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Stimulation of Porphyrinogen Oxidation by Mercuric Ion. I. Evidence of Free Radical Formation in the Presence of Thiols and Hydrogen Peroxide

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SUMMARY

The etiology of mercury-induced porphyrinuria was investigated by testing the hypothesis that mercuric ions (Hg^{2+}) promote free radical-mediated oxidation of reduced porphyrins (porphyrinogens) by compromising the antioxidant potential of endogenous thiols, particularly GSH. Studies *in vitro* demonstrated that porphyrinogens (uroporphyrinogen and coproporphyrinogen) readily undergo H_2O_2-dependent oxidization in the presence of Fe^{3+}-EDTA and that this action is attenuated by GSH at biologically relevant concentrations (0.5–10 mM). At low concentrations, Hg^{2+} complexes with GSH in a 1:2 molar ratio to decrease the antioxidant effect of GSH. However, at Hg^{2+} concentrations approaching saturation-complexation with available GSH, stimulation of porphyrinogen oxidation to 2 to 3 times that mediated by the H_2O_2/Fe^{3+}-dependent system alone is observed. Stimulation of porphyrinogen oxidation by Hg^{2+} plus GSH increases in a dose-related manner with the concentration of H_2O_2 in the reaction mixture but is independent of the presence of iron. No porphyrinogen oxidation is observed in reaction mixtures containing H_2O_2 and either Hg^{2+} or GSH alone or when Hg^{+} is substituted for Hg^{2+}. Studies with reactive oxidant scavengers and ESR spectroscopy suggest the participation of free radical species in Hg:GSH-mediated porphyrinogen oxidation. A mechanism involving ligand exchange between Hg^{2+} and GSH, which leads to formation of GS· radicals and subsequent propagation of reactive oxygen-based radical species, is proposed. These studies support the view that Hg^{2+} both compromises the antioxidant potential of GSH and promotes formation of reactive species via thiol complexation. These findings suggest a mechanistic basis underlying the porphyrinogenic as well as tissue-damaging properties of mercuric ions.

The porphyrinogenic properties of mercury compounds are well established and have been attributed largely to metal-induced alterations of the regulation of specific heme biosynthetic or degradative processes in target tissue cells (1–4). Studies on the etiology of mercury-induced porphyrinuria in rats (5, 6), for example, have demonstrated that excess urinary porphyrins excreted during prolonged exposure to mercury as MMH are largely of renal etiology, consistent with the kidney as the principal target organ of mercury compounds. Partial inhibition of renal uroporphyrinogen decarboxylase and coproporphyrinogen oxidase and secondary induction of δ-aminolevulinic acid synthetase during MMH exposure have been shown to account, in part, for the porphyrinogenic action of mercury compounds (5, 7, 8).

In recent studies (7, 9), we have determined that the magnitude of porphyrin excretion that occurs during prolonged exposure to MMH exceeds that expected on the basis of measured changes in enzyme activities alone. These observations suggest that mechanisms other than altered regulation of porphyrin metabolism also contribute to excess porphyrin accumulation and excretion observed during mercury exposure.

One such mechanism by which mercury could contribute to excess porphyrin excretion is by acting to facilitate the oxidation of reduced porphyrins (porphyrinogens) in target tissues by reactive oxidizing species that are normally produced during the course of endogenous biological oxidation reactions. Reduced porphyrins are utilized in heme biosynthesis, whereas their oxidized counterparts accumulate and are readily excreted. Previous studies from this laboratory (10, 11) and others (12–14) have demonstrated that reactive oxidants, such as (superoxide $O_2^{\cdot -}$) and (hydroxyl OH·) radicals, readily oxidize reduced porphyrins *in vitro*. Inasmuch as the tissue-damaging properties of mercury may involve free radical mechanisms (15–18), it is reasonable to postulate that oxidation of porphyrinogens in target tissue cells occurs by a comparable mecha-

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ABBREVIATIONS: MMH, methyl mercury hydroxide; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; DMPO, 5,5-dimethylpyrroline-*N*-oxide; HRP, horseradish peroxidase; GSH, glutathione.

nism, contributing to the potent porphyrinogenic effects observed during prolonged metal exposure.

A likely mechanism by which mercury could act to increase the levels of reactive intermediates in tissue cells is by depletion of cellular antioxidants, particularly GSH (10, 15, 18), which normally acts to protect cell constituents against the damaging effects of endogenous free radical species (19). The interaction of mercuric ion and GSH would be especially favored in light of the strong first-order association constant of Hg^{2+} for metal-mercaptide bond formation ($>10^{20}$) (20) and the property of mercuric ion to form a stoichiometric complex in a 1:2 molar ratio with GSH (21). The probability that the interaction between Hg^{2+} and GSH would promote porphyrinogen oxidation *in vivo* is further favored by the likelihood of this interaction occurring in or near tissue mitochondria, a principal subcellular site of mercury accumulation (22), porphyrinogen metabolism (e.g., see Ref. 23), and reactive oxidant formation (24–27).

The present studies were undertaken to investigate the hypothesis that mercury, as mercuric chloride, increases the rate of reactive oxidant-mediated porphyrinogen oxidation by compromising the availability of GSH as a free radical antioxidant. The results, derived from studies *in vitro* using a free radical-generating system, support this hypothesis and, in addition, demonstrate for the first time that mercuric ion interacts with GSH in the presence of hydrogen peroxide to stimulate, rather than compromise, porphyrinogen oxidation. The accompanying paper (28) demonstrates the potential biological relevance of these findings in describing the ability of Hg^{2+} and GSH to react with H_2O_2 produced endogenously by the mitochondrial electron transport chain of rat liver and kidney to promote porphyrinogen oxidation.

Experimental Procedures

Materials. Porphyrins (free carboxylic acids of the I isomeric configuration) were purchased from Porphyrin Products (Logan, UT). Uroporphyrin (8-carboxyl-porphyrin) was used in all studies. In preliminary experiments, it was determined that coproporphyrinogen (4-carboxyl-porphyrinogen) could be substituted for uroporphyrinogen in oxidation reactions with comparable results. Thus, although coporphyrin is of principal relevance to mercury-induced porphyrinuria, uroporphyrin was employed owing to its relative ease of reduction by sodium amalgam. GSH, $HgCl_2$, EDTA, HEPES buffer, deoxyribose, mannitol, superoxide dismutase, HRP, and DMPO were obtained from Sigma Chemical Company (St. Louis, MO). Mercurous chloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals and reagents were obtained from standard commercial sources and were of the highest available purity. All solutions were prepared with metal-free deionized water.

Preparation of iron chelates and porphyrinogens. Iron-EDTA chelates were prepared by mixing a solution of 40 mM ferric chloride or ferric nitrate with an equal volume of 41 mM EDTA, and appropriate dilutions of this solution were made with deionized water. Porphyrinogens were prepared immediately before use by reduction of the corresponding porphyrin with freshly ground 3% sodium amalgam under N_2 and were neutralized to pH 7.5 before use. A final concentration of 1 μM uroporphyrinogen was used in all studies.

