked decrease in slope (i.e. in rate of excretion) with increasing age of the birds.

The distribution of radioactivity in the excreta collected on day 1 showed that in the youngest birds 95% of the radioactivity was associated with MH (Table 3), this value being very similar to the distribution in the injected material (97·4%). However, the percentage of radioactivity associated with MH progressively fell with age, being significantly different at 12 and 18 weeks from the value at 4 weeks (Table 3). That this change was not due to bacterial breakdown in the collecting trays was shown by incubating portions of the injected solution with unlabelled excreta in acid for 24 h and then processing in the same way as used for labelled excreta (see p. 468). No change in the distribution of radioactivity was found as a result of the treatment (Table 3). Hence, the progressive change in the proportion of radioactivity associated with MH in excreta, although small, is considered real.

The radioactivity not recovered in excreta after 4 d (Table 2) was not due to losses in the recovery process since between 64 and 88% of the radioactivity unaccounted for was extracted from muscle tissue of various types (Table 4). These tissue contents were obtained by multiplying the radioactivity found in extracts of unit weights of dry tissue by the total dry weight of tissue (given in Table 6, p. 474). The total radioactivity extracted increased with the age of the birds and closely parallelled the radioactivity not accounted for in excreta. The identification of 5·5% of dose in pectoral muscle, together with the 4·3% in carcass, both from 4-week-old birds (Table 4), reinforce the claim that the recoveries of radioactivity in excreta from these birds were genuinely incomplete (Table 2) and were not due to non-specific losses.

At all ages, more than twice as much radioactivity was found in pectoral muscle as in the corresponding thigh muscles (Table 4). This ratio appeared to change with age, being five times at 4 weeks and progressively decreased to 2·1 times at 18 weeks (Table 4), even though the dry weights of the two muscles maintained a similar ratio at all ages (see Table 6, p. 474). The intermediate amounts of radioactivity retained by the remaining carcass are misleading since the weight of this tissue was between 4·5 and 7·5 times that of the thigh muscles. Thus, the radioactivity retained in the carcass per unit weight was substantially lower at all ages than that found in the corresponding mixed thigh muscles.

Table 2. *Mean daily recoveries of radioactivity (% injected dose) in hydrolysed extracts of excreta from cockerels* (n *3*)

Period after injection (d)... Age (weeks)	1	2	3	4	Total	Daily mean
4	68·5	11·2	3·4	2·0	85·1	21·3
6	76·6	9·1	2·3	1·3	88·6	22·2
9	59·6	14·5	4·9	2·1	81·1	20·3
12	37·4	12·3	7·1	3·4	60·2	15·1
18	35·3	20·5	9·3	5·2	70·4	17·6

Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	Significance ($P <$)
Age (weeks)	4	406·91	101·73	0·01
Period after injection (d)	3	27142·55	9047·52	0·005
Interaction	12	4064·90	338·74	0·005

Table 3. *Distribution of radioactivity in hydrolysed extracts of excreta from cockerels and in the injected solution before and after incubation with unlabelled excreta*

(Mean values with their standard errors for three birds)

Age (weeks)	Percentage of applied radioactivity as MH in hydrolysed extracts: Mean	SE
4	94·9	0·8
6	94·7	0·2
9	92·6	1·4
12	90·0**	1·0
18	88·6**	1·5
Injected solution (*n* 1)	97·4	
Injected solution after incubation with unlabelled excreta and then hydrolysed (*n* 1)	98·0	

MH, N^{τ}-methylhistidine

Values differed significantly from those at 4 weeks of age: $^{**}P < 0{\cdot}01$.

The radioactivity retained in tissues (Table 4) and blood, 4 d after injection of the labelled MH, was all in the perchloric acid-soluble fraction and was distributed between only two compounds, free MH and the MH-containing dipeptide, balenine (Table 5). The sum of the radioactivity in free MH and balenine often does not equal 100% (Table 5), presumably due to the low total counts (e.g. 350 counts/min) in the sample and to errors in counting the radioactivity associated with MH and balenine where each component was distributed over three to four tubes. In all samples at all ages, most (> 55%) of the radioactivity was present in balenine. Analysis of variance of the percentage of radioactivity associated with balenine showed that the differences between tissues were significant at the 0·1% level. Pectoral muscle was unusual in containing almost 90% of the radioactivity in balenine at all ages. This was significantly higher ($P < 0{\cdot}001$) than the proportion of the radioactivity

Table 4. *Site of radioactivity not recovered in excreta from cockerels after 4 d*
(Mean values with their standard errors for three birds)

