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In Vivo Formation of Methionine Sulfoximine Phosphate, a Protein-Bound Metabolite of Methionine Sulfoximine†

S. L. N. Rao and Alton Meister*

ABSTRACT: Administration of a subconvulsive dose of L-[^{35}S]-methionine sulfoximine to mice led to prompt formation in brain, liver, and kidney of protein-bound [^{35}S]methionine sulfoximine phosphate. The amounts of methionine sulfoximine phosphate formed are related to the amounts of glutamine synthetase in these tissues. The methionine sulfoximine phosphate content of the brain decreased very slowly and a significant amount remained 90 days after methionine sulfoximine was given; in contrast, more than 98% of the methionine sulfoximine phosphate disappeared from the liver within about 30 days. Restoration of liver glutamine synthetase was 90% complete after 15 days, while only about 75% of the brain glutamine synthetase returned after 90 days. About half of the brain glutamine synthetase returned within 8–12 days, while subsequent return of activity occurred much more slowly. In both liver and brain, the return of enzyme activity took place more rapidly than did removal of methionine sulfoximine phosphate.

Previous *in vitro* studies in this laboratory established that the convulsant agent methionine sulfoximine inhibits brain glutamine synthetase irreversibly and that such inhibition is associated with the conversion of methionine sulfoximine to methionine sulfoximine phosphate, which remains tightly attached to the enzyme (Ronzio and Meister, 1968; Ronzio *et al.*, 1969; Rowe *et al.*, 1969; Manning *et al.*, 1969; Rowe and Meister, 1970; Meister *et al.*, 1970). Although there is evidence that the convulsant activity of methionine sulfoximine is associated with its ability to inhibit brain glutamine synthetase (Rowe and Meister, 1970), a detailed understanding of such a relationship has not yet been achieved. In the present work, which was undertaken as a step in this direction, we have sought to determine whether the administration of methionine sulfoximine to an animal is followed by *in vivo* formation of methionine sulfoximine phosphate. We have found that administration of methionine sulfoximine to mice leads to substantial formation of protein-bound methionine sulfoximine phosphate in the brain and liver. The amounts of methionine sulfoximine formed are consistent with the amounts of glutamine synthetase in these tissues. It is of interest that the methionine sulfoximine phosphate content of the brain (in contrast to liver) decreases very slowly; thus, appreciable amounts were found in the brain 90 days after administration of a single subconvulsive dose of methionine sulfoximine.

† From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. *Received December 6, 1971.* Supported in part by the John A. Hartford Foundation, Inc.

Experimental Section

Materials

L-[^{35}S]Methionine (specific activity 1300 Ci/mole) was obtained from Schwarz BioResearch. This material was added to unlabeled carrier L-methionine and converted to L-methionine sulfoxide by the procedure of Toennis and Kolb (1939), as modified by Roper and McIlwain (1948). L-Methionine sulfoxide was converted to L-methionine (*SR*)-sulfoximine by the procedure of Bentley *et al.* (1951). L-[^{35}S]Methionine (*SR*)-sulfoximine was isolated and purified as described by Ronzio *et al.* (1969). The purified product exhibited a specific activity of 69.3 Ci/mole.

Methods

Determination of Radioactivity. Liquid samples were counted in Bray's (1960) solution with a Nuclear-Chicago scintillation counter. The insoluble residues were digested with 2 N methanolic KOH (0.5 ml/5–10 mg) for 30 min at 60° and then diluted to 2 ml with methanol; 0.2-ml aliquots were counted. The radioactivity of dry strips of Whatman No. 3MM paper was determined by scintillation counting (Rowe *et al.*, 1969).

The radioactive components present in some of the fractions were determined with an automated amino acid analyzer equipped with a Chromobeads column (120 cm) using the system of Piez and Morris (1960); radioactivity was monitored with a TriCarb liquid scintillation counter equipped with a flow-cell adapter.

