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Effect of T-2 Toxin, Fasting, and 2-Methyl-thiazolidine-4-carboxylate, a Glutathione Prodrug, on Hepatic Glutathione Levels[1,2]

ROBERT F. FRICKE AND JUAN M. JORGE

United States Army Medical Research
Institute of Infectious Diseases
Fort Detrick, Frederick, Maryland 21701-5011

Running title: HEPATIC GSH LEVELS IN T-2 TOXICOSIS

Correspondence: Robert F. Fricke
Pathophysiology Division
US Army Medical Research Institute
of Infectious Diseases
Ft. Detrick, Frederick, Maryland 21701-5011
(301) 663-7181

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The effect of T-2 toxin on hepatic glutathione content and the protective effect of 2-methyl-thiazolidine-4-carboxylate (MTCA), a glutathione prodrug, was studied in mice. Acute exposure to T-2 toxin (4 mg/kg, sc) resulted in a progressive decrease in glutathione content, reaching a minimum 4-6 hr after toxin administration. Because T-2 toxin caused decreased feed consumption, a condition known to deplete hepatic glutathione, glutathione was measured in both fed and fasted control and toxin-treated mice. Glutathione content (umol/g tissue) was 9.01 ± 0.66 (control) and 4.26 ± 0.41 (toxin) for fed mice;

4.45 ± 0.39 (control) and 2.45 ± 0.26 (toxin) for 16 hr fasted mice; and 7.18 ± 0.26 (control) and 3.76 ± 0.65 (toxin) for mice fed before, but fasted after exposure to toxin. In all cases, toxin treatment, compared to controls, resulted in significant decreases in glutathione content. Activities of glutathione-S-transferase, reductase, and peroxidase were measured at various times after administration of T-2 toxin and were not significantly different from control values. Further, treatment of T-2 intoxicated mice with MTCA (750 mg/kg, ip) not only maintained glutathione content at control levels or higher, but significantly improved survival as well. These findings indicate that, because MTCA maintained glutathione content and improved survival, the toxicity and lethality of T-2 toxin may be associated with decreased hepatic glutathione content.

Effect of T-2 Toxin, Fasting, and 2-Methyl-Thiazolidine-4-Carboxylate, a Glutathione Prodrug, on Hepatic Glutathione Levels. FRICKE, R. F. AND JORGE, J. (1986). Toxicol. Appl. Pharmacol. , - . The effect of T-2 toxin on hepatic glutathione content and the protective effect of 2-methyl-thiazolidine-4-carboxylate (MTCA), a glutathione prodrug, was studied in mice. Acute exposure to T-2 toxin (4 mg/kg, sc) resulted in a progressive decrease in glutathione content, reaching a minimum 4-6 hr after toxin administration. Because T-2 toxin caused decreased feed consumption, a condition known to deplete hepatic glutathione, glutathione was measured in both fed and fasted control and toxin-treated mice. Glutathione content (μmol/g tissue) was 9.01 ± 0.66 (control) and 4.26 ± 0.41 (toxin) for fed mice; 4.45 ± 0.39 (control) and 2.45 ± 0.26 (toxin) for 16 hr fasted mice; and 7.18 ± 0.26 (control) and 3.76 ± 0.65 (toxin) for mice fed before, but fasted after exposure to toxin. In all cases, toxin treatment, compared to controls, resulted in significant decreases in glutathione content. Activities of glutathione-S-transferase, reductase, and peroxidase were measured at various times after administration of T-2 toxin and were not significantly different from control values. Further, treatment of T-2 intoxicated mice with MTCA (750 mg/kg, ip) not only maintained glutathione content at control levels or higher, but significantly improved survival as well. These findings indicate that, because MTCA maintained glutathione content and improved survival, the toxicity and lethality of T-2 toxin may be associated with decreased hepatic glutathione content.

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T-2 toxin (3-hydroxy-4-β,15-diacetoxy-8-(3-methylbutyryloxy-12,13-epoxytrichothec-9-ene), a naturally occurring fungal metabolite, causes profound physiological, hematological, and biochemical effects on exposed animals. In addition to depression of both cardiac function (Feuerstein et al., 1985) and coagulation parameters, acute T-2 toxicosis has marked effects on protein synthesis. T-2 and other related mycotoxins are potent inhibitors of eukaryotic protein synthesis (Ueno et al., 1973; Cundliffe and Davies, 1977; Wei and McLaughlin, 1974). Although inhibition of protein synthesis may be a possible cause of cytotoxicity and lethality, other factors may play a role in either potentiating or inducing the toxic response.

