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INVOLVEMENT OF HYDROPEROXIDES AND AN ACC-DERIVED FREE RADICAL IN THE FORMATION OF ETHYLENE

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Key Word Index—*Pisum sativum*; Leguminosae; pea; ethylene biosynthesis; ACC; free radical; hydroperoxides; microsomal membranes; spin trapping.

Abstract—Microsomal membranes from etiolated peas, a model system capable of catalysing ethylene formation from 1-aminocyclopropane-1-carboxylic acid (ACC), were used to investigate the involvement of hydroperoxides and free radicals in the terminal step of ethylene biosynthesis. *t*-Butyl hydroperoxide (*t*-BuOOH) and cumene hydroperoxide increased ethylene production by up to 19- and 9-fold respectively when added to the basic reaction mixture consisting of microsomes and ACC in buffer. These enhancement effects were both heat-denaturable, and that of *t*-BuOOH also proved to be sensitive to catalase. By contrast, hydrogen peroxide inhibited ethylene production. Lipoxygenase and linolenic acid stimulated ethylene production, and removal of endogenous hydroperoxides by the addition of GSH and GSH peroxidase almost completely inhibited the formation of ethylene. Spin trapping studies with 4-(*N*-methylpyridinium)*t*-butylnitrone revealed an ACC-dependent free radical species, the production of which could be inhibited by Tiron, a disulphonated catechol that reacts with $O_2^{\cdot-}$. The data collectively suggest that the enzymatic conversion of ACC to ethylene by pea microsomal membranes is hydroperoxide-dependent, and is mediated by $O_2^{\cdot-}$ through a free radical intermediate of the substrate.

INTRODUCTION

The final step in the pathway of ethylene biosynthesis, the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC)* to ethylene, has been investigated in apple tissue [1], homogenates of etiolated pea [2], rib segments and protoplasts from Morning Glory flower tissue [3] and microsomal membranes from etiolated peas [4, 5] and carnation flowers [6]. Features of this conversion that have been established through these various model systems include a dependence on oxygen, the apparent involvement of free radicals and sensitivity to catalase. It has been suggested that the conversion of ACC to ethylene may involve a peroxidative reaction [1, 3], although recent evidence indicates that peroxidase itself does not play a direct role in the decarboxylation and ring cleavage of ACC [7, 8]. This notwithstanding, ethylene production in ripening fruits has been correlated with changes in hydroperoxide levels and peroxidase activity [9–11], and in apple ethylene production is immediately preceded by a rise in lipoxygenase activity [12].

One paradoxical feature of the enzymatically mediated conversion of ACC to ethylene in cell-free systems is the finding that it is sensitive to catalase and inhibited by hydrogen peroxide [2, 4]. In the present study, we have attempted to reconcile this discrepancy by examining the prospect that other hydroperoxides, which may also serve as substrates for catalase [13], are involved in the enzymatic conversion of ACC to ethylene by pea (*Pisum sativum* L.) microsomal membranes. Organic hydroperoxides, for example, are known to support mixed function hydroxylation reactions by serving as both a substitute for oxygen and an electron donor [14] or by facilitating the activation of molecular oxygen [15] and, in view of the putative role of $O_2^{\cdot-}$ in the conversion of ACC to ethylene [4], could be involved. Spin trapping techniques were used to investigate the molecular mechanism for ethylene production from ACC and revealed the formation of an ACC-derived free radical species analogous to that previously reported for a purely chemical system [16].

RESULTS AND DISCUSSION

The catalytic conversion of ACC to ethylene by the ethylene-forming enzyme of microsomal membranes from pea epicotyl sections is oxygen-dependent, heat-denaturable and sensitive to radical scavengers [4]. To evaluate the effect of organic hydroperoxides on this conversion, CH and *t*-BuOOH were added to the basic reaction mixture consisting of microsomal membranes and ACC in Epps buffer. The results are reported as an enhancement factor, which is the ratio of ethylene produced in the presence of added hydroperoxide relative to that produced in its absence (Figs 1 and 2). Thus, if the hydroperoxide had no effect, the enhancement factor was 1. Controls consisting of an equivalent concentration of organic hydroperoxide and ACC alone in buffer were run

*Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CH, cumene hydroperoxide; DPPH, α, α-diphenol-β-picryl hydrazyl; Epps, *N*-2-hydroxyethyl piperazine sulfonic acid; GSH, glutathione; GSH-P, glutathione peroxidase; MDA, malondialdehyde; 4-MePyBN, 4-(*N*-methylpyridinium) *t*-butyl nitrone; $O_2^{\cdot-}$, superoxide radical anion; SHAM, salicyl-hydroxamic acid; TBA, thiobarbituric acid; *t*-BuOOH, *t*-butyl hydroperoxide; Tiron, 1,2-dihydroxy benzene-3,5-disulfonic acid.

