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2,4-DIHYDROXYBENZYLAMINE: A SPECIFIC INHIBITOR OF GLUTATHIONE REDUCTASE

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Abstract—The high intracellular level of glutathione is maintained, in part, by the important redox enzyme glutathione reductase. This report describes the properties of a new inhibitor of glutathione reductase, 2,4-dihydroxybenzylamine (2,4-DHBA). The inhibition of glutathione reductase by both 2,4-DHBA and 1,3-bischloroethyl-nitrosourea (BCNU) requires the presence of the co-factor NADPH. However, the inhibition caused by 2,4-DHBA was found to occur much more rapidly. Inhibition of glutathione reductase was time dependent, involved a stoichiometric titration of the enzyme, and was not reversed by gel-filtration indicating an irreversible inhibitory mechanism. The drug interacted at two inhibitory sites as determined by a Hill-type plot analysis. 2,4-DHBA was shown to compete with the substrate oxidized glutathione, and the reducing agents, glutathione and dithioerythritol, were found to protect the enzyme from its inhibitory effect. These results suggest that the inhibition may entail a free radical effect at or near the active site. A structure–activity analysis with other meta-dihydroxybenzene derivatives revealed that the inhibition of glutathione reductase was unique to 2,4-dihydroxybenzylamine.

The metabolism of glutathione plays an important role in a variety of cellular processes, including the synthesis of DNA [1]. The activity of glutathione reductase, the enzyme which catalyzes the reduction of oxidized glutathione (GSSG)† to the reduced form (GSH), is essential in maintaining the high ratio of GSH/GSSG normally found within the cell. The mechanism of action of glutathione reductase has been studied in considerable detail [2, 3], including X-ray diffraction analyses at 0.3 nm resolution [4]. Although there is considerable interest in this enzyme because of its importance in cellular metabolism and as a potential target for chemotherapy, there are few specific inhibitors of glutathione reductase. 1,3-Bischloroethyl-nitrosourea (BCNU) has been reported to specifically inhibit this enzyme by a stoichiometric titration involving carbamylation by the nitrosourea isocyanates [5]. This inhibition is accompanied by toxicity due to other nonspecific interactions as well as the inhibition of DNA synthesis [6]. Cohen and Duvel [7] have argued that the cytotoxicity of BCNU precludes it from consideration as a potentiator of chemotherapy based on its ability to inhibit glutathione reductase. However, an agent that inhibited glutathione reductase at concentrations below that which it exhibits toxicity would be a valuable potentiator.

Our laboratory has been investigating a novel class of anticancer agents, containing the dihydroxybenzene moiety, that may be useful in the treatment of malignant melanoma. One of the most active derivatives, 3,4-dihydroxybenzylamine (3,4-DHBA) (NCS 263475), has been shown to have several biochemical effects that contribute to its potent and selective inhibition of cell growth [8, 9]. In contrast, our structure–activity studies have revealed that the meta derivative, 2,4-dihydroxybenzylamine (2,4-DHBA), has little if any cytotoxic activity towards a variety of cell lines [10]. Furthermore, our studies have indicated that, unlike 3,4-DHBA and other ortho derivatives, the meta derivative, 2,4-DHBA, is inactive as determined by a number of specific bioassays. This compound is a poor substrate for the enzyme tyrosinase [11], it is not an inhibitor of ribonucleotide reductase [12], and it has no effect on macromolecular synthesis [10]. The present investigation, however, revealed that 2,4-DHBA has a significant inhibitory effect on glutathione reductase.

