THE EFFECT OF METHIONINE ON THE REGIONAL AND INTRACELLULAR DISPOSITION OF [3H]-METHIONINE SULPHOXIMINE IN RAT BRAIN[1]

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Abstract—The intracellular disposition of the convulsant agent, methionine sulphoximine (MSO), administered as methyl-labelled [3H]MSO, was examined in rat brain. Intraperitoneal (i.p.) and intrathecal (i.th.) routes were compared. The effect of simultaneous administration of methionine on the uptake, the regional distribution and the intracellular disposition of [3H]MSO was also assessed:

(1) The peak uptake of i.p. [3H]MSO was at 2 h and amounted to about 1 per cent of the dose; the peak uptake of i.th. [3H]MSO was at 30 min post-injection and amounted to 40 per cent of the administered dose. The uptake was effectively reduced when methionine was simultaneously administered.

(2) The regional distribution of [3H]MSO as a function of time after injection revealed a rather uniform penetration of the entire brain by the drug. A maximum of 43 per cent of the tissue radioactivity was found in the cerebellum 2 h after i.p. injection, while 49 per cent accumulated in the extracortical portion of the brain 3·5 h after i.th. administration. Methionine did not affect the regional distribution of [3H]MSO.

(3) Differential centrifugation of samples of cortex and cerebellum revealed an association of [3H]MSO with intracellular particulate fractions. Since closely similar proportions of MSO occurred in the crude mitochondrial and the microsomal fractions, these fractions were analysed further: (a) [3H]MSO was bound to nerve endings sedimenting at the 1·0 M–1·2 M-sucrose interface; this binding was not abolished by prior increase of the endogenous cerebral methionine pool; and (b) [3H]MSO was released by subjecting the nerve endings to osmotic shock. However, the striking finding was that [3H]MSO could *not* be released from the nerve endings of the cerebellum from animals pre-treated with methionine.

(4) An association of [3H]MSO was observed with the membranes of the endoplasmic reticulum and specifically with its agranular component.

(5) The results implicate the cerebellum as the primary target for MSO, in confirmation of the original observations of LODIN (1958).

METHIONINE sulphoximine (MSO) has been used extensively in the study of experimental epilepsy (REINER, MISANI and WEISS, 1950; PROLER and KELLAWAY, 1962, 1965; JOHNSON, GOLDRING and O'LEARY, 1965) because it is a convulsant agent capable of eliciting after a delay of several hours, recurrent bouts of seizures interspersed with quiescent interictal periods (LODIN and KOLOUSEK, 1958a, TOWER, 1960; WOLFE and ELLIOT, 1962). As a possible biochemical correlate of the seizure phenomenon, several workers have studied in detail the specific *in vivo* and *in vitro* inhibition by MSO of cerebral glutamine synthetase (L-glutamate:ammonia ligase (ADP) EC 6.3.1.2) (PETERS and TOWER, 1959; SELLINGER and WEILER: 1963; LAMAR and SELLINGER, 1965; SELLINGER, 1967; DE ROBERTIS, SELLINGER, RODRÍGUEZ DE LORES ARNAIZ, ALBERICI and ZIEHER, 1967; LAMAR, 1968), yet these and subsequent studies have ruled out a causal relationship between the convulsant and the enzyme-inhibitory effects of the drug (SELLINGER, AZCURRA and OHLSSON, 1968). More

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Abbreviations used: MSO, methionine sulphoximine; i.p., intraperitoneal; i.th., intrathecal.

recently SELLINGER and OHLSSON (1969a) showed that after inhibition by MSO, the return to normal levels of activity of cerebral glutamine synthetase was not the same in three of the intracellular compartments with which the enzyme is known to be associated in brain tissue (SELLINGER and DE BALBIAN VERSTER, 1962, SELLINGER and DOMER, 1964; SELLINGER, DE BALBIAN VERSTER, SULLIVAN and LAMAR, 1966; SELLINGER and AZCURRA, 1968).

Even though it has been long known that methionine given together with or shortly before or after MSO prevents MSO-induced seizures (REINER et al., 1950, LODIN and KOLOUSEK, 1958b, LAMAR and SELLINGER, 1965), the chemical specificity of the seizure-protective effect has been studied only recently (SELLINGER et al., 1968). These studies revealed: (a) that several methionine analogues and derivatives are equally effective, and (b) that a reversible breakdown of a part of cerebral polyribosomes to mono- and dimeric ribosomes coincident with a transient stimulation of cerebral protein synthesis accompanies, and possibly mediates the seizure-protective action of methionine (SELLINGER and AZCURRA, 1970). LODIN and his co-workers (LODIN, FALTIN, PILNY and HARTMAN, 1968; LODIN, MULLER and FALTIN, 1968) have also recently described effects of MSO on nucleic acids and proteins in neural tissue. Previously the intracellular disposition of MSO in brain had been examined only cursorily (LAMAR and SELLINGER, 1965; DE ROBERTIS et al., 1967), mainly because MSO of sufficiently high specific radioactivity was unavailable. The present report describes the results of a study of the fate of [³H]MSO in the cerebral cortex and the cerebellum of the rat and of the effects of methionine thereon.

