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

II. ROLE IN MICROSOMAL OXIDATION OF ETHANOL

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The oxidation of ethanol by a reconstituted system containing NADPH-cytochrome P-450 reductase and cytochrome P-450 from phenobarbital-treated rats was characterized especially with regard to the role of oxygen radicals in the mechanism of ethanol oxidation. Results described in the preceding manuscript demonstrated that a strong oxidizing species with properties similar to that of the hydroxyl radical was generated by a NADPH-cytochrome P-450 reductase system. Ethanol was oxidized to acetaldehyde in the presence of the reductase-NADPH system in the absence of cytochrome P-450, similar to results found with typical hydroxyl radical scavenging agents. However, in contrast to results with the scavengers, the addition of cytochrome P-450 to the assay mixture resulted in a 2- to 3-fold increase in the rate of ethanol oxidation over that of the reductase-dependent rate. The oxidation of ethanol was dependent on both the amount of reductase and the amount of cytochrome P-450.

Ethanol oxidation by the reductase-dependent pathway was inhibited 86 and 48% by dimethyl sulfoxide and benzoate, respectively. When cytochrome P-450 was present, the ability of these competing scavengers to inhibit ethanol oxidation was attenuated such that the inhibition was accountable by the reductase-dependent component. Inhibition of ethanol oxidation by superoxide dismutase in the presence and absence of cytochrome P-450 also appeared to reflect an effect upon the reductase-dependent pathway, with no effect by superoxide dismutase on the increased rate of ethanol oxidation produced by the addition of cytochrome P-450. The reductase-dependent oxidation of ethanol was inhibited by desferrioxamine and stimulated by either EDTA or iron, suggesting that ethanol oxidation was inhibited by interaction with hydroxyl radicals generated via an iron-catalyzed Haber-Weiss reaction. Desferrioxamine did not inhibit the increase in ethanol oxidation produced by the addition of cytochrome P-450. The insensitivity of the cytochrome P-450-stimulated rate of ethanol oxidation to competing scavengers, superoxide dismutase, and desferrioxamine suggests little or no role for hydroxyl radicals in the cytochrome P-450-dependent pathway of ethanol oxidation.

Cumene and t-butyl hydroperoxide supported the oxidation of aminopyrine and ethanol or 1-butanol when added directly to cytochrome P-450 in the absence of NADPH and the reductase. The organic hydroperoxide-supported oxidation of ethanol was inhibited by metyrapone, but not by dimethyl sulfoxide. Typical hydroxyl radical scavengers were not oxidized by the hydroperoxide-supported system.

These results suggest that two independent pathways are operative in supporting NADPH-dependent microsomal oxidation of ethanol. One pathway involves hydroxyl radicals which can be generated by the reductase, whereas the other pathway requires the combined presence of both the reductase and cytochrome P-450 and appears to be independent of oxygen radicals.

The ability of isolated rat liver microsomes to oxidize methanol and ethanol was first described by Orme-Johnson and Ziegler (1). This system was subsequently characterized in detail by Lieber and De Carli (2–4). Although microsomal oxidation of alcohols was ascribed by many to be due to the peroxidatic activity of catalase (present as a contaminant in isolated microsomes) or to "microsomal" alcohol dehydrogenase (5–8), studies with reconstituted systems containing cytochrome P-450 and NADPH-cytochrome P-450 reductase clearly indicated a mechanism of ethanol oxidation independent of catalase or alcohol dehydrogenase (9–12). A major difficulty in elucidating the mechanism of ethanol oxidation by cytochrome P-450 has been the lack of stoichiometry between rates of NADPH oxidation, oxygen consumption, and product formation (acetaldehyde plus H2O2) expected of a typical mixed function oxidase activity (12, 13).

