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# EFFECT OF GLUTATHIONE DEPLETION BY BUTHIONINE SULFOXIMINE ON RAT EMBRYONIC DEVELOPMENT *IN VITRO*

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**Abstract**—The intracellular thiol glutathione has many functions within cells including protection against xenobiotic and oxidative damage, and a role in protein and DNA synthesis and amino acid transport. Consequently, glutathione might be an important substance for normal growth and development. In this study the extent of glutathione depletion by buthionine sulfoximine, an agent which depletes glutathione by inhibiting its synthesis, and the subsequent effects of the depletion on rat embryonic growth and development were assessed. Day 10.5 rat embryos were cultured in rat serum medium in the presence of L-buthionine-*S,R*-sulfoximine (0.01 to 2.0 mM) and examined for viability, malformations, growth and development 45 hr later. The glutathione concentrations of the cultured embryos and their yolk sacs were also determined. Exposure to buthionine sulfoximine produced marked and significant ($P \leq 0.05$) depletion of glutathione at a buthionine sulfoximine concentration of 0.10 mM in the embryos and 0.05 mM in the yolk sacs. Exposure to 1 mM buthionine sulfoximine depleted glutathione to less than 7% of control in both of these tissues. None of the concentrations of buthionine sulfoximine tested had a significant effect on embryo viability; however, buthionine sulfoximine caused a significant ($P \leq 0.05$) incidence of malformed embryos at concentrations of 0.25, 0.5, 1.0 and 2.0 mM. The types of defects induced by buthionine sulfoximine were blebs of the maxillary or nasal processes, prosencephalon or forelimb buds, small or misshapen heads, small prosencephalons and swollen hind brains, and tail defects. Embryonic growth was the most sensitive, of the variables assessed, to the effects of buthionine sulfoximine. Significant ($P \leq 0.05$) growth retardation was observed at buthionine sulfoximine concentrations as low as 0.01 mM. At 2.0 mM buthionine sulfoximine, the yolk sac diameter, embryo crown-rump length, head length, number of somites and morphological score were reduced to 65, 72, 77, 90 and 80% of control levels respectively. We propose that the embryotoxic effects of buthionine sulfoximine are due to glutathione depletion and, consequently, that a certain basal level of endogenous glutathione is essential to allow for normal development.

The tripeptide glutathione is found in nearly all plant and animal cells and microorganisms. Recent reviews on glutathione have discussed its role in such diverse cellular functions as protection against electrophilic or oxidative damage by xenobiotics and substances of normal cell metabolism, the metabolism of endogenous compounds such as 17$\beta$-estradiol, prostaglandins and leukotrienes, and the synthesis of proteins and DNA [1–7]. In addition, glutathione is a coenzyme for some enzymatic reactions and with its role in the $\gamma$-glutamyl cycle is involved in amino acid transport into the cell [1–7]. Though it has not yet been established, it is likely that glutathione has similar roles within the embryo and, therefore, is necessary for proper embryonic development.

Much can be learned about the functions of glutathione in the embryo and in other tissues by using depleters of endogenous glutathione to compare function in normal tissue to that in the depleted state. Buthionine sulfoximine was developed by Griffith and Meister [8] to block glutathione synthesis by specifically inhibiting its first synthetic enzyme, $\gamma$-glutamyl-cysteine synthetase. Buthionine sulfoximine successfully depletes glutathione levels within many tissues in mice and rats [8–10]. The only effect of buthionine sulfoximine other than depletion of glutathione that has been reported is inhibition of cystine uptake [11]. *In vitro*, buthionine sulfoximine decreases glutathione levels in cultured macrophages [12] and in red blood cells [13]. In human lymphoid cells, the glutathione concentration is reduced to less than 3% of control after 30 hr of culture with buthionine sulfoximine [14]. Although other agents can successfully deplete tissue glutathione concentrations (e.g. the oxidizing agent, diamide, and diethylmaleate, which covalently binds to glutathione), these substances have several disadvantages when compared to buthionine sulfoximine. They are not specific for glutathione, can have toxic and physiological effects unrelated to glutathione depletion, and their use can result in an increased rate of glutathione synthesis [15].