Age (weeks)	Mean % radioactivity not recovered in excreta (100% − values in Table 2) (% injected dose)	Radioactivity recovered (% injected dose)						
		Pectoral muscle		Thigh muscle		Remaining carcass		Sum of tissues
		Mean	SE	Mean	SE	Mean	SE	
4	14·9	5·5	1·6	1·1	0·3	4·3	1·1	10·9
6	11·4	5·5	0·8	1·2	0·2	3·3	0·6	10·0
12	39·8	11·8	3·1	3·3	0·4	8·6	0·3	23·7
18	29·7	11·1	0·8	5·3	0·5	8·3	0·3	24·7

associated with balenine in the mixed thigh muscle. The distribution of the radioactivity in carcass was similar to that in blood, the percentage in balenine being significantly less than that in pectoral and mixed thigh muscles ($P < 0{\cdot}01$).

Detectable radioactivity was present in balenine from the three muscle tissues and blood even after a relatively short period of 16 h (Table 5). The incorporation into pectoral muscle was particularly rapid so that after 16 h about 60% of the radioactivity in muscle at 4 weeks and about 20% at 18 weeks of age was associated with balenine (Table 5). These values were obtained from only one sample at each age but illustrate that measurable incorporation of MH into balenine occurred within 16 h.

The total tissue pools of balenine and MH in pectoral and thigh muscles at ages between 4 and 18 weeks are given in Table 6. The pools of balenine were not equally distributed between the two muscle types, with that in pectoral muscle being three to nine times greater than that in thigh muscle in spite of the similar tissue dry weights (Table 6). This factor is of the same order as that found for the contents of radioactivity in the two muscle types (Table 4). The ratio, balenine : free MH was markedly different between the two muscle types, being, in 18-week-old birds 8·3 : 1 in pectoral muscle and 1 : 1 in thigh muscle (Table 6). However, the total pool of free MH in thigh muscle at 18 weeks of age was twice that in the comparable pectoral muscle in spite of the similar tissue weights.

Regression analysis of MH or balenine per unit weight of muscle against age did not show any evidence that the accumulation of MH or balenine was faster or slower than the growth of muscle, as measured by increments in dry weight (Table 6). Whether or not the accumulation of MH or balenine actually increased at the same rate as muscle dry mass was inconclusive, due to scatter in the data.

The MH measured in daily collections of excreta show that while the total MH excreted increased with age, the increase was not directly proportional to body-weight since the amount excreted per kg body-weight fell progressively and significantly with increasing age (Table 7). This decrease with age would be reduced if the excreted MH was corrected for the increasing proportion of MH that was degraded with increasing age (Table 3) but the resulting values would still show a downward trend.

The $t_{\frac{1}{2}}$ of balenine in chicken pectoral and thigh muscles was estimated as described on p. 469. At 4, 6, 12 and 18 weeks of age the $t_{\frac{1}{2}}$ in pectoral muscle were 11·3, 12·1, 6·8 and 8·8 d respectively, resulting in a mean $t_{\frac{1}{2}}$ for the four age groups of 9·8 d. The comparable $t_{\frac{1}{2}}$ for the mixed thigh muscles were 7·9, 17·3, 7·1 and 6·1 d, giving a mean $t_{\frac{1}{2}}$ of 9·6 d.

Table 5. *Distribution of radioactivity in tissue extracts (% of applied radioactivity) at 4 d and at 16 h after injection of labelled N^{τ}-methylhistidine (MH)*

(Mean values with their standard errors)

Age (weeks)	No. of birds	Pectoral muscle				Thigh muscle				Remaining carcass				Blood			
		MH		Bal		MH		Bal		MH		Bal		MH		Bal	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
								At 4 d									
4	3	4·7	0·5	89·6	9·4	29·2	5·3	72·5	5·7	23·6	5·7	70·9	7·1	24·4† (19·5, 29·3)	—	79·6† (77·3, 81·8)	—
6	3	4·5	0·5	94·3	1·8	15·2	2·1	91·1	2·0	15·3	5·1	80·0	2·1	32·3	5·7	69·3	10·7
12	3	5·4	1·8	88·8	1·0	29·1	4·3	74·4	2·2	24·0	5·4	63·9	4·9	36·6† (36·0, 37·1)	—	63·1† (62·6, 63·5)	—
18	3	11·7	3·1	89·8	3·3	40·7	2·2	67·8	2·0	42·8	4·1	54·8	2·8	38·9	7·4	59·3	4·4
								At 16 h									
4	1	35·6	—	59·7	—	83·3	—	9·5	—	—	—	—	—	81·9	—	14·7	—
12	1	—	—	—	—	—	—	—	—	80·8	—	9·6	—	81·0	—	3·2	—
18	1	76·2	—	22·7	—	91·7	—	4·4	—	89·4	—	8·0	—	—	—	—	—

Bal, Balenine.

