Measurements of myofibrillar protein breakdown in newborn human infants

J. L. BURGOYNE, F. J. BALLARD, F. M. TOMAS, A. DOBOZY,† A. H. MACLENNAN,† A. FITZGERALD* AND G. W. DAHLENBURG*
CSIRO Division of Human Nutrition, Kintore Avenue, Adelaide, SA 5000, Australia, and The University of Adelaide, *Departments of Paediatrics, †Obstetrics and Gynaecology, Queen Victoria Hospital, Rose Park, SA 5067, Australia

(Received 16 December 1981/21 April 1982; accepted 7 June 1982)

Summary

1. Myofibrillar protein breakdown was calculated from the urinary excretion ratio of N7-methylhistidine (3-methylhistidine) to creatinine in newborn premature and full-term infants. Representative values were obtained from single voidings provided that the infant's metabolic status was stable.

2. N7-Methylhistidine in infant urine was measured by a rapid Auto Analyser method and shown to give similar values to those obtained by ion-exchange separation techniques.

3. The molar excretion ratio of N7-methylhistidine to creatinine averaged 0.0159 in urine samples obtained within 12 h after birth. A similar ratio was found in amniotic fluid collected at birth. It is argued that this ratio does not reflect a low rate of myofibrillar protein breakdown in the foetus, but rather a more effective transplacental passage of N7-methylhistidine than of creatinine.

4. The urinary ratio increased during the first 2 days after birth to a plateau at 0.0372. This represents a myofibrillar protein degradation rate of 3.40% day−1 in full-term infants.

5. The molar excretion ratio during the period 40–120 h after birth increased in premature infants and reflects a fractional degradation rate of 5.34% day−1 in those infants weighing less than 1 kg at birth.

6. Lower excretion ratios were found in some infants of diabetic mothers and in athyroid infants.

7. The urinary excretion ratio of N7-methylhistidine to creatinine is presented as a useful method for evaluating the breakdown rate of myofibrillar protein in neonates and can be applied to a number of abnormal nutritional or hormonal states.

Key words: amniocentesis, creatinine, prematurity, hypothyroid, 3-methylhistidine, N7-methylhistidine.

Introduction

High rates of myofibrillar protein breakdown have been reported for premature human infants, especially under conditions of inadequate nitrogen retention, acute illness or stress [1–3]. Although the N7-methylhistidine excretion method was used in these three studies, the calculated rates of protein breakdown in one study [1] were approximately twice those found by Seashore et al. [2]. In addition, Pencharz et al. [4] reported a 3-fold increase in N7-methylhistidine excretion over a 2-week period after the infants were clinically stable. In an attempt to resolve these differences, we have followed the N7-methylhistidine/creatinine excretion ratio over the 8 days after birth of full-term infants and compared these values with those obtained with premature infants.

The methods adopted permit the simple and rapid evaluation of myofibrillar protein breakdown in human infants.

Materials and methods

Subjects

Sixty-seven infants with birth weights between 550 g and 4660 g were studied. All infants were...
assessed as being appropriate weight for gestational age [5]. The choice between oral or parenteral diets was based on clinical decisions and was not influenced by any urine collections as part of this investigation. Although each diet was designed to provide adequate amounts of nutrients, no babies were excluded specifically because their food intake was reduced. Rather, urine was obtained from all infants in order to provide a representative range of values. Samples were collected in urine sample bags (Dansk Coloplast, Denmark).

Amniotic-fluid samples were obtained at amniocentesis undertaken for diagnosis of foetal abnormalities or at the birth of the infant. The project was approved by The Queen Victoria Hospital Research and Ethics Committee. Informed consent of parents was obtained.

Analytical measurements and calculations

Creatinine was determined by the alkaline picrate reaction by using the Technicon Auto Analyser method N11b.

$N^\times$-Methylhistidine was generally measured in urine samples or serum by a specific automated fluorescamine technique [6]. For the purpose of the comparison illustrated in Fig. 1, $N^\times$-methylhistidine was also measured after acid treatment and separation by ion exchange chromatography [6, 7].

Fractional rates of myofibrillar protein breakdown were calculated from the expression:

$$\text{% protein degraded per day} = 100 \times \frac{\mu\text{mol of } N^\times\text{-methylhistidine/ml}}{\mu\text{mol of creatinine/ml}} \times \frac{1000}{2.42 \times 4 \times 113}$$

In this equation 2-42 is the $\mu\text{mol of } N^\times\text{-methylhistidine per g of total protein isolated from infant muscle}$ [1], 4 is derived from the observation that 1 mg of urinary creatinine is derived from 4 g of muscle protein [8], and 113 is the molecular weight of creatinine. The assumptions involved in this calculation have been discussed previously [1, 9].

