Previous studies with irreversible β-adrenergic blockers have indicated that maximal cyclase activity can still be achieved even when a large percentage of receptors is blocked [17, 18]. This is in contrast to our *in vitro* results in Fig. 1B where maximal cyclase activity is reduced. The difference may lie in the tissue preparations that were utilized. Previously, whole cells or intact papillary muscles were used to measure hormone response while our present studies utilize tissue homogenates. In the intact cells and tissues, receptors and receptor–cyclase complexes may be re-arranged to fully active units, or receptors may be able to recycle from previously inaccessible intracellular locations subsequent to treatment with irreversible blocker and thereby illicit a maximal response. In our homogenates, the blocker may have complete access to all receptors or the homogenization process may disrupt cytoskeletal elements or energy gradients necessary for efficient coupling of occupied receptor and cyclase activation. (See Ref. 19 for a discussion of these phenomena.)

The effects of an irreversible β-adrenergic blocker, bromoacetylated alprenolol menthane (BrAcAlpM), were examined on the catecholamine-sensitive adenylate cyclase system. The results suggest that this compound is acting as an irreversible blocker of biochemically relevant β-adrenoceptors. Both *in vitro* and *in vivo* results indicate a significant inhibition of (−)isoproterenol-stimulated adenylate cyclase activity in a manner consistent with an irreversible blockade of receptor function.

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Reduction in cellular glutathione by buthionine sulfoximine and sensitization of murine tumor cells resistant to L-phenylalanine mustard*

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Murine L1210 leukemia cells resistant to the bifunctional alkylating agent L-phenylalanine mustard (L-PAM) have a 2-fold greater intracellular concentration of glutathione (GSH) and glutathione disulfide (GSSG) than do L-PAM sensitive cells [1] and convert L-PAM to its non-cytotoxic derivative 4-[bis(2-hydroxyethyl)amino]-L-phenylalanine [2]. Such L-PAM detoxification is related to the intracellular concentration of GSH and GSSG [2]. These observations prompted investigation into regimens which could be used to reduce the cellular content of GSH and GSSG and to determine whether such reduction was accompanied by altered sensitivity to the drug [1]. It was demonstrated that intracellular GSH and GSSG could be reduced by nutritional deprivation of L-cysteine. Incubation of cells in nutrient medium without L-cystine resulted in a rapid decrease in the cellular content of both glutathione disulfide and glutathione. Intracellular GSSG levels in sensitive and resistant cells decreased and became equivalent approximately 6 h following L-cysteine deprivation while the intracellular concentrations of GSH in the two cells, differing 5-fold in their sensitivity to L-PAM, became equivalent 18 h following deprivation of L-cysteine. L-PAM was equitoxic to resistant cells incubated in medium with a reduced

* A preliminary account of this work has been published: *J. cell. Biochem.* (Suppl.) 6, 375, (1982).

concentration of L-cystine and to sensitive tumor cells incubated in medium with L-cystine [1]. In an attempt to extend these observations we have initiated a series of studies with DL-buthionine-*S,R*-sulfoximine, a potent inhibitor of γ-glutamyl cysteine synthetase and, thereby, glutathione biosynthesis [3]. The present studies were undertaken to explore the possibility of employing pharmacological regimens to reduce cellular glutathione concentrations, a strategy considered potentially more applicable *in vivo* than nutritional deprivation of L-cysteine.

Materials and methods

Fetal calf serum was purchased from Flow Laboratories, Rockville, MD. and RPMI 1630 medium and Dulbecco's phosphate-buffered saline were supplied by Grand Island Biological, Grand Island, NY. Gentamicin (Schering, 50 mg/ml) was purchased from Microbiological Associates, Bethesda, MD. NADPH, Type IV glutathione reductase, and 5-5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from the Sigma Chemical Co. St. Louis, MO. 2-Vinylpyridine was obtained from the Aldrich Chemical Co., Inc., Milwaukee, WI. Glutathione and glutathione disulfide were obtained from the Calbiochem-Behring Corp., Los Angeles, CA. Unlabeled L-PAM was obtained from the Burroughs Wellcome Co., Research Triangle Park, NC, and was prepared as a 10 mM stock in 75% ethyl alcohol containing equimolar hydrochloric acid. DL-Buthionine-*S,R*-sulfoximine was purchased from Bachem Inc., Torrance, CA. ALZET mini-osmotic pumps, model 2001, were obtained from the ALZA Corp., Palo Alto, CA.