***In vitro* oxidation of porphyrinogen.** Oxidation of uroporphyrinogen at 37° was followed in 4.5 × 1 cm polystyrene cuvettes, using a Shimadzu model RF 5000U recording spectrofluorometer, by monitoring the increase in fluorescence of the oxidized porphyrin at the emission wavelength for uroporphyrin (612 nm) following excitation at 395 nm. Reaction mixtures contained 10 μM Fe^{3+}-EDTA, 2.5 mM H_2O_2 in 3 ml of 0.1 M HEPES buffer, pH 7.45, $HgCl_2$, GSH, and other components at concentrations indicated in the figures and tables. In most experiments, phosphate and Tris buffers were avoided because of the ability of these materials to chelate metals. HEPES buffer did not promote porphyrinogen oxidation itself or in the presence of H_2O_2 or metals alone. Uroporphyrinogen was added to the sample cuvette, after a 5-min incubation period, in a final concentration of 1 μM. Fluorescence was monitored for a period of 5 to 60 min, depending on the rate of porphyrinogen oxidation under specified conditions. Rates presented in the figures and tables represent the maximum rates observed under the conditions described. pH was monitored periodically using a calibrated pH meter, to ensure that a constant pH was maintained in the test sytem.

ESR studies. ESR spectroscopy was used to confirm the generation of free radical species by Hg^{2+}, GSH, and H_2O_2 under the experimental conditions employed in the porphyrinogen oxidation studies. The spectrometer was a Varian E-4 X-band instrument, with the addition of a bias arm, double-balanced mixer detector, and microwave preamplifier (29). A Zeeman field at 100 kHz was used to produce a modulated ESR signal. After detection by the microwave mixer, the signal was passed to an Ithaco Dynatrac three-phase sensitive detector to remove the modulation and produce a filtered DC output. The DC magnetic field was digitally swept under the control of a PDP 11/23 computer, and the phase detector output was digitized in synchrony with the field sweep. The resulting spectra could be added, averaged, and integrated with the computer. The experimental conditions were as follows: microwave power of 20 mW in a Varian E-231 TE_{102} ESR cavity containing a quartz Dewer insert; 100 kHz modulation amplitude of 2.5 G peak-to-peak; and output time constant of 0.4 sec, corresponding to a noise bandwidth of 0.3 Hz. The field scan range of 64 G was digitized into 1024 points; the sweep rate was controlled by the output time constant so that the DC field remained at each point for at least one time constant (0.4 s) before moving on. The aqueous sample filled the bottom 1–2-cm segment of a 100-μl capillary that was sealed at each end and put inside a 4-mm o.d. quartz sample tube that extended the length of the ESR cavitity. Samples were made up in either HEPES or phosphate buffer solutions and were composed of components at concentrations indicated in the figures. All analyses were conducted at room temperature.

Statistical analyses. Statistical differences between groups were determined by means of Student's *t* test.

Results

Glutathione attenuates H_2O_2-dependent porphyrinogen oxidation. Initial studies were conducted to demonstrate the oxidation of porphyrinogens by Fe^{3+}-EDTA plus H_2O_2 and the attenuation of this effect by GSH. Previous studies (10, 11) have shown that the rate of porphyrinogen oxidation in this system is dependent on the concentrations of both Fe^{3+}-H_2O_2-EDTA and H_2O_2 in the reaction mixture. The control system selected for the present studies contained 2.5 mM H_2O_2 and 10 μM Fe^{3+}-EDTA in 0.1 M HEPES buffer, pH 7.45, which produced an oxidation rate of 300–400 pmol/min when either uroporphyrinogen or coproporphyrinogen was added to the reaction cuvette. A comparable oxidation rate was observed in 0.1 M phosphate buffer, pH 7.5. Porphyrinogen oxidation did not occur when either Fe^{3+}-EDTA or H_2O_2 was omitted from the reaction mixture. However, comparable rates of porphyrinogen oxidation were observed when Fe^{2+}-EDTA was substituted for Fe^{3+}-EDTA in the reaction medium (data not shown). As seen in Fig. 1, GSH attenuated H_2O_2-mediated porphyrinogen oxidation in a dose-dependent manner. Addition of 5 and 10 mM GSH decreased the oxidation rate to 46 and 22% of the control rate, respectively.

Hg^{2+} reverses GSH attenuation of porphyrinogen ox-

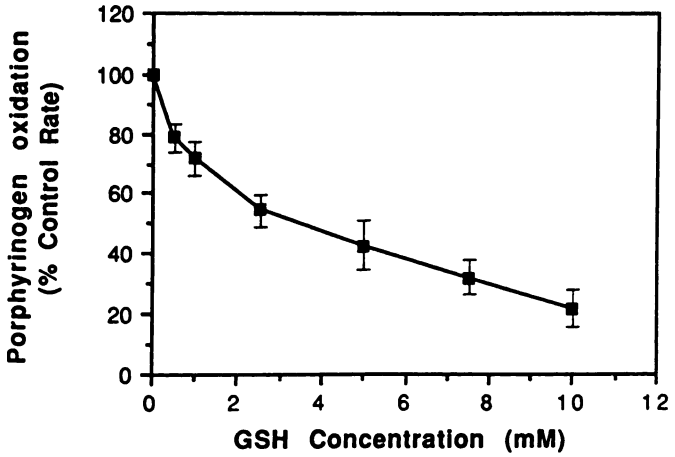

Fig. 1. Antioxidant effect of GSH on H_2O_2-dependent oxidation of uroporphyrinogen. Samples contained 2.5 mM H_2O_2, 10 μM Fe^{3+}-EDTA, and 0.1 M HEPES buffer, pH 7.45, in a total volume of 3 ml. GSH was added at the concentrations indicated. Uroporphyrinogen was added after a 5-min incubation of the reaction mixture at 37°, at a final concentration of 1 μM. Porphyrinogen oxidation was followed spectrofluorometrically, as described in Experimental Procedures. The initial rate of porphyrinogen oxidation was 356 ± 30 pmol/min. Values in this and subsequent figures are expressed as means ± standard errors of at least five replicate experiments.

