Biochemical Pharmacology, Vol. 32, No. 24, pp. 3695–3700, 1983.
Printed in Great Britain.

0006–2952/83 $3.00 + 0.00
Pergamon Press Ltd.

EFFECTS OF METHIONINE SULFOXIMINE ON THE ENZYMES OF GLUTAMATE METABOLISM IN ISOLATED ASTROCYTES OF RAT BRAIN

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(*Received* 13 *April* 1983; *accepted* 11 *July* 1983)

Abstract—The enzymes of glutamate metabolism were estimated in astrocytes isolated from brains of normal rats and those injected with the potent convulsant, methionine sulfoximine (MSO), which inhibits glutamine synthetase and induces Alzheimer type II astrocytosis. The wet weight, dry weight; contents of DNA, RNA, protein and the activities of glutamate dehydrogenase and aspartate aminotransferase were elevated following MSO administration. The metabolic effects of MSO were found to be different from those of ammonia wherein a fall in the activity of glutamate dehydrogenase and an increase in the activity of glutamine synthetase was noticed. Based on these results it is suggested that there might be an inverse relationship in the functioning of these two enzymes. Such a relationship would help in preventing the depletion of energy pools in a given cellular compartment during ammonia detoxification.

Methionine sulfoximine (MSO), unlike other convulsants has a long latent period, and induces convulsions by inhibiting glutamine synthetase [1], a key enzyme involved in the detoxification of ammonia in cerebral tissues [2], where a complete complement of urea cycle enzymes is absent [3]. Further, MSO administration results in the production of Alzheimer type II astrocytosis, akin to that produced in hyperammonemia of a different etiology [4]. Light and electron microscopic studies revealed that this glial change includes swelling and proliferation of astrocytes (without the formation of mitotic spindle), an increase in the watery cytoplasmic compartment and a proliferation of subcellular organelles in these cells [4]. These changes indicated the role played by astrocytes in hyperammonemic state which correlated well with the astroglial localization of glutamine synthetase by immunohistochemical methods [5] and the small pool of glutamate by biochemical methods [6, 7], which are pre-requisites for ammonia detoxification.

In the present communication, we report the changes in the activities of the enzymes involved in glutamate metabolism along with other biochemical parameters in the astroglial cells isolated from MSO treated rats. The results indicated a significant increase in glutamate dehydrogenase (GDH) and aspartate aminotransferase (AAT) activities following MSO administration.

MATERIALS AND METHODS

Animals. Adult albino rats of Wistar strain were chosen as experimental animals. The animals were of either sex and of same age having a body weight of 150 to 200 g. Both the controls and experimental had free access to food and water.

Drug treatment. L-Methionine-DL-sulfoximine was administered intraperitoneally using saline as a carrier. For acute treatment a dose of 300 mg/kg body weight was administered and for subacute effects the dose was reduced to 150 mg/kg body weight. The animals were decapitated at the end of 3.5 hr (acute) or 17–18 hr (subacute) and cerebral cortex was separated from rest of the brain (within 1–2 min).

Cell isolation. Astroglial cells were isolated by a modified method of Farooq and Norton [8] as reported earlier [9] which enabled us to isolate the cells by 4 hr after decapitation. The cell isolation medium and other media were same as reported by these authors. The tissue was sliced into 12 pieces and incubated at 37° in a medium containing 0.1% trypsin. At the end of 90 min, soyabean trypsin inhibitor was added and the tissue was washed twice with medium. The tissue was disaggregated by aspiration through nozzle as described in the original method. The disaggregated tissue was successively passed through monofilament nylon screens of 300, 80 and 53 μm mesh. The cell suspension was centrifuged at 720 *g* for 15 min to get a cell-rich pellet. The pellet was suspended in 7% Ficoll medium (total Ficoll 9%) and centrifuged at 720 *g* for 10 min to obtain a neuronal-enriched pellet. The supernatant was diluted 1:1.125 with medium and centrifuged at 1120 *g* for 20 min. The pellet thus obtained was suspended in 15% Ficoll (total Ficoll 17%) and centrifuged at 8500 *g* for 10 min to obtain an astrocyte-enriched pellet.

The cells were suspended in 0.32 M sucrose and an aliquot was stained with 1% methylene blue for cell counting in a hemocytometer. The cell viability was assessed by dye exclusion method using trypan blue. For enzyme assays and biochemical determinations cells were suspended in distilled water and subjected to a single cycle of freezing and thawing. Protein content was adjusted to 1 mg/ml.

