NADPH-dependent Production of Oxy Radicals by Purified Components of the Rat Liver Mixed Function Oxidase System

I. OXIDATION OF HYDROXYL RADICAL SCAVENGING AGENTS*

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Isolated microsomes catalyze an NADPH-dependent oxidation of typical hydroxyl radical scavenging agents. To determine which microsomal components participate in the oxidation of the scavengers, experiments were carried out with purified NADPH-cytochrome P-450 reductase and cytochrome P-450 isolated from phenobarbital-treated rats. The production of ethylene from 4-ketothiobutylurate or of formaldehyde from either dimethyl sulfoxide or tertiary butyl alcohol was measured in the presence of NADPH plus reductase or NADPH plus reductase plus cytochrome P-450. The reductase-dependent system itself catalyzed the oxidation of the three scavengers. Addition of cytochrome P-450 had no effect on the rates of oxidation of the scavengers.

Relative rates of oxidation of the scavengers reflected the amount of the reductase in the assay system. Varying the amount of cytochrome P-450 at a fixed concentration of the reductase did not result in rates different from that observed in the absence of cytochrome P-450. Dilauroyl phosphatidylcholine appeared to have a stimulating role in these oxidations, whereas cytochrome P-450 reductase and NADPH were obligatory components.

The reductase-dependent oxidation of the scavengers was inhibited by superoxide dismutase, catalase, and competing hydroxyl radical scavenging agents suggesting that superoxide, hydrogen peroxide, and an oxygen radical with the oxidizing power of the hydroxyl radical played a role in these oxidations. Further, these oxidations were inhibited by the iron-chelator desferoxamine but stimulated by either EDTA or by iron. These results suggest that an iron-catalyzed Haber-Weiss reaction might be involved in the mechanism by which purified cytochrome P-450 reductase mediates the oxidation of typical hydroxyl radical scavengers. The results demonstrated that NADPH-cytochrome P-450 reductase may represent an important locus of oxygen activation leading to the production of a highly oxidizing species characteristic of the hydroxyl radical.

Typical hydroxyl radical scavenging agents are oxidized during NADPH-dependent electron transport by isolated microsomes (1–5). Recently, the rate of oxidation of dimethyl sulfoxide and KTBA was found to correlate with the specific activity of NADPH-cytochrome P-450 reductase and the specific content of cytochrome P-450 in microsomes isolated from several tissues (6). Further, KTBA and ethanol were both metabolized by a reconstituted system containing purified components of the microsomal mixed function oxidase system (7). These findings suggested that oxygen radical production during microsomal electron transport are at the locus of either cytochrome P-450 or NADPH-cytochrome P-450 reductase, or both. The mechanism by which an active oxygen species is produced, resulting in the oxidation of hydroxyl radical scavengers, remains ambiguous.

Apparently, the production of superoxide represents the primary metabolic event in the sequence of reactions leading to the production of hydroxyl radicals (8–10). Two probable loci of superoxide production during microsomal electron transport are the auto-oxidation of the peroxycytochrome P-450 complex (11, 12) and/or the auto-oxidation of cytochrome P-450 reductase (13–18). Disproportionation of superoxide produces hydrogen peroxide.

\[ \text{O}_{2}^{•-} + \text{O}_{2}^{•-} \rightarrow 2\text{H}_{2}\text{O}_{2} + \text{O}_{2} \]  

Hydrogen peroxide may serve to generate the hydroxyl radical (•OH).

\[ \text{O}_{2}^{•-} + \text{H}_{2}\text{O}_{2} \rightarrow \text{•OH} + \text{•OH} + \text{O}_{2} \]  

Equation 2 is the Haber-Weiss reaction (17) which, although thermodynamically favorable, is kinetically quite slow (9, 10). Reaction 2 can be catalyzed by metals, especially iron.

\[ \text{Fe}^{2+} + \text{H}_{2}\text{O}_{2} \rightarrow \text{Fe}^{3+} + \text{•OH} + \text{OH}^{-} \]  

In view of the fact that many potential reducing agents exist in vivo that are capable of reducing ferric iron, Equation 4 or the Fenton reaction (18) may be sufficient to generate hydroxyl radicals.

