I-Methylhistidine Excretion by Vitamin E-deficient Rabbits*

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The creatinuria of rabbits maintained on the diet of Goettsch and Pappenheimer (1), which was first reported by Morgulis and Spencer (2), has been considered by most investigators to be the earliest detectable sign of nutritional muscular dystrophy. Increased urinary excretion of creatine usually is observed in vitamin E-deficient rabbits several days before the appearance of the physical signs of nutritional muscular dystrophy described by Mackenzie and McCollum (3) and can be reversed if the animals are placed on a complete diet (2) or a diet supplemented with α-tocopherol (3).

As an adjunct to studies in this laboratory dealing with metabolic changes in tissues of vitamin E-deficient rabbits, urine specimens from many of these animals were analyzed routinely for creatinine, creatine, and amino acids. A major result of these urine studies to be reported here was the observation that after about a week on the vitamin E-deficient diet, and generally before the appearance of creatinuria, the rabbits began excreting easily detectable and progressively increasing levels of 1-methylhistidine. This methylhistidinuria has been reported in preliminary papers (4, 5), and confirmed by McManus (6).

Earlier data of Dinning et al. had shown an increased urinary amino acid nitrogen excretion in nutritional muscular dystrophy of the rabbit (7) and the monkey (8), but changes in individual amino acids were not determined. Several investigators have studied urinary amino acids in human patients with progressive muscular dystrophy. Thus, Ames and Hasley (9) reported an aminoaciduria; Hurley and Williams (10) found increases in threonine, valine, leucine, arginine, and taurine in urines from dystrophic males; Blahd et al. (11) noted that dystrophic patients and their close relatives showed an increased tendency to excrete detectable levels of various ninhydrin-sensitive substances including, in female subjects, methylhistidine.

EXPERIMENTAL

New Zealand white rabbits weighing 600 to 1000 gm., housed individually in stainless steel metabolism cages, were fed the synthetic diet deficient in vitamin E described by Young and Dinning (12). Control litter mates on the deficient diet were given oral supplements of 10 mg. of α-tocopherol acetate per kg. of body weight three times weekly. The animals were weighed daily, and 24-hour urine samples were collected under toluene for routine photometric creatinine and creatine determinations with use of the Folin micro modification described by Hawk et al. (13). Small samples of the daily urine collections were stored at -20°C for subsequent chromatographic studies.

Chromatographic Techniques—For routine qualitative and semiquantitative studies of amino acid excretion by the rabbits, two-dimensional chromatograms were prepared by methods described previously (14). An aliquot representing 0.2 per cent of a total 24-hour urine specimen was applied to the filter paper in successive 25-μl portions, and the chromatogram was developed with phenol saturated with water used as the first solvent, and the upper phase from a mixture of tert-butyl alcohol, sec-butyl alcohol, and water (1:5:5.6) used as the second solvent. After reaction with ninhydrin, densitometric measurements of the colored spots were performed for estimation of the daily excretion of individual amino acids. Most of the ninhydrin-reacting material on these chromatograms could be accounted for as alanine, glycine, glutamic acid, and glutamine; and, in the vitamin E-deficient rabbit urines, an additional spot was shown to be 1-methylhistidine. The identity of this additional compound in urines of vitamin E-deficient rabbits was determined by its characteristic bluish green color reaction with ninhydrin, by comparison of RF values of the compound with those of pure 1-methylhistidine in a variety of solvents, and by cochromatography with known 1-methylhistidine.