MATERIAL AND METHODS

3,4-Dihydroxybenzylamine was prepared by demethylation of 3,4-dimethoxybenzylamine using hydrobromic acid and acetic acid following the procedure of Long and Burger [13]. 2,4-Dihydroxybenzoic acid and 2,4-dihydroxybenzaldehyde were obtained from the Aldrich Chemical Co., Milwaukee, WI. BCNU (carmustine) was from Ben Venue Laboratories Inc., Bedford, OH. G-25 Sephadex was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Glutathione reductase was obtained from the Sigma Chemical Co., St. Louis, MO (Type IV, 100–200 units/mg protein), and is a highly purified preparation that has

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† Abbreviations: GSSG oxidized glutathione; DHBA, dihydroxybenzylamine; GSH, reduced glutathione; BCNU, 1,3-bischloroethyl-nitrosourea; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); and IC_{50}, concentration resulting in 50% reduction of enzyme activity.

undergone further purification by affinity chromatography. All other drugs and reagents were also obtained from the Sigma Chemical Co.

Preparation of 2,4-dihydroxybenzylamine hydrobromide. This compound was prepared by demethylation of 2,4-dimethoxybenzylamine following the modified procedure of McOmie *et al.* [14]. 2,4-Dimethoxybenzylamine hydrochloride (20.4 g, 0.1 mol) was added to 100 mL of 1 N sodium hydroxide solution. A light yellow oil came out, which was extracted with ether (three times with 150-mL portions), dried with magnesium sulfate, and evaporated to an oil.

The above free amine was, without further purification or identification, dissolved in 250 mL of methylene chloride and cooled to −78°. Neat boron tribromide (30 mL) was added dropwise while keeping the reaction vessel dry with a calcium chloride drying tube. Stirring with a mechanical stirrer was continued overnight at room temperature. The precipitate was dissolved by the slow addition of methanol at 0°. The solution was evaporated to a gum, washed several times with ether, dissolved in methanol, decolorized with charcoal, and dried with anhydrous sodium sulfate. Evaporation of the solvent yielded 14 g (63.5%) of 2,4-dihydroxybenzylamine hydrobromide, m.p. 75–125°. ^{1}H-NMR (D_2O) δ 7.2 (d, 1H), 6.45 (s, 2H), 4.1 (s, 2H). The melting point was measured in an open pyrex capillary tube in an electrothermal melting point apparatus and is uncorrected. NMR measurements were made using a Bruker 300 mHz NMR spectrophotometer.

Glutathione reductase activity was determined using two different assays. The reaction mixture for the *NADPH assay* contained in a volume of 1.0 mL of 0.125 M potassium phosphate, pH 7.4, the following: 0.3 mM GSSG, 6.3 mM EDTA and 0.1 units glutathione reductase. These constituents along with drug were preincubated for 15 min at 30° and then 0.3 mM NADPH was added to start the reaction. The oxidation of NADPH was measured by determining the decrease in absorbance at 340 nm. The *DTNB assay* utilizes dithiobis-nitrobenzoic acid (Ellman reagent) which forms a chromophoric product when exposed to GSH (molar extinction coefficient = 13.6×10^3 at 412 nm). This assay measures the actual amount of product formed by the reduction of GSSG [15]. The assay conditions were the same as for the NADPH assay except that DTNB (0.6 mM) was added immediately after the NADPH. The formation of GSH was determined by measuring the increase in absorbance at 412 nm. The results for both assays are expressed in terms of the rate of change in absorbance over a period of 1–2 min. Each experiment was repeated at least three times with similar results. All spectrophotometric measurements were made using a Perkin–Elmer Lambda 3A spectrophotometer linked to an IBM computer for data acquisition. Each experimental sample was run simultaneously with its own control and results are expressed as percent of control. Control values for enzymatic activity were always in the range of 1 μM/sec of GSH formed, or its equivalent.