Recent electron spin resonance studies with the spin-trapping agent 5,5-dimethyl-1-pyrroline-1-oxide identified the presence of the hydroxyl radical producing during NADPH-dependent microsomal electron transport (19, 20). A characteristic DMPO:•OH signal could be detected when 12 to 20

* The abbreviations used are: KTBA, 2-keto-4-thiomethylbutyric acid; •OH, hydroxyl radical or a species with the corresponding oxidizing power of the hydroxyl radical; MeSO₂, dimethyl sulfoxide; MFO, microsomal mixed function oxidase system; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide.

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µM iron-EDTA was added to the microsomes (19, 20). In fact, DMPO was found to inhibit microsomal oxidation of ethanol and KTBA, probably by scavenging the generated ·OH (21). The DMPO-·OH signal was also observed when iron-EDTA plus NADPH was added to NADPH-cytochrome P-450 reductase purified from phenobarbital-treated rats (22). The influence of cytochrome P-450 on the intensity of the DMPO-·OH signal was not investigated. A problem recently emphasized in the interpretation of ESR spectral data with DMPO is that the DMPO-superoxide spectrum can decay to produce a DMPO-·OH spectrum (23-25). Therefore, it is possible that the ·OH signal detectable by ESR may not reflect the actual generation of ·OH during microsomal electron transport per se. The present study represents an attempt to further elucidate and define the loci at which an active oxygen species may be generated by observing the interaction of several hydroxyl radical scavengers with purified, reconstituted components of the rat liver microsomal electron transport system. The following manuscript (26) represents similar studies carried out to characterize the oxidation of ethanol by purified systems. Results demonstrating similarities as well as differences between the metabolism of ethanol and classical ·OH scavenging agents will be described.

METHODS

Isolation of Microsomes—Immature male Sprague-Dawley rats (50 to 60 g) were pretreated with sodium phenobarbital (75 mg/kg) intraperitoneally for 4 days. On day 5, the animals were killed and microsomes were prepared by differential centrifugation and were stored as a suspension in 0.25 M sucrose under nitrogen at -85°C until use.

Purification of Cytochrome P-450—The phenobarbital-inducible isozyme of cytochrome P-450 was purified as described by West et al. (27). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed a single major band (M, = 52,000). The cytochrome P-450 content of the final preparation (28) was found to be 15 nmol/mg of protein when the protein concentration was determined by the method of Lowry et al. (29).

Purification of NADPH-Cytochrome c (P-450) Reductase—NADPH-cytochrome P-450 reductase was partially purified using DEAE-Sephadex A-25 (30) and further purified by affinity chromatography. The technique was essentially that of Yasuokochi and Masters (31) except that Emulgen 911 (Kao-Atlas Co., Ltd., Tokyo) was used instead of Renex 690 as the nonionic detergent and ADP-Sepharose was replaced by 2,3,5'-ADP-agarose hexane, type 2 (P-L Biochemicals). The final preparation had a specific activity of 52,000 units/mg of protein. A unit of reductase activity is defined as an initial rate of 1 nmol of cytochrome c reduced/min at 22°C when assayed according to Phillips and Langdon (32).

Standard Assay System and Reconstitution of the Mixed Function Oxidase System—Typical incubations were carried out in test tubes (14 x 100 mm) containing 100 mM potassium phosphate buffer (pH 7.4), 10 mM MgCl₂, 1.0 mM NADP, 10 mM glucose 6-phosphate, and 7 units of glucose-6-phosphate dehydrogenase. Cytochrome P-450, NADPH-cytochrome P-450 reductase, and dihydroxyphosphatidylcholine were reconstituted at a ratio of 0.1 nmol of P-450:20 µg of dihydroxyphosphatidylcholine:1000 units of reductase (33). The reconstituting mixture was then added to the phospholipid protein mixture and gently mixed. Depending upon the experiment, the amount of the microsomal mixed function oxidase components may have varied, but this ratio of the three components was maintained whenever they were all present in the assay. Detailed assay conditions for individual experiments are presented in the respective figure or table legends. The reaction was always started by the addition of the NADPH-generating system (glucose 6-phosphate and glucose-6-phosphate dehydrogenase) and terminated by the addition of either perchloric acid (KTBA experiments) or trichloroacetic acid (all other substrates). The final reaction volume was 1.0 ml.