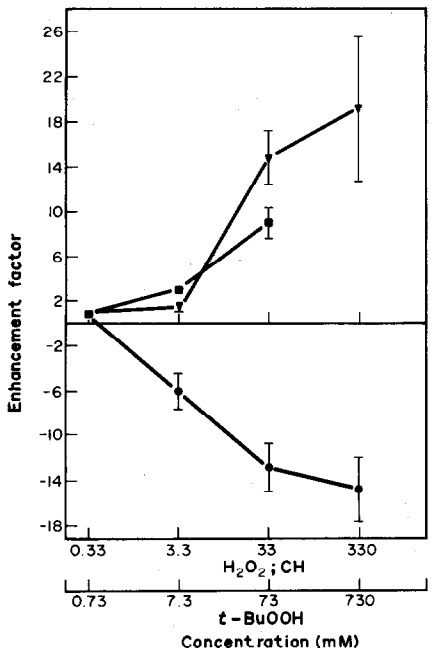

Fig. 1. Effects of hydrogen peroxide, *t*-BuOOH and CH on the conversion of ACC to ethylene by pea microsomal membranes. Final concentrations of hydroperoxide added to the basic reaction mixture (as defined in the Experimental) are indicated along the abscissa. Enhancement factor is the ratio of ethylene produced in the presence of added hydroperoxide relative to that produced in its absence. Values represent the mean ± s.e. ($n = 3$). (●) Hydrogen peroxide; (■) CH; (▲) *t*-BuOOH.

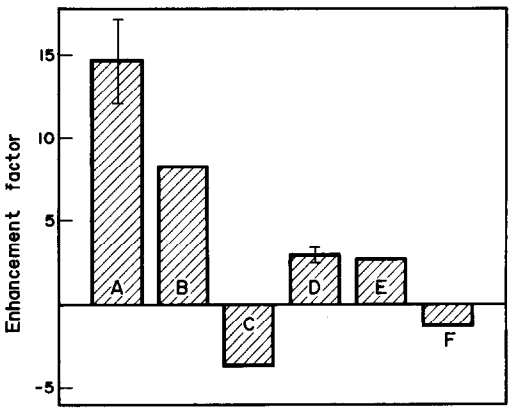

Fig. 2. Effects of catalase and heat denaturation on the hydroperoxide-induced enhancement of ethylene production from ACC by pea microsomal membranes. (A) Basic reaction mixture (as defined in the Experimental) plus 73 mM *t*-BuOOH; (B) basic reaction mixture plus 73 mM *t*-BuOOH plus 50 units/ml catalase; (C) basic reaction mixture plus 73 mM *t*-BuOOH but with heat-denatured microsomes; (D) basic reaction mixture plus 3.3 mM CH; (E) basic reaction mixture plus 3.3 mM CH plus 50 units/ml catalase; and (F) basic reaction mixture plus 3.3 mM CH but with heat-denatured microsomes. Values represent the mean ± s.e. ($n = 3$).

routinely and subtracted from the values obtained when the organic hydroperoxide was added to the full reaction mixture.

t-BuOOH had no effect on ethylene production when added to the basic reaction mixture at a concentration of 0.73 mM, but as the concentration was increased through 7.3, 73 and 730 mM, there was a progressive enhancement of ethylene production. Indeed, the amount of ethylene produced in the presence of 730 mM *t*-BuOOH was 19-fold higher than that obtained from the basic reaction mixture (Fig. 1). CH at a concentration of 33 mM, the upper limit of its solubility, also increased ethylene production by about nine-fold. As well, the enhancement effects of added organic hydroperoxides proved to be heat denaturable (Fig. 2), indicating that the increase in ethylene production is enzymatically mediated. In fact, less ethylene was obtained from the basic reaction mixture containing heat-denatured microsomes and organic hydroperoxides than from controls containing an equivalent amount of organic hydroperoxide and ACC alone and, hence, negative enhancement factors were obtained (Fig. 2). The concentrations of *t*-BuOOH and CH required to stimulate ethylene production are high, but this is presumably because they are not native substrates for the microsomal ethylene-forming enzyme. By contrast, hydrogen peroxide inhibited ethylene production when added to the basic reaction mixture at concentrations ranging from 3.3 to 330 mM (Fig. 2).