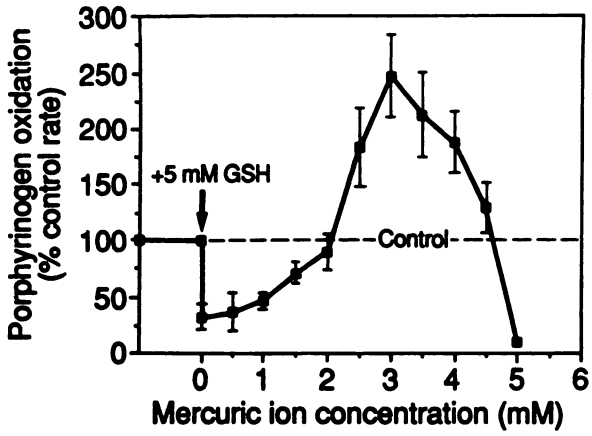

Fig. 2. Effects of Hg^{2+} and GSH on H_2O_2-dependent uroporphyrinogen oxidation. Samples contained 2.5 mM H_2O_2, 10 μM Fe^{3+}-EDTA, and 0.1 M HEPES buffer, pH 7.45, in a final volume of 3 ml. GSH (5 mM) or complexes of 5 mM GSH and $HgCl_2$ at the concentrations indicated were added to the reaction mixture before other components. Uroporphyrinogen was added after a 5-min incubation of the reaction mixture, at a final concentration of 1 μM. The initial (control) rate of porphyrinogen oxidation is that mediated by the Fe^{3+}-EDTA/H_2O_2 system alone.

idation. To test the hypothesis that mercuric ion reverses the capacity of GSH to attenuate porphyrinogen oxidation, experiments were conducted in which varying concentrations of mercuric chloride were added to the H_2O_2/Fe^{3+}-EDTA system, in which the GSH concentration was initially established at 5 mM. As seen in Fig. 2, mercury had the predicted effect of reversing GSH attenuation of porphyrinogen oxidation at concentrations in the reaction mixture ranging from 0.5 to 2 mM. In Fig. 2, the initial oxidation rate (100%) represents that elicited by the oxidizing system before addition of GSH or Hg^{2+} to the reaction mixture. This rate is attenuated by more than 70% by addition of 5 mM GSH to reaction cuvettes. The subsequent addition of mercuric chloride reversed the GSH-attenuated oxidation of porphyrinogen in a dose-related manner, with the antioxidant effect of GSH being almost entirely eliminated at a final mercury concentration of 2 mM, consistent with the stoichiometric 1:2 binding ratio of Hg^{2+} to GSH (21).

Hg^{2+} plus GSH stimulates H_2O_2-dependent porphyrinogen oxidation. It was anticipated that further addition of mercuric chloride to the reaction cuvette would completely reverse the antioxidant effect of GSH on H_2O_2-dependent porphyrinogen oxidation. Contrary to expectation, however, a substantial increase in the rate of porphyrinogen oxidation, above that mediated by H_2O_2 plus Fe^{3+}-EDTA, occurred with further increases in the mercury concentration. As further depicted in Fig. 2, the porphyrinogen oxidation rate in the presence of 2.5 mM mercuric chloride was 1.8 times that observed in the absence of mercury and GSH in the reaction mixture and increased to over 2.5 times the control rate at a mercury concentration of 3 mM.

Further studies indicated that the stimulation of porphyrinogen oxidation by mercury and GSH was a function of the Hg to GSH ratio rather than of the specific concentration of either substance in the reaction mixture. Fig. 3 demonstrates that, when the GSH concentration was increased to 10 mM in the Fe^{3+}-EDTA/H_2O_2 oxidizing system, maximal stimulation of porphyrinogen oxidation, to approximately 2.5 times the control rate, was observed at a final mercuric ion concentration of approximately 6 mM. Similarily, if the initial concentration of GSH was only 1 mM, the maximal increase in porphyrinogen oxidation was achieved at a final Hg^{2+} concentration of 600 μM (data not shown). In each case, an approximate 3:5 molar ratio of Hg^{2+} to GSH was required for maximal stimulation of porphyrinogen oxidation. Hg^{2+} alone or at a concentration in excess of a 3:5 molar ratio with GSH, even in the presence of H_2O_2 and Fe^{3+}-EDTA, caused a rapid decline in porphyrin fluorescence.

The rate of porphyrinogen oxidation in the presence of Hg^{2+} and GSH varied directly with the concentration of H_2O_2 in the reaction mixture. As seen in Fig. 4, the oxidation of porphyrinogen by Hg^{2+} plus GSH at a molar ratio of 3:5 in the reaction cuvette increased linearly with the H_2O_2 concentration between 0 and 3.5 mM. In contrast, no significant oxidation occurred in the presence of buffer alone or with buffer containing H_2O_2, H_2O_2 plus Hg^{2+}, or H_2O_2 plus GSH.

Hg plus GSH stimulates porphyrinogen oxidation independently of iron. As seen in Fig. 5, addition of Hg^{2+} in increasing concentrations to a reaction cuvette containing 2.5 mM H_2O_2 and 5 mM GSH in 0.1 M HEPES (or phosphate)

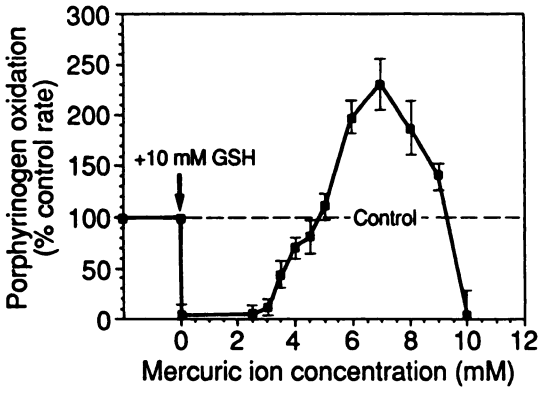

Fig. 3. Effects of Hg^{2+} on porphyrinogen oxidation in the presence of 10 mM GSH. All conditions were as described for Fig. 2.

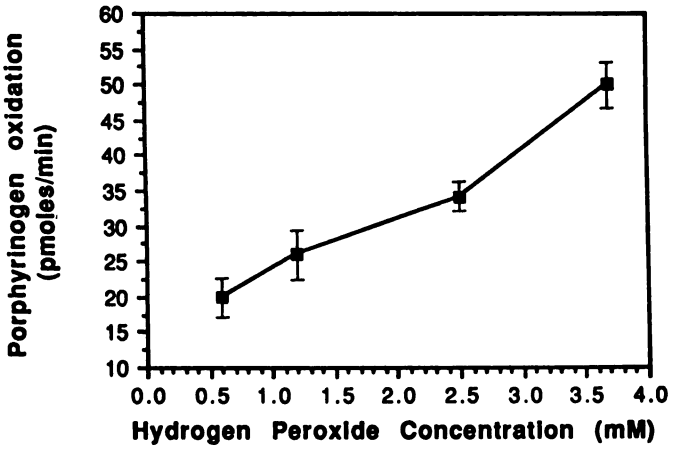

Fig. 4. Relationship of H_2O_2 concentration to Hg:GSH-promoted porphyrinogen oxidation. Samples contained 3 mM $HgCl_2$, 5 mM GSH, and 0.1 M HEPES buffer, pH 7.45, in a total volume of 3 ml. H_2O_2 was added at the concentrations indicated. Conditions were as described for Fig. 1.

