Effect of Methionine upon Utilization of DL-Homocystine by *Brucella suis* (19744)

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Although homocyst(e)ine has not been isolated from biologic systems it is accorded an intermediate position in the present concept of the L-cysteine ⇄ L-methionine transformation(1). Available evidence indicates the participation of both these latter amino acids in protein synthesis. Hence, any organism capable of utilizing either as its sole source of sulfur is assumed to be capable of converting this compound to the other according to its needs. If such an assumption is correct, it seems reasonable that such an organism would be equally capable of utilizing an exogenous supply of the common intermediates, provided these encountered no permeability barrier in passage to the centers of cell metabolism. Conversely, the failure of a compound, designated as an intermediate, to serve as the sulfur source for this organism would cast suspicion upon its projected role in the transformation(2). It has been observed that certain Brucellae which utilize either L-cyst(e)ine or L-methionine alone, under conditions which do not permit utilization of DL-homocyst(e)ine, are "stimulated" to utilize the latter in the presence of trace amounts of methionine.

Experimental results. *Brucella suis* utilizes either L-cystine, L-cystathionine, or L-methionine as sole source of sulfur in a medium containing mineral salts, essential vitamins, L-asparagine, and lactate(3). Lactate plays an important, although undefined, role in the sulfur metabolism of these organisms, since its omission from the asparagine medium creates a requirement for cyst(e)ine. The asparagine and lactate components of the medium may, however, be replaced by an oxidized casein hydrolysate(3) without altering the capacity of *B. suis* to utilize L- or DL-methionine. Since growth was somewhat more rapid in the latter medium, it was substituted for the asparagine-lactate medium in certain phases of this study.

The experimental procedures and the sources of the *B. suis* cultures have been described previously(3). Each sulfur compound was added to the basal medium in a freshly-prepared, filter-sterilized solution. Its concentration in the medium is expressed in terms of micrograms or millimicrograms of sulfur per ml. The growth response of standardized inocula (100 to 200 thousand viable cells per ml) was determined turbidimetrically after 3

TABLE I. Relation of Methionine Contamination of DL-Homocyst(e)ine to Utilization by *Brucella suis*.

Homocyst(e)ine*		Estimated methionine content (% by) Microbiological assay†	Chromatography	Homocyst(e)ine required for growth of *B. suis* 1722‡ (μg sulfur/ml)
DL-Homocystine	Sample 1	5–10	5–10	2(±)
	" 2	5–10	5–10	2(±)
	" 3	<.1	0§	>10,<25
DL-Homocysteine		.25	±‖	>5
DL-Homocysteine thiolactone		.35		>5

* The DL-homocystine and the DL-homocysteine were commercial samples. The DL-homocysteine thiolactone was kindly supplied by Dr. R. P. Wagner, Department of Zoology, University of Texas.

† Based on assay with methionine-dependent strain *Brucella melitensis* 2460.

‡ When the homocyst(e)ine was supplied as the sole sulfur source.

§ No methionine spot detected.

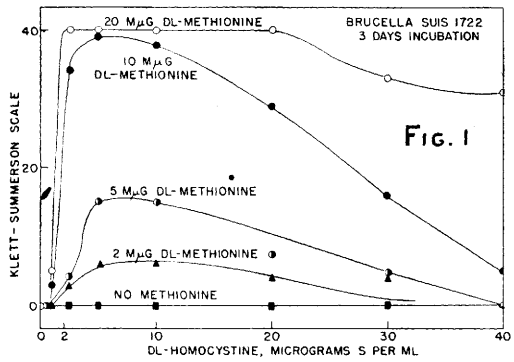
‖ Doubtful methionine spot.

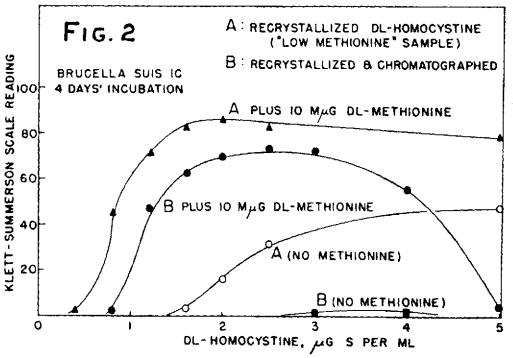
to 5 days incubation at 37°C.

