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THE INHIBITION *IN VIVO* OF CEREBRAL GLUTAMINE SYNTHETASE AND GLUTAMINE TRANSFERASE BY THE CONVULSANT METHIONINE SULFOXIMINE*

C. LAMAR, JR.† and OTTO Z. SELLINGER‡

Department of Biochemistry and the Nutrition and Metabolism Research Laboratory, Department of Medicine, Tulane University School of Medicine, New Orleans, La., U.S.A.

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Abstract—Evidence is presented for an *in vivo* interaction of the convulsant agent methionine sulfoximine (MSO) with glutamine synthetase (GS) and glutamine transferase of rat cerebral cortex. Unlike the *in vitro* interaction of MSO with GS, the *in vivo* effects of the drug on this enzyme activity were irreversible, as judged by the recovery of normal enzyme maximal velocity upon *in vitro* incubation in the presence of excess substrate (glutamate or ATP). Conversely, identity of the *in vitro* and *in vivo* effects of the drug on glutamine transferase was noted, since the inhibitory effect of MSO could be readily reversed at high *in vitro* concentrations of glutamine under both conditions.

SINCE the finding of Mellanby[1] that dogs underwent seizures after ingestion of products containing flour derived from NCl_3-treated wheat and the demonstration of methionine sulfoximine (MSO) isolated from such wheat as the toxic convulsant factor,[2–4] MSO has been shown to possess a variety of pharmacological actions. Pace and McDermott[5] studied the inhibitory action of MSO on bacterial growth and, furthermore, noted an inhibition of the synthesis of glutamine in extracts of sheep brain. Rabinovitz *et al.*[6] found an inhibitory effect of MSO on the incorporation of leucine, phenylalanine, lysine, and methionine into protein by Ehrlich ascites carcinoma cells and showed that the inhibition could be reversed by the addition of small amounts of glutamine to the medium.

In 1958, Lodin[7] described a correlation between the convulsant effects of MSO and EEG changes in dogs and rats. In rats, a latent period of approximately 4 hr following the administration of the drug was noted; during this time the animal exhibited no abnormalities. Reiner *et al.*[4] and, later, Lodin and Kolousek[8] reported that the simultaneous administration of methionine decreased seizure susceptibility. The effect of glutamine on MSO-induced convulsions in a variety of species has also been examined, with conflicting results.[8, 9] Kolousek and Babicky[10] reported that

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† Present address: McArdle Memorial Laboratory, University of Wisconsin, Medical School, Madison, Wis.

‡ Present address: University of the Republic, Department of Biochemistry, Avda. Gral. Flores 2125, Montevideo, Uruguay.

^{35}S-MSO fails to incorporate into cerebral proteins when administered *in vivo* and demonstrated the presence of the drug in the nonprotein fraction of brain in maximal amounts during seizures. In a brief report, Kolousek *et al.*[11] have also described an MSO-induced depression of the incorporation of ^{32}P into cytoplasmic RNA of rat liver, and a corresponding stimulation of the incorporation of ^{35}S-methionine into proteins of rat spleen. Peters and Tower[12] have examined in some detail the effect of MSO on cerebral metabolism by means of adding metabolites to the medium bathing cat cerebral cortex slices so as to relieve the impairment of glutamine production noted in the slices derived from MSO-treated animals.

Gershenovich *et al.*[13] used elevated oxygen tension to produce seizures in rats, a treatment resulting in an increased production of ammonia and an initial, transient increase of cerebral glutamine synthetase (GS) activity. Recently, Sellinger and Weiler[14] have reported that, *in vitro*, MSO competitively inhibits the GS activity associated with cerebral microsomes. A causal relationship between the seizure state and cerebral levels of glutamine has been questioned by Peters and Tower[12] and by Folbergrova[15], who found no direct correlation between the two parameters. The present report describes studies on the *in vivo* effects of MSO on glutamine synthetase and glutamine transferase (GT) of rat cerebral cortex. Some features of the *in vitro* and *in vivo* interactions of the drug with the enzyme activities are described, and it is suggested that MSO interacts with the enzyme protein(s) by structurally altering them, an event apparently responsible for a diminution of their functional capacity. Conditions for the reversal of the convulsant effect of MSO *in vivo* are also described, and a relationship between the state of sulfhydryl groups of GS and seizures is indicated.

METHODS

L-Glutamic acid, L- and DL-methionine, L-glutamine, DL-methionine-DL-sulfoximine (MSO), DL-methionine sulfoxide (MSI), DL-methionine sulfone (MSOne), crystalline bovine albumin, and 2,3-dimercaptopropanol (BAL) were products of the Mann Research Laboratories, New York, N.Y.; ADP was a product of Pabst Laboratories, Milwaukee, Wis.; ATP and L-glutamylhydroxamic acid were purchased from the Sigma Co., St. Louis, Mo.; 1-^{14}C-DL-methionine was obtained from Volk Radiochemical Co. of Skokie, Ill.; POPOP (*p*-bis-2-(5-phenyloxazolyl)benzene) and PPO (2,5-diphenyloxazole) were obtained from Pilot Chemical Inc., Watertown, Mass. All other reagents were of best commercial grade available.

Preparation of microsomal fraction from rat cerebral cortex. Male Holtzman rats (150–200 g) were used in all experiments. After decapitation, the brain (one or two per experiment) was exposed and the cortical layers quickly removed and placed in a tared beaker containing 1 ml of ice-cold distilled water. After weighing, the cortices were transferred to a homogenizer tube fitted with a Teflon pestle (clearance: 0·005 in) and homogenization was accomplished mechanically in a volume four times the tissue weight. The resulting homogenate was centrifuged at 25,000 *g* for 10 min (Spinco model L centrifuge, rotor no. 40), and the sedimented material was resuspended mechanically (one up-and-down stroke with the pestle) and recentrifuged at the same speed for 10 min, yielding as a sediment fraction NML. The combined supernatant fluid and washings (fraction MS) were centrifuged at 145,000 *g* for 60 min. The pellet (fraction PS) represents the microsomal fraction, as previously

defined.[14] It was suspended in 0·15 M NaCl and made to a volume ten times the original weight of the tissue, and the suspension was centrifuged at 145,000 *g* for 60 min to yield the postmicrosomal pellet (fraction P_{15}). The supernatant fluid (fraction S_{15}) contained about 85% of the GS and 75% of the GT activity present in fraction PS and was the enzyme preparation used in most assays.

Chemical determinations. Protein was determined according to Lowry *et al.*[16] with bovine serum albumine as standard.