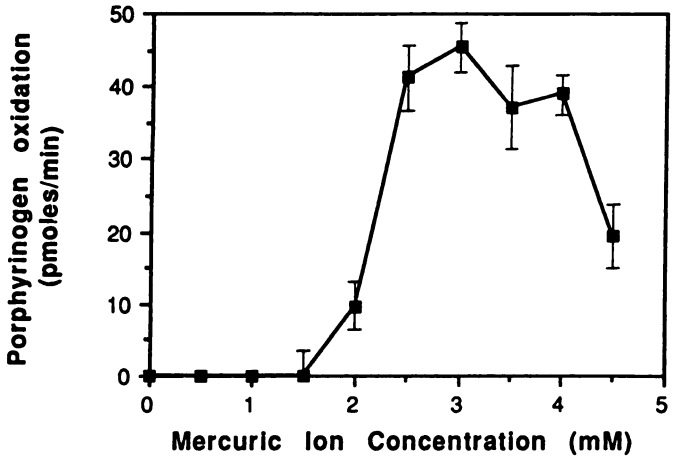

Fig. 5. Stimulation of porphyrinogen oxidation by Hg^{2+}, GSH, and H_2O_2. Reaction mixtures contained 2.5 mM H_2O_2 and 5 mM GSH in 0.1 M HEPES buffer, pH 7.45. $HgCl_2$ was mixed with GSH at the concentrations indicated and added to the reaction mixture before H_2O_2. Porphyrinogen was added at a final concentration of 1 μM following a 5-min incubation of the reaction mixture at 37°.

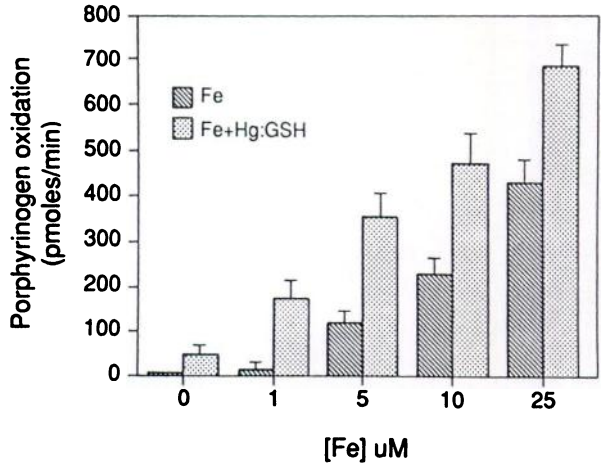

Fig. 6. Combined effects of Hg:GSH- and Fe-promoted porphyrinogen oxidation. Reaction mixtures contained 2.5 mM H_2O_2 in 0.1 M HEPES buffer, pH 7.45, 3 mM $HgCl_2$, 5 mM GSH, and Fe^{3+}-EDTA at the concentrations indicated.

TABLE 1

Attenuation of H_2O_2-mediated porphyrinogen oxidation by GSH and cysteine

Samples contained 2.5 mM H_2O_2, 10 μM Fe^{3+}-EDTA, and 0.1 M HEPES buffer, pH 7.45, in a total volume of 3 ml. $HgCl_2$, GSH, or cysteine was added to the buffer at the concentrations indicated before other components. Uroporphyrinogen was added after a 5-min incubation of the reaction mixture at 37°, at a final concentration of 1 μM. Porphyrinogen oxidation was followed spectrofluorometrically, as described in Experimental Procedures. Values are expressed as means ± standard errors of at least five replicate experiments.

Addition	Porphyrinogen oxidation rate	
	pmol/min	*% of control*
Fe^{3+}-EDTA + H_2O_2 (control)	323 ± 56	100
+ GSH		
1 mM	297 ± 32	92
5 mM	174 ± 40*	50
10 mM	48 ± 13*	15
+ Cysteine		
1 mM	229 ± 38*	71
5 mM	78 ± 29*	24
10 mM	10 ± 6*	3
Hg (3 mM) + GSH (5 mM) + H_2O_2	102 ± 34	
Hg (3 mM) + cysteine (5 mM) + H_2O_2	81 ± 16	

* $p < 0.05$ compared with control.

buffer, pH 7.45, elicited porphyrinogen oxidation with maximal rates again occurring at a final mercury concentration of 3 mM. This effect occurred in the absence of added iron in the reaction mixture. However, the stimulation of porphyrinogen oxidation by Hg^{2+} and GSH in the presence of H_2O_2 was synergistic with that mediated by Fe^{3+}-EDTA and H_2O_2, inasmuch as the combined effect of Hg plus GSH and Fe^{3+}-EDTA on H_2O_2-dependent porphyrinogen oxidation was substantially greater than the sum of the effect of either system alone. As shown in Fig. 6, Hg^{2+} and GSH in a 3:5 molar ratio significantly increased the rate of H_2O_2-dependent porphyrinogen oxidation above that produced by Fe^{3+}-EDTA alone at all Fe^{3+} concentrations between 0 and 25 μM in the reaction mixture.

Cysteine can replace GSH in Hg^{2+}-stimulated porphyrinogen oxidation. Additional studies were conducted in which cysteine was substituted for GSH in the H_2O_2/Fe^{3+}-EDTA oxidizing system, in order to confirm that stimulation of porphyrinogen oxidation resulted from the interaction of mercuric ions specifically with the sulfhydryl groups of GSH, as assumed. As shown in Table 1, cysteine was equally, if not more, effective than GSH at equivalent concentrations in attenuating H_2O_2-dependent porphyrinogen oxidation. Additionally, in reaction cuvettes containing only 2.5 mM H_2O_2 and Hg plus GSH or Hg plus cysteine in a final molar ratio of 3:5, porphyrinogen oxidation was stimulated in each case to a comparable extent. These results indicate that Hg:GSH-stimulated porphyrinogen oxidation involves the interaction of Hg^{2+} ions with the cysteinyl sulfhydryl residue of the GSH molecule and that the mechanism of oxidation is, at least in part, a thiol-mediated event.

Hg:GSH-stimulated porphyrinogen oxidation is attenuated by OH· radical scavengers. To further investigate the hypothesis that stimulation of porphyrinogen oxidation by Hg^{2+} and GSH in the presence of H_2O_2 is mediated by reactive oxidizing species, such as OH·, studies were conducted to measure the extent to which Hg:GSH-stimulated porphyrinogen oxidation could be attenuated by known OH· radical scavengers. As shown in Table 2, mannitol, deoxyribose, and dimethyl sulfoxide decreased the rate of Hg/GSH-stimulated porphyrinogen oxidation to 40, 46, and 79% of the control rate,

TABLE 2

Effects of reactive oxidant scavengers on Hg:GSH-stimulated porphyrinogen oxidation

Samples contained 3 mM $HgCl_2$, 5 mM GSH, 2.5 mM H_2O_2, oxidant scavengers at the concentrations indicated, and 0.1 M HEPES buffer, pH 7.45, in a total volume of 3 ml. Uroporphyrinogen was added after a 5-min incubation of the reaction mixture at 37°, at a final concentration of 1 μM. Porphyrinogen oxidation was followed spectrofluorometrically, as described in Experimental Procedures. Values represent means ± standard errors of at least three replicate experiments.