† Mean value from only two samples, the individual values of which are given in parentheses.

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Table 6. *Pools of free* N^{τ}*-methylhistidine (MH) and balenine in cockerel muscles*

(Mean values with their standard errors)

Age (weeks)	No. of birds	Tissue dry wt (g)				Pectoral muscle				Thigh muscle			
		Pectoral muscle		Thigh muscle		Balenine (μmol/tissue)		MH (μmol/tissue)		Balenine (μmol/tissue)		MH (μmol/tissue)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
4	4	13·4	0·4	13·1	0·8	7·0	1·3	0·9	0·2	0·8	0·2	1·3	0·2
6	3	35·9	1·7	39·2	2·2	16·0	2·8	4·3	1·0	6·5*	0·2	2·3	0·2
12	3	122·2	6·4	128·7	9·7	74·2	9·1	7·6*	0·9	18·2	7·3	8·7†	—
18	4	173·2	8·2	222·7	9·7	106·0	16·5	12·7	2·8	27·0	5·4	27·3	2·4

* Mean of two values. † One value only.

Table 7. *Mean daily excretions of* N$^{\tau}$*-methylhistidine (MH) by cockerels*
(Mean values with their standard errors)

Age (weeks)	No. of birds	Live wt (g)		Daily excretion of MH				Ratio, total non-protein-bound MH in pectoral and thigh muscles (Table 6): MH excreted daily †
				μmol/d		μmol/kg body wt		
		Mean	SE	Mean	SE	Mean	SE	
4	3	534	10	7·4	0·1	14·0	0·2	1·4
6	3	1208	40	16·0	0·6	13·4	0·6	1·9
9	3	2285	82	26·2	0·1	11·6*	0·5	—
12	2	3459	—	39·7	—	11·5	—	2·7
18	3	4583	147	53·0	0·9	11·6**	0·2	3·3

Values differed significantly from those at 4 weeks of age: * $P < 0{\cdot}05$, ** $P < 0{\cdot}001$.

† Total, non-protein-bound MH (free MH + balenine) from pectoral and thigh muscles in Table 6 were summed and compared with the daily excretion of MH (Table 7) to give the ratios shown. Thus, at 4 weeks (7·0+0·9+0·8+1·3) μmol non-protein-bound MH (Table 6), divided by 7·4 μmol MH excreted daily (Table 7), gives a ratio of 1·4 (Table 7).

DISCUSSION

One of the difficulties in interpretation of recovery values obtained in this study and that by Saunderson & Leslie (1983) is to decide whether mean recoveries of radioactivity in excreta in the range 80–90% can be regarded as complete if no supporting analyses are included. The problem was avoided in the present study by monitoring the tissues and carcass for residual radioactivity. Thus, the recoveries of 85·1, 88·6 and 81·1% from 4-, 6- and 9-week-old birds respectively (Table 2) were genuinely incomplete since most of the label not recovered in excreta was found in muscle tissue (Table 4). In the muscle the residual radioactivity was largely in the form of the dipeptide, balenine (Table 5), that appeared to be relatively stable (see p. 472). The smaller recoveries of radioactivity in excreta at 12 and 18 weeks (Table 2) were parallelled by the larger retentions found in tissues at these ages (Table 4), again demonstrating that the incomplete recoveries in excreta were not due to non-specific losses, such as incomplete collection of excreta or exhalation as carbon dioxide after oxidation.

The total recoveries obtained would not have been substantially improved by continuing excreta collection for a further 2–3 d. Table 2 shows that less than 2·2% of dose was excreted on day 4 by the 4-to 9-week-old birds, implying that only a further 4–5% at most would have been added to the cumulative totals by day 7. The 5·2% of dose excreted on day 4 by 18-week-old birds suggests that a further 5–7% would have been collected on days 5–7, resulting in a cumulative total still less than 80% over 7 d. These values were obtained by fitting an exponential to the recovery data for the first 4 d and then projecting over days 5–7. Such recoveries that progressively decreased with age are consistent, by extrapolation, with the values greater than 90% obtained in 3 d from 5-day-old chicks by Cowgill & Freeburg (1957) and suggest that recoveries approaching 100% would only be obtained in very young chicks of a few days of age. Furthermore, the recoveries of 85–90% obtained in 7 d by Saunderson & Leslie (1983) from 2-week-old broiler cocks and 16-week-old cockerels were probably also not quantitative, as claimed, and are explicable by causes other than incomplete recoveries of excreta, such as retention of some of the MH in the muscles as balenine as found in the present study (Table 4).

