Metabolism of N^{τ} -methylhistidine by mice

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Mice and rats were injected with tracer doses of radioactive N^{τ} -[Me- 14 C]methylhistidine in order to determine the recovery of the injected radioactivity and the extent of the metabolism of N^{τ} -methylhistidine. In the first 27 h after injection, 96.3, 78.0 and 97.5% of radioactivity was excreted by female mice, male mice and male rats respectively. Recovery after 5 days of collection was 98.4 and 92.8% for female and male mice respectively. However, radioactivity associated with N^{τ} -methylhistidine or its acetylated derivative accounted for 44, 86.5 and 96.0% of the excreted radioactivity for female mice, male mice and rats respectively. In female mice the remaining excreted radioactivity was associated with four major peaks of activity when the metabolites were separated by cation-exchange chromatography. In male mice there were only three of these metabolites present. After chromatographic purification, one metabolite was identified by mass spectroscopy to be 1-methylimidazole-4-acetic acid. Examination of the possible sources of this metabolite indicates that, in mice, N^{τ} -methylhistidine is decarboxylated and enters the chain of reactions common to histamine metabolism. Such extensive metabolism precludes the use of N^{τ} -methylhistidine excretion as an index of myofibrillar protein breakdown in mice.

INTRODUCTION

One residue of N^{τ} -methylhistidine is present in all actin molecules throughout the body and in all myosin heavy-chain molecules contained within white (fast, glycolytic) muscle fibres (see review by Young & Munro, 1978). The histidine residue in the protein is methylated postsynthetically, and N^{τ} -methylhistidine, which is released on degradation of the protein, is not reincorporated via protein synthesis (Young *et al.*, 1972). The consequent use of the excretion of N^{τ} -methylhistidine as an index of the rate of breakdown of myofibrillar protein of skeletal muscle has been extensively reviewed and discussed (Ward & Buttery, 1978; Young & Munro, 1978). Two of the several criteria for its use in this way are firstly that the N^{τ} -methylhistidine excretion arises largely from the skeletal-muscle myofibrillar protein, and secondly that the degree of metabolism or retention of released N^{τ} -methylhistidine is negligible. The extent to which the first criterion is met remains controversial (Ballard & Tomas, 1983; Rennie & Millward, 1983). Validation of the second criterion has assumed considerable importance, since several species have been shown either to metabolize (Saunderson & Leslie, 1983) or retain (Harris & Milne, 1980, 1981) N^{τ} -methylhistidine in the body. Thus, for each species, a rigorous examination is needed to determine whether N^{τ} -methylhistidine excretion is a valid index of the average turnover rate of the myofibrillar-protein pool.

The dystrophic mouse (129 ReJ, dy/dy) is a useful model for human muscular dystrophy (Garber *et al.*, 1980; Goldberg *et al.*, 1977). As such, the rate of excretion of N^{τ} -methylhistidine could provide valuable data in the assessment of experimental approaches to altering the rate of muscle protein turnover (Tomas &

Ballard, 1979; Ballard *et al.*, 1981). We have already validated the use of creatinine as an index of myofibrillar-protein mass in dystrophic mice (C. E. Murray *et al.*, 1981). We here present evidence relating to the validation of the N^{τ} -methylhistidine excretion technique for the assessment of the rate of myofibrillar protein breakdown in mice. The data indicate extensive metabolism of injected radioactive N^{τ} -methylhistidine, which precludes the use of the technique as an index of muscle protein turnover in mice.

MATERIALS AND METHODS

Experimental procedures

Recovery experiments. Phenotypically normal mice of the 129 ReJ, dy/dy and C57/BL/6J dy 2J /dy 2J strains aged 12 weeks and hooded Wistar rats aged 60 days were used. All animals were housed in metabolism cages and received a diet that contained 21% protein and was free of N^{τ} -methylhistidine.

Three female mice, four male mice and five male rats were injected intravenously with 1 μ Ci (18 nmol) of N^{τ} -[Me- 14 C]methylhistidine via a tail vein. Urine output was quantitatively collected from 0–12, 12–27 and 27–48 h in the mice and rats after the injection and thereafter daily for a further 3 days in the mice. The radioactive N^{τ} -methylhistidine was synthesized by one of us (A. J. M.) by using a technique based on that of Noordam *et al.* (1978) and was chromatographically pure.