Addition	Porphyrinogen oxidation rate	
	pmol/min	*% of control*
Hg^{2+} + GSH + H_2O_2 (control)	142 ± 16	100
+ Mannitol (4 mM)	57 ± 12*	40
+ Deoxyribose (10 mM)	65 ± 8*	46
+ Dimethyl sulfoxide (100 μl)	112 ± 60	79

*$p < 0.05$ compared with control.

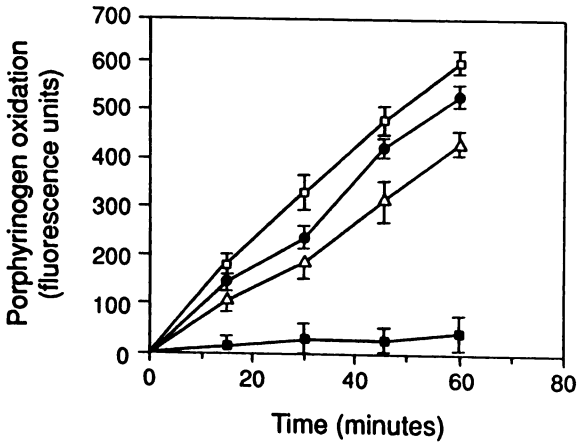

Fig. 7. Effects of reactive oxidant scavenger (deoxyribose) on Hg:GSH-promoted porphyrinogen oxidation. Reaction mixtures contained 3 mM $HgCl_2$, 5 mM GSH, 2.5 mM H_2O_2, and 1 μM uroporphyrinogen in 0.1 M HEPES buffer, pH 7.45, in a total volume of 3 ml. Deoxyribose was added at the concentrations indicated. Porphyrinogen oxidation was assessed at 15-min intervals for 1 hr. The extent of oxidation is expressed as fluorescence units. □, 0 mM deoxyribose; ●, 5 mM deoxyribose; △, 10 mM deoxyribose; ■, buffer control.

respectively, at the concentrations used. The effect of dimethyl sulfoxide; however, was not statistically significant.

Additional studies were conducted to investigate the time course of porphyrinogen oxidation by the Hg^{2+} plus GSH system and to determine the effect of the OH· scavenger deoxyribose on the oxidation rate. In these studies, the rate of oxidation of uroporphyrinogen by Hg^{2+} plus GSH at various deoxyribose concentrations was monitored at 15-min intervals over a period of 1 hr. As demonstrated in Fig. 7, porphyrinogen oxidation in the presence of Hg^{2+} plus GSH at a 3:5 molar ratio increased consistently over the 1-hr period. Oxidation was significantly attenuated by the presence of deoxyribose, in a concentration-dependent manner. Comparable results were obtained when mannitol was substituted for deoxyribose as the OH· scavenger.

In contrast to the oxidative action of Hg^{2+}, Hg^{+} did not stimulate porphyrinogen oxidation alone or in the presence of GSH and/or H_2O_2. When used in studies comparable to those described in Fig. 7, mercurous ion (Hg^{+}), if substituted for Hg^{2+}, did not promote porphyrinogen oxidation or otherwise affect the detection of oxidized porphyrin in the reaction mixture.

Hg^{2+} plus GSH and H_2O_2 promote formation of DMPO radical adducts. To substantiate the participation of reactive oxidants or other free radical species in Hg:GSH-mediated stimulation of porphyrinogen oxidation, ESR studies were undertaken using DMPO as the spin trap. Initial studies were conducted to define the parameters of the ESR signals generated by the free radical products of the Fe^{3+}-EDTA/H_2O_2 (oxygen radical-generating) and HRP/H_2O_2/GSH (thiyl radical- promoting) systems in the presence of DMPO under experimental conditions comparable to those used in the present studies. The ESR spectra produced by these systems are presented in Fig. 8. Fig. 8A shows the ESR signal produced by the DMPO adduct generated in reaction mixtures containing Fe^{3+}-EDTA, H_2O_2, and DMPO in 1 mM HEPES buffer, pH 7.45, at room temperature. The characteristic 1:2:2:1 quartet is obtained when the three-line nitrogen spectrum is split by a proton with approximately the same splitting ($a_H = a_N = 15.1$ G), giving a total spectrum width of approximately 44 G. In contrast, the ESR spectrum of the DMPO adduct derived from the HRP system, presented in Fig. 8B, has slightly different splitting constants ($a_N = 15.4$ G, $a_H = 16.2$ G) and a total spectrum width of about 46 G. These spectra are idential to those seen by Piette and Hsia (30) and by Harman *et al.* (31) for the OH· and GS· radicals, respectively.

The ESR signal produced by the DMPO adduct(s) derived from the mixture of Hg^{2+}, GSH, and H_2O_2 is shown in Fig. 9. The principal features of this spectrum are a pair of triplets and two doublets, one on either side of the pair of triplets. This spectrum is clearly more complex than that produced by either DMPO-radical adduct observed in Fig. 8 and suggests the participation of multiple radical species including, perhaps, either of those depicted in Fig. 8. In this respect, a DMPO-OH· or DMPO-GS· radical adduct could be represented (peaks

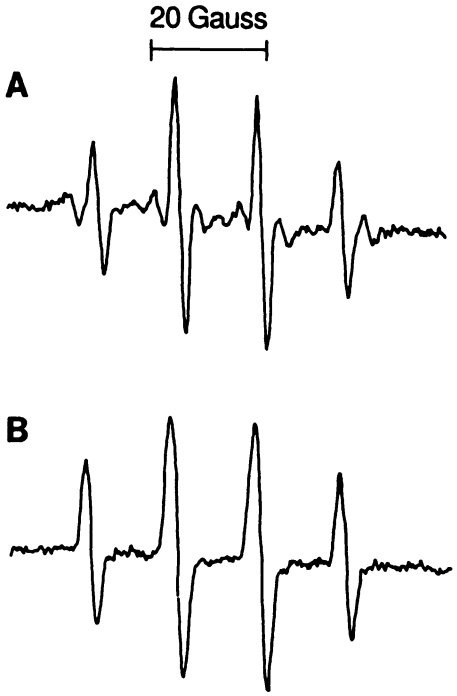

Fig. 8. ESR spectra of DMPO-OH· radical adduct (A) and DMPO-GS· adduct (B). Hydroxyl radical adducts (A) were produced in a system containing 2.5 mM H_2O_2, 25 μM Fe^{3+}-EDTA, and 90 mM DMPO in 0.1 M HEPES buffer, pH 7.45. GS· radical adducts (B) were produced in a system containing 1 mM GSH, 100 μM H_2O_2, and 0.1 mg/ml HRP in 0.1 M phosphate buffer, pH 7.5. Instrument conditions: microwave power, 20 mW; modulation amplitude, 2.5 G; time constant, 0.4 sec; scan rate, 20 G/min.

