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ENHANCEMENT OF THE EMBRYOTOXICITY OF ACROLEIN, BUT NOT PHOSPHORAMIDE MUSTARD, BY GLUTATHIONE DEPLETION IN RAT EMBRYOS *IN VITRO*

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Abstract—The intracellular thiol glutathione is known to protect cells against the toxicity of certain drugs and reactive intermediates. In this study, the role of glutathione in protecting the embryo against two embryolethal and teratogenic metabolites of cyclophosphamide, an anticancer drug, was assessed *in vitro* using the rat whole embryo culture system. Day 10.5 rat embryos were cultured in rat serum medium containing phosphoramide mustard (1, 10, or 25 μM) or acrolein (10, 25, 50 or 100 μM), with and without buthionine sulfoximine (10 or 100 μM), a compound which depletes glutathione by inhibiting its synthesis. After 45 hr, embryos were assessed for viability, malformations, growth and development, and the glutathione content of embryos exposed to buthionine sulfoximine alone was assayed. The glutathione levels of the embryos and their yolk sacs were decreased significantly by 100 μM buthionine sulfoximine, whereas 10 μM buthionine sulfoximine decreased glutathione levels in the yolk sacs only. Phosphoramide mustard alone, at concentrations of 10 and 25 μM, did not produce embryo deaths but did cause malformations and growth retardation in 100% of the exposed embryos. The addition of buthionine sulfoximine (100 μM) had no effect on the teratogenicity or growth-retarding effects of phosphoramide mustard. Acrolein alone produced a 25 and 48% incidence of embryo deaths at 50 and 100 μM, respectively, and a 46% incidence of embryo malformations, as well as significant growth retardation, among the surviving embryos at 100 μM. Buthionine sulfoximine (10 or 100 μM) significantly enhanced the embryotoxic effects of acrolein. The addition of 10 μM buthionine sulfoximine resulted in 100% embryolethality at 100 μM acrolein; this buthionine sulfoximine concentration decreased the EC_{50} values for embryo deaths and malformations to 50% of those for acrolein alone. The addition of 100 μM buthionine sulfoximine significantly potentiated the embryolethality of acrolein at 25, 50 and 100 μM; the combination of 100 μM acrolein plus 100 μM buthionine sulfoximine was 100% embryolethal. The incidence of embryo malformations was enhanced significantly at 10 and 25 μM acrolein by 100 μM buthionine sulfoximine. The EC_{50} values for embryo deaths and malformations were decreased to 50 and 20%, respectively, of those values for acrolein alone. Both buthionine sulfoximine concentrations produced significant growth retardation at all acrolein concentrations compared to either acrolein or buthionine sulfoximine alone. Thus, depletion of glutathione by buthionine sulfoximine dramatically enhanced the embryolethal, teratogenic and growth retarding effects of acrolein *in vitro*, but did not alter the embryotoxicity of phosphoramide mustard. Therefore, glutathione plays an important role in protecting the embryo against only one of the teratogenic metabolites of cyclophosphamide.

Many drugs and environmental contaminants are teratogenic to the developing embryo. Often, neither the mechanism of action of the chemical, nor the mechanisms by which embryonic cells might protect themselves and possibly detoxify the drug, are known. Adequate levels of intracellular free thiols are important in protecting many cells and organisms against drug toxicity [1, 2]. The major protective thiol in most organisms is the tripeptide glutathione; little is known about the concentrations or functions of glutathione in the embryo. However, it seems reasonable that glutathione is involved in protecting the embryo and fetus against damage from toxic chemicals and reactive intermediates.

Both Ashby *et al.* [3] and Hales [4] have found that pretreatment of pregnant rats with glutathione can protect markedly against the teratogenicity of the anticancer drug cyclophosphamide. Furthermore, Hales [4] has shown that pretreatment with diethylmaleate, a depleter of glutathione, exacerbates the *in vivo* teratogenicity of cyclophosphamide in rats. *In vitro*, Kitchin *et al.* [5] demonstrated that glutathione can protect against the embryolethality and growth-retarding effects, but not the teratogenicity, of mercuric chloride in cultured rat embryos. The effect of depletion of glutathione on the response of cultured embryos to teratogens has not, to the best of our knowledge, been reported.

