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EVALUATION OF MICROSOMAL PATHWAYS OF OXIDATION OF ALCOHOLS AND HYDROXYL RADICAL SCAVENGING AGENTS WITH CARBON MONOXIDE AND COBALT PROTOPORPHYRIN IX*

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Abstract—Rat liver microsomes catalyze the oxidation of hydroxyl radical scavenging agents by an iron-dependent process, and can oxidize alcohols by pathways dependent on, as well as independent of, ·OH. Experiments were carried out to evaluate which microsomal components participate in the production of ·OH, and in the two pathways of oxidation of alcohols. Cobalt protoporphyrin IX treatment of rats resulted in a decrease in microsomal oxidation of aminopyrine, ·OH scavengers, and alcohols. However, this treatment not only lowered the content of cytochrome P-450, but also decreased the activity of NADPH-cytochrome P-450 reductase. Carbon monoxide, metyrapone and SKF-525A also inhibited the oxidation of aminopyrine but did not affect oxidation of ·OH scavengers. Desferrioxamine, a potent iron chelator, inhibited the oxidation of ·OH scavengers but not aminopyrine. The oxidation of alcohols was partly sensitive to desferrioxamine and partly sensitive to carbon monoxide, thus showing similarities to the oxidation of ·OH scavengers and drugs. These results suggest that the desferrioxamine-sensitive, ·OH-dependent pathway of alcohol oxidation is mediated by the reductase, in analogy to results with ·OH scavengers, whereas the desferrioxamine-resistant pathway of alcohol oxidation is mediated by cytochrome P-450, in analogy to results with aminopyrine. By the use of desferrioxamine or carbon monoxide, either of the two alcohol-oxidizing pathways can be inhibited independently of each other.

Isolated rat liver microsomes can catalyze the oxidation of hydroxyl radical (·OH§) scavenging agents during NADPH-dependent electron transfer [1–3]. Azide, an inhibitor of catalase, stimulates the oxidation of ·OH scavengers which suggests that H_2O_2 may serve as the precursor of ·OH [1]. The addition of iron-EDTA or EDTA itself stimulates the oxidation of the ·OH scavenging agents, whereas the potent iron chelating agent, desferrioxamine, inhibits the oxidation of the scavengers [4, 5]. The generation of ·OH appears to involve a Fenton-type reaction between H_2O_2, produced by microsomal electron transfer, and ferrous-EDTA. Reduction of ferric iron to the ferrous state may occur either by microsomal components, e.g. NADPH-cytochrome P-450 reductase, or via superoxide anion, which is produced during microsomal electron transport [6–9].

The ability of microsomes to oxidize alcohols represents a minor pathway of ethanol metabolism [10]. Recent experiments have shown that two pathways have the potential to play a role in the microsomal system for oxidation of alcohols. One pathway appears to involve the interaction of alcohols with ·OH generated during microsomal electron transfer, whereas the other pathway appears to involve a cytochrome P-450 catalyzed reaction which is independent of oxygen radicals [4, 11, 12]. In experiments that utilize NADPH-cytochrome *c* reductase and cytochrome P-450 purified from phenobarbital-treated rats, oxidation of ·OH scavengers occurs with the reductase system alone, and there is no effect upon addition of cytochrome P-450 [phenobarbital isozyme(s)] to the reductase system [11]. With ethanol as substrate, although there is oxidation by the reductase system alone, the addition of cytochrome P-450 increases the rate of ethanol oxidation by 2- to 3-fold [12].

