CONTROVERSIES IN MEDICINE

3-Methylhistidine excretion and the urinary 3-methylhistidine/creatinine ratio are poor indicators of skeletal muscle protein breakdown

M. J. RENNIE AND D. J. MILLWARD*

Department of Medicine, University College London Medical School, The Rayne Institute, London, and
*Department of Human Nutrition, London School of Hygiene and Tropical Medicine, London

Basis of the 3-methylhistidine method and its application

Protein breakdown is more difficult to measure than protein synthesis, so even imperfect methods are welcome if they indicate the extent of and direction of possible changes in the process. We were therefore initially enthusiastic in embracing [1–3] the 3-methylhistidine method as a measure of skeletal muscle protein breakdown.

The basis of the method is that 3-methylhistidine is a constituent of actin and the heavy chain of myosin in white muscle. Thus the skeletal muscle mass comprises the largest fraction of tissue-bound 3-methylhistidine in the whole body. The amino acid is released on protein breakdown and is not re-utilized for protein synthesis. It is not metabolized in man although it is N-acetylated in the rat [4, 5] and is quantitatively excreted in urine.

It was suggested (originally by Asatoor & Armstrong [6], and independently by Young & Munro [7] who developed the method and checked many of its assumptions) that the rate of excretion of the amino acid should be proportional to the absolute breakdown of 3-methylhistidine-containing proteins, chiefly those in skeletal muscle. Since creatinine excretion is proportional to skeletal muscle mass [8] it was suggested that the urinary 3-methylhistidine/creatinine ratio should be proportional to the fractional degradation rate of myofibrillar protein [7, 9]. The method has been widely used to study the effects of dietary manipulation, growth, development, ageing, fever, trauma (both accidental and surgical) [10–20] and muscle disease (see later).

A major flaw in the theory of the method

We believe that the characteristics of 3-methylhistidine metabolism outlined above, which are commonly thought to validate the use of the method, are necessary for its successful application, but are not in themselves sufficient. A further condition has to be fulfilled (one often overlooked): i.e., not only must skeletal muscle contain most of the body's protein-bound 3-methylhistidine, but it must also contribute most to the excretion. The tissue contribution to overall excretion is determined not only by the pool size of protein-bound 3-methylhistidine but also by the turnover of that pool, i.e., the breakdown rate of the proteins. We believe that in both the rat and man small, rapidly turning over non-skeletal muscle pools of protein-bound 3-methylhistidine exist (as actin, present in all cell types), which contribute substantially to the excretion. If this is so, not only are the urinary excretion rate and 3-methylhistidine/creatinine ratio invalid as absolute indices of skeletal muscle breakdown, but even their usefulness as qualitative indices is limited. In the worst cases, such as those set out below, apparent changes indicated in skeletal muscle breakdown by 3-methylhistidine excretion are in the opposite direction to those which actually occur.

Evidence from animal studies

There are now several lines of evidence from experiments with animals that demonstrate serious
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Fig. 1. Pool sizes of protein-bound 3-methylhistidine and daily excretion rate for an adult (250 g) rat. The total pool size per whole body was $83.0 \pm 3.3 \text{(sd)} \mu\text{mol}$. Intestine pool size was obtained by direct measurement ($1.59 \pm 0.10$), and muscle pool size by measurement of the concentration in skeletal muscle samples and multiplication by a factor assuming muscle to be 45% of body weight. Urinary excretion was measured over 5 days and the mean daily excretion was $2.11 \pm 0.09 \mu\text{mol}$. The estimates of the contributions of skeletal muscle and intestine to the excretion were made by multiplying the turnover rates of skeletal muscle ($1.55 \pm 0.10\%$/day) and intestinal ($29.1 \pm 1.4\%$/day) 3-methylhistidine by the pool sizes (from [23]).

Defects in the assumptions central to the 3-methylhistidine method (Figs. 1, 2).