Statistical analysis

Correlation coefficients have been calculated between $N^\times$-methylhistidine measured by the two different techniques or between $N^\times$-methylhistidine and creatinine in untimed urine samples. Variances are compared by using Fisher's test.

Results

We have shown previously that the Auto Analyser fluorescent detection of $N^\times$-methylhistidine gives values identical with those obtained after separation of urine samples by ion-exchange chromatography [6] when the comparison involved urine from normal adult subjects or those with neuromuscular disease. We have extended this comparison to premature and full-term infants (Fig. 1) and show a close correlation ($r = 0.993$) between $N^\times$-methylhistidine measured by the two techniques. The slope and intercept of the regression line are not significantly different from 1 and 0 respectively. Since $N^\alpha$-acetyl-$N^\times$-methylhistidine does not react with fluorescamine, this modified amino acid is not detected by the Auto Analyser technique. If there were significant amounts present in the urine samples, $N^\alpha$-acetyl-$N^\times$-methylhistidine would have been measured by the ion-exchange technique because a prior acid-hydrolysis step was involved. Clearly, the low extent of $N^\times$-methylhistidine acetylation reported for adult human urine [10] also applies to urine from human infants. In subsequent experiments we have measured $N^\times$-methylhistidine by the Auto Analyser method.

A major problem which restricts the clinical use of $N^\times$-methylhistidine has been the need to obtain urine collections of 24 h or longer in order to calculate excretion rates with reasonable precision. This difficulty is particularly serious in
studies involving infants maintained under intensive care, where urine collection bags attached to the infant for long periods could lead to irritation and interfere with normal ward practices. We have suggested that, because both $N^\tau$-methylhistidine and creatinine are derived from contractile tissues, the ratio of these compounds in single urine voidings may be satisfactory for the calculation of the fractional degradation rate of myofibrillar protein [7]. Accordingly sequential urine voidings from eight infants were obtained over a period of 2 days and analysed for creatinine and $N^\tau$-methylhistidine. Fig. 2 shows that for each infant the concentrations of $N^\tau$-methylhistidine and creatinine are closely correlated. Some variability occurs between infants, but this is expected as a consequence of difference in maturity and times after birth when the collections were made. The overall correlation coefficient between $N^\tau$-methylhistidine and creatinine was 0.983 and the average molar ratio of $N^\tau$-methylhistidine to creatinine was 0.0344. The 24 h excretion rates of creatinine and $N^\tau$-methylhistidine for six of these infants aged between 2 and 18 postnatal days and weighing between 0.66 and 3.26 kg were 58.9 ± 14.4 μmol and 1.89 ± 0.28 μmol respectively.

Analysis of individual urine voidings rather than total 24 h collections is advantageous not only because of the ease by which samples are obtained but also because short-term changes in the $N^\tau$-methylhistidine/creatinine ratio may be detected. Further, urine can be obtained soon after birth, when attachment of collection bags could not be justified. Measurements of the $N^\tau$-methylhistidine/creatinine ratio in 54 urine samples from 23 infants are shown in Fig. 3. The infants investigated all weighed over 2.5 kg at birth and all were of appropriate weight for gestational age. Fig. 3 shows that urine obtained soon after birth has a low $N^\tau$-methylhistidine/creatinine ratio, which increases 3-fold to approximately 0.038 within 40 h after birth. A slight fall in the ratio may take place subsequently. Other analyses on urine from premature infants showed that the ratio was similarly low immediately after birth. However, the molar ratio of $N^\tau$-methylhistidine to creatinine in urine of premature infants eventually increased to a higher value than that found for full-term babies (Table 1). The excretion ratio for each group of infants studied between 40 and 120 h after birth decreased with increasing birth weight from 0.0584 in premature infants of less than 1 kg to 0.0352 in babies having birth weights

![Fig. 2. Correlation between $N^\tau$-methylhistidine and creatinine in sequential urine samples from eight infants. The infants weighed between 550 and 3620 g at birth and were investigated between 2 and 80 days after birth. Different symbols are used for each infant. The dashed line represents the best fit of the data by least-squares analysis.](image)

![Fig. 3. Changes in the urinary excretion ratio of $N^\tau$-methylhistidine to creatinine during the first 8 days after birth. All infants weighed over 2.5 kg at birth and were appropriate for gestational age. The dashed line is drawn by inspection.](image)

