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MECHANISMS OF SYNERGISTIC TOXICITY OF THE RADIOPROTECTIVE AGENT, WR2721, AND 6-HYDROXYDOPAMINE*

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Abstract—WR2721 is a "prodrug" for a radioprotective thiol which has been proposed for adjunctive use as a free radical scavenger in cancer chemotherapy. When used adjunctively with oxygen radical generating chemotherapeutic agents in mice, however, WR2721 produces synergistic toxicity rather than attenuation of the toxic effects of such agents. The present paper discusses potential mechanisms for such synergistic toxicity. The pathway for glutathione synthesis appeared to be inactivated in mice treated with WR2721. The disulfide metabolite of WR2721 was a potent inactivator of γ-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione synthesis. The inactivation of the enzyme by this compound was similar to that reported for cystamine, a compound known to form a mixed disulfide with a cysteine residue near the glutamic acid binding site of the enzyme. Oxygen radicals not only inactivated the synthetase, as well, but hastened the oxidation of the free thiol metabolite of WR2721 to its corresponding disulfide.

WR2721‡ is a thiophosphate "prodrug" which is cleaved *in vivo* to the radioprotective thiol, *N*-2-mercaptoethyl-1,3-diaminopropane (MDP). MDP is proposed to be a clinically useful free radical scavenger [1–3]. We demonstrated previously, however, that adjunctive use of WR2721 in mice treated with 6-hydroxydopamine, an oxygen radical generator, leads to synergistic toxicity, rather than to the obviation of free radical damage [4]. In addition, when each is administered alone to A/J mice, WR2721 and 6-hydroxydopamine lead to a decrease in total hepatic glutathione [4, 5]. The mechanism of these phenomena is not certain. Therefore, we have begun studies of the effects of WR2721, 6-hydroxydopamine, and their metabolites upon glutathione synthesis.

Two major mechanisms of interference with glutathione synthesis have been identified. Limiting the availability of cysteine, most commonly accomplished by fasting, drives hepatic glutathione levels down to 50% of control values within 24 hr [6]. We therefore first investigated the possibility that WR2721 induces anorexia in mice, and thereby limits the availability of cysteine for glutathione synthesis. In addition, a number of disulfide compounds have been shown to inactivate γ-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione biosynthesis, *in vitro* [7]. We demonstrate here that the disulfide analogue of MDP [(MDP–S–)$_2$], a putative metabolite of WR2721 [8], is one such inactivator, and that this finding has relevance *in vivo* in mice treated with WR2721. This phenomenon most likely contributes to the depletion of glutathione in mice treated with WR2721.

Oxygen radicals have been shown to inactivate several enzymes *in vitro* [9, 10]. The toxicity of 6-hydroxydopamine is mediated via the formation of oxygen radicals [11]. We therefore investigated the possibility that γ-glutamylcysteine synthetase might be inactivated by oxygen radicals generated by 6-hydroxydopamine *in situ*. Such a mechanism, should it be operative, would explain additive, but not synergistic, toxicity in A/J mice.

Oxygen radicals have been shown to oxidize free sulfhydryls to their corresponding disulfides [9, 12]. Enhancement of the formation of (MDP—S—)$_2$ from MDP by 6-hydroxydopamine would account for the synergy of its toxicity with WR2721. We therefore examined the rate of formation of (MDP—S—)$_2$ from MDP in the presence and absence of 6-hydroxydopamine, and the influence of specific oxygen radical scavengers upon this rate.

MATERIALS AND METHODS

Chemicals. WR2721 was supplied by Dr. K. Borah of Organon Pharmaceuticals (West Orange, NJ). It was converted to its free thiol analogue, MDP, by boiling for 2 min in 1 M HCl as previously described [2]. Quantitative conversion to the free thiol was demonstrated by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) [13] after adjustment to pH 7.5 with NaOH. MDP was shown to be free of other amino compounds by amino acid analysis after con-

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‡ Abbreviations: WR2721, *S*-2-(3-aminopropylamino) ethyl phosphorothioic acid; MDP, *N*-2-mercaptoethyl-1,3-diaminopropane; (MDP—S—)$_2$, *N*-2-mercaptoethyl-1,3-diaminopropane disulfide; and MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride.

version to its sulfonic acid derivative by performic acid oxidation as we have described previously [14]. Disappearance of the free thiol upon incubation at 37°, pH 7.4, was verified [13]. Retreatment of this reaction mixture with performic acid [14] resulted in an amino acid analysis profile that was identical to those obtained with MDP and WR2721. This is most consistent with the assumption that disappearance of the free sulfhydryl from solutions of MDP incubated at 37°, pH 7.4, is due to the formation of $(MDP—S—)_2$.

N-Acetyl-L-cysteine, 6-hydroxydopamine hydrobromide, glutathione reductase, superoxide dismutase, monoamine oxidase, catalase, dimethyl sulfoxide, and all reagents needed for enzymologic assays were obtained from the Sigma Chemical Co. (St. Louis, MO). *N*-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) was obtained from Research Biochemicals, Inc. (Wayland, MA).

Animals. A/J male mice were obtained from Jackson Laboratories (Bar Harbor, ME), and used between 5 and 7 weeks of age for all studies. Mice were killed by cervical dislocation. This protocol was approved by the Children's Hospital of Pittsburgh Animal Use Committee.

Mice were fed Wayne Rodent Blox (Wayne, OH) *ad lib*, except where otherwise indicated in the text, and were given unlimited access to water.

Determination of hepatic glutathione content. The total (reduced + oxidized) glutathione content of the livers of mice was determined by the method of Tietze [15]. Simultaneous controls from the same batch of mice were done for each experiment.

Partial purification and determination of γ-glutamylcysteine synthetase activity. γ-Glutamylcysteine synthetase was partially purified from mouse liver by a modification of the procedure of Seelig and Meister [16]. Mouse liver (1–5 g) was homogenized in 150 mM KCl, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol (4 ml solution/g of liver). This and all subsequent steps were carried out at 4°. The homogenate was centrifuged at 10,000 *g* for 15 min. Solid ammonium sulfate (1.252 g/g liver) was added to the supernatant fraction, and the mixture was stirred for 30 min. The sample was centrifuged at 13,000 *g* for 15 min. Solid ammonium sulfate (0.1252 g/g liver) was added to the supernatant fraction, and the mixture was again stirred for 30 min. The sample was centrifuged at 13,000 *g* for 15 min, and the supernatant fraction was discarded. The pellet was suspended in 10 mM sodium phosphate buffer (pH 8.0) (3 ml) and dialyzed against the same buffer. An aliquot (2 ml) of the sample was clarified by filtration through a 0.45-μm Millipore HA filter, and applied to a column of DEAE-5-PW. The column was prewashed with 10 mM sodium phosphate buffer, pH 8.0, and eluted with a 70-min linear gradient from 0 M to 1 M NaCl in 10 mM sodium phosphate buffer. A simultaneous pH gradient from pH 8.0 to pH 6.5 was run. Elution was performed using a Waters semi-preparative high pressure chromatographic system with a flow rate of 8 ml/min. Fractions of 8 ml were collected. Fractions were assayed for γ-glutamylcysteine synthetase activity, and the active fraction was dialyzed against 50 mM Tris–HCl, pH 7.4.