Oxidation of Substrates—The methods used to measure the oxidation of various scavengers used in these present studies are described elsewhere (1, 2, 4). Essentially, the scavengers were incubated in the standard assay system described above. Ethylene production from the oxidation of KTBA was monitored by measuring directly a head space aliquot from the reaction vessel by gas chromatography.

Me-SO and t-butyl alcohol oxidation, as well as the N-demethylation of aminopyrine (34), was determined by measuring the formation of formaldehyde produced according to a fluorimetric modification of the method of Nash (35). NADPH oxidation was determined spectrophotometrically by following the change in absorbance at 340 nm.

Chelexing of Buffers and Water—CONTES columns (15 x 3 cm) were packed with Chelex 100 (Bio-Rad). After equilibration with distilled H₂O, the phosphate buffer was loaded onto the column and two void volumes were allowed to go to waste before collecting material for assay purposes. The Chelex treatment did not alter the pH of the buffers. All other solutions were prepared with chelexed water.

Reagents and Enzymes—All reagents, enzymes, resins, and chemicals were of the highest grade available from established commercial sources.

RESULTS

NADPH-dependent Oxidation of Hydroxyl Radical Scavengers—In order to determine whether the hydroxyl radical scavenging agents used in this study were, in fact, oxidized by the reconstituted mixed function oxidase system, the generation of products over a time course of 30 min was followed. Fig. 1 shows that KTBA, Me-SO, and t-butyl alcohol were all oxidized by the complete system. Ethylene production from KTBA appeared to be linear for about 5 min, whereas formaldehyde production from Me-SO and t-butyl alcohol appeared to be linear for essentially 10 and 15 min, respectively. Thus, the reconstituted system oxidizes these three ·OH scavengers to products previously shown to result from the interaction of the scavengers with ·OH generated by model ·OH-

![Fig. 1. Time course for the oxidation of 10 mM KTBA, 30 mM Me-SO, and 32 mM t-butyl alcohol by purified, reconstituted components of the liver mixed function oxidase system of phenobarbital-pretreated rats. Assays were carried out in the presence (x-x-x) and absence (O-O-O) of cytochrome P-450. In experiments with KTBA and Me-SO, the reconstituted system contained 0.5 nmol of cytochrome P-450 (when present), 5000 units of reductase, and 100 µg of dihydroxyphosphatidylcholine in a final reaction volume of 1.0 ml, as described under "Methods." Experiments with t-butyl alcohol were conducted under the same conditions except the ratio of cytochrome P-450:reductase:phosphatidylcholine was 0.25 nmol:2500 units:50 µg, respectively.](http://www.jbc.org/)
generating systems and isolated microsomes (1, 2). A comparison of the rate of oxidation of the ‘OH scavengers by NADPH plus reductase relative to rates found with NADPH plus reductase plus cytochrome P-450 is shown in Fig. 1. With all three ‘OH scavengers, the rate of oxidation found in the presence of the reductase alone was not changed by the addition of cytochrome P-450 to the system, at all time points tested. The oxidation of KTBA and Me,S0 was completely dependent upon the presence of both NADPH and the reductase (not shown). Heat-denatured reductase failed to mediate the production of oxidative products from the respective substrates. The role of phospholipid in the assay appeared to be stimulatory, as indicated by a loss of approximately 20% of the activity in its absence. All subsequent experiments contained phospholipid in the system.