Wet weight: Approximately 0.5×10^6 cells were

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suspended in Krebs Ringer phosphate (NaCl 128 mM, KCl 5 mM, $MgSO_4$ 1.3 mM $CaCl_2$ 1.0 mM, Na–phosphate buffer 10 mM, (pH 7.4), glucose 10 mM) and filtered on millipore membrane filter (0.45 μm mesh) under negative pressure and weighed. The difference in the weights of filters with and without cells was taken as the weight of cells and weight of a single cell was calculated.

Dry weight: The filters with and without cells were dried at 60° to a constant weight. The difference between wet weight and dry weight was used for calculating cell water content.

DNA, RNA and protein. DNA and RNA were estimated by the methods of Schmidt and Thaunnhauser [10] and protein content by the method of Lowry *et al.* [11].

Enzyme assays. Glutamate dehydrogenase (GDH) was assayed by the method of Chee *et al.* [12] and aspartate and alanine aminotransferases (AAT, AlAT) by the method of Bergmayer and Bernt [13]. Glutamine synthetase (GS) was determined as described by Rowe *et al.* [14] and glutaminase as described by Nimmo and Tipton [15].

As the astroglia swell following MSO administration their buoyant density decreases. It was observed that these cells failed to sediment in 15% Ficoll. Hence the procedure was slightly modified. The incubation time was reduced to 60 min and after disaggregation the cell suspension was passed through 300 and 80 μm nylon screens. The time of centrifugations was increased by 5 min and final centrifugation at 8500 *g* was performed after suspending the crude astrocyte pellet in 10% (final Ficoll concentration 12%) instead of 15% Ficoll.

All the enzyme activities were expressed per milligram protein and per cell. Statistical evaluations were performed using Student *t*-test.

RESULTS

Behavioural changes. The time period required for the production of behavioural changes varied with the administered dose of MSO, though the pattern was same for both acute and subacute groups. However, the changes were more conspicuous and well pronounced in acute group of animals than in subacute. The pattern of changes observed was similar to those reported by us for acute [16] and for subacute animals [18]. The time period for acute animals was 3.5 hrs and for subacute it was 17–18 hr.

Cellular parameters. The number of cells isolated from normal animals and from experimental animals by employing the modified method was highly reproducible (Table 1; Fig. 1). The number increased significantly after treatment with MSO (Table 2; Figs. 1 and 2). The cells from experimental animals had less buoyant density as they could be sedimented only in 10% Ficoll but not in 15% which was used for control group.

Swelling of astrocytes was adjudged from wet weight and water content of the cells. The water content increased by 3-fold in acute animals and 10-fold in subacute group. However, the wet weight of the cells increased by 5-fold in acute group and by 7.5-fold in subacute animals (Table 2).

The magnitude of increase in dry matter was more in acute animals than in subacute group (Table 2).

DNA, RNA and protein. DNA content of the astroglial cells from control animals was similar to the value reported earlier [8]. The values for RNA content in control animals were also similar to those reported by these authors. Both the DNA and RNA contents increased after MSO administration and the magnitude being greater in the cells from subacute animals than acute. The percent increase in the protein content of the astrocytes was two times more in acute conditions than in the subacute state. The ratio of protein to DNA, which is considered as an indicator of cell size [17], increased in acute state but decreased in subacute animals (Table 2).

Enzymes of glutamate metabolism. Specific activity. As expected MSO inhibited the activity of GS below the level of detection both in acute and subacute conditions. Similarly, the activity of AlAT was inhibited, the percentage of inhibition being more in acute than in subacute. There was no change in the specific activity of the GDH in the astrocytes isolated from animals injected with an acute dose of MSO while in the cells isolated from animals receiving the subacute dose of MSO, there was a 2.5-fold increase in the specific activity of this enzyme. The activity of AAT increased more or less to the same extent in both acute and subacute animals. A similar change was noticed with the activity of glutaminase (Table 3).

Cellular activity. The profile exhibited by GS remained the same as above while the magnitude of inhibition in the AlAT activity was more in subacute than in acute. The activity of GDH increased both in acute and subacute, the percent increase being more in the latter group. A similar pattern was noticed with respect to the activity of AAT. However, glutaminase activity was elevated in acute state but not in subacute state (Table 3).