Of particular interest is the finding that the *t*-BuOOH-induced enhancement of ethylene production from ACC can be partially inhibited by the addition of 50 units/ml of catalase (Fig. 2). This inhibition most likely reflects a reaction involving catalase and the organic hydroperoxide, since low MW aliphatic hydroperoxides are known to serve as substrates for this enzyme [13]. Catalase had no perceptible effect on the CH-supplemented system (Fig. 2), an observation that may reflect steric hindrance attributable to the benzene ring of CH. Ethylene production by the basic reaction mixture is also inhibited by catalase [4], and inasmuch as hydrogen peroxide, the more conventional substrate for catalase, actually reduced the conversion of ACC to ethylene by microsomes [4], this effect of catalase may reflect depletion of an endogenous organic hydroperoxide which is essential for ethylene production from ACC.

In situ, organic hydroperoxides are derived, in part, from lipids, and to test the prospect that products of lipid peroxidation may facilitate the formation of ethylene from ACC, soybean lipoxygenase (EC 1.13.11.12) was added directly to the basic reaction mixture. This enzyme mediates the formation of hydroperoxides from unsaturated fatty acids [17], shows increased activity in response to wounding [18] and has been implicated as a contributing factor in the formation of wound ethylene and ethane [19]. When lipoxygenase was added to the basic reaction mixture, ethylene production increased by *ca* 33% (Table 1). When linolenic acid was added as well, ethylene production was increased still further, reaching values 144% higher than the level produced by the basic reaction mixture (Table 1). Heat-denatured microsomes produced only very low levels of ethylene in the presence of lipoxygenase, and lipoxygenase and linolenic acid alone with ACC also yielded very little ethylene (Table 1). Thus, the enhancement observed with the basic reaction mixture is a consequence of the interaction between a product of the lipoxygenase reaction, presumably a hydroperoxide

Table 1. Effect of lipoxygenase on the conversion of ACC to ethylene by pea microsomal membranes

Treatment	nl C_2H_4/ml·hr	Δ% control
Control (A)*	1.91 ± 0.10	—
A + 8500 units lipoxygenase	2.55 ± 0.20	33.5
A + 8500 units lipoxygenase + 400 μM linolenic acid	4.66 ± 0.58	144.0
Lipoxygenase (8500 units/ml), 400 μM linolenic acid and 1 mM ACC in 50 mM Epps, pH 8.5	0.38 ± 0.14	—
A (heat denatured) + 8500 units lipoxygenase	0.35 ± 0.07	—

*Control is the basic reaction mixture as defined in the Experimental. Ethylene was assayed after 2 hr of closure at 31°. Means ± s.e. ($n = 3$).

derivative of linolenic acid, and the microsomal ethylene-forming enzyme. However, when microsomal lipid peroxidation was induced by adding NADPH, an electron donor for cytochrome P-450 [14], to the basic reaction mixture, ethylene production was inhibited by 63% (Table 2). NADPH added to the basic reaction mixture in the absence of ACC did not produce any detectable ethylene (Table 2). The incidence of lipid peroxidation in these experiments was scored by measuring the formation of MDA, and in the presence of 4 mM NADPH, MDA levels increased by 64% over the 90 min period during which ethylene was collected in the basic reaction mixture and by 40% in the absence of ACC (Table 2). It is not immediately clear why NADPH-induced peroxidation inhibited ethylene production whereas lipoxygenase-induced peroxidation stimulated ethylene production, although it is conceivable that the microsomal ethylene-forming enzyme is damaged in the presence of NADPH, for MDA is known to cross-link free amino groups of proteins [20].

To further test the prospect that organic hydroperoxides are required for activity of the ethylene-forming enzyme, GSH peroxidase, an enzyme that catalyses the reduction of hydroperoxides [21], was used. Ethylene production from ACC was reduced by 84% in the presence of GSH and GSH peroxidase (Table 3), suggesting that endogenous hydroperoxides, presumably of lipid origin, are required for the conversion. GSH peroxidase alone also inhibited the conversion of ACC to ethylene presumably by utilizing endogenous GSH present in the microsomes to reduce the lipid hydroperoxides (Table 3). Ethylene production also proved sensitive to GSH alone and to sodium dithionite (Table 3), both of which are strong reducing agents, and these observations together

Table 2. Effect of NADPH-induced lipid peroxidation on the conversion of ACC to ethylene by pea microsomal membranes

Treatment	Δ%* C_2H_4 (nl/ml·hr)	Δ%* MDA (nmol MDA/mg microsomal protein)
Basic reaction + NADPH (4 mM)	−63.0 ± 7.9	64.2 ± 11.5
Basic reaction − ACC + NADPH (4 mM)	−100	40.9 ± 4.8

*Relative to levels produced by the basic reaction mixture as defined in the Experimental. Incubation time was 90 min. Means ± s.e. ($n = 4$).

