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POSSIBLE ROLE OF AN IRON–OXYGEN COMPLEX IN 4(*S*)-4-HYDROXYOCHRATOXIN A FORMATION BY RAT LIVER MICROSOMES

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Abstract—Rat liver microsomes were examined for their ability to oxidize the mycotoxin ochratoxin A (OTA) to 4(*R*)-4-hydroxyochratoxin A [(*R*)-4-OH-OTA] and 4(*S*)-4-hydroxyochratoxin A [(*S*)-4-OH-OTA] and to induce OTA-dependent lipid peroxidation. Microsomes isolated from rats pretreated with pregnenolone-16α-carbonitrile greatly induced both (*R*)-4-OH-OTA and (*S*)-4-OH-OTA formation whereas isoniazid pretreatment primarily induced (*S*)-4-OH-OTA. (*R*)-4-OH-OTA and (*S*)-4-OH-OTA formation showed significant differences with respect to pH optima, effect of antioxidants, and iron chelators. (*R*)-4-OH-OTA showed a pH optimum of 6.5 and was not inhibited by the antioxidants butylated hydroxyanisole or *N*,*N*-diphenyl-1,4-phenylenediamine or the iron chelators Desferal or bathophenanthrolinedisulfonic acid. In contrast, both (*S*)-4-OH-OTA and lipid peroxidation showed a pH optimum of 7.0 and both activities were sensitive to inhibition by the above antioxidants and iron chelators. Lipid peroxidation was not involved in (*S*)-4-OH-OTA formation since addition of linoleic acid hydroperoxide to microsomes did not give rise to (*S*)-4-OH-OTA. Cytochrome P450 appeared to be essential since other hemoproteins like horseradish peroxidase and hemoglobin were ineffective in metabolizing OTA in the presence of hydroperoxides. The results suggest that (*R*)-4-OH-OTA is formed by normal mixed-function oxidation but that (*S*)-4-OH-OTA formation may involve free iron. It is likely that an active Fe^{2+}–oxygen complex, formed via NADPH-cytochrome P450 reductase and cytochrome P450-dependent reduction of free Fe^{3+} followed by oxygen binding, serves as the species inducing lipid peroxidation and at least part of (*S*)-4-OH-OTA formation.

Ochratoxin A (OTA)† is a mycotoxin produced by various species of the fungal genera *Aspergillus* and *Penicillium* and is a common contaminant in feed and food stuffs [1, 2]. It consists of a dihydroisocoumarin moiety linked by a peptide bond to L-β-phenylalanine and a chlorine atom at position 5 (Fig. 1). Several studies have shown OTA to be nephrotoxic and hepatotoxic to a variety of animal species [3–5] and to be a renal carcinogen in mice and rats [6, 7]. OTA has also been associated with Balkan endemic nephropathy (BEN), a disease that affects residents of select regions of Bulgaria, Romania and Yugoslavia where OTA contaminates their crops [8]. In these infected areas, OTA was isolated from the sera of residents and those who had high OTA levels often also had urinary tract tumors and BEN [9, 10].

Recently, we showed that OTA, when administered to rats *in vivo* or when added to liver or kidney microsomes *in vitro*, was able to greatly enhance lipid peroxidation [11]. In a subsequent study [12], using a reconstituted microsomal lipid peroxidation system, we demonstrated that OTA induced lipid peroxidation by chelating Fe^{3+} and that the resulting OTA–Fe^{3+} chelate was more readily reducible by the flavoprotein NADPH-cytochrome P450 reductase to an OTA–Fe^{2+} complex which, in the presence of oxygen, provided the active species that initiated lipid peroxidation. In a third study [13], we demonstrated the involvement of cytochrome P450 in OTA-stimulated lipid peroxidation.

Ochratoxin A is known to be metabolized to 4(*R*)-4-hydroxyochratoxin A [(*R*)-4-OH-OTA] and 4(*S*)-4-hydroxyochratoxin A [(*S*)-4-OH-OTA] (Fig. 1) by rat, pig and human liver microsomes in the presence of NADPH [14]. Here we show that formation of (*R*)-4-OH-OTA occurred by the normal mixed-function oxidase route but that an iron–oxygen complex may be at least partly involved in (*S*)-4-OH-OTA formation.

MATERIALS AND METHODS

Chemicals. Ascorbate, bathophenanthrolinedisulfonic acid (sodium salt) (BPS), butylated hydroxyanisole (BHA), catalase, $FeCl_3$, $FeSO_4$, H_2O_2, isocitric acid, isocitric dehydrogenase, isoniazid (INH), horseradish peroxidase (HRP), mannitol, NADPH, pregnenolone-16α-carbonitrile (PCN), superoxide dismutase (SOD) and thiobarbituric acid

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† Abbreviations: BHA, butylated hydroxyanisole; BPS, bathophenanthrolinedisulfonic acid, sodium salt; CHP, cumene hydroperoxide; DPPD, *N*,*N*-diphenyl-1,4-phenylenediamine; Hb, hemoglobin; HRP, horseradish peroxidase; (*R*)-4-OH-OTA, 4(*R*)-4-hydroxyochratoxin A; (*S*)-4-OH-OTA, 4(*S*)-4-hydroxyochratoxin A; INH, isoniazid; LAHP, linoleic acid hydroperoxide; MDA, malondialdehyde; OTA, ochratoxin A; PCN, pregnenolone-16α-carbonitrile; SOD, superoxide dismutase; and TBA, thiobarbituric acid

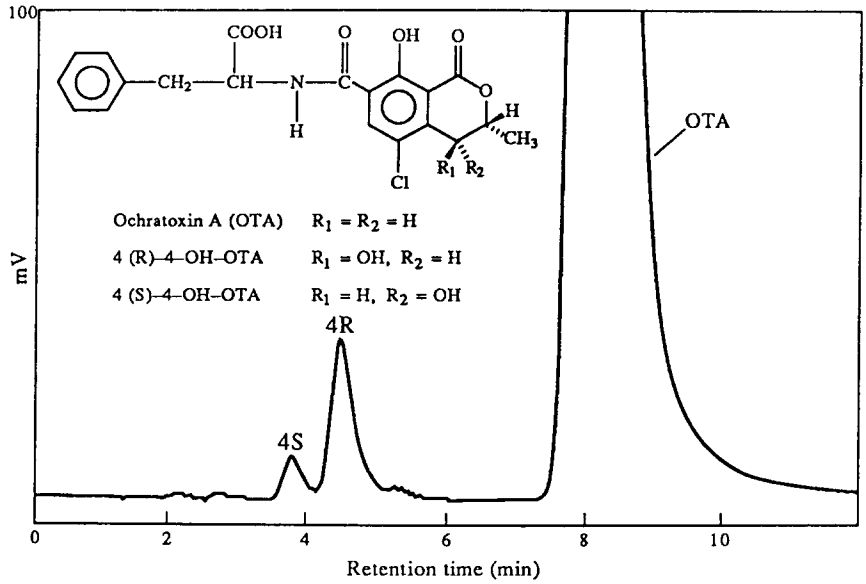

Fig. 1. Structures and HPLC profile of OTA and its metabolites. A typical HPLC separation profile of OTA and its metabolites following incubation of OTA with PCN-pretreated microsomes is shown. Details of the incubation, extraction and HPLC conditions are as described in Materials and Methods.