In vitro *growth of murine L1210 leukemia cells and evaluation of drug cytotoxicity*. The L-PAM sensitive murine L1210 leukemia was obtained under contract from the Mason Research Institute, Boston, MA, and was maintained in female DBA/2 mice by weekly intraperitoneal injection of 10^5 cells. An L-PAM resistant tumor was developed at the Southern Research Institute, Birmingham, AL, and was also maintained in female DBA/2 mice by weekly intraperitoneal injection of 10^6 cells. Mice bearing the L-PAM resistant tumor received an intraperitoneal injection of L-PAM (7.5 mg/kg) 2 days after tumor inoculation. The L-PAM resistant tumor (L1210/L-PAM$_1$) and its sensitive counterpart (L1210) have been maintained *in vitro* in RPMI 1630 medium supplemented with 16% heat-inactivated fetal calf serum and 50 μM β-mercaptoethanol. The effect of DL-buthionine-*S,R*-sulfoximine on the cellular content of glutathione was evaluated following exposure of cells to the inhibitor at concentrations of 1–500 μM for periods of 1–72 hr. Incubations were performed in RPMI 1630 medium containing 20% fetal calf serum. The cytotoxicity of L-PAM was evaluated following a 2-day exposure to the drug in the respective experimental medium. Cells were harvested following exposure to L-PAM and washed in fully supplemented RPMI 1630 medium; cell survival was estimated by growth of cells in soft-nutrient agar for 2 weeks according to the procedure of Chu and Fischer [4]. All growth medium utilized for studies described in this communication was supplemented with 50 μM β-mercaptoethanol and 50 μg/ml gentamicin. The intracellular concentration of glutathione was determined by the method of Griffith [5] as described previously [1].

In vivo *antitumor experiments*. The L-PAM sensitive and resistant tumors were passaged as described above. Male CDF$_1$ mice were inoculated intraperitoneally with 1×10^5 cells and used for all antitumor experiments described in this communication.

The model 2001 ALZET mini-osmotic pump was utilized in selected experiments in order to evaluate the effect of sustained administration of DL-buthionine-*S,R*-sulfoximine on the antitumor activity of L-PAM. The pump has a capacity of 225 μl and a pumping rate of 1.0 μl/hr. The inhibitor was prepared at a concentration of 50 mg/ml in phosphate-buffered saline. The pumps, containing 11.2 mg of inhibitor, were implanted intraperitoneally into 28–30 g male CDF$_1$ mice anesthesized with 65 mg/kg sodium pentobarbital. Mice were inoculated intraperitoneally with 1×10^5 cells 24 hr following implantation of the pump. Therapy was initiated 24 hr following tumor inoculation.

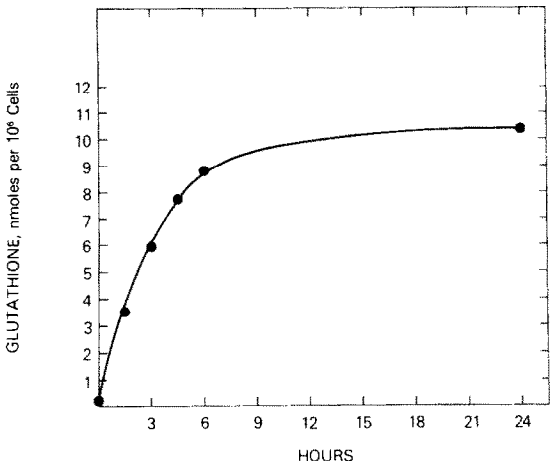

Fig. 1. L-Cystine and regeneration of cellular glutathione. L1210/L-PAM$_1$ cells were harvested from RPMI 1630 medium containing 20% fetal calf serum, washed in RPMI 1630 medium with a reduced concentration of L-cystine [1], and suspended in the same medium at a concentration of 2.5×10^5 cells/ml. Twenty-four hours later, 1×10^7 cells were harvested, and the cellular content of glutathione was determined by the method of Griffith [5] as described previously [1]. The remaining cells were suspended in RPMI 1630 medium containing 20% fetal calf serum and 100 mg/l L-cystine. Aliquots (1×10^7 cells) were removed at 1.5, 3.0, 4.5, 6.0 and 24 hr following resuspension in medium containing L-cystine and prepared for glutathione analysis as described previously [1].