Synthetase activity was determined by coupling of the generation of ADP by the synthetase to the production of NAD by the actions of pyruvate kinase and lactic dehydrogenase [16]. Activity was determined in a double beam spectrophotometer using a "blank" containing all of the assay components except α-aminobutyric acid, one of the substrates of γ-glutamylcysteine synthetase in this assay. This procedure controls for the presence of nonspecific ATPases in the enzyme preparation.

The enzyme preparation was also assayed for alanine transaminase activity (Ektochem slide method, Kodak, Rochester, NY), to assess the likelihood that one of its substrates, pyruvate, which is generated by the action of pyruvate kinase in the NAD-linked assay, might be shunted away from the actions of lactic dehydrogenase.

The results of the NAD-linked assay were verified by measuring the generation of inorganic phosphate [17] by the synthetase. A control sample containing all of the assay components except α-aminobutyric acid was assayed simultaneously to control for other ATPase activities which might be present. This assay could not be used in experiments involving preparations of MDP or $(MDP—S—)_2$ derived from WR2721, because of the presence of phosphate in these preparations (see "Chemicals").

Where indicated in the text, preincubation of the enzyme with 6-hydroxydopamine, with WR2721 or its metabolites, or with MPTP was carried out in 50 mM Tris–HCl, pH 7.4, at 37°. For these experiments, control samples of enzyme alone were preincubated for the same length of time, and enzyme activity is expressed as percent of the activity of the control sample at that time point. This would account for the possible loss of activity due to enzyme instability at 37°. All samples were open to the air and were vigorously agitated periodically during the incubation. Because of the potential for variability among batches of enzyme, in the oxygen content of solutions of 6-hydroxydopamine, and among batches of superoxide dismutase and catalase, all experiments shown represent the results of simultaneously prepared and run samples and controls. Solutions of 6-hydroxydopamine were prepared immediately prior to use.

Determination of glutathione reductase activity. Glutathione reductase activity was determined by the method of Tietze [15] using 40 μg/ml oxidized glutathione as a substrate. Where indicated in the text, preincubation of the enzyme with 6-hydroxydopamine was carried out in 100 mM sodium phosphate buffer, 5 mM EDTA, pH 7.5, at 37°. Controls consisted of enzyme alone preincubated for the same length of time.

Glutathione precursor supplementaion. N-Acetylcysteine was administered intraperitoneally to mice as a solution in distilled water brought to pH 7.0 with 10 N NaOH. The dose of 1000 mg/kg was chosen for these studies because it represents a dose sufficient to supplement glutathione even after the most massive overdoses (1500 mg/kg) of acetaminophen [18]. All experimental protocols were performed such that *N*-acetylcysteine was given between 8:00 and 10:00 a.m.

For studies involving the ability of *N*-acetyl-

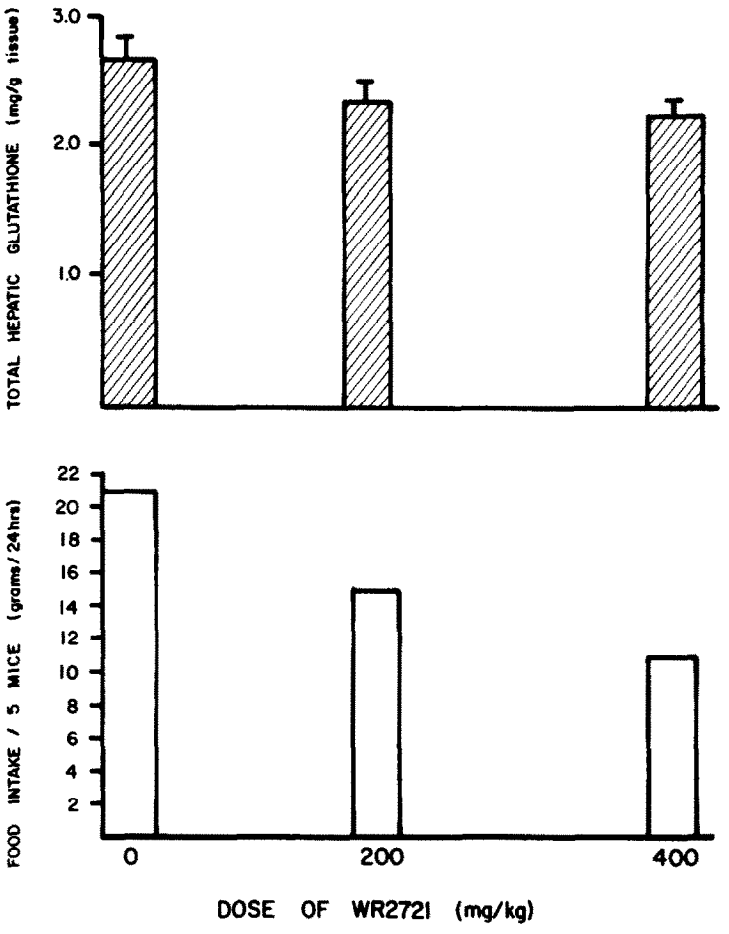

Fig. 1. Effects of treatment with WR2721 upon the 24-hr food intake and hepatic glutathione content of male A/J mice. Mice were treated with 0, 200, or 400 mg/kg WR2721 by intraperitoneal injection (N = 5 for each group). Twenty-four hours later, their food intake was determined. The mice were then killed, and their hepatic glutathione content was determined. The error bars in this and subsequent figures represent the standard error of the mean.

cysteine to restore the glutathione content of animals that were glutathione depleted by fasting, A/J mice (N = 30) were denied food but given water *ad lib.* for 12 hr. All but five of the mice were then given 1000 mg/kg *N*-acetylcysteine as an intraperitoneal injection. Groups of five mice were killed at 0, 2, 4, 6, and 8 hr after the *N*-acetylcysteine dose, and their hepatic glutathione was measured. The significance of the difference between control fasted and treated fasted animals was assessed by Student's *t*-test.

For studies involving the ability of *N*-acetylcysteine to restore the glutathione content of animals that were glutathione depleted by treatment with WR2721, A/J mice (N = 30) were given 400 mg/kg WR2721 as an intraperitoneal injection. Two hours later, all but five of the mice were given 1000 mg/kg *N*-acetylcysteine as an intraperitoneal injection. Groups of five mice were killed at 0, 2, 4, 6, and 8 hr after the *N*-acetylcysteine dose, and their hepatic glutathione was measured. The significance of the difference between animals treated with WR2721 alone and animals supplemented with *N*-acetylcysteine after WR2721 treatment was assessed by Student's *t*-test.

For studies involving the *in vivo* effects of 6-hydroxydopamine upon glutathione synthesis from *N*-acetylcysteine, A/J mice (N = 10) were given 400 mg/kg 6-hydroxydopamine as an intraperitoneal injection. Two hours later, half of the mice were given 1000 mg/kg *N*-acetylcysteine as an intraperitoneal injection. Six hours later, all of the mice were killed, and their hepatic glutathione was measured. The significance of the difference between animals treated with 6-hydroxydopamine alone and those supplemented with *N*-acetylcysteine was assessed by Student's *t*-test.