Using rat whole embryo culture, it was demonstrated recently that buthionine sulfoximine added to the culture medium potentiates the embryolethality, teratogenicity and growth retardation caused by the reactive aldehyde, acrolein [16]. It was further found that 0.10 mM buthionine sulfoximine itself causes slight but statistically significant decreases in embryo growth and development as assessed by the yolk sac diameter, crown-rump length, head length, number of somites and morphological score, indicating that

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depletion of glutathione itself may affect rat embryonic growth and development *in vitro* [17]. Further evidence for a role for glutathione in development has been provided by Calvin and Grosshans [18] who reported that preweanling mice administered buthionine sulfoximine display retarded growth, severe cataracts and untidy fur.

The purpose of the present study was to assess the effects of buthionine sulfoximine on the glutathione content and on the growth and development of cultured rat embryos.

## METHODS

*Chemicals.* L-Buthionine-*S,R*-sulfoximine was provided by Dr. Alton Meister (Cornell University, New York, NY). Tyrode's saline, Hanks' Balanced Salt Solution, and penicillin/streptomycin (10,000 units/ml and 10,000 μg/ml respectively) were purchased from Gibco Laboratories (Burlington, Ontario). Glutathione reductase for the assay of glutathione was purchased from Boehringer Mannheim (Laval, Quebec).

*Animals.* Timed-gestation pregnant Sprague–Dawley rats (180–200 g) were purchased from Charles River Canada, Inc. (St. Constant, Quebec). The day on which spermatozoa were found in the vaginal smear was considered day zero of pregnancy. Rats were housed in the McIntyre Animal Centre (McGill University, Montreal, Quebec) and given Purina rat chow and water *ad lib.*

*Embryo culture procedure.* The embryo culture procedure used in this study was based on the system described by New [19]. The uteri of etherized pregnant rats were removed on the morning of day 10 of gestation, and the embryos were dissected free of maternal tissue and the Reichert's membrane, leaving the ectoplacental cone and yolk sac intact. The dissection was done in Hanks' Balanced Salt Solution under aseptic conditions. The embryos were placed in sterile 60-ml culture bottles containing medium consisting of 80% heat-inactivated filtered rat serum, 20% Tyrode's saline and penicillin/streptomycin (final concentrations were 50 units/ml and 50 μg/ml respectively). Each bottle contained two to four embryos with a serum to embryo ratio of 1.6 ml serum medium per embryo. The contents of the bottles were gassed with a mixture of 20% $O_2$, 5% $CO_2$, 75% $N_2$, prior to the addition of buthionine sulfoximine (0.01 to 2.0 mM). The bottles were placed in a rotator (New Brunswick Scientific Co., Edison, NJ) at 25 rpm, and the embryos were cultured for 45 hr at 37°. After the first 24 hr the embryos were regassed, with 95% $O_2$, 5% $CO_2$.

At the end of the culture period all embryos were removed and examined for viability. Only those embryos with yolk sac circulation (a score of one or greater by the scoring system of Brown and Fabro [20]) and a heart beat were further evaluated. The viable embryos were classified as normal or abnormal, the abnormalities were documented, and representative photographs were taken. Embryo growth was assessed by measurements of the yolk sac diameter, crown-rump length and head length, and the number of somites. The embryo scoring system of Brown and Fabro [20] was used to determine a total morphological score for each embryo.