EXPERIMENTAL PROCEDURE

Materials. L-Methionine-DL-sulphoximine, D-methionine and ninhydrin were obtained from Pierce Chemical Co. (Rockford, Ill.); α-methyl-DL-methionine was purchased from Cyclo Chemical Corp. (Los Angeles, Calif.); and L-methionine was obtained from Nutritional Biochemicals (Cleveland, Ohio). Crystalline bovine serum albumin was a product of Calbiochem (Los Angeles, Calif.) and sucrose was the enzyme grade obtained from Mann Research Laboratories (New York City), 2,5-diphenyloxazole (PPO) and 1,4 bis-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) were purchased from Packard Instrument Co. (Downers Grove, Ill.) and Biosolv-3 was from Beckman Instrument Co. (Palo Alto, Calif.). The [³H]MSO, labelled in the methyl group was custom-synthesized by Tracerlab (Boston, Mass.). As shipped (5·1 mCi/mmol), the product was about 65% [³H]MSO and 35% [³H]methionine sulphoxide. The procedure to remove the sulphoxide is described below. Cellulose MN 300 for thin-layer chromatography was a product of Brinkman (Westbury, N.Y.).

Animals. Male rats, (Sprague–Dawley; 17–20 days old), were used throughout the study.

Preparation and sampling of animals. Both intrathecal (i.th.) and intraperitoneal (i.p.) injections of [³H]MSO were administered. Unless stated otherwise, methionine and α-methyl-DL-methionine were injected i.p. The doses of drugs and the amounts of radioactivity administered varied and are specified individually in the legends to the Tables and Figures. The animals were killed by decapitation, and the excised brain areas were placed in preweighed beakers containing 0·25 M-sucrose.

Primary and gradient fractionation of brain samples. The brain samples were homogenized and the subcellular fractions were isolated by the differential and gradient centrifugation schedules of DE ROBERTIS and co-workers (DE ROBERTIS, PELLEGRINO DE IRALDI, RODRÍGUEZ DE LORES ARNAIZ and SALGANICOFF, 1962; DE ROBERTIS, RODRÍGUEZ DE LORES ARNAIZ, SALGANICOFF, PELLEGRINO DE IRALDI and ZIEHER, 1963). The osmotic shock of the crude mitochondrial fraction was carried out as recently described (SELLINGER, DOMINO, HAARSTAD and MOHRMANN, 1969).

Isolation of polyribosomes. The free polyribosomes (not attached to the endoplasmic reticulum) were isolated according to CAMPAGNONI and MAHLER (1967) with the modifications recently described (SELLINGER and OHLSSON 1969b; SELLINGER and AZCURRA, 1970). The polyribosomes attached to the granular endoplasmic reticulum and the ribosome-free agranular endoplasmic reticulum were isolated according to published techniques (SELLINGER and AZCURRA, 1968; SELLINGER et al., 1968). Additional details are provided in the appropriate legends.

Isolation of ribosomal subunits. The free and the attached polyribosomes were treated with 67 mM-EDTA (AZCURRA and SELLINGER, 1967) and the suspensions were centrifuged in 15–40% (w/v) linear sucrose gradients in the Spinco SW-25.1 rotor for 13 h at 60,000 g to separate the large and small ribosomal subunits.

Analytical. The absorbance of the polyribosomes and of the ribosomal subunits was monitored at 254 nm by pumping the density gradients (proportioning pump; Technicon, Chauncey, N.Y.) through the bottom of the centrifuge tube and through a rectangular flow-cell (0·4 cm light path) into fraction-collector tubes (LKB Instruments, Rockville, Md.) at a rate of 7 drops/tube. Protein was determined according to LOWRY, ROSEBROUGH, FARR and RANDALL (1951) with bovine serum albumin as standard. RNA was determined according to FLECK and BEGG (1965).

Radioactivity. All measurements were carried out with a Unilux II scintillation spectrometer (Nuclear Chicago Co., Des Plaines, Ill.). Quenching corrections were applied using the channels ratio method. A PDP-8 computer (Digital Computer Corp., Maynard, Mass.) equipped with a program to correct for differences in quenching between individual samples, converted c.p.m. into d.p.m.

The samples were prepared for counting as follows: portions of suspensions of the subcellular fractions or of contents of gradient tubes were pipetted into scintillation vials to which water (up to 1 ml) followed by Biosolv-3 (2 ml) were added and the vials placed on a 37°C bath for 1 h to bring the samples completely into solution. The scintillation counting fluid was 10 ml of toluene containing 4 g of PPO and 0·1 g of dimethyl POPOP/l. Radioactivity of paper chromatograms was determined by counting 1 cm-wide paper rectangles totally submerged in the scintillation fluid. No decrease in counting efficiency over that of counting eluates was noted.

