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The Generation and Subsequent Fate of Glutathionyl Radicals in Biological Systems*

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Horseradish peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of either glutathione (GSH), cysteine, or *N*-acetylcysteine led to the production of the appropriate thiyl radical which could be observed using EPR spectroscopy in conjunction with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide. This confirms earlier work using acetaminophen (Ross, D., Albano, E., Nilsson, U., and Moldéus, P. (1984) *Biochem. Biophys. Res. Commun.* 125, 109–115). The further reactions of glutathionyl radicals (GS•), generated during horseradish peroxidase-catalyzed oxidation of *p*-phenetidine and acetaminophen in the presence of GSH, were investigated by following kinetics of oxygen uptake and oxidized glutathione (GSSG) formation. Oxygen uptake and GSSG generation were dependent on the concentration of GSH but above that which was required for maximal interaction with the primary amine or phenoxy radical generated during peroxidatic oxidation of *p*-phenetidine or acetaminophen, suggesting that a secondary GSH-dependent process was responsible for oxygen uptake and GSSG production. GSSG was the only product of thiol oxidation detected during peroxidatic oxidation of *p*-phenetidine or acetaminophen in the presence of GSH, but under nitrogen saturation conditions its production was reduced to 8 and 33% of the corresponding amounts obtained under aerobic conditions in the cases of *p*-phenetidine and acetaminophen, respectively. Nitrogen saturation conditions did not affect horseradish peroxidase-catalyzed metabolism. This shows that the main route of GSSG generation in such reactions is not by dimerization of GS• but via mechanism(s) involving oxygen consumption such as via $GSSG^{\cdot-}$ or via GSOOH.

Both *p*-phenetidine and acetaminophen, primary metabolites of phenacetin, have been suggested to be involved in the pathogenesis of the nephrotoxicity of phenacetin (1–4). The proposed mechanism involves peroxidase-catalyzed activation of these two metabolites to reactive species. Horseradish peroxidase-catalyzed metabolism of acetaminophen occurs via the generation of a phenoxy free radical (5), and indirect evidence obtained using a spin probe supports the involvement of amine radicals during peroxidatic oxidation of *p*-phenetidine by horseradish peroxidase (6). Glutathione (GSH[1]) is a tripeptide among whose functions is the detoxification of reactive electrophilic species by the formation of glutathione conjugates which can then be excreted from the cell. Ohnishi *et al.* (7) have reported that GSH causes a lag phase in the generation of chlorpromazine free radicals during horseradish peroxidase-catalyzed metabolism of this compound, suggesting that GSH can reduce the chlorpromazine radical, but there are few data concerning the interaction of other free radicals with glutathione. We have been investigating the interaction of thiols with xenobiotic-derived free radicals and have demonstrated that acetaminophen-derived radicals generated during peroxidatic oxidation of acetaminophen are reduced by thiols such as GSH, thus generating thiyl radicals (8). We have in this study investigated the interaction of GSH with products of the peroxidase-catalyzed oxidation of both acetaminophen and *p*-phenetidine and confirm that peroxidase-catalyzed oxidation of *p*-phenetidine proceeds by a one-electron mechanism. As the fate of thiyl radicals in biological systems is poorly understood, we have examined in detail the further reactions of the glutathionyl radical, a species whose production in biological systems is of considerable toxicological relevance. This was performed by investigating the kinetics of oxygen uptake and oxidized glutathione formation, two reactions of glutathionyl radicals which are known to occur from studies of the radiolysis of thiol containing solutions (9–14). Our data suggest that the major end product resulting from glutathionyl radical generation is GSSG but that this reaction occurs mainly under aerobic conditions via an oxygen-consuming reaction and not from a simple dimerization process.