(TBA) were purchased from the Sigma Chemical Co. (St. Louis, MO). Desferal and *N,N*-diphenyl-1,4-phenylenediamine (DPPD) were obtained from Ciba-Geigy (Montreal, Quebec) and the Aldrich Chemical Co. (Milwaukee, WI), respectively. Linoleic acid (LAHP) was prepared and purified as described by O'Brien [15]. All other chemicals were of the highest grade commercially available.

Treatments of rats. Male Sprague–Dawley rats (200–250 g), obtained from Canadian Breeding Farms, Halifax, Nova Scotia, were used in all experiments. Animals were maintained on a 12-hr light and dark cycle and had free access to standard laboratory chow and water. INH was given as a 0.1% solution in drinking water for 10 days and rats were killed on day 11 [16]. PCN was administered by gastric intubation (100 mg/kg in 1 mL of 1% Tween 80) once daily for 4 days and rats were killed 24 hr after the last dose [17]. Liver microsomes were isolated by differential centrifugation of liver homogenates as described earlier [18]. Cytochrome P450 levels were measured as described by Omura and Sato [19]. Protein was measured by the method of Lowry *et al.* [20].

Incubations. Unless otherwise indicated, incubations were carried out in duplicate at 37° for 30 min in 0.1 M potassium phosphate buffer (pH 7.4) and contained in a total volume of 1 mL: 2 mg microsomal protein, 125 nmol OTA and an NADPH-regenerating system (consisting of 0.4 μmol $NADP^+$, 5 μmol $MgCl_2$, 5 μmol DL-isocitrate and 0.65 U of isocitric dehydrogenase). Catalase (800 U), SOD (35 U), mannitol (11 mM), BHA (10 μM), DPPD (10 μM), Desferal (50 μM) or BPS (100 μM), when included, were added prior to initiating the reaction with NADPH. At the end of 30 min, a 0.5-mL aliquot from each incubation was withdrawn for measurement of OTA metabolites and the remaining 0.5 mL was used to measure lipid peroxidation. Time-course studies were carried out in an identical manner except that the incubation volume was 10 mL and, at timed intervals, two 0.5-mL aliquots were withdrawn, one each for measurement of OTA metabolites and lipid peroxidation.

Incubations with hemoproteins/hydroperoxides were carried out in duplicate in 0.5 mL of 0.1 M potassium phosphate buffer (pH 7.4) and contained (i) OTA (62.5 nmol), (ii) hemoprotein-PCN-microsomes (1 mg; 1.2 nmol cytochrome P450) or hemoglobin (Hb) (640 μg; 10 nmol) or hematin (2.5 nmol) or HRP (400 μg; 10 nmol) and (iii) hydroperoxide-cumene hydroperoxide (CHP) (0.5 mM) or H_2O_2 (5 mM) or LAHP (65 μM). Incubations were terminated after 30 min and analyzed for OTA metabolites as described below.

Analysis of OTA metabolites. Aliquots (0.5 mL) were removed from incubations and the reaction was terminated by addition of 1 M HCl (0.1 mL) followed by saturated NaCl (0.5 mL). The mixtures were extracted with chloroform (2 × 2 mL), and the two chloroform extracts from each incubation were combined and dried under nitrogen. The residues were dissolved in 500 μL of methanol and 50 μL of each sample were analyzed by HPLC on a Partisil 10 ODS-2 column (0.45 cm × 25 cm) using a solvent system consisting of (i) a mixture of acetonitrile:methanol (1:1, v/v) 65% and (ii) 5 mM sodium acetate:acetic acid (500:14, v/v) 35%. The flow rate was 1.5 mL/min. Ochratoxin A and its metabolites were detected fluorimetrically with excitation at 340 nm and emission at 465 nm. (*S*)-4-OH-OTA, (*R*)-4-OH-OTA and OTA eluted at 3.8, 4.5 and 8.2 min, respectively. Metabolites were identified and quantitated using standards provided by Dr. M. Castegnaro, IARC, Lyon, France.

Table 1. Effects of INH- and PCN-pretreatments on hepatic cytochrome P450 levels, OTA metabolism and malondialdehyde formation

Pretreatment	Cyt. P450	(R)-4-OH-OTA (nmol/mg protein)	(S)-4-OH-OTA	MDA
None	0.85 ± 0.04	0.015 ± 0.004	0.014 ± 0.006	17.29 ± 1.21
INH	0.83 ± 0.07	0.022 ± 0.030 (1.5)	0.093 ± 0.031 (7)	35.49 ± 1.95
PCN	1.32 ± 0.09	0.816 ± 0.150 (55)	0.194 ± 0.040 (14)	31.32 ± 1.89

Pretreatment of rats and measurement of cytochrome P450 levels and OTA metabolites were carried out as described in Materials and Methods. Values are means ± SD of duplicate incubations with microsomes prepared from individual rat livers (N = 4 per treatment group). Numbers in parentheses represent fold-increase in metabolites in the two treatment groups relative to no pretreatment. The pretreatment regimen was repeated once, and results obtained were within 15% of those reported above.

Lipid peroxidation. Lipid peroxidation was estimated by measuring malondialdehyde (MDA) levels [11]. For this purpose, 0.5 mL of 30% trichloroacetic acid and 50 μL of butylated hydroxytoluene (2% in ethanol) were added to 0.5 mL of each incubation. Finally, 0.5 mL of 50 mM TBA was added and the mixtures were placed in a boiling water bath for 15 min. After centrifugation for 5 min at ~1500 *g* (lab top centrifuge), the absorbance of the MDA–TBA complex in the supernatant was read at 535 nm ($E_{535} = 156\ mM^{-1}\ cm^{-1}$) [21].

