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Identification of L-Methionine-*S*-Sulfoximine as the Convulsant Isomer of Methionine Sulfoximine*

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Abstract. The convulsant agent methionine sulfoximine inhibits brain glutamine synthetase irreversibly and the inhibitor becomes bound to the active site of the enzyme as methionine sulfoximine phosphate. Only one of the four isomers of methionine sulfoximine, L-methionine-*S*-sulfoximine, inhibits glutamine synthetase. In the present work, D-methionine-*SR*-sulfoximine, and highly purified preparations of L-methionine-*S*-sulfoximine and L-methionine-*R*-sulfoximine were tested in mice for convulsant activity; only L-methionine-*S*-sulfoximine produced convulsions. The finding that only one of the four optical isomers of methionine sulfoximine induces convulsions, and that only this same isomer inhibits glutamine synthetase, lends support to the conclusion that these two effects of methionine sulfoximine are closely connected.

It has been known for some time that administration of methionine sulfoximine produces convulsions in a number of animal species.[1–12] The ability of methionine sulfoximine to inhibit glutamine synthetase, first reported in 1952,[13] has been amply confirmed and there has been interest in the possibility that inhibition of brain glutamine synthetase by methionine sulfoximine is related to its convulsant action.[14–32] Although plausible hypotheses have been put forth, it is still not clear as to exactly how these phenomena are connected; indeed, it has been suggested that they may not be directly related,[18,26,27] and, therefore that the convulsant action of methionine sulfoximine might be mediated through its effect on another system or perhaps by a metabolite of methionine sulfoximine.

In the course of work in this laboratory on the mechanism of inhibition of glutamine synthetase by methionine sulfoximine, it was found that this amino acid becomes bound to the active sites of the enzyme as methionine sulfoximine phosphate and thereby irreversibly inhibits the enzyme.[28,30,31] Subsequent study revealed that only one of the four isomers of methionine sulfoximine (the L-*S* isomer) inhibits the enzyme and is phosphorylated.[32] This finding has led us to inquire as to whether a similar or a different specificity exists in relation to the convulsant action of methionine sulfoximine. In the present work, D-methionine *SR*-sulfoximine and highly purified preparations of L-methionine-*S*-sulfoximine and L-methionine-*R*-sulfoximine were tested in mice for convulsant activity; their effects on the glutamine synthetase activity of the brain and liver were also studied. We conclude that the same isomer (L-methionine-*S*-sulfoximine)

that inhibits glutamine synthetase is also responsible for the convulsant effect of this amino acid.

Materials. L-Methionine-*SR*-sulfoximine and the corresponding D-form were obtained as described.[31] (+)-Camphor-10-sulfonic acid was obtained from Eastman Kodak Co., and recrystallized from glacial acetic acid. Glutamine synthetase was isolated from sheep brain.[33] Mice (male; 15–25 gm) of the AH/J and C 57 black strains were used.

Preparation of the *S*- and *R*-diastereoisomers of L-methionine sulfoximine: The procedure used is based on that of Christensen *et al.*[34] L-Methionine-*SR*-sulfoximine (10 mmoles) and (+)-camphor-10-sulfonic acid (20.2 mmoles) were dissolved in 50 ml of hot *n*-propanol. The solution was filtered and allowed to stand at 24°C for 18 hr. A few seed crystals of the camphor sulfonate salt of L-methionine-*R*-sulfoximine were added and the solution was placed at 4°C for 24–48 hr. The crystals which formed were filtered off and dissolved in about 20 ml of hot *n*-propanol. (After recrystallization the camphor sulfonate salt of L-methionine-*R*-sulfoximine was worked up as described below.)

The filtrate was reduced to 25 ml by evaporation *in vacuo* and then placed at −15°C for several days; any crystals which formed during this time were filtered off and discarded. The clear solution was evaporated to dryness *in vacuo* and the residue was dissolved in 15 ml of 0.01 *N* HCl. This solution was placed on a column (10 × 2.5 cm) of Dowex 50 (H^+) and 2 col. volumes of water were added to remove the camphor sulfonic acid. The free amino acid was eluted with 2 *N* NH_4OH; the eluate was evaporated to dryness *in vacuo*, and the residue was dissolved in the minimum volume of warm water. Two volumes of ethanol were added and the solution was placed at 4°C for several hours. The crystalline free amino acid thus obtained was recrystallized from ethanol-water. This preparation of L-methionine-*S*-sulfoximine was subjected to two additional camphor sulfonic acid fractionations which were performed as described above.

The recrystallized camphor sulfonate salt of L-methionine-*R*-sulfoximine obtained as described above, was converted to the free amino acid as described for the *S*-isomer; it was found to contain about 8% of the *S*-isomer as determined by the enzymatic method described below. After this material was subjected to two additional purification cycles, a product containing only about 0.5% of the *S*-isomer was obtained.