MATERIALS AND METHODS

Reduced glutathione (GSH), oxidized glutathione (GSSG), acetaminophen, horseradish peroxidase (Type IV), and hydrogen peroxide were obtained from Sigma. *p*-Phenetidine and [^{14}C]*p*-phenetidine were synthesized as described previously (15). Catalase was obtained from Boehringer-Mannheim. Glutathione sulfonic acid was synthesized according to Ref. 16.

Incubations with Horseradish Peroxidase—Incubations were performed in 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA. Reactions were performed at 25 °C, initiated by the addition of peroxide and terminated by the addition of catalase (0.05 ml, 1500 units/ml).

Oxygen Uptake—Oxygen uptake was monitored at 25°C with a Clarke electrode (Yellow Springs Instrument Co.) and rates were calculated from the linear part of the graph.

Quantitation of p-Phenetidine Consumption and Generation of p-Phenetidine Metabolites—Measurement of *p*-phenetidine consumption was performed as described previously using high pressure liquid chromatography (HPLC). Alternatively, [^{14}C]*p*-phenetidine was used and both *p*-phenetidine and its metabolites generated during peroxi-

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[1] The abbreviations used are: GSH, glutathione; GSSG, oxidized glutathione; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; HPLC, high pressure liquid chromatography; GSOO•, glutathione peroxysulphenyl radical; GSOOH, glutathione sulphenyl hydroperoxide.

datic oxidation were measured using TLC. This was performed essentially as described previously (6). Aliquots of the aqueous reaction mixtures were mixed with catalase (0.05 ml, 1500 units/ml) and subjected to TLC analysis without prior extraction using silica plates (Merck) and a mobile phase of chloroform:methanol, 19:1. The portions of the chromatogram corresponding to the various colored metabolites were scraped off and mixed with scintillant and radioactivity was counted.

Measurement of GSH and Products of GSH Oxidation—This was performed using HPLC according to the method of Reed *et al.* (17). All data presented showing GSSG formation during horseradish peroxidase-catalyzed metabolism has been corrected for the oxidation of GSH by hydrogen peroxide in the absence of cosubstrate.

EPR Studies—These were performed at room temperature using a Varian E9 spectrometer in the presence or absence of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) 100 mM. Instrument conditions were: microwave power, 10 milliwatts; modulation amplitude, 0.2 G; receiver gain, 2×10^4; time constant, 0.3 s; and scan time, 2 min. Reactions were started by addition of hydrogen peroxide and spectra recorded 1 min later.

Experiments under an Atmosphere of Nitrogen—These were performed in 25-ml flasks equipped with self-sealing rubber insert caps. The buffer used was bubbled with nitrogen for 15 min prior to use. All reactants were added to the flask and the mixture was again bubbled with nitrogen for 2 min. The flasks were then sealed and reactions were initiated by adding hydrogen peroxide using a Hamilton syringe inserted through the rubber caps. Experiments were terminated by adding catalase (0.05 ml, 1500 units/ml) by the same route.

RESULTS

We have previously shown that horseradish peroxidase-catalyzed oxidation of acetaminophen in the presence of thiols leads to the generation of thiyl radicals (8). Similarly horseradish peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of thiols led to the production of the appropriate thiyl radical which could be observed using EPR spectroscopy in conjunction with the spin trap DMPO (Fig. 1). The EPR spectra of the DMPO-thiyl radical adducts observed during peroxidatic oxidation of *p*-phenetidine in the presence of GSH (a^N = 15.0 G, a^H = 16.3 G), cysteine (a^N = 15.2 G, a^H = 17.0 G), and *N*-acetylcysteine (a^N = 15.0 G, a^H = 16.8 G) are shown in Fig. 1. The hyperfine splitting constants of the DMPO-cysteinyl radical adduct are in agreement with previous studies (18, 19) and almost identical spectra were obtained during horseradish peroxidase-catalyzed oxidation of acetaminophen in the presence of these three thiols (8). Thus, this data together with previous work shows that thiols can reduce xenobiotic-derived radicals generated during peroxidatic oxidation causing the production of thiyl radicals.