Recent experiments (Tsuchida et al., 1984) revealed that acute, oral administration of T-2 toxin stimulated lipid peroxidation. The peroxidation of polyunsaturated fatty acids present in membranes has been proposed as a mechanism responsible for toxicity of many foreign compounds (Mead, 1976; Recknagel and Glende, 1973). An important cellular defense against peroxidative damage is the presence of glutathione and its use as an enzyme substrate or cofactor. Even though intracellular glutathione concentration is in the millimolar range (Kosower and Kosower, 1978), there are conditions which lead to depletion of this peptide. Cellular damage induced by foreign compounds is potentiated under conditions of glutathione depletion and ameliorated with agents that lead to increased glutathione resynthesis (Williamson et al., 1982; Nagasawa et al., 1982).

Because there are few documented effects of T-2 toxin on glutathione levels and related enzymes, this study was conducted to establish the effect of acute administration of T-2 toxin on hepatic glutathione levels and the activities of glutathione-S-transferase, peroxidase, and reductase. Further, the effect of methyl-thiazolidine-4-carboxylate (MTCA), a glutathione prodrug, was investigated as a hepatoprotective agent.

METHODS

Animals. Male, Swiss ICR mice (Buckburg Laboratory Animals, Tompkins Cove, N.Y.), weighing 22-27 g, were used in these studies. All animals were acclimated for at least 1 week before use and were allowed free access to both feed (Zeigler Bros., Inc., Gardners, Penn.) and water. Before use, animals were weighed and randomly assigned to experimental and control groups. Depending on the specific experimertal protocol, some mice were fasted either before or during the experiment.

Preparation and administration of T-2 toxin and MTCA. High purity (> 99%) T-2 toxin (Myco-Labs, Chesterfied, Mo.) was prepared as a stock solution of 25 mg/ml in absolute ethanol and stored at 4°C. The stock toxin solution was diluted with propylene glycol:ethanol (90:10) to yield the desired dose in 100 µl injection volume. Control mice received vehicle only. For determination of LD50 values, six equally spaced, logarithmical doses (1 to 5 mg/kg) were prepared and injected sc (100 µl) into mice (10/dose group). The prodrug, MTCA, (Dr. H.T. Nagasawa, University of Minnesota, Minneapolis, Minn.) was dissolved in 0.1% sodium bicarbonate, neutralized (approximately pH 7) with concentrated NaOH, and injected ip in a volume of 100 µl.

Hepatic glutathione assay. After decapitation of the mice, the livers were quickly removed, rinsed with ice-cold normal saline, and weighed. The tissue was homogenized in 10% (w/v) 5-sulfosalicylic acid (Griffith, 1980) to give a final tissue concentration of 10% (w/v), and centrifuged (5000 g, 5 min). The protein-free supernatant was assayed for total glutathione content (GSH +

GSSG) by a modification of the 5,5'-dithiobis-(2-nitrobenzoic acid)/glutathione reductase recycling method of Tietz (1969). This method[3] was specifically modified for automated analysis on a Cobas-Bio Automated Chemistry Analyzer (Roche Analytical Instruments, Inc.). The reagents used in this assay were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Preliminary experiments revealed that oxidized glutathione (GSSG), as measured by the 2-vinylpyridine method (Griffith, 1980) in the above assay system, did not change and was consistently less than 1 percent of the total glutathione content. Total glutathione (GSSG + GSH) content was therefore measured rather than the distribution of reduced and oxidized.

Hepatic glutathione enzyme assays. GSH-S-transferase, GSH peroxidase, and GSSG reductase were measured in the 100,000 g supernatant fraction (Guengerich, 1982) of mouse liver. GSH-S-transferase activity was assayed by measuring the rate of conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione (Habig and Jakoby, 1981). GSH peroxidase activity was assayed with cumene hydroperoxide and reduced glutathione as primary substrates and indirectly measuring the rate of oxidized glutathione formed by glutathione reductase and NADPH (Wendel, 1981). GSSG reductase activity was measured with oxidized glutathione and NADPH as substrates (Carlsberg and Mannervik, 1975).

All enzymatic assays were specifically modified for automated analysis on a Cobas-Bio Automated Chemistry Analyzer. Enzymatic activity was determined in the linear range for both time and protein. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as reference standard. Reagents used in these assays were all obtained from Sigma Chemical Co.

Statistical analysis. Statistical differences between groups were determined by either unpaired Student's t-test or Duncan's multiple range test for multiple comparisons (Steel and Torrie, 1960).