The above experiments suggest that, in reconstituted systems, the reductase could serve as a locus for the production of ·OH. The current report concerns experiments carried out to evaluate whether, in intact microsomes, the reductase was responsible for the production of ·OH, since it is possible that uncoupling of the reductase from efficient electron transfer to cytochrome P-450 occurs during purification. In addition, the ratio of reductase to cyto-

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§ Abbreviations: ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; Me_2SO, dimethyl sulfoxide; KTBA, 2-keto-4-thiomethylbutyric acid; cobalt IX, cobalt protoporphyrin IX; and $O_2^{\cdot}$, superoxide anion radical.

chrome P-450 used in the purified systems was different than the ratio found in intact microsomes and, in general, decomposition of an oxygenated cytochrome P-450 complex is usually implicated as the major source of $O_2^{\cdot}$ in microsomal systems [9, 13]. Experiments were carried out with rats treated with cobalt protoporphyrin IX (cobalt IX) since it has been reported recently that this treatment markedly reduces the content of rat liver microsomal cytochrome P-450 [14]. In other experiments, the effects of carbon monoxide and other inhibitors of cytochrome P-450 catalyzed reactions on the oxidation of alcohols, ·OH scavengers, and aminopyrine (as a representative drug substrate) were evaluated.

MATERIALS AND METHODS

Rat liver microsomes were prepared from male, Sprague–Dawley rats (200–250 g body wt) as previously described [4]. In some experiments, microsomes were prepared from rats that had been injected with cobalt IX (125 μg/kg body wt) 72 hr prior to the time of sacrifice as described by Drummond and Kappas [14], except that KOH was used instead of NaOH to dissolve the cobalt IX. Control rats received the saline vehicle 72 hr prior to being killed. In all cases, rats were starved overnight. The microsomes were washed once and suspended in 125 mM KCl. Protein was determined by the method of Lowry *et al.* [15]. The content of cytochrome P-450 was determined as described by Omura and Sato [16], while the activity of NADPH-cytochrome *c* reductase was assayed at 23° as described by Phillips and Langdon [17].

The oxidation of the substrates was assayed at 37° in 25-ml Erlenmeyer flasks containing 100 mM potassium phosphate, pH 7.4, 10 mM sodium pyrophosphate, 10 mM $MgCl_2$, 10 mM glucose-6-phosphate, 2.3 units of glucose-6-phosphate dehydrogenase, 0.4 mM $NADP^+$, 0.1 mM EDTA, 1.0 mM azide, and about 2.5 mg of microsomal protein in a final volume of 1.0 ml. EDTA was added to promote the production of ·OH by the microsomes [5], while azide was added to inhibit catalase activity of isolated microsomes. Final concentration of substrates was: ethanol, 50 mM; 2-butanol, 33 mM; Me_2SO, 33 mM; 2-keto-4-thiomethylbutyric acid (KTBA), 10 mM; and aminopyrine, 5 mM. The reactions were initiated by the addition of glucose-6-phosphate plus glucose-6-phosphate dehydrogenase and were terminated by the addition of HCl (final concentration of 300 mM) for the experiments with ethanol, 2-butanol and KTBA or by trichloroacetic acid (final concentration of 4.5%, w/v) for the experiments with Me_2SO or aminopyrine. In some experiments, cumene hydroperoxide (final concentration of 1 mM) was used in place of the NADPH-generating system to catalyze the oxidation of aminopyrine or the alcohols. The production of acetaldehyde from ethanol, of 2-butanone from 2-butanol, of ethylene from KTBA, and of formaldehyde from either Me_2SO or aminopyrine was determined by methods previously described [4]. All values were corrected for zero-time controls in which acid was added before the addition of microsomes.

For those experiments which involved the addition of carbon monoxide or nitrogen, the procedure utilized was as follows. The flasks were sealed with serum stoppers, 10 cc of air was removed with a syringe, and then 10 cc of either CO or N_2 was added via syringe. Reactions were initiated and terminated via injections made through the serum stopper.

All chemicals were of the highest grade available. The buffers (except $MgCl_2$) were passed through a Chelex-100 column (Bio-Rad Laboratories, Richmond, CA) to remove contaminating iron. Desferrioxamine (Desferal) was obtained from the CIBA Pharmaceutical Co. (Summit, NH). All values refer to mean ± S.E.M. Statistical analysis was performed by Student's *t*-test.