Metabolite isolation and identification. In order to increase the amount of urine for analysis, a further seven female mice aged 13–14 months were housed and fed as above. After collection of about 300 ml of urine, each

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Fig. 1. Cumulative excretion of injected radioactivity after injection of N^7 -[Me- 14 C]methylhistidine into female (●) and male (▲) mice and male rats (■)

(a) Shows the excretion of total radioactivity, and (b) shows the excretion of label associated with N^7 -methylhistidine and N -acetyl- N^7 -methylhistidine. Values are means $\pm$ S.E.M. for three to five animals.

mouse received an intravenous injection of N^7 -[Me- 14 C]methylhistidine and urine was then collected for a further 24 h. The radioactive urine samples were pooled with the previously collected 300 ml of urine.

Analytical procedures

Samples of the injected material and of the daily urine collections for each animal were mixed with a scintillant (Picofluor-30; Packard Instrument Co., La Grange, IL, U.S.A.) and radioactivity was measured in a Searle 1196 liquid-scintillation counter. Rat urine samples were pooled within each collection period. These pooled samples and the remaining individual mouse urine samples (all 0–12 and 12–27 h samples and the 27–48 h samples for males) were dried under vacuum, reconstituted in sodium citrate buffer, pH 2.2, filtered (0.45 μ m-pore-size filter) and chromatographed on Aminex-A9 cation-exchange resin contained in a 6 mm $\times$ 300 mm column fitted to a modified LKB 4101 amino acid analyser. The resin was eluted with sodium citrate buffer (0.4 M, pH 4.35, 1.5 ml/min) and the eluate collected in a fraction collector. The fractions were subsampled for measurement of N^7 -methylhistidine by autoanalysis (A. J. Murray *et al.*, 1981) and of radioactivity. Fractions containing peaks of radioactivity were pooled, a portion of each was hydrolysed under mild (2 M-HCl, 100 $^{\circ}$ C for 1 h) or strong (5 M-HCl, 105 $^{\circ}$ C for 24 h) acid conditions and rechromatographed after drying and reconstituting in buffer as above. Chromatograms of Pierce 'B' amino acid standards and individual amino acids were also run for comparison of elution times and identification of peaks.

Further characterization of metabolites not hydrolysable to N^7 -methylhistidine was performed on the bulk urine collection. The urine was adjusted to pH 1.73 with concentrated HCl and filtered through a Millipore prefilter followed by a glass-fibre filter (Whatman GF/A).

A small portion was taken for radioactivity measurement and the remainder loaded on to a 2.5 cm $\times$ 60 cm column of AG50W resin (Bio-Rad; X8, 200–400 mesh, H $^{+}$ form) with a bed volume of 250 ml and washed overnight with 2 litres of glass-distilled water. Less than 5% of the radioactivity loaded was eluted during loading and washing of the column. The column was eluted sequentially with 3.1 litres of 0.2 M-pyridine, 2.7 litres of 1.0 M-pyridine, 0.7 litres of aq. 3.0 M-NH $_3$ and 2.6 litres of 0.2 M-NaOH. Eluent was collected in 100 ml fractions in which the radioactivity was determined. Fractions containing major peaks of radioactivity were pooled, dried, reconstituted in buffer and further chromatographed by using an amino acid analyser. Fractions from this second chromatography step, which contained substantial radioactivity, were desalted and further processed by t.l.c. Separation of radioactive metabolites was obtained by using a silica-gel/Mylar plate with butan-1-ol/acetic acid/water (5:2:3, by vol.) as the solvent. The metabolite in the radioactive band was eluted and dried, a portion counted for radioactivity and the remainder subjected to mass spectroscopy for identification by both chemical-ionization and electron-impact techniques.

RESULTS

The cumulative excretion of the injected radioactivity is shown in Fig. 1. After 27 h, 96.3 ± 1.4 , 78.0 ± 8.1 and $97.5 \pm 0.7\%$ of the injected radioactivity had been excreted by female mice, male mice and male rats respectively. After 5 days, male mice excreted only $92.9 \pm 4.4\%$ of injected label, compared with $98.4 \pm 1.3\%$ for female mice. The rats excreted $98.6 \pm 0.7\%$ of the injected label in 48 h. There was notably more variation in the rates of excretion of the radioactivity by male mice than by either female mice or rats.

Table 1. Percentage distribution of radioactivity in compounds excreted in mouse and rat urine and separated by ion-exchange chromatography

Values are means $\pm$ S.E.M. for the numbers of animals shown in parentheses.