**Turnover of skeletal muscle 3-methylhistidine**

Evidence collected from isotopic labelling studies in rats indicates that the turnover of 3-methylhistidine in skeletal muscle is too slow to account for more than about half of the excretion rate [21-24]. The component of protein turnover of interest here is breakdown, the measurement of which is beset by difficulties well illustrated by the attempts of Nishizawa's group to measure 3-methylhistidine turnover in muscle [25]. In that work, often cited as showing that non-skeletal muscle sources account for only 17% of the excretion, no net loss (i.e., no apparent turnover) of labelled 3-methylhistidine in skeletal muscle was observed after pulse-labelling with $[\text{methyl-}^{14}\text{C}]$-methionine, possibly due to persistence of the label in the methyl pool. We attempted to overcome this problem by the measurement of the synthesis rate of 3-methylhistidine in the steady state, when it should be equal to the degradation rate [21-23].

We recognize that other potentially serious problems remain; these include the assumption of the steady state, the problem of the actual value of precursor labelling, and the assumption that methylation rates of histidine are comparable with protein synthetic rates. The first of these is not a serious problem in fed adult rats in which the growth rate is near zero, so that a steady state, in the fed state, be likely to lead to an underestimate of protein synthesis, and thus of degradation; this would result in the apparent turnover of skeletal muscle 3-methylhistidine being artificially high, leading to an understate-
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precursor labelling could have been a source of error in the first labelling study [21] if S-adenosylmethionine, the precursor, was compartmented in sarcoplasm. We have no evidence that this was in fact the case, and we believe it was not; if so, our initial measurements would remain valid. Nevertheless, in our recent studies [22-24] we have adopted a different technique, using incorporation of [14C]histidine, which, more likely than not, labels a common pool for both presumptively methylated and non-methylated histidine incorporated into muscle protein.

Let us now deal with the last problem, frequently raised by critics of our conclusions. Everyone agrees that for an unchanging composition of muscle the overall turnover rates of 3-methylhistidine, histidine and the methyl group substituent, must be identical, since demethylation apparently does not occur. In other words, the methylation rate of histidine (e.g. in actin) is equal to the rate of protein (i.e. actin) synthesis. What about the possibility of a lag between protein synthesis and histidine methylation? If the processes were not synchronous the mismatch would give rise to a falsely low labelling ratio in 3-methylhistidine to histidine after pulse injection of [14C]histidine, especially noticeable soon after injection of the tracer. This is, of course, a testable hypothesis, and we have checked to see whether a lag in the labelling ratio exists. The turnover of 3-methylhistidine in skeletal muscle protein was 35% of the overall rate of mixed muscle protein synthesis irrespective of whether it was measured 1 h or 4-5 h after injection of tracer [23, 24]. This confirms the results of others, who have shown no differences between rates of methylation and overall protein synthesis [26, 27].

A further possible criticism of our measurement is that they have been made on one group of muscles (i.e. those of the hindlimbs), which may be unrepresentative of the entire musculature. We feel that this is unlikely, since the fibre-type distribution of leg muscles is similar to that of the whole-body musculature in rodents [28], and measurements of protein synthesis in rat quadratus lumborum and psoas, muscles of the trunk, have given values similar to those obtained for leg muscles [29].

Turnover of non-skeletal muscle 3-methylhistidine (Fig. 1)

There is now solid evidence, again from isotopic studies, that small pools of 3-methylhistidine in non-skeletal muscle tissues of the rat do in fact turn over sufficiently rapidly to contribute disproportionately to whole-body excretion. Our initial measurements of turnover in skin and intestine suggested that although turnover in these tissues was faster than in skeletal muscle, they did not appear to be major sources of urinary 3-methylhistidine [21]. It now appears that we initially underestimated 3-methylhistidine turnover in non-skeletal muscle tissues, particularly in intestine, most likely as a result of precursor compartmentation. We applied the histidine labelling method [22-24], coupled with accurate measurements of overall turnover rates of mixed protein by the large-dose phenylalanine method [30], to the measurements of intestinal 3-methylhistidine turnover in adult rats (Fig. 1). By these methods turnover of 3-methylhistidine in intestine is 20 times faster (at 29%/day) than in skeletal muscle, so even though intestine contains only 2% of the whole-body pool, it accounts for over 20% of the excretion. Independently, other workers [31] have recently come to similar conclusions after showing that, on the basis of 3-methylhistidine release from perfused intestine and hemicorpus preparations, intestine could produce two-thirds as much 3-methylhistidine as skeletal muscle [31]. It seems to us that there is every reason to expect that other smooth muscle-containing tissue (e.g. skin, the vascular bed and the lungs), together with the wide variety of non-muscle cells containing actin, could account for the 20-30% of 3-methylhistidine excretion not due to skeletal muscle and intestine.