The major non-protein thiol present in mammalian systems is glutathione (GSH). Thus, the further reactions of glutathionyl radicals generated during interaction of GSH with xenobiotic-derived radicals is of considerable toxicological relevance and we decided to investigate the fate of glutathionyl radicals by using the horseradish peroxidase-catalyzed oxidation of acetaminophen and *p*-phenetidine in the presence of GSH as a model system. Extensive radiolysis work has shown that thiyl radicals cause the generation of oxidized thiol and induce oxygen consumption (9–14). That these two reactions occurred during the horseradish peroxidase-catalyzed oxidation of both acetaminophen and *p*-phenetidine was confirmed and these results are shown in Table I.

The reduction of *p*-phenetidine radicals generated during horseradish peroxidase-catalyzed oxidation by GSH should lead to a regeneration of *p*-phenetidine via Equation 1

$$R^{\cdot} + GSH \rightarrow RH + GS^{\cdot} \quad (1)$$

and this was indeed found to be the case (Fig. 2), the amount of regeneration depending on GSH concentration in agreement with the early work of Ohnishi *et al.* (7). The amounts of GSSG generated (after subtraction of values obtained in control incubations without *p*-phenetidine) and oxygen uptake observed increased with increasing concentrations of

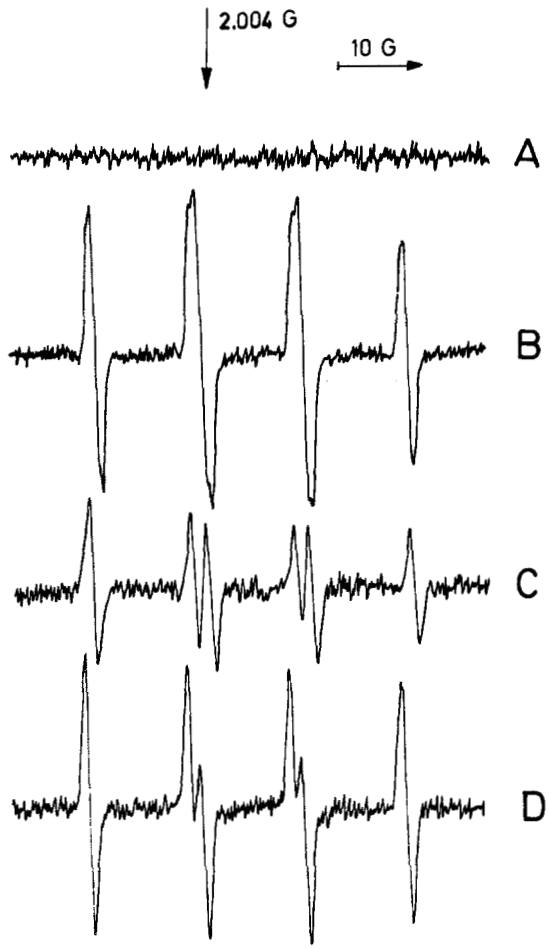

FIG. 1. **DMPO-thiyl radical adducts generated during horseradish peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of thiols and DMPO.** Reactions contained: *A*, *p*-phenetidine (0.5 mM), horseradish peroxidase (0.2 μg/ml), hydrogen peroxide (0.25 mM), and DMPO (100 mM); *B*, as *A* but in the presence of GSH (5 mM); *C*, as *A* but in the presence of cysteine (5 mM); *D*, as *A* but in the presence of *N*-acetylcysteine (5 mM). Instrument settings are detailed under "Materials and Methods." The signals obtained were enzyme-, *p*-phenetidine-, and thiol-dependent.

TABLE I

Formation of GSSG and oxygen uptake during horseradish peroxidase-catalyzed oxidation of p-phenetidine and acetaminophen in the presence of GSH

Reactions were performed at 25 °C for 5 min in the case of GSSG determination and until uptake was complete in the case of oxygen uptake.

