The Biochemical Conversion of 2-Hydroxy-4-methylthiobutyric Acid into Methionine by the Rat *in vitro*

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1. The conversion of 2-hydroxy-4-methylthiobarbituric acid into methionine requires a flavine coenzyme, which can be partially replaced by NAD or NADP, and glutamine, which can be partially replaced by asparagine. The system also requires aerobic conditions. 2. There does not appear to be a requirement for a metal ion: Mn^{2+} is slightly inhibitory and Cu^{2+} is strongly inhibitory. 3. Of the kidney, liver, brain, small intestine and muscle, the kidney possesses the greatest conversion activity/g. of tissue, but on a total organ basis the liver is the more important organ. Within the liver cells most of the activity is present in the supernatant fraction.

It has been well established (Block & Jackson, 1932; Machlin & Gordon, 1959; P. György, personal communication) that MHA† can effectively replace methionine as a supplement in the diet of the chicken and the rat. This dietary equivalence indicates that MHA is converted into methionine. Little has been done, however, to elucidate the biochemical processes involved in such a conversion. This paper presents results on the cofactor and metal ion requirements and organ and intracellular location of the system responsible for this conversion.

MATERIALS AND METHODS

2-Hydroxy-4-methylthiobutyric acid. The MHA used in these studies was DL-$(MHA)_2Ca$ and was supplied through the courtesy of Dr K. Maddy, Monsanto Chemical Co., St Louis, Mo., U.S.A.

Preparation of tissue for assay. (*a*) Cofactor studies. Livers were obtained from rats (Sprague–Dawley strain) that had been maintained on a commercial laboratory diet. The livers were washed, blotted dry, weighed and homogenized with 4 ml. of 0·05 M-sodium phosphate buffer (pH 7·2)/g. of tissue for 2 min. at full speed in a Servall Omni-Mixer at 0°. The homogenate was then centrifuged at 37000*g* for 3 hr. (Servall RC–2 centrifuge, SS–34 head; 17500 rev./min.). Then 15 ml. of the supernatant was subjected to gel filtration on a column (4·5 cm. diam. × 55 cm. long) of Sephadex G-25 with 0·05 M-phosphate buffer as eluent. The fractions containing the protein were pooled and used as the source of enzymes.

(*b*) Metal ion-requirement studies. The protein obtained as described in section (*a*) was treated further by incubating it with 0·1 μmole of EDTA/mg. of protein at 25° for 1 hr. At the end of this incubation period the reaction mixture was subjected to an additional gel filtration, identical with that previously employed. The protein fractions were the source of enzymes.

(*c*) Organ location studies. The organs utilized in this series of investigations were the liver, brain, muscle (pectoralis), kidney and small intestine of the rat. The segment of small intestine was obtained by cutting at a point 2 cm. distal to the pyloric valve and again at a point 10 cm. distal to the first cut. Each system was prepared for assay as described by Langer (1964).

(*d*) Intracellular distribution studies. The procedures used for homogenation and centrifugal fractionation of the liver cells were as described by Hogeboom (1955).

(*e*) Other studies. The other studies dealing with the relationship of enzyme activity to enzyme concentration and the effects of anaerobic atmospheric conditions utilized, as a source of enzyme, livers that had been prepared as in section (*a*) except that the gel-filtration step was eliminated.

Reaction mixture. The reaction mixture generally used consisted of DL-$(MHA)_2Ca$ (5 μmoles), FAD (1 μmole) and L-glutamine (10 μmoles) dissolved in 2 ml. of 0·05 M-tris–maleate buffer, pH 7·2. This buffer was used because a precipitate was formed if more than 1 ml. of 0·05 M-phosphate was present in the reaction mixture. As this precipitate was noted when 5 μmoles of Ca^{2+} were added to 1 ml. of 0·05 M-phosphate buffer, it was assumed that the precipitate was calcium phosphate. A 1 ml. portion of enzyme solution (in 0·05 M-phosphate buffer) containing about 5 mg. of protein completed the reaction mixture. In all studies this 2:1 ratio of buffers was maintained.

All experiments were conducted at 37° for 3 hr. in air,

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† Abbreviation: MHA, 2-hydroxy-4-methylthiobutyric acid, the α-hydroxy analogue of methionine.