RESULTS

Effect of WR2721 upon food intake of A/J mice. Three groups of mice housed five per cage and injected intraperitoneally with 0, 200, or 400 mg/kg WR2721, respectively, were given free access to 110 g of food and to water *ad lib.* for the 24-hr period following injection. As is shown in Fig. 1, the 24-hr food intake of mice given WR2721 at a dose of 400 mg/kg was about half that of control mice. These mice exhibited a 15% decrease in their hepatic glutathione content relative to simultaneous controls

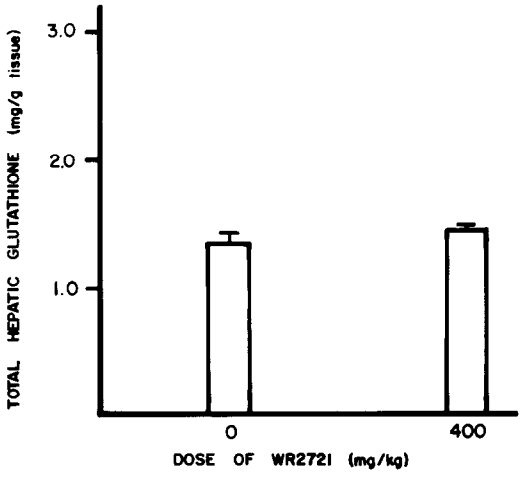

Fig. 2. Effects of WR2721 upon the hepatic glutathione content of food-deprived A/J mice. Mice were fasted for 24 hr following the administration of 0 or 400 mg/kg WR2721 by intraperitoneal injection (N = 5 for each group). The mice were then killed, and their hepatic glutathione content was determined.

($P < 0.05$, Student's *t*-test). All of the mice in all groups appeared clinically normal.

Effect of fasting upon glutathione depletion by WR2721. Mice injected intraperitoneally with 0 or 400 mg/kg WR2721 were deprived of food from the time of injection until sacrifice at 24 hr post-injection. Figure 2 shows the hepatic glutathione content of these mice. Fasting abolishes the decline in glutathione content induced by WR2721, suggesting that the same pool is depleted in both phenomena and that it has already been depleted by 24 hr of fasting.

Effect of N-acetylcysteine supplementation upon the hepatic glutathione content of mice treated with WR2721. If in fact the depletion of glutathione seen with WR2721 was the result of the drug-induced anorexia we had observed, replacement of the incurred "cysteine deficit" by parenteral supplementation with *N*-acetylcysteine would be expected to lead to the obviation of glutathione depletion.

The diet of our mice contained 0.4% cysteine by weight. We therefore compared the hepatic glutathione contents of mice treated in one of three ways: (1) control mice: fed *ad lib.* and untreated; (2) mice fed *ad lib.* and treated with 400 mg/kg WR2721 as an intraperitoneal injection 24 hr prior to sacrifice; and (3) mice fed *ad lib.*, treated with 400 mg/kg WR2721 as an intraperitoneal injection 24 hr prior to sacrifice, and supplemented with *N*-acetylcysteine at a dose calculated to compensate for the cysteine deficit induced by anorexia (12 mg *N*-acetylcysteine/day; see Fig. 1). For group (3), the daily dose of *N*-acetylcysteine was given as four equal intraperitoneal doses 6 hr apart. The first injection was given immediately preceding the WR2721 dose. Sacrifice and measurement of total glutathione level were performed 6 hr after the last dose of *N*-acetylcysteine. The repletion of glutathione with intraperitoneal *N*-acetylcysteine after fasting or toxin ingestion [19] reaches peak levels 6 hr after the *N*-acetylcysteine dose.

As is shown in Fig. 3, supplementation of WR2721-treated mice with *N*-acetylcysteine failed to alter their hepatic glutathione content.

Inactivation of γ-glutamylcysteine synthetase by (MDP—S—)$_2$. The elution profile of γ-glutamylcysteine synthetase from the DEAE column is shown in Fig. 4. The isolation procedure resulted in between 125 and 150 units of the synthetase per mg of protein, as assayed by the NAD-linked assay of Seelig and Meister [16]. This represents a 100-fold purification from the ammonium sulfate precipitation. Because the activity as measured by this assay might be artifactually lowered by the presence of alanine transaminase in the preparation, and because the preparation was shown to contain 0.37 units/mg protein alanine transaminase, the preparation was assayed as well for glutamate- and α-aminobutyrate-dependent ATPase activity as described in Materials and Methods. The enzyme preparation produced 100 μmole of inorganic phosphate per hour per mg protein. These results confirm the values obtained for synthetase specific activity using the NAD-linked assay.

The K_m values for glutamic acid and α-aminobutyric acid were 1.5 and 1.0 mM respectively. These values are in concert with those previously reported for this enzyme [16].

Figure 5 shows the relationship of the degree of inactivation of γ-glutamylcysteine synthetase to the concentration of (MDP—S—)$_2$ in the incubation mixture. The synthetase was 90% inactivated by incubation for 15 min with 1 mM (MDP—S—)$_2$. If all of a 400 mg/kg dose of WR2721 given intraperitoneally to mice were converted to (MDP—S——)$_2$, the theoretical serum concentration of (MDP—S—)$_2$ would be 1.5 mM [2, 4]. Figure 5 also compares such data for (MDP—S—)$_2$

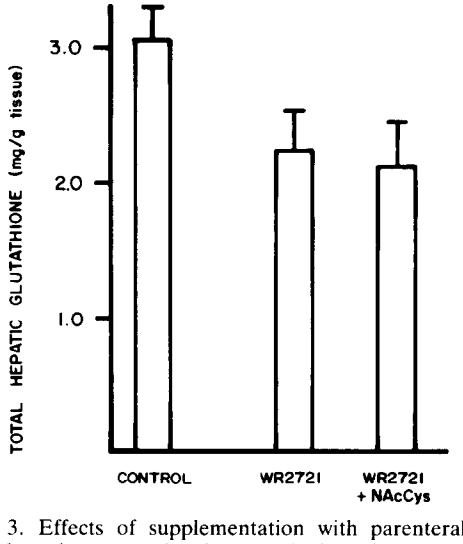

Fig. 3. Effects of supplementation with parenteral *N*-acetylcysteine upon the decrease in hepatic glutathione content caused by WR2721. Mice were either untreated, treated with 400 mg/kg WR2721 alone, or treated with 400 mg/kg WR2721 and supplemented with 12 mg/day *N*-acetylcysteine. The dose of *N*-acetylcysteine was calculated to compensate for the cysteine deficit incurred as a result of the decrease in food intake caused by WR2721 (N = 5 for each group).