	GSSG formation[a]	O_2 uptake[b]
	μM	
p-Phenetidine[c]	663	192
Acetaminophen[d]	417	120

[a] After subtraction of control values obtained in incubations minus *p*-phenetidine or acetaminophen.

[b] No uptake was observed minus *p*-phenetidine or acetaminophen, minus GSH, minus H_2O_2. A small oxygen uptake was observed minus horseradish peroxidase and the results are corrected for this.

[c] Conditions were: 0.5 mM substrate, 2 μg/ml horseradish peroxidase, 0.25 mM H_2O_2, and 5 mM GSH.

[d] As Footnote c but 25 μg/ml horseradish peroxidase was used.

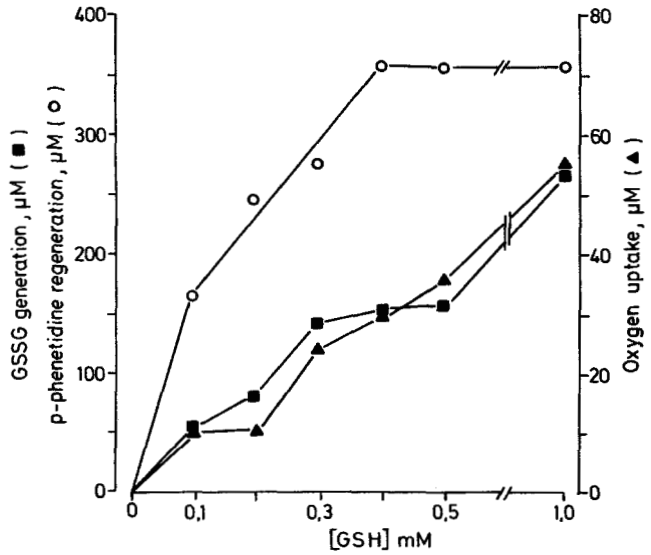

FIG. 2. **Oxygen consumption (▲), GSSG generation (■) and *p*-phenetidine regeneration (○) during horseradish peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of varying concentrations of GSH.** Conditions were: horseradish peroxidase (0.2 μg/ml), *p*-phenetidine (0.5 mM), and hydrogen peroxide (0.2 mM). *p*-Phenetidine regeneration was calculated from the difference between the amount of *p*-phenetidine removed in the absence of thiol minus the amount of *p*-phenetidine removed in the presence of thiol.

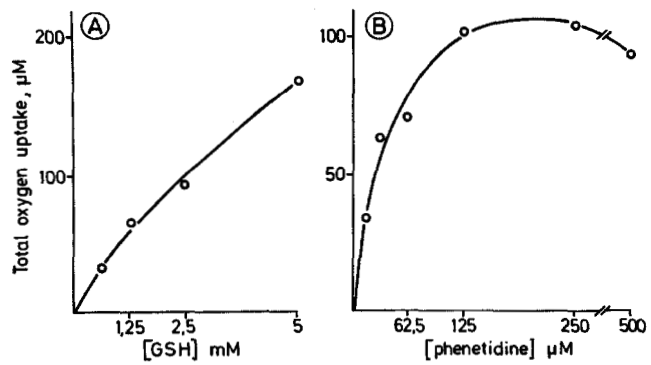

FIG. 3. **Oxygen uptake during horseradish peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of GSH.** *A*, *p*-phenetidine (0.5 mM), horseradish peroxidase (0.2 μg/ml), hydrogen peroxide (0.25 mM), and GSH (0–5 mM). *B*, *p*-phenetidine (0–0.5 mM), horseradish peroxidase (0–2 μg/ml), hydrogen peroxide (0–0.25 mM), and GSH (2.5 mM).