Results

Sensitization of L-phenylalanine mustard resistant tumor cells to L-PAM was reversible. Previous studies [1] demonstrated that reduction in cellular GSH and GSSG by nutritional deprivation of L-cysteine results in sensitization of the resistant tumor cell to L-PAM. These results prompted further studies designed to determine whether restoration of the cellular glutathione pool was accompanied by reacquisition of resistance to L-PAM. As can be seen in Fig. 1, cells rapidly replenished their glutathione pool following exposure to medium containing L-cystine. Cellular glutathione pools were nearly completely replenished 6 hr following exposure of the glutathione-deficient tumor cell to L-cystine.

Increases in the cellular glutathione pools following exposure of cells to L-cystine were accompanied by reacquisition of resistance to L-PAM (Fig. 2) although the latter consistently lagged behind the time course for regeneration of cellular glutathione. These results clearly indicate that cellular resistance to L-PAM is reversible and that small increases in the cellular glutathione pools are accompanied by large decreases in cytotoxicity.

Reduction in cellular glutathione by DL-buthionine-S,R-sulfoximine was accompanied by sensitization of the resistant tumor cell. The results described above indicate that both cellular glutathione and, consequently, resistance to L-PAM were increased markedly by extracellular L-cystine. The potential difficulty of reducing the availability of cysteine to cells *in vivo* prompted studies with DL-

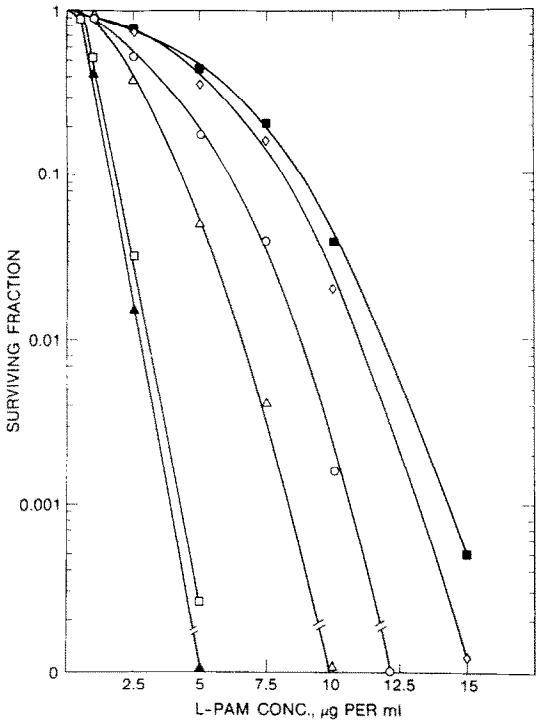

Fig. 2. Reversal of sensitization of L-phenylalanine mustard resistant tumor cells to L-PAM. L1210/L-PAM$_1$ cells were harvested from RPMI 1630 growth medium containing 20% fetal calf serum, washed in RPMI 1630 medium with a reduced concentration of L-cystine [1], and suspended in the same medium at a concentration of 2.5×10^5 cells/ml. Twenty-four hours later the indicated concentration of L-PAM was added to one aliquot of the cells (□). The remaining L1210/LPAM$_1$ cells were harvested, washed twice in RPMI 1630 medium containing 100 mg/l L-cystine, and suspended in the same medium at a concentration of 2.5×10^5 cells/ml. The indicated concentrations of L-PAM were then added 1 hr (△), 6 hr (○) or 24 hr (◇) following resuspension of cells in medium containing 100 mg/l L-cystine. All cells were harvested 48 hr following exposure to LPAM and washed in RPMI 1630 medium containing 20% fetal calf serum; cell survival was assessed by clonal growth of surviving cells in soft-nutrient agar for 2 weeks [4]. Dose–response curves are shown for the L-PAM sensitive L1210 (▲) and L1210/L-PAM$_1$ (■) exposed to the drug in RPMI 1630 containing 100 mg/l L-cystine.

buthionine-*S*,*R*-sulfoximine, a potent inhibitor of γ-glutamyl cysteine synthetase [3].