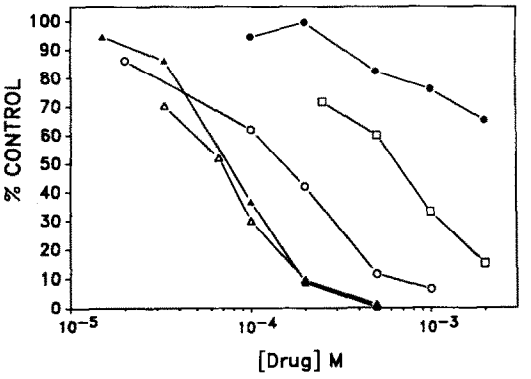


Fig. 1. Concentration–response for the inhibition of glutathione reductase by BCNU and 2,4-DHBA preincubated with and without NADPH. Open symbols represent experiments using the DTNB assay and the closed symbols are experiments that used the NADPH assay. Key: 2,4-DHBA preincubated with GSSG (—○— and —●—) or NADPH (—△— and —▲—); BCNU preincubated with NADPH (—□—).

RESULTS

Figure 1 shows the concentration–response curves for the inhibition of glutathione reductase by 2,4-DHBA and BCNU using two different assay procedures with and without preincubation with NADPH. No inhibition of glutathione reductase by BCNU was observed with either assay when NADPH was omitted from the preincubation mixture (results not shown). Interestingly, the two assays gave contrasting results for the inhibition of glutathione reductase by 2,4-DHBA when not preincubated with NADPH. While there was only a slight inhibitory effect as measured with the NADPH assay, there was greater inhibition as determined by the DTNB assay. Preincubation with NADPH greatly increased the inhibitory effect of both BCNU and 2,4-DHBA (Fig. 1). This requirement of NADPH for BCNU inhibition of glutathione reductase is in agreement with results reported previously [4]. It has been reported that preincubation of glutathione reductase with NADPH can inhibit enzyme activity [16]. In our studies, however, we did not observe any significant inhibition of glutathione reductase after preincubation with NADPH for up to 90 min.

We examined the effects of several other dihydroxybenzene derivatives on glutathione reductase activity. Table 1 reveals that only 2,4-dihydroxybenzylamine had any significant inhibitory effect. This result demonstrates that both the meta orientation of the hydroxy groups and the side chain have important roles in the inhibitory activity of this compound.

The results of Hill-type plot analysis [17] (Fig. 2), where the slopes of the curves indicate the number of inhibitor interaction sites, revealed that there are two inhibitor binding sites for 2,4-DHBA per molecule of glutathione reductase. Inhibitory reactions involving two binding sites usually arise when two molecules of the inhibitor are attacking sites on the enzyme that are physically linked [17].

Table 1. Effect of meta-dihydroxybenzene derivatives on glutathione reductase activity

Drug	IC_{50}* (μM) +NADPH	−NADPH
2,4-Dihydroxybenzylamine	74	151
2,4-Dihydroxybenzaldehyde	NE†	NE
2,4-Dihydroxybenzene	NE	NE
2,4-Dihydroxybenzoic acid	NE	NE
2,4-Dihydroxybenzamide	NE	NE
3,4-Dihydroxybenzylamine	NE	NE
1,3-Bischloroethyl-nitrosourea	646	NE

* IC_{50} = concentration of drug causing 50% reduction in enzyme activity when preincubated in the presence (+NADPH) or absence (−NADPH) of NADPH.
† NE = no effect.

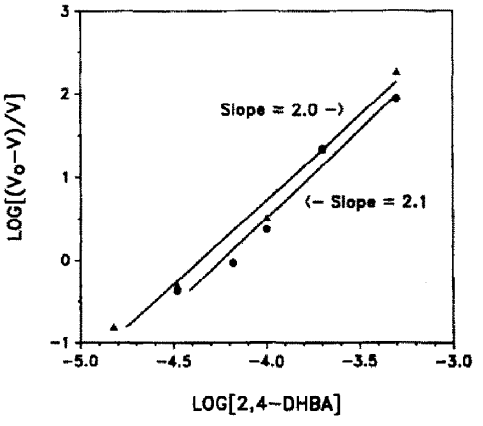


Fig. 2. Hill-type plot of the inhibition of glutathione reductase by 2,4-DHBA preincubated with NADPH. The experiment was performed using the NADPH assay (—▲—) and the DTNB assay (—●—).