Time course of urine and tissue 3-methylhistidine labelling (Fig. 2)

The existence of substantial non-muscle sources of 3-methylhistidine is also indicated by the time course of the labelling of urinary and tissue 3-methylhistidine after a pulse of [methyl-14C]-methionine. We initially analysed the urinary decay curve in terms of the contributions from various pools [21], and it appears that by frankly stating that our analysis was not likely to produce reliable absolute estimates, we caused some confusion in the minds of our critics [32]. In fact the importance of the data lies not in the magnitude of the pool sizes obtained from an analysis of the curve, but in the fact that the urinary labelling falls to much lower levels than in either muscle or intestinal 3-methylhistidine [23, 24]. This can only be explained on the basis of substantial non-muscle sources.

One criticism of our work is that we have reported a variety of measured values for the rates of turnover of 3-methylhistidine and the contributions to urinary excretion (e.g. [21-24]) so that there is inconsistency in our published data. Such an inconsistency has been suggested between our
In the case of the above example, for instance, it must be recognized that there are considerable errors (as shown in Fig. 2) for the actual values for the labelling of the three pools of 3-methylhistidine; taken together with theoretical considerations relating to the kinetics and mechanism of protein breakdown [21, 24] these factors suggest that it is fruitless to make any attempt to fit the data to a rigid quantitative scheme. However, this caveat does not influence our main conclusions. The general concurrence of information from different studies should in itself be seen to provide strong support for our views.

Taking all the isotopic evidence together we believe that it is consistent with the existence of physiologically important contributions to the whole-body production of 3-methylhistidine from fast turning-over, non-skeletal muscle pools; the results cannot be explained simply as methodological artifacts. There is certainly no justification for arbitrarily increasing our measured values for rat muscle 3-methylhistidine turnover and consequently raising the presumed skeletal muscle contributions, as adopted by one advocate of the case for 3-methylhistidine excretion [32].

Other evidence from animal studies

The question of most importance for confident use of the method is not whether muscle is the sole source or not of 3-methylhistidine but whether changes in urinary 3-methylhistidine give a useful (i.e. reliable) indication of at least the direction of changes in skeletal muscle breakdown. In some cases the method does appear to give a reasonable qualitative description of changes in muscle protein breakdown. For example, in protein deficiency there is a fall in 3-methylhistidine excretion [33] and also in skeletal muscle protein breakdown [34]. Also, in rats treated with large doses of corticosterone we have confirmed the previously reported [35] transient increase in 3-methylhistidine excretion, and this is synchronous with a parallel increase in skeletal muscle protein breakdown (Odedra et al. [35a]). However, circumstances also exist in which the output of 3-methylhistidine alters in the opposite direction to the change in skeletal muscle protein breakdown. Such paradoxical increases (Fig. 3) in 3-methylhistidine excretion are observed in young starving rats, in which measurements of skeletal muscle protein content and protein synthetic rate indicate a fall in both muscle protein synthesis and breakdown over the first 3 days of starvation, during which time 3-methylhistidine production (i.e. excretion and the increase in the body free pool of 3-methylhistidine) almost doubles [36]. Results from current research indi-
cate that the increase in whole-body 3-methylhistidine production is partly due to an increase in the rate of breakdown of 3-methylhistidine-containing proteins in gut tissues. At the time of body, it is possible to measure a significant net output of 3-methylhistidine in blood draining the gut but there is no detectable efflux of 3-methylhistidine from leg muscle (L. Cotellese, P. W. Emery & M. J. Rennie, unpublished results). In these circumstances interpretation of increased 3-methylhistidine excretion as an increase in skeletal muscle protein breakdown would be totally misguided.