Table 3. Effects of GSH, GSH peroxidase and various inhibitors on the conversion of ACC to ethylene by pea microsomes

Treatment	Δ%
A + 0.08 mM GSH	−33.0 ± 8.7
A + 0.16 mM GSH	−58.2 ± 1.1
A + 40 units GSH-P	−32.2 ± 2.4
A + 40 units GSH-P + 0.08 mM GSH	−84.1 ± 0.4
A + 2.5 mM sodium dithionite	−94.7 ± 0.9
A + 2.5 mM SHAM	−96.5 ± 0.7
A + 1 mM cytochrome *c*	−50.6 ± 3.4

*Relative to levels produced by the basic reaction mixture as defined in the Experimental. Ethylene was assayed 1–2 hr after closure at 31°. Means ± s.e. ($n = 3$).

with the strong inhibitory effect of the hydroxamic acid SHAM (Table 3), suggest the involvement of a transition metal. Hydroxamic acids are known to possess strong chelating action for metal ions [22], and inasmuch as the conversion of ACC to ethylene is likely mediated by an oxidase [23], the involvement of a transition metal is not unexpected.

Hydroperoxides support $O_2^{\cdot-}$-mediated reactions and are thought to do so by facilitating the activation of oxygen [24]. In the light of spin trapping evidence indicating involvement of $O_2^{\cdot-}$ in the conversion of ACC to ethylene by these membranes [4], it is conceivable that hydroperoxides promote ethylene production through a similar mechanism. A role for $O_2^{\cdot-}$ is supported by the observation that cytochrome *c*, a scavenger of $O_2^{\cdot-}$ [25], strongly inhibits ethylene formation from ACC (Table 3). Using the diagnostic spin trap 4-MePyBN, it has also proved possible to detect a free radical in the complete reaction mixture for ethylene production, which appears to require ACC, $O_2^{\cdot-}$ and hydroperoxides for its formation. A spectrum of the 4-MePyBN spin adduct of this radical is shown in Fig. 3(C), and it is clear that upon heat denaturation of the microsomal membranes or in the absence of ACC, the radical species is not formed (Fig. 3A, B). This spectrum is closely similar to that obtained in a chemical system for ethylene production in which the conversion of ACC to ethylene is driven by hydroxyl radicals formed through the Fenton reagent (Fig. 3C, D; ref. [16]). Additionally, its hyperfine splitting constants are distinctly different from those for the corresponding spin adducts of $O_2^{\cdot-}$ and $OH^{\cdot}$, indicating that neither superoxide nor the hydroxyl radical is the species being trapped, and they are similar to those for spin adducts of CH_2OH and the radical species formed in the chemical system for ethylene production from ACC (Table 4). These observations collectively suggest that the radical species formed in the microsomal system for ethylene production is derived from ACC and may well be a carbon-centered radical of ACC.

Formation of the 4-MePyBN adduct in the microsomal system also proved sensitive to Tiron (Fig. 3E). Tiron is known to react with $O_2^{\cdot-}$ to form the semiquinone (Fig. 3F; ref. [4]) and thus serves as an effective scavenger of superoxide. Accordingly, its ability to block formation of the 4-MePyBN adduct in the microsomal system indicates that formation of the ACC-derived radical is dependent upon prior generation of $O_2^{\cdot-}$. In addition, a 46% reduction in the amplitude of the ACC-derived spectrum was observed when GSH (0.08 mM) and GSH-P (40 units/ml) were added (data not shown), indicating that the formation of the ACC-derived radical also requires hydroperoxides. 4-MePyBN also reacts with $O_2^{\cdot-}$, but the superoxide adduct of this spin trap rapidly disproportionates at pH values of 8.0 and higher [4] and thus does not register in the spectrum recorded for the microsomal system (Fig. 3C). The Tiron semiquinone, formed when Tiron reacts with $O_2^{\cdot-}$ (Fig. 3F), is not seen when Tiron (10 mM) and 4-MePyBN (160 mM) are both added to the microsomal system (Fig. 3E) presumably because the higher concentration of 4-MePyBN out-competes Tiron for the available $O_2^{\cdot-}$. This contention is supported by the fact that 4-MePyBN is able to inhibit the enzymatic conversion of ACC to ethylene by pea microsomes [4].