Phosphoramide mustard and acrolein are toxic and reactive metabolites of the widely used anticancer drug and known teratogen, cyclophosphamide [6]. Both phosphoramide mustard and acrolein are teratogenic and embryolethal in rabbits and rats *in vivo* when administered intraamniotically [7–9] and *in vitro*, in whole rat embryo culture [10, 11]. At least one of these compounds, acrolein, is known to be very reactive toward thiols and can form conjugates

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with glutathione both *in vivo* and *in vitro*; these reactions can occur nonenzymatically and enzymatically catalyzed by the glutathione-*S*-transferases [12–14]. Additionally, acrolein can deplete hepatic glutathione levels in rats *in vivo* [15] and in cultured hepatocytes *in vitro* [16].

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 [17] to block glutathione synthesis by specifically inhibiting one of the synthetic enzymes, γ-glutamylcysteine synthetase. Buthionine sulfoximine is less toxic and more specific in its action than either diethylmaleate or diamide, two widely used glutathione-depleting agents. In addition, when synthesis is the target for inhibition, inhibition should be sustained longer, since rebound synthesis is less likely to occur. Buthionine sulfoximine successfully depletes glutathione levels within many tissues in adult mice and rats when administered orally or subcutaneously, with no observable adverse effects [18–20]. *In vitro*, buthionine sulfoximine decreases glutathione levels in cultured macrophages [21], and in human lymphoid cells to less than 3% of control after 30 hr of culture [22].

In the present study, we investigated, using rat whole embryo cultures, the extent of glutathione depletion by buthionine sulfoximine within embryos and their yolk sacs and the effects of this depletion of glutathione on the embryolethality and teratogenicity of phosphoramide mustard and acrolein, the two teratogenic metabolites of cyclophosphamide.

METHODS

Chemicals. Acrolein (99% pure) was purchased from the Aldrich Chemical Co. (Montreal, Quebec). Phosphoramide mustard (ASTA-5317) was provided by Professor N. Brock (Asta-Werke, Bielefeld, Germany) and L-buthionine-*S,R*,-sulfoximine by Dr. A. 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 of New [23] as modified in our laboratory [11]. Pregnant rats were etherized 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 embryos were placed in sterile 60 ml culture bottles containing medium consisting of 80% heat-inactivated 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 1.6 ml of medium per embryo. The bottles were gassed with a mixture of 20% O_2, 5% CO_2, 75% N_2, prior to the addition of phosphoramide mustard alone (final concentration of 1, 10 or 25 μM), acrolein alone (final concentration of 10, 25, 50 or 100 μM), or phosphoramide mustard or acrolein in combination with buthionine sulfoximine (10 or 100 μM). 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 1 or greater by the scoring system of Brown and Fabro [24]) and a heart beat were evaluated further. The embryos were classified as normal or abnormal, and the abnormalities were documented. The yolk sac diameter, crown–rump length, and head length were measured, and the number of somites was counted. The embryo scoring system of Brown and Fabro [24] was used to determine a total morphological score for each embryo. Embryos and their yolk sacs were individually frozen at −80° for subsequent assay of glutathione and protein content. Total glutathione content of the homogenized samples was measured by the method of Tietze [25] as modified by Brehe and Burch [26] and expressed as nmol glutathione equivalents/mg protein (level of detection of 0.016 nmol glutathione/mg protein). Protein content was measured by the method of Lowry *et al.* [27].

Statistics. All data on embryo deaths and malformations were analyzed by the Fisher Exact Test [28]. In addition, comparisons of the dose–response curves for acrolein-induced embryo deaths and malformations in the absence and presence of buthionine sulfoximine were done by probit analysis with EC_{50} determinations [29]. Comparisons of the glutathione content, yolk sac diameter, crown–rump length, head length, number of somites, and morphological score were done by the one-way ANOVA with the F-Test used to isolate the differences between groups [30]. The level of significance used throughout was $P \leq 0.05$.

RESULTS

Effect of buthionine sulfoximine on embryo and yolk sac glutathione concentrations. The effects of the addition of buthionine sulfoximine on the glutathione levels within the cultured embryos and yolk sacs are shown in Fig. 1, A and B. Culture with 10 μM buthionine sulfoximine had no effect on the glutathione content of the embryo but significantly decreased the glutathione content within the yolk sacs. However, exposure to 100 μM buthionine sulfoximine markedly decreased the glutathione concentrations in both the embryos and yolk sacs to 16 and 17% of control respectively.