Recent studies have implicated a role, at least in part, for 'OH, or a species with the oxidizing power of 'OH, in the mechanism whereby microsomes oxidize ethanol. Ethanol oxidation was inhibited by a series of 'OH scavengers in a competitive fashion (14–16). The addition of iron-EDTA, which increases the production of 'OH by microsomes, increased the oxidation of ethanol (17). The ability of the microsomes to oxidize several typical 'OH scavengers constituted chemical evidence for the production of 'OH by microsomes during NADPH-dependent electron transport (16). Similar experiments were also carried out with purified reconstituted systems. We previously found that ethanol oxidation...
by cytochrome P-450 purified from phenobarbital-treated rats was inhibited by a series of 'OH scavengers (18). At that time, it was noted that considerable oxidation of ethanol occurred even in the absence of cytochrome P-450 (23 to 50% of the rate found with the complete system) (18). The oxidation of ethanol by cytochrome P-450 purified from chronic ethanol-fed rats was also inhibited by 'OH scavengers, but not by superoxide dismutase (19). In contrast, a recent study demonstrated that the oxidation of ethanol by cytochrome P-450 purified from phenobarbital-treated rats was inhibited by both 'OH scavengers as well as superoxide dismutase (20). In the latter study, it was concluded that ethanol oxidation reflects the interaction of ethanol with 'OH which is generated via an iron-catalyzed Haber-Weiss reaction (21). The cytochrome P-450 was presumed to be the iron catalyst. However, as described in detail in the preceding paper (22), the addition of cytochrome P-450 did not increase the rate of oxidation of 'OH scavengers produced by the reductase alone.

Can the mechanism for the microsomal oxidation of alcohols be explained solely by a 'OH-dependent pathway? Recently, we observed that the potent iron-chelator, desferrioxamine, inhibited the NADPH-dependent oxidation of several hydroxyl radicals scavengers by more than 90%, whereas the oxidation of either 1-butanol or ethanol was inhibited approximately 40 to 60%, even at very high levels of desferrioxamine (23). Further, desferrioxamine did not inhibit aminopyrine demethylase activity in those studies, suggesting that cytochrome P-450 iron was not affected by this chelator. The desferrioxamine-insensitive rate of oxidation of alcohols was not inhibited by competitive 'OH scavengers, suggesting that 'OH was not involved in the mechanism. There are reports that organic hydroperoxides (which react directly with cytochrome P-450) can support the oxidation of ethanol (12, 24) and that antibody to cytochrome P-450 blocks NADPH-dependent ethanol oxidation (12). Moreover, cytochrome P50 isolated from chronic ethanol-fed rats had a higher turnover number with regard to ethanol oxidation than control cytochrome P-450 (the same preparation of reductase was used for both preparations of cytochrome P-450 (9-11). All the above point to a role for cytochrome P-450 in the mechanism of ethanol oxidation. The current report describes studies characterizing the oxidation of ethanol by NADPH-cytochrome P-450 reductase in the absence and presence of cytochrome P-450. Evidence is presented to demonstrate that ethanol oxidation involves both a 'OH-dependent (reductase) as well as a 'OH-independent (cytochrome P-450) mechanism.

METHODS

Treatment of the rats with phenobarbital, isolation of microsomes, and purification of cytochrome P-450 and NADPH-cytochrome P-450 reductase were carried out as described in the preceding paper (22).

Typical incubations were carried out in test tubes (14 x 100 mm) containing 100 mM potassium phosphate, pH 7.4, 10 mM MgCl2, 1.0 mM NADP+, 10 mM glucose-6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, the substrate, 200 μg of dialuoyl phosphatidylcholine, 10,000 units of NADPH-cytochrome P-450 reductase, and, when present, 1.0 nmol of cytochrome P-450, in a final volume of 1.0 ml. The cytochrome P-450, reductase, and phosphatidylcholine were reconstituted as described in the preceding paper (22). In some experiments, the absolute amount of either cytochrome P-450 or reductase was varied, whereas in other experiments, the total amount of the components was varied but the basic ratio of cytochrome P-450:reductase:phosphatidylcholine described above was maintained. Reactions were initiated at 37 °C by the addition of glucose 6-phosphate plus glucose-6-phosphate dehydrogenase and terminated by the addition of either perchloric acid or trichloroacetic acid.

The following reactions were assayed: oxidation of ethanol or 1-butanol to acetaldehyde or 1-butyraldehyde, respectively; production of ethylene from KTBA; production of formaldehyde from either aminopyrine, t-butyl alcohol, or Me2SO. Ethanol oxidation was measured by two different techniques. When NADPH-dependent reactions were conducted, ethanol oxidation was measured by gas chromatographic analysis of acetaldehyde in the head space of a reaction vessel, as previously described (18). When organic hydroperoxides were used to mediate the oxidation of ethanol, or when NADPH-dependent oxidation of butanol was studied, the reactions were carried out in 25-ml center well flasks which contained 15 mM semicarbazide HCl in the center well. Following termination of the reaction by acid, the acetaldehyde or 1-butyraldehyde produced was allowed to diffuse into the center wells for a period of 24 h. An aliquot of the center well contents was then removed and assayed for the formation of the acetaldehyde or 1-butyraldehyde semicarbazone complex, spectrophotometrically at 224 nm. An extinction coefficient of 9.4 mM cm−1 was used to calculate the amount of aldehyde produced. The production of ethylene from KTBA or of formaldehyde from aminopyrine, t-butyl alcohol, or Me2SO, and the method of Chelex treatment of the phosphate buffer, solutions and the H2O are described in the preceding paper (22).