Injection of Mice and Preparation of Tissues. In the experiments in which methionine sulfoximine phosphate was iso-

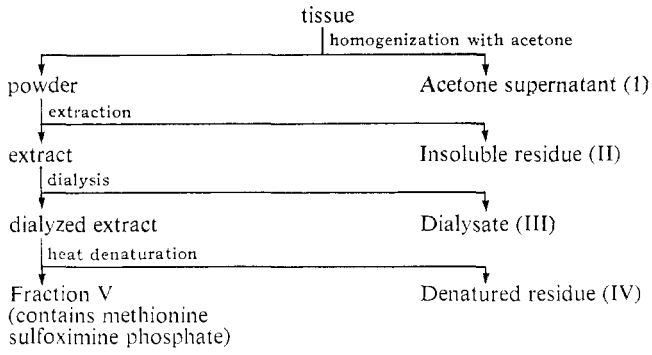

FIGURE 1: Outline of the tissue fractionation procedure (see the text).

lated, L-[^{35}S]methionine sulfoximine (specific activity 38.4 Ci/mole) was injected intraperitoneally into mice (male, HA/ICR, 23–25 g); the dose was 1 mg (4×10^8 cpm). The glutamine synthetase activity of liver and brain was determined (see below) under identical conditions using mice of the same strain, sex, age, and weight and unlabeled methionine sulfoximine. It was not practicable to determine methionine sulfoximine phosphate and glutamine synthetase activity on the same tissue samples, because of the different procedures required and because of the difficulty in obtaining identical anatomical samples. However, glutamine synthetase activity was determined in several experiments in which [^{35}S]methionine sulfoximine phosphate was isolated, and the values were, within experimental error, the same as those obtained in the studies in which unlabeled methionine sulfoximine was injected. The animals were killed by decapitation and the livers and brains were quickly excised and processed either for preparation of an acetone powder or assayed for glutamine synthetase activity (see below).

Determination of Glutamine Synthetase Activity. The tissues were homogenized with 10 volumes of a solution consisting of 0.15 M KCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA (adjusted to pH 7.2 by addition of NaOH). The homogenates were centrifuged at 40,000*g* for 15 min at 0° and the supernatant solutions were assayed for glutamine synthetase activity by the γ-glutamyl hydroxamate assay method (Wellner and Meister, 1966). The assay mixture consisted of sodium glutamate (0.05 M), NaATP (0.02 M), 2-mercaptoethanol (0.01 M), magnesium chloride (0.04 M), hydroxylamine (0.1 M), imidazole-HCl buffer (pH 7.2, 0.1 M), and 0.2 ml of homogenate; final volume 1 ml. After incubation for 15 min at 37°, the reaction was terminated by addition of 1.5 ml of ferric chloride reagent (Lipmann and Tuttle, 1945) and the formation of γ-glutamyl hydroxamate was determined at 535 nm. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of hydroxamate under these conditions. Homogenates of liver and brain from controls exhibited, respectively, 55 ± 5 and 35 ± 3 units per g of tissue.

Fractionation of Liver and Brain; Isolation of Bound Methionine Sulfoximine Phosphate. The freshly excised tissues were immediately minced and then homogenized with 10 volumes of acetone (at −10°) in a glass homogenizer. The suspension was centrifuged at 3000*g* for 3 min; the residue was washed four times by centrifugation with 5-ml portions of acetone (−10°). The acetone fractions were combined and saved for the determination of radioactivity. The acetone powder was dried in a vacuum desiccator and then weighed. The powder was fractionated as follows (see Figure 1). The powder (50 mg) was extracted with efficient stirring for 30 min with 3 ml of a solution containing 0.1 M KCl, 1 mM EDTA, and 5 mM 2-mercaptoethanol (adjusted to pH 7.2 with NaOH). The mixture was centrifuged to yield the acetone powder extract and an insoluble residue (II). The acetone powder extract was dialyzed against three changes of 250 ml each of 5 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 0.01 M 2-mercaptoethanol. The final dialysate contained negligible amounts of radioactivity. The dialyzed extract was denatured by placing it at 100° for 2.5 min. The denatured extract was evaporated to dryness in a vacuum desiccator over P_2O_5 and the dried material was extracted thoroughly with 2 ml of water. This mixture was centrifuged to yield fraction V and the denatured residue (IV). Fraction V was subjected to paper electrophoresis at pH 2.6 according to the procedure of Ronzio *et al.* (1969) which effectively separates methionine sulfoximine phosphate from methionine sulfoximine, methionine, methionine sulfone, and methionine sulfoxide. Carrier amounts of these compounds (unlabeled) were added prior to paper electrophoresis. After electrophoresis, the paper was dried and scanned for radioactivity; more than 90% of the radioactivity was found in the methionine sulfoximine phosphate area. Occasionally very small amounts of label were found in the methionine sulfoximine area.