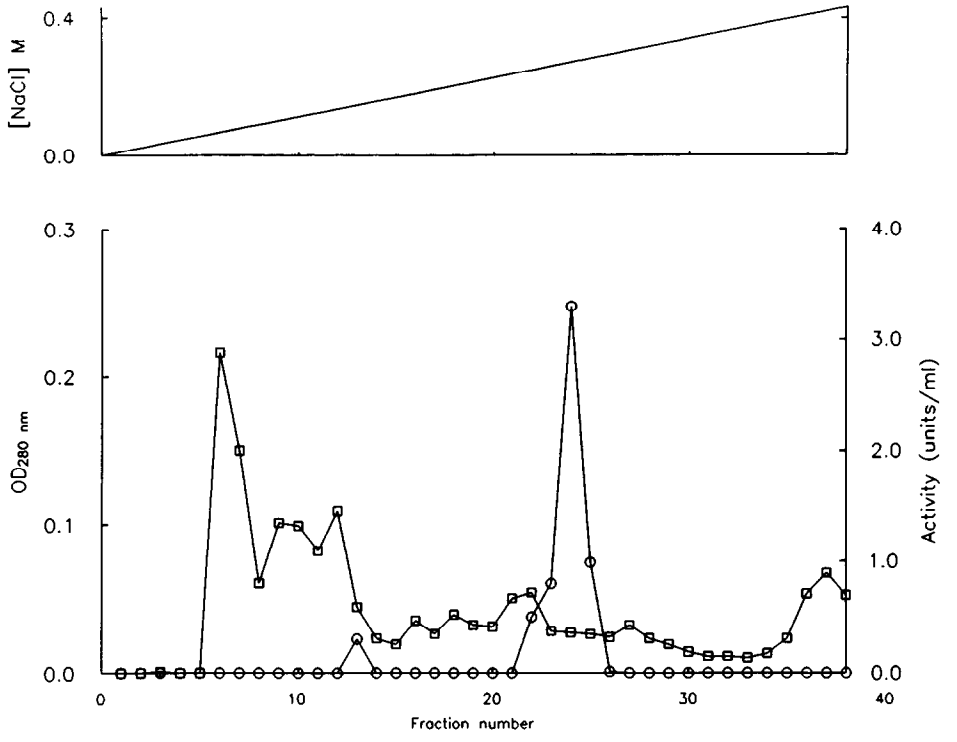

Fig. 4. Elution of protein (O.D.$_{280\,nm}$; □) and γ-glutamylcysteine synthetase activity (○) from DEAE-5-PW. Specific column conditions are detailed in Materials and Methods. The yield of activity was 81%. The specific activity of the enzyme in tube 24 was 125 units/mg protein. The NaCl gradient is illustrated above.

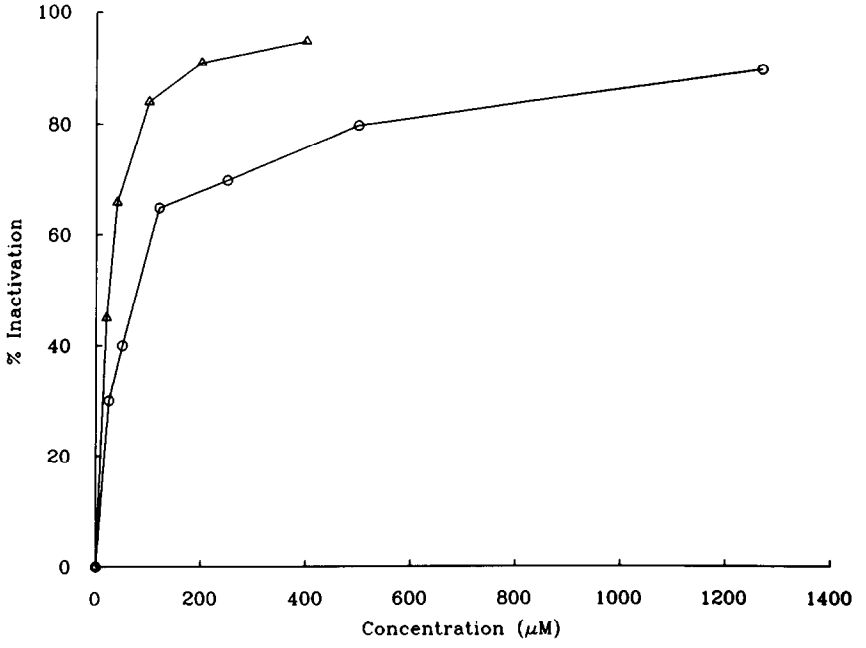

Fig. 5. Inactivation of γ-glutamylcysteine synthetase by (MDP—S—)$_2$. Aliquots containing 0.6 units of enzyme were incubated for 15 min at 37° in 50 mM Tris–HCl, pH 7.4, with various amounts of (MDP—S—)$_2$. Control activity was that of an equivalent aliquot of enzyme incubated for 15 min without (MDP—S—)$_2$. A representative experiment is shown (○). For comparison, similar data for inactivation by cystamine (△), plotted from Table 3 of Ref. 7, are shown.

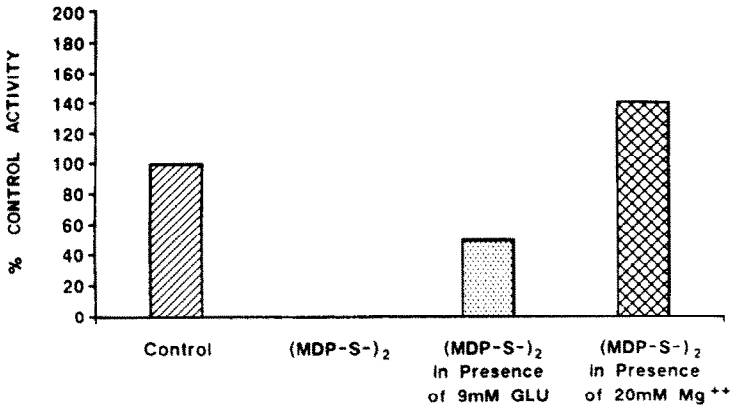

Fig. 6. Effects of addition of Mg^{2+} (20 mM) or glutamic acid (9 mM) to the preincubation buffer upon inactivation of γ-glutamylcysteine synthetase (0.6 units) by (MDP—S—)$_2$ (6.44 mM). Samples were preincubated at 37° and pH 7.4 for 15 min. A representative experiment is shown.

Table 1. Inactivation of γ-glutamylcysteine synthetase by (MDP—S—)$_2$

Length of incubation (min)	Inactivation (%)
5	<10
10	20
12	56
15	90

All incubations were carried out in 50 mM Tris–HCl, pH 7.4, at 37°. The activity of enzyme incubated with 1 mM (MDP—S—)$_2$ was compared with that of enzyme incubated for an equivalent length of time without (MDP—S—)$_2$. A representative experiment is shown.

with that obtained by Griffith *et al.* [7] for cystamine, the most effective inactivator identified to date.

WR2721, at concentrations of up to 12.88 mM, had no effect upon the synthetase under the conditions detailed in Fig. 5. The theoretical serum concentration of WR2721 administered intraperitoneally to mice at a dose of 400 mg/kg is approximately 3 mM [2, 4]. The sulfhydryl compound, MDP, produced 50% inactivation at 15 min when incubated at a concentration of 6.44 mM with the synthetase. This degree of inactivation may be due to formation of (MDP—S—)$_2$ *in situ*, as has been shown for cystamine [7], or to some intrinsic activity of the sulfhydryl compound.

The time course of inactivation of γ-glutamylcysteine synthetase by 1 mM (MDP—S—)$_2$ is shown in Table 1. The results of a representative experiment are given. The inactivation nears completion by 15 min of incubation.

The inactivation of an 800-μl sample of synthetase by 6.44 mM (MDP—S—)$_2$ was not reversed by dialysis against two changes of 50 mM Tris–HCl (pH 7.4; 1 liter and 2 hr per change). This implies covalent modification of the enzyme by (MDP—S—)$_2$.