Initial studies using DL-buthionine-*S*,*R*-sulfoximine were designed to determine whether the inhibitor, at concentrations which were non-cytotoxic, could reduce the intracellular content of glutathione sufficiently to sensitize L-PAM resistant cells to the drug. As can be seen in Fig. 3, concentrations of the inhibitor of 10–50 μM produced only a slight decrease in the surviving fraction of cells. Incubation of cells with the inhibitor at a concentration of 50 μM decreased the intracellular concentration of glutathione from 5 nmoles to 2.5 nmoles per 10^6 cells after approximately 18 h (Fig. 4). Reduction in the cellular concentration of glutathione by exposure of cells to the inhibitor for 24 hr prior to L-PAM resulted in sensitization of the resistant tumor cells to the drug (Fig. 5). Incubation of cells with inhibitor either for periods of less than 18–24 h prior to exposure to L-PAM (Fig. 6) or removal of the inhibitor at the time of addition of L-PAM resulted in L-PAM dose–

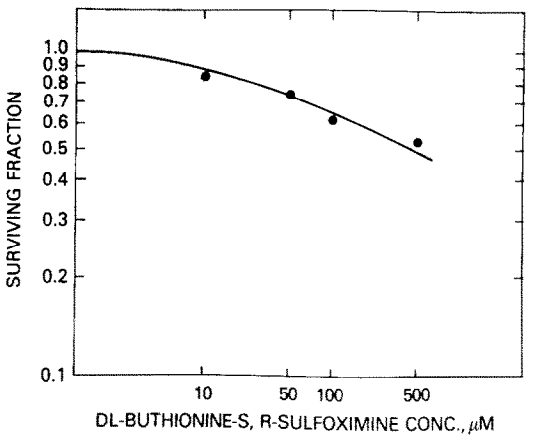

Fig. 3. Cytotoxicity of DL-buthionine-*S*,*R*-sulfoximine toward murine L1210 leukemia cells resistant to L-phenylalanine mustard. L1210/L-PAM$_1$ cells were harvested from RPMI 1630 medium containing 20% fetal calf serum, washed twice in the same medium, and suspended in growth medium containing the indicated concentration of DL-buthionine-*S*,*R*-sulfoximine at a cell concentration of 2.5×10^5 cells/ml. Twenty-four hours later the cells were harvested, washed in growth medium containing the indicated concentration of the inhibitor, and incubated for an additional 48 hr at 37°. The cells were then harvested and washed in fresh growth medium without inhibitor, cell survival was assessed by clonal growth of surviving cells in soft-nutrient agar for 2 weeks [4].

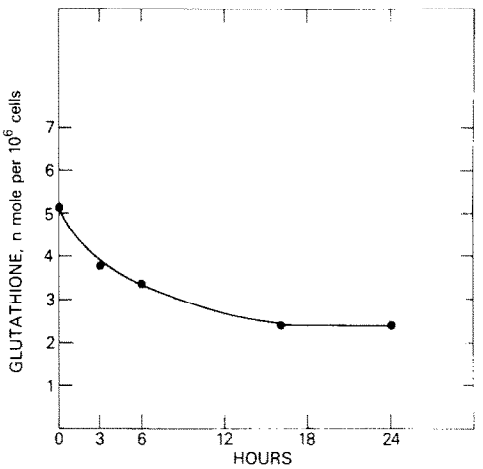

Fig. 4. Reduction in cellular glutathione by DL-buthionine-*S*,*R*-sulfoximine. L1210/L-PAM$_1$ cells were harvested from RPMI 1630 growth medium containing 20% fetal calf serum, washed twice in fresh growth medium, and suspended in it at a cell concentration of 2.5×10^5 cells/ml. DL-Buthionine-*S*,*R*-sulfoximine was added to a final concentration of 50 μM, aliquots of 1×10^7 cells were removed at the indicated time points, and the cellular content of glutathione was determined by the method of Griffith [5] as described previously [1].

