Sex- and phenotype-dependent metabolism of *N*^τ-methylhistidine by mice

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The metabolism of *N*^τ-[Me-¹⁴C]methylhistidine by mice was influenced by both the sex and the phenotype of the animal. After subcutaneous injection of labelled *N*^τ-methylhistidine, recoveries of radioactivity in excreta were incomplete, and the distributions of radioactivity showed that different mouse phenotypes degraded *N*^τ-methylhistidine *in vivo* to different extents. Hence the excretion of *N*^τ-methylhistidine by mice cannot be used as an index of protein breakdown *in vivo*.

INTRODUCTION

Lean and obese strains of mice have been extensively studied in order to gain insight into the control of the partition of dietary energy into protein and/or fat. Differences between the lean and obese phenotypes have been described for both lipid and carbohydrate metabolism, but little information is available on lean tissue mass and its turnover. Bergen *et al.* (1975) found that obese mice accumulated less muscle in their hindlimbs than did lean littermates, and Trostler *et al.* (1979) reported values for the turnover of mixed muscle in male lean and obese phenotypes. In these latter studies it was assumed that excretion of *N*-MeHis in the urine would provide an index of muscle protein turnover. However, Trostler *et al.* (1979) did not establish that *N*-MeHis was excreted in the urine of mice quantitatively and without degradation, as in rats (Young *et al.*, 1972). The study now reported was undertaken to provide this information.

The completeness of the excretion of *N*-MeHis was determined from the recovery of radioactivity in excreta after subcutaneous injection of labelled *N*-MeHis, and evidence of metabolic conversion of *N*-MeHis was assessed from the proportion of radioactivity that remained associated with excreted *N*-MeHis on ion-exchange chromatography. The investigation was extended to lean and obese phenotypes of both sexes, when it was found that urinary *N*-MeHis was extensively degraded by female white mice, but was altered to only a small extent by the males. Some of these findings have been presented in a preliminary communication (Harris *et al.*, 1982).

MATERIALS AND METHODS

Animals

White mice, subsequently referred to as normal mice (strain MF-1, originally purchased from Olac Ltd., Shaw's Farm, Blackthorn, Bicester, Oxford OX6 0TP, U.K.), were bred in the Institute's animal house, and six lean and six obese littermates (C57BL/6-*ob/ob*/Ola) of both sexes were also obtained from Olac. Animals were housed at 30 °C in a 12 h-light/12 h-dark schedule and fed on pasteurized rat breeding diet (Oxoid, Basingstoke,

Hants., U.K.). A mature male rat (Rowett Hooded Lister strain), approx. 500 g, was also used, but was housed at 20 °C.

¹⁴C-labelled *N*^τ-methylhistidine

N^τ-[Me-¹⁴C]Methylhistidine was obtained by custom synthesis from Amersham International, Amersham, Bucks., U.K., and was injected subcutaneously as a solution in 0.2 ml of sterile saline (9 g of NaCl/l) at a dose of 12 μCi/100 g body wt. Some animals were injected more than once, but at different ages.

Collection and processing of urine

The mice were housed in small metabolic cages (Rucklidge & McKenzie, 1980), with plastic collecting funnels that did not separate urine and faeces; the excreta were collected in 20 ml of 1.0 M-HCl. The wire-mesh cage base and the collecting funnel of the cage were washed several times in each 24 h period with distilled water to prevent degradation of urinary components by faecal bacteria, since the mucous coating of the faecal pellets caused adhesion to the surface of the funnel. Excreta samples were collected daily for a total of 3 days after injection and, after centrifuging at 2000 *g* for 15 min at 4 °C, samples were taken for the measurement of total radioactivity recovered. The distribution of radioactivity was measured on the first 24 h collection from each animal. Urine from a large rat was collected into 1 M-HCl in an all-glass Metabowl (Jencons Scientific, Leighton Buzzard, Beds. LU7 8UA, U.K.) after subcutaneous administration of labelled *N*-MeHis. As with the mice, the wire-mesh base and the collecting funnel of the cage were washed several times each day.

Urine samples were not desalted, since they were already considerably diluted in 1.0 M-HCl plus water washings and because of the danger of losing labelled components that would otherwise appear in the distribution of radioactivity. Distributions of radioactivity were measured after chromatography on a 0.6 cm × 21 cm bed of Beckman M72 resin (Beckman-RIIC, High Wycombe, Bucks. HP12 4JL, U.K.) thermostatically maintained at 30 °C in sodium citrate buffer (0.38 M in Na⁺), pH 4.18, at a flow rate of 30 ml/h.