Because the inactivation of the synthetase by other disulfide compounds has been shown to be attenuated in the presence of Mg^{2+} or glutamic acid [7], the effects of incubation of γ-glutamylcysteine synthetase with (MDP—S—)$_2$ (6.44 mM) in the presence of Mg^{2+} or glutamic acid upon enzyme activity were examined. Incubation with (MDP—S—)$_2$ in the presence of Mg^{2+} (20 mM) enhanced the activity of the synthetase. Incubation of the enzyme with 20 mM Mg^{2+} in the absence of (MDP—S—)$_2$ had no effect on activity. Glutamic acid (9 mM) decreased the degree of inactivation by a factor of two. A representative experiment is shown in Fig. 6.

Because the inactivation of the synthetase by other disulfide compounds has been shown to be reversed by treatment of the inactivated enzyme with disulfide reducing agents such as dithiothreitol [7], the effect of dithiothreitol upon enzyme inactivated by (MDP—S—)$_2$ was examined. The enzyme was inactivated completely by 6.44 mM (MDP—S—)$_2$ under the conditions described in Fig. 5. Dithio-

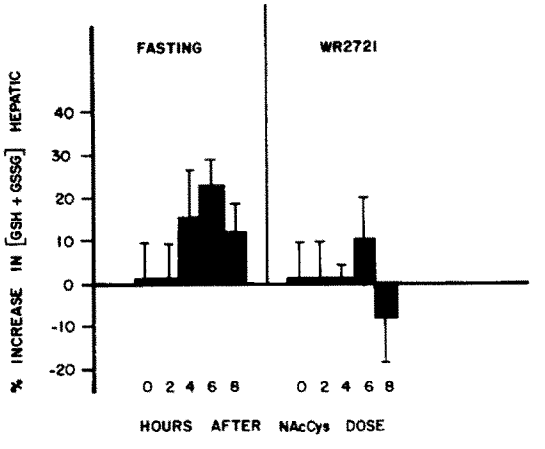

Fig. 7. Effects of *N*-acetylcysteine (1000 mg/kg) upon the hepatic glutathione content of A/J mice rendered glutathione-deficient by 12 hr of fasting or by treatment with WR2721. WR2721 (400 mg/kg) was given as an intraperitoneal injection 2 hr prior to the *N*-acetylcysteine dose (N = 5 at each time point). The value for fasted, treated animals at 6 hr after treatment was significantly different from the corresponding control value ($P < 0.05$). No statistically significant difference was seen between animals treated with WR2721 and *N*-acetylcysteine and those treated with WR2721 alone.

threitol (10 mM) restored 100% of the activity of inactivated γ-glutamylcysteine synthetase within 15 sec at 37°. This is in accord with the hypothesis that (MDP—S—)$_2$ forms a mixed disulfide with γ-glutamylcysteine synthetase. Treatment of native enzyme with 10 mM dithiothreitol alone for 1 min at 37° resulted in no measurable change in activity. Treatment with dithiothreitol alone for longer periods resulted in a loss, rather than an increase, of enzyme activity.

Dithiothreitol (10 mM) did not restore the 50% loss of activity seen when MDP was added to the synthetase. This suggests that the inactivation caused by MDP is due not to *in situ* formation of (MDP—S—)$_2$, but rather to some intrinsic property of MDP itself. Incubation of the synthetase with dithiothreitol or 2-mercaptoethanol (1.25 mM) for 15 min at 37° resulted in identical activity reduction to that seen with MDP (1.25 mM) under the same conditions (data not shown).

Effect of treatment with WR2721 upon the ability of mice to synthesize glutathione from N-acetylcysteine. The results described above suggested that, although WR2721 does induce anorexia in mice, decreased food intake is not the sole mechanism for glutathione depletion by this drug. Because our previous studies had indicated that (MDP—S—)$_2$ paralyzes the *in vitro* synthesis of glutathione from its precursors by inactivating γ-glutamylcysteine synthetase, we sought to examine the *in vivo* effects of treatment with WR2721 upon the synthetase. Direct measurement of changes in hepatic γ-glutamylcysteine synthetase activity after administration of WR2721 to mice is technically difficult, since the enzyme purification procedure entails homogenization of the liver with 2-mercaptoethanol [16], a treatment which has been shown previously to reverse inactivation of the synthetase by disulfides [7]. For this reason, we examined the activity of this enzyme indirectly by examining the ability of mice treated with WR2721 to make glutathione when provided with a single massive dose of *N*-acetylcysteine.

Animals treated with WR2721 alone and killed at 4 hr after the dose had hepatic glutathione levels that were 70% of those of control mice ($P < 0.001$, Student's *t*-test). Figure 7 shows that even massive doses of *N*-acetylcysteine failed to increase significantly the hepatic glutathione levels of mice treated with WR2721. For comparison, the effects of 1000 mg/kg *N*-acetylcysteine upon the glutathione levels of mice with glutathione depletion induced by 12 hr of food deprivation are also shown in Fig. 7. Food deprivation led to glutathione levels that were 50% of those of control mice ($P < 0.001$, Student's *t*-test).

Inactivation of γ-glutamylcysteine synthetase by 6-hydroxydopamine. As shown in Fig. 8, incubation of γ-glutamylcysteine synthetase with 0.67 mg/ml 6-hydroxydopamine hydrobromide resulted in complete inactivation of the enzyme by 2 hr after addition of the drug. This concentration of 6-hydroxydopamine was chosen because it is the theoretical serum concentration obtained when 400 mg/kg 6-hydroxydopamine, a known toxic dose [4], is administered to mice, and because the concentration of 6-hydroxydopamine in those cells which specifically take it up is unknown. The inactivation was not reversed by dialysis against 50 mM Tris–HCl, pH 7.4. This implies covalent modification of the enzyme.

Superoxide radical is known to be formed by the

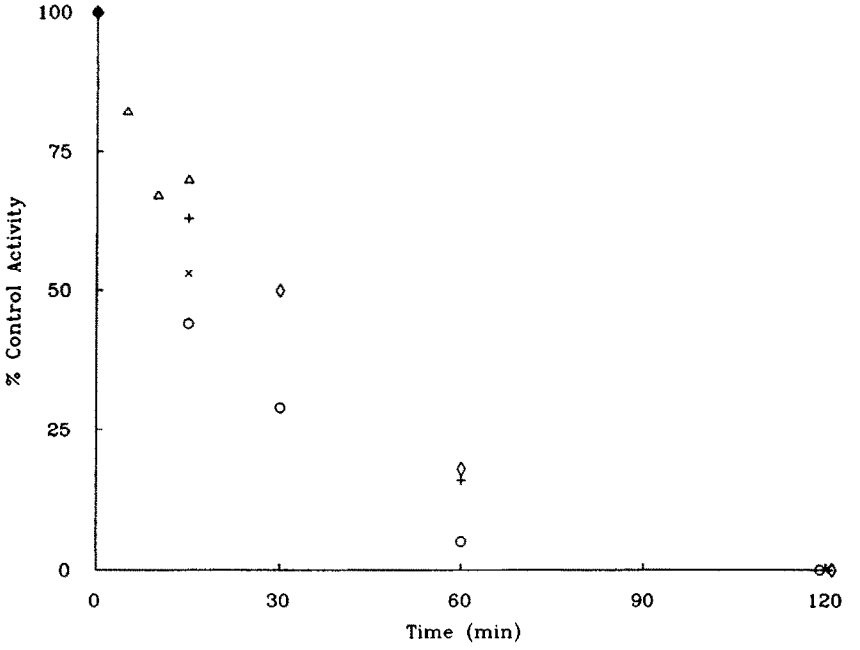

Fig. 8. Inactivation of γ-glutamylcysteine synthetase (0.6 units) by 0.67 mg/ml 6-hydroxydopamine hydrobromide in 50 mM Tris–HCl, pH 7.4, at 37°. For each time point in each experiment, the control to which each sample was compared was a simultaneously prepared and incubated aliquot of the synthetase without 6-hydroxydopamine. The results of five experiments are shown.