GSH even after maximal (~70%) regeneration of substrate had occurred (Fig. 2). These data indicated that oxygen uptake and oxidized thiol formation were occurring at high GSH concentrations via a process independent of the initial reaction between a *p*-phenetidine radical and GSH generating a thiyl radical which could then either dimerize or interact with oxygen. This secondary process associated with GSSG generation and oxygen uptake, however, was still dependent on GSH concentration.

The thiol concentration dependence of oxygen uptake observed during horseradish peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of GSH is shown in Fig. 3*A*. This experiment was performed by using hydrogen peroxide concentrations of one-half that of the cosubstrate *p*-phenetidine. As hydrogen peroxide oxidizes horseradish peroxidase from its resting Fe^{3+} form to Fe^{5+} (20) and the reduction of the oxidized form of the enzyme occurs via a one-electron mechanism (Fig. 1), this ratio ensures stoichiometry between the amounts of *p*-phenetidine and hydrogen peroxide used. If the amount of peroxide used was in excess and the concentration of *p*-phenetidine varied, the total oxygen uptake did not change but the rate of uptake was dependent on *p*-phenetidine concentration, showing that under these conditions enzymatic activity was dependent on the concentration of cosubstrate (data not shown). As a concentration of 0.5 mM *p*-phenetidine was used and therefore a maximum value of 0.5 mM radical generated a concentration of GSH equal to 1.25 mM was more than enough to ensure maximal regeneration of amine assuming that 1 mol of GSH reacted with 1 mol of amine radical. The oxygen uptake observed however (Fig. 3*A*) was not maximal at GSH = 1.25 mM but increased with increasing concentrations of GSH up to 5 mM proving that GSH was inducing oxygen uptake via a secondary mechanism independent of thiyl radical formation during *p*-phenetidine regeneration. By maintaining the concentration of hydrogen peroxide at one-half of the concentration of *p*-phenetidine, the amount of *p*-phenetidine-derived radicals generated by horseradish peroxidase could be varied (*i.e.* [*p*-phenetidine] = 2x, [hydrogen peroxide] = x) and the oxygen uptake observed at a fixed concentration of GSH (Fig. 3*B*). This data showed that maximal oxygen uptake was not directly related to *p*-phenetidine radical concentration but reached a plateau value with increasing concentrations of amine. This suggested that other reactions of the primary *p*-phenetidine radical, such as dimerization, may become more important with increasing radical concentrations. An alternative explanation may be that once sufficient concentrations of *p*-phenetidine-derived radical had been generated to initiate the secondary GSH-dependent route of oxygen uptake, then no more were required to ensure maximal uptake. Similar results were observed with acetaminophen (Fig. 4*A*), the oxygen uptake observed increasing with increasing concentrations of GSH (→10 mM) much above that required for maximal regeneration of acetaminophen (0.125 mM). The curve relating the dependence of oxygen uptake on acetaminophen radical concentration (at fixed GSH concentration) did not plateau to an acetaminophen concentration of 0.5 mM, suggesting that in the case of acetaminophen the primary reactions resulting from substrate regeneration are more important, relative to the case of *p*-phenetidine, to oxygen uptake.

Whether the two parameters, oxygen uptake and GSSG formation, observed during horseradish peroxidase-catalyzed oxidation of *p*-phenetidine and acetaminophen were related was investigated by performing the enzymatic reactions under