LD50 values were calculated by probit regression analysis (Finney, 1971). If the slopes of the probit regression lines were not significantly different from each other by X^2 analysis, the best fit common slope was used to calculate the LD50 values and relative potency values (LD50 (treated)/LD50 (untreated). Comparisons for statistical significance between untreated and treated groups were carried out by least significant difference analysis on the pooled variance of the LD50 values (Steel and Torrie, 1960).

RESULTS

Effect of T-2 toxin on weight gain and feed and water intake. Weight gain and consumption of feed and water were studied in vehicle- and T-2 toxin-injected mice. After an initial lag of about 6 hr, the toxin-treated mice (2 mg/kg, sc) showed progressive and significant decrease in body weight lasting up to 36 hr; after this time, the change in weight stabilized (Fig. 1, Panel A). The decrease in body weight of the toxin-treated mice is in direct contrast to the steady weight gain of the control mice throughout the experiment. The loss of body weight of the toxin-treated mice appears to be a direct reflection of decreased consumption of both feed (Panel B) and water (Panel C). From 6-18 hr after toxin treatment, the exposed mice did not eat or drink feed or water. After 18 hr, however, the consumption rates increased, largely a reflection of surviving mice recovering from the effects of toxin.

Effect of vehicle on hepatic glutathione levels. To determine what effect vehicle might have on hepatic glutathione levels, mice were not injected (naive controls) or injected (100 µl, sc) with one of the following vehicles: 90% propylene glycol, 10% ethanol; 90% propylene glycol, 10% normal saline; 10% ethanol, 90% normal saline; or 100% normal saline. Glutathione levels measured 6 hr post-injection were 5.26 ± 0.75 (saline), 5.46 ± 0.40 (ethanol:saline), 5.79 ± 0.42 (propylene glycol:saline), and 6.07 ± 0.43 (propylene glycol:ethanol). The differences were not significantly different from the uninjected control value of 5.62 ± 0.75.

Time course of hepatic glutathione levels. At various times after administration of T-2 toxin (4 mg/kg, sc), livers were removed and assayed for total glutathione content. After an initial lag of approximately 1 hr, toxin-treated mice showed a progressive, time-dependent decline in the hepatic glutathione content, reaching a minimum of approximately 4.0, at 6-8 hr of approximately 4.0, compared to approximately 6.8 for controls.

Effect of feeding and fasting on hepatic glutathione levels. To determine if decreased hepatic glutathione levels were due to T-2 toxin, or secondarily to toxin-induced decrease in feed consumption, hepatic glutathione levels were measured in fed and fasted mice after treatment with T-2 toxin. Hepatic glutathione levels, determined 6 hr after administration of T-2 toxin, were, in all cases, significantly lower in toxin-treated mice (Fig. 3). Although fasting by itself significantly lowered hepatic glutathione levels, administration of T-2 toxin resulted in a further decrease.

Effect of T-2 toxin on glutathione-S-transferase, reductase, and peroxidase activities. At various times after the administration of T-2 toxin (4 mg/kg, sc), livers were removed and assayed for GSH-S-transferase, GSH peroxidase, and GSSG reductase activities. At the time points measured, there were no significant differences in the enzymatic activities between control and toxin-treated mice. Enzyme activities (μmol/min/mg protein) were 16.9 ± 0.69 (control) and 15.4 ± 0.69 (toxin) for GSH-S-transferase; 0.277 ± 0.0091 (control) and 0.292 ± 0.011 (toxin) for GSSG reductase; and 1.92 ± 0.053 (control) and 1.95 ± 0.043 (toxin) for GSH peroxidase.

Protective effect by MTCA. The protective effect of the glutathione prodrug, MTCA, in reversing toxin-induced glutathione depletion was studied in fasted (16 hr) mice (Fig 4). Mice were first treated with MTCA or drug vehicle, then 2 hr later by either T-2 toxin or toxin vehicle. Hepatic glutathione levels of control mice showed a gradual, time-dependent decrease throughout the experiment. MTCA significantly increased glutathione levels, which, after peaking at 2 hr, decreased to control levels at 5 hr. In the two groups of animals that received T-2 toxin, hepatic glutathione levels decreased significantly. For mice receiving both MTCA and T-2 toxin, hepatic glutathione levels, although decreased, were still significantly higher than those in the toxin only treatment group.