RESULTS

OTA metabolism by liver microsomes from untreated (control) rats was quite small giving rise to (*R*)-4-OH-OTA and (*S*)-4-OH-OTA levels that averaged 0.015 and 0.014 nmol/mg protein, respectively, over a 30-min incubation period (Table 1). PCN pretreatment elevated P450 levels by 55% and increased (*R*)-4-OH-OTA and (*S*)-4-OH-OTA formation 55- and 14-fold, respectively, over controls. In contrast, INH pretreatment did not alter substantially the levels of P450 or (*R*)-4-OH-OTA but increased (*S*)-4-OH-OTA levels by 7-fold over controls (Table 1). Lipid peroxidation, as measured by MDA levels, was also elevated 1.8- to 2-fold by both pretreatments. Figure 1 shows the structures of OTA, (*R*)-4-OH-OTA and (*S*)-4-OH-OTA together with a typical HPLC profile (showing their separation) from an incubation of OTA with PCN-microsomes.

Figure 2 shows the time course of OTA metabolism and lipid peroxidation using microsomes from INH-pretreated rats. There was very little (*R*)-4-OH-OTA formation which reached a maximum of 0.017 nmol/mg protein at 30 min. In contrast, the level of (*S*)-4-OH-OTA continued to increase steadily and was 5 times higher than that of (*R*)-4-OH-OTA reaching a maximum of 0.080 nmol/mg protein at 40 min, the final time point tested (Fig. 2A). In parallel measurements, MDA levels also increased steadily reaching 16 nmol/mg protein by 40 min (Fig. 2B). In contrast to INH-microsomes, PCN-microsomes efficiently catalyzed the formation of (*R*)-4-OH-OTA, the levels of which steadily increased to ~1 nmol/mg protein at 40 min, the final time point tested. (*S*)-4-OH-OTA levels also increased steadily with time to reach 0.17 nmol/mg protein by 40 min (Fig. 3A). As in the case of INH-microsomes, MDA levels also increased with time reaching 20.5 nmol/mg protein at 40 min (Fig. 3B).

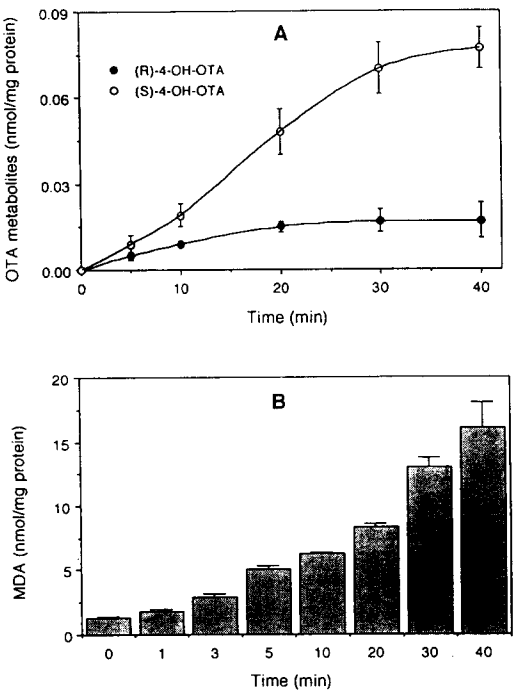

Fig. 2. Time course of OTA metabolism (A) and lipid peroxidation (B) by liver microsomes from INH-pretreated rats. Incubations were carried out at 37° in 0.1 M potassium phosphate buffer (pH 7.4) and contained per mL; microsomal protein (2 mg), 125 nmol OTA and an NADPH-regenerating system. At timed intervals, two 0.5-mL samples were withdrawn, one for HPLC analysis of OTA metabolites and the other for measurement of MDA levels. Details are described in Materials and Methods. Results are means ± SD of duplicate incubations from two separate experiments.

The pH optima for (*S*)-4-OH-OTA formation

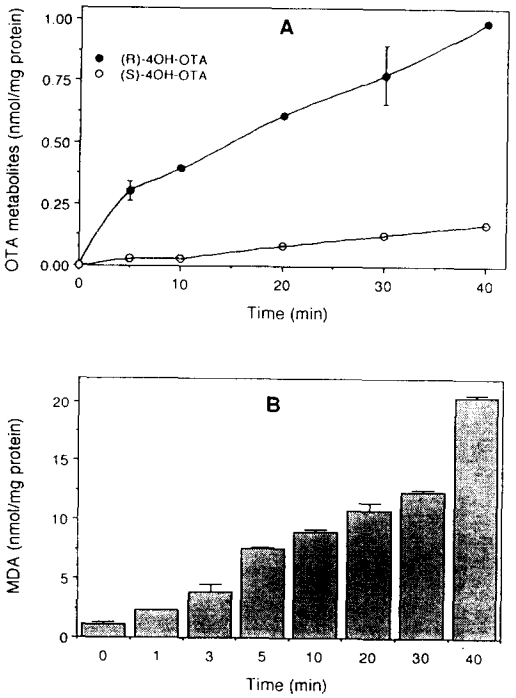

Fig. 3. Time course of OTA metabolism (A) and lipid peroxidation (B) by liver microsomes from PCN-pretreated rats. Incubations were carried out exactly as described in the legend of Fig. 2. Results are means ± SD of duplicate incubations from two separate experiments.

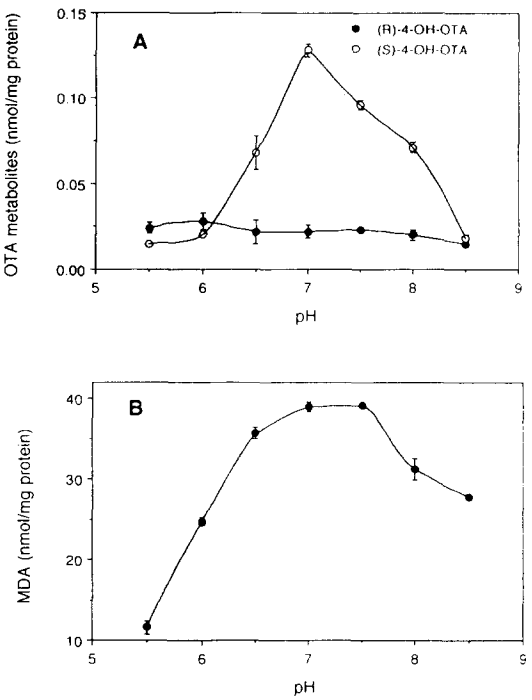

Fig. 4. Effect of pH on OTA metabolism (A) and lipid peroxidation (B) by liver microsomes from INH-pretreated rats. Incubations were carried out at 37° in 0.1 M potassium phosphate (pH 5.5 to 8.5) and contained in a total volume of 1 mL: microsomal protein (2 mg), 125 nmol OTA and an NADPH-regenerating system. At the end of 30 min, 0.5 mL from each incubation was withdrawn for HPLC analysis of OTA metabolites and the remaining 0.5 mL was used for measurement of MDA levels. Details are described in Materials and Methods section. Results are means ± SD of duplicate incubations from two separate experiments.

