Studies on Methylmalonic Acid in Humans. I. Concentrations in Serum and Urinary Excretion in Normal Subjects after Feeding and during Fasting, and after Loading with Protein, Fat, Sugar, Isoleucine, and Valine

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Determination of methylmalonic acid (MMA) in serum or urine for evaluation of tissue cobalamin (vitamin B_{12}) deficiency is becoming an important diagnostic procedure. Here I present the first investigation of dietary influence on concentrations of MMA in serum and urine. Everyday meals caused an increase in urinary excretion, whereas the concentration in serum was not increased significantly. It is difficult to prime the accumulation of MMA in normal subjects by stressing the metabolic pathway; after loading subjects with 100 mmol of isoleucine or valine, the absolute amount of MMA excreted increased by only about 3 μmol. Its concentration in serum tended to decrease and its urinary excretion declined after lack of protein intake for more than 15 h. Although a linear relationship was demonstrated, for the first time, between concentrations in serum and urinary excretion, my results indicate that patients with early evidence of cobalamin deficiency and normal subjects may best be differentiated by measurements in serum, especially in the case of nonfasting (i.e., ambulatory) patients.

Additional Keyphrases: cobalamin status • metabolism

The clinical value of cobalamin estimations is controversial. Some have argued (1–3) that cobalamin concentrations in serum may be normal or high despite a functional cobalamin deficiency. The need for an ancillary diagnostic test in many patients with low or low-normal serum cobalamin has been emphasized, and testing for methylmalonic acid (MMA) in urine (4, 5) or in serum (6) has been recommended. Others claim that serum cobalamin is the simplest, least expensive, and most useful measure of cobalamin status. Considerable debate on these divergent points has ensued (7–11).

Intracellular accumulation of methylmalonyl-CoA results when the conversion to succinyl-CoA by methylmalonyl-CoA mutase, mediated by 5′-deoxyadenosylcobalamin, becomes impaired. MMA is then released into the blood and is excreted in the urine. An increased concentration of MMA in serum and its excessive urinary excretion are believed to be direct measures of tissue stores of cobalamin (12) and thus provide the first indication of cobalamin deficiency (4, 8). If this is true—particularly given the view that cobalamin deficiency is a continuum, with anemia and macrocytosis occurring only in the final stage (13), so that delay of treatment may have serious consequences—then measurement of MMA becomes an important diagnostic tool in clinical chemistry.

A prerequisite for the interpretation of MMA measurements is knowledge of the magnitudes of possible preanalytical sources of variation. To my knowledge, no investigation on the effect of food intake on concentrations of MMA in blood or urine has yet been reported, but earlier investigators (14–17), applying various colorimetric and chromatographic methods of analysis, were mostly unable to prime the accumulation of MMA in normal human subjects after loading them with substances that yield MMA, such as valine and isoleucine. However, quantifying normal concentrations of MMA requires the use of the recently described gas chromatographic–mass spectrometric procedure in the selected-ion monitoring mode, with deuterated MMA as an internal standard (18, 19). Surprisingly, no studies on the variability and dietary dependence of urinary excretion and blood concentrations of MMA, as measured with this new technique, have as yet been reported. Furthermore, in the recently reported normal reference values for MMA (19, 20), the preanalytical conditions—such as previous intake of food—were not stated.

Therefore, as part of a prospective clinical evaluation of cobalamin deficiency in hospitalized and ambulatory patients, I had to establish whether intake of food before sample collection could stress the enzymatic conversion of methylmalonyl-CoA into succinyl-CoA in normal humans and thereby increase the biological "noise" that might otherwise conceal an important biological "signal" of cobalamin deficiency. This question is important, because the experience at my hospital suggests that high-normal or only slightly increased concentrations of MMA may provide an early clue to the existence of cobalamin deficiency.

Using a mass spectrometer in the selected-ion monitoring mode, I have recently developed a reliable and rapid stable-isotope-dilution method for determination of MMA in serum and urine, suitable for routine use in the clinical laboratory (20). I now report the use of this method to address the above question. My findings suggest that dietary influences in normal subjects are minimal in serum, whereas the urinary excretion of MMA significantly increases after meals.