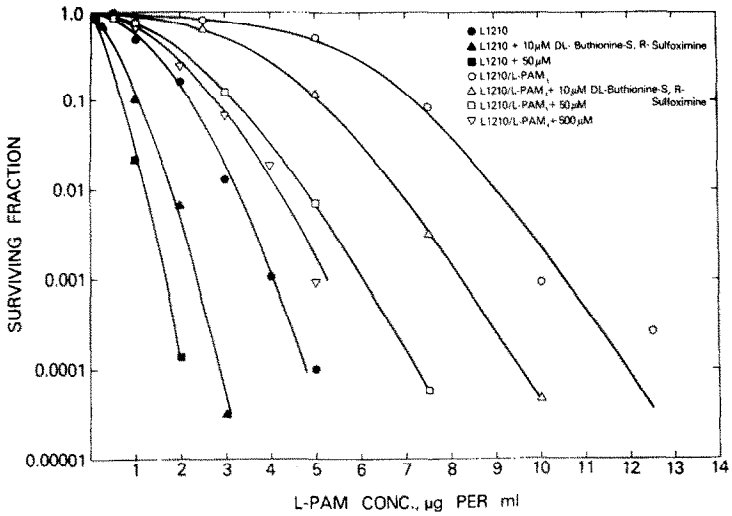

Fig. 5. Sensitization of murine L1210 leukemia cells to L-phenylalanine mustard by DL-buthionine-*S*,*R*-sulfoximine. Murine L1210 leukemia cells sensitive to L-PAM (closed symbols) and their resistant counterparts (open symbols) were harvested from growth medium, washed twice in fresh medium, and suspended in it at a cell concentration of 2.5×10^5 cells/ml. The indicated concentration of DL-buthionine-*S*,*R*-sufloximine was added and the incubation continued for 24 hr. Cells were then harvested and washed in fresh medium containing the indicated concentration of the inhibitor with or without L-PAM as indicated. The cells were harvested 48 hr later, and cell survival was assessed by clonal growth in soft-agar nutrient [4]. Data were corrected for the appropriate reduction in cell survival caused by DL-buthionine-*S*,*R*-sulfoximine alone.

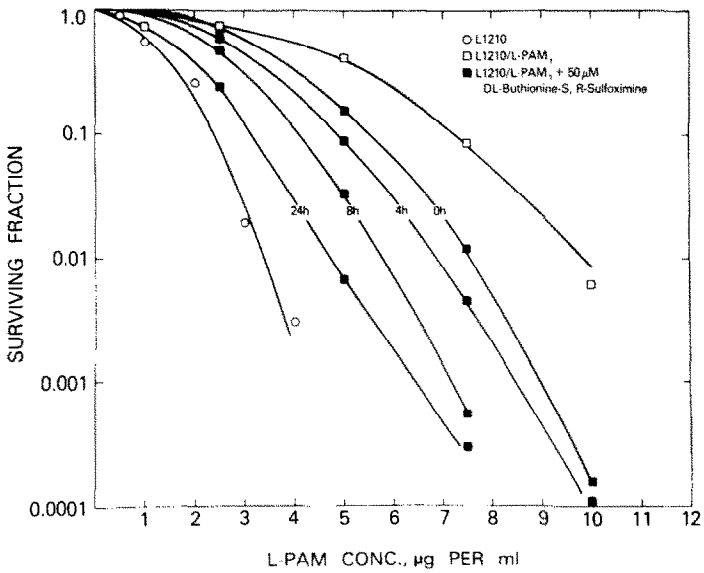

Fig. 6. Sensitization of L-PAM resistant tumor cells and preincubation time with DL-buthionine-*S*,*R*-sulfoximine. L1210 (○) and L1210/L-PAM$_1$ (□) murine L1210 leukemia cells were harvested from growth medium, washed twice in fresh medium, and suspended in it at a concentration of 2.5×10^5 cells/ml. Medium (○,□) or DL-buthionine-S,R-sulfoximine (50 μM) was added with L-PAM (0 hr), 4 hr prior to L-PAM (4 hr), 8 hr prior to L-PAM (8 hr) or 24 hr prior to L-PAM (24 hr). The incubation was continued for an additional 48 hr. Cells were harvested, and cell survival was assessed by clonal growth of surviving cells in soft-nutrient agar [4].

Table 1. Evaluation of DL-buthionine-*S,R*-sulfoximine as a potential adjuvant to L-PAM therapy

Expt.	L-PAM (13 mg/kg)	DL-Buthionine-*S,R*-sulfoximine	T/C (%)	Ascites at death*
1†	Day 2	None	106	++++
2‡	Day 2	Single injections, 4.5 mmoles/kg days 1 and 2	116	++++
3§	Day 2	0.2 mmoles/kg/day continuous infusion	126	None
	None	0.2 mmoles/kg/day continuous infusion	100	++++

* The relative amount of ascites was estimated at the time of death of the mice on a scale of none to ++++, the latter being an extremely bloody ascites.