**Table 1. $N^\tau$-Methylhistidine to creatinine excretion ratios in 40–120 h neonates as a function of birth weight**

<table>
<thead>
<tr>
<th>Birth weight (g)</th>
<th>$N^\tau$-Methylhistidine/creatinine molar ratio</th>
<th>Myofibrillar protein degraded (% day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>&lt;1000 (12)</td>
<td>0.0584 ± 0.0033</td>
<td>5.34</td>
</tr>
<tr>
<td>&gt;1000 &lt;1500 (16)</td>
<td>0.0525 ± 0.0036</td>
<td>4.80</td>
</tr>
<tr>
<td>&gt;1500 &lt;2500 (6)</td>
<td>0.0421 ± 0.0013</td>
<td>3.85</td>
</tr>
<tr>
<td>&gt;2500 &lt;3000 (10)</td>
<td>0.0352 ± 0.0010</td>
<td>3.22</td>
</tr>
<tr>
<td>&gt;3000 (13)</td>
<td>0.0388 ± 0.0014</td>
<td>3.55</td>
</tr>
</tbody>
</table>
between 2.5 and 3 kg. The ratio was slightly higher in infants weighing more than 3 kg at birth. It is possible that the fractional degradation rate of myofibrillar protein in foetuses can be calculated from amniotic fluid analyses. Accordingly we have measured $N^\tau$-methylhistidine and creatinine concentrations in samples obtained by amniocentesis as well as at term (Fig. 4). The results show a slight fall in amniotic fluid concentrations of $N^\tau$-methylhistidine from mid-gestation to term, a substantial increase in creatinine and thus a very marked fall in the molar ratio of these two metabolic products. For eight infants we have compared the concentrations of $N^\tau$-methylhistidine and creatinine as well as the ratio of these compounds in amniotic fluid obtained at delivery with the appropriate measurements in the babies' first urine. Whereas the concentrations of $N^\tau$-methylhistidine and creatinine in urine, averaging 0.061 mM and 3.95 mM respectively, are much higher than those found in amniotic fluid (Fig. 4), the molar ratios are remarkably similar. These are indicated as open circles in Fig. 4 joined to solid circles representing the $N^\tau$-methylhistidine/creatinine ratio in the appropriate amniotic-fluid samples.

Although the data reported in Fig. 3 and Table 1 are derived from normal full-term or premature infants, the analyses can also be applied to infants with hormonal and other abnormalities. Accordingly we have measured the $N^\tau$-methylhistidine/creatinine excretion ratio in eight babies whose mothers were diabetic (Fig. 5). These urine samples taken between 40 and 120 h after birth are compared with the normal range of all infants which were appropriate for gestational age, weighed more than 2.5 kg at birth and studied during the same post-natal period. It is evident that, although the children of diabetic mothers had an average excretion ratio within the normal range, three infants were below and one was above the 95% confidence limits calculated for the normal group. When compared by Fisher's test, the variances of the two groups differed significantly ($F = 4.72, P < 0.05$).

Three athyroid infants tested between 2 and 3 weeks after birth had molar excretion ratios which were approximately 80% of normal (Fig. 5). Infants in the normal group used for comparison had ages ranging from 8 to 105 days (mean 28 days). One athyroid infant was tested a second

![Fig. 4. Measurement of $N^\tau$-methylhistidine and creatinine in amniotic fluid samples obtained at birth or by amniocentesis (●). The molar ratio of these metabolites in the first urine of some infants is indicated (○).](image)

![Fig. 5. The $N^\tau$-methylhistidine/creatinine molar ratio in urine from infants of diabetic mothers (●) and in athyroid infants (○). Separate age-matched groups are given (mean ± 2 SD in each case) because the infants of diabetic mothers were studied between 40 and 120 h after birth whereas the athyroid infants were investigated between 2 and 6 weeks after birth. A second excretion ratio is indicated for one athyroid infant 4 weeks after commencing replacement therapy.](image)
time when 6 weeks old, 4 weeks after commencing thyroid replacement therapy. By that time his excretion ratio had increased to 107% of the mean value for normal infants.