At the present time, the pea microsomal enzyme can only be regarded as a model system for studying the enzymatic conversion of ACC to ethylene. The model system has provided evidence for the involvement of hydroperoxides, a concept initially proposed by Adams and Yang [1], and at least two radical species, $O_2^{\cdot-}$ and an ACC-derived free radical. The formation of $O_2^{\cdot-}$ is not ACC-dependent, although scavenging of this reactive species inhibits ethylene production as well as formation of the free radical derivative of ACC (Fig. 3; ref. [4]). It would seem reasonable to propose that the ethylene-forming enzyme in this model system mediates the formation of an ACC-derived free radical intermediate prior to ethylene formation in a manner that is dependent upon the presence of $O_2^{\cdot-}$, and that hydroperoxides support this reaction by facilitating the activation of oxygen to superoxide in accordance with a mechanism identified previously [24].

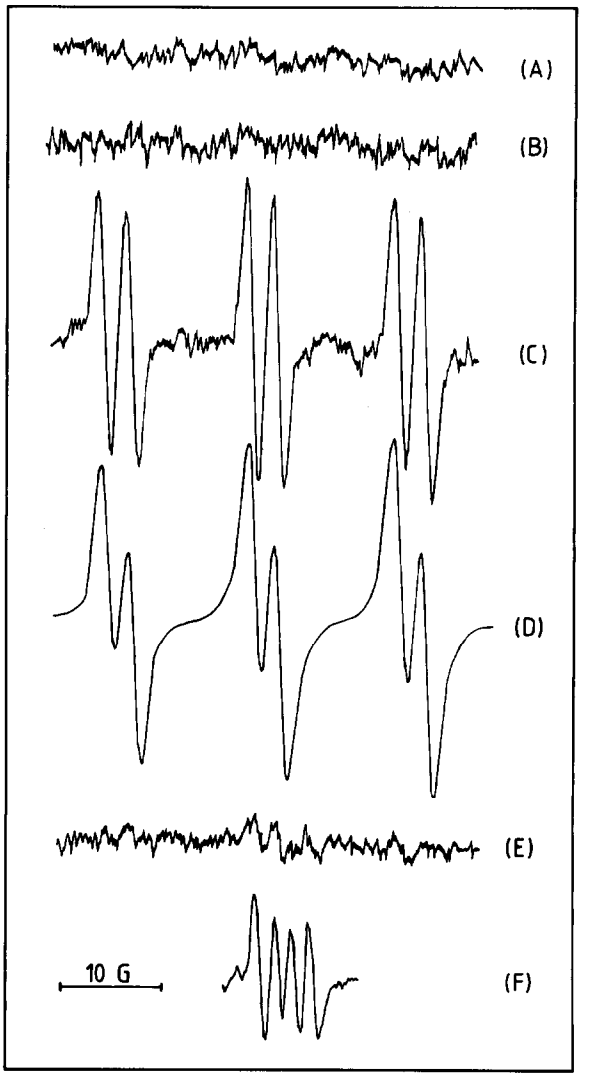

Fig. 3. ESR spectra of spin adducts of 4-MePyBN. (A) Microsomes plus 4-MePyBN; (B) heat-denatured microsomes plus ACC plus 4-MePyBN; (C) microsomes plus ACC plus 4-MePyBN; (D) ACC and Fenton reagent plus 4-MePyBN; (E) microsomes plus ACC plus 10 mM Tiron plus 4-MePyBN; and (F) microsomes plus ACC plus 10 mM Tiron. For concentrations of components other than Tiron see legend of Table 4.

Table 4. Hyperfine splitting constants for various adducts of 4-MePyBN*

Treatment	a_N†	a_β^H†	g-value
(1) Microsomes with ACC	14.77 ± 0.02	2.72 ± 0.01	2.0089
(2) O_2^-	13.78	1.65	2.0091
(3) Fenton reagent (·OH)	14.39 ± 0.03	1.42 ± 0.06	2.0068
(4) Fenton reagent + EtOH (·CH_2OH)	14.72 ± 0.06	2.5 ± 0.0	2.0054
(5) ACC and Fenton reagent (chemical system for converting ACC to ethylene, see ref. [16])	14.61 ± 0.07	2.70 ± 0.21	2.0056

*In gauss at room temperature.