(Fig. 4A) and lipid peroxidation (Fig. 4B) by INH-microsomes were found to be 7.0 and 7.0 to 7.5, respectively. In contrast, (*R*)-4-OH-OTA did not show a distinct pH optimum, possibly because of low levels of formation. The corresponding pH optima for (*R*)-4-OH-OTA formation (Fig. 5A) and lipid peroxidation (Fig. 5B) with PCN-microsomes were 6.5 and 7.0 to 7.5, respectively. In this instance, no distinct pH optimum for (*S*)-4-OH-OTA formation was observed probably again due to very low yields (Fig. 5A).

Table 2 shows the effects of SOD, catalase, mannitol, BHA, DPPD, Desferal and BPS addition on OTA metabolism and lipid peroxidation by microsomes from INH- and PCN-pretreated rats. With INH-microsomes, (*S*)-4-OH-OTA formation was inhibited 100% by BHA, over 90% by DPPD, Desferal and BPS, ~30% by mannitol and ~15% by SOD and catalase. In contrast, none of these agents inhibited (*R*)-4-OH-OTA formation with the exception of DPPD which exerted a 40% inhibitory effect. In fact, both Desferal and BPS *increased* the yield of (*R*)-4-OH-OTA by 60–100%. Parallel determinations of lipid peroxidation indicated that BHA, DPPD, Desferal and BPS all inhibited MDA formation by >90%; mannitol was slightly inhibitory (15%) whereas catalase and SOD had no effect. When microsomes from PCN-pretreated rats were used, both antioxidants (BHA and DPPD) inhibited (*S*)-4-OH-OTA formation by 50%, whereas BPS exerted a 35% inhibitory effect but SOD, catalase and Desferal *increased* (*S*)-4-OH-OTA levels by 35, 50 and 30%, respectively. Mannitol was without effect. (*R*)-4-OH-OTA formation was not affected significantly except by catalase, Desferal and BPS, which increased it by 30, 100 and 170%, respectively. As expected, MDA formation was strongly inhibited by BHA, DPPD, Desferal and PBS (>90%), whereas both catalase and mannitol were mildly inhibitory (15–20%).

Figure 6 shows that various concentrations of BHA inhibited both (*S*)-4-OH-OTA formation and lipid peroxidation by INH-microsomes to roughly the same extent. Thus, 2.5 and 5 μM BHA were required to completely inhibit both (*S*)-4-OH-OTA (Fig. 6A) and MDA (Fig. 6B) at 10 and 30 min, respectively. Lower concentrations of BHA were correspondingly less inhibitory. In contrast, (*R*)-4-OH-OTA formation was not inhibited (data not shown). Incubation of INH- or PCN-microsomes with NADPH and OTA for 30 min resulted in the destruction of P450 by 40 and 75%, respectively. Inclusion of Desferal provided complete protection against P450 loss in the case of INH-microsomes and

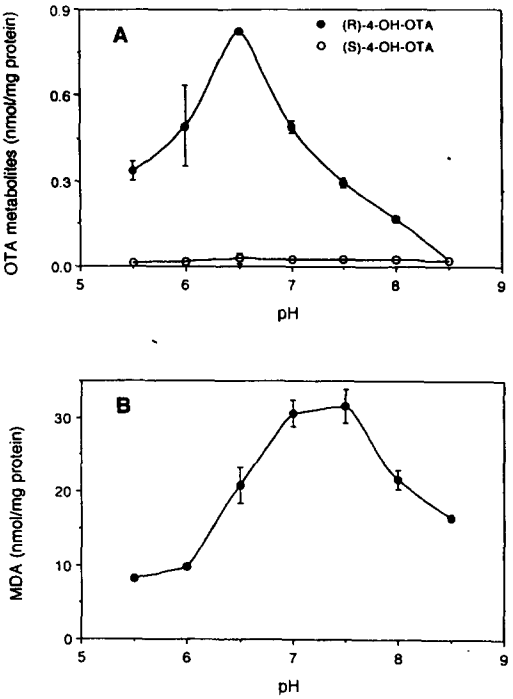

Fig. 5. Effect of pH on OTA metabolism (A) and lipid peroxidation (B) by liver microsomes from PCN-pretreated rats. Incubations were carried out exactly as described in the legend of Fig. 4. Results are means ± SD of duplicate incubations from two separate experiments.

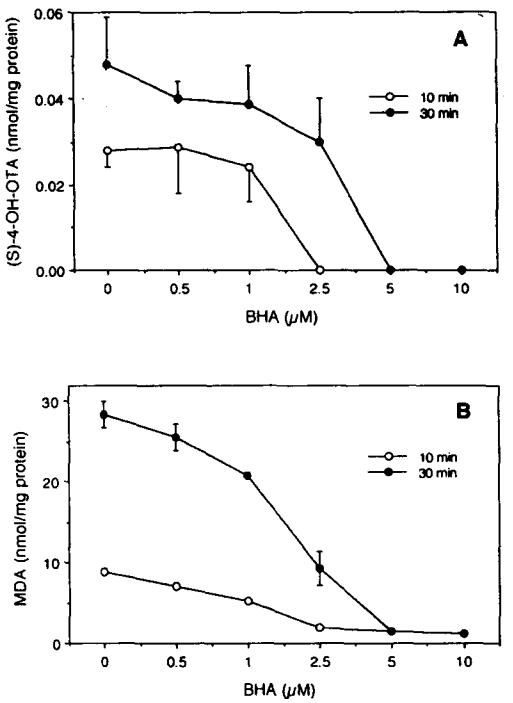

Fig. 6. Effect of various BHA concentrations on (A) (S)-4-OH-OTA metabolism and (B) lipid peroxidation by liver microsomes from INH-pretreated rats. Incubations were carried out for 10 and 30 min in the presence of 0–10 μM BHA exactly as described in the legend of Fig. 2. Details are given in Materials and Methods. Results are means ± SD of duplicate incubations from two separate experiments.