DISCUSSION

In Alzheimer type II glial change, associated with hyperammonemia, the astrocytes are known to enter into a reactive phase. Gutierrez and Norenberg [4] made a detailed ultrastructural study of this glial change in MSO induced hyperammonemia. They reported a 42% increase in the number of astrocytes in about 7½ hr after MSO administration. The cells had a swollen perikaryon with an enlarged cytoplasmic compartment along with a large number of membrane bound vacuoles (hydropic change). In addition, there was a 7-fold increase in the content of both smooth and rough endoplasmic reticulum and a pronounced accumulation of glycogen granules. The number of mitochondrial profiles increased by about three fold. But for these ultrastructual studies, no attempts were made to elucidate the biochemical changes in the glial cells under these conditions.

In the present study an attempt was made to follow the biochemical changes in the astrocyte with an assumption that the post-mortem changes and other changes due to isolation procedure would be the same both in the cells isolated from control and

Table 1. Cell number, DNA, RNA and protein content and enzymes of glutamate metabolism in bulk separated astrocytes from rat brain

	Activity (μmoles per mg protein)	Activity per cell (nmoles)	Values from literature**
Cell number × 10^6		7.4 ± 1.4 (12)	8.3 [8]
DNA*		6.98 ± 1.0 (8)	7.7 [8]; 11.2 [22]; 6.7 [23]
RNA*		6.90 ± 0.9 (7)	8.1 [8]; 29.1 [22]
Protein*		219 ± 40 (12)	195 [8]; 307 [22]; 411 [24]
RNA/DNA		0.99 ± 0.15 (8)	1.1 [8]; 1.6–2.8 [23]
Protein/DNA		31.3	26 [8]; 27 [22]; 63 [23]
Glutamate dehydrogenase (NADH (oxidized)/hr)	7.24 ± 0.98 (8)	2.05 ± 0.32 (8)	0.69 [25]; 0.78 [26]
Glutamine synthetase (γ-glutamyl hydroxamate/hr)	2.6 ± 0.2 (7)	0.62 ± 0.06 (7)	0.84 [25]; 1.38 [26]
Aspartate aminotransferase (NADH oxidized/hr)	115 ± 8 (5)	27.5 ± 4.2 (5)	5.75 [25]; 11.28 [26]
Alanine aminotransferase (NADH oxidized/hr)	5.6 ± 1.4 (5)	1.3 ± 0.3 (5)	—
GABA: α-ketoglutarate aminotransferase (succinic seminaldehyde/hr)	2.64 ± 0.25 (6)	0.58 ± 0.7 (6)	—
Carbonic anhydrase (CO_2 liberated/hr)	2300 ± 900 (6)	702 ± 20 (4)	3.0 [29]
Butyrylcholinesterase (butyrylcholine iodide hydrolyzed/hr)	607 ± 14 (4)	147 ± 3.5 (2)	—

* pg/cell.

** Values for cell number (×10^6); for DNA, RNA and protein (pg/cell); for enzyme activity the values are μmoles/mg protein/hr (recalculated where necessary).

Values are mean ± S.D. Number in parenthesis indicate number of determinations made from different cell preparations.

Table 2. Effects of acute and subacute dose of MSO on the biochemical parameters of astrocytes isolated from rat brain

	Control	Acute	(%) change over control	Subacute	(%) change over control
Cell number $\times 10^6$	7.4 ± 1.4 (12)	8.4 ± 0.6 (6) $P < 0.05$	+14	8.8 ± 2.0 (15) $P < 0.05$	+19
Wet weight*	3.4 ± 0.4 (3)	16.4 ± 1.1 (4) $P < 0.0001$	+352	26.0 ± 3.0 (4) $P < 0.0001$	+665
Dry weight*	1.6 ± 0.3 (3)	10.1 ± 1.4 (4) $P < 0.0001$	+537	3.8 ± 0.5 (4) $P < 0.0001$	+137
Water content**	2.02 ± 0.14 (3)	6.2 ± 1.5 (4) $P < 0.0001$	+208	22.4 ± 3 (4) $P < 0.0001$	+1000
DNA***	6.98 ± 1.0 (8)	9.0 ± 1.9 (6) $P < 0.005$	+29	15.1 ± 1.8 (4) $P < 0.0001$	+118
RNA***	6.9 ± 0.9 (7)	11.9 ± 1.4 (6) $P < 0.001$	+72	15.6 ± 1.6 (4) $P < 0.001$	+126
Protein***	219 ± 40 (12)	548 ± 98 (11) $P < 0.001$	+150	367 ± 61 (13) $P < 0.001$	+68
Protein/DNA	31	61	+95	24	−22

* ng/cell; ** ml/cell; *** pg/cell.
The number in parenthesis indicates the number of individual observations made.
+ Indicates an increase over control value and − a decrease from the control value.
All the values are mean ± S.D.