Evidence from studies in man

The relative contribution of muscle to the lean body mass is greater in man than in the rat. Thus it may have been expected that problems of interpretation of 3-methylhistidine excretion data would be less in human studies. There is growing evidence that this is not so. For example, Afting and co-workers showed that there was considerable residual excretion of 3-methylhistidine and creatinine in a paralysed patient, who on later autopsy was declared to have no detectable skeletal muscle [37]. The 3-methylhistidine excretion could have accounted for between 20 and 80% of the range of excretion in normal subjects. There are many difficulties in the interpretation of these values and we do not wish to use them to derive a 'correct' value for the contribution of skeletal muscle to the excretion rate of 3-methylhistidine; rather, we believe this kind of information to be highly suggestive of a significant contribution of non-skeletal muscle pools of 3-methylhistidine, thereby ruling out the possibility of the method providing a definitive measure of muscle protein breakdown in man.

The most obvious application of the 3-methylhistidine method is to circumstances in which there is gross muscle wasting. It is not surprising, therefore, that the method was rapidly taken up for studies of muscle protein breakdown in a variety of muscle diseases, most notably Duchenne muscular dystrophy. Many groups of workers have now shown that although the daily excretion of 3-methylhistidine is lower than normal in affected children, the urinary 3-methylhistidine/creatinine ratio is elevated [38-44]. This has been interpreted as showing that the fractional breakdown of myofibrillar protein is elevated, with the absolute rate of breakdown (i.e. grams of muscle protein degraded per unit time) being depressed because of a similar muscle mass.

However, we cannot accept the validity of the 3-methylhistidine/creatinine ratio as an expression of fractional breakdown in circumstances of reduced muscle mass. There is evidence from measurement of the excretion of creatine, creatinine and guanidino compounds that the rate of synthesis by liver and kidney of creatine (which becomes trapped in muscle as creatine phosphate) is controlled to match the muscle mass in patients with wasting diseases [45]. Thus if the loss of lean tissue is largely confined to muscle such a loss will cause a disproportionately greater fall in the excretion of creatinine than of 3-methylhistidine, assuming that a constant amount of 3-methylhistidine is contributed from non-skeletal muscle sources. It can be calculated, for example, that with the muscle mass only 25% of normal, the 3-methylhistidine/creatinine ratio would double without any alteration in turnover rate of any muscle proteins at the time of measurement of the urine 3-methylhistidine/creatinine ratio.

More concrete reasons for doubting the interpretations based on the urinary ratio values is provided by measurements of synthesis rate of mixed muscle protein taken in needle biopsy samples after infusions of L-[1-13C]leucine [44]. In normal children who are growing, skeletal muscle protein synthesis ought to be greater than in adult men, but we found the rate in mixed muscle from boys with Duchenne muscular dystrophy (n = 11, aged 5-18 years) to have a mean value only one-third of the adult value [44,46]. It is apparent both from clinical inspection and from measurement of the muscle mass on the basis of creatinine excretion and 40K measurements that muscle mass in these children does not fall markedly beyond the age of 5 or 6 years [47]. Thus an elevated rate of muscle protein breakdown can only be reconciled with the lack of ongoing muscle wasting if muscle protein synthesis is elevated. We have examined our methods for measurement of muscle protein synthesis in these children and can find no easily identifiable error which would justify the threefold correction necessary to increase the value obtained for dystrophic muscle to the normal value in adult men.

Particular criticisms of values for protein synthesis obtained in children with muscle disease have been levelled: these include the possible interference in the method from collagen-synthesizing cells and our assumption that the labelling of the precursor pool for muscle-protein synthesis was similar to that of blood α-ketoisocaproate. In answer to the first criticism we must re-emphasize that our measurements were made on alkali-soluble protein extracted from muscle biopsy samples after gross dissection of fat and connective tissue.
tissue from the frozen sample. In any case there is evidence that the synthesis of collagen by muscle taken from affected patients proceeds faster than the synthesis of other classes of protein [48]. As far as the precursor pool is concerned we have made a limited number of measurements of the labelling of the free pool of leucine and α-ketoisocaprate in muscle biopsy samples. The labelling of the free amino acids from muscle was about 85% of the labelling in plasma and was very similar to the labelling of the α-ketoisocaprate in blood; the labelling of the α-ketoisocaprate in blood and muscle were identical [48, 49].

We therefore feel that the discrepancy between the measured rate of muscle protein degradation (as the difference between net wasting and protein synthesis) and the assumed rate inferred from the 3-methylhistidine/creatinine ratio can only be reconciled if the 3-methylhistidine/creatinine ratio is not a good index of muscle protein breakdown. In addition to the effect of reduced muscle mass discussed above, we have some reason to think that the turnover in non-skeletal muscle tissue in children with Duchenne muscular dystrophy is more rapid than usual [44], and this would exacerbate the elevation of the urinary 3-methylhistidine/creatinine ratio.