Further identification of methionine sulfoximine phosphate was established by carrying out its hydrolysis with yeast acid phosphatase as described by Ronzio *et al.* (1969). In these studies the extract (0.125 ml) was incubated with 8×10^{-5} unit of yeast acid phosphatase (kindly furnished by Dr. Gerhard Schmidt) in 0.2 ml of solution containing 0.1 M sodium acetate buffer (pH 4.0); incubation was carried out for 30 min at 37°. After incubation, paper electrophoretic studies indicated that all of the radioactivity moved with methionine sulfoximine and none remained in the methionine sulfoximine phosphate area. Additional confirmation of the identity of methionine sulfoximine phosphate was established by chromatography on the automated amino acid analyzer (Manning *et al.*, 1969) using an authentic sample of methionine sulfoximine phosphate (Rowe *et al.*, 1969) as standard.

Pronase Digestion. The insoluble residue (II) and the denatured residue (IV) were suspended in 3 ml of 0.2 M ammonium bicarbonate (adjusted to pH 7.8 with NH_4OH); Pronase (10 mg; Calbiochem) was added along with a few drops of toluene, and the reaction mixture was stirred at 37° for 24 hr. After 14 hr, additional Pronase (5 mg) was added. After 24 hr, perchloric acid (final concentration, 4%) was added and the precipitated material was removed by centrifugation. The supernatant solution was neutralized with 2 N KOH and the precipitated potassium perchlorate was removed by centrifugation. The supernatant solution was examined on the automated amino acid analyzer equipped with scintillation counter and flow-cell adapter.

Tryptic Digestion. The insoluble residue (II) and denatured residue (IV) were suspended in 2 ml of 0.02 M Tris-HCl (pH 7.8) buffer containing 1 mM $CaCl_2$. Trypsin (5 mg) was added and the suspension was stirred mechanically at 37° for 24 hr. After digestion the pH was brought to 2.5 by addition of 4 N acetic acid; after centrifugation, the supernatant solution was analyzed on the automated amino acid analyzer.

Results

Formation of Methionine Sulfoximine Phosphate in Liver and Brain after Injection of Methionine Sulfoximine. A group of mice were injected with a subconvulsive dose of L-[^{35}S]-

TABLE I: Radioactivity (cpm/g of Tissue) of the Fractions Obtained from Liver and Brain.[a]

Time	Acetone Supernatant (I)		Insoluble Residue (II)		Dialysate (III)		Denatured Residue (IV)		Methionine Sulfoximine Phosphate (V)	
	Liver	Brain	Liver	Brain	Liver	Brain	Liver	Brain	Liver (% of Total)	Brain (% of Total)
5 hr	3,970,000	2,250,000	1,000,000	306,000	15,000,000	2,250,000	543,000	45,000	476,000 (2.3)	435,000 (8.2)
24 hr	390,000	266,000	751,000	208,000	2,050,000	598,000	445,000	85,000	303,000 (7.7)	334,000 (22)
6 days	56,000	50,000	462,000	369,000	240,000	66,000	255,000	21,000	154,000 (13)	390,000 (44)
15 days	12,000	17,000	41,000	105,000	57,000	42,000	51,000	17,000	60,500 (27)	250,000 (58)
30 days	7,500	12,500	16,000	35,000	18,000	32,000	25,000	0	8,000 (11)	157,000 (67)
60 days	200	1,500	13,000	40,000	1,300	7,000	0	0	0	150,000 (76)
90 days	0	0	2,200	42,000	0	800	0	0	0	176,000 (80)

[a] Each figure represents the mean of determinations on tissues of two animals.

methionine sulfoximine and at various intervals the animals were killed and their livers and brains were fractionated and analyzed for [^{35}S]methionine sulfoximine phosphate as described under Methods. Two animals were worked up separately at intervals varying from 1 hr to 90 days; the data obtained are summarized in Figure 2. In both brain and liver maximal formation of methionine sulfoximine phosphate was observed 5 hr after injection of methionine sulfoximine. Thus, about 5 nmoles of methionine sulfoximine phosphate was found per g of brain and a similar, perhaps slightly higher, content was found in the liver. The methionine sulfoximine phosphate content of liver decreased relatively rapidly; thus, about two-thirds of the methionine sulfoximine phosphate disappeared after 6 days. After 30 days methionine sulfoximine phosphate was still detectable, but after 60 and 90 days none could be found. In contrast, the methionine sulfoximine phosphate content of the brain decreased much more slowly. As indicated in Figure 2, the methionine sulfoximine phosphate in the brain decreased about 20% from the first to the 15th day after injection of methionine sulfoximine. The values obtained after 30, 60, and 90 days were, within experimental error, about the same, *i.e.*, about 2 nmoles/g or close to 40% of the maximal (5 hr) value.