The protective effect of MTCA in decreasing lethality was determined in T-2 intoxicated mice. The LD50 (± 95% limits) values for T-2 toxin, measured at 48 hr, were 1.57 (1.18-2.14) for untreated controls and 2.06 (1.58-3.94) for MTCA-treated (750 mg/kg, ip) mice. The LD50 value for the MTCA-treated mice was significantly ($p<0.001$) greater than untreated controls.

DISCUSSION

Results obtained in this study indicate that T-2 toxin caused acute changes in hepatic glutathione levels. T-2 toxin, when administered to mice, caused a time-dependent decrease in the level of hepatic glutathione. A similar reduction in hepatic glutathione concentration was observed after treatment with aflatoxin B_1 or benzo(a)pyrene (Emerole et al., 1978). While the latter compounds did cause an increase in GSH-Transferase activity, T-2 toxin had little effect on the activities of GSH-S-transferase, GSH peroxidase, or GSSG reductase.

Feed deprivation results in decreased hepatic glutathione levels (Lauterburg and Mitchell, 1981). Since the above studies show that T-2 toxin causes decreased feed consumption (Fig. 1), the decreased hepatic glutathione levels observed after administration of T-2 toxin may be due to decreased feed consumption, rather than a direct effect of the toxin. Therefore, experiments were carried out to determine the combined effect of fasting and T-2 toxin on hepatic glutathione levels. Depletion of glutathione was due, in part, to toxin-induced anorexia. If the depleting effect of T-2 toxin was due solely to decreased feed consumption, then the glutathione levels of control and toxin-treated mice fasted after the start of the experiment (Group B, Fig. 3) should have been closer in value. However, the glutathione levels in this group of toxin-treated mice were significantly lower than fasted controls. This decrease was seen most markedly in animals fasted before and after toxin treatment (Group C, Fig. 4). In this case, even though fasting severely depleted hepatic glutathione content, T-2 toxin caused further, significant depletion.

Aflatoxin B_1 (Emerole et al., 1978), benzo(a)pyrene (Gelboin, 1980), and acetaminophen (Moldeus, P., 1978) undergo metabolic activation to reactive intermediates. These reactive intermediates conjugate with glutathione, leading to depletion. At present, however, there is little evidence to suggest that T-2 toxin undergoes similar metabolic activation. Metabolism studies with T-2 toxin indicate that the main secondary metabolites are glucuronide conjugates (Pace et al., 1985; Roush et al., 1985; Gareis et al., 1986; Pace, 1986). No significant nonenzymatic or enzymatic conjugation occurs between T-2 toxin and glutathione. The observed decrease in hepatic glutathione levels in T-2 toxicosis may therefore be due to other metabolic or physiological factors.

The mechanism for T-2 toxin-induced depletion of hepatic glutathione was not investigated in this report. Tsuchida et al. (1984) demonstrated that acute exposure to T-2 toxin led to increased lipid peroxidation, which was ameliorated by pretreatment with vitamin E. Further, Segal et al. (1983) revealed that high concentrations of T-2 toxin causes increased hemolysis in vitro. The addition of GSH to the media significantly reduced the amount of hemolysis. These data suggest that T-2 toxicosis is accompanied by the generation of reactive intermediates, capable of lipid peroxidation and membrane damage.

Physiologically, acute T-2 toxicosis leads to the development of a shock-like state (Fuerstein et al. 1985). Several investigators have reported that the content of glutathione in various tissues decreases in shock (Beck et al., 1954; Sakguchi et al., 1981; Yamada, 1977), and, indeed, that the administration of GSH to experimentally shocked animals improves survival (Kosugi et al., 1983). In the studies presented here, MTCA treatment prevented the T-2 toxin-induced decrease in hepatic glutathione levels. The ability of glutathione prodrugs to maintain hepatic glutathione levels may account for the

improved survival of T-2-intoxicated mice after treatment with either MTCA and oxothiazolidine-4-carboxylate, another glutathione prodrug (Fricke et al., 1984). Glutathione prodrugs, which are effective in treatment of acetaminophen intoxications (Nagasawa et at., 1982; Williams et al., 1982), may also be effective in treatment of toxin-induced shock.

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FOOTNOTES

1. Presented in part at the 23rd annual meeting of the Society of Toxicology in March, 1984, Atlanta, Ga.

2. In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The Facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

FIGURES

FIG. 1. Effect of T-2 toxin on body weight and intake of feed and water. Mice were treated with either vehicle (circles) or T-2 toxin (2 mg/kg, sc) (squares). At 6 hr intervals, the change in body weights (Panel A) and the cumulative feed (Panel B) and water (Panel C) intake were measured for control and toxin-treated groups. Values represent the mean ± S.D. (n = 20, control; n = 40, T-2 toxin). Significant difference from corresponding control values is indicated by * ($p < 0.05$). From 12 to 48 hr, the change in weight and feed and water intake for toxin-treated mice were all significantly lower ($p < 0.001$) than corresponding controls.