To further verify that the reductase, and not cytochrome P-450, was playing the major role in the ability of the complete reconstituted system to oxidize the ‘OH scavengers, titration experiments were performed in which the oxidation of KTBA and t-butyl alcohol was measured as a function of varying concentrations of one of the components in the presence of a fixed concentration of the other. As shown in Table I, the rate of KTBA and t-butyl alcohol oxidation was dependent solely upon the addition of reductase to the assay mixture. In one experiment, Me,S0 oxidation also showed a direct dependence upon reductase but not cytochrome P-450 concentration (data not shown). Aminopyrine demethylase activity, as expected, was dependent upon the presence of cytochrome P-450 in the reconstituted system and was proportional to increasing amounts of cytochrome P-450, reductase, and phospholipid when relative proportions were kept constant (not shown).

Effect of Superoxide Dismutase, Catalase, and Competing Scavengers on Oxidation of KTBA—To elucidate the mechanism by which the hydroxyl radical scavenging agents were oxidized by the reductase, the effects of superoxide dismutase and catalase on the reductase-dependent oxidation of KTBA were investigated. Table II demonstrates that this activity was sensitive to both superoxide dismutase and catalase which suppressed the production of ethylene from KTBA 40 and 70%, respectively. Thus, there is an apparent role for both superoxide and hydrogen peroxide in the reductase-dependent oxidation of KTBA. A nonspecific protein effect by superoxide dismutase or catalase can be ruled out since the addition of bovine serum albumin had no effect on the oxidation of KTBA (Table II).

The sensitivity to both superoxide dismutase and catalase suggests that the production of ethylene reflects an interaction of KTBA with hydroxyl radicals generated from H$_2$O$_2$ (Equations 2 to 4). It follows that competing ‘OH scavengers should inhibit the production of ethylene. Ethylene production from KTBA was, in fact, inhibited by three competing scavengers, Me,S0, benzoate, and ethanol, in a dose-related manner (Table III).

### Table I

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Units of reductase x 10$^{-5}$</th>
<th>Nanomoles of cytochrome P$_{450}$</th>
<th>Nanomoles of product per 30 min</th>
</tr>
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<tbody>
<tr>
<td>KTBA</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.5</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.5</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.5</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0</td>
<td>20.0</td>
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<td></td>
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<td>0.1</td>
<td>16.5</td>
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<td></td>
<td>10.0</td>
<td>0.3</td>
<td>21.0</td>
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<tr>
<td></td>
<td>10.0</td>
<td>0.5</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1.0</td>
<td>18.0</td>
</tr>
<tr>
<td>t-Butyl alcohol</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>0.3</td>
<td>3.0</td>
</tr>
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<td></td>
<td>2.5</td>
<td>0.3</td>
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<td>0.3</td>
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<td>2.5</td>
<td>0</td>
<td>12.0</td>
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<td>10.5</td>
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<td>0.3</td>
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<td></td>
<td>2.5</td>
<td>0.5</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

* Ethylene from 10 mM KTBA, mean of 2 determinations.
* Formaldehyde from 32 mM t-butyl alcohol, mean of 2 determinations.

### Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Nanomoles of ethylene min$^{-1}$</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.27</td>
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<tr>
<td>Superoxide dismutase</td>
<td>0.16</td>
<td>-41</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.08</td>
<td>-70</td>
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<tr>
<td>Bovine serum albumin</td>
<td>0.25</td>
<td>-7</td>
</tr>
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</table>

* Values represent mean of 4 determinations.

### Table III

<table>
<thead>
<tr>
<th>Additions</th>
<th>Nanomoles of ethylene min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.15</td>
</tr>
<tr>
<td>Me,S0</td>
<td>0.07</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.04</td>
</tr>
<tr>
<td>Benzoate</td>
<td>0.09</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.07</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Results represent the mean of 2 experiments in duplicate.
Oxidation of \( \cdot \text{OH} \) Scavengers by Purified MFO Components

xanthine oxidase (9, 10). In contrast, the latter blocks iron-catalyzed \( \cdot \text{OH} \) production in several systems (36, 37), including the oxidation of \( \cdot \text{OH} \) scavengers by microsomes (38).