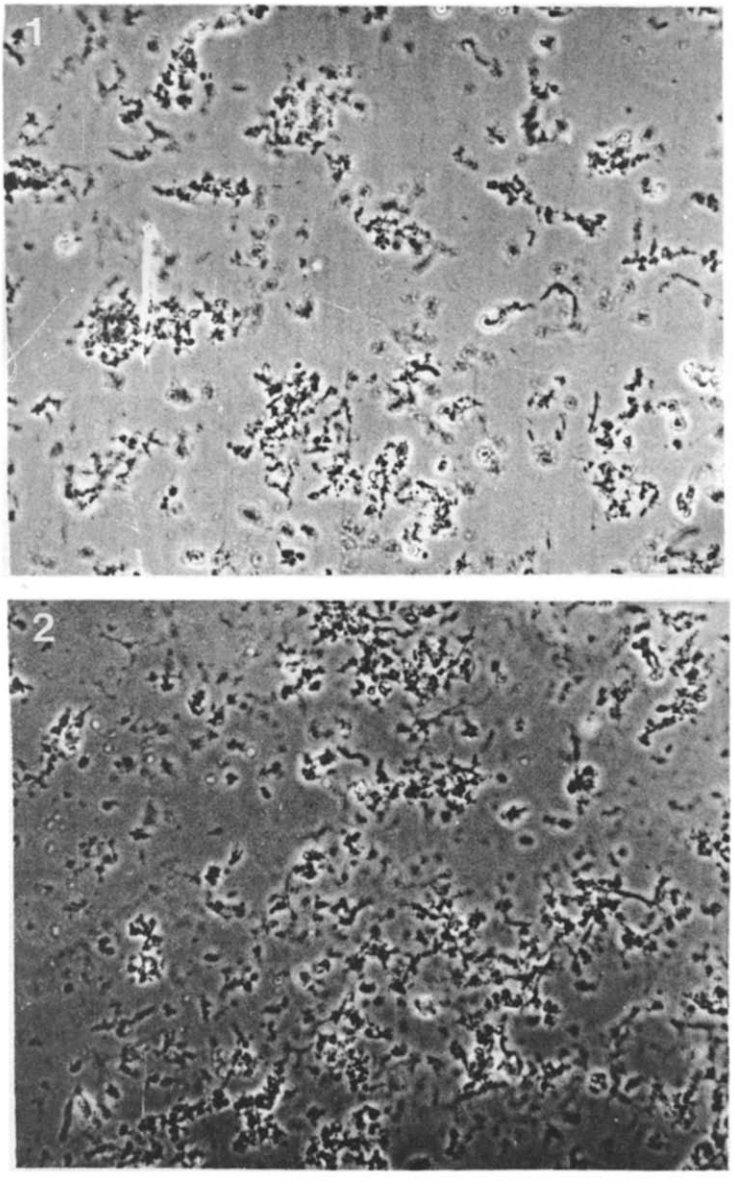
experimental animals and the changes produced as a result of drug action would be retained during cell isolation. In the following discussion, emphasis will be given to enzyme activity per cell than to specific activity (activity/mg protein) as the latter parameter would be governed by cellular protein content (enzymic + nonenzymic). Any variation in the enzyme activity would then be masked by changes in the total protein content of the cell and with the amount of available material and techniques it is highly difficult to distinguish the amount of enzymic and nonenzymic proteins. Similar problems were encountred when the enzyme activity was expressed per mg wet weight or dry weight of the cell.

One of the major characteristic features of Alzheimer change is the proliferation of glial cells. Fol-

Table 3. Effect of acute and subacute dose of MSO on the enzymes of glutamate metabolism in astrocytes isolated from rat brain