RESULTS

Experiments with cobalt protoporphyrin IX. The effect of cobalt IX on the NADPH-dependent microsomal oxidation of various substrates is shown in Table 1. Among the substrates tested, aminopyrine was utilized as a typical drug substrate, ethanol and 2-butanol as substrates for the microsomal alcohol oxidizing system, and Me_2SO and KTBA as typical ·OH scavengers. The oxidation of aminopyrine was depressed 80% after the cobalt IX treatment, which is consistent with the observation that an acute dose of cobalt IX decreases the activity of ethylmorphine demethylase and aniline hydroxylase by 88 and 63% respectively [14]. In an analogous manner, the microsomal oxidation of alcohols was decreased by 70% after cobalt IX treatment (Table 1). The oxidation of Me_2SO to formaldehyde or of KTBA to ethylene gas was decreased by about 60% after the cobalt IX treatment (Table 1). Hence, the cobalt IX treatment produced a corresponding decrease in the oxidation of drugs, alcohols and ·OH scavengers.

The above experiments were repeated in the presence of desferrioxamine, an iron chelator which decreases the generation of ·OH by a variety of systems [18–20] including the microsomes [4]. Table 1 shows that desferrioxamine did not block drug (aminopyrine) oxidation under conditions in which the oxidation of ·OH scavengers (Me_2SO and KTBA) was decreased by 90%. The microsomal oxidation of alcohols was partly (40–65% inhibition) sensitive to desferrioxamine (Table 1), suggesting that the alcohol oxidation pathway involved both a desferrioxamine-sensitive, i.e. an ·OH-dependent component and a desferrioxamine-insensitive, i.e. an ·OH-independent component. The latter may be similar to the typical mixed function oxidase system which oxidizes drugs. Indeed, the rates of oxidation of aminopyrine, ethanol and 2-butanol in the presence of desferrioxamine were inhibited to similar extents after cobalt IX treatment (Table 1).

Subtraction of the desferrioxamine-insensitive rates of oxidation of substrates from the total rates of oxidation in the absence of desferrioxamine yields the ·OH-dependent oxidation rates of the substrates. These values are shown in Table 1, experiment C. There was little oxidation (10%) of aminopyrine by the ·OH-dependent pathway, whereas Me_2SO and KTBA were oxidized only (90%) by the ·OH pathway. Alcohols (40–60%) were oxidized in part by the ·OH pathway. The ·OH-dependent oxidation of

Table 1. Effect of cobalt protoporphyrin IX on the oxidation of various substrates in the absence and presence of desferrioxamine*

Experiment	Substrate	Rate of product formation (nmoles/min/mg microsomal protein)		Inhibition %
		Saline	Cobalt IX	
(A) Total activity	Aminopyrine	4.9 ± 0.6	0.9 ± 0.2	82†
	Ethanol	19.1 ± 3.3	6.2 ± 0.5	68†
	2-Butanol	20.5 ± 2.9	6.0 ± 0.7	71†
	Me_2SO	2.4 ± 0.5	1.0 ± 0.1	58‡
	KTBA	2.3 ± 0.4	1.0 ± 0.1	57§
(B) Desferrioxamine	Aminopyrine	4.4 ± 0.5	0.7 ± 0.1	84†
	Ethanol	6.7 ± 0.9	2.3 ± 0.2	66†
	2-Butanol	12.3 ± 1.0	2.8 ± 0.5	77†
	Me_2SO	0.2 ± 0	0.2 ± 0	
	KTBA	0.2 ± 0	0.1 ± 0	
(C) ·OH-dependent	Aminopyrine	0.5 ± 0.2	0.2 ± 0.1	60
	Ethanol	12.4 ± 2.6	3.9 ± 0.4	69§
	2-Butanol	8.2 ± 2.0	3.2 ± 0.5	61§
	Me_2SO	2.2 ± 0.4	0.8 ± 0.1	64§
	KTBA	2.1 ± 0.1	0.9 ± 0.1	57§

* The oxidation of 5 mM aminopyrine, 50 mM ethanol, 33 mM 2-butanol, 33 mM Me_2SO and 10 mM KTBA by microsomes isolated from rats treated with cobalt IX, or from saline controls, was assayed as described in Materials and Methods. Experiment B refers to experiments in which 0.25 mM desferrioxamine was present. Results in experiment C were calculated by subtracting the values in experiment B from the corresponding values found in experiment A (no desferrioxamine present). Results are from six or seven pairs of rats.
† $P < 0.001$.
‡ $P < 0.02$.
§ $P < 0.01$.

the alcohols and the ·OH scavengers was decreased significantly by the cobalt IX treatment (Table 1).