Glutamine synthetase assay. The previously described[17] incubation mixture (3 ml) and assay procedure were used. Incubation time (37°) was 30 min. A standard curve was prepared with L-glutamylhydroxamic acid (GHA). A unit of enzyme activity refers to the formation of 1 μmole GHA/30 min.

Glutamine transferase. The 3 ml of the incubation mixture contained (in final concentration at pH 7·2): 80 mM Tris buffer, 50 mM freshly prepared and neutralized hydroxylamine HCl, 80 mM potassium glutamine, 0·14 mM ADP, 2 mM $MnCl_2$, 14 mM $NaHAs_2O_4$, 0·5 mM BAL, and usually 0·10 ml of the enzyme. In the control tubes the enzyme solution was replaced by water. After 15 min of incubation at 37° the reaction was terminated and the color developed as for GS.[17] Enzyme units are defined as above for GS.

Preparation of 1-^{14}C-DL-*methionine*, DL-*sulfoximine.* The preparation of radioactive MSO was accomplished as described by Bentley *et al.*,[18] with 1-^{14}C-DL-methionine as the starting material. The specific activity of the final product, which was determined to be more than 90% pure by chromatography (contaminant: unreacted MSI), was 34 μc/m mole.

Preparation and chromatography of the cortical free amino acids. Approximately 1 g of brain was homogenized in 10 ml of 1% (w/v) picric acid, and the suspension was centrifuged at 2,000 rpm for 10 min. The supernate was drawn off and the picric acid removed by passage through a Dowex 2-×10 (50-100 ×) column (0·5 × 2·0 cm) in the chloride form. The effluent and washings (2 × 4 ml of 0·002 N HCl) were lyophilized and the residue taken up in 0·5 ml of water. Aliquots (50μl) were chromatographed (Whatman no. 1) in two dimensions by the descending technique. The first solvent was sec. butanol : formic acid (85%) : water (75·0 : 14·5 : 15·5, v/v). The paper was dried and then exposed to the second solvent; aq. phenol (80%) : conc. NH_4OH (99 : 1,v/v). The thoroughly dry chromatogram was developed with ninhydrin (0·2%,w/v, in acetone).

Measurement of radioactivity. The determination of the radioactivity of ^{14}C-labeled MSO and MSI was carried out as described by Bray[19] in a Packard Tri-Carb liquid scintillation counter system, model 5000, and an ethylene glycol–dioxane medium. The radioactivity of ^{14}C-MSO on one-dimensional chromatograms was evaluated by means of a Nuclear Chicago 4π strip counter. The radioactivity of ^{14}C-MSO spots developed by two-dimensional chromatography (see above) was estimated after suspension of the ninhydrin-stained spot in 2 ml of water followed by thorough shaking in 15 ml phosphor and counting.

RESULTS

In-vivo administration of MSO.

Effect of time of administration. MSO was administered (i.p.) at the dose (400 mg/kg) reported by Lodin[7] to cause convulsions in 4 to 6 hr. The rats were sacrificed at

TABLE 1. THE INHIBITION BY METHIONINE SULFOXIMINE (MSO) OF GLUTAMINE SYNTHETASE AND GLUTAMINE TRANSFERASE IN CEREBRAL SUBCELLULAR FRACTIONS: EFFECT OF TIME OF ADMINISTRATION

Subcellular fraction	Enzyme	Time* of sacrifice after MSO, (min)					
		15	30	60	80	160	240
SS	GS	86		66	56	33	32
	GT	92		55	33	12	3
PS	GS	100		59	47	24	18
	GT	88	62	45	43	11	7
P_{15}	GS	100	100	70	44	25	15
	GT	98	99	55	24	12	7
S_{15}	GS	90	84	55	37	25	27
	GT	94	74	46	23	7	8

* Values are averages obtained from pooled brains of at least 3 animals per experiment, except at 15 min period (2 animals).

All values are in % residual activity as compared, at times specified to corresponding saline-injected animals. For definition of fractions, see text. GS = glutamine synthetase; GT = glutamine transferase.

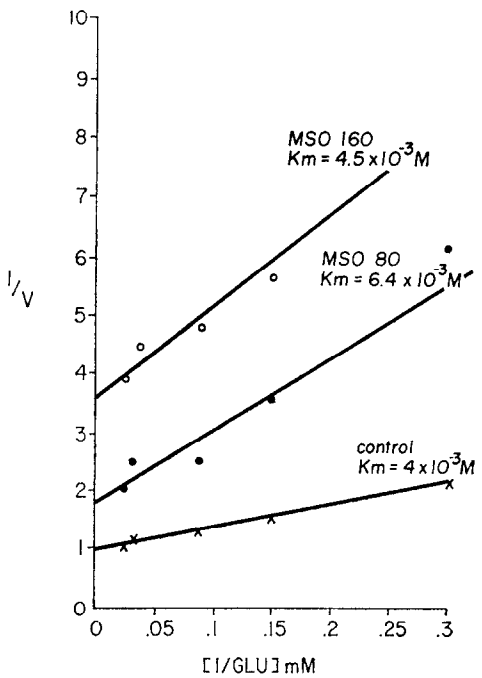

FIG. 1. Inhibition of cerebral glutamine synthetase by the *in-vivo* administration of DL-methionine-DL-sulfoximine (MSO). The enzyme preparation was isolated in soluble form (fraction S_{15}, see Methods) from rats treated *in vivo* (i.p.) with 400 mg MSO/kg, respectively, 80 and 160 min before sacrifice. Controls received equal volume of saline. Enzyme activity was determined as a function of L-glutamate concentration, and the results are plotted according to Lineweaver and Burk.[20] Glu = glutamate; V = μmoles of glutamylhydroxamate/30 min.

different times after the administration of MSO, and the cerebral cortex was fractionated by differential centrifugation. Perceptible inhibition of both enzyme activities occurred within 30 min. (Table 1). The inhibition of GT was more severe than that of GS, and the difference in severity of the inhibition of the two activities persisted throughout the fractionation. The extent of the inhibition was progressive with time and the animals not sacrificed shortly after 4 hr, i.e. at a time when residual enzyme activity was almost nil, invariably underwent seizure episodes.