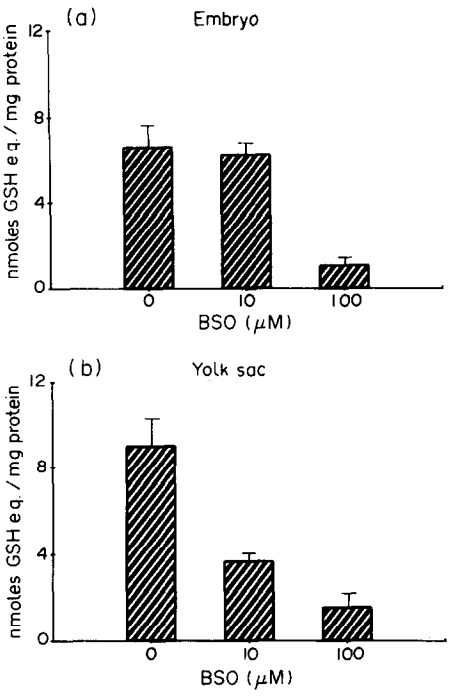

Fig. 1. Effects of buthionine sulfoximine on the glutathione content of cultured rat embryos (a) and their yolk sacs (b). The bars represent means ± SEM.

Effects of phosphoramide mustard and buthionine sulfoximine on embryo deaths, malformations and growth. There were no embryo deaths among the control embryos or those exposed to phosphoramide mustard concentrations of 1, 10 or 25 μM, whereas exposure to 50 μM phosphoramide mustard was 100% embryolethal. There were no malformations among the control embryos or those exposed to 1 μM phosphoramide mustard, yet all the embryos exposed to phosphoramide mustard concentrations of 10 or 25 μM were malformed. In the presence of buthionine sulfoximine (100 μM), none of the control embryos or low dose (1 μM) phosphoramide mustard-treated embryos were either dead or malformed. There was also no significant effect of the addition of buthionine sulfoximine on the embryos exposed to 10 or 25 μM phosphoramide mustard. As observed with phosphoramide mustard alone, all of the embryos treated with phosphoramide mustard (10 μM) and buthionine sufloximine (100 μM) were alive and malformed; of the embryos exposed to 25 μM phosphoramide mustard and buthionine sulfoximine, 23% were dead while the surviving embryos were all abnormal.

The malformations observed after exposure of the embryos to phosphoramide mustard included hypoplasia and ventrolateral protrusions of the prosencephalon, hypoplastic limb buds, abnormal mandibular arches and blunted tails. There were no differences in the types or the severity of malformations between those embryos exposed to phosphoramide mustard alone or phosphoramide mustard plus buthionine sulfoximine.

The effects of phosphoramide mustard alone and in combination with buthionine sulfoximine on parameters of embryo growth and development are shown in Fig. 2. Concentrations of phosphoramide mustard of 10 and 25 μM significantly decreased the yolk sac diameter, crown–rump length, head length and somite number of cultured embryos compared to control. At 25 μM phosphoramide mustard, these parameters were 74, 60, 63 and 59% of control respectively. Buthionine sulfoximine (100 μM) alone also significantly decreased the yolk sac diameter, crown-rump length and head length of cultured embryos, to 84, 72 and 76% of control, respectively, yet had not effect on the somite number. Interestingly, there was no significant increase in the growth retardation caused by phosphoramide mustard when buthionine sulfoximine was added. Thus, although both phosphoramide mustard and buthionine sulfoximine alone caused growth retardation, these effects were clearly not additive.

The morphological score of control embryos was 47.2 ± 0.8. Exposure to phosphoramide mustard concentrations of 10 or 25 μM decreased the morphological score of cultured embryos to 37.2 ± 0.5 and 21.8 ± 0.6 respectively. Buthionine sulfoximine alone (100 μM) significantly decreased the morphological score of cultured embryos of 42.8 ± 1.5, but had no significant effect on the morphological scores of embryos concomitantly exposed to either 10 or 25 μM phosphoramide mustard.

In summary, the addition of buthionine sulfoximine did not alter the embryolethality, teratogenicity or growth retardation caused by phosphoramide mustard.