Table 2. Effects of active oxygen scavengers, antioxidants and iron chelators upon NADPH-dependent OTA metabolism and lipid peroxidation

Addition	(R)-4-OH-OTA (nmol/mg protein)	(S)-4-OH-OTA (nmol/mg protein)	MDA (nmol/mg protein)
INH-microsomes			
None	0.014 ± 0.003	0.100 ± 0.008	26.64 ± 0.34
SOD (35 U)	0.015 ± 0.004	0.085 ± 0.010*	26.83 ± 0.95
Catalase (800 U)	0.015 ± 0.004	0.087 ± 0.008*	25.38 ± 1.48
Mannitol (11 mM)	0.014 ± 0.003	0.071 ± 0.016‡	22.79 ± 0.06‡
BHA (10 μM)	0.013 ± 0.003	0.000†	1.99 ± 0.08†
DPPD (10 μM)	0.008 ± 0.004	0.011 ± 0.001†	0.70 ± 0.03†
Desferal (50 μM)	0.028 ± 0.004†	0.006 ± 0.002†	1.23 ± 0.11†
BPS (100 μM)	0.022 ± 0.004‡	0.008 ± 0.001†	0.85 ± 0.06†
PCN-microsomes			
None	0.67 ± 0.06	0.20 ± 0.02	23.91 ± 1.18
SOD (35 U)	0.72 ± 0.10	0.27 ± 0.04‡	21.18 ± 3.28
Catalase (800 U)	0.86 ± 0.12‡	0.31 ± 0.04†	20.67 ± 0.19‡
Mannitol (11 mM)	0.76 ± 0.10	0.24 ± 0.05	19.45 ± 0.48‡
BHA (10 μM)	0.67 ± 0.03	0.10 ± 0.02†	2.15 ± 0.34†
DPPD (10 μM)	0.76 ± 0.03	0.10 ± 0.01†	0.85 ± 0.05†
Desferal (50 μM)	1.35 ± 0.15†	0.26 ± 0.03‡	1.42 ± 0.31†
BPS (100 μM)	1.80 ± 0.09†	0.13 ± 0.01‡	1.05 ± 0.06†

Incubations were carried out for 30 min as described in Materials and Methods. Results are means ± SD of duplicate incubations from two separate experiments.

* $P < 0.05$.

† $P < 0.001$.

‡ $P < 0.01$.

Table 3. Protection of microsomal cytochrome P450 by Desferal during OTA metabolism

System	Cytochrome P450 (nmol/mg protein)	Loss (%)
INH-microsomes		
Prior to incubation	0.83	0
No addition	0.49	40
Desferal (50 μM)	0.83	0
PCN-microsomes		
Prior to incubation	1.32	0
No addition	0.33	75
Desferal (50 μM)	0.82	37

Incubations were carried out, and cytochrome P450 levels were determined as described in Materials and Methods. Desferal, when included, was added prior to the NADPH-regenerating system. In the case of "No addition," Desferal (50 μM) was added at the end of the incubation just prior to cytochrome P450 determination.

reduced the P450 loss from 75% to 37% in the case of PCN-microsomes (Table 3).

Replacement of NADPH by ascorbate/Fe^{2+} almost completely eliminated (*R*)-4-OH-OTA formation by PCN-microsomes but did not affect (*S*)-4-OH-OTA formation by either PCN- or INH-microsomes (Table 4). As expected, the inclusion of BHA strongly inhibited (*S*)-4-OH-OTA as well as MDA formation (>90%) by both types of microsomes. Addition of mannitol did not appreciably change the levels of (*S*)- and (*R*)-4-OH-OTA or MDA.

CHP could effectively replace NADPH in catalyzing (*R*)-4-OH-OTA but not (*S*)-4-OH-OTA formation by liver microsomes (Table 5). By comparison, H_2O_2 was much less effective while LAHP was totally ineffective. Very little or no OTA metabolism was seen with hemoglobin, hematin or HRP in combination with either CHP, H_2O_2 or LAHP (Table 5).

DISCUSSION

In these studies, we have examined the ability of microsomes from INH- and PCN-pretreated rats to metabolize OTA and to induce OTA-dependent lipid peroxidation. A variety of cytochrome P450 inducers including clofibrate, erythromycin, isosafrole, INH, 3-methylcholanthrene, phenobarbital and PCN were examined for OTA metabolism. From these, INH- and PCN-pretreated microsomes were selected for the present study because PCN pretreatment increased (*R*)-4-OH-OTA formation >50-fold over the control while INH-pretreatment did not substantially increase (*R*)-4-OH-OTA formation (Table 1). In contrast, both PCN and INH pretreatments increased (*S*)-4-OH-OTA formation 14- and 7-fold, respectively. Control microsomes were not used because of their relatively poor ability to catalyze OTA metabolism (Table 1). OTA hydroxylation activity and its inducibility appear to vary with the strain of rat used. Hietanen *et al.* [22] observed OTA 4-hydroxylation rates of ~2.5 and ~0.7 pmol/min/mg protein with liver microsomes from female Lewis and DA rats, respectively. Moreover, this activity was inducible ~2.3-fold in Lewis rats but ~15-fold in DA rats upon phenobarbital pretreatment. On the other hand, Stormer and Pedersen [23] found a very high rate of OTA 4-hydroxylation (92 pmol/min/mg protein) in liver microsomes from male Wistar rats and this activity was inducible 3.4-fold upon phenobarbital pretreatment. In the above studies, it is not indicated whether just (*R*)-4-OH-OTA (the major metabolite) or both epimers were measured. In comparison, we observed an OTA 4-hydroxylation rate of ~1 pmol/min/mg protein (Table 1; combined (*R*)- and (*S*)-metabolites) which is more in line with the values observed by Hietanen *et al.* [22]. In our hands, microsomes from Wistar rats were only about twice as efficient as those from Sprague–Dawley rats used in the present study (data not shown). The effects of INH and PCN pretreatments on OTA metabolism have not been examined previously. However, the inability of INH pretreatment to induce (*R*)-4-OH-OTA formation suggests that cytochrome P450 2E1, the major isoform induced by INH [24], is unable to catalyze its formation.