Abbreviation used: *N*-MeHis, *N*^τ-methylhistidine.

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Samples in solution were hydrolysed by mixing with an equal volume of conc. HCl and heating in sealed tubes for 16 h at 110 °C. Acid was removed *in vacuo* and samples were redissolved in 0.1 M-HCl.

Isolation of the major acid-labile metabolite of *N*-MeHis from extracts of excreta from female fat *ob/ob* mice

(a) Samples (5 ml) of supernatants from excreta were eluted from 0.9 cm × 6 cm beds of Amberlite CG-120 (100–200 mesh, H⁺ form; B.D.H., Poole, Dorset, U.K.) with 0.2 M-sodium citrate, pH 3.2 at room temperature. Fractions 4–8 (each 3 ml) containing the radioactive metabolites were pooled and acidified to pH 2.5. This procedure separated the labelled metabolites from unchanged labelled *N*-MeHis.

(b) The labelled metabolites from (a) were desalted on columns of Amberlite CG-120 as used in (a), with freshly prepared 1 M-NH₃ for elution.

(c) Pooled fractions from (b) were diluted with an equal volume of water and applied to 1.0 cm × 8 cm columns of Dowex 1 × 8 (200–400 mesh) (equilibrated with 1 M-acetic acid and washed to neutrality with water). The column was washed with 10 ml of distilled water, and then a linear gradient was generated from 75 ml each of distilled water and 0.05 M-acetic acid. Fractions (2 ml) were collected, and the large peak eluted at around tube 40, monitored at 225 nm, coincided with the radioactivity. Samples were concentrated by freeze-drying and, after acid hydrolysis (see above), the distribution of radioactivity was determined.

Radioactivity counting procedures

Radioactivity was measured by using Lumax scintillator (LKB Instruments, Selsdon, South Croydon, Surrey CR2 9PX, U.K.) in a Beckman model LS 345 spectrometer.

Statistics

Results are presented as means ± S.E.M., with the numbers of observations in parentheses. The significance of difference between groups was assessed by Student's *t* test.

RESULTS AND DISCUSSION

Recoveries of radioactivity in excreta

The total radioactivity recovered in excreta over 3 days ranged from 76 to 90% of the injected dose, suggesting that these recoveries may not be complete: the lowest values of dose recovered (76 and 81%) were clearly not quantitative (Table 1). These recoveries would not have been improved by prolonging the period of collection, since, at most, only 3.5% of dose was excreted on day 3. In most cases (39 of 49 recoveries) less than 2% of dose was collected on day 3. The 3-day recoveries did not show any trend with age within a single group, and so the results presented are mean values for a given age range (Table 1).

Losses of the labelled *N*-MeHis injected may be due to a number of factors, such as the collection system itself, retention on fur coupled with ingestion during grooming, or to metabolism while passing through the animal in the period between injection and excretion. Losses in the recovery system owing to retention of radioactive urine on the cage or its wire-mesh base are unlikely because these were washed several times a day (see the Materials

Table 1. Cumulative recoveries of radioactivity in excreta during 3 days after subcutaneous injection of mice with *N*-[Me-¹⁴C]MeHis

Excreta were collected daily for 3 days after subcutaneous injection of a solution of labelled *N*-MeHis. Each collection of excreta was centrifuged and the total radioactivity in the supernatant expressed as a percentage of that injected. Recoveries did not differ significantly (*P* > 0.05).

Phenotype	No. of recoveries	Age range (weeks)	Total radioactivity recovered (% of injected dose)
Male normal	9	5–19	75.9 ± 3.2
Male lean <i>ob/ob</i>	12	6–38	85.7 ± 2.4
Male fat <i>ob/ob</i>	11	5–38	86.4 ± 2.6
Female normal	8	4–32	87.7 ± 5.4
Female lean <i>ob/ob</i>	4	7–26	89.8 ± 6.3
Female fat <i>ob/ob</i>	5	7–26	81.4 ± 2.7