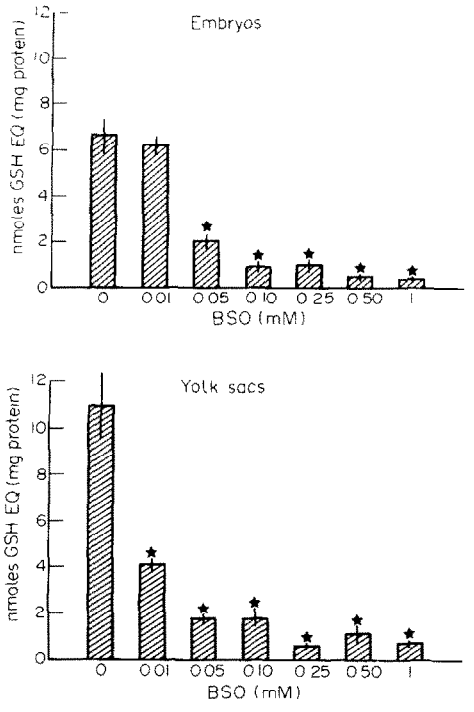


Fig. 1. Effect of buthionine sulfoximine on the glutathione content of cultured rat embryos (top) and their yolk sacs (bottom). The bars represent the means ± SEM (N = 5–13). Key: (★) significantly depleted compared to control ($P \leq 0.05$ by one-way ANOVA with the F-Test).

Embryos and their yolk sacs were individually frozen at −80° for subsequent assay of glutathione and protein content. The tissues were homogenized in 1 ml of 0.01 N HCl, and the total glutathione content (reduced and oxidized) of the homogenized samples was measured by the spectrophotometric method of Tietze [21], as modified by Brehe and Burch [22], and expressed as nmoles glutathione equivalents/mg protein. The level of detection of the glutathione assay was 0.016 nmole glutathione/mg protein. Protein content was measured by the method of Lowry *et al.* [23]. In addition, a time course of the glutathione depletion with exposure to buthionine sulfoximine was done. Embryos were exposed to 0.10 mM buthionine sulfoximine for 2, 4, 8, 12, 24 or 48 hr and frozen for analysis of the glutathione concentrations and comparisons with time-matched controls.

*Statistics.* All data on embryo deaths and malformations were analyzed by the Fisher Exact Test [24]. Comparisons of the yolk sac diameter, crown-rump length, head length, number of somites, morphological score, glutathione and protein contents were done by the one-way ANOVA with the F-Test to isolate the differences [25]. The level of significance used throughout was $P \leq 0.05$.

## RESULTS

The effects of exposure to buthionine sulfoximine (0.01 to 1.0 mM) on the glutathione content of the embryos and their yolk sacs are shown in Fig. 1. In

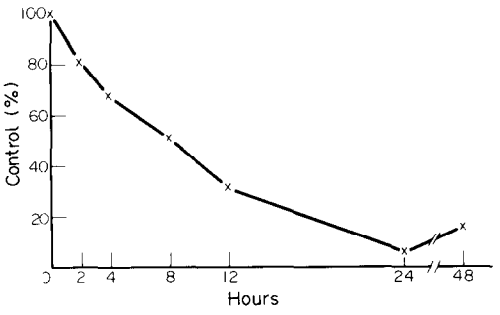


Fig. 2. Time course of glutathione depletion in the whole conceptus after exposure to 0.10 mM buthionine sulfoximine. The results, expressed as percent of control at each time point, are the means of five determinations.

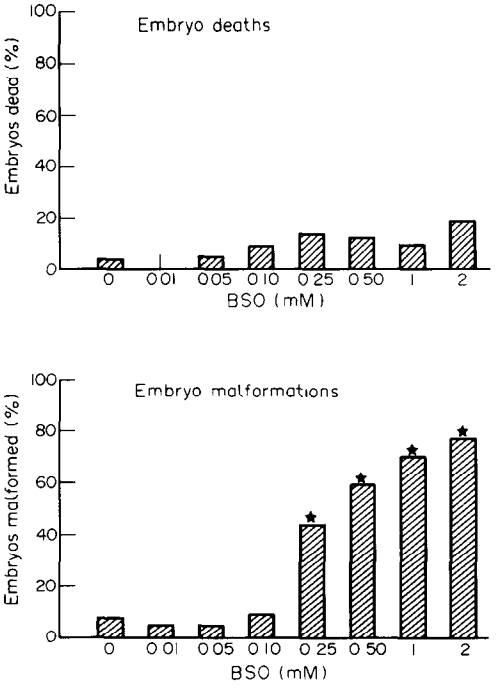


Fig. 3. Effects of buthionine sulfoximine on the incidence of embryo deaths (top) and malformations (bottom). Embryo deaths are quantitated as the percent of embryos surviving out of the total number cultured. Embryo malformations are quantitated as the percent of embryos malformed of the number which survived. Key: (★) significantly greater compared to control ($P \leq 0.05$ by the Fisher Exact Test).