The use of MH to assess muscle protein breakdown requires that this amino acid is not only excreted rapidly and quantitatively but is in an unchanged form. This has not been

monitored by any previous authors and Table 3 shows that a small but increasing proportion of MH was degraded with increasing age and that this degradation was exclusively the result of physiological events in the bird. The site or cause of such degradation has not been established in the present study, but at least two possibilities exist. Degradation may occur internally, i.e. in tissues such as liver, and may involve enzymes normally concerned with detoxification processes or normal metabolism. As an example, some phenotypes of mice are now known to degrade extensively MH in the tissues before excretion (Harris *et al.* 1986). Alternatively, and perhaps more likely in fowl, there is the well-established external process, i.e. not in the tissues, of refluxing of urine from the cloaca into both the colon and, more importantly, the caeca which are repositories of a rich microflora (Akester *et al.* 1967; Skadhauge, 1968). The caeca periodically expel most of their contents into the colon and refill with liquid by retroperistalsis (Olson & Mann, 1935).

The radioactivity retained in the tissues (Table 4) and blood was found only in free MH and in balenine (Table 5), with the majority in balenine, particularly in pectoral muscle. This rapid incorporation of labelled MH into balenine (Table 5) has also been found in sheep and pigs (Harris & Milne, 1980, 1981*b*) where the labelled peptide, once formed, seemed to be relatively stable so that the radioactivity was then released only slowly over a period of weeks. This relative stability is further illustrated by the $t_{\frac{1}{2}}$ of 9·8 and 9·6 d now estimated for balenine in chicken pectoral and thigh muscles, implying that little breakdown of labelled balenine would be expected in the 4 d period during which excreta were collected (Table 2). A $t_{\frac{1}{2}}$ of 29 d has been measured for the analogous peptide carnosine in rats (Tamaki *et al.* 1980).

Although balenine was identified in chicken blood it is essentially absent from the blood of mammals. The erythrocytes of avian and amphibian blood are characteristically nucleated and are known to contain the dipeptide, carnosine (Van Balgooy *et al.* 1974), synthesized by carnosine synthase (*EC* 6.3.2.11). This enzyme has been purified from chicken erythrocytes by Ng & Marshall (1976) and catalysed the incorporation of MH into dipeptides at 75% of the rate of histidine, thus demonstrating that both balenine and carnosine could be synthesized by the erythrocytes. No such synthesis occurs in mammalian erythrocytes and the dipeptides balenine, carnosine and anserine are either absent or present only in trace amounts in normal mammalian blood.

The majority of the radioactivity in blood (> 59%) was associated with balenine at 4 d after injection (Table 5). This distribution may be due to leakage from tissues since a generally similar distribution was found in blood, thigh muscle and remaining carcass (Table 5), or it may result from the active synthesis of peptides due to the presence in chicken erythrocytes of carnosine synthase. The latter view seems more probable since, although the erythrocytes were not isolated from the whole blood used in the present study, the demonstration of balenine as well as elevated concentration of carnosine and anserine in perchloric acid extracts of whole blood is consistent with the active synthesis of such dipeptides by erythrocytes. Thus, the blood from 18-week-old birds contained, as molar ratios, thirty-six times as much anserine and fifty-two times as much carnosine as balenine (C. I. Harris and G. Milne, unpublished results). The possibility that these dipeptides remain sequestrated within the erythrocytes in vivo must be considered since they are not mentioned in the many published analyses of amino acids in chicken plasma. Richardson *et al.* (1965), in a comparative study of free amino acids in chicken tissues, were unable to detect carnosine or anserine in plasma although both peptides were identified in muscle.

The distribution of total non-protein-bound MH between free MH and balenine shows markedly different ratios in pectoral and thigh muscles (Table 6). This more active synthesis of balenine in pectoral muscle is in agreement with the fivefold difference in carnosine-synthesizing activities of chick pectoral and gastrocnemius muscles (Ng & Marshall, 1976)

and may be associated with the fibre composition which is predominantly type II in pectoral muscle (Aberle & Stewart, 1983) rather than the mixed fibre population found in thigh muscles.