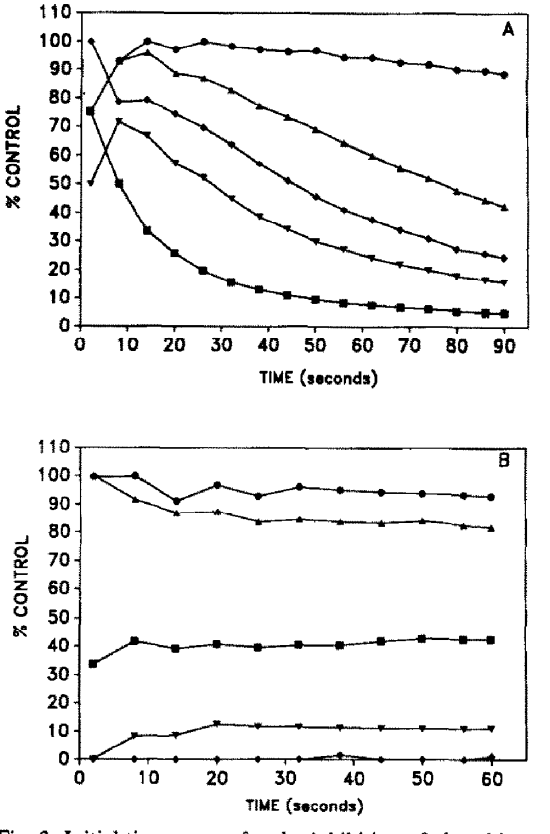


Fig. 3. Initial time course for the inhibition of glutathione reductase by 2,4-DHBA. The reaction was monitored continuously (DTNB assay) but only selected times have been plotted. The value for the percent control was obtained by dividing each time point by the control value for that time point. (A) preincubated without NADPH; 20 μM (—●—), 100 μM (—▲—), 200 μM (—◆—), 500 μM (—▼—) and 1.0 mM (—■—) 2,4-DHBA. (B) Preincubated with NADPH; 33 μM (—●—), 66 μM (—▲—), 100 μM (—■—), 200 μM (—▼—) and 500 μM (—◆—), 2,4-DHBA.

Figure 3 compares the initial time courses for the inhibition of glutathione reductase by 2,4-DHBA when preincubated in the absence or presence of NADPH. The results indicated a rapid increasing inhibitory effect (Fig. 3A) when NADPH was not present in the preincubation mixture. When 2,4-DHBA was preincubated with NADPH (Fig. 3B), there was no change in inhibition during the 1- to 2-min observation. This result suggests that the inhibition observed using the DTNB assay begins only upon addition of NADPH which precipitates a rapid attack on glutathione reductase by 2,4-DHBA.

Figure 4 shows a time course for the inhibition of glutathione reductase by 2,4-DHBA and BCNU when preincubated with NADPH for various lengths of time (0–90 min). The inhibitory effects for both drugs were found to increase with time. This result is consistent with an irreversible inhibition of the enzyme. It can also be seen that the inhibition of glutathione reductase by BCNU occurred more slowly (approximately 15 min) than the inhibition by 2,4-DHBA (immediate).

A plot of the enzyme activity versus the enzyme concentration is shown in Fig. 5. As shown in this Ackermann–Potter plot, 2,4-DHBA completely inhibited glutathione reductase activity at low enzyme concentrations. However, above a threshold concentration of enzyme (*ca.* 0.03 units/mL) the enzyme activity increased at a rate that was

Table 2. Effect of gel-filtration on glutathione reductase activity following exposure to 2,4-DHBA and BCNU

	Glutathione reductase activity* (% control)	
	Unfiltered	Filtered
2,4-DHBA, 1 mM (+NADPH)†	1.5	0.0
2,4-DHBA, 1 mM (−NADPH)	4.1	101.9
BCNU, 1 mM (+NADPH)	19.0	23.1
BCNU, 1 mM (−NADPH)	80.0	97.3

* Glutathione reductase was exposed to drug for 20 min. One-half the sample was filtered through a G-25 Sephadex column (see Materials and Methods). Enzyme activity was measured using the DTNB assay and compared to samples that were treated similarly but without drug.