Kinetic behavior of GS and GT in soluble postmicrosomal preparations isolated from the brain of rats treated with MSO.

a. GS with glutamate. Fraction S_{15} (see Methods) was isolated from the brains of rats sacrificed at different times after MSO, and the activity of GS was determined *in vitro* at different glutamate concentrations. Double reciprocal plots[20] characteristic of and denoting noncompetitively inhibited GS were obtained (Fig. 1). The K_m values for glutamate of the enzyme isolated from the control rats and from rats given MSO 80 to 160 min prior to sacrifice were calculated as $4{\cdot}0 \times 10^{-3}$ M, $6{\cdot}4 \times 10^{-3}$ M, and $4{\cdot}5 \times 10^{-3}$ M, respectively.

b. GS with ATP. The above experiment was repeated, and cerebral GS activity from a control rat and from a rat treated with MSO 160 min before sacrifice was determined at different ATP concentrations. A Lineweaver-Burk plot[20] of the data

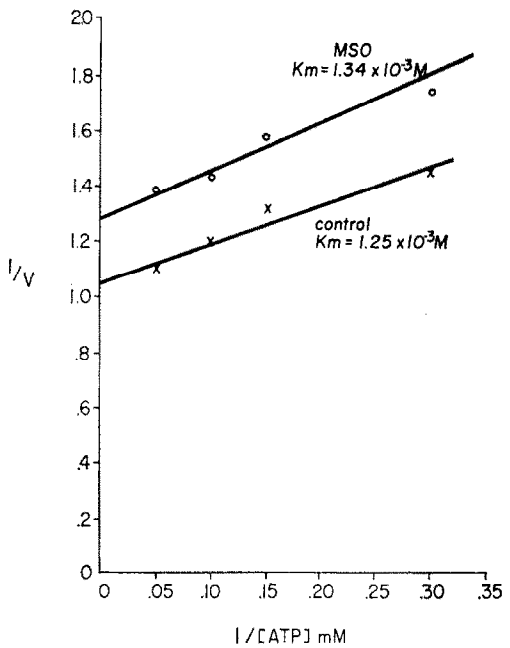

FIG. 2. Conditions as in Fig. 1 except that GS activity was determined *in vitro* as a function of ATP concentration.

is shown in Fig. 2. The K_m for ATP of the control enzyme was calculated to be $1{\cdot}25 \times 10^{-3}$ M and the K_m for the inhibited enzyme was found to be $1{\cdot}34 \times 10^{-3}$ M.

c. Effect of MSO dosage on the kinetics of GS. Four rats were given respectively (i.p.) saline or 100, 200, or 400 mg of MSO/kg. All animals were sacrificed 3 hr later.

Fraction S_{15} was isolated and the GS activity determined as a function of the concentration of glutamate. Lineweaver-Burk plots[20] of the data are shown in Fig. 3: the degree of inhibition of GS increased with the dose, and the inhibition resisted reversal by glutamate *in vitro*, even after the lowest *in vivo* dose of MSO (100 mg/kg).

d. GT with glutamine. A plot of the kinetic behaviour of GT at different glutamine concentrations yielded a pattern characteristic of a competitively inhibited enzyme (Fig. 4). The calculated K_m for glutamine was 5×10^{-2} M for the enzyme from the

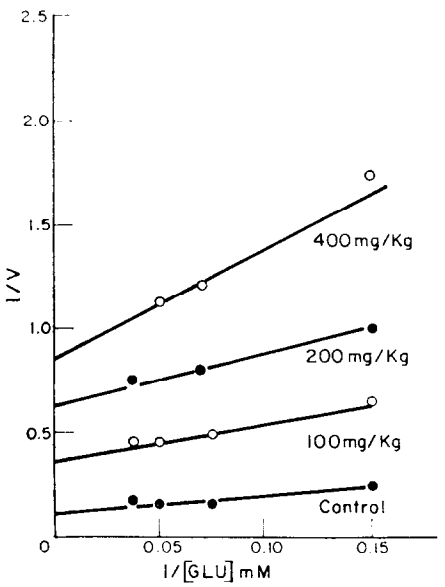

FIG. 3. Effect of increasing doses of MSO on cerebral glutamine synthetase. Conditions for isolation of enzyme (3 hr after administration of MSO) and determination of *in-vitro* activity as in Fig. 1

control rat and 2×10^{-1} M for that from the animal killed 160 min after the administration of MSO (400 mg/kg).

Thus there is an interesting difference between the mode and the severity of inhibition exerted by MSO upon two distinct enzyme activities* heretofore deemed to be associated with the same protein.[21]

Effect of MSO on other cerebral enzymes. It seemed important to assess the specificity of MSO by testing its effects on other cerebral enzymes. Accordingly, lactic dehydrogenase,[22] ATPase,[23] and esterase (substrate: *o*-nitrophenyl acetate)[24] activities were measured in microsomal and soluble preparations obtained from brains of control and MSO-treated animals, and no apparent inhibition of these enzymes was noted. The possibility that the drug inhibits other enzymes, in particular those whose substrates it resembles, is, of course, by no means ruled out.

* Preliminary evidence has been obtained suggesting that the activities of GS and GT reflect the existence of distinct protein entities, inasmuch as differential centrifugation of cerebral homogenates yielded diverging intracellular distributions of the two activities (C. Lamar, Jr. and O. Z. Sellinger, unpublished observations).

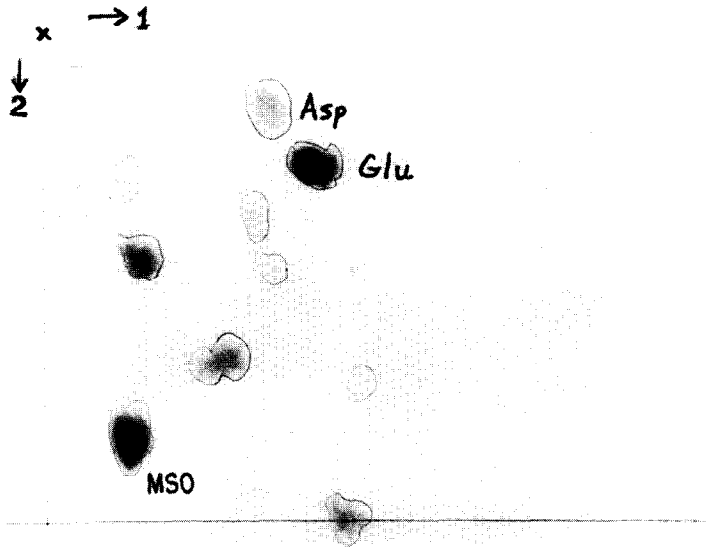

FIG. 5. Bidimensional chromatogram of an extract of rat cerebral cortex, prepared as described in Methods. Authentic MSO was added to the extract before chromatography at starting, marked ×. Arrows indicate direction of chromatography development; 1 and 2 refer to solvents 1 and 2, as listed in Methods. Glu = glutamate; Asp = aspartate.