Materials and Methods

In experiment 1, concentrations of MMA in urine and serum were monitored during a 24-h period of normal Danish intake of protein—i.e., about 100 g—and during fasting over the subsequent 24 h.

In experiment 2, the subjects were loaded with protein, fat, carbohydrate, and isoleucine or valine to establish whether MMA accumulation can be primed by stressing the metabolic pathway.
In experiment 3, MMA in serum was measured in 10 subjects on two days in which they were fed a commonplace diet and on two days in which they continued overnight fasting.

Subjects

In experiments 1 and 2, three volunteers were studied, two women (L and A) and one man (J), all college students. History and physical examination failed to reveal any abnormality. In experiment 3, serum specimens were obtained from 10 apparently healthy volunteers (three men and seven women, ages 40–57 years).

In all 13 subjects, concentrations of cobalamin in serum, erythrocyte counts, leukocyte counts, differential leukocyte counts, platelet counts, and concentrations of hemoglobin were all within normal limits, as were results of renal-function and liver-function tests. All were in good nutritional state, with a normal protein intake. None took drugs.

Collection and Storage of Specimens

Blood. In experiments 1 and 2, blood samples were taken from an indwelling cannula inserted into the antecubital vein. A few milliliters of isotonic saline was used to keep the cannula patent. After coagulation at room temperature for 1 h, serum was separated by centrifugation and stored at −20°C.

Urine. Subjects L, A, and J were familiar with the problems of collecting timed urine specimens, but strict instructions were reiterated. Urine was stored at room temperature during the collection period and then, after the volume was measured, at −20 °C.

Experimental Procedures

Except for the amino acid loads, the meals and loads were isocaloric, 3000 kJ each, appropriate for the individual’s age and body weight. The proportion of protein, carbohydrate, and fat was determined and the content of the two most important precursors of MMA, valine and isoleucine, was calculated after a detailed dietary assessment of the edible portion of the meals and loads (21). The subjects were asked to drink plenty of tap water between meals and loads, to promote diuresis. Unsweetened coffee and tea, but no other liquid, food, or smoking was allowed during experiments 1 and 2. The subjects were kept under conditions of moderate physical activity.

The protocol began with an overnight (12-h) fast. Half an hour after midnight the subjects voided a urine sample that was discarded. The first blood sample was drawn at 0600 h and urine was collected (sample 0) immediately before breakfast, which was served at 0635–0700 h.

Experiment I. Urine was collected from subject L every 6 h throughout the study. Blood was collected every hour until midnight, again at 0200 h, and at 3-h intervals thereafter (see Figure 1).

Fig. 1. Concentrations of MMA in serum (O) and urinary excretion relative to creatinine (bars) in healthy volunteers during a 24-h period with normal intake of food and during fasting over the following 24 h (subject L), or after stress tests (see Procedures) of the metabolic pathway (subjects A and J).

Each data point is the mean of duplicate determinations.

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The breakfast (21 g of protein) consisted of milk, bread, butter, cheese, and a boiled egg. Valine and isoleucine content was 12.3 and 8.4 mmol, respectively. The lunch (juice, open-faced sandwiches, and fruit), from 1235 to 1300 hours, contained 27 g of protein (11.9 mmol of valine and 7.9 mmol of isoleucine). The dinner (seafood, lean meat, vegetables, and cheese), from 1835 to 1900 h, contained 56 g of protein (28.2 mmol of valine and 22.1 mmol of isoleucine). Thereafter, subject L was fasted for the following 36 h.

Experiment 2. Subjects A and J participated in the study. After the first urine sampling on the morning (sample 0), they collected 12 3-h urine samples. Blood was collected every hour until midnight, during the night at 0200 and 0500 h, and at 1-h intervals from 0700 to 1800 h (see Figure 1).