Initial investigations of the availability of DL-homocystine* for 10 strains of *B. suis* yielded inconsistent results which were related to the different commercial samples under test. By paper chromatography, and by assay with a strain of *Brucella melitensis* which has an absolute requirement for methionine, a direct correlation was established between methionine content of the DL-homocystine samples and their utilization by *B. suis* 1722 (Table I). After recrystallization several times sample No. 3 yielded a product which, in the absence of added methionine, supported no growth of the test strain after 3 days' incubation with amounts up to 40 μg DL-homocystine sulfur per ml (Fig. 1). Other more sensitive strains, as *B. suis* 1C, required more rigid purification of homocystine in order to eliminate appreciable growth response in the absence of the methionine supplement (Fig. 2). Although DL-homocysteine and the thiolactone were not subjected to purification, their activity was no greater than DL-homocystine of comparable methionine content.

When 10 millimicrograms of DL-methionine per ml were added to the medium containing purified DL-homocystine, a striking growth response ensued (Fig. 1). This effect was detectable with as little as 1 millimicrogram of methionine sulfur, although for maximum response in the presence of 2 to 5 μg of DL-homocystine sulfur, 10 to 20 millimicrograms of methionine sulfur per ml were required. Although utilization of the impure preparations of DL-homocysteine and its thiolactone was stimulated by a supplement of 10 millimicrograms of methionine, utilization of lower concentrations was somewhat inferior to that obtained with DL-homocystine under comparable conditions.

The growth response to homocystine was roughly proportional to the concentration of methionine within the range from 1 to 10 millimicrograms (Fig. 3). It is significant that other known biological effects of amino acids can be detected only in quantities 10 to 100 times greater.





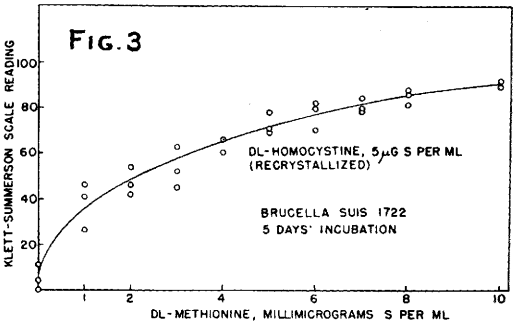


FIG. 1. Effect of methionine concentration on utilization and zonal toxicity of DL-homocystine. Tested in .1% oxidized casein hydrolysate medium.

FIG. 2. Effect of purification of DL-homocystine, and of added methionine, on growth in .1% oxidized casein hydrolysate medium.

FIG. 3. Relation of methionine concentration to utilization of DL-homocystine. Basal medium is .1% oxidized casein hydrolysate. 10 mμg DL-methionine alone produced no visible growth.

One of the unusual aspects of the growth response to DL-homocystine and DL-homocysteine in the presence of the methionine supplement was the form of the dose response curves. Relatively high concentrations of homocyst(e)ine were required before growth

* Commercial preparations, probably diasteroisomeric mixtures approximating 25% D-D, 25% L-L, and 50% D-L.

was initiated, and the subsequent response seldom was in direct proportion to increased concentrations of homocyst(e)ine. In some experiments it appeared as though there was a threshold concentration of homocystine below which it was not utilized, and above which assimilation was complete (Fig. 1). Although higher concentrations of methionine increased the sensitivity of response to homocystine, even an amount sufficient to produce detectable growth when used alone (about 20 millimicrograms per ml) failed to alter the sigmoidal character of the homocystine response curve. Moreover, at low concentrations of methionine the response curve indicates a zonal utilization of homocystine. If the concentration of homocystine exceeded that which produced the maximum response, a "toxic" effect became evident. This inhibition could be reversed by increasing the methionine concentration (Fig. 1). Riesen and colleagues (4) encountered a similar response of *Lactobacillus pentosus* to DL-homocystine.