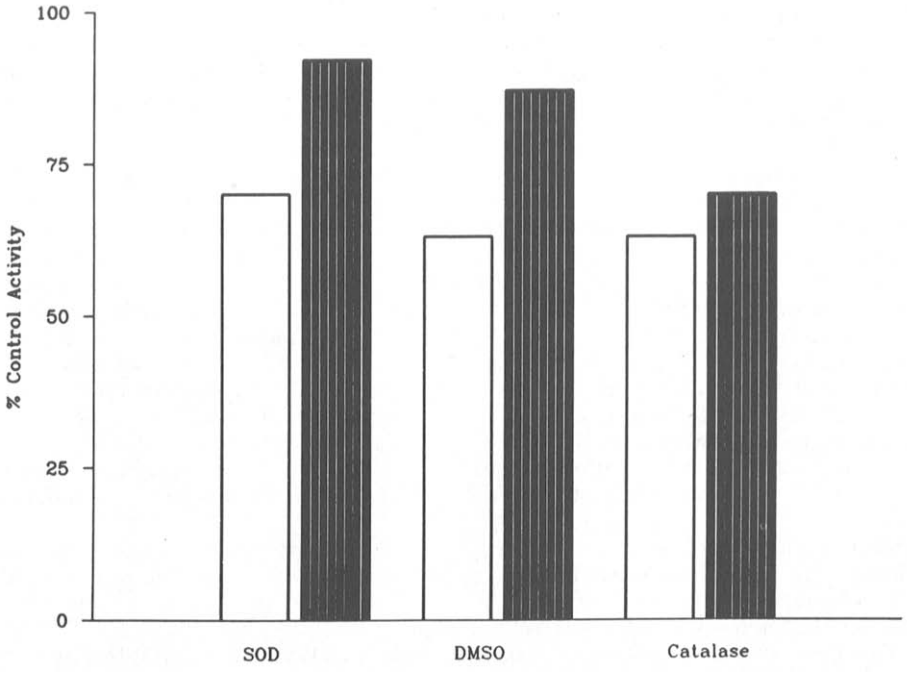

Fig. 9. Effect of adding superoxide dismutase (SOD; 1500 units), catalase (50 units), or dimethyl sulfoxide (DMSO; 1 mM) to the incubation mixture upon inactivation of γ-glutamylcysteine synthetase (0.6 units) by 6-hydroxydopamine hydrobromide (6-OHDA; striped bars). The incubation mixture contained 0.67 mg/ml 6-OHDA. Samples were incubated for 15 min. A representative set of experiments is shown. For each experiment, a sample treated with 6-OHDA alone was simultaneously assayed (open bars). The small difference seen with the addition of catalase was consistently seen.

spontaneous oxidation of 6-hydroxydopamine in aqueous solution [20]. As shown in Fig. 9, superoxide dismutase (1500 units), a superoxide radical scavenger, decreased the rate of inactivation of γ-glutamylcysteine synthetase by 6-hydroxydopamine. The data shown in the figure are for 15 min of incubation at 37°. This implies a role for superoxide radical in the inactivation process. Hydrogen peroxide and hydroxyl radical are also formed in aqueous solutions of 6-hydroxydopamine [11]. As also shown in Fig. 9, catalase (50 units), a peroxide-metabolizing enzyme, and dimethyl sulfoxide (1 mM), a hydroxyl radical scavenger, offered smaller degrees of protection against inactivation.

Oxygen radicals have been shown to oxidize protein sulfhydryls [12]. However, the inactivation of the synthetase was not reversed by the addition of 10 mM dithiothreitol, a disulfide reducing agent, either before or after dialysis.

Oxidation of 6-hydroxydopamine in 50 mM Tris–HCl (pH 7.4) at 37°. Because the incubation conditions used in the experiments described here are different from the conditions under which the autooxidation of 6-hydroxydopamine with generation of its quinone was originally described [20], the formation of the quinone was monitored over time by monitoring the $O.D._{490\,nm}$ of a solution of 0.67 mg/ml 6-hydroxydopamine hydrobromide in 50 mM Tris–HCl.

Figure 10 shows that the reaction was complete after 120 min of incubation, and that in the presence of 1500 units of superoxide dismutase, the formation of quinone lagged behind that found in the absence of superoxide dismutase. Catalase (50 units) and dimethyl sulfoxide (1 mM) increased the rate of quinone formation. Despite this, they, like superoxide dismutase, attenuated synthetase inactivation (see Fig. 9). The effect of 6-hydroxydopamine upon γ-glutamylcysteine synthetase activity therefore, was due not to its generation of the corresponding quinone, an organic free radical, but rather to its generation of oxygen radicals.

Effect of incubation with the neurotoxic organic free radical generator, MPTP, upon γ-glutamylcysteine synthetase activity. MPTP has a spectrum of neurotoxicity similar to that of 6-hydroxydopamine. Although both agents generate highly reactive organic free radicals *in vivo*, MPTP, unlike 6-hydroxydopamine, does not generate oxygen radicals [21]. Therefore, the effect of this compound upon the activity of γ-glutamylcysteine synthetase was tested.

Incubation of γ-glutamylcysteine synthetase for up to 30 min with 0.64 mM MPTP or with MPTP which had been preincubated with 0.1 units monoamine oxidase to generate its neurotoxic metabolite, the organic free radical, *N*-methyl-4-phenylpyridinium [21] resulted in no change in enzyme activity. This concentration of MPTP is the theoretical serum concentration of the drug in mice given 80 mg/kg intraperitoneally, a known toxic dose [21]. The finding that this pyridinium radical, an organic free radical, did not inactivate γ-glutamylcysteine synthetase is consistent with the notion that inactivation of the synthetase proceeds via its interaction with oxygen radical species, and not via the formation of adducts