FIG. 2. Total hepatic glutathione content in vehicle- (control) (circles) and T-2 toxin-treated (4 mg/kg, sc) (squares) mice. Values represent the mean ± S.E. (n = 5) for each time point. Values significantly different from vehicle control are indicated by * ($p < 0.5$), ** ($p < 0.01$), and *** ($p < 0.001$).

FIG. 3. Effect of T-2 toxin (4 mg/kg, sc) on total hepatic glutathione levels of fed and fasted mice. Treatment groups are: (A) Mice fed throughout the entire experiment; (B) fed before, but fasted after treatment; and (C) fasted before (16 hr) and after treatment. Glutathione levels were measured 6 hr after injection of either vehicle controls (open bars) or T-2 toxin (cross-hatched bars). Values represent mean ± S.E. with the number of observations shown in parenthesis. Significant differences for the bracketed comparisons are indicated by * ($p < 0.05$) and *** ($p < 0.001$), with N.S. indicating no significant difference.

FIG. 4. The time course for the effect of MTCA and T-2 toxin on total hepatic glutathione levels (mean $\pm$ S.E., n = 5) was determined. At 0 hr, two groups of fasted (16 hr) mice were injected with either vehicle (open symbols) or MTCA (750 mg/kg, ip) (closed symbols). After 2 hr, the mice were injected with either toxin vehicle (circles) or T-2 toxin (4 mg/kg, sc) (squares). Values significantly different from control are indicated by * ($p<0.05$), ** ($p<0.01$), or *** ($p<0.001$).