On the basis of studies in a variety of neuromuscular diseases (see Table 1) as well as in cancer and malnutrition we have to come to the conclusion that a depression of protein synthesis is the main cause of wasting in man, and that protein breakdown in muscle is not elevated, despite the occurrence of an elevated 3-methylhistidine/creatinine urinary ratio. That these elevations in the ratio are artifactual is strongly suggested by recent information obtained by Röthig et al. [50]. They showed that by correcting the measured excretion of 3-methylhistidine and creatinine (on the basis of the excretion of these substances from their patient without detectable skeletal muscle) there was no longer any evidence of an elevation in the ratio measured in urine from patients with a variety of muscle diseases [50]. This is strong corroborative evidence of the artifactual nature of the elevation in the ratio.

Perhaps the most direct evidence that urinary 3-methylhistidine is not a valid index of skeletal muscle production of 3-methylhistidine is from recent measurements we have made in comparing urinary excretion of 3-methylhistidine with the production from the leg of 3-methylhistidine, measured by estimation of the difference between arterial and femoral venous 3-methylhistidine concentration with concomitant measurement of leg blood flow. As shown by others [51], surgical trauma results in an increased excretion of 3-methylhistidine, which falls during postoperative nutritional support, and we confirmed this. Despite this increase in whole-body production rate, the efflux of 3-methylhistidine from legs of patients studied 1 day after major abdominal surgery showed a depression by 50% when compared with values obtained before surgery (Fig. 4). The patients were on a meat-free diet before surgery and were sustained by total parenteral nutrition after surgery. All measurements were made after overnight fast. Tyrosine efflux from muscle, an indication of net amino acid balance, increased as a result of the operation, in contrast with the fall in 3-methylhistidine. These results together indicate that there was an increase in the net loss of

### Table 1. Muscle protein synthetic rate in healthy adult men and patients with wasting diseases of muscle

<table>
<thead>
<tr>
<th></th>
<th>Skeletal muscle protein synthetic rate</th>
<th>10^4 × Urinary 3-methylhistidine/creatinine molar ratio</th>
<th>Muscle mass (% of normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal men, fed (n = 7)</td>
<td>0.198 ± 0.055</td>
<td>19.0 ± 2.4</td>
<td>96.2 ± 12.5</td>
</tr>
<tr>
<td>Normal men, fasted 18 h (n = 7)</td>
<td>0.098 ± 0.043</td>
<td>17.6 ± 1.9</td>
<td>30.6 ± 15.9</td>
</tr>
<tr>
<td>Boys with Duchenne muscular dystrophy (n = 9)</td>
<td>0.055 ± 0.033</td>
<td>49.1 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Adult limb girdle dystrophy (n = 1)</td>
<td></td>
<td></td>
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<tr>
<td>quadriiceps</td>
<td>0.120</td>
<td>26.2</td>
<td>57</td>
</tr>
<tr>
<td>calf</td>
<td>0.020</td>
<td></td>
<td></td>
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<tr>
<td>Myotonic dystrophy, adults (n = 6)</td>
<td>0.086 ± 0.041</td>
<td>27.2 ± 7.8</td>
<td>58 ± 21.0</td>
</tr>
</tbody>
</table>
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We have also shown that in malnourished and cachectic patients, and in patients with acute infection, 3-methylhistidine efflux is depressed from normal when tyrosine efflux is elevated [52].

Conclusions

These last-mentioned studies of leg exchange and urine output of 3-methylhistidine provide the most unequivocal demonstrations to date of the discrepancy between urinary excretion and skeletal muscle protein degradation. We believe that such demonstrations, taken with the evidence previously presented above, leave little room for doubt that the use of 3-methylhistidine production in urine or of the urinary 3-methylhistidine/creatinine ratio as indices of skeletal muscle protein breakdown should be discontinued. However, we believe that the measurement of arteriovenous differences of 3-methylhistidine across skeletal muscle can be used to provide useful information about the regulation of myofibrillar protein breakdown, and this method deserves wider use.

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