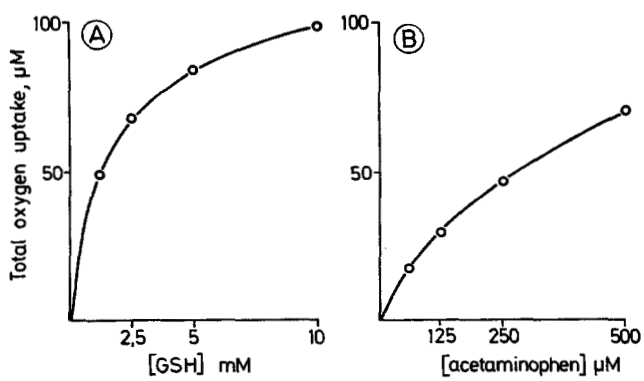

FIG. 4. **Oxygen uptake during horseradish peroxidase-catalyzed oxidation of acetaminophen in the presence of GSH.** *A*, acetaminophen (0.25 mM), horseradish peroxidase (25 μg/ml), hydrogen peroxide (0.125 mM), and GSH (0–10 mM). *B*, acetaminophen (0–0.5 mM), horseradish peroxidase (25 μg/ml), hydrogen peroxide (0–0.25 mM), and GSH (5 mM).

an atmosphere of nitrogen. That nitrogen did not inhibit horseradish peroxidase-catalyzed metabolism is shown in Fig. 5 where the removal of *p*-phenetidine (Fig. 5*A*) and appearance of its metabolites (Fig. 5*B*) were monitored under aerobic and nitrogen saturation conditions. The results of the experiment measuring GSSG formation are shown in Fig. 6. Under aerobic conditions much more GSSG was formed than under an atmosphere of nitrogen, particularly during horseradish peroxidase catalyzed oxidation of *p*-phenetidine. This shows that the major proportion of GSSG formed during horseradish peroxidase-catalyzed oxidation of acetaminophen and *p*-phenetidine under aerobic conditions does not, as previously supposed, occur via direct thiyl radical dimerization. The GSSG formed under conditions of nitrogen saturation is presumably a reflection of the latter process and is quantitatively of minor importance representing 8 and 33% of the total amount of GSSG generated in the cases of the oxidation of *p*-phenetidine and acetaminophen, respectively.

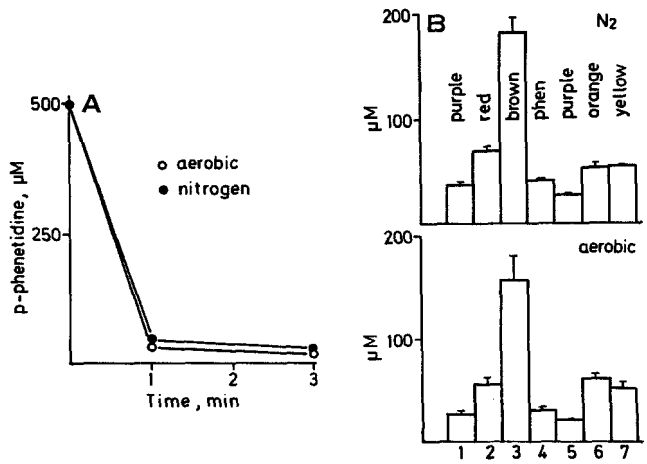

FIG. 5. **Removal of *p*-phenetidine (*A*) and generation of metabolites (*B*) during the horseradish peroxidase-catalyzed oxidation of *p*-phenetidine under aerobic and nitrogen saturation conditions.** Conditions were: [^{14}C]*p*-phenetidine (0.5 mM), horseradish peroxidase (2 μg/ml), and H_2O_2 (0.25 mM). The reactions were treated with catalase (0.05 ml, 1500 units/ml) at t = 1 and 3 min. The *p*-phenetidine removal was measured either using HPLC or TLC, and the appearance of metabolites was monitored by TLC (see "Materials and Methods"). *phen*, *p*-phenetidine.