Purification of [³H]MSO. TLC on cellulose-coated plates developed in n-butanol:acetic acid:water (12:3:5, by vol.) successfully separated MSO (R_F: 0·26) and methionine sulphoxide (R_F: 0·35). The area containing MSO was transferred into 10 ml of water in a 30-ml centrifuge tube fitted with a Teflon-lined screw-cap. Elution of the [³H]MSO from the cellulose was by vigorous sonication of the tube for 2 min on a Vortex Junior mixer. The resulting slurry was centrifuged (12,000 g, 30 min) and the supernatant fluid was concentrated *in vacuo*.

Isolation of [³H]MSO *from tissue homogenates.* The tissue was homogenized in 1% (w/v) picric acid and centrifuged at 12,000 g for 10 min. The pellet was washed once and the suspension was re-centrifuged as before. The pooled supernatant fluids were applied to a column (1 × 20 cm) of Dowex-AG2 × 10 (50–100 mesh) in the Cl⁻ form. [³H]MSO was quantitatively eluted with about 45 ml of 0·002 M-HCl.

Paper chromatography. Whatman 3 MM paper was used. The chromatograms were developed ascending in n-butanol:acetic acid:water (12:3:5, by vol.).

RESULTS

Recovery of [³H]MSO *from brain and effect of route of injection.* A rat injected i.p. with [³H]MSO was killed 4 h later. The radioactivity found in homogenates of the brain (including the cerebellum) was 0·41 per cent of the injected dose (or 2·30 × 10⁵ d.p.m.). The free amino acids were isolated as described in Methods, and a small portion of the eluate from Dowex-AG-2 was streaked on a strip of Whatman 3 MM paper. After chromatographic development (see Methods), the area of radioactivity on the paper strip that corresponded in R_F value to authentic [¹²C]MSO was cut out and its radioactivity was counted. Recovery was quantitative (2·64 × 10⁵ d.p.m.). Identical results were obtained for brain samples taken 2 h after i.p. injection of [³H]MSO. In a similar experiment, [³H]MSO and L-[¹²C]methionine were injected together and the animal killed 2 h later. Again results were similar.

LAMAR and SELLINGER (1965) found that approximately 20 per cent of the [¹⁴C]-MSO injected intracisternally into adult rats could be recovered in the cerebral cortex about 2 h after injection; conversely, less than 0·02 per cent of an intraperitoneal dose resided there 3 h post-injection. These findings were extended by DE ROBERTIS *et al.* (1967), who showed that about 3 per cent of an i.th. dose could be recovered in the cerebral cortex at about 1·5 h post-injection. A more extensive comparison of the effectiveness of the route of injection on the uptake of MSO by the brain showed
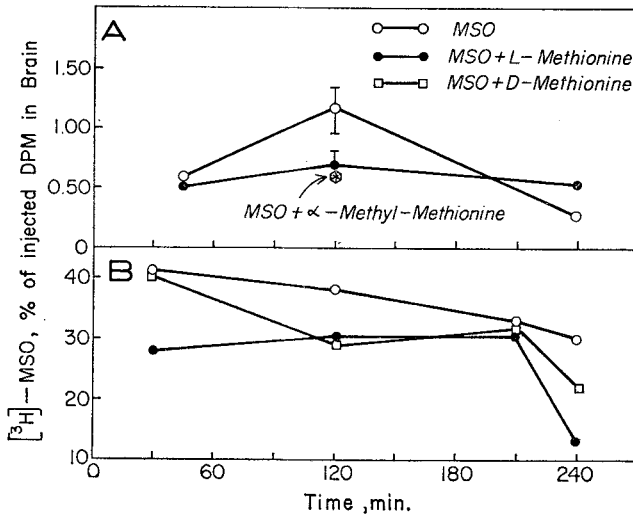
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FIG. 1.—*The uptake by rat brain of intraperitoneally- (A) or intrathecally- (B) administered* [³H]MSO.

(*A*) [³H]MSO (9·0 × 10⁶ d.p.m.–5·7 × 10⁷ d.p.m. in the various experiments) together with carrier [¹²C]MSO to provide a dose of 0·94 mmol/kg was injected i.p. and the animals were killed at the times indicated. The radioactivity measured in brain represented the sum of the d.p.m. present in the cerebral cortex, the cerebellum and the rest of the brain. The vertical bars for points at 120 min after injection indicate the full or upper range of values, respectively. When administered simultaneously, the i.p. dose of methionine or of α-methyl-DL-methionine was 4·7 mmol/kg. Each point is an average of values obtained from at least three individual determinations, except for the experiment with α-methyl-DL-methionine (two determinations).

(*B*) In a representative experiment, [³H]MSO (2·4 × 10⁵ d.p.m.) together with 0·37 μmol of [¹²C]MSO and when indicated, 2·2 μmols of L- or D-methionine, were injected i.th. Each point is the average of two separate determinations.