RESULTS

NADPH-dependent Oxidation of Ethanol—Initial experiments compared the rate of ethanol oxidation catalyzed by the complete reconstituted hepatic mixed function oxidase system to the rate which occurs in the absence of cytochrome P-450 (reductase-dependent system). It was observed that either in the presence or absence of cytochrome P-450, the rate of acetaldehyde production was essentially linear over the time course of 30 min (Fig. 1). No activity was observed in the absence of an NADPH-generating system. The absence of phospholipid in the system resulted in 12 to 20% loss of the activity observed in its presence. While significant oxidation of ethanol occurred in the reductase-dependent system, the addition of cytochrome P-450 resulted in approximately a 2-fold increase in the rate of ethanol oxidation (Fig. 1). This enhancement was also noted in several other experiments that were conducted at single time points; there was always a 2- to 3-fold increase in ethanol oxidation in the presence of cytochrome P-450 at ratios of P-450 to reductase shown in Fig. 1.

![Fig. 1. Time course for the oxidation of ethanol by purified, reconstituted components of the liver mixed function oxidase system from phenobarbital-pretreated rats in the presence (● or absence (○) of cytochrome P-450. The complete reaction mixture contained 1.0 nmol of cytochrome P-450 (when present), 10,000 units of NADPH-cytochrome c reductase, 200 μg of dialuoyl phosphatidylcholine, and 53 mM ethanol in a final reaction volume of 1.0 ml as described under “Methods.”](http://www.jbc.org/content/1515/8/1515/f1.large.jpg)
Thus, the reductase alone can account for approximately 30 to 50% of the ethanol oxidized by a reconstituted system consisting of components purified from phenobarbital-induced rats, when the ratio of the components is held at 1 nmol of P-450 to 10,000 units of reductase to 200 μg of dilauroyl phosphatidylcholine.

For typical drugs or xenobiotics that are oxidized (hydroxylated or dealkylated) via the microsomal mixed function oxidase system, there is an obligatory dependency upon the presence of cytochrome P-450. The cytochrome P-450 reductase is not sufficient to mediate the oxidation of a xenobiotic substance such as aminopyrine (22). It is possible that there may be two loci of oxygen radical production in the reconstituted system, one dependent on the reductase, the other on decomposition of an oxycytochrome P-450 complex. However, as described in detail in the preceding paper (22), the addition of cytochrome P-450 did not change the rate of oxidation of typical ·OH scavengers such as KTBA, Me₂SO, or t-butyl alcohol over the rate catalyzed by the reductase itself. Hence, unique differences exist between the oxidation of ethanol (which appears to involve both a reductase-dependent and a cytochrome P-450-dependent component) and either typical ·OH scavengers (reductase-dependent only) or classical drug substrates (cytochrome P-450-dependent).

The present data suggested that ethanol oxidation might be occurring via two independent pathways. If this was in fact the case, then it would be possible to have two rate-limiting components mediating a single event, i.e. ethanol oxidation. The data presented as Fig. 2 show the results of a typical titration experiment in which either the reductase or the cytochrome P-450 was varied as a function of a fixed concentration of the other. It can be seen that ethanol oxidation increased as a linear function of both components. The essentially 2-fold greater slope generated by the reductase titration curve compared to the cytochrome P-450 titration curve demonstrates a greater dependency of the reaction upon added reductase to the system.