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Identification of MSO in acid-soluble extract of rat brain. An extract of brain containing free amino acids was prepared (see Methods) and a portion was chromatographed. To a second portion 250 μg MSO was added before chromatography and its migration characteristics determined (Fig. 5). Chromatography of cerebral extracts prepared 4 hr after either the i.p. or the intracisternal (i.c.) administration of MSO revealed a spot migrating identically with authentic MSO. In separate experiments with 1-^{14}C MSO significant radioactivity was present in the MSO spot 3 hr after parenteral or cisternal administration.

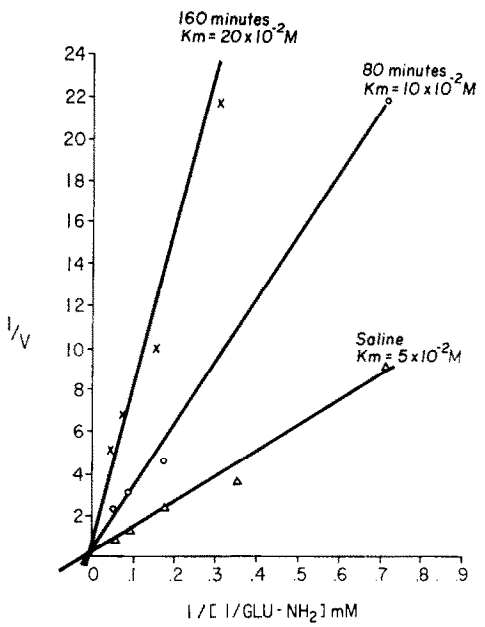

FIG. 4. Inhibition of cerebral glutamine transferase by the *in-vivo* administration of MSO. The enzyme preparation was derived as in Fig. 1, from the same animals on which GS activity was determined (Fig. 1). Enzyme activity was determined *in vitro* as a function of L-glutamine concentration. V = μmoles of glutamylhydroxamate/15 min.

Attempts to reverse the in vivo *inhibition of GS by MSO. By BAL*: In view of the possibility that MSO may have brought about a structural change in the GS and GT molecule(s), as suggested by the altered kinetic behavior of the activities isolated from MSO-treated rats, it was thought that a suitable pretreatment of the animal might protect from the action of MSO. Accordingly, BAL was administered (20 mg/kg, s.c.) at various times in relation to MSO. Although the results were not entirely consistent in that the administration of BAL afforded no protection from inhibition in 2 of 12 experiments, the remainder of the experiments provided definite evidence of protection of both GS and GT. In a typical experiment, the activity of cerebral GS isolated from rats treated with BAL 15 min prior to the administration of MSO and sacrificed 60 min later was compared to that derived from rats given MSO alone or saline alone (Fig. 6, 7). Almost no inhibition of GS and GT was noted 45 min after pretreatment with BAL whereas a 15-min pretreatment resulted in 50% protection.

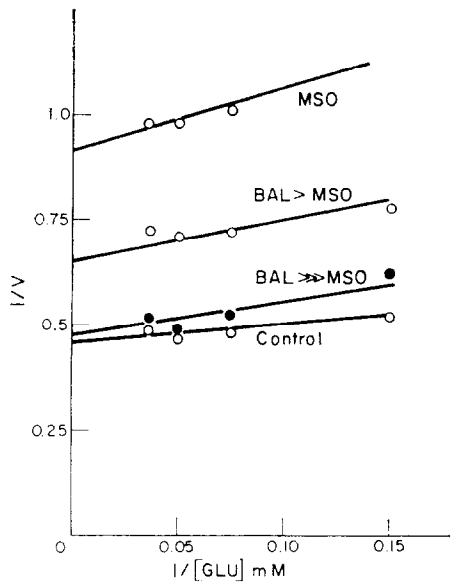

FIG. 6. Protection of cerebral glutamine synthetase from the *in vivo* inhibition by MSO; pre-treatment with BAL. For the conditions of administration of BAL, see text; > and >>> refer to the administration of BAL 15 and 45 mins before MSO respectively.

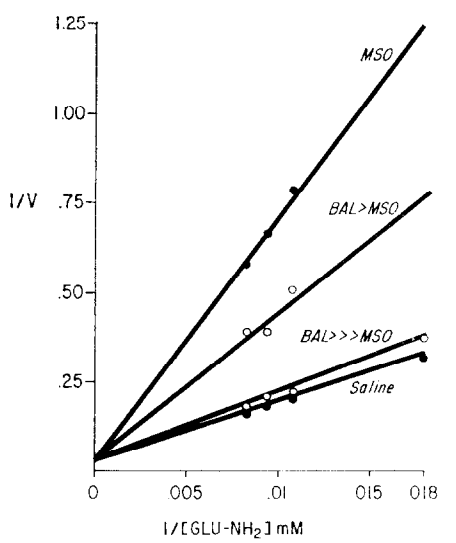

FIG. 7. Protection of cerebral glutamine transferase from the *in-vivo* inhibition by MSO. Pretreatment with BAL. See legend to Fig. 6 for details.

These findings demonstrate that the administration of BAL successfully thwarted the inhibitory action of MSO. Hence it seems possible that the structural damage inflicted by MSO in the GS and GT molecule(s) involves the enzyme sulfhydryl or dithiol[25] residues and that BAL thus served as an effective protective agent for them.

By glutamine. It seemed possible that the MSO-elicited depletion of cerebral glutamine[15, 26] might result in an arrest of the synthesis of proteins, including the GS–GT protein. To test this, L-glutamine (600 mg/kg) was administered to rats 1 hr after MSO, and the animals were sacrificed an hour later. Half the dose of glutamine was administered i.p. and half s.c. After isolation of fraction S_{15} from animals given MSO or MSO + glutamine, and from controls given saline, the GS activity at different glutamate concentrations was determined. There was no apparent reversal by glutamine of the inhibition caused by MSO. Furthermore, in agreement with the findings of Lodin and Kolousek[8], glutamine afforded no protection from seizures.

By methionine. Methionine, in a molar ratio to MSO of 5 : 1·7,[8] was administered simultaneously with, 1 hr before, and 1 hr after MSO, and the animals were sacrificed 2 hr after MSO. The GS activity of fraction S_{15} was determined in the usual manner. When methionine was administered simultaneously with MSO, but not otherwise, no apparent inhibition of GS was noted. The results of the kinetic experiments verifying this observation are shown in Fig. 8.