(Fig. 1) that a distinctly higher proportion of the [³H]MSO reached the brain when administered i.th. than when given i.p. Methionine partially blocked the entry of MSO into the brain. The amounts of [³H]MSO in the brain were lower when the two substances were given together than when the drug was given alone, an effect particularly evident 2 h after the i.p. injection. The dependence of levels of cerebral [³H]MSO on the molar ratios of methionine to MSO is illustrated in Table 1.

TABLE 1.—EFFECT OF THE RATIO OF METHIONINE TO [³H]MSO ON THE UPTAKE OF [³H]MSO BY THE BRAIN

Ratio: L-Methionine / [³H]MSO	Number of animals	[³H]MSO in brain*
5·0	5	0·71 ± 0·08†
2·0	2	0·86; 0·76
0·5	2	0·86; 1·10
0	5	1·15 ± 0·18†

Extent of uptake was determined 2 h after the simultaneous i.p. injection of both substances. MSO was administered in a dose of 094· mmol/kg, including 5 × 10⁶ d.p.m. of [³H]MSO; L-Methionine was administered in the molar excess to MSO, as indicated.

* Expressed as % of the injected dose. Values represent means ± S.D. or individual values.

† These two sets of values are significantly different (*P* < 0·025).

The regional distribution of [³H]MSO *in brain.* [³H]MSO was measured in the cerebral cortex, the cerebellum and the rest of the brain in order to detect any gross differences in its regional distribution. Peak levels of [³H]MSO were reached in these three regions 2 h after injection (Fig. 2), but were prevented if a five-fold molar
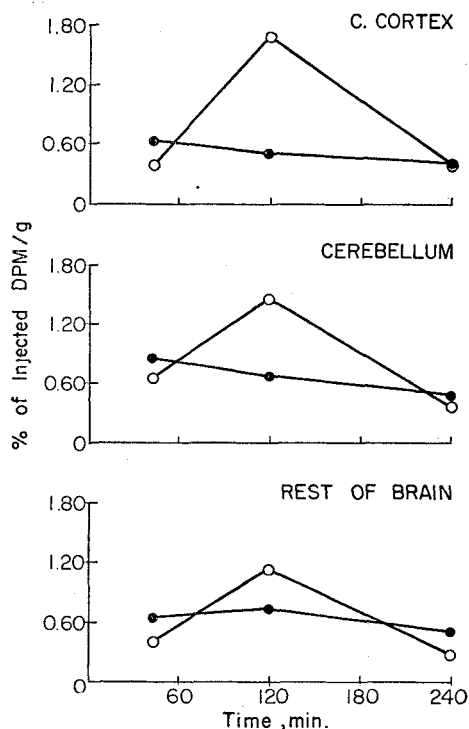

FIG. 2.—*The regional uptake of intraperitoneally administered* [³H]MSO *as a function of time after injection.* The radioactive drug with carrier was administered as described in the legend of Fig. 1 (*A*). At the times indicated, the animals were killed and the desired brain region was dissected out and homogenized (see Methods). The radioactivity was determined on portions of the homogenate and was corrected to unit weight to permit expression as concentration. O— — — —O: [³H]MSO only; ●— — — —●: [³H]MSO + L-methionine.

excess of L-methionine was injected simultaneously. However, at early times after the injection of MSO + methionine (0–45 min) more MSO was found in the brain than when the drug was given alone, indicating a rapid *initial* tissue uptake of MSO. Subsequently the uptake of MSO slowed as methionine progressively flooded the tissue. The highest uptake of [³H]MSO was by the cerebral cortex in which 1·65 per cent of the injected dose had accumulated by 2 h (Fig. 2).

In experiments involving i.p. administration of [³H]MSO, the pattern of [³H]-MSO distribution did not change appreciably over the subsequent 4-h period or in the three regions examined and was not influenced by methionine or by its analogue, α-methyl-DL-methionine (Table 2). Thus, cortical [³H]MSO remained at about 25 per cent of the total tissue radioactivity, the cerebellar [³H]MSO at about 35–45 per cent, and the subcortical levels of the drug varied between 30 and 40 per cent. However, upon i.th. administration, the flux of [³H]MSO into the various brain parts was

TABLE 2.—THE EFFECT OF METHIONINE ON THE REGIONAL DISTRIBUTION OF [³H]MSO IN RAT BRAIN

Brain region	[³H]MSO			[³H]MSO + L-methionine		
	After intraperitoneal administration					
	45 min	120 min	240 min	45 min	120 min	240 min
Cerebral cortex	28·4	23·5	26·0	27·2	25·8 23·8*	26·5
Cerebellum	41·0	43·0	36·2	32·5	35·2 35·9*	35·6
Rest of brain	30·6	33·5	37·8	40·3	39·0 40·3*	37·9
	After intrathecal administration					
	30 min	120 min	240 min	30 min	120 min	210 min
Cerebral cortex	31·3	21·6	19·5	15·0	22·0 26·5†	26·2
Cerebellum	22·0	32·2	31·5	37·4	44·5 44·6†	38·5
Rest of brain	46·7	46·2	49·0	47·6	33·5 28·9†	35·3

Results are expressed as percentage of total brain d.p.m.