The formation of (*R*)-4-OH-OTA and (*S*)-4-OH-

Table 4. Effects of BHA and mannitol upon ascorbate-dependent OTA metabolism and lipid peroxidation

Addition	(*R*)-4-OH-OTA	(*S*)-4-OH-OTA (nmol/mg protein)	MDA
INH-microsomes			
None	0.023 ± 0.007	0.140 ± 0.022	34.50 ± 1.80
Mannitol (11 mM)	0.017 ± 0.004	0.167 ± 0.023	31.80 ± 3.20
BHA (10 μM)	0.017 ± 0.005	0.020 ± 0.011*	2.73 ± 0.32*
PCN-microsomes			
None	0.030 ± 0.001	0.133 ± 0.005	30.80 ± 3.70
Mannitol (11 mM)	0.029 ± 0.012	0.130 ± 0.011	27.60 ± 2.10
BHA (10 μM)	0.023 ± 0.004†	0.012 ± 0.006*	0.79 ± 0.03*

Incubations were carried out for 30 min as described in Materials and Methods. Results are means ± SD of duplicate incubations from two separate experiments.

* $P < 0.001$.

† $P < 0.01$.

Table 5. Ability of various hemoproteins/hydroperoxides to metabolize OTA

System	(R)-4-OH-OTA (nmol/nmol heme)	(S)-4-OH-OTA (nmol/nmol heme)
PCN microsomes/CHP	0.533 ± 0.003	0.028 ± 0.017
PCN microsomes/H_2O_2*	0.015 ± 0.001	0.013 ± 0.002
PCN microsomes/LAHP	0	0
Hb/CHP	<0.001	<0.001
Hb/H_2O_2	<0.001	<0.001
Hb/LAHP	0	0
HRP/CHP or H_2O_2 or LAHP	0	0
Hematin/CHP	0.006 ± 0.002	0.010 ± 0.005
Hematin/H_2O_2	0.007 ± 0.002	0.003 ± 0.001
Hematin/LAHP	0	0

Incubations were carried out for 30 min as described in Materials and Methods. Results are means ± SD of duplicate incubations from two separate experiments.
* Hydroxylamine (150 μM) was included to inhibit contaminating catalase.

OTA shows significant differences with respect to pH optima, effect of antioxidants and iron chelators. The pH optimum for (R)-4-OH-OTA formation by PCN microsomes was 6.5 and at pH 7.5 there was a 60% decrease in (R)-4-OH-OTA formation (Fig. 5). The pH optimum for cytochrome P450-dependent mixed-function oxidation of most substrates is around 7.5 so it is unusual that (R)-4-OH-OTA formation exhibits a low pH optimum. One possible explanation for this could be that at the higher pH of 7.5 there is increased lipid peroxidation (Fig. 5B) and a consequent greater destruction of cytochrome P450 which would lead to lower (R)-4-OH-OTA levels. In keeping with this, we observed a 75 and 50% destruction of cytochrome P450 after 30 min incubation of PCN-microsomes with OTA at pH 7.5 (Table 3) and 6.5 (data not shown), respectively. Similar results were also observed with microsomes isolated from isosafrole- and 3-methylcholanthrene-pretreated rats. Additionally, since the pK_a of the phenolic hydroxyl of OTA is 7.1 [25], it may be that OTA with an unionized hydroxyl binds preferentially to cytochrome P450, thus possibly yielding more (R)-4-OH-OTA below this pH. The pH optimum of 6.5 obtained by us for (R)-4-OH-OTA formation is the same as that observed by Ueno [26] for liver microsomes from 3-methylcholanthrene-induced rats but differs significantly from the value of 7.5 observed by Stormer and Pedersen [23] for liver microsomes from phenobarbital-pretreated Wistar rats. This difference in pH optima was not due to the different pretreatments since we observed the same pH optimum for (R)-4-OH-OTA formation with liver microsomes from phenobarbital- as well as 3-methylcholanthrene-pretreated rats (data not shown).

In contrast to (R)-4-OH-OTA, the pH optimum of 7.0 observed for (S)-4-OH-OTA formation with INH microsomes also coincided with the pH optimum of MDA formation in this system (Fig. 4), suggesting that the two processes may be linked. Furthermore, an intact mixed-function oxidase system is required for the formation of (R)-4-OH-OTA but not (S)-4-OH-OTA or lipid peroxidation since replacement of NADPH by ascorbate abolished the first but not the latter two. The ability of ascorbate to stimulate microsomal lipid peroxidation is well known [27]. A correlation between lipid peroxidation and (S)-4-OH-OTA formation is further confirmed by the finding that both processes [but not (R)-4-OH-OTA formation] are remarkably sensitive to inhibition by the antioxidants BHA and DPPD (Tables 2 and 4). In fact, in the presence of various concentrations of BHA, both lipid peroxidation and (S)-4-OH-OTA formation were inhibited in parallel (Fig. 6), suggesting that either lipid peroxides are responsible for (S)-4-OH-OTA formation or that a common species mediates the formation of both.

As expected, the iron chelators Desferal and BPS virtually eliminated both (S)-4-OH-OTA formation and lipid peroxidation by INH-microsomes. However, Desferal increased by 30% and BPS decreased by 35% (S)-4-OH-OTA formation by PCN-microsomes (Table 2). The reason for the differing actions of Desferal and BPS on (S)-4-OH-OTA formation is not apparent. Both Desferal and BPS also increased (R)-4-OH-OTA formation by 60–170% with both types of microsomes (Table 2). The induction of lipid peroxidation [28] and active oxygen species [29] by OTA can lead to the destruction of cytochrome P450 [28] which is responsible for (R)-4-OH-OTA formation. Desferal, by chelating iron, would prevent the formation of the active oxygen species responsible for lipid peroxidation and thus the destruction of cytochrome P450. This was indeed observed (Table 3). It is therefore likely that the protective effect of Desferal on cytochrome P450 is responsible for the elevated (R)-4-OH-OTA levels (Table 2). It is not readily apparent why Desferal increased (S)-4-OH-OTA formation with PCN-microsomes and dramatically decreased it with INH-microsomes.

The inability of microsomes to produce (S)-4-OH-OTA in the presence of LAHP suggests that lipid peroxides may not be involved (Table 5). In contrast, microsomes in the presence of CHP gave rise to substantial amounts of (R)-4-OH-OTA and also some (S)-4-OH-OTA (Table 5) although the latter was much less than that observed with NADPH (Table 2). Cytochrome P450 is known to function

as a peroxidase and the ability of CHP to catalyze cytochrome P450-dependent metabolism of xenobiotics has been well documented [30, 31]. A variety of hemoproteins including HRP [32, 33] and Hb [34] are also known to oxidize xenobiotics. However, these hemoproteins as well as hematin were unable to catalyze OTA oxidation in the presence of a variety of hydroperoxides, suggesting that cytochrome P450 is perhaps essential.