Examination of the two-dimensional chromatograms indicated that one-dimensional chromatography in phenol would move methylhistidine to an area relatively free from overlapping bands representing the common urinary amino acids; this would permit duplicate analyses of urine together with amino acid standards on the same chromatogram. The remainder of the urinary amino acids could be almost entirely accounted for by analysis of three bands containing glutamic acid, glycine, and alanine plus glutamine. Accordingly, various volumes of the urine specimens (2 to 10 μl., based on estimates of amino acid levels from the two-dimensional chromatograms) were spotted on sheets of filter paper, with spot sizes corresponding to those of 5-μl aliquots of standard amino acid solutions interspersed among the urine samples. After an ascending development of approximately 17 cm. in phenol saturated with water, followed by drying at room temperature for 24 hours, the chromatograms were sprayed with ninhydrin and the intensity of the spots was measured densitometrically. Under the conditions of time and temperature employed for color development, the ninhydrin color reaction for methylhistidine was, on a molar basis, only about one-fifth as sensitive as that for alanine and other common amino acids. The concentrations of the amino acids were estimated from curves obtained with the standards.

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RESULTS

Representative urinary amino acid chromatograms are shown in Fig. 1 for two rabbits maintained on the vitamin E-deficient diet for different lengths of time. Those in Fig. 1a were selected for a rabbit with no physical symptoms of muscular dystrophy when killed at 16 days and those in Fig. 1b, for a rabbit that developed clinical signs of dystrophy during the interval between the 24th and 30th days. There was often a pronounced transitory elevation of glutamic acid (see Fig. 1b), but the major change, clearly evident in these chromatograms, is the intensification of the methylhistidine spot. In the first chromatogram shown for each of the two series methylhistidine is barely detectable, and the remaining chromatograms, representing progressively longer periods on the deficient diet, illustrate the steady increase in the excretion of this amino acid, until it becomes the dominant spot on the chromatogram even before correction for the very low chromogenicity of methylhistidine in the ninhydrin reaction. A slight methylhistidinuria has sometimes been detected as early as the 4th day after initiation of the tocopherol-deficient diet; but one rabbit included in our studies did not commence excretion of this amino acid until the 20th day, and another had developed no signs of vitamin E-deficiency when killed on the 22nd day. Most of the animals, however, began to excrete easily detectable amounts of methylhistidine after 9 to 16 days on the unsupplemented diet.

Tallan et al. (15) reported species differences in urinary methylhistidine, with the normal rabbit excreting low levels of the compound. In our studies of many control urine samples from 22 rabbits fed the basal diet supplemented with α-tocopherol, urinary methylhistidine in appreciable amounts was detected in only two rabbits, and in these irregularly. Of the 31 experimental animals which received no α-tocopherol supplements,

Fig. 1a. Chromatograms of urinary amino acids during a developing vitamin E deficiency. Those in Fig. 1a were for a rabbit killed after 16 days, and those in Fig. 1b (below) for another killed after 35 days on the tocopherol-free diet. Urine (0.2 per cent of 24-hour specimen) was applied at the position indicated by the cross at the upper right corner of the chromatogram and phenol was run from right to left, followed by a butyl alcohol mixture as the second solvent. Here the ninhydrin color reaction for 1-methylhistidine (Me-Hist.) was, on a molar basis, only one-fifth as sensitive as that for the other amino acids specified on the figures.
TABLE I

<table>
<thead>
<tr>
<th>Days on -E diet</th>
<th>Rabbit 47*</th>
<th>Rabbit 92†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylhistidine</td>
<td>Creatine: creatinine</td>
<td>Methylhistidine</td>
</tr>
<tr>
<td>3</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Trace</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>1+</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>2+</td>
<td>0.5</td>
</tr>
<tr>
<td>13</td>
<td>3+</td>
<td>1.6</td>
</tr>
<tr>
<td>14</td>
<td>4+</td>
<td>2.1</td>
</tr>
<tr>
<td>15</td>
<td>5+</td>
<td>1.8</td>
</tr>
<tr>
<td>16</td>
<td>20-22</td>
<td>Trace</td>
</tr>
<tr>
<td>23</td>
<td>3+</td>
<td>0.5</td>
</tr>
<tr>
<td>24</td>
<td>4+</td>
<td>2.2</td>
</tr>
<tr>
<td>30</td>
<td>5+</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* See Fig. 1a (killed at 16 days).
† See Fig. 1b (killed at 35 days).

on the other hand, methylhistidinuria developed in all but five. (These five were among those killed before the appearance of creatinuria or physical signs of vitamin E-deficiency; four were killed after only 4 to 11 days on the deficient diet but the fifth still appeared normal after 22 days).