† Drug was incubated in the presence (+NADPH) or absence (−NADPH) of the reducing co-factor NADPH.

proportional to the control. This type of plot is characteristic of irreversible inhibitors that titrate the enzyme [18].

To determine whether the inhibitory effects of BCNU and 2,4-DHBA were reversible, glutathione reductase (0.25 units/mL) was incubated in the presence of a 4 mM concentration of each compound for 20 min at 22° in the presence and absence of NADPH. One-half of the sample was passed through a 2-mL column of G-25 Sephadex equilibrated with 125 mM phosphate, pH 7.4. The enzyme was eluted with the same buffer, and the first 0.4-mL fraction following the void volume was collected and the amount of glutathione reductase activity determined using the DTNB assay. The results, shown in Table 2, confirmed the initial findings shown in Figs. 4 and 5 that the inhibition caused by the 2,4-DHBA derivative was irreversible, since enzyme activity was not restored following passage of the drug-treated enzyme through the Sephadex G-25 column. The results shown in Table 2 also indicate that NADPH is essential for the inhibitory effect. When glutathione reductase was incubated with 2,4-DHBA or BCNU in the absence of NADPH, gel filtration of the sample resulted in complete recovery of enzyme activity. This finding would indicate that in the absence of NADPH these drugs do not inhibit the enzyme.

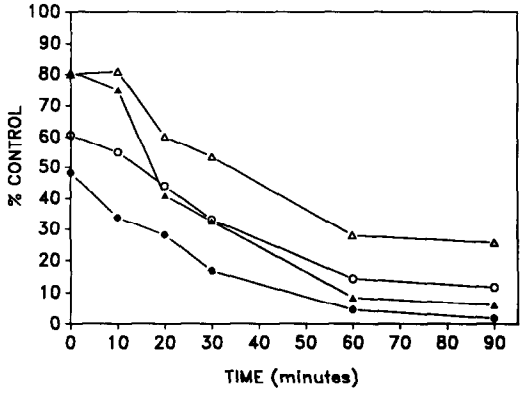


Fig. 4. Time course for the inhibition of glutathione reductase by BCNU (DTNB assay [1 mM (—△—) or 500 μM (—▲—)] and 2,4-DHBA [100 μM (—○—) or 150 μM (—●—)].

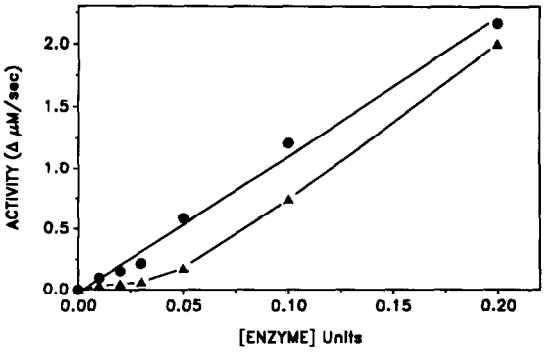


Fig. 5. Ackermann–Potter plot of the activity of glutathione reductase (DTNB assay) at various enzyme concentrations. Key: control (—●—) and 100 μM 2,4-DHBA (—▲—). The activity unit is expressed as the rate of formation of product (GSH) per second.

The effects of the product, GSH, and another reducing agent, dithioerythritol (DTE), on the inhibition of glutathione reductase by 2,4-DHBA were examined. The results shown in Fig. 6 indicate that at concentrations which cause no product inhibition (data not shown) GSH and DTE protected the enzyme against the inhibitory effect of 2,4-DHBA. Figure 6 reveals that concentrations of drug that caused complete inhibition of the enzyme in the absence of the reducing agent produced no inhibitory effect in the presence of the reducing agent.