the control embryos, the mean glutathione content was 6.7 nmoles glutathione equivalents/mg protein. There was a significant decrease in the glutathione content of the embryos at buthionine sulfoximine concentrations of 0.05 mM and higher. Control yolk sacs contained 10.9 nmoles glutathione/mg protein. There was a significant decrease in the yolk sac glutathione content at buthionine sulfoximine concentrations of 0.01 mM and above. At 1.0 mM buthionine sulfoximine the glutathione levels of both the embryos and yolk sacs were less than 7% of control.

The glutathione content (nmoles/mg protein) of control embryos and embryos exposed to 0.1 mM buthionine sulfoximine was determined on the whole conceptus (embryo plus yolk sac) at different time intervals. The average glutathione concentration of a control conceptus prior to culture was 11.3 nmoles/mg protein. After 2 hr of culture in the absence of drug, the glutathione content was decreased significantly to 5.8 nmoles/mg protein among control embryos. After 24 or 48 hr of culture the glutathione concentration was not significantly different than at 2 hr. Figure 2 illustrates the glutathione concentration of embryos exposed to 0.10 mM buthionine sulfoximine as a percent of control at each time point. The glutathione levels of buthionine sulfoximine exposed embryos were decreased to 50% of those of control embryos after 8 hr and 7% of control embryos after 24 hr of exposure to buthionine sulfoximine.

The effects of exposure to buthionine sulfoximine on the incidences of embryo deaths and malformations are shown in Fig. 3. The incidence of deaths among control embryos was 4%. None of the concentrations of buthionine sulfoximine tested produced a significant change in embryo deaths compared to control. Among surviving control embryos, 9% were malformed. Exposure to concentrations of buthionine sulfoximine of 0.25 mM and above produced a significant increase in the incidence of malformed embryos. At 0.25, 0.50, 1.0, and 2.0 mM buthionine sulfoximine, 40, 59, 76, and 79% of the embryos were malformed respectively. Fifty-two of the 154 surviving embryos from all the buthionine sulfoximine-treated embryos were malformed. The types of malformations most frequently induced by buthionine sulfoximine were blebs of the maxillary or nasal processes, forelimb buds or forebrain (39 embryos), defects of the brain region such as swollen hind brains and hypoplastic forebrains (28 embryos), and tail defects (11 embryos). Some of these malformations are illustrated in Fig. 4. Embryo A is a control embryo. Embryos B (side view) and C (top view) were exposed to 1.0 mM buthionine sulfoximine.

The effects of exposure to buthionine sulfoximine on rat embryo growth and development *in vitro* are shown in Table 1. Exposure of embryos to buthionine sulfoximine produced growth retardation in a dose-dependent manner. The yolk sac diameter, crown-rump length, head length, number of somites and morphological score were all decreased significantly at buthionine sulfoximine concentrations of 0.05 mM and above; the crown-rump length was also decreased at 0.01 mM buthionine sulfoximine, the lowest concentration of this drug used. Thus, embryonic growth is a more sensitive measure of the effects of glutathione depletion by buthionine sulfoximine than is the presence of malformations. At 2.0 mM buthionine sulfoximine, the yolk sac diameter, crown-rump length, head length, number of somites and the morphological score were only 65, 72, 77, 90 and 80% of control respectively.