†Means ± s.e. ($n = 3$). Final concentrations in reaction mixtures: (1) microsomes (4.6 mg protein/ml); 0.1 M ACC; 160 mM 4-MePyBN. (2) values taken from [29]. (3) 3% H_2O_2; 50 μM $FeSO_4$; 160 mM 4-MePyBN. (4) 10% EtOH; 3% H_2O_2; 50 μM $FeSO_4$; 160 mM 4-MePyBN. (5) 3% H_2O_2; 50 μM $FeSO_4$; 160 mM 4-MePyBN; 0.1 M ACC. All reagents were suspended in 50 mM Epps buffer, prepared with twice distilled water, pH 8.5.

EXPERIMENTAL

Materials. ACC was obtained from Calbiochem-Behring, La Jolla, California. H_2O_2, CH and TBA were from Canadian Laboratory Supplies, Toronto, Canada, and *t*-BuOOH from Aldrich, Canada. 4-MePyBN was obtained from Edward G. Janzen, Department of Chemistry, University of Guelph, Ontario, Canada. All other chemicals and enzymes were from Sigma.

Growing conditions and microsomal membrane isolation. Pea seeds (*Pisum sativum* L. cv Alaska) were surface-sterilized for 10 min in 5% NaClO, washed and soaked in aerated H_2O for 6 hr. Seedlings were grown under etiolating conditions at 29° for 6 days, after which the plumular hook was removed and the top 20 mm of the epicotyl were harvested. The excised epicotyl sections were homogenized in cold 0.01 M Epps buffer, pH 8.5, at a tissue–buffer ratio of 1:1 (w/v). The homogenate was filtered through six layers of cheesecloth and centrifuged at 10 000 *g* for 20 min. The resulting supernatant was dialysed overnight at 4° against 2 mM Epps buffer, pH 8.5, with two changes, and then centrifuged at 90 000 *g* for 70 min. The resulting pellets of microsomal membrane were combined and either stored under N_2 at −65° or resuspended directly in 2 mM Epps buffer, pH 8.5 (1.0 mg microsomal protein/ml or *ca* 50 mg wet wt/ml) for immediate use.

Ethylene analysis. Measurements of ACC conversion to C_2H_4 were carried out in 12 × 100 mm test tubes. The basic reaction mixture consisted of: (a) 0.1 ml 0.05 M Epps buffer, pH 8.5; (b) 0.2 ml microsomal membrane suspension containing 200 μg protein; (c) 0.6 ml 2 mM Epps buffer, pH 8.5, containing additional reagents as specified; and (d) 0.1 ml 0.01 M ACC. After initiation of the reaction by the addition of ACC, the tubes were closed under aerobic conditions with rubber serum stoppers and incubated at 31° in a shaking water bath for 1–3 hr. At the end of this period, the C_2H_4 in 1 ml gas samples taken from the headspace was analysed by GC as previously described [6]. Measurements were conducted in triplicate for each individual expt. For expts employing heat denatured microsomes, the microsomal suspension (1 mg protein/ml) was immersed in a boiling water bath for 15 min.

Purification and assay of GSH peroxidase. GSH peroxidase was partially purified from rat liver (4 week, immature female Wistar rats) by the procedure of ref. [26]. GSH peroxidase activity was assayed according to the method of ref. [17]. A unit of activity was taken to be the amount of enzyme that gave a difference of 0.001 per min in the log of GSH concn under the assay conditions. The sp. act. of the isolated enzyme preparation was 2840 units/mg protein.

Spin trapping. Radical species were detected using the diagnostic spin trap 4-MePyBN. Spectra of the spin trap adducts were recorded at room temp. with a Varian E-12 spectrometer; *g*-values were determined using DPPH as a standard for which $g = 2.0037$. All components of the reaction mixture were prepared in twice distilled H_2O, and their concns are given in the figure legends. Components of the reaction mixtures were mixed in 6 × 50 mm test tubes; spin trap was added last. Sample (100 μl) was then placed in a glass capillary and spectra recorded immediately.

Assays. Microsomal protein was determined by the method of ref. [27]. MDA was assayed spectrophotometrically at 535 nm using a modified TBA test and a ε value of 1.56×10^5 [28]. None of the reagents in the reaction mixture formed a TBA-colour complex.

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