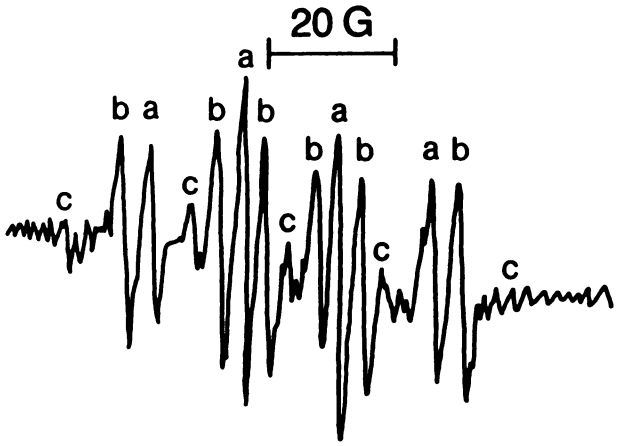

Fig. 9. ESR spectrum of DMPO-radical adducts formed from Hg plus GSH. The mixture contained 3 mM $HgCl_2$, 5 mM GSH, 0.2 mM H_2O_2, and 90 mM DMPO in 0.1 M phosphate buffer, pH 7.5. Instrument conditions were as described for Fig. 8.

labeled *a*), based on the observed splitting constants and the total spectral width. Additionally, a DMPO-carbon-centered radical adduct with splitting constants a_N = 15.8 G and a_H = 23.3 G appears to be present (peaks labeled *b*). A smaller signal (a_N) = 15.6 G, a_H = 21.1 G) (peaks labeled *c*), representing yet a third DMPO-radical adduct, may also be represented in the spectrum. This spectrum took approximately 1 hr to develop to maximum size and then slowly decayed, a much slower process than required for the generation of the DMPO-radical adduct signals depicted in Fig. 8. The time course of development of the maximum ESR signal is consistent with the time required for the maximum rate of porphyrinogen oxidation by Hg^{2+} plus GSH, as observed spectrofluorometrically (Fig. 7). Further work is in progress to analyze the time dependence and nature of the radical species present.

Discussion

The results of the present studies demonstrate that reduced porphyrins are highly susceptible to oxidation by reactive oxidant species *in vitro* and that GSH readily attenuates this effect at biologically relevant concentrations. Additionally, it was found that mercuric ion reverses the antioxidant action of GSH, apparently as a result of formation of metal mercaptide bonds with the sulfhydryl groups of the cysteinyl moiety of the GSH molecule. These findings are consistent with the known property of Hg^{2+} to complex with sulfhydryl groups on a 1:2 molar basis (18) and with the observed high first-order binding constant of Hg^{2+} for SH^- moieties ($k_1 = 10^{20}$) (20), the strongest of any metal. Previous studies (10) have demonstrated that reduction in the concentration of GSH via metal complex formation increases the rate of free radical-mediated oxidation of reduced porphyrins *in vitro*.

The mechanisms by which Fe^{3+}-EDTA mediates H_2O_2-dependent porphyrinogen oxidation in the present studies are not immediately apparent, inasmuch as reduced iron (Fe^{2+}), rather than Fe^{3+}, is ordinarily required to promote the formation of OH· radicals via the Fenton reaction (32). In the present reaction system, OH· could be formed as result of the interaction of H_2O_2 with Fe^{2+}, subsequent to the reduction of Fe^{3+} by the reduced porphyrin itself. Such a mechanism is consistent with the observation that Fe^{3+}-EDTA, when substituted for Fe^{3+}-EDTA in reaction mixtures, promotes precisely the same rate of H_2O_2-dependent porphyrinogen oxidation as observed with Fe^{3+}-EDTA. This view is supported by recent observations by Yamazaki and Piette (33), which demonstrate that the stoichiometry of OH· formation favors very low Fe^{2+} concentrations relative to that of H_2O_2 as would be expected from the reduction of Fe^{3+} by the porphyrinogen in the present reaction system. On the other hand, the formation of a comparably reactive iron-oxygen species, such as a ferrous-dioxygen-ferric chelate complex, as proposed by Bucher *et al.* (34), might be responsible for the oxidation reaction observed. As another possibility, De Matteis (12) has suggested the Fe^{3+} could act similarily to a peroxidase in the presence of H_2O_2, leading to the rapid oxidation of porphyrinogen observed. The one-electron oxidation of porphyrins by peroxidases has been recently described (35). Any of these mechanisms has potential significance physiologically, inasmuch as the predominant form of iron *in vivo* is as the ferric chelate (36). Identification of the precise mechanism or species involved, however, requires further investigation.

The finding that Hg^{2+} interacts with GSH to stimulate oxidation of reduced porphyrins is of considerable interest with respect to the mechanisms of mercury-induced porphyrinuria as well as cell injury. The reaction utilizes H_2O_2 or, possibly, other reduced oxygen species but is apparently independent of the presence of transition metals such as iron, normally required for Fenton-type O_2 reduction reactions (32). The interaction of Hg^{2+}, GSH, and H_2O_2 appears to involve free radical generation, as suggested by both studies of oxygen radical scavengers and ESR spectroscopy. The ESR signal generated by the interaction of Hg^{2+} and GSH in the presence of H_2O_2 suggests a complex system consistent with the production of multiple reactive species derived, possibly, from reduced oxygen as well as from GSH constituents. Although the generation of reactive species resulting from the interaction of Hg^{2+}, GSH, and H_2O_2 has not been previously proposed, free radical chain reactions involving organomercurials have been well defined (37).

A possible mechanism by which the prooxidant action of Hg^{2+} in the presence of GSH (or other thiols) and H_2O_2 could be explained is suggested from the coordination chemistry of Hg^{2+} compounds, which are known to participate in extremely rapid ligand-exchange reactions when bound in coordination complexes with thiols or other nucleophiles. Thus, although the stoichiometry of Hg^{2+} and GSH predominantly involves linear two-coordination with Hg^{2+} bound between the thiol groups of two GSH molecules, Hg^{2+} may add third and fourth donor atoms in the presence of excess ligands, which undergo rapid exchange in and out of the metal ion coordination sphere (38). Under such circumstances, substitution of inner sphere water in Hg^{2+} complexes occurs with a rate constant of 10^9 sec^{-1}, as fast as any metal ion known (39). This rapid metal ion exchange among mercury donor atoms in the presence of excess GSH could lead to formation of Hg^+-GS complexes (40), which could then decompose to GS· radicals.[3] This sequence is depicted in reaction (1).

$$Hg^{2+} + GSH \rightarrow [Hg^+ \cdot GS] \rightarrow Hg^+ + GS\cdot + H^+ \quad (1)$$