Chromatogram peak no.	Distribution (%)			Identity of compound
	Mice		Rats	
	Female (3)	Male (4)	Male (5)	
1	4.0 $\pm$ 1.5	1.6 $\pm$ 0.3	1.8*	Unknown
2	21.4 $\pm$ 0.7	14.7 $\pm$ 0.8	69.3	<i>N</i> -Acetyl- <i>N</i> ⁷ -methylhistidine†
3	18.5 $\pm$ 0.8	6.0 $\pm$ 0.9	0.9	Unknown
4	15.9 $\pm$ 0.8	6.0 $\pm$ 0.9	1.3	<i>N</i> ⁷ -Methylimidazol-acetic acid
5	28.0 $\pm$ 4.5	71.8 $\pm$ 2.5	26.7	<i>N</i> ⁷ -Methylhistidine
6	11.8 $\pm$ 3.7	0	0	Unknown‡

* Analyses on pooled urine samples from five rats.

† About 25% of this radioactivity in female mice urine does not transfer to peak 5 on mild-acid hydrolysis (see the text and Fig. 1).

‡ Part of this peak in female mice urine is transferred to peak 2 on strong-acid hydrolysis (see the text).

Although the rate of excretion of radioactivity was lower in male than in female mice, a higher proportion of this activity was associated with *N*⁷-methylhistidine (Fig. 1, Table 1). Activity in *N*⁷-methylhistidine or *N*-acetyl-*N*⁷-methylhistidine (i.e. that which was co-eluted with the *N*⁷-methylhistidine peak after acid hydrolysis) accounted for 44, 86.5 $\pm$ 1.8 and 96.0% of the excreted radioactivity for female mice, male mice and rats respectively.

Fig. 2 shows a typical example of the distribution of radioactivity in the major metabolites from a female-mouse urine sample separated by ion-exchange chromatography.

The average distribution data from all samples is shown in Table 1. The metabolite eluted in peak one was contained in the early dump of non-retained acid-neutral fluorogenic compounds at the column front and its identity is unknown. Since it constituted only a small portion of the total activity and was probably heterogeneous, no attempt was made at identification.

Female-mouse urine peak 2 contained at least two metabolites. Upon mild acid hydrolysis, about 75% of the radioactivity was transferred and added to that of the *N*⁷-methylhistidine peak (peak 5). This suggested its identity as *N*-acetyl-*N*⁷-methylhistidine, and, in fact, authentic *N*-acetyl-*N*⁷-methylhistidine was eluted in the same position. The non-hydrolysable material contained within peak 2 from female mice has not been identified. In rat urine, about 60–70% of the total radioactivity was found in this peak and it was completely transferred to peak 5 upon hydrolysis. The peak-2 radioactivity in urine from male mice was also transferred to peak 5 after mild acid hydrolysis.

The substance eluted at peak 3 is stable to acid hydrolysis. Attempts to characterize it by g.l.c.–m.s. after derivation were unsuccessful, possibly due to g.l.c. column absorption or failure to form a volatile derivative. Further purification on silica-gel plates and direct introduction into a mass spectrometer also failed to produce a satisfactory spectrum. There was insufficient sample for further attempts at identification.

The metabolite associated with peak 4, which was also acid-stable, was isolated and purified and subjected to mass-spectroscopic analysis. The fragmentation pattern produced by both chemical ionization and electron impact (Fig. 3) was identical with that of authentic 1-methylimidazole-4-acetic acid (*N*⁷-methylimidazol-acetic acid) with a mass number of 140 for the molecular ion.

Authentic *N*⁷-methylhistidine was eluted coincidentally with peak 5. This was the only radioactive peak to contain material measurable in the autoanalysis method of A. J. Murray *et al.* (1981), specific for *N*⁷-methylhistidine.

Fig. 2. A typical elution pattern of radioactivity obtained by column chromatography of female-mouse urine

The mouse was previously injected with *N*⁷-[Me-¹⁴C]methylhistidine. The sample was loaded on to a 6 mm $\times$ 300 mm column containing Aminex-A9 cation-exchange resin and eluted with sodium citrate buffer (0.45 M, pH 4.35) at 1.5 ml/min. Details of the numbered peaks are given in the text and in Table 1.

Fig. 3. Electron-impact mass spectra of material eluted in peak 4 prepared as described in the text (a) and of authentic *N*^r-methylimidazole acetic acid hydrochloride (b)

The molecular ion has a mass of 140, and the mass numbers 96 and 81 correspond to the loss of CO₂ and [CO₂ + CH₃] respectively.