The dose of i.p. MSO was 0·94 mmol/kg; this included 2–5 × 10⁷ d.p.m. of [³H]MSO. When given, the i.p. dose of L-methionine was 4·7 mmol/kg. A convulsant dose of intrathecal MSO was used. In the various experiments, this ranged between 1 and 1·5 × 10⁵ d.p.m. and included between 0·36 and 0·52 μmol of [¹²C]MSO carrier. When injected intrathecally, L-methionine was in five to seven-fold molar excess to MSO.

* α-Methyl-DL-methionine, equimolar with methionine was administered.

† D-Methionine was administered in lieu of the L-isomer.

much more time-dependent and could be readily influenced by methionine. In the cerebellum 44·5 per cent of the tissue [³H]MSO was present at 2 h after the injection of [³H]MSO plus methionine, whereas only 32·2 per cent was found when [³H]MSO alone was given. The subcortical levels exhibited the opposite changes (i.e., 46·1 per cent and 33·5 per cent respectively) when [³H]MSO was given alone or together with methionine. Interestingly, the cortical share of [³H]MSO was unaffected under all conditions tested.

The subcellular distribution of [³H]MSO *administered intrathecally.* Cortical and cerebellar homogenates were fractionated according to the procedure of DE ROBERTIS *et al.* (1962, 1963) and four primary fractions (see legend, Table 3) and five subfractions of the particulate fraction NEML were obtained. The procedure was not modified for the cerebellum, a brain region whose subcellular fractions have recently been examined by DEL CERRO, SNIDER and OSTER (1969). We also included an experiment in which the subcellular distribution of [³H]MSO was examined in brains of animals injected i.p. with L-methionine (4·7 mmols/kg) 90 min before i.th. injection of [³H]-MSO to assess the influence of an elevated tissue pool of methionine on the subcellular disposition of [³H]MSO.[1] At 30 min post-injection there were no regional differences in the subcellular distribution of [³H]MSO among the primary fractions of the two brain regions; about 70 per cent of the administered dose appeared in the soluble fraction (S). The 13–14 per cent which sedimented in the particulate fraction (NEML) was concentrated in gradient fractions 1·2 M and 0·8 M (Table 3). High levels of

TABLE 3.—THE EFFECT OF HIGH LEVELS OF CEREBRAL METHIONINE ON THE INTRACELLULAR DISTRIBUTION OF [³H]MSO IN THE CEREBRAL CORTEX AND THE CEREBELLUM

Subcellular fraction*	Cerebral cortex				Cerebellum			
	[³H]MSO		[³H]MSO + methionine		[³H]MSO		[³H]MSO + methionine	
	%	SBR†	%	SBR†	%	SBR†	%	SBR†
N	3	0·17	3	0·19	6	0·19	6	0·22
NEML	13	0·48	11	0·43	14	0·52	7	0·38
Mic	14	0·53	15	0·45	11	0·67	5	0·23
S	70	1·49	71	1·84	69	1·68	82	1·90
Recovery	95·5		95		84		98	
0·8 M	21	1·32	18	1·50	15	0·72	20	0·60
1·0 M	14	1·01	9	1·32	10	0·92	19	1·57
1·2 M	44	2·19	50	1·33	36	1·37	30	1·20
1·4 M	16	0·45	16	0·44	27	0·94	10	0·41
Pellet ('p')	5	0·22	7	0·37	12	0·68	21	0·78
Recovery	78		88		85		70	

L-Methionine (4·7 mmols/kg) was given i.p. 90 min before [³H]MSO; 1 × 10⁸ d.p.m. of [³H]MSO with no carrier were administered intrathecally. The animals were killed 30 min later.

Values represent means of three experiments. Individual recoveries in primary fractions [(d.p.m. in fractions/d.p.m. in homogenate) × 100] were: cortex: 91, 98 and 98 per cent; cerebellum: 71, 94 and 87 per cent in the three successive experiments. NEML subfraction recoveries were: cortex, 70, 78 and 86 per cent; cerebellum; 94, 70 and 91 per cent. The MSO + methionine values are from a single experiment. See text for details of fractionation procedures.

* N = Nuclei and cell debris; NEML = nerve endings, mitochondria and lysosomes, Mic = microsomes, S = soluble cell sap.

† SBR: specific binding ratio: d.p.m.⁻¹ mg of protein⁻¹ in fraction/d.p.m.⁻¹ mg of total protein⁻¹.

tissue methionine did not affect the subcellular distribution of [³H]MSO in the cerebral cortex, whereas in the cerebellum, a diminution of the proportion of particulate [³H]MSO from 31 per cent to 18 per cent of the dose in fractions N + NEML + Mic was noted (Table 3).