Figure 7 shows the effect of various concentrations of the substrate, GSSG (oxidized glutathione), on the inhibition of glutathione reductase by 2,4-DHBA. Although the GSSG offered less protection than the reducing agents, oxidized glutathione did appear to compete with 2,4-DHBA. This result would suggest that 2,4-DHBA is acting at or near the active site.

DISCUSSION

The identification of a new inhibitor of an

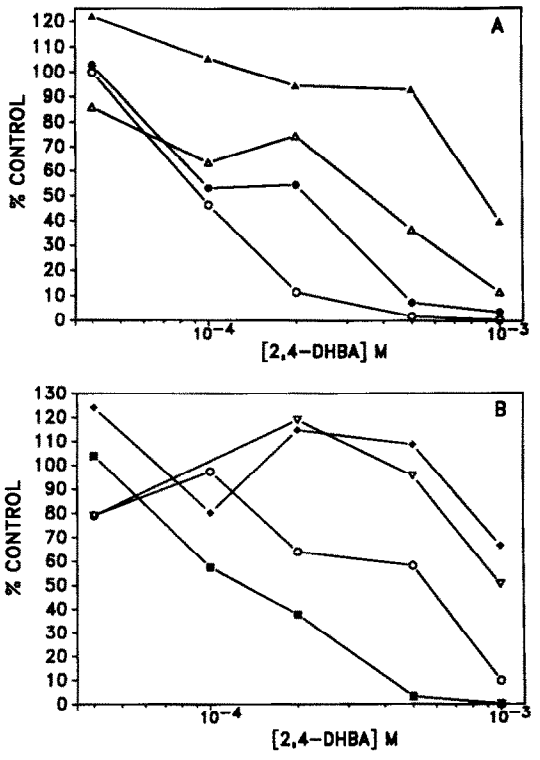
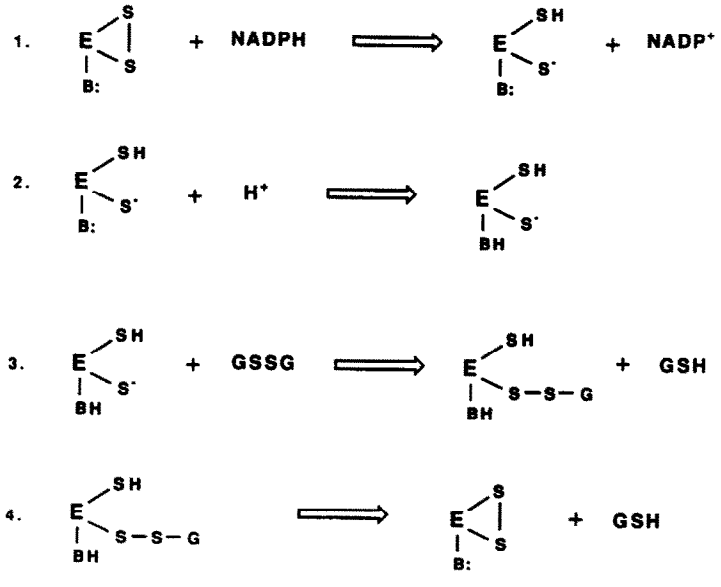


Fig. 6. Protection of glutathione reductase from inhibition by 2,4-DHBA (DTNB assay). (A) GSH, 1 mM (—▲—), 300 μM (—△—), and 100 μM (—●—); no GSH (—○—). (B) DTE, 1 mM (—◆—), 300 μM (—▽—), and 100 μM (—○—); no DTE (—■—).