INDEX TERMS

T-2 toxin

Trichothecene mycotoxin

Glutathione

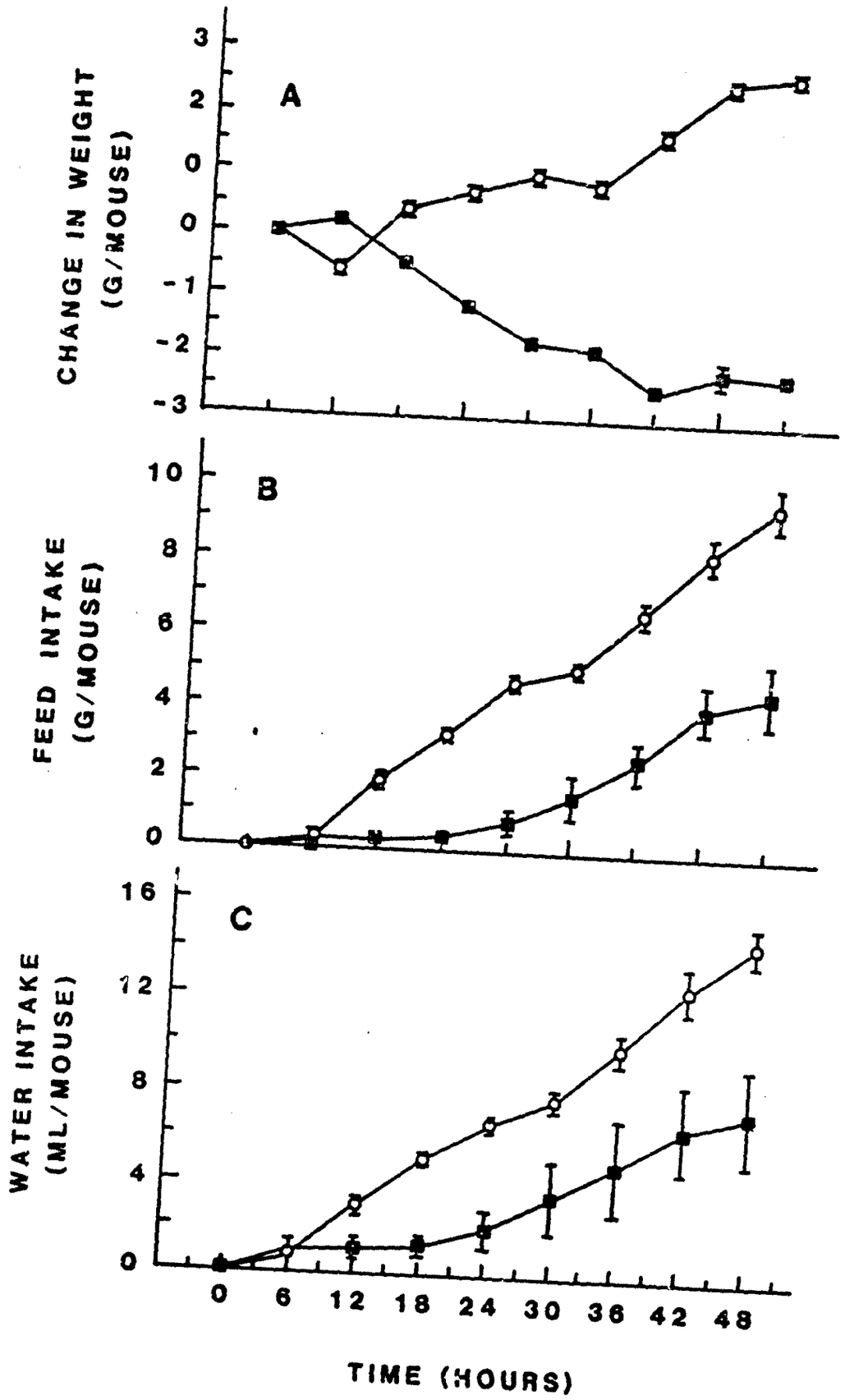
L-2-methyl-thiazolidine-4-carboxylate

Glutathione prodrugs

Glutathione reductase

Glutathione peroxidase