GS· radicals, in turn, could interact with GSH and H_2O_2, as

[3] Glen A. Russell, Ph.D., Department of Chemistry, Iowa State University, personal communication.

suggested by reactions 2 and 3, to promote formation of additional free radical species.

$$GS\cdot + GSH \rightarrow GSSG\cdot^{-} + H^{+} \quad (2)$$

$$GS\cdot + H_2O_2 \rightarrow GSSG + OH\cdot + OH^{-} \quad (3)$$

Although reactions 2 and 3 have not been confirmed in the present studies, evidence that these reactions take place under the experimental conditions used here has been provided by numerous investigators (36, 41–44). The formation of highly reactive OH· radicals in the present system, as could occur by reaction 3, is supported by the OH· scavenger studies described in Table 2 and Fig. 7. Further evidence of the presence of OH· is derived from the ESR pattern depicted in Fig. 9, which appears to have spectral characteristics (peaks labeled *a*) comparable to those produced by DMPO-OH· adducts observed in Fig. 8A. Should OH· be formed in this manner, the further interaction of OH·, either with DMPO or GSH, could give rise to a carbon-centered radical adduct (peaks labeled *b*), as well as other undefined species (peaks labeled *c*), as is suggested from the ESR spectrum depicted in Fig. 9. Thus, the complex spectrum generated by the interaction of Hg^{2+}, GSH, and H_2O_2 could be explained by the reaction sequence proposed above and is consistent with the idea that the initiating species is a single radical, such as GS·, as suggested. Further work is required to confirm these findings, as well as to identify the nature of the species involved and the mechanisms by which they arise.

The present findings are of interest in suggesting that glutathione, generally viewed as an endogenous antioxidant, can interact with mercury (as Hg^{2+} but not Hg^{+}) to facilitate oxidation of reduced porphyrins. Although the formation of thiyl radicals from GSH and other thiol compounds in the presence of transition metals has been described by numerous investigators (41, 45–47), this is the first report, to our knowledge, suggesting the formation of free radicals from the interaction of GSH and Hg^{2+} ions in the presence of H_2O_2 as well as the promotion of the oxidation of biological constituents arising from this interaction. These findings may have physiological significance with respect to the underlying basis of Hg-induced cell injury, inasmuch as ligand exchange between Hg^{2+} and GSH, suggested herein as the basis of free radical formation, accounts principally for the rapid partitioning of mercury among sulfhydryl groups in tissues. Preliminary studies in this regard provide evidence for the formation of thiobarbiturate-reactive intermediates as well as conjugated dienes consequent to Hg^{2+}, GSH, and H_2O_2 treatment of liver and kidney lipids *in vitro* and in the kidney after prolonged MMH exposure. The present findings suggest, therefore, that Hg^{2+} may interact with GSH to promote formation of reactive species that oxidize not only reduced porphyrins but also other cell constituents. As described in the following paper (28), stimulation of porphyrinogen oxidation by Hg:GSH complexes in mitochondrial systems at Hg^{2+} concentrations observed to occur in renal tissue during prolonged mercury exposure attests to the likely physiological significance of this effect. Should further research show this to be the case, a common mechanistic basis underlying the etiology of both the porphyrinogenic and tissue-damaging properties of mercury compounds would be identified.

In summary, the present findings indicate that Hg^{2+} and GSH interact in the presence of H_2O_2 to promote formation of multiple free radical species that oxidize reduced porphyrins *in vitro*. This effect appears to be independent of the presence of Fe but is synergistic with the Fe-mediated oxidant reactions. Should these reactions occur *in vivo*, these observations could explain the porphyrinogenic as well as oxidative tissue-damaging properties of mercury compounds.

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References

1. Woods, J. S. Mechanisms of metal-induced alterations of cellular heme metabolism. *Comments in Toxicology* **3**:3–25 (1989).
2. Marks, G. S. Exposure to toxic agents: the heme biosynthetic pathway and hemoproteins as indicator. *Crit. Rev. Toxicol.* **15**:151–179 (1988).
3. Fowler, B. A., A. Oskarrson, and J. S. Woods. Metal and metalloid-induced porphyrinurias: relationships to cell injury. *Ann. N. Y. Acad. Sci.* **514**:172–182 (1987).
4. Maines, M. D. Metals as regulators of heme metabolism. *Science* (*Washington D. C.*) **198**:1215–1219 (1977).
5. Woods, J. S., and B. A. Fowler. Renal porphyrinuria during methyl mercury exposure. *J. Lab. Clin. Med.* **90**:266–273 (1977).
6. Fowler, B. A., and J. S. Woods. Ultrastructural and biochemical changes in renal mitochondria following chronic methyl mercury exposure: the relationship to renal function. *Exp. Mol. Pathol.* **27**:402–412 (1977).
7. Woods, J. S., and M. P. Southern. Studies on the etiology of trace metal-induced porphyria: effects of porphyrinogenic metals on coproporphyrinogen oxidase in rat liver and kidney. *Toxicol. Appl. Pharmacol.* **97**:183–190 (1989).
8. Woods, J. S., D. L. Eaton, and C. Lukens. Studies on porphyrin metabolism in the kidney: effects of trace metals and glutathione on renal uroporphyrinogen decarboxylase. *Mol. Pharmacol.* **26**:336–341 (1984).
9. Woods, J. S. Regulation of porphyrin and heme metabolism in the kidney. *Semin. Hematol.* **25**:336–348 (1988).
10. Woods, J. S. Attenuation of porphyrinogen oxidation by glutathione *in vitro* and reversal by porphyrinogenic trace metals. *Biochem. Biophys. Res. Commun.* **152**:1428–1434 (1988).
11. Woods, J. S., and C. A. Calas. Iron stimulation of free radical-mediated porphyrinogen oxidation by hepatic and renal mitochondria. *Biochem. Biophys. Res. Commun.* **160**:101–108 (1989).
12. De Matteis, F. Role of iron in the hydrogen peroxide-dependent oxidation of hexahydroprophyrins (porphyrinogens): a possible mechanism for the exacerbation by iron of hepatic uroporphyria. *Mol. Pharmacol.* **33**:463–469 (1988).
13. Francis, J. E., and A. G. Smith. Oxidation of uroporphyrinogens by hydroxyl radicals: evidence for nonporphyrin products as potential inhibitors of uroporphyrinogen decarboxylase. *FEBS Lett.* **233**:311–314 (1988).
14. Jacos, J. M., P. R. Sinclair, R. W. Lambrecht, and J. F. Sinclair. Effects of iron-EDTA on uroporphyrinogen oxidation by liver microsomes. *FEBS Lett.* **250**:349–352 (1989).
15. Christie, N. T., and M. Costa. *In vitro* assessment of the toxicity of metal compounds. IV. Disposition of metals in cells: interactions with membranes, glutathione, metallothionein and DNA. *Biol. Trace Element Res.* **6**:139–158 (1984).
16. Cantoni, O., R. M. Evans, and M. Costa. Similarity in the acute cytotoxic response of mammalian cells to mercury (II) and X-rays: DNA damage and glutathione depletion. *Biochem. Biophys. Res. Commun.* **108**:614–619 (1982).
17. Yonaha, M., Y. Ohbayshi, T. Ichinose, and M. Sagai. Lipid peroxidation stimulated by mercuric chloride and its relation to the toxicity. *Chem. Pharmacol. Bull.* **30**:1437–1443, (1982).
18. Stacey, M. H., and H. Kappus. Cellular toxicity and lipid peroxidation in response to mercury. *Toxicol. Appl. Pharmacol.* **63**:29–35 (1982).
19. Meister, S., and M. E. Anderson. Glutathione. *Annu. Rev. Biochem.* **52**:711–760 (1983).
20. Gurd, F. R. N., and P. E. Wilcox. Complex formation between metallic cations and proteins, peptides and amino acids. *Adv. Protein Chem.* **11**:311–427 (1956).
21. Rabinstein, D. L., R. Guevremont, and C. A. Evans. Glutathione and its metal complexes, in *Metal Ions in Biological Systems* (H. Sigel, ed.), Vol. 9. Marcel Dekker, New York, 103–141 (1979).
22. Goyer, R. A., and B. C. Rhyne. Toxic changes in mitochondrial membranes and mitochondrial function, in *Pathology of Cell Membranes* (B. F. Trump and A. U. Arstila, eds), Vol. I. Academic Press, New York, 383–428 (1975).
23. Tait, G. H. The biosynthesis and degradation of heme; in *Heme and Hemoproteins* (F. De Matteis and W. N. Aldridge, eds.). Springer-Verlag, Berlin, 1–48 (1978).
24. Georgellis, A., M. Tsirigotis, and J. Rydstrom. Generation of superoxide anion and lipid peroxidation in different cell types and subcellular fractions of rat testis. *Toxicol. Appl. Pharmacol.* **94**:362–373 (1988).
25. Loschen, G., L. Flohe, and B. Chance. Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. *FEBS Lett.* **18**:261–264 (1971).
26. Kennedy, C. H., J. M. Dyer, D. F. Church, G. W. Winston, and W. A. Pryor.