The metabolite eluting at peak 6 was found only in female mice. This substance contained a substantial proportion of the radioactivity, but has not been identified. The peak was unaffected by mild-acid hydrolysis, which indicates it is not a riboside. Upon strong-acid hydrolysis, radioactivity from peak 6 was eluted predominantly with the column front in the acid-neutral-amino-acid 'dump', but a portion was transferred to peak 2.

DISCUSSION

The data clearly show that *N*^r-methylhistidine is extensively metabolized in mice, confirming our preliminary findings (Tomas *et al.*, 1981). In accord with previous observations on rats, we did not find significant metabolism of *N*^r-methylhistidine in this species (Young *et al.*, 1972).

Our identification of 1-methylimidazole-4-acetic acid (*N*^r-methylimidazolacetic acid) as an excretory product of *N*^r-methylhistidine in mice indicates that decarboxylation to *N*^r-methylhistidine followed by oxidative deamination occurs. This sequence of reactions has been shown to occur in mice and rats by Schwartz *et al.* (1972, 1973), and is shown schematically in Scheme 1. The alternative pathway for histamine metabolism, direct oxidative deamination via diamine oxidase (Maslinski, 1975), is also shown.

The decarboxylation of *N*^r-methylhistidine has been shown to be catalysed by histidine decarboxylase, which was prepared as a crude extract from either rat or mouse stomach (Schwartz *et al.*, 1973), but not by bacterial histidine carboxylase. However, the affinity constant of the enzyme for *N*^r-methylhistidine is very much less than for histidine. Since we found very little radioactivity excreted by rats other than in *N*^r-methylhistidine, we must assume that this reaction is quantitatively unimportant in that species. This is consistent with the limited capacity by rats to oxidize methylhistamine to methylimidazolacetic acid (Westling & Wetterquist, 1962; Doshi & Edwards, 1980) and the relatively minor role in rats of the methylation pathway compared with oxidative

Scheme 1. Major pathways of histamine metabolism in mammals and links with histidine metabolism

Enzymes catalysing the numbered reactions shown in the Figure are: (1) histidine decarboxylase (EC 4.1.1.22); (2) histamine methyltransferase (EC 2.1.1.8); (3) diamine oxidase (EC 1.4.3.6); (4) Type B monoamine oxidase (EC 1.4.3.4).

deamination. In mice, *N*⁷-methylhistamine is further metabolized to *N*⁷-methylimidazolacetic acid, which in this species is the normal excretory end product of histamine metabolism after methylation of histamine. These pathways for histamine metabolism also occur in man. However, there is virtually no metabolism of *N*⁷-methylhistidine in man (Long *et al.*, 1975), which suggests that the primary species difference must lie in the specificity of the histidine decarboxylase.

The differences between male and female mice are intriguing, as they indicate additional pathways of *N*⁷-methylhistidine metabolism by female mice. Ample evidence exists to show sex differences in rats and mice in terms of histamine metabolism (Marshall, 1961; Bjuro *et al.*, 1963; Doshi & Edwards, 1980), differences that are linked to the circulating sex hormones. Although male mice metabolize *N*⁷-methylhistidine to a lesser extent than do female mice, the rate of excretion of the radioactivity in males is also much lower. This longer retention time and the incomplete recovery suggest that a substantial balenine (β -alanyl-*N*⁷-methylhistidine) pool may exist. Such a pool occurs in sheep and pigs (Harris & Milne, 1980, 1981), and if the pool is expanding, a portion of the *N*⁷-methylhistidine released from protein would be stored in this pool rather than excreted in the urine. The excretion data from the male mouse are consistent with such an expanding pool. However, no measurements were made of carcass balenine nor of the amount and distribution of radioactivity retained within the carcass.

We conclude that the use of *N*⁷-methylhistidine as an index of myofibrillar breakdown cannot be extended to mice, on account of its substantial retention in males and its extensive metabolism, especially in females. The excretion of the metabolic end product, *N*⁷-methylimidazolacetic acid, suggests that decarboxylation of *N*⁷-methylhistidine to *N*⁷-methylhistamine has occurred. It seems likely that this pathway may also occur in species such as poultry (Saunderson & Leslie, 1983; Harris *et al.*, 1983; Tomas *et al.*, 1984), which are known to metabolize *N*⁷-methylhistidine. Thus the end products of histamine metabolism would seem to be a good point from which to search for metabolic products of *N*⁷-methylhistidine in those species where such metabolism occurs.

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