In addition to affecting yolk sac diameter, exposure to buthionine sulfoximine also impaired yolk sac circulation. The mean score for the yolk sac circulatory system (possible scores are 0–4 [20]; an

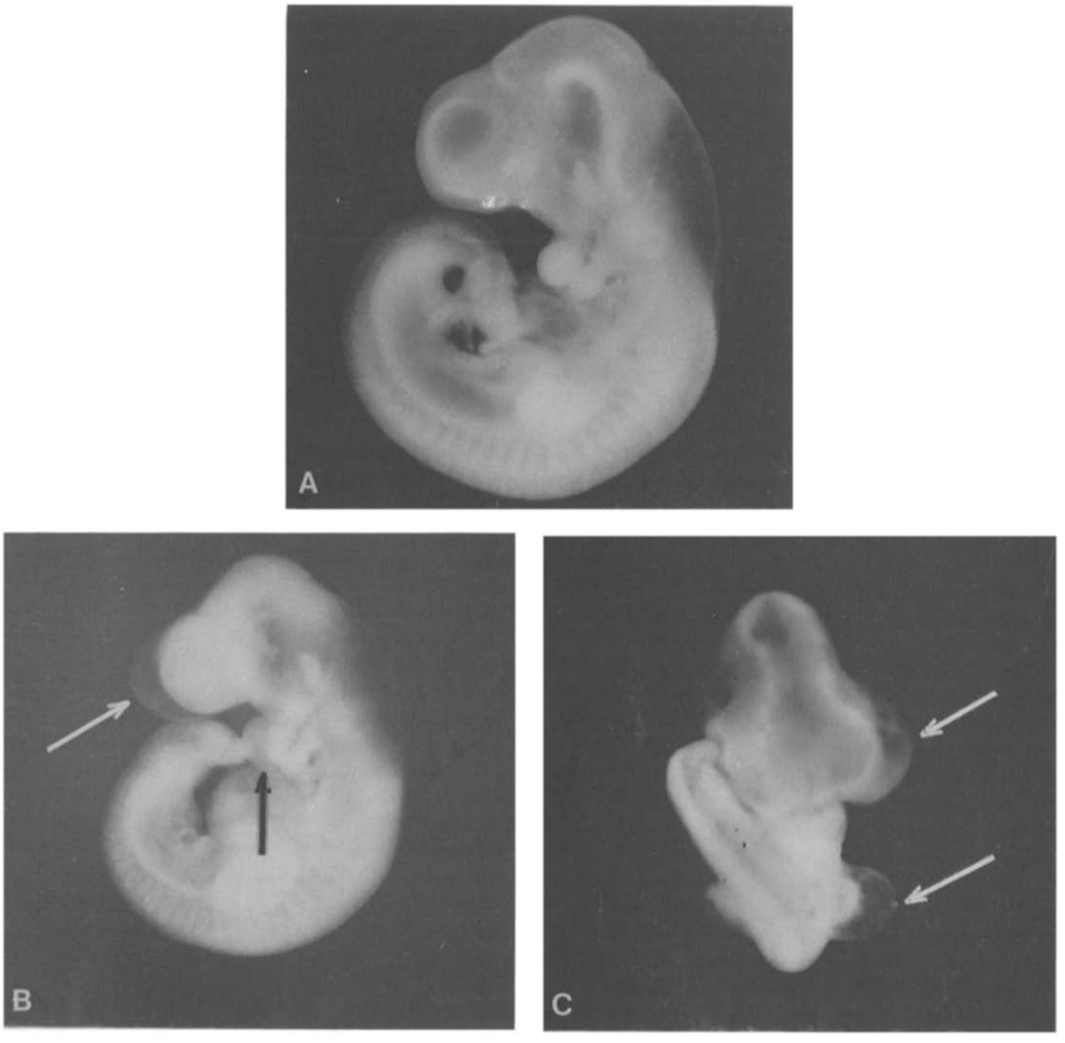


Fig. 4. Effects of buthionine sulfoximine on embryo morphology. Embryo A is a control; embryos B (side view) and C (top view) were exposed to 1.0 mM buthionine sulfoximine. The arrows point to blebs of the prosencephalon, brachial bars, and forelimb buds. Original magnification, 16×.