† Experiment 1: Male CDF_1 mice (10) were inoculated intraperitoneally with 1×10^5 L-PAM resistant murine L1210 leukemia cells (day 0). L-PAM (13 mg/kg) was administered as a single intraperitoneal injection 48 hr later.

‡ Experiment 2: Male CDF_1 mice (10) were inoculated intraperitoneally with 1×10^5 L-PAM resistant murine L1210 leukemia cells. Mice were given single intraperitoneal injections of DL-buthionine-*S,R*-sulfoximine (4.5 mmoles/kg) 24 and 48 hr later. L-PAM (13 mg/kg) was administered as a single intraperitoneal injection 2 hr following the second injection of the sulfoximine.

§ Experiment 3: Model 2001 ALZET mini-osmotic pumps containing DL-buthionine-*S,R*-sulfoximine were implanted intraperitoneally into male CDF_1 mice as described in Materials and Methods. Mice (10) were inoculated intraperitoneally 24 hr later with 1×10^5 L-PAM resistant tumor cells. L-PAM (13 mg/kg) or saline was administered intraperitoneally 24 hr following tumor inoculation.

response curves intermediate between those obtained for L-PAM sensitive and resistant tumor cells. These observations can be attributed to the fact that, in the former instance, intracellular glutathione had not been decreased sufficiently to allow for maximal sensitization (Fig. 4) and, in the latter instance, cellular glutathione was being rapidly replenished. These observations prompted a series of studies utilizing DL-buthionine-*S,R*-sulfoximine in tumor-bearing mice (Table 1). The results suggest that the inhibitor was therapeutically ineffective as an adjuvant to L-PAM therapy when given as single injections. These results were not unexpected based on our *in vitro* studies and prompted a series of antitumor experiments employing continuous infusion of DL-buthionine-*S,R*-sulfoximine into tumor-bearing mice from ALZET mini-osmotic pumps. The results of these experiments (Table 1) indicate that there was a small increase in the lifespan of tumor-bearing animals treated with L-PAM and receiving the inhibitor by continuous infusion over that observed with animals treated either with L-PAM alone or with L-PAM and single injections of the inhibitor. We have observed a consistent absence of ascites at the time of death of tumor-bearing animals, treated with L-PAM, which had received the inhibitor by continuous infusion (Table 1). We interpret these results to suggest that sensitization of L-PAM resistant tumor cells within the peritoneal cavity had occurred.

Discussion

The results described in this communication indicate that sensitization of the resistant tumor cell to L-PAM by reducing cellular glutathione was reversible, i.e.

$$\text{Resistant} \underset{-GSH}{\overset{-GSH}{\rightleftharpoons}} \text{Sensitive}$$

and was influenced markedly by the availability of L-cysteine.

These results also indicate that DL-buthionine-*S,R*-sulfoximine, an inhibitor of glutathione biosynthesis [3], is capable of sensitizing murine L1210 leukemia cells resistant to L-phenylalanine mustard. Sensitization of the resistant tumor cells to L-PAM occurred at inhibitor concentrations that were minimally cytotoxic, required a sufficient exposure time to the inhibitor to reduce the cellular content of glutathione prior to treatment with L-PAM, and occurred in the presence of amino acid precursors utilized for glutathione biosynthesis. A detailed comparison in this laboratory of DL-buthionine-*S,R*-sulfoximine with other pharmacological agents that reduce intracellular glutathione, e.g. diethylmaleate [6] and two other inhibitors of γ-glutamylcysteine synthetase, methionine sulfoximine [7] and L-3-amino-1-chloro-2-pentanone [8], revealed that these agents, at concentrations which were minimally cytotoxic, were incapable of reducing intracellular glutathione to levels which result in sensitization of resistant cells to L-PAM (unpublished observations).

The observation that resistance to L-PAM was reversible has distinct consequences when possible inclusion of DL-buthionine-*S,R*-sulfoximine into therapeutic regimens is considered. We believe that the results of our *in vivo* studies suggest that sustained administration of DL-buthionine-*S,R*-sulfoximine resulted in sensitization of resistant tumor cells within the peritoneal cavity to L-PAM. The failure to observe significant increases in the lifespan of these animals is due to metastatic invasion and growth of the tumor in host organs, notably the liver. The failure to observe sensitization of the resistant tumor cell to L-PAM when the inhibitor was given as a single injection, either prior to and/or with L-PAM, most probably can be attributed to failure of maintaining pharmacologically effective concentrations of the inhibitor within the resistant tumor cell for a time period sufficient to both reduce the cellular glutathione content and to maintain it at reduced levels long enough for L-PAM to exert its critical cytotoxic lesion(s).