The effect of hypotonicity on the release of particulate [³H]MSO. The fractions from 0·8 M to 1·4 M isolated as gradient bands were centrifuged at 269,000 g for 45 min and recovered as pellets. Aqueous suspensions of these pellets and of fraction 'p' (Table 3) were homogenized at about 1300 rev./min for 1 min (SELLINGER et al., 1969). The particulate remnants of the osmotically-shocked particles were obtained by centrifugation at 269,000 g for 60 min. The supernatant fluid represented the intraparticulate contents and, in the case of fraction 0·8 M, presumably included material solubilized from myelin by the centrifugal recycling procedure. The partition of [³H]MSO and of protein between the particulate and soluble phases was determined (Table 4). [³H]MSO resisted solubilization from the cortical and cerebellar fraction 'p', indicating that the low levels of radioactivity found in this fraction (Table 3; 5 and 12 per cent, respectively, of the cortical and cerebellar fraction NEML) reflected a true association of [³H]MSO, presumably with mitochondria and/or lysosomes (SELLINGER and NORDRUM, 1969). In view of the high value for relative specific

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¹ In separate experiments it could be shown that maximal levels of methionine were reached in the brain about 2 h after i.p. injection: 0·128 μmol/g contrasted with 0·058 μmol/g in non-injected controls (GHITTONI and SELLINGER, unpublished observations).

Table 4.—The effect of high tissue levels of methionine on the release of particulate [³H]MSO by osmotic shock

Gradient subfraction	Cerebral cortex		Cerebellum	
	[³H]MSO RSR*	[³H]MSO + methionine RSR*	[³H]MSO RSR*	[³H]MSO + methionine RSR*
0·8 M, soluble	0·77	1·22	0·61	1·36
0·8 M, particulate	1·68	0·29	2·26	0·36
1·0 M, soluble	1·33	1·25	0·91	0·98
1·0 M, particulate	0·50	0·17	1·18	1·05
1·2 M, soluble	2·20	2·56	1·30	0·44
1·2 M, particulate	0·24	0·13	0·50	3·56
1·4 M, soluble	2·62	4·78	1·16	0·38
1·4 M, particulate	0·45	0·17	0·73	6·60
Pellet, soluble	0·79	1·14	0·73	1·13
Pellet, particulate	1·55	0·25	1·83	0·21

Gradient subfractions from the NEML primary subcellular fraction (Table 3, and text) were centrifuged at 269,000 g for 45 min. The pellets plus pellet 'p' (Table 3) were suspended in H_2O, homogenized for 1 min (Sellinger et al., 1969) and re-centrifuged at 269,000 g for 60 min. The partition of [³H]MSO and of protein between particulate and soluble (intraparticulate) phases are tabulated. See text for discussion.

* RSR = relative specific radioactivity: % of total d.p.m./% of total protein.
For description of fractions, see De Robertis et al. (1963).

radioactivity obtained for the particulate component of fraction 0·8 M, an interaction of [³H]MSO with myelin was also suggested. On the other hand, fractions 1·0 M, 1·2 M and 1·4 M, particularly in the cerebral cortex, released most of their radioactivity upon exposure to hypotonic conditions, as indicated by the high values for relative specific radioactivity of the soluble component. There was also an effect of the high tissue pools of methionine on the release of particulate [³H]MSO that was apparently specific for the cerebellum. Thus, equal proportions of [³H]MSO were released from the control and 'high methionine' cortical nerve endings, whereas the release of the drug in the cerebellum occurred readily from the control but not from the 'high methionine' nerve endings (Table 4).

The submicrosomal disposition of [³H]MSO. Since 14 and 13 per cent of the total tissue radioactivity in the cortex and the cerebellum, respectively exhibited a microsomal localization (Table 3), this fraction was separated into its two principal membranous components, the granular endoplasmic reticulum studded with polyribosomes and the polyribosome-free agranular endoplasmic reticulum by means of a centrifugal procedure modelled after that of Dallner (1963) and described in detail in previous publications from this Laboratory (Sellinger and Azcurra, 1968; Sellinger et al., 1968; Sellinger and Ohlsson, 1969b; Sellinger and Azcurra, 1970). The association of [³H]MSO with free polyribosomes (unattached to the endoplasmic reticulum) and with ribosomal subunits was also assessed. The results (Table 5) indicate that a large proportion of the microsomal [³H]MSO was adsorbed to the agranular endoplasmic reticulum, since recycling of this fraction by suspension and recentrifugation resulted in the release of about 80 per cent of its radioactivity (Table 5, Expt. 1). The interesting feature of these findings was the significantly smaller concentration of [³H]MSO found associated with the granular than with the agranular endoplasmic reticulum. This preferential association of MSO with the agranular endoplasmic reticulum could be accentuated even further by expressing the specific

TABLE 5.—THE ASSOCIATION OF [³H]MSO WITH SUBMICROSOMAL COMPONENTS

Experiment	Subcellular fraction*	Cerebral cortex			Cerebellum		
		d.p.m./g	d.p.m./mg of protein	d.p.m./µg of RNA/g	d.p.m./g	d.p.m./mg of protein	d.p.m./µg of RNA/g
1	Microsomes	19,200	6500	37	33,150	3900	119
	Agranular ER	2050	3300	186	2920	5340	185
	Granular	840	770	1·9	1300	2600	11·4
2	Granular ER	2900	1600	6·4	1450	3100	12·7
	Polyribosomes†	242	340	0·8	—	—	—
	ER-Membranes†	2690	5600	—	—	—	—
3	Free polyribosomes	550	2040	3·6	83	615	2·0

Rats were injected intrathecally with 1×10^8 (carrier-free) [³H]MSO and killed 15 min later.