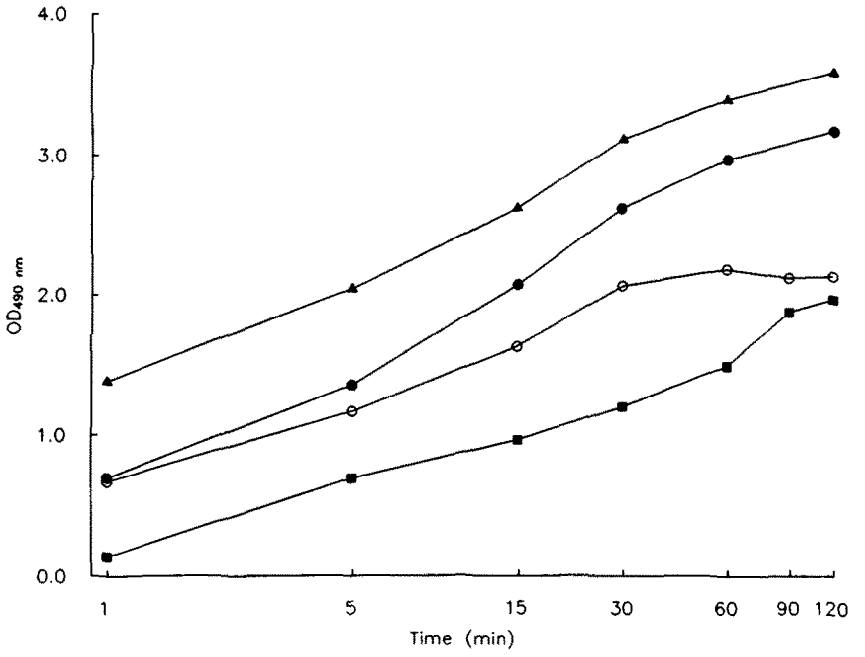

Fig. 10. Formation of the quinone of 6-hydroxydopamine in 50 mM Tris–HCl, pH 7.4, at 37° in the presence of superoxide dismutase (1500 units; ■), catalase (50 units; ▲), and dimethyl sulfoxide (1 mM; ●). The formation of the quinone was monitored at its absorption maximum of 490 nm. The curve for 6-hydroxydopamine alone is shown with open circles.

with concomitantly formed organic radicals, such as the quinone free radical.

Effect of incubation with 6-hydroxydopamine upon the activity of glutathione reductase. To test whether the effect of 6-hydroxydopamine upon the synthetase was an entirely nonspecific effect of oxygen radicals upon all proteins, the effect of this compound upon the activity of glutathione reductase was studied. Incubation of glutathione reductase with 0.67 mg/ml 6-hydroxydopamine hydrobromide for up to 15 min resulted in no change in enzyme activity.

Evidence for in vivo *inactivation of γ-glutamylcysteine synthetase by 6-hydroxydopamine.* 6-Hydroxydopamine alone decreased the hepatic glutathione to 17% of the control value for A/J mouse liver in our laboratory, which is standardized for time of day and nutritional status ($P < 0.001$, Student's *t*-test). *N*-Acetylcysteine increased the hepatic glutathione content of the livers of mice that had been glutathione depleted by fasting (see Fig. 7) or by acetaminophen poisoning [19] within 4–6 hr of its administration. As is shown in Fig. 11, there was no significant increase in glutathione at 6 hr in those animals given *N*-acetylcysteine after 6-hydroxydopamine relative to those given 6-hydroxydopamine alone. In addition, the 24- and 48-hr mortality rates of animals treated with *N*-acetylcysteine using the dosage schedule above were identical (60 and 100% respectively) to those of animals given 6-hydroxydopamine alone. Dividing the *N*-acetylcysteine dose into four equal doses with the first dose given 2 min before the administration of 6-hydroxydopamine was also ineffective in obviating toxicity. Thus, mice whose glutathione is depleted by 6-hydroxydopamine appear to be unable to replete their glutathione after administration of the glutathione precursor, *N*-acetylcysteine.

Facilitation of formation of (MDP—S—)$_2$ from MDP by 6-hydroxydopamine. If the oxidation of MDP to (MDP—S—)$_2$ is hastened by 6-hydroxydopamine, the administration of 6-hydroxydopamine to animals treated with WR2721 would be expected to produce synergistic depletion of glutathione. For this reason, the rate of disappearance of free sulfhydryl from solutions of MDP was monitored in the presence and absence of 0.67 mg/ml 6-hydroxydopamine. This is a measure of the rate of oxidation of MDP to (MDP—S—)$_2$. The assay was unaffected by the presence of 6-hydroxydopamine.

As is shown in Fig. 12, 6-hydroxydopamine markedly increased the rate at which MDP was oxidized. Figure 12 also shows that catalase and superoxide dismutase greatly attenuated this rate, again implicating oxygen radicals in the process.

DISCUSSION

Hepatic glutathione levels decrease in animals treated with WR2721. The potential mechanisms for such a decrease include limitation of precursor availability and inactivation of enzymes responsible for glutathione synthesis. The findings reported herein suggest that, although WR2721 induces anorexia, its glutathione-depleting effect is not simply the result of decreased precursor availability. Had a decreased cysteine pool alone been responsible for glutathione depletion, replacement of that pool with parenteral *N*-acetylcysteine should have repleted the glutathione stores. Parenteral *N*-acetylcysteine, given in doses sufficient to replete even the most

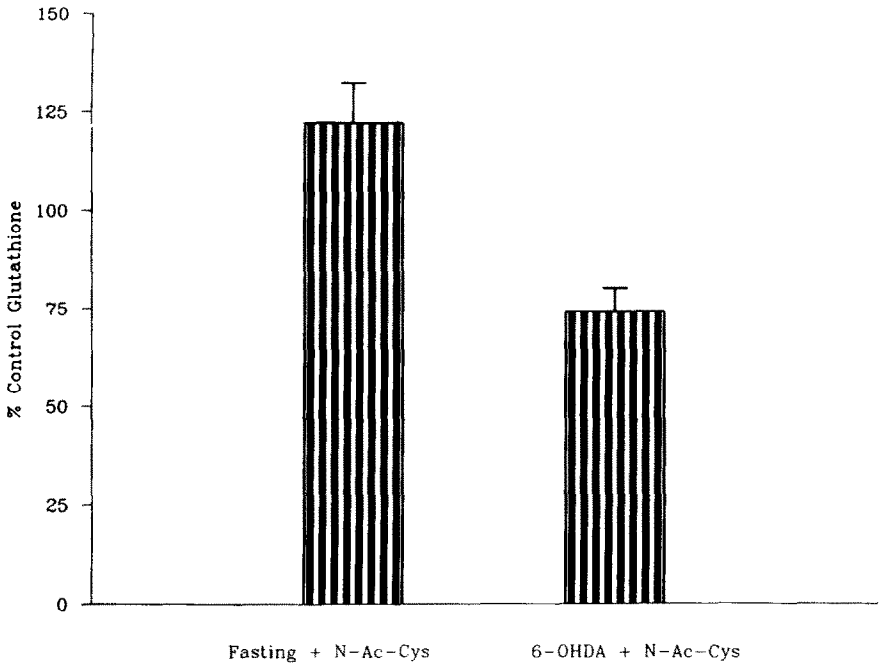

Fig. 11. Effect of treatment with *N*-acetylcysteine (*N*-Ac-Cys; 1000 mg/kg) upon the glutathione content of the liver of A/J mice made glutathione-deficient by 12 hr of fasting or by treatment with 6-OHDA (400 mg/kg). The control group for *N*-acetylcysteine treatment after fasting was a similar group of untreated, fasted mice. The control group for *N*-acetylcysteine treatment after 6-OHDA was a similar group of mice treated with 6-OHDA alone. Data are expressed as percent glutathione relative to their respective controls. The control value for fasted animals was 0.88 ± 0.09 mg glutathione/g tissue, and the control value for 6-OHDA-treated animals was 1.62 ± 0.14 mg glutathione/g tissue. For each group, N = 5 mice. The glutathione content of fasted mice given *N*-acetylcysteine was increased significantly relative to control fasted mice with a P maximally 0.05. The glutathione content of mice treated with 6-OHDA plus *N*-acetylcysteine was not statistically different from that of mice treated with 6-OHDA alone ($P > 0.05$).

severe of glutathione deficiencies [18], however, was ineffective in obviating or ameliorating the glutathione deficiency caused by treatment with WR2721. These results imply that WR2721 and/or its metabolites impair the synthesis of glutathione from *N*-acetylcysteine.