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Mechanism of metallic mercury oxidation *in vitro* by catalase and peroxidase

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Kudsk [1] found that ethyl alcohol inhibited the uptake of metallic mercury by blood *in vitro* and *in vivo*. Ogata *et al.* [2] described the oxidation of metallic mercury by human red blood cells having different catalase activities, hypocatalasemia and acatalasemia, with or without hydrogen peroxide. The peroxidase in animals occurs as glutathione peroxidase in red blood cells, as myeloperoxidase in white blood cells, and as lactoperoxidase in milk, while the peroxidase in plants is of the horseradish type. Horseradish peroxidase is reported by Ikeda *et al.* [3] to be capable of oxidizing metallic mercury. Lactoperoxidase, which is very similar to horseradish peroxidase in regard to oxidizing phenols and aromatic amines in the presence of hydrogen peroxide, was used in experiments reported in this paper. The present authors recently demonstrated [4] the oxidation of metallic mercury by lactoperoxidase.

In experiments *in vivo* with acatalasemia mice exposed to metallic mercury, the oxidation rates of metallic mercury in the lung and blood were shown to be greater than those in normal mice, with increased levels also seen in the liver [5]. The results were considered as indicating that a peroxidase, other than catalase, in the liver is possibly involved in the oxidation of metallic mercury. Ogata *et al.* [6] demonstrated previously, through the use of proteins such as lactoperoxidase and ferritin, that the oxidation of metallic mercury by blood is so related.

The present report deals with the mechanism of oxidation of metallic mercury *in vitro* by the peroxidative enzymes, catalase and lactoperoxidase or horseradish peroxidase, using ethyl alcohol, L-dopa and pyrogallol as auxilliary substrates in the presence of hydrogen peroxide.

Crystalline bovine liver catalase (2x crystallized, 33,900 Sigma units/mg protein) and lactoperoxidase from cow's milk (79 units/mg protein using the pyrogallol method) were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Horseradish peroxidase (100–150 units/mg protein) was obtained from the Wako Chemical Co., Osaka, Japan. All other reagents, including ethyl alcohol, L-β-(3,4-dihydroxyphenyl)alanine (L-dopa), pyrogallol, and hydrogen peroxide, were of analytical grade.

The reaction mixture was placed in the main chamber of a 15.ml Warburg flask with 0.1 ml of metallic mercury or metallic mercury vapor generated by the addition of stannous chloride to mercuric chloride in the side arm and 0.1 ml of hydrogen peroxide in the center well. Phosphate buffer was added to give a final volume of 3 ml. Incubation was conducted at 37° for 90 min with shaking at 80 cycles/min. The amount of mercury present in the main chamber after incubation was determined by an elemental mercury analyzer (Hitachi, model 207) with circulating air which contained mercury vapor, as described in our previous report [7].

The oxidation of metallic mercury by bovine liver catalase in the presence of hydrogen peroxide tended to increase with increased activity of catalase [8]. When ethyl alcohol was added, as an inhibitor of catalase activity and metallic mercury oxidation, to the catalase–H_2O_2 system at a fixed concentration of hydrogen peroxide, the oxidation of metallic mercury decreased as the concentration of ethyl alcohol was increased. The result is shown in Table 1. The oxidation of metallic mercury by catalase in the absence of ethyl alcohol was 1.23 μg/ml of incubation mixture. The competitive nature of the inhibition by ethyl alcohol was shown by Lineweaver–Burk plots [9], with two straight lines having different slopes with or without ethyl alcohol and crossing at the same intercept on the axis (Fig. 1). The result suggests that the reacting sites on the catalase for mercury and ethyl alcohol are very close to each other, or identical.

The oxidation of metallic mercury by lactoperoxidase increased with increased concentration of hydrogen peroxide [10] under the experimental conditions employed. When L-dopa was added to the lactoperoxidase–H_2O_2 system, the oxidation of metallic mercury by lactoperoxidase at a fixed concentration of hydrogen peroxide decreased as the concentration of L-dopa was increased. The result is shown in Table 2. The L-dopa used in this experiment may have served as a substrate [11] and/or a reducing agent for lactoperoxidase. The Lineweaver–Burk plots for mercury oxidation by lactoperoxidase with or without L-dopa