and methods section). This procedure not only lessened the transfer of urine from the cage wire base to fur (and subsequent losses during grooming), but also washed faecal pellets and small drops of urine from the collecting funnel into the acid solution of the reservoir. This ensured that bacterial breakdown of urinary constituents, including *N*-MeHis, was decreased to negligible values, if not prevented. If grooming or retention on the fur was a significant contributor to the observed losses, then the fat *ob/ob* phenotypes might be expected to show poorer recoveries, owing to their larger size and area of fur, but this was not observed (Table 1). Parallel experiments on lean and obese Zucker rats resulted in recoveries of radioactivity exceeding 95% (C. I. Harris & G. Milne, unpublished work), suggesting that losses owing to the recovery system and to grooming were negligible. Thus recoveries between 76 and 90% of dose from the several phenotypes of mice were probably genuinely incomplete (Table 1).

These losses of radioactivity to not appear to be due to incorporation into the *N*-MeHis-containing dipeptide balenine found in muscle, as occurs in sheep, pigs and chickens (Harris & Milne, 1980a, 1981b; Harris *et al.*, 1983). Analysis of mixed thigh muscles from female white mice (*n* = 8) showed in most cases a complete absence of the peptide, although trace amounts, too low to quantify, may have been present in two samples (results not shown).

Distributions of radioactivity in excreta

Evidence that *N*-MeHis was degraded by mice was obtained from comparisons of the distributions of radioactivity in solutions of the labelled *N*-MeHis used for injection with those in excreta. In the injected solution, the 98.4% of the radioactivity associated with *N*-MeHis did not change after incubation with unlabelled excreta in 1 M-HCl for 24 h at room temperature (to simulate the conditions of collection of excreta) and subsequent hydrolysis (Table 2). In contrast, the excreta showed lower percentages of radioactivity associated with *N*-MeHis (Table 2). Partial degradation of *N*-MeHis was inferred from the distribution of radioactivity if

Table 2. Distribution of radioactivity in the injected solution and in extracts from excreta collected during 24 h after subcutaneous injection of mice with *N*-[Me-¹⁴C]MeHis

The distribution of radioactivity was determined by ion-exchange chromatography before and after acid hydrolysis. For each sample, the radioactivity associated with *N*-MeHis is expressed as a percentage of the total radioactivity in the unfractionated sample. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with normal males; †*P* < 0.001 compared with normal females.

	No. of recoveries	Percentage of radioactivity as <i>N</i> -MeHis in unhydrolysed extract	Percentage of radioactivity as <i>N</i> -MeHis in hydrolysed extract	Increment in percentage of radioactivity as <i>N</i> -MeHis
Injected solution	2	98.4	98.0	—
Mouse phenotype				
Male normal	9	62.8 ± 1.8	77.8 ± 1.6	15.0 ± 1.4
Male lean <i>ob/ob</i>	12	55.9 ± 2.5*	73.0 ± 1.8	17.1 ± 1.0
Male fat <i>ob/ob</i>	11	66.0 ± 2.5	85.9 ± 1.1**	19.9 ± 1.9*
Female normal	8	50.3 ± 2.5**	60.6 ± 2.9***	10.3 ± 0.8
Female lean <i>ob/ob</i>	4	25.8 ± 3.0***†	37.5 ± 3.7***†	11.7 ± 3.1
Female fat <i>ob/ob</i>	5	55.4 ± 3.5	84.3 ± 1.9†	28.9 ± 3.0***†

Fig. 1. Distribution of radioactivity in extracts of excreta from a female lean *ob/ob* mouse

Untreated (—) and acid-hydrolysed (---) extracts of excreta were analysed by ion-exchange chromatography (see the Materials and methods section). The '%' values given on the various peaks represent radioactivity (c.p.m.) as a percentage of the total radioactivity in the sample before analysis. Fractions 4–9 represent material not retained by the resin, fractions 26–30 are free *N*-MeHis and fractions 39–44 contained components eluted during regeneration of the resin with 0.3 M-NaOH. The 18% loss of radioactivity from fractions 4–9 after hydrolysis appeared as increases in fractions 26–30 (10%), 39–44 (5%) and 18–20 (4%).

some of the ¹⁴C-labelled material failed to revert to the position of *N*-MeHis after acid hydrolysis.