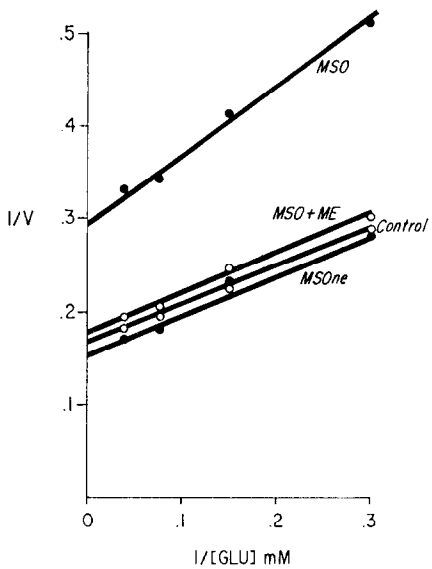

FIG. 8. Protection of cerebral glutamine synthetase from the *in-vivo* inhibition by MSO, by the simultaneous administration of L-methionine. Methionine (ME) was administered in a molar ratio of 5 to 1·7 with respect to MSO. For details of the isolation and determination of GS activity, see legend to Fig. 1.

As reported by Lodin and Kolousek,[8] the simultaneous administration of MSO and methionine is known to diminish MSO-provoked EEG abnormalities in the rat, presumably because less MSO enters the brain as a result of a competition between the two amino acids for passage across the blood-brain barrier.[27] Further evidence for this suggestion was obtained in an experiment in which radioactive MSO ($13{\cdot}5 \times 10^7$ cpm, 400 mg/kg) was administered to two animals of equal weight, one of which received methionine concurrently; the radioactivity present in the brain was determined 3 hr later. The total radioactivity in the brain homogenate of the animal given MSO alone was 19,269 cpm; that in the brain of the animal given MSO + methionine was 9,200 cpm.

Specificity of MSO: in vivo *effects of MSI and MSOne*. When either MSI or MSOne was injected (i.p.) in amounts equimolar with MSO and the kinetics of GS in fraction S_{15} examined, no inhibition of GS was noted.

The interaction of MSO with glutamine synthetase and glutamine transferase. a. In vitro. In analogy to the studies of Wieland *et al.*[28] and Krishnaswamy *et al.*,[29, 30] which demonstrated the formation of a bond between glutamine synthetase and glutamate, it was considered germane to obtain evidence for binding of MSO to the enzyme. Accordingly, samples of fraction S_{15} enzyme were preincubated with 1-^{14}C-MSO or in its absence at 37° or 0° for 30 min under the individual conditions described in the legend to Table 2. At the completion of the preincubation period, 0·5-ml

TABLE 2. THE *IN-VITRO* BINDING OF MSO TO GLUTAMINE SYNTHETASE

Tube	Preincubation	Incubation	Radioactivity	
	GHA formed (μmoles/30 min)	GS activity (units/ml)	Second dialyzate (cpm/ml)	Non-dialyzable (total cpm)
1	0·37	58·0	3·7	192
2	12·0	43·5	9·2	187
3	24·5	77·0		
4	1·04	0·0	13·0	378

Enzyme, fraction S_{15} (see Methods) was used. Aliquots (4 ml) were added to tubes 1 to 4, containing as follows: tubes 1 and 2, Tris buffer (pH 7·2) 120 μmoles; Na–ATP, 80 μmoles, MgCl, 120 μmoles; freshly neutralized hydroxylamine, 240 μmoles; BAL; 1·5 μmoles; L-glutamate, 240 μmoles; and 1-^{14}C-MSO (34 μc/m mole), 55 μmoles. Tubes 3 and 4 as tubes 1 and 2, with MSO omitted in tube 3 and L-glutamate omitted in tube 4. Final preincubated volume: 6·1 ml. Preincubation temperature was 0° for tube 1 and 37° for tubes 2 to 4. Preincubation time was 30 min. The amount of GHA formed was determined (column 1), 0·5-ml aliquots withdrawn and the GS activity (column 2) determined in the assay medium described previously.[17] The activity of a control, non-preincubated sample of fraction S_{15} was 84 units/ml. The remaining enzyme preparation was exhaustively dialyzed until the radioactivity of the dialyzate reached the levels in column 3.

aliquots of the mixture were withdrawn from all tubes and the amount of glutamyl-hydroxamate formed during preincubation was determined. All tubes were then placed in ice to arrest GHA formation. Aliquots (0·3 ml) were removed and assayed in the absence of MSO for residual GS activity (30 min, 37°) to assess the effect of MSO and/or preincubation on the stability of the enzyme. The remaining tube contents were dialyzed, each in a separate container, against 6 liters of 0·002 M Tris buffer, pH 7·2, for 4 hr at 4°. The buffer was replaced, and further dialysis against 6 liters of buffer was carried out for an additional 12 hr. The contents of the dialysis bag were taken for radioactivity determinations and for determination of enzyme kinetics. The results indicate that preincubation (Table 2, column 1) resulted in inhibition of half the GS activity when glutamate was present (tube 2), and in virtually total inhibition when glutamate was absent (tube 4). In agreement with the preincubation findings, the enzyme activity assays (Table 2, column 2) confirmed the virtual loss of GS as a result of exposure to MSO (tube 4). Of interest are the results obtained after preincubation with MSO at 0°, a treatment resulting in virtual arrest of enzyme activity but in only a moderate loss of the catalytic potential of the enzyme. The total radioactivity present in the incubation mixture containing MSO was

determined before and after dialysis, and the radioactivity that resisted dialysis and remained associated with the bag contents is shown in Table 2, column 4.*

Further to assess the nature of the MSO-elicited inactivation of GS, the exhaustively dialyzed enzyme preparations from tubes 1–4 (Table 2) were tested in kinetic experiments. The results revealed the irreversibility of the enzyme–MSO interactions which ensued as a result of preincubation in the absence of glutamate (Table 2, tube 4), inasmuch as no activity could be recovered in this preparation upon incubation at high glutamate concentrations. The plots derived from data obtained with the remaining preparations (Fig. 9) suggest irreversible inhibition by MSO as a result of a

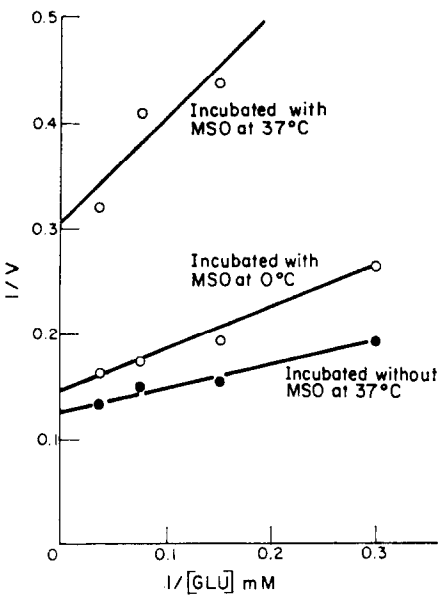

FIG. 9. *In-vitro* preincubation of cerebral glutamine synthetase with MSO: effect on enzyme kinetics. For details of preincubation and incubation conditions see legend to Table 2.