Effects of acrolein and buthionine sulfoximine on embryo deaths, malformations and growth. The effects of buthionine sulfoximine and acrolein alone, and in combination, on the incidence of embryo deaths are illustrated in Fig. 3. There was one embryo death among control embryos, no deaths among embryos exposed to 10 μM buthionine sulfoximine, and two embryo deaths (11%) at 100 μM buthionine sulfoximine. There were no embryo deaths among embryos exposed to either 10 or 25 μM acrolein, but significant embryolethality was observed at 50 μM (25%) and 100 μM (48%) acrolein. The addition of 10 μM buthionine sulfoximine had no significant effect on the incidence of embryo deaths at 10, 25 or 50 μM acrolein; however, the combination of 10 μM buthionine sulfoximine plus 100 μM acrolein was 100% embryolethal. With 100 μM buthionine sulfoximine, the embryolethality of acrolein was enhanced significantly at 25 μM (from 0 to 26%) and 50 μM acrolein (from 25 to 75%). The combination of 100 μM buthionine sulfoximine and 100 μM acrolein was 100% embryolethal.

The effects of acrolein and buthionine sulfoximine alone, and in combination, on the incidence of malformed embryos are shown in Fig. 4. Among the control group, 9% of the surviving embryos were malformed. No embryos were malformed following exposure to 10 μM buthionine sulfoximine, and only one embryo was malformed after exposure to 100 μM buthionine sulfoximine. Acrolein alone did not produce any malformed embryos at 10 μM and did not produce a significant number of malformed embryos at 25 or 50 μM. At 100 μM acrolein, however, 46% of the surviving embryos were malformed. The

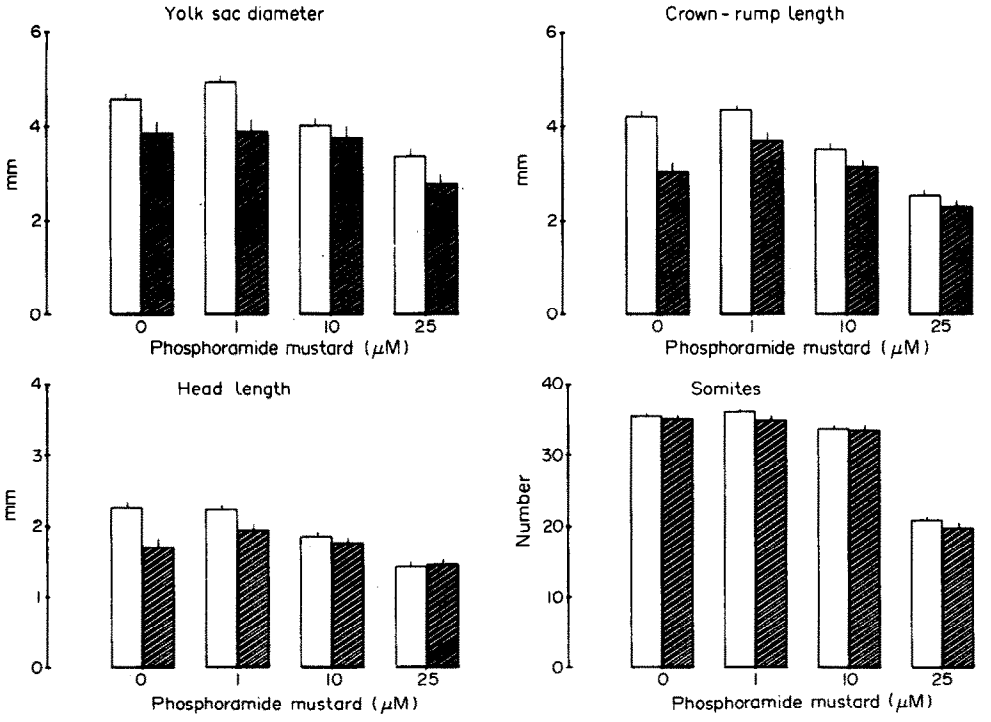

Fig. 2. Effects of phosphoramide mustard alone and in combination with buthionine sulfoximine on embryo growth *in vitro*. Key: (□) embryos not exposed to a drug (0) or exposed to phosphoramide mustard (1, 10 or 25 μM) alone; (▨) embryos exposed to phosphoramide mustard and 100 μM buthionine sulfoximine. Values represent means ± SEM.

addition of 10 μM buthionine sulfoximine had no significant effect on the incidence of malformed embryos at any of the acrolein concentrations evaluated. However, with the addition of 100 μM buthionine sulfoximine, 39% of the embryos exposed to an acrolein concentration of only 10 μM were malformed. This is one-tenth the concentration of acrolein which produced significant teratogenicity in the absence of buthionine sulfoximine. There was also significant enhancement of the teratogenicity of acrolein at a concentration of 25 μM with the addition of 100 μM buthionine sulfoximine. [The combination of 100 μM acrolein with either concentration of buthionine sulfoximine could not be evaluated for malformations as it was 100% embryolethal (Fig. 3)].