The lack of substantial inhibition by SOD or catalase suggests that the superoxide anion or H_2O_2 are not directly involved in (*S*)-4-OH-OTA or MDA formation by INH-microsomes. Both SOD and catalase increased OTA metabolism with PCN-microsomes possibly by protecting cytochrome P450 from radical-induced damage. Previously, we have shown [29] that the presence of OTA stimulated superoxide and hydroxyl radical formation by liver microsomes. Mannitol, a known scavenger of hydroxyl radicals, did inhibit (*S*)-4-OH-OTA by ~30% in the case of INH-microsomes indicating that hydroxyl radicals may play a role. However, mannitol did not inhibit either the NADPH-dependent (PCN-microsomes) or the ascorbate-dependent (INH- and PCN-microsomes) metabolism of OTA or lipid peroxidation, indicating that hydroxyl radicals are probably not involved in either of these processes. It is already known that hydroxyl radicals do not play a significant role in NADPH-dependent microsomal lipid peroxidation [35].

The fact that both Desferal and BPS inhibited (*S*)-4-OH-OTA and MDA formation (at least in INH-microsomes) but not (*R*)-4-OH-OTA formation suggests that free iron is involved in their formation. This, together with observations that cytochrome P450 is essential and that free (unbound) reactive oxygen species are probably not involved, suggests that an active Fe^{2+}–oxygen complex may be responsible for both (*S*)-4-OH-OTA formation and lipid peroxidation. The results are less clear with PCN-microsomes since BHA, DPPD, BPS and Desferal all strongly inhibited lipid peroxidation but only the first three partially inhibited (*S*)-4-OH-OTA formation. This suggests that with PCN-microsomes at least a part of (*S*)-4-OH-OTA may arise via the active Fe^{2+}–oxygen complex. The lack of (*S*)-4-OH-OTA formation with CHP would also be explained as free Fe^{2+} would be oxidized by the CHP.

The precise mechanism by which such a hydroxylation occurs is presently not known. We have already demonstrated [12] that OTA induced lipid peroxidation by chelating Fe^{3+} and that the resulting OTA–Fe^{3+} chelate is more readily reducible by the flavoprotein NADPH-cytochrome P450 reductase to the OTA–Fe^{2+} complex which, in the presence of oxygen, provides the active species that initiates lipid peroxidation. Cytochrome P450 is required presumably to facilitate the reduction of the OTA–Fe^{3+} complex to the OTA–Fe^{2+} complex [13]. The exact nature of the initiating species is presently not known and different investigators have implicated the perferryl ion [36, 37], the ferryl ion [38] and the Fe^{2+}–O_2–Fe^{3+} complex [39]. A similar species could also be responsible for (*S*)-4-OH-OTA formation. In keeping with this, we have observed that formation of both (*R*)- and (*S*)-4-OH-OTA is reduced by >90% in the absence of oxygen, suggesting that the hydroxyl oxygen in both enantiomers may come from molecular oxygen rather than water. However, absolute proof of this must await experiments using ^{18}O-labeled O_2 and H_2O. It is not clear why a small initiating species as suggested above would have the specificity to give rise to (*S*)-4-OH-OTA formation. Since OTA has been shown to bind iron [12], it is possible that the geometry of the OTA-bound active species influences the specificity of the reaction.

It is not known with certainty if OTA is toxic *per se* or requires metabolic activation prior to exerting its toxic effects. If cytochrome P450-dependent metabolism is primarily a detoxication mechanism, then induction of P450 by agents such as PCN should enhance OTA clearance and hence decrease its toxicity. On the other hand, if OTA is activated by cytochrome P450, then induction of the latter should enhance toxicity. The dramatic increase in OTA metabolism seen upon PCN or phenobarbital pretreatment of rats should allow us to distinguish between these two possibilities.

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REFERENCES

1. Krogh P and Nesheim S, Ochratoxin A, Introduction. In: *Environmental Carcinogens. Selected Methods of Analysis* (Eds. Stoloff L, Castegnaro M, Scott P, O'Neill IK and Bartsch H), Vol. 5, pp. 247–259. IARC Scientific Publications, No. 44, Lyon, 1982.
2. Harwig J, Ochratoxin A and related metabolites. In: *Mycotoxins* (Ed. Purchase IFH), pp. 345–368. Elsevier, Amsterdam, 1974.
3. Purchase IFH and Theron JJ, The acute toxicity of ochratoxin A to rats. *Food Cosmet Toxicol* **6**: 479–483, 1968.
4. Peckham JC, Doupnik B Jr and Jones OH Jr, Acute toxicity of ochratoxin A and B in chicks. *Appl Microbiol* **21**: 492–494, 1971.
5. Krogh P, Ochratoxins. In: *Mycotoxins in Human and Animal Health* (Eds. Rodricks JV, Hesseltine CW and Mehlman MA), pp. 489–498. Pathotox Publishers, New York, 1978.
6. Bendele AM, Carlton WW, Krogh P and Lillehoj EB, Ochratoxin A carcinogenesis in the (C57BL/6JXC3H)F1 mouse. *J Natl Cancer Inst* **75**: 733–739, 1985.
7. Boorman G, *NTP Technical Report on the Toxicology and Carcinogenesis Studies of Ochratoxin A*. NTP TR358, NIH Publication No. 88-2813. Research Triangle Park, NC, 1988.
8. Krogh P, Mycotoxic porcine nephropathy—a possible model for Balkan (endemic) nephropathy. In: *Proceedings of the Second International Symposium on Endemic Nephropathy, Sofia* (Ed. Pulchev A), pp. 266–270. Bulgarian Academy of Sciences, Sofia, 1974.
9. Hult K, Plestina R, Habazin-Novak V, Radic B and Ceovic S, Ochratoxin A in human blood and Balkan endemic nephropathy. *Arch Toxicol* **51**: 313–321, 1982.
10. Petkova-Bocharova T, Chernozemsky IN and Castegnaro M, Ochratoxin A in human blood in relation to Balkan endemic nephropathy and urinary system