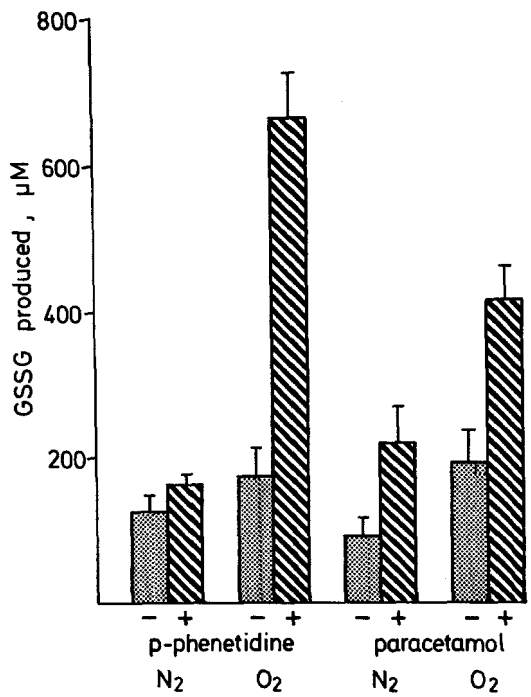

FIG. 6. **GSSG generated during horseradish peroxidase-catalyzed oxidation of acetaminophen and *p*-phenetidine in the presence of GSH under aerobic and nitrogen-saturation conditions.** For *p*-phenetidine, conditions were: *p*-phenetidine (0.5 mM), horseradish peroxidase (2 μg/ml), and H_2O_2 (0.25 mM). For acetaminophen, conditions were: acetaminophen (0.5 mM), horseradish peroxidase (25 μg/ml), and H_2O_2 (0.25 mM). GSH = 5 mM in both cases.

DISCUSSION

We have confirmed in this study that horseradish peroxidase-catalyzed oxidation of *p*-phenetidine occurs via a one-electron mechanism by utilizing GSH as a hydrogen donor in these reactions and observing the thiyl radical generated using EPR spectroscopy in conjunction with the spin trap DMPO (Fig. 1). More importantly this data confirms that the interaction of xenobiotic radicals such as those derived from horseradish peroxidase-catalyzed oxidation of acetaminophen (8) and *p*-phenetidine, with GSH, generates glutathionyl radicals and documents the further reactions of these thiyl radicals. Extensive investigations of the radiolysis of thiol-containing solutions has demonstrated the generation of oxidized thiol and the uptake of oxygen associated with thiyl radical generation (9–14). The concept of thiols such as GSH participating in electron and hydrogen atom transfer reactions has also been well-documented in studies on radioprotective mechanisms (reviewed in Ref. 21) and recent work has suggested that GSH can act as a catalyst linking rapid hydrogen atom transfer reactions in chemical systems (21, 22). On the basis of this work and our data presented here, we propose the scheme shown in Fig. 7 as representative of the further reactions of glutathionyl radicals.

After xenobiotic radical reduction by GSH, the thiyl radical ($GS^{\cdot}$) generated may either dimerize, interact with oxygen, or react with GSH (9–14). Under conditions where the amount of GSH is more than adequate to reduce all of the xenobiotic radicals generated, oxygen uptake still occurs via a mechanism dependent on the concentration of GSH (Figs. 2–4). This may reflect one or a mixture of two mechanisms. The thiyl radical can react with glutathione anion to generate glutathione anion radical ($GSSG^{-\cdot}$) which in turn can interact with oxygen (9). Thus, this route of oxygen consumption would be dependent on GSH concentration. Alternatively the glutathione peroxysulphenyl radical ($GSOO^{\cdot}$), generated by addition of oxygen to the glutathionyl radical, may abstract hydrogen from the excess GSH present to form glutathione sulphenyl hydroperoxide (GSOOH). This would regenerate a thiyl radical which could then go through the reaction sequence again causing a cyclical reaction which would consume oxygen in a manner dependent on the concentration of the reactant which drives the process, *i.e.* GSH. This pathway, due to its cyclical nature, represents a route whereby extensive thiol oxidation can occur as a result of a comparatively small amount of xenobiotic radical production. There are other possible reactions of $GSOO^{\cdot}$ such as termination reactions involving dimerization and interaction with glutathionyl radicals, which have been suggested to be sources of singlet molecular oxygen (23), and further oxidative reactions to generate the sulfonic acid of glutathione (23). We were not able to detect the latter product in this system using HPLC, and the absence of sulfonate is in agreement with radiolysis studies of GSH containing solutions at neutral pH (10, 11).