The values measured here for free MH in pectoral muscle at 4 weeks of age (67·2 nmol/g dry weight or 16·8 nmol/g wet weight if dry wt is 25% of wet wt) are very similar to those found by Hillgartner *et al.* (1981) in 4-week-old, non-dystrophic male birds (18·8 nmol/g wet weight). However, the findings in Table 6 clearly show these values to be considerable underestimates of the total pool of non-protein-bound MH (i.e. free MH + balenine) which, in pectoral muscle from 4-week-old birds, was 590 nmol/g dry weight (148 nmol/g wet weight). This value is almost eight times the concentration of free MH found by Hillgartner *et al.* (1981) and 8·8 times the content of free MH found in the present study. The magnitude of these factors emphasize the importance of including balenine in the pool of non-protein-bound MH. This large pool of non-protein-bound MH still does not compare with the value of 63·8 μmol MH/g wet pectoral muscle found by Fisher *et al.* (1975) in cockerels that were much older (2·5 years) than those used in the present or similar studies and had been fed on a diet containing 480 g fish meal/kg, a source of MH, for 2 months before sampling. The observation by Fisher *et al.* (1975) refers to free MH only and did not consider any contribution from balenine. This may imply that the concentration of non-protein-bound MH in pectoral muscles of cockerels continues to increase with age or are influenced by dietary sources of MH, or both, but these points require further study.

The decreasing excretion of MH per unit body-weight with increasing age (Table 7) is consistent with the general, age-related excretion patterns found in other (mammalian) species, such as the rat (Haverberg *et al.* 1975), man, (Tomas *et al.* 1979) and cattle (Harris & Milne, 1981*c*). The decrease with age found in the present study, at least for the 4- and 6-week-old groups (Table 7), is very similar to that reported by Hayashi *et al.* (1985), also for broiler cocks, at 21 and 42 d of age. However, the agreement does not extend to the 9-week-old (Table 7) and 63-d-old groups (Hayashi *et al.* 1985) in spite of the similarity in mean body-weights, due, possibly, to an abnormally low excretion in the latter group.

For MH to be useful as an index of protein degradation, the pool of free MH in tissues must be small in relation to the excretion rate. This is true for the chicken but the presence of high concentrations of balenine means that the pool of total non-protein-bound MH is effectively quite high (see above). Although the pool of total non-protein-bound MH did not increase at the same rate as the muscle mass (dry weight, Table 6), the rate of increase was still greater than that of the daily excretion of MH with age (Table 7), suggesting that the ratio of flux of MH (through the MH pool):pool size progressively decreased with increasing age. We have not identified whether the flux rate increased with age but at a slower rate than the change in pool size, remained constant or was due to an actual decrease in flux. It is clear, however, that the MH excreted each day became a progressively decreasing proportion of the muscle pool of non-protein-bound MH (Table 7). Thus, even at 4 weeks of age, the muscle pool of non-protein-bound MH was already 1·4 times the mean daily excretion of MH and that by 18 weeks, the muscle pool had more than doubled relative to the excretion. These factors are understimates since the contribution of the remaining muscle in the carcass has not been included. This relative change in pool size thus explains the decrease in excreted radioactivity observed with age (Table 2) which may be attributed to the progressive dilution of label in an expanding pool. Such an interpretation is valid provided that the production rate of MH did not also increase with age since the rate of excretion of labelled MH is, in theory, a measure of the turnover rate of the pool rather than its size. Whether the production rate of MH remains constant, in view of the much higher growth rate of the pectoral muscle in young birds relative to the rate of total

body growth (Latimer, 1925; Maruyama *et al.* 1978; Hayashi *et al.* 1985) and an increasing content of protein-bound MH which, in broiler cockerels, did not reach the levels found in layer cocks until at least 9 weeks of age (Hayashi *et al.* 1985), has still to be established.

The findings reported here for cockerels and the more limited information presented by Saunderson & Leslie (1983) are in general agreement over the pattern of recoveries of labelled MH in excreta. However, the additional evidence provided in the present paper indicates that, contrary to the hypotheses of Saunderson & Leslie (1983), the pool of balenine is not small and that it does interact with labelled MH, resulting in measurable incorporation of label into balenine. Furthermore, the pool of balenine was not constant and progressively expanded with age. As a consequence, the recoveries of labelled MH in excreta were not complete and, in fact, progressively decreased with increasing age of the birds. These findings contradict the conclusions of Saunderson & Leslie (1983) that labelled MH was recovered quantitatively in excreta and can provide a measure of muscle protein degradation. The conclusion of this paper is that excretion of MH by growing cockerels more than 4 weeks old cannot be used as an index of muscle protein breakdown in vivo.

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