D-, DL-, and L-methionine have been found equally effective in promoting utilization of homocystine in the asparagine-lactate medium. In the oxidized casein hydrolysate medium, however, D-methionine was inactive. By the addition of supplements of peroxide-treated casein hydrolysate to the asparagine-lactate medium it was demonstrated that the former inhibits utilization of D-methionine by *B. suis* 1722 and 1C. This inhibition can be reproduced with 100 μg of DL-methionine sulfone. From quantitative considerations, however, this sulfone cannot be considered as the only, or even the most potent, inhibitory substance in peroxide-treated casein hydrolysate. It appears likely that the oxidized casein inhibits conversion of D- to L-methionine, since *B. melitensis* 2460 utilized only DL- or L-methionine in its presence, or in the presence of DL-methionine sulfone. In the asparagine-lactate medium, however, the utilization of D-methionine by this organism is equal to or slightly superior to the utilization of L-methionine. Somewhat similar observations on the inhibitory effect of oxidized casein and methionine sulfone upon D-methionine assimilation have been reported for *Lactobacillus arabinosus* by Camien and Dunn(5).

The availability of DL-homocystine as a sulfur source in the asparagine medium was found to be dependent upon both the lactate and the trace methionine supplements. If either is omitted growth fails to occur. Oxidized casein hydrolysate, glutamic acid, and pyruvic acid substituted for lactate in promoting the utilization of homocystine to the same degree that they stimulated utilization of L-cystathionine and methionine. These results are consistent with the previous suggestion that lactate is linked specifically to a reaction transforming L-cystathionine to L-cysteine (3).

Another mechanism enabling *B. suis* to utilize DL-homocystine has been observed. Occasionally cultures incubated with recrystallized DL-homocystine produced delayed growth after 8 to 14 days. Transplants from such cultures utilize DL-homocystine promptly in the absence of methionine, although the growth shows the characteristic concentration-response lag of the "unadapted" culture (Fig. 4). This acquired capacity to utilize homocystine was retained during transfer in the absence of homocystine. It appears probable that a mutant capable of prompt utilization of homocystine was selected in the absence of methionine. On the other hand, transfers from cultures containing homocystine and as much as 8 millimicrograms methionine sulfur were no more efficient in utilizing methionine-free homocystine than was the parent culture. When the concentration of methionine in the original culture was reduced to 1 to 2 millimicrograms per ml the resulting growth contained a mixture of unadapted and mutant cells. Apparently the rate and quantity of growth in the original culture, as determined by its methionine content, is the principal factor operating for or against selection of the mutants capable of utilizing homocystine.

Consideration has been given to several possible explanations of the failure of purified DL-homocystine to function in the metabolism of *B. suis*. For example, inability to reduce the disulfide linkage, as observed with certain *Neurospora* and *Bacillus subtilis*(6,7) mutants, apparently cannot account for its inactivity for *B. suis*, since it is utilized no less efficiently than DL-homocysteine at compara-

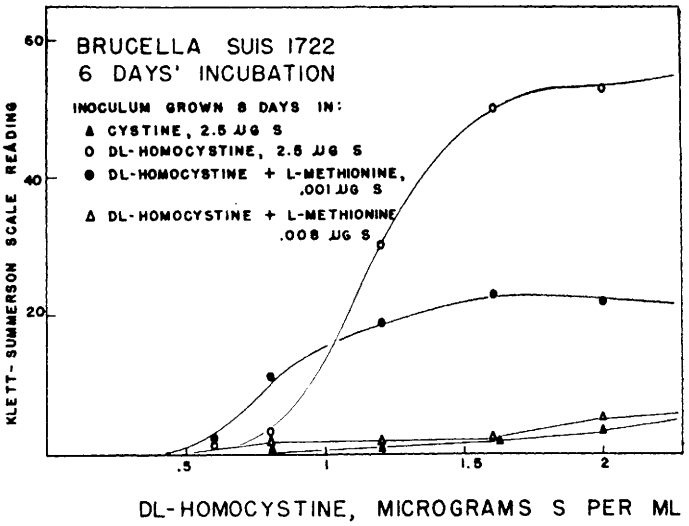


FIG. 4. Effect of inoculum source on utilization of DL-homocystine in a basal medium containing .1% L-asparagine and .01% lactic acid.

ble concentrations of methionine. Although membrane impermeability to pure homocystine cannot be ruled out, this explanation would require the difficult assumptions of an extreme degree of membrane selectivity for the next lower homologue, cystine, and the elimination of this selectivity by a minute amount of methionine. Moreover, it may be noted that homocystine utilization was not promoted by varying the pH of the medium over the range from 6.3 to 8.3, within which the response to cystine remain unaltered.