In addition, we have reported previously synergistic toxicity associated with concomitant use of WR2721 and 6-hydroxydopamine [4]. The present findings demonstrate that γ-glutamylcysteine synthetase is inactivated by 6-hydroxydopamine at concentrations of the drug which are theoretically obtainable in animals. The inactivation appears to be mediated through the generation of superoxide, peroxy, and hydroxyl radicals. All of these are produced by the oxidation of 6-hydroxydopamine in aqueous solutions [11]. Although superoxide might exert its action by catalyzing the formation of the quinone of 6-hydroxydopamine [22], an organic free radical, the attenuation of inactivation brought about by the peroxide scavenger, catalase, and the hydroxyl radical scavenger, dimethyl sulfoxide, both of which increase quinone formation, and the lack of effect of MPTP, a neurotoxin which generates a reactive organic radical without oxygen radical generation, strongly suggest that oxygen radical species, and not the quinone itself, are responsible for the inactivation. The increase in quinone formation brought about by catalase has been reported previously [23]. The inactivation is not reversed by the disulfide reducing agent, dithiothreitol, and is therefore probably not due to synthetase-synthetase disulfide formation. This distinguishes this reaction from that of oxygen radicals with glutathione peroxidase [9].

If 6-hydroxydopamine and WR2721 each produced independent inactivation of the synthetase, without potentiating each other's effect, additive, but not synergistic, toxicity would be expected from adjunctive administration of the two compounds. The present study demonstrates, however, that, in addition to its own effect upon γ-glutamylcysteine synthetase, 6-hydroxydopamine hastened the oxidation of MDP to $(MDP—S—)_2$. This finding provides a possible mechanism for the observed synergistic toxicity.

$(MDP—S—)_2$ is a potent inactivator of γ-glutamylcysteine synthetase *in vitro*. The prevention of inactivation by Mg^{2+} or glutamic acid and the reversal of this inactivation by dithiothreitol point to the similarity of the inactivation effected by $(MDP—S—)_2$ to that produced by cystamine. The latter has been shown to be due to the formation of a mixed disulfide with a cysteine residue near the glutamic acid binding site of the enzyme. $(MDP—S—)_2$ is a less potent inactivator of the synthetase than is cystamine. Comparison of $(MDP—S—)_2$ with other

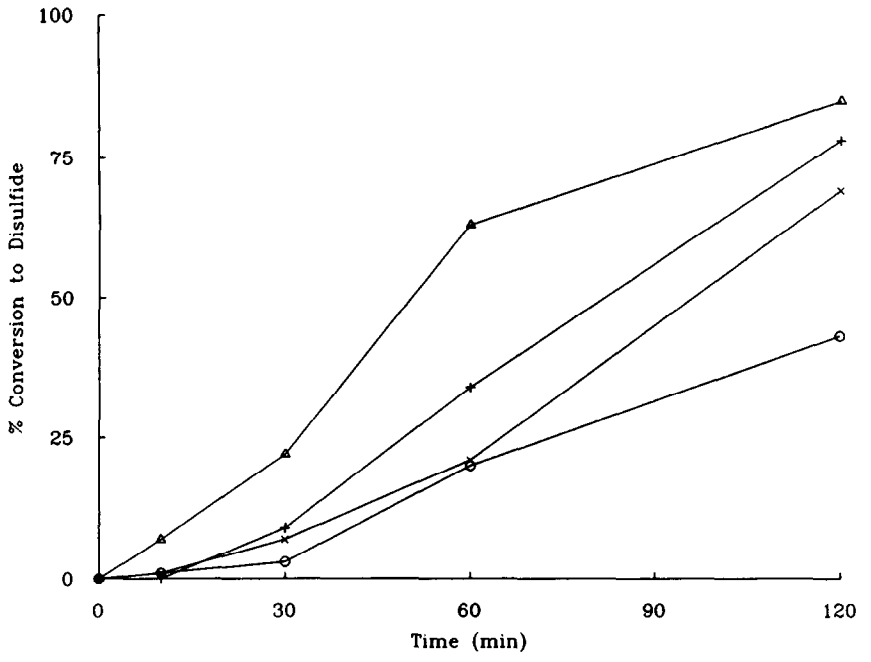

Fig. 12. Oxidation of MDP to (MDP—S—)$_2$ in the presence (△) and absence (○) of 0.67 mg/ml 6-hydroxydopamine. The initial solution contained 10 mM MDP and 50 mM Tris–HCl at pH 7.4, and was incubated at 37°. The effects of the addition of superoxide dismutase (800 units/ml; +) or catalase (50 units/ml; ×) upon the enhancement of oxidation by 6-hydroxydopamine, are shown as well.

known inactivators of the enzyme places it between cystamine and thiocholine disulfide in potency [7]. The mechanism of enhancement of enzyme activity by preincubation with Mg^{2+} in the presence of (MDP—S—)$_2$ is unclear. Divalent cations such as Mg^{2+} and Mn^{2+} have been shown to have variable effects upon inactivation of γ-glutamylcysteine synthetase at the glutamate site by α-chloroketones [24], but in no case did addition of Mg^{2+} to the preincubation mixture enhance enzyme activity. The inactivation of the synthetase by MDP was clearly not due to the formation of (MDP—S—)$_2$ *in situ*, since it was not reversed by dithiothreitol. It is most likely akin to the inactivation reported for prolonged incubation with other sulfhydryl compounds, such as dithiothreitol [7, 25]. The inability of mice treated with WR2721 to synthesize glutathione from *N*-acetylcysteine is most consistent with the inactivation of the synthetase *in vivo* by this compound.

A previous report warned about the potential danger of adjunctive use of WR2721 as a free radical scavenger with chemotherapeutic agents which themselves deplete glutathione [4]. The results presented herein further caution against the adjunctive use of WR2721 with drugs, such as bleomycin which are, or are metabolized to, oxidizing agents. The lack of effect of the organic free radical generated by MPTP or of the quinone generated by 6-hydroxydopamine upon synthetase activity suggests that agents such as cyclophosphamide, which generate organic free radicals, might be less likely to cause synergistic toxicity with WR2721. This is consistent with the previous report of protection from cyclophosphamide toxicity in humans by WR2721 [26].

In addition to its implications for therapy with 6-hydroxydopamine, the inactivation of γ-glutamylcysteine synthetase by oxygen radicals has wider import as regards the toxicity of such radicals generated, for example, by reperfusion injury after stroke and myocardial infarction [27, 28]. Since the reduction of such radicals by glutathione plays a role in their detoxification *in vivo* [29], paralysis of glutathione synthesis would be expected to impair physiologic handling of oxygen radicals. Inactivation of γ-glutamylcysteine synthetase by oxygen radicals could perpetuate the toxic effects of such radicals by paralyzing their detoxification.

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