important step in the generation of reduced glutathione may have significant chemotherapeutic implications. A number of studies have shown that glutathione reductase activity can confer drug resistance [19] and, conversely, that inhibition of this enzyme can increase the effectiveness of certain drugs which cause oxidative damage [20]. The only drug that inhibits glutathione reductase with some specificity is BCNU. However, BCNU has a variety of cytotoxic consequences which supercede its inhibitory effect against glutathione reductase. Our finding that the meta-dihydroxybenzylamine derivative, 2,4-DHBA, was a potent inhibitor of this enzyme is of particular interest, since it is known to have minimal toxicity. 2,4-DHBA may be capable of potentiating the cytotoxic effect of other chemotherapeutic agents as a result of its effects on glutathione metabolism.

In addition to its potential chemotherapeutic importance, 2,4-DHBA may be an important probe for examining the biochemical mechanism of action for glutathione reductase. Schema I shows a summary of the catalytic steps involved in the reduction of oxidized glutathione. Our results demonstrate that there is greater inhibition of glutathione reductase by 2,4-DHBA, as determined by measuring the effect on the production of product, glutathione (DTNB assay), than when the assay measured the loss of the reducing co-factor NADPH (Fig. 1). This suggests that 2,4-DHBA is less effective at inhibiting the initial step in the reduction reaction, i.e. the transfer of an electron from NADPH to the redox active disulfides (step 1, Schema I) than the final stage, i.e. transfer of electrons to GSSG (steps 3 and 4, Schema I). One possible interpretation of these results is that the drug interacts at a stage which is distal to the step at which the coenzyme NADPH interacts with the enzyme. This explanation is consistent with our findings demonstrating that the inhibition of glutathione reductase by 2,4-DHBA requires the presence of NADPH. An alternative explanation that NADPH may be activating 2,4-DHBA can be ruled out, since preincubation of the



Schema I

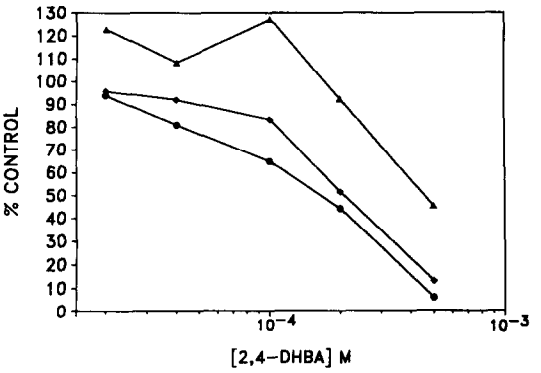


Fig. 7. Protection of glutathione reductase by GSSG from inhibition by 2,4-DHBA (NADPH assay). Key: 500 μM (—▲—), 250 μM (—◆—), and 100 μM (—●—) GSSG.

drug with NADPH before adding to the enzyme did not enhance its inhibitory effect.

Our results also suggest a possible mechanism for the interaction of 2,4-DHBA with glutathione reductase. The Hill plot analysis (Fig. 2) indicated that 2,4-DHBA attacks at two independent but physically linked binding sites. In this situation one of two possibilities may exist: (1) either glutathione reductase with one molecule of 2,4-DHBA is totally active, or (2) the K_i for the second molecule of 2,4-DHBA becomes so low that essentially any enzyme with one molecule of inhibitor will have a second molecule attached [17]. Furthermore, the results shown in Fig. 7 indicated that 2,4-DHBA competes with the substrate GSSG, suggesting that it is binding at or near the active site. Based on these findings, the following sequence could reasonably explain the interaction of 2,4-DHBA with glutathione reductase. NADPH binds to the enzyme producing the deprotonated thiol at the active site (steps 1 and 2, Schema I). One molecule of 2,4-DHBA then binds to the deprotonated thiol (step 3, Schema I) blocking the binding of the substrate GSSG which normally binds to this thiol group [2]. Concurrently, a second molecule of 2,4-DHBA interacts with the neighboring protonated thiol group.

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