functional alteration of the enzyme molecule. In four separate experiments, when enzyme mixtures similar to those contained in tubes 1 and 2 (Table 2), but preincubated with 1-^{14}C-MSO at 0° and 37° for only 20 minutes, were subjected to exhaustive dialysis, the nondialyzable radioactivity was at least 10 times higher than the radioactivity of the dialysis fluid (cpm/ml). However, when the dialyzed mixtures were subjected to chromatography on DEAE cellulose,† the radioactivity was recovered for the most part in the break-through effluent and no radioactivity accompanied enzyme activity. These findings are interpreted as indicating that the nondialyzable radioactivity became dissociated from its particulate attachment upon passage through the DEAE cellulose column. Further evidence for this was obtained in an experiment in which two enzyme preparations that had been preincubated with

* In separate experiments it could be shown that dialysis of MSO or MSO + bovine serum albumin, under the conditions employed, resulted within 12 hr at 0° in complete equilibration of the radioactivity between the dialysis fluid and the contents of the bag.

† C. Lamar, Jr. and O. Z. Sellinger, unpublished observations.

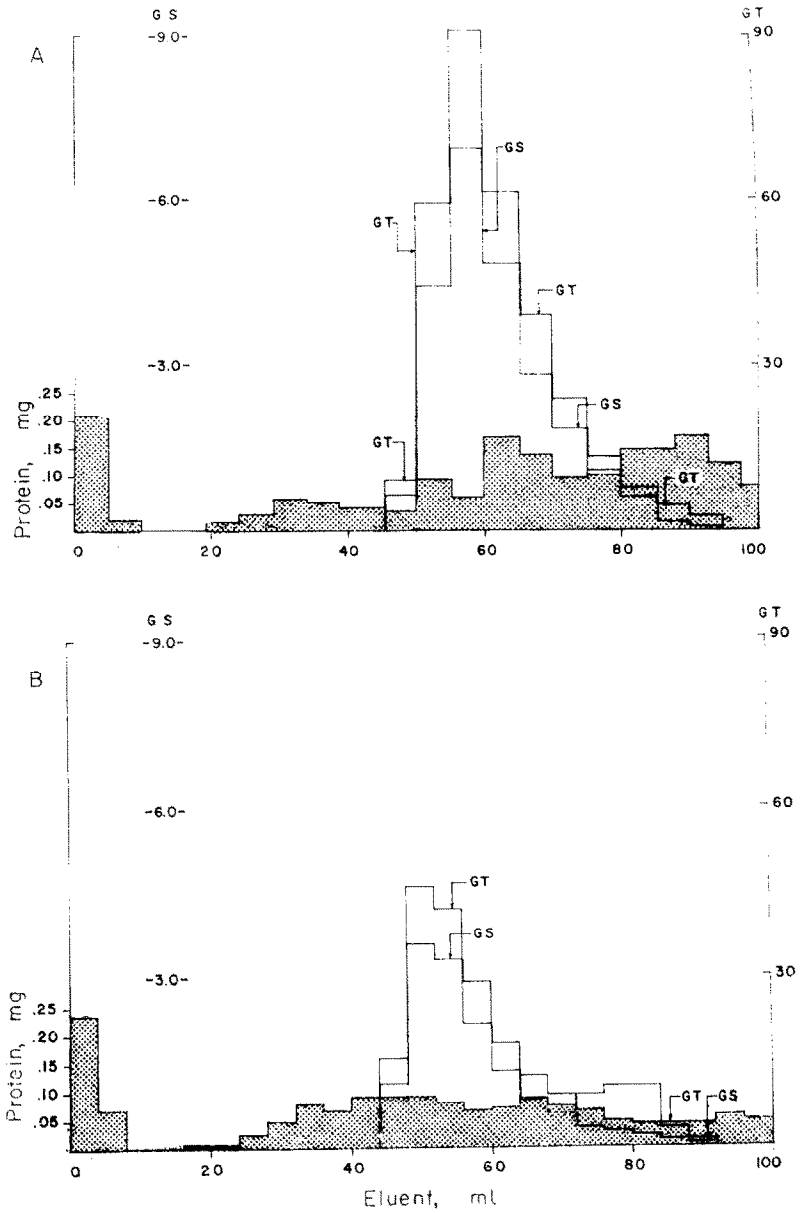

FIG. 10. A. Elution profile of cerebral glutamine synthetase and glutamine transferase activities isolated from the cerebral cortex of normal rats: chromatography on DEAE cellulose. Protein (shaded profile) refers to mg/tube; GS and GT activity is expressed in units/ml of effluent. Fraction S_{15} (see Methods) was concentrated by ultrafiltration (LKB ultrafilter) to contain at least 0·1 mg protein/ml. To each 30 ml of enzyme solution were added, before ultrafiltration, 2·4 ml of 0·1 M ATP (pH 7·2 Na Salt) and 0·6 ml of 0·6 M $MgCl_2$. The resulting ultrafiltrate was dialyzed against 3 liters of 0·003 M Tris buffer, pH 7·0. The column (a 5-ml graduated pipet was used) was packed with Whatman powder DE 50 and was equilibrated at 4° with 500 ml of 0·002 M Tris buffer, pH 7·2. An increasing linear gradient of NaCl was used for elution. The mixing chamber contained 250 ml of 0·004 M Tris buffer (pH 6·8) to which 0·05 ml of mercaptoethanol was added. The reservoir contained the same components but was made 1 M in NaCl; 5 ml aliquots were collected.

B. Elution profile of cerebral glutamine synthetase and glutamine transferase activities isolated from the cerebral cortex of MSO-injected rats. MSO was administered at the convulsive dose of 400 mg/kg and the animals sacrificed 3·5 hr later. For details of chromatographic procedure and definition of units, see above.

MSO, one at 37° and the other at 0°, were compared kinetically after dialysis and simultaneous passage of each through a separate and identical DEAE cellulose column. The two preparations now exhibited an identical kinetic behavior, both with respect to glutamate and to glutamine.

b. In vivo. Soluble preparations of GS (fraction S_{15}) derived from the cerebral cortex of rats treated with MSO were subjected to overnight dialysis and were then chromatographed on DEAE cellulose. Eluates containing GS and GT activity were compared to similar preparations derived from control animals. The elution characteristics of the inhibited and noninhibited enzyme activities were similar except that the enzyme and specific activity peaks of the former were smaller for both GS and GT (Fig. 10 A and B). The kinetics of the peak effluent tubes were determined as a function of the concentration of the respective substrate, L-glutamate or L-glutamine, (Fig. 11 and 12) and the results demonstrate that the inhibition by MSO *in vivo*, in clear contrast to that *in vitro*, resisted reversal by the DEAE cellulose treatment.

