METHYLMALONIC ACIDURIA AND VITAMIN B₁₂ DEFICIENCY IN THE RAT

M. ORLANDO, A. FIORI, and M. COSTA

Instituto Superiore di Sanità, Rome and Institute of Biological Chemistry, University of Rome
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Methylmalonic aciduria is a metabolic defect characterized by an excessive excretion of methylmalonic acid in the urine. The present paper reports the results obtained on rats fed a vitamin B₁₂-deficient diet to which were administered propionate or the amino acids L-valine, L-isoleucine, L-methionine, L-threonine. Detection of metabolites was carried out by gas chromatography. The results obtained showed that besides propionate only L-valine and L-isoleucine cause an increase in the daily excretion of methylmalonic acid, while L-methionine and L-threonine have no effect on the rat.

Vitamin B₁₂ deficiency is known to cause a metabolic defect in man and other animals unable to synthesize the vitamin. Characterized by methylmalonic aciduria, this defect leads to pernicious anemia when sufficiently severe (1-3). The metabolic pathway involved is the conversion of propionate to succinate. This reaction, catalysed by the coenzymatic form of vitamin B₁₂, entails the interconversion of methylmalonyl CoA and succinyl CoA in the presence of methylmalonyl CoA mutase (4-7).

The complete metabolic pathway is shown in Fig. 1, in which it is seen why vitamin B₁₂ deficiency could prevent or cause great reduction of methylmalonyl CoA conversion to succinyl CoA, with the consequent excretion of methylmalonic acid (8).

Two variants of methylmalonic aciduria have been described. Aside from the form caused by vitamin B₁₂ deficiency, there exists a second variant which does not regress following the administration of the cobamide coenzyme. In this case, the defect appears to reside in the defect of the apoenzyme of methylmalonyl CoA mutase, as concluded from studies by Morrow et al. (9) on the liver of deceased patients presenting normal quantities of coenzyme B₁₂.

Experiments conducted on rats with dietetically induced vitamin B₁₂ deficiency have furnished further information on the biochemical lesion produced by cobamide deficiency (10,11). The present study was carried out using rats fed
Methylmalonic acid was identified using a new gas chromatographic method, which is more sensitive than the commonly applied colorimetric methods and allows for the distinction of various levels of deficiency, as will be explained in further detail below.

MATERIAL AND METHODS

Animals. Male rats (Wistar strain) of variable ages and weighing 250 g were used. The rats were divided in groups of ten each. One group was fed a standard laboratory diet, while the other (experimental rats) received a vitamin B$_{12}$-deficient diet (12). This diet (Table 1) was administered for varying periods of time, as will be explained in the results, beginning when the rat had reached a weight of 100 g.

Table 1. Percentage composition of Fatterparker's vitamin B$_{12}$-deficient diet (12).

| Ingredients                        | Percentage |
|------------------------------------|------------|
| Peanut extracted with alcohol      | 46%        |
| Corn meal                          | 44%        |
| Peanut oil with lipid-soluble vitamins | 5%      |
| B vitamins in sucrose              | 1%         |
| Hegsted salt mixture               | 2%         |
| Succinylsulfathiazone              | 2%         |

The experimental rats were analysed for methylmalonic acid and then received intraperitoneal injection of 100 mg of sodium propionate or separately of single aminoacids dissolved in water. They were then placed in metabolic cages, and urine samples were collected daily and stored under toluene at 0°C. During the period of urine collection the animals were fasted but were left free to drink.
In order to avoid coprophagy, specially constructed cages with double floor nets were used.

**Material.** The following amino acids were used, all purchased from the Kock-Light Co.: L-valine, L-isoleucine, L-threonine, and L-methionine.

Sodium propionate was provided by Sigma.

Trimethylsilylation was carried out using trimethylchlorosilane and hexamethyldisilazane (BDH), and pyridine was furnished by C. Erba.

**Urine extraction and identification of methylmalonic acid by gas chromatography.** Methylmalonic acid was mechanically extracted three times using four volumes of ethyl ether, following acidification (pH 1) with concentrated hydrochloric acid and controls of pH acidity upon each extraction. Centrifuging was occasionally necessary, especially at the beginning, in order to obtain sufficient separation between the two phases. The combined ether extracts, containing 90% methylmalonic acid, were evaporated to dryness in a bath at 40°C under a stream of nitrogen. The dry residue was then redissolved in pyridine and trimethylsilylated, adding hexamethyldisilazane and trimethylchlorosilane (0.5: 0.25: 0.25) at room temperature.