Their protein-rich breakfast (107 g of protein, corresponding to 57.4 mmol of valine and 42.6 mmol of isoleucine, i.e., a total of 100 mmol) was served from 0635 to 0700 h. This protein load included 200 mL of buttermilk, 25 g of bread with 15 g of butter, a boiled egg, 150 g of drained canned shrimps, 115 g of caviar, 100 g of boiled haddock, 50 g of uncreamed cottage cheese, and 50 mL of yogurt dressing. Of the 3000 kJ energy requirement, 62% was met by protein, 24% by fats, and 14% by carbohydrates.

After 12 h, subject A was fed 186 g (544 mmol) of white beet sugar (saccharose) dissolved in 200 mL of water and flavored with lemon. Subject J was fed 195 mL of 39% cream containing 75.5 g of fat.

The next morning subjects A and J were given an oral load of amino acids: 100 mmol of L-valine (11.7 g) and 100 mmol of L-isoleucine (13.1 g), respectively, dissolved in 400 mL of water.

Experiment 3. Serum specimens were obtained during a two-week period. On day 1 and day 3, the 10 subjects continued overnight fasting for a total of 13 to 15 h; on day 2 and day 4, blood was sampled 5 h after an everyday breakfast (coffee with Danish pastry was allowed approximately 3 h after the breakfast).

Determinations

MMA in serum and urine was determined by stable-isotope-dilution with solid-phase extraction of the samples (20). MMA from urine (an aliquot equivalent to 1 μmol of creatinine) and from 550 μL of serum was quantitatively extracted, together with added methyl-d3-malic acid (internal standard), onto a small, disposable, strong-anion-exchange column. The sorbent counter-ion was formate. Neutral and basic compounds were washed out with water, and the retained organic acids were eluted with 18 mol/L formic acid. The formic acid was removed by evaporating the resulting extract under a stream of nitrogen at room temperature. After a methanol rinse, the residue was dissolved in hydrogen chloride, 1.5 mol/L in cyclohexanol, and incubated at 115 °C for 15 min. The cyclohexanol was evaporated almost to dryness at 70 °C under a gentle stream of nitrogen, and the residue was dissolved in methanol. The dicyclohexyl derivatives were measured by the gas chromatographic–mass spectrometric procedure, with the mass spectrometer in the selected-ion monitoring mode (20). The total analytical imprecision of my method is 0.025 μmol/L (SD) at a mid-normal concentration in serum of 0.32 μmol/L (CV = 7.9%). For urine, the total analytical imprecision is 0.08 mmol per mole of creatinine (20). Creatinine was determined by the Technicon automated alkaline picrate method (22).

Results and Discussion

Compared with normal reference values I had determined for blood donors, ranging from 0.08 to 0.56 μmol/L, the initial concentrations of MMA in serum were low in experiments 1 and 2: 0.11–0.13 μmol/L, exceeding the analytical sensitivity of the method (0.026 μmol/L), defined as the value at which the assay curve deviates from linearity (20), by only four- to fivefold. Therefore, on the basis of the 30 determinations in duplicate, ranging from 0.026 to 1.00 μmol/L (see Figure 1), I determined the low-level imprecision (SD) to be 0.026 mmol/L. As a result of the increased concentration of MMA in urine relative to serum, the total analytical imprecision of the measurements on urine did not exceed 0.08 mmol per mole of creatinine.

Figure 1 shows concentrations in serum and urinary excretion of MMA during experiments 1 and 2.

In Tables 1 and 2 the mean values for concentrations of MMA in serum during the urine collection periods are reported, together with the measured amounts of MMA per mole of creatinine, the calculated values for the concentrations in urine, and the urinary excretions calculated hourly.

Table 3 gives the values for concentrations of MMA in serum from the 10 subjects in experiment 3.

Effect of Feeding

This study presents the first investigation of dietary dependence of concentrations of MMA in serum and urine in normal subjects. By the 3 × CV criterion, it is evident from Figure 1 and Table 1 that the concentration of MMA in serum from subject L was not appreciably altered by ingestion of the diet given on the first day. In contrast, the meals caused a significant stepwise increase in the urinary excretion of MMA during the following hours, doubling after dinner, suggesting that there had been increased formation of this acid. The effect of protein intake may be long lasting. Thus, the effect of ingestion of the protein-rich dinner on the urinary concentration of MMA was still apparent 12 h later.