* Subfractions were isolated in a sucrose-free medium (20 mM-tris, pH 7·2, and 10 mM-Mg^{2+}) according to the procedures of SELLINGER *et al.* (1968) and SELLINGER and OHLSSON (1969b).

† These subfractions were obtained by treating the granular ER with 0·3% (w/v) deoxycholate and centrifuging at 269,000 g for 50 min. The soluble portion represents ER-membranes, the pellet consists of polyribosomes.

Abbreviation: ER, endoplasmic reticulum.

radioactivity as d.p.m.$^{-1}$ µg of RNA^{-1}, —g^{-1} and by comparing fraction Mic to the granular endoplasmic reticulum (Table 5, Expt. 1). The [³H]MSO was associated with the membrane component of the granular endoplasmic reticulum rather than with the ribosomes (Table 5, Expt. 2). The ribosomal pellet was analysed further by gradient sedimentation (Fig. 3) and a definite association of a portion of the radioactivity with the highly aggregated polyribosomes was demonstrated (Fig. 3A and 3B); however, the bulk of the radioactivity appeared in the upper region of the gradient where soluble and/or membranous contaminants of this fraction accumulate (MURTY and HALLINAN, 1969). A gradient-sedimentation analysis of the large and small ribosomal subunit obtained by dissociating the ribosomes with EDTA (AZCURRA and SELLINGER, 1967) did not reveal any preferential accumulation of, [³H]MSO with either subunit (Fig. 3C).

DISCUSSION

In confirmation of our previous observations (LAMAR and SELLINGER, 1965), the methyl-labelled [³H]MSO used in the present study underwent no biotransformation in the brain, and could be quantitatively recovered 4 h after administration. Furthermore, the present results also indicate that the administration of methionine failed to induce or otherwise influence MSO metabolism, a finding which certainly weakens the hypothesis that methionine would prevent seizures induced by MSO by promoting the biotransformation of MSO to pharmacologically inert metabolites.

Although the amounts of MSO entering the brain after i.p. injection were less than 0·5 per cent of the dose administered, they were sufficient to elicit seizures. We calculate that MSO levels of the order of 0·02 µmol/brain were attained. Less MSO was present in the brain when injected together with methionine, particularly subsequent to 45 min after administration (Fig. 1; Table 1), possibly because the high cerebral levels of methionine (reached within 2 h after its injection) were maintained above control levels over the entire 4 h preconvulsive period (GHITTONI and SELLINGER, unpublished observation) and thus blocked further accumulation of MSO. An
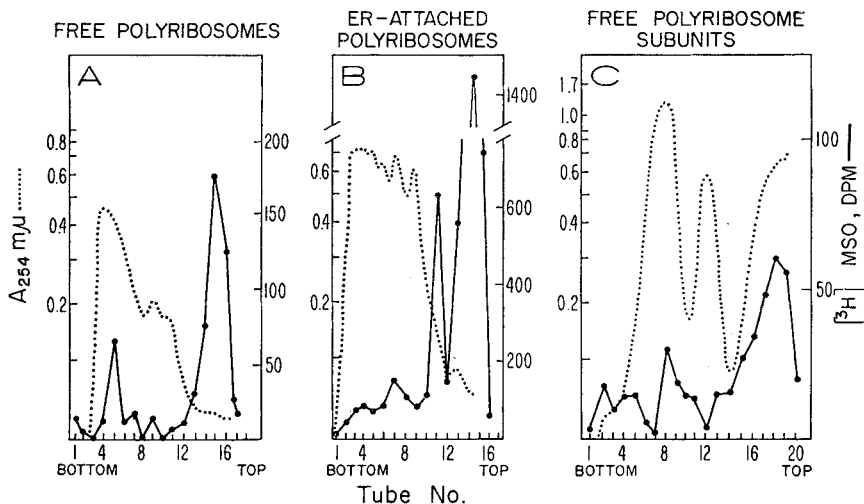
FIG. 3.—*The association of* [³H]MSO *with ribosomes*. Three rats were injected i.th. with
1 × 10⁸ d.p.m. of [³H]MSO and killed 15 min later. The ultraviolet absorbance profile
(A₂₅₄ nm; − − − −) has been plotted together with the radioactivity profile (————).
(*A*) Free polyribosomes were isolated as modified by SELLINGER and OHLSSON
(1969*b*). Note the peak of radioactivity corresponding to the polyribosomes of highest
aggregation. (*B*) The polyribosomes attached to endoplasmic reticulum (ER) were iso-
lated by the procedure of SELLINGER *et al.* (1968). Note that a portion of the radioactivity
was associated with polyribosomes of high aggregation; the bulk of the radioactivity,
however, appeared near or at the top of the sucrose gradient. (*C*) The free poly-
ribosomes (see above under *A* for preparation) were suspended in 0·5 ml of 20 mM-tris
(pH 7·2), and 25 mM-KCl and 0·5 ml of 0·134 M-Na-EDTA (pH 7) were added. After
standing for 10 min at 0°C, the suspension was layered on a 15–40% (w/v) linear sucrose
gradient and centrifuged for 13 h at 60,000 *g*. The ultraviolet absorbance profile
indicated dissociation of the polyribosomes into the large (tubes 5–10) and small (tubes
11–14) ribosomal subunits.