Discussion

The molar excretion ratio of $N^\tau$-methylhistidine to creatinine increased markedly during the first 2 days after birth before reaching a plateau value (Fig. 3). Apparent fractional degradation rates of myofibrillar protein were $1.45\%$ day$^{-1}$ calculated from urine collected within the first 12 h after birth and $3.40\%$ day$^{-1}$ in samples obtained between 40 and 120 h after birth. It is important to establish whether this change truly represents an increase in myofibrillar protein degradation from a low rate in utero. Since $N^\tau$-methylhistidine/creatinine excretion ratios as well as absolute excretion rates of $N^\tau$-methylhistidine in premature infants are increased during infection [2] or when infants are in negative nitrogen balance [1, 2], it is possible that the observed post-natal increase in the ratio reflects a combination of birth associated trauma and poor nutrition in the post-natal period. However, the excretion ratio at birth is much lower than found in well-nourished infants who are gaining weight (Fig. 5; see also [1]) and even in children aged between 8 and 10 years old [7]. A gradual decrease in muscle protein breakdown with age rather than an increase would be expected in concert with the fall in total body protein breakdown reported for humans [11] and in agreement with the fall in muscle protein breakdown in growing rats [12].

An alternative explanation for the low $N^\tau$-methylhistidine/creatinine ratio in the first urine after birth is that transport of $N^\tau$-methylhistidine from foetus to mother is more effective than creatinine transport. We have observed a similar ratio of $N^\tau$-methylhistidine to creatinine in first urine as in amniotic fluid (Fig. 4). This suggests that urine is simply a more concentrated amniotic fluid, at least with respect to these two metabolites. Also the $N^\tau$-methylhistidine concentrations in cord serum obtained at birth from three infants were $5.31$, $5.22$ and $5.73 \mu M$, only slightly higher than the concentration in amniotic fluid (Fig. 4) and twice that in maternal serum ($2.55 \pm 0.19 \mu M$, mean $\pm$ SEM for 16 samples). On the other hand, creatinine concentrations in amniotic fluid at birth (Fig. 4) averaged $192 \mu M$, considerably greater than in the cord serum of the same three infants described above ($84$, $72$ and $70 \mu M$, respectively) and nearly four times that in maternal serum ($52 \pm 3\mu M$). These differences are best interpreted as a substantial creatinine gradient from amniotic fluid to cord serum to maternal serum. We consider that amniotic fluid or urine obtained at birth is not therefore representative of a foetal excretion pool, but a pool modified by the different elimination rates of $N^\tau$-methylhistidine and creatinine from foetus to mother. The post-natal change in the urinary ratio reflects a gradual attainment of urine as a true excretion pool.

The higher excretion ratio of $N^\tau$-methylhistidine to creatinine in very premature infants as compared with infants weighing 2.5 kg or more (Table 1) may be a consequence of a gradually decreasing protein breakdown rate with increasing gestational age. However, the gestational age comparison is not particularly satisfactory, because the very small infants are frequently ill as a consequence of the inability to adapt rapidly to an extra-uterine environment. Thus it is well established that muscle protein breakdown is increased after trauma in adults [13–16]. Also higher excretion ratios have been reported in stressed premature infants than in rapidly growing infants [1, 2]. However, it is likely that a sickness or stress component can only account for part of the higher calculated rates of muscle protein breakdown in premature infants, because the excretion ratio for the 23 early premature infants studied previously who were gaining weight was $0.0438 \pm 0.0009$ (mean $\pm$ SEM) [1]. This value is somewhat higher than that found for full-term infants in the present study (Fig. 5).

Protein breakdown in cultured cells is invariably decreased by insulin provided that the medium does not contain serum [17], whereas myofibrillar protein breakdown assessed by the $N^\tau$-methylhistidine technique is increased in spontaneously diabetic rats [18] but only marginally in juvenile diabetics [19]. It was therefore of interest to measure the urinary $N^\tau$-methylhistidine/creatinine excretion ratios in infants of diabetic mothers during the immediate post-natal period. At this stage low ratios might be expected to accompany hyperinsulinaemia. As described above, however, it is likely that urinary measurements do not reflect rates of myofibrillar protein breakdown until about 2 days after birth. By this stage the eight infants of diabetic mothers had excretion ratios which averaged in the normal range, even though the variance of the data was greater than for the normal group. It remains possible that excretion ratios below the $95\%$ confidence limits of the normal in three of eight affected children represent a residual low rate of myofibrillar protein breakdown caused by elevated insulin concentrations. This supposition will need to be tested in more detail.