Most of the radioactivity (60–86%) in excreta was associated with two components, free *N*-MeHis and an acid-labile form that reverted to free *N*-MeHis on acid hydrolysis (Table 2). The female lean *ob/ob* group was an exception to this general pattern in that more than 50% of radioactivity in excreta was associated with components other than *N*-MeHis (Table 2, Fig. 1). Free

N-MeHis in excreta (unhydrolysed extract, Table 2) showed, at most, only 66% of radioactivity as *N*-MeHis in the male fat *ob/ob* group, whereas for female lean *ob/ob* mice the comparable value was only 26%. The lean *ob/ob* phenotype of both sexes was associated with a lower proportion of radioactivity as *N*-MeHis, but, in general, this change appeared to be more characteristic of the female (normal and lean *ob/ob* groups, unhydrolysed and hydrolysed extracts, Table 2) than the male. Acid hydrolysis increased the proportion of radioactivity as total free *N*-MeHis in all groups to, at most, 86% of total radioactivity. All values were lower than that of the injected solution (98%), suggesting that some metabolic conversion of *N*-MeHis had occurred before excretion in all groups. The proportion of radioactivity associated with *N*-MeHis was highest for both sexes of the fat *ob/ob* phenotype, indicating that obesity in mice was associated with minimal metabolic conversion of *N*-MeHis.

These differences between groups are thought to represent degrees of metabolism *in vivo* as influenced by the mouse phenotypes. If the patterns of radioactivity resulted from bacterial breakdown of *N*-MeHis after excretion, the distributions should show a random scatter. This did not happen, and Table 2 shows that a segregation into dissimilar groups occurred (cf. female lean and fat *ob/ob* phenotypes). Hence the differences observed between groups appear to result from differential metabolism of *N*-MeHis in the mouse before excretion. Although most of the radioactivity injected was recovered (Table 1), the losses, up to 24% of dose, may represent more extensive metabolism of *N*-MeHis to, e.g., ¹⁴CO₂, which would be excreted via the lungs.

If metabolism of *N*-MeHis by the mouse is the most likely cause of loss of radioactivity, then all the mouse phenotypes metabolize *N*-MeHis, at least to a limited extent, whereas in two groups the alteration was much more extensive (Table 2). This interpretation implies that the 83% recovery of radioactivity from male non-dystrophic mice in 2 days (Tomas *et al.*, 1981) is also indicative of limited metabolism of the *N*-MeHis before excretion. A revision of some of the rates of protein breakdown calculated by Trostler *et al.* (1979) from the excretion of *N*-MeHis may also be necessary, because of

the differential metabolism of *N*-MeHis by male lean and obese *ob/ob* mice (Table 2).

These above observations are of importance from at least two aspects. Firstly, they demonstrate that gross recoveries of radioactivity, even if essentially complete, do not prove that the labelled *N*-MeHis injected is unchanged when excreted. Secondly, but more important, some of the phenotypes of mice examined actively metabolized *N*-MeHis to a considerable extent. This is the first species identified to do so, and contrasts with earlier studies of a range of species. Thus rats, humans, rabbits and cattle all rapidly excrete *N*-MeHis in the undegraded form (Harris & Milne, 1981*a*), whereas sheep and pigs retained a proportion of the dose in a large body pool of the *N*-MeHis-containing dipeptide balenine (Harris & Milne, 1980*b*, 1981*c*). All of these species do not appear to degrade the excreted *N*-MeHis. To these two groups, identified on the basis of metabolism of *N*-MeHis and the rate of excretion of a dose of labelled *N*-MeHis, must now be added a third group, represented at present only by mice, that extensively alter the *N*-MeHis before excretion.

Products of the metabolic alteration of *N*-MeHis

The consistently high recoveries of total radioactivity (Table 1) demonstrate that, when metabolism of *N*-MeHis does occur (Table 2), the products are also largely recovered in the extracts of excreta, as shown by the distribution of radioactivity in Fig. 1. These profiles for a female lean *ob/ob* mouse show that, in addition to the unchanged labelled *N*-MeHis (24–26% of the applied radioactivity; fractions 26–30), there is a large heterogeneous peak of radioactive material (65–70% of applied radioactivity; fractions 4–9), part of which (10–12% of applied radioactivity) was acid-labile and reverted to *N*-MeHis on hydrolysis (Table 2, Fig. 1). The remaining components in fractions 4–9 (approx. 50% of applied radioactivity) show little or no change in elution position, even though the radioactive profile showed a clear doublet after hydrolysis. The fact that the elution position of these latter compounds did not change after hydrolysis cannot be used as evidence of the absence of molecular alteration, since changes in elution from the ion-exchange system used here will only be apparent if the compound(s) become more basic after hydrolysis. Such a change does occur in the case of the acid-labile component that reverts to free *N*-MeHis.