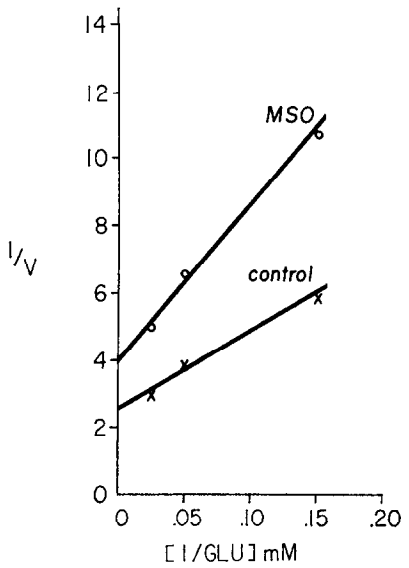

FIG. 11. Kinetics of cerebral glutamine synthetase isolated from the cerebral cortex of the control and MSO-injected rats described in Fig. 10A and B: persistence of irreversible inhibition pattern after DEAE cellulose chromatography. For details see text.

Subcellular Disposition of in vivo 1-^{14}*C-MSO*

Initially it was desired to assess the subcellular distribution of a substance structurally related to MSO but known not to inhibit GS. Radioactive MSI was selected for this purpose. The distribution among six subcellular fractions of 0·115 mg of MSI (44,000 cpm) added to a brain homogenate may be seen in Table 3. A striking feature of the presumably random distribution of MSI was the negligible amount (330 cpm, 0·75%) of radioactivity found associated with the microsomal pellet (fraction PS). Overall recoveries ranging between 90 and 110%, (MS + NML/BH and PS + SS/MS) were noted, indicating that the counting procedures were sufficiently reliable. Particular care was taken to achieve statistically significant counts

in all cases, and samples with low radioactivity and background were counted for a minimum of 300 min.

Radioactive MSO was given to three separate groups of animals and the partitioning of the radioactivity throughout the cerebral subcellular fractions can be seen in

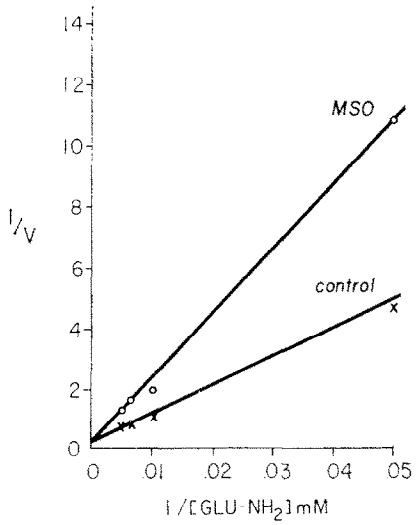

FIG. 12. Kinetics of cerebral glutamine transferase isolated from the cerebral cortex of the control and MSO-injected rats, described in Fig. 10A and B: persistence of reversible inhibition pattern after DEAE cellulose chromatography. For details see text.

TABLE 3. DISTRIBUTION OF RADIOACTIVE METHIONINE SULFOXIDE (MSI) AND METHIONINE SULFOXIMINE (MSO) AMONG THE SUBCELLULAR FRACTIONS OF RAT CEREBRAL CORTEX*

Fraction	MSI added to BH		MSO i.c.		MSO i.p.		MSO & ME i.p.	
	(Total cpm)	% of BH (cpm)	(Total cpm)	% of BH (cpm)	(Total cpm)	% of BH (cpm)	(total cpm)	% of BH (cpm)
BH	44,000	100	95,000	100·0	19,268	100·0	8,410	100·0
NML	7,500	17	25,000	26·0				
MS	35,000	80	80,000	84·0				
SS	32,000	73	77,000	81·0				
PS	300	0·75†	2,620	2·8†	1,700‡	13·0	1,000†	12·0
S	194	0·44	1,120	1·2	350	2·6	250	3·0
P	20	0·045	950	1·0	870	6·6	210	2·5

* BH = brain homogenate; ME = L-methionine; i.c. = intracisternal; i.p. = intraperitoneal.
† PS pellet was resuspended in 0·15 M NaCl and was recentrifuged to yield fractions S and P.
‡ PS pellet was resuspended in water and was recentrifuged to yield fractions S and P.
For definition of fraction symbols, see Methods; for further details, see text.

Table 3. In the first experiment, radioactive MSO (0·2 mg, 74,000 cpm/rat) in a volume of 0·10 ml was introduced i.c. to each of a group of six rats (weight 200 g). The animals were permitted to convulse just prior to sacrifice as evidence that the administration had been properly accomplished. The brains were pooled, homogenized, and

fractionated as described in Methods. The other two experiments each involved a single animal (weight 80 g) to which 1-^{14}C-MSO (400 mg/kg) was administered i.p. ($13{\cdot}6 \times 10^7$ cpm). One of the animals also received unlabeled methionine (ME : MSO, 5 : 1·7). The results point to a nonrandom distribution of radioactivity, inasmuch as in the three experiments with radioactive MSO, fraction PS concentrated, respectively, 2·8, 12 and 13% of the counts originally present in the unfractionated homogenate, as compared to 0·75% of MSI retained in analogous, albeit not identical, experiments. Moreover, when the radioactive microsomal pellet obtained from the MSO, but not the MSI, experiment was suspended either in 0·15 M NaCl (column 2, Table 3) or water (columns 3 and 4, Table 3) and the suspension was recentrifuged, the postmicrosomal pellets contained an appreciable percentage of the recovered counts. It is to be noted, furthermore, that in the experiment in which MSO was given i.p. without methionine and in which the microsomal pellet was washed with water (column 3, Table 3), 72% of the microsomal radioactivity was retained in particulate form, as compared to 45% so retained when the pellet was washed with 0·15 M NaCl (column 4, Table 3). Since this pattern of release of radioactivity and the simultaneously ascertained pattern of release of glutamine synthetase[31] were in good mutual agreement, these findings provide additional evidence for tangible binding of MSO to the glutamine synthetase protein.

DISCUSSION

In this paper evidence is presented which demonstrates an *in-vivo* interaction of the convulsant agent, methionine sulfoximine, with glutamine synthetase and glutamine transferase of rat cerebral cortex. Unlike the *in vitro* interaction of MSO with GS described previously,[14] the effects of the drug *in vivo* on this enzyme activity were found to be irreversible, by the criterion of recovery of normal enzyme maximal velocity upon incubation *in vitro* in the presence of excess substrate (glutamate or ATP, Fig. 1 and 2). Conversely, identity of the *in vitro*[32] and the *in vivo* effects of the drug on glutamine transferase (GT) was noted, for, under both conditions, the inhibitory effect of MSO could be readily reversed at high concentrations of glutamine *in vitro*.