Gas chromatography of the silyl derivatives was carried out approximately for 30 min, but not more than 6 hr, following the reaction. Stainless steel columns, 6 feet × 1/8 inch, containing 3% OV 17 on Chromosorb W and maintained

![Gas-chromatogram of the TMS derivatives of methylmalonic and succinic acid.](image)

Fig. 2. Gas-chromatogram of the TMS derivatives of methylmalonic and succinic acid. See the test for the details. TMS, trimethylsilyl derivatives; C, concentration.
at 110°C were used. The peaks were shown using a flame ionization detector with variable attenuation, and quantitation was obtained by comparing under identical attenuation the peak weights of the unknowns with standards in which recovery was considered.

A typical chromatogram, showing retention times of the silyl derivatives of methylmalonic acid and succinic acid, is illustrated in Fig. 2.

RESULTS AND DISCUSSION

The first group of rats, 4 months old, fed a normal diet and weighing approximately 250 g, were examined. No trace of methylmalonic acid was found in the urine, but a different acid was identified on the basis of the retention time as succinic acid. The same results are obtained when the rats were administered intraperitoneal injection of abnormal quantities of sodium propionate, which was thus shown to be completely metabolized by the normal cycle.

Different results were obtained from one group of rats fed the FATTERPARKER's diet (12). After four months of the diet, methylmalonic acid was not present in the urine, but there was succinic acid. Following administration of sodium propionate (100 mg intraperitoneal injection), however, a certain amount of methylmalonic acid appeared, reaching a mean value of 2.7 mg/24 hr, even though the succinic acid was still present. This finding would suggest a slight deficiency state in which under normal conditions the rat is still capable of normal metabolism, while, under abnormal conditions (as evoked by the injection of 100 mg of propionate) only part of the substance follows the normal metabolic cycle and is converted to succinic acid; the rest is transformed into methylmalonic acid.

Following 10 months of the vitamin B_{12}-deficient diet, the amount of succinic acid in the urine was greatly reduced, while there was a proportional increase in methylmalonic acid. This finding was further evidenced when these rats were treated with amino acids, which present methylmalonyl CoA as an intermediary in their metabolism.

The amino acids L-valine, L-isoleucine, L-methionine, and L-threonine were administered at doses of 100 mg each. The results (Table 2) show no significant differences between L-valine and L-isoleucine, which actually cause an increase in the daily excretion of methylmalonic acid, of approximately the same equimolecular quantity. This result confirms the findings for the same amino acids reported by LUHBY (13) and by ROSENBERG (6) in man, and for valine alone as reported by CARDINALE (10) in the pig and by STOKKE (3) and OBERHOLZER (2) in man. As also reported by LUHBY no increase in the daily excretion of methylmalonic acid was found following the administration of an equal quantity of L-methionine. LUHBY's findings were, however, different with regard to L-threonine administration, which in the present study do not cause an increased excretion
of the acid. Therefore by comparing our results with those obtained by the above-mentioned authors it would seem that on the rat there is a different situation. Indeed while in the man L-valine, L-isoleucine, L-methionine, L-threonine, form methylmalonic acid as intermediate in their metabolism, in the rat this pathway can be demonstrated only for L-valine and L-isoleucine.

The gas chromatographic method used, was able to reach very high limits of sensitivity (up to 1 μg), and further increases were made possible by varying attenuation. In addition, there is no need of the preliminary treatment of the urine with ion-exchange chromatography, required in the colorimetric methods (14) for removing substances which may interfere with colour development and which are not always removable, as in the case of malonate. The gas chromatographic method also allows for the distinction of the various metabolites on the basis of their retention times, as well as for their quantitative analysis, thus providing a useful element in establishing varying degrees of deficiency. Due to its simplicity, the method is recommended for clinical application to identify and distinguish anemia caused by vitamin B12 deficiency from that caused by folic acid deficiency. In this case, the only differential method is the study of the metabolites, that is of methylmalonic acid and formiminoglutamic acid, respectively.
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