inhibitory effect of methionine on the rate of uptake by the brain of DL-DOPA has
recently been reported by SHAH, KAMANO, GLISSON and CALLISON (1968). Whether
the decrease in cerebral MSO observed in the face of high tissue pools of cerebral
methionine is the result of direct competition between these two substances for ident-
ical transport mechanisms or whether it depends on complex interplays involving
other amino acids (DANIEL and WAISMAN, 1969) cannot be stated at this time. We
approached the question of the regional partition of MSO by measuring levels of
[³H]MSO in the cerebral cortex, the cerebellum and in the rest of the brain. Follow-
ing i.p. administration (Fig. 2), MSO exhibited a transient preference for cortical
sites, while by 2 h after its i.th. administration, 21·6 per cent of the tissue radioactivity
was in the cerebral cortex, 46·1 per cent in the subcortical regions and 32·3 per cent
in the cerebellum. The administration of methionine redirected the flux of MSO
toward the cerebellum, as early as 30 min post-injection, when the cerebellum accounted
for 37·4 per cent of the tissue radioactivity in brains with high endogenous methion-
ine, in comparison to only 22·0 per cent in control brains (Table 2).

[³H]MSO reached the cerebellum earlier when [³H]MSO was administered with
methionine than when it was given alone (Fig. 2). When [³H]MSO was injected
i.th. 60 min after giving i.p. L-methionine, there was a shift (13 per cent) of the cere-
bellar [³H]MSO from the particulate fractions to the soluble supernatant fraction (S)

and within fraction NEML from subfraction 1·2 M to subfraction 1·0 M (Table 3). There was also a specific association of [³H]MSO with nerve endings sedimenting to the 1·2 M-sucrose interface (Table 3; specific binding ratio (SBR) 2·19 in the cortex and 1·37 in the cerebellum) (DE ROBERTIS et al., 1963; DEL CERRO et al., 1969). A secondary peak could be discerned in the cortex, but not in the cerebellum, in the fraction replete with myelin fragments (0·8 M); this peak was, however, less pronounced (SBR: 1·32) than the 1·2 M SBR peak and involved only 20 per cent of the radioactivity of the particulate fraction NEML. When subjected to osmotic shock, all cortical nerve endings lost particulate radioactivity (fractions 1·0 M, 1·2 M and 1·4 M) and the loss was more pronounced in methionione-pretreated animals than in controls (Table 4). The loss of [³H]MSO from nerve endings of the cerebellum was moderate in magnitude, and methionine pretreatment appeared to 'fix' the particulate MSO to the remnants of fractions 1·2 M and 1·4 M which exhibited relative specific radioactivities eight and 17 times higher, respectively, than those of the soluble components.

In the microsomal fraction [³H]MSO was preferentially bound to the membranes of the endoplasmic reticulum. Moreover, the interaction between [³H]MSO and endoplasmic reticulum seemed to favour the agranular membrane component (four- and two-fold higher specific radioactivity in the cortex and the cerebellum, respectively; Table 5). Within the granular fraction, 92 per cent· of the radioactivity associated with the membranes and 8 per cent with the ribosomes, but only a small portion of the ribosomal radioactivity sedimented with the highly aggregated polyribosomes. These studies complement our previous observation of a more rapid inhibition in vivo by MSO of the agranular than of the granular endoplasmic reticulum component of the cortical glutamine synthetase (SELLINGER et al., 1968). Thus, both sets of results are consistent with a stepwise involvement of the endoplasmic reticulum with MSO, the first step being an association of the drug with the agranular portion. The interpretation of the effect of methionine on the intracellular distribution of MSO and, hence, on its action as a convulsant agent, appears complex, particularly since the effect differed in the two parts of the brain examined. The principal feature was the ability of methionine to decrease particulate binding of [³H]MSO in the cerebellum (Table 3) while strengthening its specific association with some of the nerve-ending membranes in the cerebellum. As early as 1958, LODIN suggested an origin for MSO-elicited seizures other than in cerebral cortex. Subsequent reports from LODIN's laboratory have demonstrated injurious effects of MSO on the granular and molecular cells of the cerebellum and on cerebellar metabolism (FOLBERGROVÁ, 1964; FOLBERGROVÁ, PASSONNEAU, LOWRY and SCHULZ, 1969; LODIN, HARTMAN, FALTIN and MALAN-OWSKI, 1968).

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