Evidence for an interaction of MSO with both enzyme activities could be obtained as early as 30 min after the i.p. administration of a convulsant dose of the drug (Table 1); i.e. at a time when certainly less than 0·1% of the administered dose was present in the cortex.[33] The inhibition of both activities was progressive with time and was generally of the same magnitude, at equal times, in each of four subcellular fractions tested, indicating that intracellular relocation of the enzyme does not represent a major feature of the drug's action. The progressive nature of the irreversible drug–enzyme interaction is further supported by the results shown in Fig. 3, in which a gradual decline of the extrapolated maximal velocities of GS could be seen to follow the administration of increasing doses of the drug.

The lack of reversal of the GS inhibition by glutamine provides some support for the reported absence of a causal correlation between the low levels of cerebral glutamine,[12, 15, 26] known to follow the administration of MSO, and the occurrence of seizures. Further supporting evidence for the concept that low glutamine levels in the brain per se do not cause convulsive states has recently been reported by

Hathway and Mallinson[31], who showed that after the administration of Telodrin* cerebral glutamine levels are highest just preceding the convulsive period. Also Stone *et al.*[35] have noted no change in glutamine in the cortical tissue of dogs injected with convulsing amounts of pentylenetetrazol. However, the possibility that the low post-MSO levels of cerebral glutamine may be partially the result of a greater than normal efflux of glutamine from the brain, is not entirely precluded, particularly in view of the recent findings of Folbergrova[36] that glutamine levels remain low even after the joint administration of MSO and methionine, a situation resulting in no seizures, no inhibition of cerebral GS, and consequently no impairment of glutamine formation. Roth *et al.*[33] have shown that the administration of MSO together with ^{35}S-methionine augments *in vivo* the hepatic uptake of the latter when compared to the uptake of methionine in the absence of MSO. In this connection, Tews and Stone[26] have recently shown that the *in vivo* administration of MSO results in a depression of the levels of free cerebral methionine. In the present experiments it could be shown that the simultaneous administration i.p. of ^{14}C-MSO and ^{12}C-L-methionine resulted in a 50% decrease of uptake of ^{14}C-MSO by the brain, when compared to the uptake of ^{14}C-MSO administered alone. Moreover, evidence was obtained which suggests that the amount of MSO that did reach the brain when given together with methionine failed to interact irreversibly with GS, as shown by the results plotted in Fig. 8.

However, since other results (Fig. 3) point to a strong inhibition of GS by subconvulsive doses of MSO—i.e. by cerebral MSO levels which, presumably, should be similar to those present in the brain after giving a convulsive dose (400 mg/kg) of MSO together with methionine—a discrepancy arises. There must be explained, on one hand, the observed inhibition of GS by subconvulsive doses of MSO given alone and, on the other, the complete lack of any effect on the enzyme of convulsive doses of the drug accompanied by methionine. This contradictory situation emphasizes the special role of methionine as a modulator of the effects of MSO on brain metabolism and function. Additional evidence that methionine counteracts the effects of MSO could be secured on the subcellular level by showing (Table 3) that water-washed microsomal pellets (P_{H_2O}) prepared from animals receiving ^{14}C-MSO alone retained about 26% more radioactivity (presumed to be in association with GS protein), than similar pellets isolated from animals receiving MSO + methionine. Thus, it appears possible to maintain the concept that methionine exerts a protective action against the effects of MSO upon GS at the enzyme site(s) which may be vulnerable to MSO attack. Some evidence for the nature of the *in vitro* requirements for GS–MSO interaction as well as of the enzyme site(s) that might be affected by MSO *in vivo* was obtained in the present study. Thus, from the results of the *in vitro* binding experiments (Table 2), it is apparent that exposure of the enzyme to MSO, in the absence of glutamate, led to loss of catalytic function of the enzyme and resulted in maximal binding of MSO radioactivity. The latter occurred, presumably, because of the structural similarity of MSO and glutamate and is thus in support of the previously established role of glutamate as a competitive inhibitor of GS *in vitro*.[11] The *in vitro* experiments further revealed that the interaction of MSO with GS is not dependent on enzyme catalysis, for equal amounts of MSO radioactivity remained in association with the enzyme, irrespective of the temperature of preincubation. Moreover, as the

* (1, 3, 4, 5, 6, 7, 8, 8-octachloro-1, 3,-3α, 4, 7, 7α-hexahydro-4, 7-methanoisobenzofuran).

interaction of MSO with the protein endowed the inhibited enzyme with altered kinetics toward glutamate, a structural modification of the enzyme is suggested as best representing the *in vitro* effect of MSO on the enzyme. On the other hand, it could be shown that the alteration wrought by MSO *in vitro*, unlike that *in vivo*, is not totally irreversible; thus by chromatographing the inhibited enzyme on DEAE cellulose, it was possible to achieve a return of the kinetics to pre-MSO values.

The experiments in which rats were pretreated with BAL 45 min before MSO and protection of GS and GT from MSO was achieved (Fig. 6, 7) indicate that the MSO-interacting site of GS may be the latter's dithiol component.[25] Thus a suitably timed administration of BAL would afford the brain an SH pool of sufficient magnitude and possessing a requisite lifetime to counteract the damaging effects of MSO on the enzyme. The mechanism of the action of MSO *in vivo* on the enzyme activities studied may thus relate to an attack of the drug on the functionally essential group(s) of the GS protein. Although the detailed features of such a process are at present unknown, model experiments on the reaction of aliphatic dithiols with sulfoxides[37] yielding dithianes suggest the possibility of analogous dithiol–sulfoximine interactions. This proposed relationship could equally account for the inhibitory, yet readily reversible effects of MSO on GT, an enzymic activity known not to depend on SH groups. Thus, it could be assumed that the state of the SH groups of GS may in some manner reflect upon the conformation and, hence, the activity of the GT protein[32] or, as the case may be, of the GT "head" of the GS protein,[21] but that this interaction produces a less severe and, therefore, reversible functional alteration than that resulting from the direct attack of MSO on GS. Whether such considerations may also apply to the effects of MSO on other species, e.g. the dog and the rabbit, in which small doses of MSO (3–5 mg/kg) are convulsant, is at present difficult to evaluate. Species differences in the nature of the MSO–glutamine synthetase interaction may, however, be expected, in view of the recently described species differences of intracellular location and of structural attachment of the enzyme in a number of cerebral regions examined.[38]

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