Radical production in liver mitochondria by peroxidic tumor promoters. *Biochem. Biophys. Res. Commun.* **160**:1067–1072 (1989).
27. Forman, H. J., and A. Boveris. Superoxide radical and hydrogen peroxide in mitochondria, in *Free Radicals in Biology* (W. A. Pryor, ed.), Vol. 5. Academic Press, New York, 65–90 (1982).
28. Woods, J. S., C. A. Calas, and L. D. Aicher. Stimulation of porphyrinogen oxidation by mercuric ion. II. Promotion of oxidation from the interaction of mercuric ion, glutathione, and mitochondria-generated hydrogen peroxide. *Mol. Pharmacol.* **38**:261–266 (1990).
29. Mailer, C., J. D. S. Danielson, and B. H. Robinson. Computer controlled pulsed EPR spectrometer. *Rev. Sci. Inst.* **56**:1917–1925 (1985).
30. Piette, L. H., and J. C. Hsia. Spin labeling in biomedicine, in *Spin Labeling—Theory and Applications* (L. J. Berliner, ed), Vol. 2. John Wiley, New York, 247–290 (1979).
31. Harman, L. S., D. K. Carver, J. Schreiber, and R. P. Mason. One- and two-electron oxidation of reduced glutathione by peroxidases. *J. Biol. Chem.* **261**:1642–1648 (1986).
32. Aust, S. D., L. A. Morehouse, and C. E. Thomas. The role of metals in oxygen radical reactions. *Free Radicals Biol. Med.* **1**:3–25 (1985).
33. Yamazaki, I., and L. H. Piette. ESR spin-trapping studies on the reaction of Fe^{2+} ions with H_2O_2–reactive species in oxygen toxicity in biology. *J. Biol. Chem.* **265** (1990). (Still in press)
34. Bucher, J. R., M. Tien, and S. D. Aust. The requirement for ferric in the initiation of lipid peroxidation by chelated ferrous iron. *Biochem. Biophys. Res. Commun.* **111**:777–784 (1983).
35. Morehouse, K. M., H. J. Sipe, and P. R. Mason. The one-electron oxidation of porphyrins to porphyrin *pi*-cation radicals by peroxidases: an electron spin resonance investigation. *Arch. Biochem. Biophys.* **273**:158–164 (1989).
36. Miller, D. M., G. R. Buettner, and S. D. Aust. Transition metals as catalysts of "autooxidation" reactions. *Free Radicals Biol. Med.* **8**:95–108 (1990).
37. Russell, G. A. Free radical chain reaction involving alkyl- and alkenylmercurials. *Acc. Chem. Res.* **22**:1–8 (1989).
38. Martin, R. B. Bioinorganic chemistry of metal ion toxicity, in *Metal Ions in Biological Systems* (H. Sigel, ed.), Vol. 20. Marcel Dekker, New York, 21–54 (1986).
39. Dieber, H., M. Eigen, G. Ilgenfritz, G. Maas, and R. Winker. Kinetics and mechanism of reactions of main group metal ions with biological carriers. *Pure Appl. Chem.* **20**:93–115 (1969).
40. Stricks, W., and I. M. Kolthoff. Polarography of glutathione. *J. Am. Chem. Soc.* **74**:4646–4653 (1952).
41. Saez, G., P. Thornalley, H. Hill, R. Hems, and J. Bannister. The production of free radicals during the autooxidation of cysteine and their effects on radiated rat hepatocytes. *Biochim. Biophys. Acta* **719**:24–31 (1982).
42. Ross, D. Glutathione, free radicals and chemotherapeutic agents: mechanisms of free radical-induced toxicity and glutathione-dependent protection. *Pharmacol. Ther.* **32**:231–249 (1988).
43. Subrahmanyam, V. V., and P. J. O'Brien. Peroxidase catalyzed oxygen activation in arylamine carcinogens and phenol. *Chem. Biol. Interact.* **50**:185–199 (1985).
44. Munday, R., and C. C. Winterbourn. Reduced glutathione in combination with superoxide dismutase as an important biological antioxidant defence mechanism. *Biochem. Pharmacol.* **38**:4349–4352 (1989).
45. Keller, R. J., R. A. Coulombe, R. P. Sharma, T. A. Grover, and L. H. Piette. Oxidation of NADH by vanadium compounds in the presence of thiols. *Arch. Biochem. Biophys.* **271**:40–48 (1989).
46. Rowley, D. A., and B. Halliwell. Superoxide-dependent formation of hydroxyl radicals in the presence of thiol compounds. *FEBS Lett.* **138**:33–36 (1982).
47. Tsen, C. C., and A. L. Tappel. Catalytic oxidation of glutathione and other sulfhydryl compounds by selenite. *J. Biol. Chem.* **233**:1230–1232 (1958).

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