Table 1. Effects of buthionine sulfoximine (BSO) on embryo growth and development *in vitro*

| | N | Yolk sac diameter (mm) | Crown-rump length (mm) | Head length (mm) | No. of somites | Morphological score |
|---|---|---|---|---|---|---|
| Control | 49 | 5.12 ± 0.08 | 4.84 ± 0.07 | 2.60 ± 0.04 | 36.22 ± 0.21 | 52.67 ± 0.46 |
| 0.01 mM BSO | 20 | 5.02 ± 0.11 | 4.54 ± 0.09* | 2.50 ± 0.06 | 35.45 ± 0.27 | 51.53 ± 0.43 |
| 0.05 mM BSO | 19 | 4.74 ± 0.09* | 4.33 ± 0.06* | 2.32 ± 0.03* | 35.37 ± 0.29* | 49.83 ± 0.47* |
| 0.10 mM BSO | 35 | 4.34 ± 0.10* | 4.17 ± 0.06* | 2.24 ± 0.04* | 34.74 ± 0.17* | 49.02 ± 0.35* |
| 0.25 mM BSO | 25 | 4.03 ± 0.16* | 3.87 ± 0.09* | 2.14 ± 0.09* | 34.12 ± 0.27* | 46.85 ± 0.64* |
| 0.50 mM BSO | 22 | 3.49 ± 0.13* | 3.57 ± 0.11* | 2.14 ± 0.09* | 33.73 ± 0.27* | 45.11 ± 1.19* |
| 1.0 mM BSO | 20 | 3.79 ± 0.15* | 3.83 ± 0.10* | 2.25 ± 0.12* | 33.68 ± 0.27* | 46.13 ± 0.64* |
| 2.0 mM BSO | 13 | 3.34 ± 0.21* | 3.47 ± 0.13* | 2.00 ± 0.08* | 32.54 ± 0.34* | 42.27 ± 1.04* |

Values are the means ± SEM.
* Significantly less than control ($P \leq 0.05$ by one-way ANOVA with the F-Test).

embryo with a score of zero was considered dead) was decreased significantly at buthionine sulfoximine concentrations of 0.05 mM and above (control = 3.48 ± 0.08; 0.05 mM buthionine sulfoximine = 2.55 ± 0.09).

Thus, exposure of cultured rat embryos to buthionine sulfoximine results in significant teratogenicity and growth retardation, but not in significant embryo death.

## DISCUSSION

The objective of this study was to determine the extent of glutathione depletion by buthionine sulfoximine in rat embryos, and the effect of such depletion on embryonic growth and development *in vitro* using rat whole embryo culture. Cultured control rat embryos were found to contain glutathione concentrations approximately one-tenth of those found in the adult rat liver [21], the main organ of drug biotransformation and detoxification. Due to the small size of the embryo, no attempt was made to measure the concentrations of glutathione in different parts of the embryo.

Relatively low levels of buthionine sulfoximine were able to produce profound depletion of glutathione which was more pronounced in the yolk sac than in the embryo proper. This could be a result of the yolk sac being exterior to the embryo and thus exposed to buthionine sulfoximine first, or it may be the result of differing tissue sensitivities to buthionine sulfoximine. The extent of depletion of glutathione by buthionine sulfoximine in a particular tissue is thought to be dependent on the glutathione turnover rate in that tissue [8]. Thus, the turnover rate of glutathione in the yolk sac may be higher than in the embryo.