The most frequently observed malformations with acrolein and buthionine sulfoximine were similar to those found in the embryos exposed to acrolein

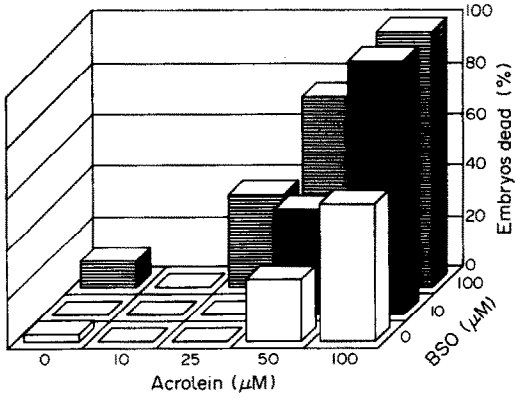

Fig. 3. Effects of acrolein alone and in combination with buthionine sulfoximine on the incidence of deaths among cultured rat embryos. Key: (□) embryos not exposed to a drug (0) or exposed to acrolein (10, 25, 50 or 100 μM) alone; (■) embryos exposed to no drug or to acrolein and 10 μM buthionine sulfoximine; and (▤) embryos exposed to no drug or to acrolein and 100 μM buthionine sulfoximine.

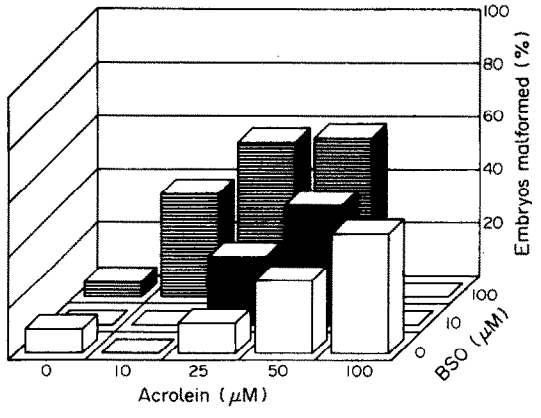

Fig. 4. Effects of acrolein alone and in combination with buthionine sulfoximine on the incidence of malformations among cultured rat embryos. Key: (□) embryos not exposed to a drug (0) or exposed to acrolein (10, 25, 50 or 100 μM) alone; (■) embryos exposed to no drug or to acrolein and 10 μM buthionine sulfoximine; and (▤) embryos exposed to no drug or to acrolein and 100 μM buthionine sulfoximine.

Table 1. EC_{50} values for the embryolethality and teratogenicity of acrolein alone and combined with buthionine sulfoximine (BSO)

	EC_{50} (μM)	
	Embryo deaths	Embryo malformations
Acrolein alone	89	101
Acrolein + 10 μM BSO	52*	48
Acrolein + 100 μM BSO	32	19

* An EC_{50} could not be calculated since there was only one dose which was not a 0% or 100% response. This is an estimate determined from linear regression of the log concentration–response curve.

alone. These malformations were of the brain region such as swollen hindbrains and hypoplastic forebrains, facial abnormalities such as blebs or protrusions of the maxillary and nasal processes, indentations of the somites, usually at the forelimb bud level, cardiac abnormalities such as an enlarged pericardium and/or underdeveloped heart, and abnormal flexion [11].