The various fractions obtained from liver and brain at different time intervals after injection of [^{35}S]methionine sulfoximine contained substantial amounts of radioactivity; representative data are given in Table I. As indicated in the right-hand column of Table I, the fraction of the total radioactivity present in the brain that could be accounted for as methionine sulfoximine phosphate increased progressively from 8.2% (after 5 hr) to 80% (after 90 days). Much lower values were obtained with liver. Efforts were made to determine whether any of the other fractions obtained from liver and brain contained methionine sulfoximine phosphate. We found that the radioactivity present in the insoluble residue fraction (fraction II) and the denatured residue (fraction IV) could not be extracted into solution with hot water, 4% perchloric acid, or 0.4 N KOH. On the other hand, when these insoluble residues were digested with Pronase as described under Methods, all of the radioactivity was released in a soluble form. Analysis of this material on the automated amino acid analyzer failed to reveal the presence of methionine sulfoximine phosphate; more than 80% of the radioactivity was associated with methionine sulfoximine. When the insoluble fractions (II and IV) were digested with trypsin, about half of the initial radioactivity was released in a soluble form; analysis indicated that all of the soluble ^{35}S was present as methionine sulfoximine. When the insoluble fractions were suspended in 2% sodium dodecyl sulfate at 60° for 0.5–4 hr (with or without addition of carrier unlabeled methionine sulfoximine and methionine sulfoximine phosphate), and then treated with perchloric acid, about 50% of the radioactivity was released; analysis of the soluble material showed that all of the radioactivity was associated with methionine sulfoximine. Several unsuccessful attempts were made to find labeled peptides after proteolytic digestion. No evidence for the incorporation of methionine sulfoximine into proteins was obtained, although this possibility cannot be unequivocally excluded. The findings indicate that virtually all of the radioactivity present in the insoluble fractions (II and IV) can be accounted for as methionine sulfoximine, which appears to be tightly bound.

Chromatographic analysis of the acetone supernatant fraction (fraction 1) and the dialysate (fraction III) did not reveal the presence of methionine sulfoximine phosphate. However, large amounts of methionine sulfoximine and seven additional radioactive compounds were found which have not as yet been identified. One of these exhibited chromatographic behavior identical with a compound believed to be the α-keto analog of methionine sulfoximine. The latter compound is currently under investigation in this laboratory;

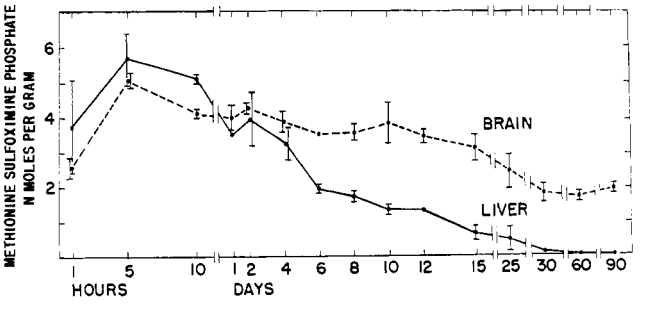

FIGURE 2: Methionine sulfoximine phosphate content of brain and liver after administration to mice of a subconvulsive dose of methionine sulfoximine. The curves are drawn through points indicating the average values found in two separate isolations (indicated by the vertical lines) from liver and from brain; other details are given in the text.

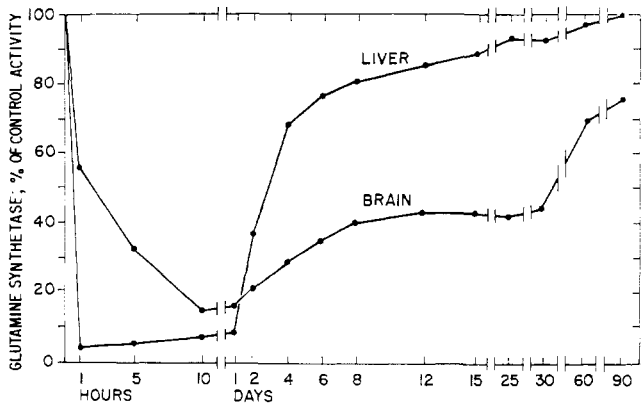

FIGURE 3: Glutamine synthetase activity of brain and liver after administration to mice of a subconvulsive dose of methionine sulfoximine. The experimental conditions were identical to those given in Figure 2; the values plotted are the average (SD ± 10%) of two determinations on each of two animals (see the text).