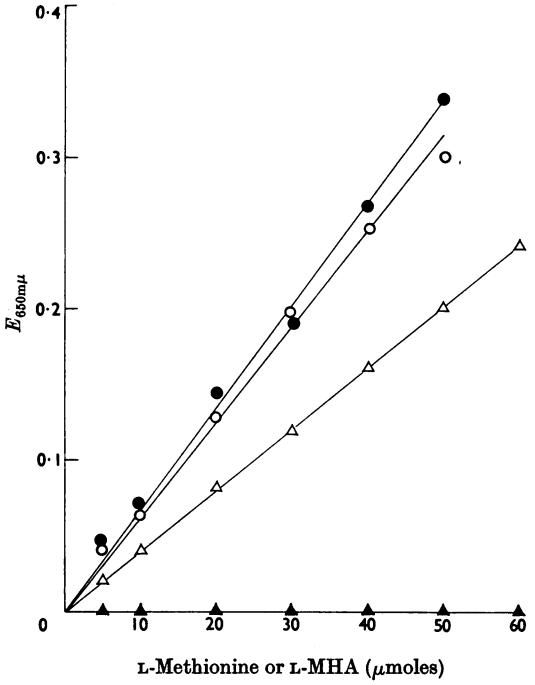


Fig. 1. Growth response of *L. mesenteroides* to L-methionine (△), L-MHA (▲), L-methionine (●), and L-methionine+ 100 μmoles of DL-MHA (○). Two separate experiments (▲, △, and ●, ○) are presented in this Figure.

with the exception of the anaerobic experiments, which were incubated under nitrogen.

Protein determination. The protein content of the enzyme preparations was determined spectrophotometrically by the procedure of Kalckar (1947).

Methionine determination. At the end of the incubation period, the reaction vessels were autoclaved for 5 min. at 120°. The coagulated protein was removed by centrifugation and portions of the supernatant were assayed for methionine with *Leuconostoc mesenteroides* P-60 (A.T.C.C. 8042), as described by Langer & Kratzer (1964). This micro-organism does not respond to MHA and the presence of MHA does not interfere in the assay for methionine (Fig. 1). The difference in methionine content between the boiled zero-time controls and the experimental tubes was considered to be the net amount of methionine synthesized by the system.

RESULTS

The results of the cofactor study (Table 1) show that the system responsible for the conversion of MHA into methionine requires a flavine as an oxidizing agent and glutamine as the source of the amino group. FAD could be replaced by FMN, but the replacement with NAD or NADP only partially restored activity. The replacement of glutamine by glutamate, aspartate or ammonium sulphate did not restore activity, but asparagine could partially replace glutamine. Generally, the inclusion of pyridoxal phosphate did not improve the utilization of the amino donors with the possible exception of the utilization of asparagine.

The experiments on the metal ion requirement of

Table 1. *Cofactor requirements for the conversion of MHA into methionine by rat-liver preparations*

The preparation of the liver involved homogenation, centrifugation and gel filtration. Details are given in the Materials and Methods section. Each value is the average ± S.E.M. of two experiments, each experiment being conducted in duplicate. The specific activity of the complete system is 31·2 ± 0·3 mμmoles of methionine/mg. of protein/hr. The total synthesis of the complete system is 618 ± 242 mμmoles of methionine in 3 hr. The large variations are due to differences in protein content in each experiment.

Constituents	Activity (% of control)
Complete system [5 μmoles of DL-$(MHA)_2Ca$, 1 μmole of FAD, 10 μmoles of glutamine]	(100·0)
Complete system with boiled enzyme	5·3 ± 1·3
Complete system − MHA	2·9 ± 0·8
Complete system − FAD	52·5 ± 6·8
Complete system − glutamine	5·2 ± 0·6
Complete system + pyridoxal phosphate (1 μmole)	96·9 ± 3·0
Complete system − FAD + FMN	93·7 ± 1·5
Complete system − FAD + NAD (1 μmole)	73·5 ± 4·5
Complete system − FAD + NADP (1 μmole)	79·3 ± 0·3
Complete system − glutamine + glutamate	4·4 ± 0·7
Complete system − glutamine + glutamate + pyridoxal phosphate	7·4 ± 0·1
Complete system − glutamine + aspartate	5·9 ± 1·5
Complete system − glutamine + aspartate + pyridoxal phosphate	7·5 ± 1·0
Complete system − glutamine + asparagine	16·5 ± 1·8
Complete system − glutamine + asparagine + pyridoxal phosphate	21·0 ± 1·0
Complete system − glutamine + $(NH_4)_2SO_4$	5·5 ± 2·8
Complete system − glutamine + $(NH_4)_2SO_4$ + pyridoxal phosphate	5·2 ± 2·3