Therefore, a stimulation by EDTA with a concomitant inhibition by desferrioxamine of substrate oxidation would be consistent with the presence of iron in these systems. Table IV shows that the reductase-dependent oxidation of KTBA, Me\( \text{SO}_2 \), and \( t \)-butyl alcohol was stimulated 157, 55, and 66% respectively, by 50 \( \mu \text{M} \) EDTA, whereas desferrioxamine inhibited the same reactions by approximately 60% in parallel experiments.

In view of the above, the addition of iron would be expected to increase the generation of \( \cdot \text{OH} \) and subsequently augment the oxidation of \( \cdot \text{OH} \) scavengers. The data in Fig. 2 show the relative stimulation of scavenger oxidation produced by the addition of known amounts of Fe-EDTA. In all cases, Fe-EDTA enhanced the oxidation of the scavengers in a dose-related manner. Using the relative increase in activity by the added iron-EDTA in these reactions enabled the extrapolations shown in Fig. 2 to be made. These extrapolations should represent reasonable approximations of the amount of adventitious iron in the system. In spite of the stringent Chelex 100 treatment (see "Methods") of water and phosphate buffers in these experiments, it can be seen that iron was present at levels of between 1 and 2 \( \mu \text{M} \).

### DISCUSSION

Rat liver microsomes were previously shown to catalyze the oxidation of a variety of \( \cdot \text{OH} \) scavengers during NADPH-dependent electron transport (1-5). Azide stimulated the oxidation of the \( \cdot \text{OH} \) scavengers, suggesting that \( \text{H}_2\text{O}_2 \) alone was not effective in promoting scavenger oxidation, indicating that reducing equivalents were also required. In view of the stimulation of scavenger oxidation by external iron-EDTA and the inhibition by chelators such as desferrioxamine (6, 38), reducing equivalents may be required to reduce microsomal iron to the ferrous state. A Fenton reaction between \( \text{Fe}^{2+} \) and \( \text{H}_2\text{O}_2 \) could produce \( \cdot \text{OH} \), that subsequently oxidizes the \( \cdot \text{OH} \) scavengers. Microsomal components involved in the production of superoxide and subsequently \( \text{H}_2\text{O}_2 \) may include the NADPH-cytochrome P-450 reductase or cytochrome P-450 (11-16). The microsomal iron catalyst may be adventitious iron present in the microsomes or iron present in hemoproteins such as cytochrome P-450 or \( h_b \). Indeed, in view of the reactive nature of \( \cdot \text{OH} \), the actual oxidizing species could be the bound kinetic equivalent of \( \cdot \text{OH} \), the ferryl radical.

To understand the role that various components play in the oxidation of \( \cdot \text{OH} \) scavengers by microsomes, the interaction of typical hydroxyl radical scavenging agents with purified, reconstituted components of the microsomal mixed function oxidase system has been investigated. The data presented herein demonstrate that an active oxygen species with the characteristic of a \( \cdot \text{OH} \) can be generated by NADPH-cytochrome P-450 reductase. The production of ethylene from KTBA is found to be sensitive to inhibition by superoxide dismutase, catalase, competitive \( \cdot \text{OH} \) scavengers, and by desferrioxamine, but is stimulated by iron-EDTA and by EDTA itself. These results suggest that an iron-catalyzed Haber-Weiss type of reaction is responsible for production of \( \cdot \text{OH} \):

\[
\text{NADPH} + \text{reductase} \rightarrow \text{reduced reductase} + \text{O}_2 \tag{a}
\]

Reduced reductase \( + \text{Fe}^{2+} \rightarrow \text{oxidized reductase} + \text{Fe}^{3+} + \text{O}_2 \tag{b} \]

\( \text{O}_2^- + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{O}_2 \tag{c} \]

\( \text{O}_2^- + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \tag{d} \]