Cumene hydroperoxide can support the cytochrome P-450-dependent oxidation of drugs and alcohols in the absence of NADPH, molecular oxygen and NADPH-cytochrome P-450 reductase [21, 22]. Cumene hydroperoxide did not support the oxidation of ·OH scavengers nor was there any role for ·OH in the cumene hydroperoxide-supported oxidation of alcohols [23]. Table 2 shows that the cumene hydroperoxide-dependent oxidation of aminopyrine, ethanol and 2-butanol was decreased after cobalt IX treatment.

Taken as a whole, the above results suggest that the cobalt IX treatment resulted in a decrease in both the cytochrome P-450 as well as the ·OH-dependent oxidation of alcohols, and produced a significant decrease in the production of ·OH by the microsomes. In view of the decreased oxidation of ·OH scavengers, it seemed possible that the cobalt IX treatment could affect the activity of the reductase, in addition to lowering the content of cytochrome P-450. Table 3 confirms that the cobalt IX treatment lowers the content of cytochrome P-450 [14]. In addition, it can be seen that the activity of the reductase was also greatly diminished after cobalt IX treatment (Table 3).

Experiments with carbon monoxide. To determine if the cytochrome P-450 pathway of oxidation of alcohols could be inhibited without affecting the

Table 2. Effect of cobalt protoporphyrin IX on the cumene hydroperoxide-dependent oxidation of aminopyrine and alcohols*

Substrate	Rate of product formation (nmoles/min/mg microsomal protein)		Inhibition (%)
	Saline	Cobalt-IX	
Aminopyrine	3.3 ± 0.8	0.8 ± 0.3	76†
Ethanol	3.3 ± 0.6	1.6 ± 0.4	52†
[illegible]anol	5.9 ± 0.9	2.4 ± 1.0	59†

[illegible]idation of aminopyrine, ethanol and 2-butanol was assayed as [illegible]n Materials and Methods. Reactions were initiated by the oxidation [illegible]e hydroperoxide in place of the NADPH-generating system. Results [illegible]a seven pairs of rats.
[illegible] < 0.001.

Table 3. Effect of cobalt protoporphyrin IX on the content of cytochrome P-450 and activity of NADPH-cytochrome *c* reductase*

Treatment	Content of cytochrome P-450 (nmoles/mg protein)	Activity of NADPH-cytochrome *c* reductase (nmoles/min/mg protein)
Saline	1.21	104 ± 15
Cobalt IX	0.26 ± 0.02	46 ± 6

* The enzymatic assays were carried out as described in Materials and Methods.

·OH pathway, experiments with inhibitors of cytochrome P-450 catalyzed reactions were carried out. CO is a classic inhibitor of drug metabolism and it was considered that addition of CO may inhibit the cytochrome P-450-dependent oxidation of alcohols, but perhaps not the ·OH-dependent pathway. Microsomal oxidation of a variety of alcohols was inhibited by 40–50% in an atmosphere containing $CO:O_2$ at a ratio of 10:1 [24], whereas NADPH-dependent H_2O_2 generation was inhibited 35% at a $CO:O_2$ ratio of 9:1 [25]. Because oxygen is required for the formation of radicals such as $O_2^{\cdot-}$ and ·OH, it was important to avoid anaerobic conditions. Reactions were thus studied under a 30% CO/70% air atmosphere ($CO:O_2$ of 2:1). In addition, for comparative purposes, assays were also carried out under a 30% N_2/70% air atmosphere. Table 4 shows that all substrates tested were oxidized to the same extent, whether they were incubated under a 30% N_2/70% air mixture or if they were incubated under 100% air (controls). The oxidation of aminopyrine was decreased 47% by the 30% CO:70% air mixture, while the oxidation of Me_2SO and KTBA was not affected by CO (Table 4). A trend towards some inhibition by CO against the oxidation of alcohols was observed (Table 4). Previous experiments [26] have suggested that 2-butanol, relative to ethanol, appears to be a particularly effective substrate for the cytochrome P-450-dependent oxidation of alcohols; this may contribute to the greater sensitivity of 2-butanol oxidation to CO.