tumors in Bulgaria. *Food Addit Contam* **5**: 299–301, 1988.
11. Rahimtula AD, Bereziat J-C, Busscchini-Griot V and Bartsch H, Lipid peroxidation as a possible cause of ochratoxin A toxicity. *Biochem Pharmacol* **37**: 4469–4477, 1988.
12. Omar RF, Hasinoff BB, Mejilla F and Rahimtula AD, Mechanism of ochratoxin A stimulated lipid peroxidation. *Biochem Pharmacol* **40**: 1183–1191, 1990.
13. Omar RF, Rahimtula AD and Bartsch H, Role of cytochrome P-450 in ochratoxin A-stimulated lipid peroxidation. *J Biochem Toxicol* **6**: 203–209, 1991.
14. Stormer FC, Hansen CE, Pederson JI, Hvistenhahl G and Aasen AJ, Formation of 4(*R*)- and 4(*S*)-4-hydroxyochratoxin A from ochratoxin A by liver microsomes from various species. *Appl Environ Microbiol* **42**: 1051–1056, 1981.
15. O'Brien PJ, Intracellular mechanisms for the decomposition of a lipid peroxide. I. Decomposition of a lipid peroxide by metal ions, heme compounds, and nucleophiles. *Can J Biochem* **47**: 485–492, 1969.
16. Ryan DE, Ramanathan L, Iida S, Thomas PE, Haniu M, Shively JE, Lieber CS and Levin W, Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. *J Biol Chem* **260**: 6385–6393, 1985.
17. Graves PE, Kaminsky LS and Halpert J, Evidence for functional and structural multiplicity of pregnenolone-16α-carbonitrile-inducible cytochrome P-450 isozymes in rat liver microsomes. *Biochemistry* **26**: 3887–3894, 1987.
18. Rahimtula AD, Zachariah PK and O'Brien PJ, Differential effects of antioxidants, steroids and other compounds on benzo(a)pyrene 3-hydroxylase activity in various tissues of rat. *Br J Cancer* **40**: 105–112, 1979.
19. Omura T and Sato R, The carbon monoxide-binding spectrum of liver microsomes. *J Biol Chem* **239**: 2379–2384, 1964.
20. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
21. Jordan RA and Schenkman JB, Relationship between malondialdehyde production and arachidonate consumption during NADPH-supported microsomal lipid peroxidation. *Biochem Pharmacol* **31**: 1393–1400, 1982.
22. Hietanen E, Malaveille C, Camus A-M, Bérézíat J-C, Brun G, Castegnaro M, Michelon J, Idle JR and Bartsch H, Interstrain comparison of hepatic and renal microsomal carcinogen metabolism and liver S9-mediated mutagenicity in DA and Lewis rats phenotyped as poor and extensive metabolizers of debrisoquine. *Drug Metab Dispos* **14**: 118–126, 1986.
23. Stormer FC and Pedersen JI, Formation of 4-hydroxyochratoxin A by rat liver microsomes. *Appl Environ Microbiol* **39**: 971–975, 1980.
24. Yang CS, Yoo J-SH, Ishizaki H and Hong J, Cytochrome P-450IIE1: Roles in nitrosamine metabolism and mechanisms of regulation. *Drug Metab Rev* **22**: 147–159, 1990.
25. Chu FS, Interaction of ochratoxin A with bovine serum albumin. *Arch Biochem Biophys* **147**: 359–366, 1971.
26. Ueno Y, Biotransformation of mycotoxins in the reconstituted cytochrome P-450 system. *Proc Jpn Assoc Mycotoxicol* **22**: 28–30, 1985.
27. Ernster L and Nordenbrand K, Microsomal lipid peroxidation: Mechanism and some biomedical implications. In: *Lipid Peroxides in Biology and Medicine* (Ed. Yagi K), pp. 55–79. Academic Press, New York, 1982.
28. Khan S, Martin M, Bartsch H and Rahimtula AD, Perturbation of liver microsomal calcium homeostasis by ochratoxin A. *Biochem Pharmacol* **38**: 67–72, 1989.
29. Hasinoff BB, Rahimtula AD and Omar RF, NADPH-cytochrome-P-450 reductase promoted hydroxyl radical production by the iron (III)–ochratoxin A complex. *Biochim Biophys Acta* **1036**: 78–81, 1990.
30. Rahimtula AD and O'Brien PJ, Hydroperoxide catalyzed liver microsomal aromatic hydroxylation reactions involving cytochrome P-450. *Biochem Biophys Res Commun* **60**: 440–447, 1974.
31. Rahimtula AD and O'Brien PJ, Hydroperoxide dependent O-dealkylation reactions catalyzed by liver microsomal cytochrome P-450. *Biochem Biophys Res Commun* **62**: 268–275, 1975.
32. Corbett MD and Corbett BR, HRP-catalyzed bioactivation of carcinogenic hydroxamic acids. The greater reactivity of glycolyl- versus acetyl-derived hydroxamic acids. *Chem Biol Interact* **63**: 249–264, 1987.
33. O'Brien PJ, Radical formation during the peroxidase catalyzed metabolism of carcinogens and xenobiotics: The reactivity of these radicals with GSH, DNA, and unsaturated lipid. *Free Rad Biol Med* **4**: 169–183, 1988.
34. Catalano CE and Ortiz de Montellano PR, Oxene transfer, electron abstraction and cooxidation in the epoxidation of stilbene and 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by hemoglobin. *Biochemistry* **26**: 8373–8380, 1987.
35. Gutteridge JMC, The role of superoxide and hydroxyl radicals in phospholipid peroxidation catalyzed by iron salts. *FEBS Lett* **150**: 454–458, 1982.
36. Tien M, Svingen BA and Aust SD, Initiation of lipid peroxidation by perferryl complexes. In: *Oxygen and Oxy-radicals in Chemistry and Biology* (Eds. Rodgers MAJ and Powell EL), pp. 147–152. Academic Press, New York, 1981.
37. Ursini F, Maiorino M, Hochstein P and Ernster P, Microsomal lipid peroxidation: Mechanisms of initiation. The role of iron chelators. *Free Radic Biol Med* **6**: 31–36, 1989.
38. Koppenol WH, The reaction of ferrous-EDTA with hydrogen peroxide: Evidence against hydroxyl radical formation. *J Free Radic Biol Med* **1**: 281–285, 1985.
39. Bucher JR, Tien M and Aust SD, The requirement for ferric in the initiation of lipid peroxidation by chelated ferrous iron. *Biochem Biophys Res Commun* **111**: 777–784, 1983.