		Control	Acute	(%) over control	Subacute	(%) over control
Glutamine synthetase*	a	2.6 ± 0.2 (6)	N.D.	−100	N.D.	−100
	b	0.62 ± 0.06 (6)	N.D.	−100	N.D.	−100
Alanine aminotransferase**	a	5.6 ± 1.4 (5)	2.2 ± 0.3 (6) $P < 0.001$	−61	1.5 ± 0.1 (4) $P < 0.001$	−73
	b	1.3 ± 0.3 (5)	1.02 ± 0.2 (6) $P < 0.05$	−20	0.49 ± 0.06 (6) $P < 0.0005$	−62
Glutamate dehydrogenase**	a	7.2 ± 1.0 (8)	6.6 ± 0.7 (6) $P < 0.2$	−9	16.6 ± 1.5 (6) $P < 0.001$	+130
	b	2.05 ± 0.3 (8)	4.05 ± 1.5 (6) $P < 0.0025$	+98	6.65 ± 1.3 (6) $P < 0.0001$	+176
Aspartate aminotransferase**	a	115 ± 8 (5)	143 ± 9 (6) $P < 0.001$	+24	155 ± 8 (3) $P < 0.001$	+35
	b	27.5 ± 4.2 (5)	67 ± 18 (6) $P < 0.0001$	+144	49 ± 8 (4) $P < 0.001$	+77
Glutaminase**	a	17.2 ± 1.4 (5)	10.5 ± 1.3 (4) $P < 0.001$	−40	9.5 ± 0.7 (4) $P < 0.001$	−45
	b	3.8 ± 0.3 (5)	4.5 ± 1.3 (4) $P < 0.05$	+18	3.6 ± 0.2 (4) NS	−4

a: Activity (μmoles)/mg protein/hr; b: activity (nanomoles)/cell/hr.
* γ-Glutamylhydroxamate formed; ** NADH oxidized.
All the values are mean ± S.D. The number in parenthesis indicate the number of individual observations made.
+ Indicates increase while − indicates a decrease in relation to control.
N.D. Not detectable.



Figs. 1 and 2. Phase contrast photomicrographs of astrocytes isolated from rat brain cortex (×320). Fig. 1. From normal rat; Fig. 2. From rats treated with MSO (15 mg/100 g body wt). The astrocytes isolated from rats treated with MSO (30 mg/100 g body wt) also have similar appearance.

lowing MSO administration, more cells could be isolated from the same amount of cortex, the number being more in subacute group than in acute. Because of a hydropic change, the cells were more fragile, hence at least some of them might not have survived the conditions employed for cell isolation.

An enormous increase in the wet weight and water content following MSO administration suggested swelling of astrocytes. This is further evidenced by sedimentation properties of the cells during isolation as the vacuolated cells fail to sediment in a medium with a higher density. Increase in dry matter also indicated proliferation and accumulation of biochemical components.

The observed increase in the contents of DNA, RNA and protein in the astrocytes following MSO administration suggested an increase in the translational and transcriptional activity. This observation correlated well with the reported increase in the proliferation of cells as well as cellular organelles. In spite of a higher nucleic acid content, the accumulated protein was less in the astrocytes isolated from rats injected with a subacute dose of MSO. This could be due to an increased rate of degradation of proteins under these conditions as the cells enter a degenerative state after an initial reactive phase. Further, increased protein content in the cells following MSO administration would also alter the

buoyant density of the cells [30]. The increase in the protein content presently reported correlated well with our earlier reports in brain homogenates [18]. Due to a greater change in the DNA content than protein, the ratio of protein to DNA decreased under chronic conditions inspite of an enormous swelling in the cells.

An increase in GDH activity in astrocytes observed following the administration of MSO facilitates ammonia detoxification as the equilibrium constant of the reaction is in favour of glutamate formation [12]. Since the activity of GS was inhibited by MSO, this reaction might become an important ammonia detoxification mechanism. The glutamate, so formed, would accumulate instead of being converted to glutamine. This suggestion is in accord with the increased brain glutamate levels following MSO administration [19]. Further, this reaction would then interfere with the operation of citric acid cycle by depleting α-ketoglutarate in this cellular compartment.

The increased activity of AAT would facilitate the transport of glutamate from mitochondria [20] and the reducing equivalents from cytoplasm [21]. This reaction would then facilitate GDH reaction by supplying reducing equivalents inspite of an interference in the operation of citric acid cycle. Further, it would maintain a favourable redox state in the cytoplasm for glycolysis to continue.

These effects of MSO were found to be different from those of ammonia [9]. In acute conditions of ammonia toxicity the activity of GS was found to be elevated and that of GDH decreased. In MSO-toxicity these changes were reversed. These results suggested that ammonia disposal by astroglial cells under normal conditions might be by glutamine formation during which glutamate formation in the same cellular compartment would be inhibited, thereby preventing a major drain on energy sources. The necessary precursors would be obtained from exogenous sources such as nerve endings. When glutamine synthetase is inhibited, GDH reaction would be increased as a compensatory mechanism. Such a reciprocal relationship between these two enzymes might be of physiological importance as it conserves energy in a given cellular compartment.

Acknowledgements—We are grateful to Indian Council of Medical Research for their financial assistance in the form of a grant [5/3-1(2)/79-BMS]. We thank Dr. Aparna Ray for the kind help in microscopy work.

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