Since the generation of ·OH, as reflected by the oxidation of Me_2SO or KTBA, was not affected by CO, the small effect by CO (at this $CO:O_2$ ratio of 2) on the oxidation of alcohols could reflect the possibility that the alcohols were being oxidized to a large extent by the ·OH-dependent pathway. The experiments with desferrioxamine indicated that about 60% of ethanol oxidation and 40% of 2-butanol oxidation appeared to be ·OH dependent (Table 1). Since CO produced 45% inhibition of aminopyrine oxidation, it can be calculated that the oxidation of ethanol could be inhibited by about 18% by CO (0.4 × 0.45) while the oxidation of 2-butanol could be inhibited by about 27% by CO (0.6 × 0.45). The observed inhibition by CO was 19% for ethanol and 30% for 2-butanol (Table 4). To study the effect of CO on the cytochrome P-450 pathway of alcohol

Table 4. Effect of carbon monoxide on the oxidation of various substrates in the absence and presence of desferrioxamine*

Experiment	Substrate	Rate of product formation (nmoles/min/mg microsomal protein)			Effect of CO (%)
		Air	N_2	CO	
(A) Total activity	Aminopyrine	6.5 ± 0.6	6.8 ± 0.6	3.6 ± 0.2	−47†
	Ethanol	10.3 ± 1.3	10.3 ± 0.9	8.3 ± 1.1	−19
	2-Butanol	14.2 ± 2.1	13.2 ± 2.0	9.3 ± 1.8	−30
	Me_2SO	2.1 ± 0.4	1.8 ± 0.3	2.1 ± 0.5	+17
	KTBA	1.7 ± 0.3	1.6 ± 0.3	1.4 ± 0.2	−12
(B) Desferrioxamine	Aminopyrine	6.3 ± 0.6	6.4 ± 0.5	3.3 ± 1.8	−48†
	Ethanol	5.3 ± 0.6	5.5 ± 0.5	3.5 ± 1.5	−36‡
	2-Butanol	10.8 ± 0.2	8.3 ± 0.4	4.6 ± 0.9	−45‡
(C) ·OH-dependent	Aminopyrine	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	−25
	Ethanol	5.0 ± 0.9	4.8 ± 0.4	4.8 ± 0.9	0
	2-Butanol	3.4 ± 0.6	4.9 ± 0.9	4.7 ± 0.8	−4

* The oxidation of the various substrates was assayed as described in Materials and Methods, in the presence of 100% air, or a 70% air–30% N_2 mixture, or a 70% air:30% CO mixture. Experiment B reflects experiments in which 0.25 mM desferrioxamine was present. Results in experiment C were calculated by subtracting the values in experiment B from the corresponding values found in experiment A (no desferrioxamine present). Results are from five to seven experiments.

† $P < 0.001$.

‡ $P < 0.05$, as compared to N_2:air mixture

oxidation, the experiments were repeated in the presence of desferrioxamine. Table 4, experiment B, shows that the oxidation of ethanol and 2-butanol was now as sensitive to CO as was the oxidation of aminopyrine. Thus, the cytochrome P-450, but not the ·OH-dependent pathway of alcohol oxidation, was inhibited by CO. Subtraction of the desferrioxamine-resistant rates of alcohol oxidation from the total rates of oxidation confirmed that the ·OH-dependent oxidation of alcohols was not inhibited by CO (Table 4, experiment C).