Glutathione-S-transferase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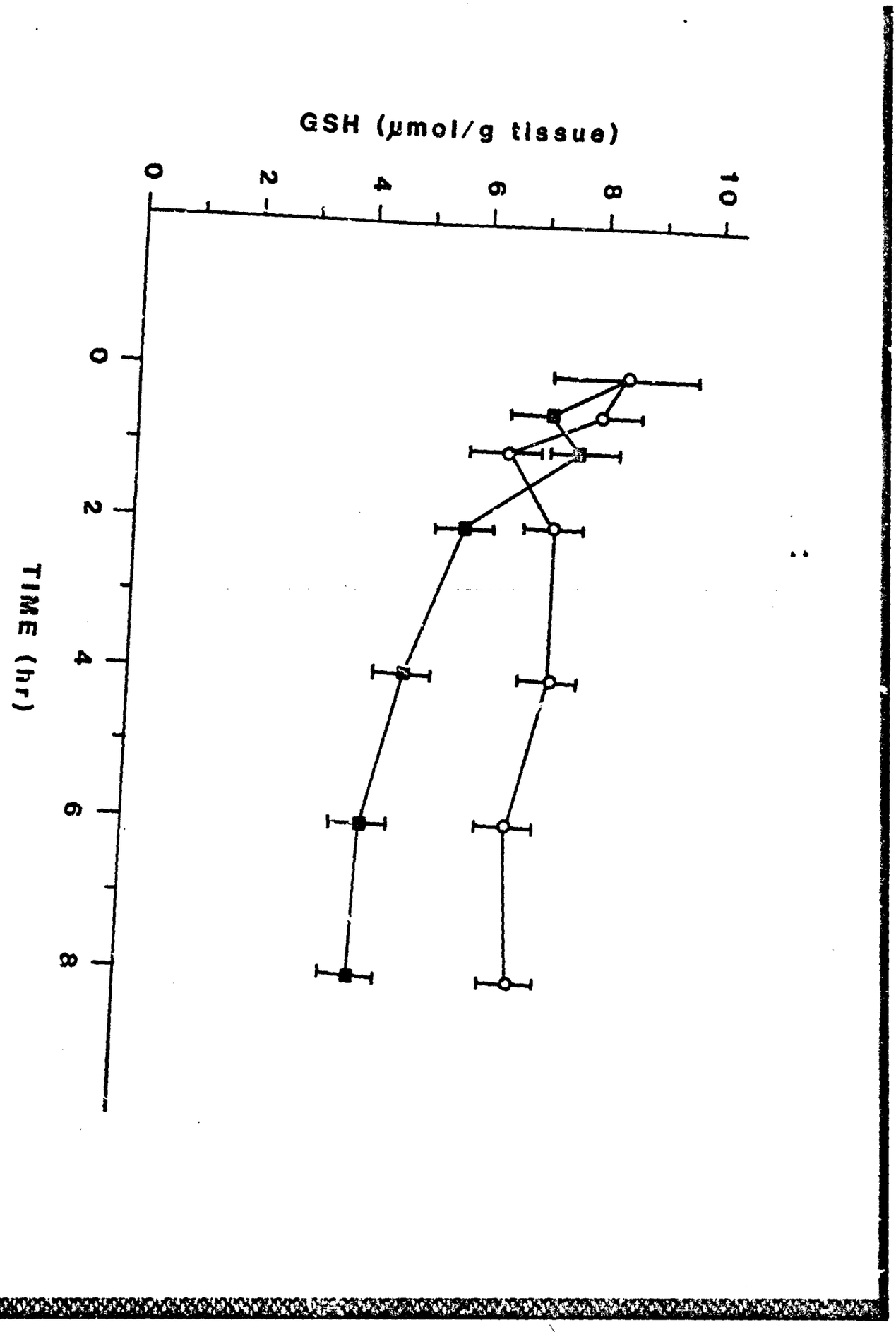
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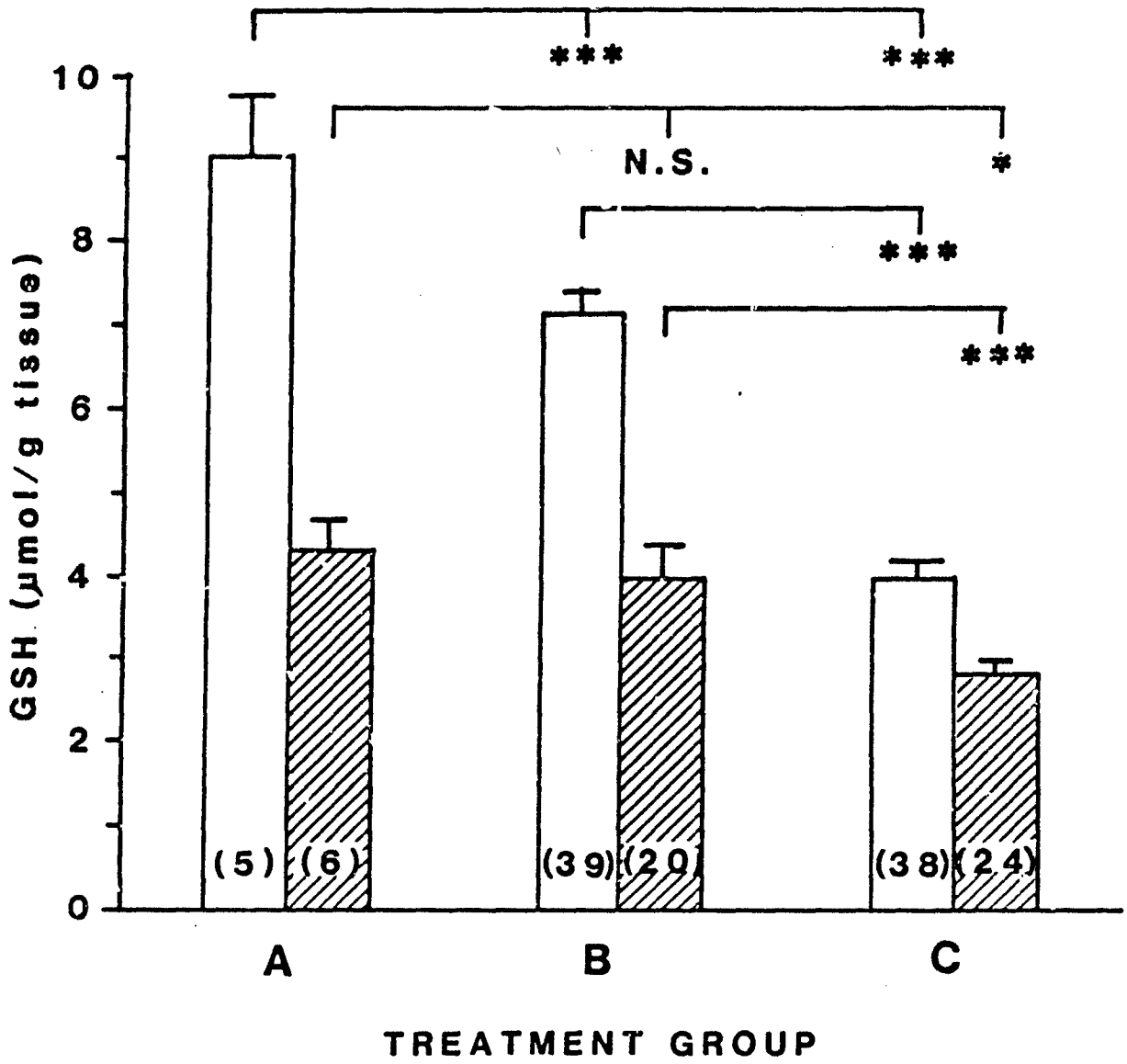