\( \text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{OH}^- + \text{Fe}^{2+} \tag{e} \]

Superoxide dismutase will prevent reaction \( c \) by removal of \( \cdot \text{OH} \), while catalase prevents reaction \( e \) by removal of \( \text{H}_2\text{O}_2 \). Chelation of iron by desferrioxamine or by EDTA will decrease or increase reactions \( c \) and/or \( e \), respectively. The results with desferrioxamine and EDTA suggest that, despite rigorous treatment with Chelex 100 resin, sufficient iron persisted to catalyze the production of \( \cdot \text{OH} \). Calculations presented in the following manuscript suggest that 10\( ^{-5} \) \( \mu \text{M} \) iron is more than sufficient to account for the reductase-dependent rates of oxidation of the \( \cdot \text{OH} \) scavengers. The rate of NADPH

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**Table IV**

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Additions</th>
<th>Activity</th>
<th>Effect of addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>KTBA</td>
<td>None</td>
<td>0.14(^\text{a})</td>
<td>(%)</td>
</tr>
<tr>
<td></td>
<td>Desferrioxamine</td>
<td>0.06</td>
<td>-57</td>
</tr>
<tr>
<td>Me( \text{SO}_2 )</td>
<td>EDTA</td>
<td>0.36</td>
<td>+157</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0.58(^\text{b})</td>
<td>(%)</td>
</tr>
<tr>
<td></td>
<td>Desferrioxamine</td>
<td>0.23</td>
<td>-90</td>
</tr>
<tr>
<td>t-Butyl alcohol</td>
<td>EDTA</td>
<td>0.90</td>
<td>+55</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0.27(^\text{b})</td>
<td>(%)</td>
</tr>
<tr>
<td></td>
<td>Desferrioxamine</td>
<td>0.10</td>
<td>-63</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>0.45</td>
<td>+66</td>
</tr>
</tbody>
</table>

\(^\text{a}\) KTBA, Me\( \text{SO}_2 \), and t-butyl alcohol were present at 10, 30, and 32 \(\mu\text{M} \), respectively.

\(^\text{b}\) Desferrioxamine and EDTA were present at 330 and 50 \(\mu\text{M} \), respectively.

\( \mu \text{M} \) Me\( \text{SO}_2 \) and t-butyl alcohol were added to the reaction mixture to achieve the final concentrations presented in the figure. The final reaction mixture contained 2,500 units of NADPH-cytochrome P-450 reductase and 50 \( \mu \text{g} \) of dilauroyl phosphatidylcholine in the standard reaction mixture, as described under "Methods."

\( \text{KTBA} \) is found to be sensitive to inhibition by superoxide dismutase, catalase, competitive \( \cdot \text{OH} \) scavengers, and by desferrioxamine, but is stimulated by iron-EDTA and by EDTA itself. These results suggest that an iron-catalyzed Haber-Weiss type of reaction is responsible for production of \( \cdot \text{OH} \):
oxidized was about 1.5 to 2.5 nmol/min/2,500 units of reductase. Since the oxidation of the 'OH scavenger varied between 0.15 and 0.59 nmol/min/2,500 units of reductase, about 4 to 10 equivalents of NADPH were oxidized per 'OH generated. According to the above scheme, at least 3 superoxide anion radicals are required to generate 1 'OH. This suggests that a minimum of 3 eq of NADPH must be oxidized to generate 1 'OH. Since iron stimulates the oxidation of the 'OH scavengers (Fig. 2), H2O2 may have accumulated in the reaction medium. This would suggest that the higher estimate of NADPH equivalents oxidized per 'OH generated is the more probable value.