Two other inhibitors of certain cytochrome P-450-catalyzed reactions were also studied, metyrapone and SKF-525A. Table 5 shows that the oxidation of aminopyrine was inhibited by either metyrapone or SKF-525A. However, similar to results with CO, the oxidation of ·OH scavengers was not affected by these inhibitors (Table 5). Several other compounds were tested; the addition of 0.5 mM *p*-chloromercuribenzoate produced > 80% inhibition of the oxidation of aminopyrine, ·OH scavengers and ethanol. Disruption of the microsomal membrane by either cholate or deoxycholate also resulted in >80% inhibition of all the substrates (data not shown).

DISCUSSION

Rat liver microsomes can catalyze the oxidation of ·OH scavengers and alcohols during NADPH-dependent electron transfer [1–3]. Experiments carried out with azide, added H_2O_2, ferric-EDTA, and desferrioxamine suggested that a Fenton reaction between ferrous-EDTA and H_2O_2 could produce the ·OH that subsequently oxidizes ·OH scavengers and alcohols. Microsomal electron transfer was responsible for the production of H_2O_2 and for the reduction of iron to the ferrous state. Microsomal components involved in the production of superoxide and therefore H_2O_2 may include NADPH-cytochrome *c* (P-450) reductase or cytochrome P-450 [6–8, 13, 27, 28]. Iron could be reduced to the ferrous state either by $O_2^{\cdot-}$ or directly by microsomal enzymes such as the reductase. Although experiments with purified reconstituted systems showed that the reductase by itself could promote the oxidation of ·OH scavengers [11, 12], it was necessary to determine which microsomal components play a role in the oxidation of ·OH scavengers by intact microsomes, and in the two pathways responsible for the oxidation of alcohols.

The oxidation of typical ·OH scavengers is insensitive to CO treatment. A rather low ratio of CO:O_2 (2:1) was employed in these experiments rather than the 5:1 or 10:1 ratios usually employed, in order to avoid anaerobiosis which, by itself, would decrease oxygen radical production. Indeed, preliminary experiments showed that, while greater inhibition of aminopyrine oxidation occurred using these latter ratios of CO:O_2, there was also a considerable decrease in ·OH production. The latter was also apparent when N_2 was used instead of CO, implying that low oxygen tension was responsible for inhibition of ·OH production. Metyrapone and SKF-525A both inhibited the oxidation of aminopyrine, whereas the oxidation of Me_2SO and KTBA was insensitive to these inhibitors. These inhibitors, however, block certain but not all cytochrome P-450-catalyzed reactions and do not significantly inhibit microsomal oxidation of ethanol [10, 12]. These results suggest that, in intact microsomes, the reductase appears to be an important locus for the production of ·OH. Recent experiments have shown that the reductase can directly reduce ferric EDTA by a superoxide dismutase-insensitive reaction [29]. Autoxidation of the reductase or the ferrous EDTA apparently can produce the $O_2^{\cdot-}$ and, subsequently, the H_2O_2 required for the production of ·OH.

Alcohols, on the other hand, show properties which are, in part, similar to those found with the ·OH scavengers and, in part, similar to the oxidation of aminopyrine. The oxidation of alcohols was inhibited in part (40–60%) by desferrioxamine. Thus, the sensitivity to desferrioxamine was ·OH

Table 5. Effect of metyrapone and SKF-525A on the oxidation of aminopyrine and ·OH scavengers*

Addition	Concn (mM)	Rate of substrate oxidation (nmoles/min/mg microsomal protein)		
		Aminopyrine	KTBA	Me_2SO
Control		8.86 ± 1.13	2.50 ± 0.16	3.85 ± 0.14
Metyrapone	0.33	5.87 ± 1.17†	2.38 ± 0.10	3.81 ± 0.23
	0.67	5.40 ± 1.07†	2.69 ± 0.15	3.89 ± 0.29
	1.0	4.35 ± 0.85‡	2.76 ± 0.17	3.82 ± 0.14
Control		6.64 ± 0.57	3.09 ± 0.14	2.76 ± 0.32
SKF-525A	0.1	4.83 ± 0.48‡	3.15 ± 0.11	2.65 ± 0.32
	0.5	4.04 ± 0.15§	3.36 ± 0.15	2.71 ± 0.26
	1.0	2.89 ± 0.50§	3.36 ± 0.15	2.43 ± 0.29

* The oxidation of aminopyrine, KTBA and Me_2SO was assayed as described in Materials and Methods. Results are from three experiments.