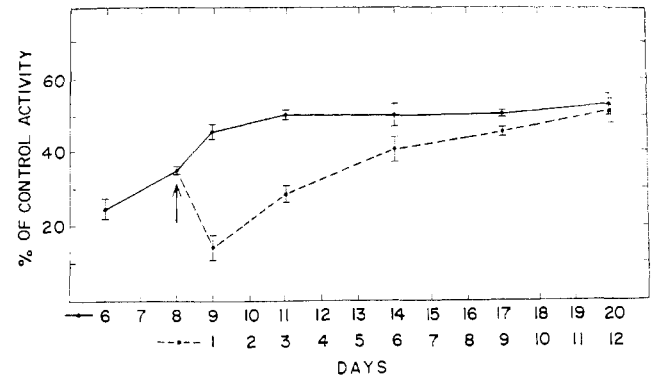

FIGURE 4: Glutamine synthetase activity of brain after administration of a single subconvulsive dose of methionine sulfoximine (upper curve) and after administration of an additional dose (see arrow; lower curve). The values plotted are the average of two determinations on each of two animals; the range of values obtained is indicated by the vertical lines.

its formation and properties will be described in a subsequent publication.

A few determinations of methionine sulfoximine phosphate were carried out on kidney. Analyses done 1, 5, and 24 hr after injection of methionine sulfoximine revealed the presence of 1.3, 1.1, and 0.85 nmoles of methionine sulfoximine phosphate per g. These values are about one-fourth of those obtained for brain; the glutamine synthetase activity of kidney is about 10 units/g or about 20–30% of that of brain.

Effect of Methionine Sulfoximine on Liver and Brain Glutamine Synthetase Activity. Figure 3 summarizes determinations of glutamine synthetase on homogenates of liver and brain obtained from animals injected with methionine sulfoximine. In both tissues the activity decreased to 15% or less of the control activity soon after injection of methionine sulfoximine. The subsequent increase of glutamine synthetase activity was much more rapid in liver than brain; thus, about 70 and 90% of the control activity of liver was observed after 4 and 15 days, respectively. In contrast, brain glutamine synthetase activity increased relatively slowly reaching a value of about 45% of the control after 12 days. This was followed by a slower increase of activity leading to restoration of about 75% of the control brain glutamine synthetase activity after 90 days.

Since these data indicate that about half of the brain glutamine synthetase activity returns within 8–15 days after injection of methionine sulfoximine, while subsequent restoration of activity occurs at a much slower rate, it became of interest to determine the effect of a second injection of methionine sulfoximine. In the experiment described in Figure 4, a group of mice were given a second injection of methionine sulfoximine 8 days after receiving the initial dose. After the second injection, brain glutamine synthetase activity decreased immediately and then increased over a period of 6–12 days reaching approximately the same level as that exhibited by the control, *i.e.*, animals which had been given only a single dose of methionine sulfoximine.

Discussion

The present findings, which indicate that methionine sulfoximine phosphate is a major metabolite of methionine sulfoximine in the brain and liver, are in accord with the *in vitro* studies on glutamine synthetase which elucidated the mechanism of inhibition of this enzyme by methionine sulfoximine (Ronzio and Meister, 1968; Ronzio *et al.*, 1969, Rowe *et al.*, 1969). It is notable that substantial amounts of the radioactivity of [^{35}S]methionine sulfoximine were accounted for as protein-bound methionine sulfoximine phosphate; furthermore, no free methionine sulfoximine phosphate was found. The maximal amounts of methionine sulfoximine phosphate found in brain, liver, and kidney seem to reflect the relative amounts of glutamine synthetase present in these tissues. The glutamine synthetases of mouse tissues have not yet been isolated in purified form; however, the published data on purified rat liver (Tate and Meister, 1971) and sheep brain (Rowe *et al.*, 1970) glutamine synthetases for molecular weight, specific activity, and binding of methionine sulfoximine phosphate lead to values (3–6 nmoles/g of tissue) that are in the same range as was observed in the present studies (Figure 2).