To confirm the observed dietary independence of serum MMA in normal subjects, I performed experiment 3. Analysis of results in Table 3 by two-way analysis of variance with two observations per cell (23) showed significant variation between the subjects (P < 0.01), but no significant variation between the treatments (P > 0.05). Therefore, for each individual, whether fasting or not, the concentration of MMA in serum was consistent within the two-week period.

Effect of Prolonged Fasting

After subjects had fasted for about 15 h, the concentration of MMA in serum tended to decrease, and the urinary excretion declined to a low, apparently constant, value. As mentioned earlier, MMA is an intermediary in the catabolism of the two branched-chain amino acids isoleucine and valine. Thus the decrease may be a consequence of depressed concentrations of valine and isoleucine. Snyderman et al. (24) found that the most striking changes in the plasma aminogram after reduction in intake of protein were depression of the branched-chain amino acids. During more prolonged starvation, the plasma concentrations of the branched-chain amino acids are known to increase (25).

Effect of Loading

Protein. Even the protein-rich meal given subjects A and
Table 1. Effect of Feeding and Fasting on Concentrations in Serum and Urinary Excretion of Methylmalonic Acid (Experiment 1; Subject L)

<table>
<thead>
<tr>
<th>Time of sample collection</th>
<th>MMA in serum,μmol/L (0.028)</th>
<th>MMA in urine</th>
<th>mmol/mol creatinine (0.08)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0030-0630</td>
<td>0.120</td>
<td>5.09</td>
<td>0.22</td>
</tr>
<tr>
<td>Breakfast from 0635 h</td>
<td>0.123</td>
<td>4.81</td>
<td>0.39</td>
</tr>
<tr>
<td>0630-1230</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lunch from 1235 h</td>
<td>0.142</td>
<td>2.73</td>
<td>0.45</td>
</tr>
<tr>
<td>1230-1830</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinner from 1835 h</td>
<td>0.165</td>
<td>3.95</td>
<td>0.48</td>
</tr>
<tr>
<td>1830-0030</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-term fasting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0030-0630</td>
<td>0.145</td>
<td>3.85</td>
<td>0.34</td>
</tr>
<tr>
<td>0630-1230</td>
<td>0.140</td>
<td>4.73</td>
<td>0.28</td>
</tr>
<tr>
<td>1230-1830</td>
<td>0.085</td>
<td>2.38</td>
<td>0.22</td>
</tr>
<tr>
<td>1830-0030</td>
<td>0.105</td>
<td>1.43</td>
<td>0.25</td>
</tr>
<tr>
<td>0030-0630</td>
<td>0.090</td>
<td>8.90</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*The first fasting value is from a single determination in duplicate at 0600 h. The values after the three meals represent the means of six determinations in duplicate. The values during long-term fasting are means of two determinations in duplicate (see Fig. 1). Determinations in duplicate. Total analytical imprecision (SD); see Results and Discussion.

J caused only a minor "noisy" increase of the MMA concentration in serum (Figure 1 and Table 2), whereas the increase in urinary excretion exceeded the total analytical imprecision by at least 10-fold. Oberbolzer et al. (14), using a colorimetric method of limited specificity, reported a decrease in plasma after a load containing about 27 g of protein was given to an eight-year-old girl suffering from an inherited block in the metabolic conversion of methylmalonyl-CoA to succinyl-CoA. Despite this decrease in plasma MMA, they noted a significant increase in the rate of urinary excretion of MMA (data were not stated).