The acid-labile component was isolated from the excreta of female obese mice, the group in which this component was most abundant (Table 2). The component largely reverted to *N*-MeHis after acid hydrolysis ($84.2 \pm 2.5\%$ of radioactivity as *N*-MeHis, $n = 5$). A similar labile component was also purified from the urine of a mature male rat, where the identity of the compound was already known as α -*N*-acetyl-*N*-MeHis and where the component comprised up to 80% of the total *N*-MeHis in urine (Young *et al.*, 1972). Hence, by analogy, the acid-labile component from mouse urine is

tentatively identified as α -*N*-acetyl-*N*-methylhistidine. Table 2 shows that this component is produced by all groups of mice. However, it is significant (factually and statistically) that the two groups in which considerable degradation of *N*-MeHis occurred (normal and lean *ob/ob* females) have lower proportions of the putative *N*-acetyl derivative. This is perhaps predictable in female lean *ob/ob* mice, since only 38% of the excreted radioactivity remained as *N*-MeHis even after acid hydrolysis, but is more surprising in the female normal mice, where almost twice as much radioactivity is present in *N*-MeHis (61%), although the percentage of radioactivity as the putative *N*-acetyl derivative is unchanged (Table 2).

The implications of this work for the use of mice in studies of protein metabolism are generally disappointing, since it was hoped that some phenotypes of mice would show rapid and quantitative excretion of the labelled *N*-MeHis, thereby allowing useful information to be obtained on general protein turnover from the urinary excretion of *N*-MeHis. This is evidently not the case, and it is clear that normal or lean female *ob/ob* mice cannot be used under any circumstances, since these groups metabolize a large proportion of the *N*-MeHis (Table 2, Fig. 1). The same two phenotypes of male mice also appear to metabolize *N*-MeHis, but to a much lowered extent compared with their female counterparts (Table 2). The remaining two groups, i.e. male and female fat *ob/ob* mice, appear the most promising, since the groups both show the highest total recoveries of radioactivity as well as the highest contents of radioactivity as *N*-MeHis, even if these values indicate a limited alteration of *N*-MeHis. However, the usefulness of these obese phenotypes for studies of protein turnover *in vivo* is severely limited without the comparable lean littermates. The overall conclusion is that excretion of *N*-MeHis by the various phenotypes of mice is of little value as an index of protein turnover *in vivo*.

REFERENCES

- Bergen, W. G., Kaplan, M. L., Merkel, R. A. & Leveille, G. A. (1975) *Am. J. Clin. Nutr.* **28**, 157–161
- Harris, C. I. & Milne, G. (1980*a*) *Br. J. Nutr.* **44**, 129–140
- Harris, C. I. & Milne, G. (1980*b*) *Biochem. Soc. Trans.* **8**, 552
- Harris, C. I. & Milne, G. (1981*a*) *Br. J. Nutr.* **45**, 411–422
- Harris, C. I. & Milne, G. (1981*b*) *Br. J. Nutr.* **45**, 423–429
- Harris, C. I. & Milne, G. (1981*c*) *Biochem. Soc. Trans.* **9**, 315P
- Harris, C. I., Rucklidge, G. J., Milne, G. & McDiarmid, R. (1982) *Abstr. Int. Congr. Biochem.* 12th Abstr. POS 003–100
- Harris, C. I., Milne, G. & McDiarmid, R. (1983) *Protein Metab. Nutr. Int. Symp.* 4th **11**, 61–64
- Rucklidge, G. J. & McKenzie, J. D. (1980) *Lab. Anim.* **14**, 213–216
- Tomas, F. M., Murray, C. E. & Murray, A. J. (1981) *Proc. Nutr. Soc. Aust.* **6**, 99
- Trostler, N., Romsos, D. R., Bergen, W. G. & Leveille, G. A. (1979) *Metab. Clin. Exp.* **28**, 928–933
- Young, V. R., Alexis, S. C., Baliga, B. S., Munro, H. N. & Muecke, W. (1972) *J. Biol. Chem.* **247**, 3592–3600