The possibility has been considered that the utilization of DL-homocystine is dependent upon an adaptive enzyme which requires a trace of preformed methionine for its synthesis. It is difficult, however, to reconcile this explanation with the prompt utilization of methionine-free L-cystine, which is assumed to pass through the homocysteine intermediate in its conversion to methionine. The same objection could be raised to the possible role of "active methionine"(8,9), functioning in a catalytic manner to convert homocyst(e)ine to methionine, thereby generating a continuous supply of methionine. It might be supposed that an "active homocyst(e)ine" is involved in the cysteine $\rightleftarrows$ methionine transformation effected by *B. suis*. Thus the mutant *B. suis* may possess an enzyme system for transforming DL-homocystine to the active form, whereas this mechanism is deficient or lacking in the wild type. This hypothesis has the merit of offering a possible explanation for the zonal toxicity of DL-homocystine in the presence of suboptimal concentrations of methionine, since it might be expected that an excess of DL-homocystine would compete with the "active intermediate" being formed slowly at low methionine concentration. However, it is also possible that the L-L form is the only isomer used in the diastereoisomeric complex, and that the other unnatural isomers (D-D and D-L) complete with the L-L form for essential enzyme systems. This inhibition might then be reversed by methionine, particularly within the "toxic" range of homocystine concentration.

Regardless of the explanation of the stimulatory effect of methionine upon utilization of homocystine by *B. suis*, it may be significant that other workers(10,11) have found that the amount of homocyst(e)ine required to produce maximum growth responses of different microorganisms exceeds by several fold the quantity of methionine or cystine required. It is possible that the excess was required to introduce sufficient methionine contamination to provide the stimulation necessary for efficient utilization of homocyst(e)ine. We have observed, for example, that the "methionineless" *Escherichia coli* mutant 58-161(11) grew

more promptly and at lower concentrations of "purified" DL-homocystine in the presence of 10 millimicrograms of methionine, although some delayed growth occurred in its absence. Certainly, conclusions relative to the metabolic utilization of homocystine by microorganisms must take into account the stimulatory effect of traces of methionine.

Summary. *Brucella suis* utilized either L-cystine, L-cystathionine, or L- or D-methionine as sole sulfur source in an asparagine-lactate medium. DL-homocystine, DL-homocysteine, and DL-homocysteine thiolactone also were utilized under the same conditions, but only to a degree related to their methionine content. Purified preparations of DL-homocystine were not utilized except in the presence of small quantities of supplementary methionine. D-, L-, and DL-methionine were equally effective in promoting utilization of DL-homocystine, and their effect increased to a maximum over a range from 1 to 20 millimicrograms of methionine sulfur per ml. Oxidized casein hydrolysate and methionine sulfone inhibited the activity of D-methionine. The mode of action of methionine and the possible significance of these observations are considered from the standpoint of the intermediacy of homocyst(e)ine in sulfur transformations.

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Received June 30, 1952. P.S.E.B.M., 1952, v80.

Immunization of Mice with Inactivated Type 2 Poliomyelitis Virus Against Types 2 and 3 Viruses. (19745)

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Homotypic immunization of mice with inactivated suspensions of Type 2 (Lansing) poliomyelitis virus has been reported(1-3). The amount of vaccine needed for definite protection of a mouse was derived from about 40 mg, or more, of infected central nervous system (CNS) tissue; Schwerdt *et al.*(4) however, immunized cotton rats with less material, about 10 mg (Loring *et al.*(5)).

Shortly after the MEF1 strain, Type 2, of poliomyelitis virus had been successfully adapted to newborn mice(6,7), and after it was found that sera from persons having Type 1 (Brunhilde) virus infection but no past or concurrent infection with Type 2 virus reacted with a complement-fixing antigen prepared with the adapted MEF1 strain(8), the present studies were begun. They concerned the possibility of an increased immunizing power developed by the newborn-mouse adapted MEF1 virus and of cross-protection after vaccination with it against types other than Type 2. In the studies reported here, therefore, vaccines were prepared using both the newborn-mouse adapted MEF1 strain and the standard, unadapted, virus. The MEF1 standard (Type 2) and the Leon (Type 3) were used as challenge agents. The reason for selecting Type 3 as the heterotypic agent was that of expediency, namely, the Leon strain can be propagated in mice by the intraspinal

* Dr. Brown's work was aided by a Fellowship from The National Foundation for Infantile Paralysis.