Determination of the purity of L-methionine-*R*-sulfoximine: A sample of the preparation of L-methionine-*R*-sulfoximine to be tested (0.02 to 10 mM; final concentration) was preincubated at 37°C in a reaction mixture (final volume, 0.1 ml) containing glutamine synthetase (5 μg), ATP (10 mM), $MgCl_2$ (20 mM), 2-mercaptoethanol (2.5 mM), and imidazole-HCl buffer (50 mM; pH 7.2). A control without methionine sulfoximine was also tested. After 15 min of preincubation, 0.3 ml of a solution containing Na glutamate (50 μmoles), imidazole-HCl buffer (50 μmoles; pH 7.2), NH_2OH (100 μmoles), ATP (10 μmoles), and 2-mercaptoethanol (25 μmoles) were added together with sufficient water to bring the final volume to 1.0 ml. After incubation for 15 min at 37°C, 1.5 ml of ferric chloride reagent was added and the formation of γ-glutamyl-hydroxamate was determined from the absorbance at 535 mμ.[33] The amount of L-methionine-*S*-sulfoximine in a sample of methionine sulfoximine was calculated from the observed inhibition and a reference inhibition curve. The reference inhibition curve was constructed by performing a series of experiments in which 0.02–0.20 mM L-methionine-*SR*-sulfoximine was added to the preincubation mixture. The reciprocals of the activity values obtained were plotted against the L-methionine-*SR*-sulfoximine concentration; the curve was linear over the range 0.01–0.2 mM. In this calculation, it was assumed that L-methionine-*SR*-sulfoximine consists of an equal mixture of both diastereoisomers. This assumption is consistent with earlier data in which inhibition by L-methionine-*S*-sulfoximine and L-methionine-*SR*-sulfoximine were compared.[32] Application of the method described above to the best preparation of L-methionine-*R*-sulfoximine indicated a purity of 99.5%. This material was used in the experiments described in Table 2.

Methods. Amino acids were injected intraperitoneally in dilute solution in Earle's balanced salt solution. After 2 to 6 hr, the animals were decapitated and the brain

and liver were immediately excised. The tissues were homogenized with a Teflon pestle homogenizer in ice-cold 5 mM potassium phosphate buffer (pH 7.2) containing 0.1 *M* KCl. A volume of 2.5 ml of buffer was used for each brain; 5 ml of buffer were used for each liver. The homogenates were centrifuged at 30,000 *g* at 4°C for 30 min and the supernatant solutions obtained were used for determination of glutamine synthetase by the γ-glutamylhydroxamate method;[33] blanks in which ATP was omitted were employed. Protein was determined as described by Lowry *et al.*[35]

Results. In the studies summarized in Table 1 mice of the AH/J strain were injected with L-methionine, D-methionine-*SR*-sulfoximine, and the separate *S*- and *R*-diastereoisomers of L-methionine sulfoximine. The most significant ob-

TABLE 1. *Glutamine synthetase activity of the brain and liver of mice after injection of methionine sulfoximine isomers.*

Expt.	Amino acid injected (dose, mmoles/kg)	Behavior	Glutamine Synthetase Activity* Brain (units/mg protein)	Liver (units/mg protein)
1	L-Methionine (1.0) (control)	Normal	0.94	0.45
2	D-Methionine-*SR*-sulfoximine (1.0)	Normal	1.04	0.32
3	L-Methionine-*R*-sulfoximine/† (1.0)	Normal	0.47	0.04
4	L-Methionine-*S*-sulfoximine (1.0)	Convulsions (140 min)	0.10	0.03
5	L-Methionine-*S*-sulfoximine (0.1)	Irritable (240 min)	0.47	0.04
6	L-Methionine-*S*-sulfoximine (0.05)	Normal	0.48	0.05

* Enzyme activity was determined 6 hr after injection on the pooled tissues of two mice (Strain AH/J) and is expressed as units/mg of protein.

† This preparation contained 96% of the L-*R* isomer and 4% of the L-*S* isomer.

servation was that L-methionine-*S*-sulfoximine (expt. 4) produced convulsions while L-methionine-*R*-sulfoximine (expt. 3) did not. In other experiments involving 30 mice, injection of L-methionine-*SR*-sulfoximine and L-methionine-*S*-sulfoximine (1 mmole/kg) invariably produced convulsions, while the same dose of L-methionine-*R*-sulfoximine did not produce convulsions. In the experiments described in Table 1 there was a marked decrease of liver and brain glutamine synthetase activity after injection of L-methionine-*S*-sulfoximine. Although L-methionine-*R*-sulfoximine did not exhibit convulsant activity, it produced a substantial decrease of liver glutamine synthetase activity and some decrease of the brain activity. The preparation of L-methionine-*R*-sulfoximine used in these studies was known to contain about 4% of the *S*-isomer. It is, therefore, pertinent to note that injection of close to this amount of *S*-isomer (expt. 6) led to levels of enzyme activity that are similar to those found after injection of the L-methionine-*R*-sulfoximine preparation (expt. 3).