Although there is general correlation between the data on the disappearance of methionine sulfoximine phosphate (Figure 2) and those on the reappearance of glutamine synthetase activity (Figure 3), it is clear that the two phenomena do not take place in exactly parallel fashion. Thus, liver glutamine synthetase increased from about 10 to 70% of the control from day 1 to 4 after injection of methionine sulfoximine; during the same period, liver methionine sulfoximine phosphate decreased only slightly. Brain glutamine synthetase increased from about 15% of the control on day 1 to about 45% of the control on day 12; the decrease in brain methionine sulfoximine phosphate over the same period was only about 10–15%. Furthermore, while about 75% of the brain glutamine synthetase activity had returned after 90 days, about 40% of the maximal level of methionine sulfoximine phosphate was still present in the brain. Therefore, it appears that glutamine synthetase activity returns more rapidly than bound methionine sulfoximine phosphate disappears both in the liver and in the brain. A number of unsuccessful attempts have been made *in vitro* to reverse the inhibition by methionine sulfoximine (Ronzio *et al.*, 1969). We cannot exclude the possibility that an *in vivo* reactivation mechanism exists; however, if there is such a mechanism it would appear that the methionine sulfoximine phosphate removed from glutamine synthetase is transferred intact to another protein. On the other hand, a more reasonable interpretation of the findings is that new enzyme is synthesized at a more rapid rate than that of the removal of inactivated enzyme. *In vitro* stud-

ies on glutamine synthetase in our laboratory indicate that the enzyme is much less susceptible to the action of trypsin after inactivation by methionine sulfoximine; quite possibly a similar phenomenon occurs *in vivo*. It is also possible that a considerable fraction of the inactivated glutamine synthetase remains attached to cell membrane components which turn over relatively slowly.

The present findings show that brain glutamine synthetase activity is restored to normal levels very slowly after injection of methionine sulfoximine. This observation, which is in general agreement with studies carried out on the injection of methionine sulfoximine to rats (Lamar, 1968; Sellinger *et al.*, 1968; Sellinger and Ohlsson, 1969), has led to an additional finding which is illustrated by the experiment described in Figure 4. This experiment shows that about half of the glutamine synthetase activity returns relatively rapidly as compared to the remainder of the activity, suggesting that there may be two types of glutamine synthetases, distinguished here by virtue of their greatly different rates of regeneration. The findings might reflect differences in the subcellular localization of glutamine synthetase (see, for example, DeRobertis *et al.*, 1967). Ghittoni *et al.* (1970) and Sellinger *et al.* (1971) have reported on the intracellular disposition of radioactive label after administration of *methyl*-^{3}H-labeled methionine sulfoximine to rats. However, the data published by these investigators do not permit conclusions concerning the distribution of methionine sulfoximine as compared to methionine sulfoximine phosphate; the methods used do not appear to have been capable of distinguishing between these compounds. The present studies, which indicate that both methionine sulfoximine and methionine sulfoximine phosphate are present in brain and liver after injection of methionine sulfoximine to mice, suggest that it might be of interest to carry out detailed studies of the subcellular distribution of glutamine synthetase activity, methionine sulfoximine, and methionine sulfoximine phosphate. Quite apart from these considerations, the data indicate that methionine sulfoximine phosphate (and presumably also the inactivated enzyme) remains in the brain for a long time after administration of methionine sulfoximine, and it is reasonable to suspect that this might lead to permanent changes in brain structure and function.

In relation to the evidence that the convulsant action of methonine sulfoximine is associated with its effect on glutamine synthetase, the possibility was considered previously that methionine sulfoximine might have another, as yet undiscovered, action which causes convulsions (Rowe and Meister, 1970). In this connection it is worthy of note that in the present study appreciable amounts of methionine sulfoximine were bound tightly to components present in fractions II and IV in both liver and brain. The nature and significance of this bound methionine sulfoximine remains to be explored.

Finally it may be noted that the methionine sulfoximine–glutamine synthetase system may be of value in studies on the synthesis of this enzyme and on its intracellular localization in various tissues. Although there are significant differences between the glutamine synthetases of different cells, inhibition by methionine sulfoximine appears to be a general property of this enzyme (Tate and Meister, 1971). The fact that methionine sulfoximine phosphate binds very tightly to the enzyme offers a potentially useful marker for histochemical and cell fractionation studies.

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