The oxidation of the 'OH scavengers can occur independently of the presence of cytochrome P-450 in the assay mixture. Several lines of results previously suggested that oxidation of 'OH scavengers could be disassociated from the typical mixed function oxidase activity of cytochrome P-450. It was noted that competitive 'OH scavenging agents inhibited the oxidation of other scavengers but do not inhibit the metabolism of typical drug substrates (1, 39). Desferrioxamine inhibits nearly completely the oxidation of benzoic acid, MeSO2, and KTBA by microsomes, but fails to inhibit the oxidation of aminopyrine in parallel experiments (38). Furthermore, organic hydroperoxides, which can replace NADPH in mediating the oxidation of several drugs by cytochrome P-450 (40–42), could not support the oxidation of benzoate (3) or MeSO2 in microsomal systems nor of KTBA or 3-butanal alcohol in reconstituted systems (26). Taken as a whole, these results tend to disassociate the oxidation of typical substrates for the microsomal mixed function oxidase system from the oxidation of typical hydroxyl radical scavengers. The results suggest that either the role of cytochrome P-450, in promoting drug oxidations, differs from its role in catalyzing 'OH production (e.g. acting as a catalyst of the Fenton reaction) or that cytochrome P-450 plays little or no role in the generation of 'OH.

These results do not exclude production of H2O2 at the loci of cytochrome P-450, but merely suggest that sufficient H2O2 can be produced by the reductase and the sequelae of additional events leading to the production of 'OH can occur at this locus. It is recognized that, because we used the phenobarbital-inducible isozyme of cytochrome P-450 in these studies, some caution should be exercised in interpreting these results. One could envision, for instance, that the 'OH scavengers might serve as more suitable substrates for other isozymes of cytochrome P-450. However, as will be described in the following manuscript, the reductase-dependent oxidation of ethanol is increased by the addition of the phenobarbital-derived cytochrome P-450. Thus, this isozyme can promote the oxidation of alcohols and drugs, but not 'OH scavengers. Another possibility is that, when the cytochrome P-450 is added to the reductase, a shift in the locus of 'OH production occurs, i.e. the cytochrome P-450, by mediating an efficient electron transfer, would serve to reduce the probability of auto-oxidation of the reductase. Auto-oxidation of oxyxycytochrome P-450 would then result in the production of oxy radicals. This possibility would necessitate that the diminishment of oxy radical production arising at the locus of the reductase is completely compensated by the production of oxy radicals by the cytochrome P-450, since oxidation of the 'OH scavengers is exactly the same in the absence and presence of cytochrome P-450. It should be pointed out that this similar rate of oxidation of 'OH scavengers is maintained even when the concentration of cytochrome P-450 is widely varied in the presence of two different fixed concentrations of reductase (Table I). However, a linear dependence of the oxidation of 'OH scavengers on the concentration of reductase is readily observed (Table I).

Although a shift in the locus of oxy radical production upon the addition of cytochrome P-450 to the reductase cannot be rigorously excluded, the following experiment tends to suggest that cytochrome P-450 iron does not catalyze the production of 'OH or its equivalent. The oxidation of MeSO2 was studied in the absence and presence of cytochrome P-450. Table IV shows that desferrioxamine inhibits the reductase-dependent oxidation of MeSO2 by 60%. Desferrioxamine does not inhibit typical mixed function oxidase activity (38). If, in fact, the addition of cytochrome P-450 results in diminishing the reductase contribution to the formation of an oxidizing species while augmenting its own contribution, then one would anticipate observing a differential effect of desferrioxamine on the oxidation of MeSO2 by reconstituted systems in the presence and absence of cytochrome P-450. Table V shows that the effect of desferrioxamine is essentially identical in a complete system as it is in just a reductase-dependent system. Further, neither SKF-525A nor metyrapone, typical inhibitors of mixed function oxidase activity, have any effect on the oxidation of MeSO2 at concentrations which strongly inhibit the oxidation of aminopyrine (Table V). This is consistent with the observation of Lai and Piette (20) that neither metyrapone nor CO blocked the DMPO-'OH ESR signal in a microsomal system supplemented with iron-EDTA. Taken as a whole, these results tend to minimize the role of cytochrome P-450 in the oxidation of 'OH scavengers and suggest that, at least under these reaction conditions, the reductase itself can serve as a sufficient generator of oxy radicals in the presence of iron.

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