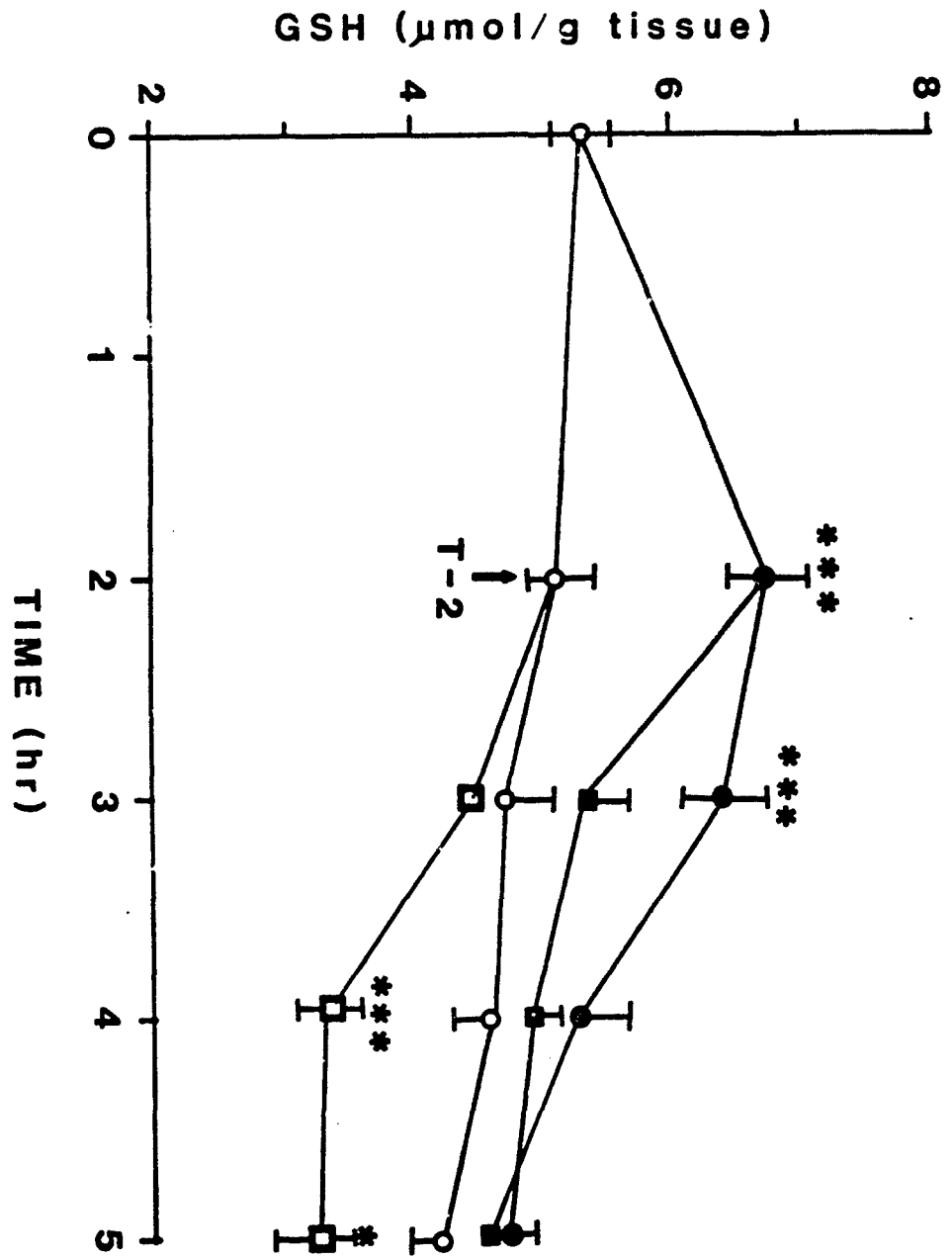

***
***
N.S.
*
***
***
10
8
6
4
2
0
GSH (μmol/g tissue)
(5)
(6)
(39)
(20)
(38)
(24)
A
B
C
TREATMENT GROUP


GSH (µmol/g tissue)
2
4
6
8
0
1
2
3
4
5
TIME (hr)
T-2
***
***
***
*