Table 2. *Metal ion requirement for the conversion of MHA into methionine by rat-liver preparations*

The preparation of the liver involved homogenation, centrifugation, gel filtration, treatment with EDTA and a second gel filtration. Details are given in the Materials and Methods section. Each value is the average ± S.E.M. of two experiments, each experiment being conducted in duplicate. The specific activity of the complete system treated with EDTA is 38·4 ± 1·6 mμmoles of methionine/mg. of protein/hr. (that of the complete system not treated with EDTA is 33·5 mμmoles/mg./hr.). The total synthesis of the treated complete system is 409 ± 11 mμmoles of methionine in 3 hr.

Constituents	Activity (% of control)
Complete system (treated with EDTA) [5 μmoles of DL-$(MHA)_2Ca$, 1 μmole of FAD, 10 μmoles of glutamine]	(100·0)
Complete system with boiled enzyme	5·6 ± 0·4
Complete system + Zn^{2+} (1 μmole)	97·5 ± 2·0
Complete system + Mg^{2+} (1 μmole)	99·8 ± 2·3
Complete system + Mn^{2+} (1 μmole)	86·6 ± 3·1
Complete system + MoO_4^{2-} (1 μmole)	99·9 ± 3·4
Complete system + Fe^{2+} (1 μmole)	96·8 ± 6·0
Complete system + Fe^{3+} (1 μmole)	94·4 ± 1·2
Complete system + Cu^{2+} (1 μmole)	32·2 ± 1·1
Complete system + Co^{2+} (1 μmole)	95·3 ± 1·3

Table 3. *Tissue location of the system that converts MHA into methionine*

The details for the preparation of the tissue for assay are completely described in section (*c*) of the first part of the Materials and Methods section and by Langer (1964). Each value is the average ± S.E.M. of two experiments, each experiment being conducted in duplicate.

Tissue	Net synthesis of methionine (mμmoles/mg. of protein/hr.)	Net synthesis of methionine (mμmoles/g. of tissue/hr.)
Kidney	104·3 ± 7·8	10680 ± 1010
Liver	47·4 ± 14·3	4220 ± 370
Small intestine	7·8 ± 3·9	724 ± 137
Brain	3·3 ± 0·6	104 ± 80
Muscle	1·1 ± 0·3	51 ± 34

the system (Table 2) clearly indicate that none of the metal ions tested was required. On the contrary, Mn^{2+} appeared slightly inhibitory, and Cu^{2+} was severely inhibitory. Treatment of the enzyme preparation with EDTA had no deleterious effect on activity (Table 2).

Of the kidney, liver, small intestine, brain and muscle, the kidney had the highest amount of activity/g. of tissue, with the others, as listed, possessing a decreasing amount of activity (Table 3). Fractionation of the liver-cell components indicates that the enzyme system responsible for the conversion of MHA into methionine is located primarily in the supernatant fraction (Table 4), with some activity in other cell fractions. The amount of enzyme activity was a linear function of protein concentration.

Incubation of the experimental vessels in a nitrogen atmosphere resulted in an activity that was only 56·7 ± 6·5% of that of aerobically incubated control vessels, indicating that the system is aerobic.

Table 4. *Liver intracellular location of the system that converts MHA into methionine*

The procedures used for the enzyme preparation were those described by Hogeboom (1955). Each value is the average ± S.E.M. of two experiments, each experiment being conducted in duplicate.