Dimerization of glutathionyl radicals (GS$^{\cdot}$) is one of the possible mechanisms of GSSG generation during these reactions. In this study we obtained substantial decreases in the amount of GSSG generated under anaerobic conditions, suggesting that the presence of oxygen is essential for maximal levels of thiol oxidation (Fig. 6). Indeed, quantitatively the aerobic route leading to GSSG generation was by far the major mechanism of oxidized thiol production representing as much as 93% in the case of the horseradish peroxidase-catalyzed oxidation of *p*-phenetidine. Thus, thiyl radical dimerization under these conditions appears to play a minor role in the generation of GSSG despite the fact that the rate constant for this reaction calculated from radiolysis experiments is high (Fig. 7) (14). A possible explanation for this may be that the latter was obtained at a pH of 3.9 and that a lower rate constant is associated with the pH used here.

The only end product of GSH oxidation detected in this study was GSSG and this is again in agreement with radiolysis studies of GSH solutions which have reported 85 and 90% conversion into GSSG at neutral pH (10, 11). Both of the two routes discussed in connection with the GSH-dependent oxygen uptake observed, *i.e.* via GSSG$^{\cdot-}$ and via GSOO$^{\cdot}$, can produce GSSG. From the glutathionyl anion radical (GSSG$^{\cdot-}$), interaction with oxygen generates the oxidized thiol. The second route leading to GSSG generation is more complex (see Fig. 7) and involves interaction of GSOOH with either water (10) or GSH generating the hypothetical intermediate glutathione sulphenic acid (GSOH) which can then interact with GSH to produce GSSG or be further oxidized to the sulfinate. Which of these two mechanisms contributes most to the further reactions of the thiyl radical generated in this system cannot be deduced unequivocally from our data. In the case of the route via GSSG$^{\cdot-}$, the oxygen uptake observed should depend on the amount of cosubstrate radical generated as this has a theoretical 1:1 ratio to the amount of oxygen consumed. This was not the case for *p*-phenetidine (Fig. 3*B*) as the oxygen consumption observed did not increase with increasing concentrations of cosubstrate radical above 0.25 mM. This may reflect alternative reactions of the primary radical at higher concentrations or the fact that, during horseradish peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of GSH, the major mechanism underlying oxygen uptake and GSSG generation is via GSOOH and the GSH-dependent cyclical generation of thiyl radical shown in Fig. 7. In the case of acetaminophen the oxygen uptake observed did increase with increasing cosubstrate radical concentrations, suggesting that the major propagating reaction is via the glutathione anion radical (GSSG$^{\cdot-}$).

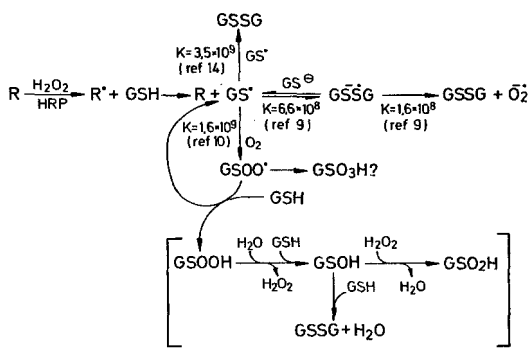

FIG. 7. **Proposed scheme for the interaction of GSH with xenobiotic radicals (R$^{\cdot}$) and the subsequent fate of the glutathionyl radical.** Rate constants are shown in $M^{-1} s^{-1}$.

In summary we have observed the interaction of GSH with xenobiotic-derived radicals and followed the subsequent fate of the glutathionyl radical thus generated. We show that the product of the further reactions of the thiyl radical is GSSG but that this is produced mainly via mechanism(s) involving oxygen consumption and not by a simple thiyl radical dimerization process.

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