The EC_{50} values for embryo deaths and malformations with acrolein in the presence and absence of buthionine sulfoximine are shown in Table 1. With acrolein alone the EC_{50} for embryo malformations was higher than that for embryo deaths. With the addition of 10 μM buthionine sulfoximine, the EC_{50} values for deaths and malformations were approximately equal, whereas with the addition of 100 μM buthionine sulfoximine the EC_{50} for embryo deaths was nearly twice that for embryo malformations. Thus, it appears that with depletion of glutathione by buthionine sulfoximine there is a shift from acrolein being primarily an embryolethal agent to a more teratogenic one. A test for parallelism [31] of the concentration–response curves for embryo deaths and malformations found the slopes for both embryo deaths and embryo malformations to be significantly different between acrolein alone and acrolein plus either 10 or 100 μM buthionine sulfoximine ($P < 0.05$, one-tailed).

The effects of acrolein and buthionine sulfoximine alone, and in combination, on rat embryo growth and development are shown in Fig. 5. Neither 10, 25 or 50 μM acrolein induced growth retardation as assessed by the yolk sac diameter, crown–rump length, head length or number of somites. The lower concentration of buthionine sulfoximine itself had no effect on any of the measured parameters of embryo growth and development, yet inclusion of this buthionine sulfoximine concentration with acrolein in the culture medium resulted in significant decreases in the embryo head length at 10 μM acrolein and in the yolk sac diameter, crown-rump length, head length or number of somites at 25 μM acrolein or 50 μM acrolein compared to embryos exposed to these concentrations of acrolein alone. All of these decreases were also significant compared to the embryos exposed to 10 μM buthionine sulfoximine alone. Exposure to 100 μM buthionine sulfoximine alone resulted in slight yet statistically significant decreases in all four measurements of embryo growth

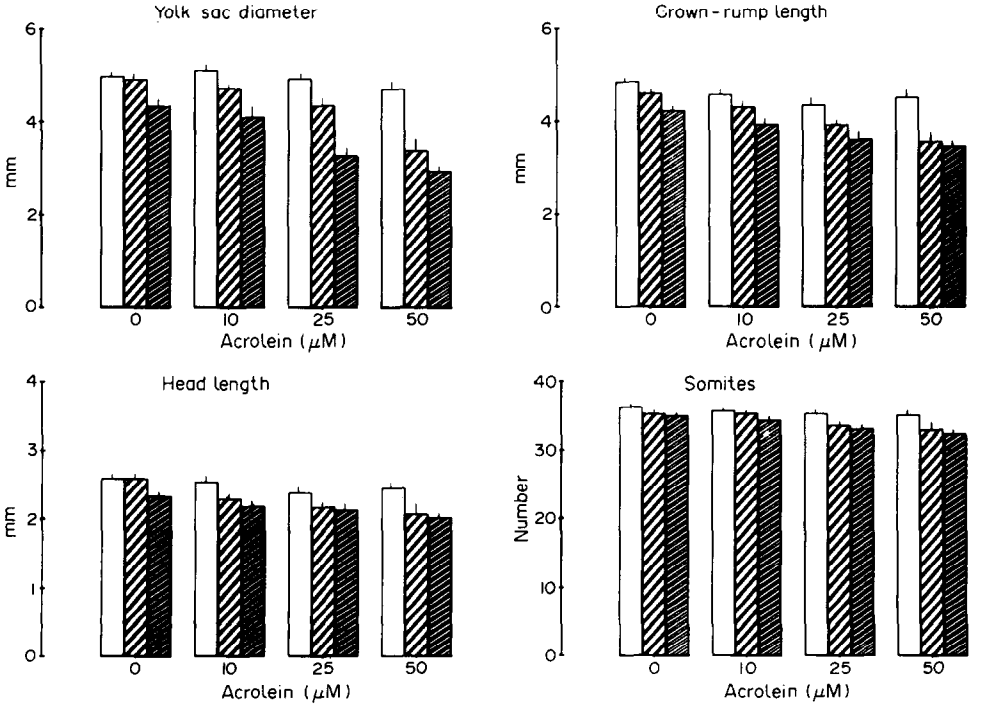

Fig. 5. Effects of treatment with acrolein alone or in combination with buthionine sulfoximine on embryo growth *in vitro*. Key: (□) embryos not exposed to a drug (0) or exposed to acrolein (10, 25 or 50 μM) alone; (▨) embryos exposed to no drug or to acrolein and 10 μM buthionine sulfoximine; and (▨) embryos exposed to no drug or to acrolein and 100 μM buthionine sulfoximine. The values represent means ± SEM.

and development. When embryos were exposed to 100 μM buthionine sulfoximine plus acrolein, there was a significant decrease in all four measurements of embryo growth and development at 10 and 25 μM acrolein and in the yolk sac diameter, crown–rump length and number of somites at 50 μM acrolein compared to acrolein alone. All of these decreases were significantly different from embryos exposed to 100 μM buthionine sulfoximine alone. The magnitude of the growth retardation produced by the two drugs in combination was greater than the sum of the decreases caused by the drugs individually. Thus, as for embryo deaths and malformations, acrolein and buthionine sulfoximine act synergistically with respect to their effects on growth retardation in cultured rat embryos.