Buthionine sulfoximine was found to be teratogenic and growth retarding but not embryolethal in the rat whole embryo culture system. Even with depletion of glutathione levels to less than 7% of control, the embryos, although some were malformed, were alive. The effect of buthionine sulfoximine on the different variables of embryo growth that were measured correlated well with the extent of glutathione depletion; in contrast to this finding, very significant depletion of the embryonic and yolk sac glutathione concentration was observed at lower buthionine sulfoximine concentrations than those which produced significant teratogenicity. This could mean that the embryo has a surplus of glutathione, and malformations are seen only after profound depletion. The steep concentration–response curve for malformations could indicate a threshold effect whereby there is a critical concentration below which development does not take place normally. However, the differences in the embryo and yolk sac glutathione concentrations at buthionine sulfoximine concentrations of 0.10 mM and below (non-teratogenic) and buthionine sulfoximine concentrations of 0.25 mM and above (teratogenic) are small compared to the differences in the incidence of malformations. Another explanation could be that more than one pool of glutathione exists within the embryo. These pools could have different turnover rates and thus differing sensitivities to depletion by buthionine sulfoximine. Two kinetically distinct pools of glutathione, cytoplasmic and mitochondrial, are reported to exist in the rat liver [26, 27].

The mechanism(s) by which buthionine sulfoximine produces embryonic growth retardation and malformations is (are) not known, but it seems likely that glutathione depletion by this chemical is involved. Glutathione serves many functions within the cell; perturbation of any of these functions might result in adverse effects on embryonic development. For example, glutathione, with the enzyme glutathione peroxidase, provides protection against cell damage from peroxides generated by normal cell processes [28]; when glutathione is depleted, peroxides could cause cell damage and death leading to embryonic growth retardation and/or malformations. The oxygen requirement is extremely high for the embryos after the first 24 hr of culture; it is possible that reactive metabolites of oxygen mediate embryotoxic effects when glutathione is depleted. Glutathione is also involved in the formation of deoxyribonucleotides for DNA synthesis [29]. In the absence of adequate concentrations of glutathione, DNA synthesis might be impaired. Such impairment of DNA synthesis during a time when many embryonic structures are undergoing rapid growth and formation could lead to growth retardation and/or malformations. Additionally, since one mechanism of amino acid transport is coupled to the catabolism of glutathione [30], depletion of glutathione could impair the transport of amino acids necessary for proper embryonic growth and development.

Depletion of glutathione by buthionine sulfoximine has been demonstrated to be cytotoxic to human lymphoid cells *in vitro* [14]. Exposure to 1.0 mM buthionine sulfoximine for 48 hr decreases the viability of cultured T-cells to 73% of control cells cultured without this drug [14]; the glutathione concentrations of these cells are less than 1% of the control cells. However, mouse lymphoma cells can be adapted to long-term culture in the presence of concentrations of buthionine sulfoximine as high as 10 mM [31]. The glutathione concentrations of these adapted cells are 10% of control cells and they proliferate at a similar rate to that of nondepleted cells [31].

It has not been clearly proven that the effects observed in this study in cultured rat embryos or in previous investigations with cultured cells [14] are due specifically to depletion of glutathione. However, in view of the information available on the mechanism of action of buthionine sulfoximine and on its selectivity, we feel that these effects are very likely to be due to glutathione depletion. Buthionine sulfoximine is a selective inhibitor of the enzyme γ-glutamylcysteine synthetase [8]. Methionine sulfoximine, an analog of buthionine sulfoximine which also depletes glutathione, causes convulsions and death in mice, apparently due to its ability to inhibit the enzyme glutamine synthetase [32]. Buthionine sulfoximine does not inhibit glutamine synthetase and is not a convulsant [8]. Buthionine sulfoximine also does not inhibit glutathione synthetase, the second enzyme in the pathway of glutathione synthesis, γ-glutamylcyclotransferase or γ-glutamyltranspeptidase, two enzymes of the γ-glutamyl cycle

of glutathione degradation.* *In vivo*, buthionine sulfoximine administered to mice in single doses of 32 mmoles/kg and in multiple doses totalling 72 mmoles/kg in 27 hr has no toxic effects [8, 10]. Mice have consumed drinking water containing 20 mM buthionine sulfoximine for 45 days without toxic effects [9]. Furthermore, buthionine sulfoximine added to the drinking water of adult mice for 28 days produces no differences in a number of measured biochemical variables [33].

In view of the lack of observed toxicity in adult mice, the teratogenicity and growth retardation in the developing rat embryo were unexpected. We suggest that endogenous glutathione is essential in normal embryonic development.

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