In Table I, the ratios of creatine to creatinine excretion are listed for the two vitamin E-deficient rabbits whose urinary amino acid chromatograms are shown in Fig. 1. Included also is a rough classification of the intensity of the methylhistidinuria, as estimated from two-dimensional chromatograms. In studies with serial urines from 20 rabbits on the vitamin E-deficient diet, methylhistidinuria was observed before creatinuria in 85 per cent of the animals. Two rabbits (10 per cent) excreted detectable methylhistidine and creatine simultaneously and one animal showed detectable levels of urinary creatine 1 day before the appearance of methylhistidine.

Dinning et al. have reported that dystrophic rabbits may excrete as much as 13.5 mg. of amino acid nitrogen (equivalent to about 1000 μmoles of amino acids) per kg. of body weight per day, which is about 3 times the level found for the controls (7). Summation of our chromatographic analyses of the in-
individual free amino acids in urine from dystrophic rabbits revealed a similar total, averaging about 700 μmoles per kg. per day (range, 400 to 1000), of which methylhistidine represented about 500 μmoles or 75 per cent (range, 65 to 80 per cent). Further elucidation of the metabolic role of anserine revealed a similar total, averaging about 700 μmoles per kg. per methylhistidinuria, and adequate assessment of its biological significance in nutritional muscular dystrophy may require further elucidation of the metabolic role of anserine.

**Discussion**

1-Methylhistidine may apparently be formed either by direct methylation of histidine (6, 16) or by methylation of carnosine (β-αlanylhistidine) (16, 17), followed by enzymatic hydrolysis of the resulting anserine (β-αlanyl-1-methylhistidine) (6, 18). Conversely, several species, excluding the rat, can utilize 1-methylhistidine for synthesis of anserine (16, 19), and this dipeptide contains most, if not all, of the methylhistidine present in muscle (20). Accordingly it seems likely that the massive methylhistidinuria found in the present studies might either effect or reflect abnormalities in the metabolism of anserine or carnosine, and these dipeptides, in turn, may have important roles to play in the proper functioning of the muscle (21–25).

Vitamin E deprivation in animals does lead to a reduced anserine content of the muscle (26, 27), but in our tissue studies (unpublished) the decrease did not appear to occur early enough in the developing deficiency or to be of sufficient magnitude to permit the large quantity of urinary methylhistidine to be considered simply as a breakdown product of preformed anserine. Serum levels of methylhistidine were too low for accurate study by the methods employed, but such data as could be obtained tended to rule out a lowered renal threshold as a primary cause of the methylhistidinuria. More detailed metabolic studies will be required in order to evaluate other possible causes of the methylhistidinuria, and adequate assessment of its biological significance in nutritional muscular dystrophy may require further elucidation of the metabolic role of anserine.

Regardless of the causative mechanisms, however, the loss of large amounts of methylhistidine might by itself produce secondary effects such as a significant drain on the body’s reserve of the essential amino acid, histidine, and on the precursors of the methyl group (19, 28, 29). This latter drain would be augmented by the increased loss of single-carbon units in urinary creatine and allantoin during vitamin E-deficiency (12, 30, 31).

**Summary**

Urinary amino acids were analyzed chromatographically during a developing vitamin E-deficiency in rabbits. A transitory rise in glutamic acid excretion was often noted, but the most pronounced change involved 1-methylhistidine. This compound usually appeared in easily detectable levels a week after the rabbits had been placed on the deficient diet, and its excretion increased progressively until it became the major amino acid in the urine. Methylhistidinuria could usually be detected a few days earlier than creatinuria and preceded by a week or two the appearance of physical symptoms of muscular dystrophy.

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**References**

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