As mentioned, the concentration of MMA in serum appears to be particularly labile after ingestion of protein, especially in subject J, a trend similar to that observed after the protein-rich dinner in experiment 1, in contrast to the stable value during prolonged fasting (Figure 1). A likely explanation might be differences in the readiness with which the proteins from egg, milk, meat, and vegeta-

Table 2. Effect of Various Stress Tests on Concentrations in Serum and Urinary Excretion of Methylmalonic Acid (Experiment 2; Subjects A and J)

<table>
<thead>
<tr>
<th>Time of sample collection</th>
<th>MMA in serum,μmol/L (0.028)</th>
<th>MMA in urine</th>
<th>mmol/mol creatinine (0.08)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0030-0630</td>
<td>0.135</td>
<td>15.8</td>
<td>0.37</td>
</tr>
<tr>
<td>High-protein meal from 0635 h</td>
<td>0.106</td>
<td>16.9</td>
<td>0.36</td>
</tr>
<tr>
<td>0630-0930</td>
<td>0.134</td>
<td>7.02</td>
<td>0.63</td>
</tr>
<tr>
<td>0930-1230</td>
<td>0.144</td>
<td>3.34</td>
<td>1.07</td>
</tr>
<tr>
<td>1230-1530</td>
<td>0.136</td>
<td>2.49</td>
<td>0.83</td>
</tr>
<tr>
<td>1530-1830</td>
<td>0.128</td>
<td>2.09</td>
<td>0.67</td>
</tr>
<tr>
<td>Sugar (subject A) and fat (subject J) at 1835 h</td>
<td>0.175</td>
<td>3.71</td>
<td>0.59</td>
</tr>
<tr>
<td>1830-2130</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2130-0330</td>
<td>0.109</td>
<td>1.56</td>
<td>0.65</td>
</tr>
<tr>
<td>0030-0330</td>
<td>0.092</td>
<td>3.39</td>
<td>0.40</td>
</tr>
<tr>
<td>0330-0630</td>
<td>0.061</td>
<td>11.5</td>
<td>0.31</td>
</tr>
<tr>
<td>Valine (subject A) and isoleucine (subject J) at 0635 h</td>
<td>0.139</td>
<td>1.43</td>
<td>0.46</td>
</tr>
<tr>
<td>0630-0930</td>
<td>0.042</td>
<td>1.39</td>
<td>0.46</td>
</tr>
<tr>
<td>0930-1230</td>
<td>0.055</td>
<td>2.38</td>
<td>0.65</td>
</tr>
<tr>
<td>1230-1530</td>
<td>0.065</td>
<td>2.68</td>
<td>0.65</td>
</tr>
<tr>
<td>1530-1830</td>
<td>0.071</td>
<td>4.05</td>
<td>0.56</td>
</tr>
</tbody>
</table>

*The first set of fasting values is from single determinations in duplicate at 0600 h. The following values are the means of three determinations in duplicate, except the two sets of night-values (0030-0330 and 0330-0630 h), which are single determinations in duplicate (see Fig. 1). Determinations in duplicate. Total analytical imprecision (SD). Below the limit of detection (<0.026 μmol/L).
Tables in the mixed diet were digested.

Fat and sugar. In accordance with the findings by Oberholzer et al. (14), the concentration of MMA in serum decreased and the amount of MMA excreted in sweat markedly after ingestion of sugar or fat. The decline was most likely the result of lack of protein intake for more than 15 h (see Effect of Prolonged Fasting).

Valine and isoleucine. Loading with either 100 mmol of valine or 100 mmol of isoleucine gave increases of MMA in serum; at the most, however, these amounts to only 4 SD (see Table 2), but the trend is clearly present (see Figure 1). In contrast to the "noisy" increase after protein intake, this increase continued for only 2 to 3 h. This short-lived response may reflect the known rapidity with which valine and isoleucine are absorbed from the gastrointestinal tract (24) and the fact that their concentrations in plasma appear to vary directly with intake (26).

The excess in urinary excretion amounted only to about 3 μmol (see below). Probably because of the insensitivity of the various chromatographic methods used, Chanarin et al. (17), in accordance with earlier investigators (15, 16), noted no increase in excretion in 39 control subjects after an oral dose of 85 mmol of valine or isoleucine, except for one subject whose MMA excretion was 50% above baseline after oral isoleucine.