Cell fraction	Net synthesis of methionine (mμmoles/mg. of protein/hr.)
Complete homogenate	24·6 ± 8·7
Nuclei + cell debris	9·9 ± 1·6
Mitochondrial fraction	10·4 ± 4·8
Microsomal fraction	4·1 ± 0·7
Supernatant	34·4 ± 9·2

DISCUSSION

Cofactor study. As has been demonstrated previously (Gordon, Maddy, Jenkins & Sizer, 1954; R. S. Gordon, personal communication), MHA is

readily converted into the α-oxo analogue of methionine, which is, in turn, aminated to form methionine. This is supported by our observations that there is a requirement for a flavine (FAD or FMN) and oxygen, as well as glutamine. The glutamine requirement has been indicated previously (R. S. Gordon, personal communication) for the rat. However, the flavine requirement has not been noted. An examination of the flavine-containing enzymes reveals that the amino acid oxidases do possess the ability to convert α-hydroxy acids into their α-oxo analogues (Blanchard, Green, Nociti-Carroll & Ratner, 1945, 1946), indicating the possibility of a role for these enzymes in the conversion of MHA into methionine.

The partial restoration of the activity of the system by NAD and NADP indicates the possibility of a second oxidative pathway that requires these cofactors. An example of a system that oxidizes an α-hydroxy acid to its α-oxo analogue is lactate dehydrogenase.

The utilization of glutamine and asparagine as amino donors in the formation of methionine from 4-methylthio-2-oxobutyric acid in rat-liver preparations has been shown by Meister & Tice (1950) and Meister, Sober, Tice & Fraser (1952). The present results clearly indicate a requirement for glutamine that can partially be replaced by asparagine. Glutamic acid, aspartic acid and ammonium sulphate did not replace the glutamine as an amino donor, thereby confirming the observations of Meister & Tice (1950) and Meister *et al.* (1952), in which it was shown that glutamate and aspartate were poor amino-group sources for the amination of the α-oxo analogue of methionine.

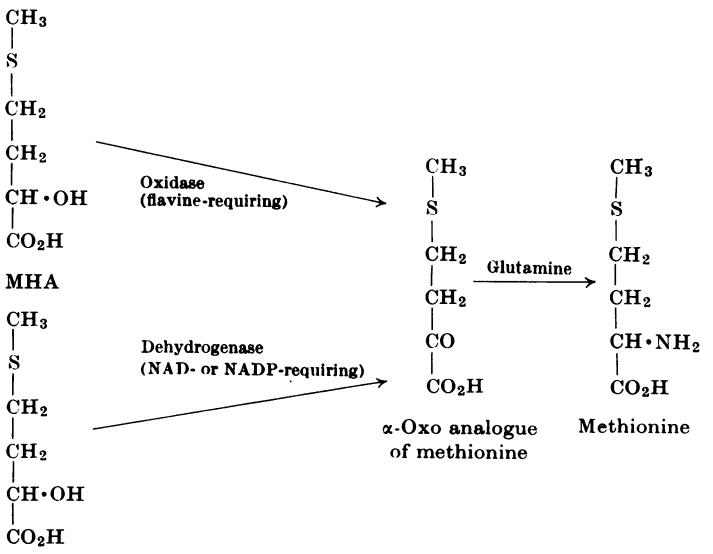
Pyridoxal phosphate did not significantly affect the utilization of glutamine, glutamate, aspartate or ammonium sulphate, and therefore appears not to be required by the system. Although it did improve the utilization of asparagine slightly, the improvement was not enough to allow replacement of the glutamine by asparagine and pyridoxal phosphate. The role, if any, of pyridoxal phosphate in the utilization of asparagine is not understood, and Meister *et al.* (1952) and Meister & Tice (1950) did not note any need for it in the donation of an amino group by asparagine or glutamine.

Metal ion requirement. The ions Zn^{2+}, Mg^{2+}, MoO_4^{2-}, Fe^{2+}, Fe^{3+} and Co^{2+} have no significant effect on the conversion of MHA into methionine; Mn^{2+} appears to be slightly inhibitory, and Cu^{2+} severely inhibits the conversion.

Organ and intracellular location. Though the kidney contains the greatest amount of activity/g. of tissue, the liver, because of its much greater weight, must play a predominant role in converting MHA into methionine in the intact animal.

The supernatant fraction, obtained by differential centrifugation of disrupted liver cells, contains the greatest amount of conversion ability. The other cell fractions did, however, possess some activity, but this was considerably less than that found in the supernatant.

From the observations presented above, it is



Scheme 1. Postulated pathway for the conversion of MHA into methionine.

postulated that the conversion of MHA into methionine takes place in the manner illustrated in Scheme 1.

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