The lower ratio of $N^\tau$-methylhistidine to
creatinine in urine from athyroid infants and the restoration of this ratio to normal in the one infant tested provide further evidence that thyroid status has an important regulatory function on myofibrillar protein breakdown. Thus thyrotoxic adults have myofibrillar protein breakdown rates substantially above normal [16, 20, 21], whereas hypothyroid conditions are associated with low rates of myofibrillar protein breakdown [16, 20]. In both hyperthyroid and hypothyroid states the protein breakdown rates return to normal after treatment [20, 21]. Whereas the need for a meat-free diet complicates the use of N\(^{-}\)methylhistidine for monitoring treatment of abnormal thyroid states in adults, this is not a problem for infants, whose diet would not contain N\(^{-}\)methylhistidine. Accordingly the ratio of N\(^{-}\)methylhistidine to creatinine in untimed urine voidings could be a useful parameter for the assessment of thyroid replacement therapy in infants.

We have interpreted the excretion ratio of N\(^{-}\)methylhistidine to creatinine as a measure of myofibrillar protein breakdown and have inferred in our calculations that the myofibrillar protein is exclusively present in skeletal muscle. The latter point is an oversimplification, because small amounts of actin, and accordingly N\(^{-}\)methylhistidine, are present in all cells. It has been calculated that 72–80% of the urinary N\(^{-}\)methylhistidine is derived from skeletal muscle in adult rats [22], rabbits [23] and a single human subject [24], with most of the remaining N\(^{-}\)methylhistidine being produced by skin. Published data on the contributions of skin and muscle to total body weight [25] as well as the nitrogen content of both tissues [25, 26] suggest that the ratio of skin protein to muscle protein is similar in the newborn human to that in the adult. In the absence of information on the content and turnover rate of skin actin it seems reasonable to assume therefore that skeletal muscle also accounts for the same large proportion of the N\(^{-}\)methylhistidine excretion in the human neonate as in the adult. Nevertheless we recognize that skin or intestinal sources of N\(^{-}\)methylhistidine may contribute to the excretion of this amino acid in the human neonate. Should the contribution be significant, it would result in an overestimation of the calculated rate of myofibrillar protein breakdown.

A second interpretational problem arises if creatinine excretion rates do not reflect the mass of myofibrillar protein, because the relationship that 1 g of creatinine excreted daily represents a 20 kg muscle mass [8] was not derived from infant studies. Measurements on six infants aged between 2 and 18 days for whom 24 h urine collections were available (see Fig. 2) averaged 58.9 μmol of creatinine excreted per 24 h per kg body weight, equivalent to a body muscle content of 11.8% [8]. Although this value is only half the reported muscle content of newborn babies [27], it closely reflects the amount of contractile tissue because the proportion of myofibrillar protein in human neonatal muscle is half that of adult muscle [29]. We have not been able to test the relationship between creatinine excretion and muscle mass in infants younger than 2 days old. Accordingly we recognize that, whereas creatinine excretion rates provide a satisfactory index of muscle mass in the older infants studied, the method may be less accurate during the first day after birth.

Myofibrillar protein breakdown rates calculated from N\(^{-}\)methylhistidine excretion rates reported here and by us previously [1] are twice those found by Seashore et al. [2]. Also Pencharz et al. [4] report a 3-fold increase in myofibrillar protein breakdown between infants studied once they were clinically stable and the same infants 2 weeks later. Their lower rate lies between values reported by Seashore et al. [2] and our measurements, whereas their higher rate of 2.9 g of myofibrillar protein degraded day\(^{-1}\) kg\(^{-1}\) is much above other reported rates. We have not observed a late post-natal increase analogous to that reported by Pencharz et al. [4], since the few infants that we have investigated who were more than 3 weeks old ([13]; see also Figs. 2 and 5) have slightly lower N\(^{-}\)methylhistidine excretion rates or excretion ratios than are found in the period from 2 to 8 days after birth. It is possible that low ratios of N\(^{-}\)methylhistidine to creatinine could be incorrect, owing to conversion of excreted creatine into creatinine, such as occurs if urine is collected with acid as preservative. We can offer no explanation of how anomalously high ratios or N\(^{-}\)methylhistidine excretion rates could occur except as a result of co-elution of N\(^{-}\)methylhistidine with other amino acids during column chromatography. Clearly a resolution of these anomalies must be an important goal of future research.

Acknowledgments

We thank the Muscular Dystrophy Association (U.S.A.), the National Health and Medical Research Council of Australia and the Channel 10 Children's Medical Research Foundation for support of this research. We also thank Dr A. J. Murray for assistance with the Auto Analyser methods, as well as Miss K. Shepherd and Miss L. Pope for technical assistance.
References