Relationships between Concentration of MMA in Serum and Urinary Excretion

Using the coefficient of linear regression to establish the relationships between the parameters listed in Tables 1 and 2, I found good correlation between the measurements of mean concentration of MMA in serum and MMA in urine (millimoles per mole of creatinine), in each of the three subjects L, A, and J (r = 0.88, 0.75, 0.77; P < 0.01, <0.01, <0.005; and n = 9, 13, 11, respectively) with an overall correlation of r = 0.63 (P < 0.005; n = 33). These data agree well with the linear relationship (r = 0.98, P <0.001) between concentrations in serum and urine (range: 0.05–34.2 μmol/L) obtained from 48 subjects (volunteers and patients with cobalamin deficiency) and 24-h urinary excretion of MMA relative to creatinine, as reported in the accompanying paper (27). The overall correlation between MMA in serum and the urinary excretion rate calculated hourly was weaker (r = 0.41, P <0.01, n = 33). No correlation was found between concentration of MMA in serum and in urine (r = 0.05, P >0.35, n = 33), undoubtedly because of variations in diuresis. This lack of correlation, together with the much broader range for the urinary concentrations of MMA over 24 h as compared with the range of MMA per mole of creatinine (see Table 1), should discourage the practice of expressing amounts of MMA per volume of untimed urine specimens.

Excess Excretion of MMA after Feeding and Loading

The urinary excretion of MMA by subject L during the initial fasting period (sample 0) and during the prolonged fasting period appears similar (Table 1). When the initial fasting value was regarded as baseline excretion, the excess excretion in the 6-h periods after breakfast, lunch, and dinner was 1.02, 1.35, and 1.56 μmol, respectively. Therefore, although the meals contained a total of 91 mmol of the two major precursors for methylymalonyl-CoA, isoleucine and valine, the absolute amount of MMA excreted increased by only about 4 μmol.

Similarly, the excess excretion in subject A during the 12-h periods after loading with protein and valine was 5.16 and 1.98 μmol, respectively (Table 2). In subject J the excess excretion during the 12-h periods after loading with protein and isoleucine amounted to 8.37 and 0.69 μmol, respectively. However, the excretion during the 3-h period before loading with amino acid was lower than the initial baseline excretion, especially in subject J (see Table 2). If this preload excretion was regarded as baseline, the excess excretion of MMA after valine in A and after isoleucine in J amounted to 2.70 and 3.09 μmol, respectively.

Unless some MMA was metabolized via unknown pathways, the above observations suggest that in healthy persons only a tiny fraction (about 3 × 10⁻⁶) of the methylmalonyl-CoA molecules formed in the mitochondria escape conversion to succinyl-CoA. Therefore, it seems worthwhile to study further the possible usefulness of an amino acid-loading test in distinguishing between cobalamin-deficient patients and healthy persons.

Finally, the present study gives reason to believe that the current normal reference values for MMA in serum are too high. This assumption rests on two points. First, the concentrations of MMA in serum from my 13 healthy subjects were lower than the normal reference values reported, ranging from 0.16 to 0.64 μmol/L in the U.S.A. (19) and from 0.08 to 0.56 μmol/L in Denmark (20), both calculated on the basis of determinations on 50 blood donors; however, the donors had neither been assessed for sufficient cobalamin status. My observation is in agreement with the relatively low range (0.05–0.28; mean 0.16 μmol/L) noted (27) for serum from 28 healthy volunteers, ages 22–86, who all had a normal concentration of cobalamin in serum. Second, the finding that MMA in serum was not appreciably increased by stressing the biochemical pathway makes it unlikely that the high values encountered in some of the blood donors were due to previous intake of food. In my laboratory, we are currently attempting studies on apparently normal subjects before and after injections of cobalamin, in an attempt to define the normal concentration of MMA in serum of non-cobalamin-deficient persons.

In conclusion, my set of data implies that it is unlikely that the concentration of MMA in serum in normal subjects is
appreciably influenced by previous intake of food. In contrast, its urinary excretion, best expressed relative to that of creatinine, is significantly increased. Therefore, distinction between patients with early evidence of cobalamin deficiency and normal subjects may best be achieved by measurements of serum, especially for nonfasting, i.e., ambulatory, patients.

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References