Similar experiments in which more highly purified (99.5%) L-methionine-*R*-sulfoximine was used are described in Table 2. Again, only the L-*S* isomer (expt. 3) produced convulsions. In these studies injection of the L-*R* isomer (expt. 2) produced no effect on brain glutamine synthetase; the moderate decrease in brain activity observed in experiment 3 (Table 1) may very probably be ascribed to the higher content of *S*-isomer of the L-methionine-*R*-sulfoximine used in that study. It is of interest that injection of 1.0 mmole/kg of 99.5% pure L-methionine-*R*-sulfoximine (Table 2, expt. 2) led to markedly decreased

TABLE 2. *Glutamine synthetase activity of the brain and liver of mice after injection of* L-*methionine sulfoximine diastereoisomers.*

Expt.	Amino acid injected (dose, mmoles/kg)	Behavior	Glutamine Synthetase Activity* Brain (units/mg protein)	Liver (units/mg protein)
1	L-Methionine (1.0) (control)	Normal	0.59, 0.64	0.36, 0.46
2	L-Methionine-*R*-sulfoximine† (1.0)	Normal	0.53, 0.60	0.04, 0.08
3	L-Methionine-*S*-sulfoximine (1.0)	Convulsions‡ (150 min)	0.05, 0.07	0.004, 0.006
4	L-Methionine-*S*-sulfoximine (0.005)	Normal	0.59, 0.68	0.07, 0.18
5	L-Methionine-*R*-sulfoximine (0.005)	Normal	0.59, 0.75	0.48, 0.53

* Each value represents a determination of the pooled tissues of two mice (Strain AH/J) performed 5½ hr after injection.

† This preparation contained 99.5% of the L-*R* isomer and 0.5% of the L-*S* isomer.

‡ The determinations of activity were made 210 min after injection. Convulsions typically occur 4–5 hr after injection of 1.0 mM/kg of L-methionine-*SR*-sulfoximine.

liver glutamine synthetase. Presumably this effect reflects the small amount of *S*-isomer in the preparation; thus, when a dose of 0.005 mmole/kg of L-methionine-*S*-sulfoximine was given (Table 2, expt. 4), the liver activity was similarly affected. In these studies, the liver appears to protect the brain enzyme against inhibition by L-methionine-*S*-sulfoximine injected intraperitoneally. If we assume that the liver and brain glutamine synthetases have the same activity and capacity to bind methionine sulfoximine phosphate, it may be calculated that a large fraction of the L-methionine-*S*-sulfoximine injected in experiment 4 (Table 2) and (as a contaminant) in experiment 2 (Table 2) may be bound by the liver glutamine synthetase.

In the course of these studies, eight mice of the C57 black strain were injected with a dose (1 mmole/kg) of L-methionine-*SR*-sulfoximine which has been found to produce convulsions in all (more than 30) AH/J mice tested. We found that this dose was insufficient to produce convulsions in the C57 black mice, but that larger doses (2.5 mmoles/kg) of L-methionine-*SR*-sulfoximine did produce convulsions in all of eight animals injected. We subsequently found that the glutamine synthetase activity of the brain and liver of these animals is substantially higher than that of the corresponding tissues of AH/J strain mice (Table 3).

TABLE 3. *Glutamine synthetase activity of the brain and liver of mice.*

Strain	Glutamine Synthetase Activity Brain (units/mg protein)	Liver (units/mg protein)
C57 black	1.77 ± 0.47	0.82 ± 0.27
AH/J	0.75 ± 0.19	0.45 ± 0.12

Discussion. The finding that only one of the four optical isomers of methionine sulfoximine induces convulsions, and that only this same isomer inhibits glutamine synthetase irreversibly seems to strengthen the argument that these two effects of methionine sulfoximine are closely connected.[36] The observations on the levels of brain glutamine synthetase and on the amounts of methionine sulfoximine required to produce convulsions in AH/J and C57 black mice are also consistent with this view. There is now an impressive list of enzyme ac-

tivities that are not significantly affected by methionine sulfoximine. Thus, it has been observed that brain choline acetylase,[24,37] acetylcholinesterase,[24,37] monoamine oxidase,[24] glutamate dehydrogenase,[24] glutamate-aspartate transaminase,[24,37] γ-aminobutyrate-glutamate transaminase,[24] tryptophan hydroxylase,[37] tyrosine hydroxylase,[37] glutaminase,[27] glutamine transaminase,[38] ornithine transaminase,[27] β-acetylaminodeoxyglucosidase,[40] and acid phosphatase[40] are not inhibited by methionine sulfoximine. Slight[24] and no[39] inhibition of glutamate decarboxylase has been observed. Inhibition of glutamate-alanine transaminase was reported[24] but no inhibition of this activity has been observed in our laboratory.[38] *S*-Adenosyl methionine synthetase is not inhibited by methionine sulfoximine,[27] a finding confirmed by the present authors.