† $P < 0.05$.

‡ $P < 0.01$.

§ $P < 0.001$.

scavengers > alcohols > aminopyrine. The oxidation of alcohols was depressed by CO to a greater extent than the oxidation of ·OH scavengers, but to a lesser extent than the oxidation of aminopyrine, i.e. the sensitivity to CO was aminopyrine > alcohols > ·OH scavengers. The desferrioxamine-sensitive pathway of alcohol oxidation was not inhibited by CO which suggests that this pathway of alcohol oxidation is mediated by the reductase, in analogy to results found with typical ·OH scavengers, whereas the desferrioxamine-resistant pathway of alcohol oxidation was as sensitive to CO as was aminopyrine, suggesting oxidation mediated by cytochrome P-450.

Competitive ·OH scavengers block the oxidation of other scavengers, but not the oxidation of aminopyrine or aniline [1, 30]. Iron-EDTA stimulates, while desferrioxamine inhibits, the oxidation of ·OH scavengers, but not the oxidation of drugs [4, 31]. Organic hydroperoxides can support metabolism of drugs and ethanol [21, 22] but not the oxidation of ·OH scavengers [23]. These various examples which tend to disassociate the metabolism of ·OH scavengers from the typical mixed function oxidase activity of cytochrome P-450 can probably be explained by the fact that different enzymes play a role in the oxidation of ·OH scavengers and of drugs.

These studies do not relate to which microsomal components are primarily responsible for the production of $O_2^{\cdot}$ and H_2O_2 during electron transfer, but merely suggest that sufficient $O_2^{\cdot}$ and H_2O_2 can be produced by the reductase, under these conditions, to promote the oxidation of ·OH scavengers. It is also possible that a shift in the locus of oxygen radical production could occur when the cytochrome P-450 reaction is blocked by CO or other inhibitors, i.e. under normal conditions autoxidation of reduced cytochrome P-450 results in the production of $O_2^{\cdot}$ and H_2O_2 required to produce ·OH, and that efficient electron transfer (coupling) from the reductase to cytochrome P-450 serves to reduce the probability of autoxidation of the reductase. When this efficient electron transfer is disturbed, e.g. by inhibitors, the locus of oxygen radical production may shift to the reductase. Since superoxide dismutase does not inhibit the oxidation of ·OH scavengers [5, 29] nor the reduction of ferric EDTA by the reductase [29], reduction of ferric iron is probably not mediated by $O_2^{\cdot}$ produced from autoxidation of oxy-cytochrome P-450 or the reductase, but rather directly by the reductase (or, perhaps, ferro cytochrome P-450).

Experiments with cobalt protoporphyrin IX confirmed that this agent decreased the content of cytochrome P-450 and inhibited the oxidation of drugs [14]. The oxidation of the ·OH scavengers and the oxidation of alcohols by the ·OH-dependent pathway were also inhibited by the cobalt IX treatment. It was anticipated that little or no effect on ·OH production would occur if the only major consequence of the cobalt IX treatment was reduction of the content of cytochrome P-450. However, the cobalt IX treatment also decreased the activity of the reductase, which would explain the decreased production of ·OH. While this work was in progress, Cheeseman [32] reported that cobalt IX decreases the content of cytochrome P-450 and the activity of NADPH cytochrome *c* reductase. Thus, the effects of cobalt IX on microsomal reactions are complex since lowering of drug metabolism and the oxidation of alcohols probably reflect perturbation of the activity of the reductase, in addition to the decreased content of cytochrome P-450.

In summary, the production of ·OH appears to be mediated primarily by the reductase in intact microsomal preparations. The ·OH-dependent pathway of oxidation of alcohols occurred at this locus while the ·OH-independent pathway of alcohol oxidation was catalyzed by cytochrome P-450. By the use of desferrioxamine or CO, either of the two alcohol-oxidizing pathways can be inhibited independently of each other.

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