DISCUSSION

The present results demonstrate that cultured rat embryos and their yolk sacs contain measurable amounts of glutathione and that buthionine sulfoximine, when added to the embryo culture medium, does deplete glutathione in both the embryo and its yolk sac. However, the addition of buthionine sulfoximine had no effect on the embryotoxicity or growth retardation induced by phosphoramide mustard. In contrast to the results with phosphoramide mustard, the *in vitro* embryolethality, teratogenicity and growth retardation of acrolein were dramatically enhanced by the addition of buthionine sulfoximine to the culture medium. Acrolein is known to be reactive toward thiols, whereas phosphoramide mustard is less reactive and has been reported to be an order of magnitude less effective than acrolein or cyclophosphamide in depleting hepatic glutathione [15]. In consequence, the administration of thiol compounds has been reported previously to protect against the toxic effects of cyclophosphamide that are mediated by acrolein (e.g. urotoxicity) but not those mediated by phosphoramide mustard (e.g. leukopenia) [15, 32, 33].

Acrolein has a narrow teratogenic range and steep dose–response curves for embryolethality and teratogenicity *in vivo* when administered intraamniotically [8, 9], and *in vitro* in whole embryo culture [11]; this may be indicative of a threshold effect whereby the embryotoxic effects are not seen until a critical acrolein concentration is achieved. For this threshold to be overcome, the mechanism(s) producing the cell damage leading to teratogenesis must occur at a greater rate than the mechanism(s) involved in protecting the embryo. Since depletion of glutathione by buthionine sulfoximine markedly potentiated the embryotoxicity of acrolein, it seems apparent that glutathione is involved in protecting the embryo against acrolein-mediated toxicity and may play a role in the threshold phenomenon.

Exposure to buthionine sulfoximine itself, in higher concentrations, has been reported to cause malformations in cultured rat embryos [34] and to produce dense cataracts in weanling mice administered the drug between 9 and 12 days after birth [35]. When human lymphoid cells are glutathione depleted by buthionine sulfoximine, they exhibit increased sensitivity to irradiation [21]. Exposure of tumor cells to buthionine sulfoximine increases their susceptibility to oxidative cytolysis [36, 37]. Treatment of murine L1210 leukemia cells resistant to the anticancer drug L-phenylalanine mustard with buthionine sulfoximine lowers the abnormally high glutathione levels within these cells and resensitizes them to this alkylating agent [38]. Thus, it is particularly interesting that the presence of buthionine sulfoximine had no effect on the teratogenicity of the metabolite of cyclophosphamide with high alkylating activity, phosphoramide mustard. In fact, there is probably an antagonistic effect because there was no difference with respect to embryo growth and development between those embryos exposed to phosphoramide mustard alone and those exposed to phosphoramide mustard and buthionine sulfoximine, yet buthionine sulfoximine alone did cause growth retardation.

It is interesting that on a nmol/mg basis the embryonic yolk sacs contained higher concentrations of glutathione than the embryo itself and were clearly more sensitive than were the embryos to glutathione depletion by buthionine sulfoximine. These findings may reflect a sensitivity of the yolk sac to chemical alterations and drug toxicity as well as the importance of the integrity of the yolk sac to embryonic growth and development. Exposure to acrolein *in vitro* does result in a decrease in yolk sac circulation and circulatory system development [11]. The yolk sac may be a target of the action of acrolein, whereas the embryo proper may be the site of action of phosphoramide mustard.

In summary, the present findings demonstrate that marked glutathione depletion can be produced within cultured rat embryos using buthionine sulfoximine. This depletion synergistically enhanced the embryolethal, teratogenic and growth-retarding effects of acrolein but not phosphoramide mustard *in vitro*. Thus, there are fundamental differences with respect to the effects of these two reactive teratogens on rat embryos in culture.

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