A number of studies suggest that glutamate and γ-aminobutyrate are respectively excitatory and inhibitory transmitters in the central nervous system (see, for example, refs. 41 and 42). Thus the possibility that the effect of methionine sulfoximine is mediated through alterations in the metabolic interrelationships between glutamate, glutamine, and γ-aminobutyric acid seems plausible. The available evidence indicates that L-methionine-*S*-sulfoximine inhibits glutamine synthetase, but not glutamine, γ-aminobutyrate, or glutamate transaminases or glutamate decarboxylase; indeed, it is notable that L-methionine-*S*-sulfoximine appears to inhibit glutamine synthetase by virtue of its ability to serve as an analog of the tetrahedral intermediate[43] (formed by reaction between ammonia and enzyme-bound γ-glutamyl phosphate) rather than of glutamate itself. Most of the brain glutamine synthetase is localized in the microsomal fraction; a significant portion is concentrated in subcellular fractions containing nerve endings.[23,24,29,44–46] Administered methionine sulfoximine is bound to the subcellular fractions that contain nerve endings, and electron microscope studies have shown that methionine sulfoximine causes swelling of nerve endings and loss of synaptic vesicles especially of the noncholinergic type.[24] The attractive possibility exists that a particular fraction of brain glutamine synthetase functions in certain synapses to remove glutamate in a manner analogous to the function of acetylcholine esterase in cholinergic synapses. Inhibition of glutamine synthetase by methionine sulfoximine might then be expected to disturb the normally balanced excitatory effect of glutamate.

On the other hand, several workers have concluded that inhibition of glutamine synthetase by methionine sulfoximine is not directly related to the onset of seizures.[18,26,27] The ability of methionine (and other amino acids) to protect against methionine sulfoximine-induced seizures has been noted in several laboratories.[5,9,21,26] Sellinger *et al.*[26] found that inhibition of glutamine synthetase by methionine sulfoximine "was not directly seizure-related," that rats "could be protected against seizures (by methionine) in the face of a virtual block of the enzyme, and conversely that seizures could be elicited in the face of relatively elevated enzyme levels." The protective effect of methionine may probably be ascribed to the effect of this amino acid on the transport of methionine sulfoximine. The data[26] indicate that intraventricularly administered methionine has little protective effect when methionine sulfoximine is administered intraperitoneally or intraventricularly. It is not unreasonable to suppose

(in the absence of data at this time) that methionine sulfoximine and methionine share an active transport system. Folbergrova[18] failed to find a correlation in rats between seizures and brain glutamine concentration, and Lamar[27] reported similar findings. Changes in ammonia concentration in the brain do not correlate well with the onset of seizures,[49] nor do changes in the concentration of glutamate[49] or other amino acids. While these observations must be considered carefully, it is important to note that in the reported studies the determinations of metabolites and enzyme activity represent over-all values for the entire brain or of particular subdivisions of the brain. It is possible that chemical stimulation of a relatively limited number of neurons in one or more areas of the brain might elicit a generalized increase in neuronal activity perhaps by a mechanism similar to that which produces spreading depression.[12,50,51]

It is of related interest that methionine sulfoximine protects animals against the toxic effects (coma, seizures) of ammonia.[47] Toxic doses of ammonia deplete ATP and phosphocreatine at the base of the brain, but not in the cortex;[48] presumably this effect on basilar energy metabolism is related to the increased utilization of ATP catalyzed by glutamine synthetase, and this is prevented by methionine sulfoximine. Thus, ammonia and methionine sulfoximine seem to produce seizures by different mechanisms affecting different regions of the brain.

It is quite possible that phenomena other than inhibition of glutamine synthetase are involved in the production of seizures by L-methionine-*S*-sulfoximine—and it cannot yet be excluded that this amino acid has another as yet undiscovered action which leads directly to seizures. However, although a detailed mechanism cannot be shown at this time, the weight of evidence seems to favor the view that inhibition of glutamine synthetase is a major factor in methionine sulfoximine-induced seizures. The finding that the enzyme acts specifically on L-methionine-*S*-sulfoximine to yield the corresponding (enzyme-bound) sulfoximine *N*-phosphate may offer new experimental approaches to some of the many remaining questions.

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