**Effect of Hydroxyl Radical Scavengers, Superoxide Dismutase, and Catalase on Ethanol Oxidation**—Because the reductase alone was shown to catalyze the oxidation of ·OH scavengers (22), the reductase-dependent pathway of ethanol oxidation probably reflects the interaction of ethanol with ·OH generated from the reductase during NADPH-dependent electron transfer. When cytochrome P-450 was present in the system, there was no enhancement of the reductase-dependent oxidation of the ·OH scavengers (22), suggesting that the cytochrome P-450-dependent pathway of ethanol oxidation may not involve oxygen radicals. The effect of competitive ·OH scavengers on the rate of ethanol oxidation catalyzed by the reductase and by the complete reconstituted system, was therefore evaluated. Table I shows that ethanol oxidation by the reductase-dependent pathway was inhibited 66 and 46% by Me₂SO and benzoate, respectively. It should be recalled from the preceding paper that the production of ethylene from KTBA, as catalyzed by the reductase, was also sensitive to inhibition by competitive ·OH scavengers, including ethanol. Thus, ethanol inhibits the oxidation of a ·OH scavenger whereas ·OH scavengers block the oxidation of ethanol. Clearly, a role for ·OH in the reductase-dependent pathway of ethanol oxidation can be discerned. By contrast, when cytochrome P-450 was added to the incubation mixture (note the increase in rate of ethanol oxidation), the ability of Me₂SO and benzoate to act as inhibitors of ethanol oxidation was attenuated (Table I). In fact, most of the decrease in ethanol oxidation produced by Me₂SO and benzoate in the complete system appears to be due to inhibition of the reductase-dependent activity.

The initial event in the sequelae leading to the production of the oxidizing species capable of oxidizing ethanol is believed to be formation of the superoxide anion radical O₂⁻. Dismutation of O₂⁻ produces H₂O₂, which is probably the precursor of ·OH in biological systems. Indeed, azide (which inhibits the catalase present as a contaminant in isolated microsomes) was found to increase the oxidation of ·OH scavengers by allowing microsomal H₂O₂ to accumulate (16, 25, 26). The production of ·OH from H₂O₂ may occur either via a Fenton reaction or an iron-catalyzed Haber-Weiss type of reaction. It follows that removal of O₂⁻ by superoxide dismutase, or removal of H₂O₂ by catalase, should decrease the generation of ·OH and, hence, decrease the oxidation of substrates dependent on interacting with the generated ·OH. As can be seen from the data in Table II, superoxide dismutase inhibited the reductase-dependent oxidation of ethanol by 48%, whereas ethanol oxidation by the complete system was inhibited only 22%. As with the competitive ·OH scavengers, the inhibition of the complete system by superoxide dismutase appears to reflect the inhibition of the reductase-dependent component. Thus, the cytochrome P-450-potentiated activity was essentially insensitive to superoxide dismutase (and ·OH scavengers). Bovine serum albumin had no effect on ethanol oxidation by either system (Table II) ruling out the possibility that inhibition by

![Fig. 2. Ethanol oxidation as a function of varying the amount of NADPH-cytochrome P-450 reductase (A) and cytochrome P-450 (B). Cytochrome P-450 was held constant at 0.25 nmol/flask in the reductase titration experiments (A). The reductase was held constant at 2500 units in the cytochrome P-450 titration experiments (B). The final reaction mixture was 1.0 ml as described under "Methods." Dilauroyl phosphatidylcholine was maintained at 100 μg/flask.](http://www.jbc.org/figs/10.1074/jbc.151615115)

**TABLE 1**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Reductase-dependent activity change</th>
<th>Reductase + P-450-dependent activity change</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.7 vs 2.7 (103 ± 1)</td>
<td>6.5 vs 6.5</td>
</tr>
<tr>
<td>Me₂SO (30 mM)</td>
<td>0.9 vs 0.9 (103 ± 1)</td>
<td>5.4 vs 5.4</td>
</tr>
<tr>
<td>Benzoate (30 mM)</td>
<td>1.5 vs 1.5 (103 ± 1)</td>
<td>5.2 vs 5.2</td>
</tr>
</tbody>
</table>

The final reaction mixture was 1.0 ml as described under "Methods," and contained 5000 units of NADPH-cytochrome P-450 reductase, 0.5 nmol of cytochrome P-450 (when present), and 100 μg of dilauroyl phosphatidylcholine. Ethanol was present at a final concentration of 53 mM.
superoxide dismutase was a nonspecific protein effect, e.g., scavenging of 'OH instead of O₂•⁻. Catalase produced a significant increase in the rates of ethanol oxidation in the absence and presence of cytochrome P-450 (Table II). This probably reflects the peroxidatic activity of catalase H₂O₂ with ethanol to produce acetaldehyde (27). The increase in ethanol oxidation produced by catalase indicates that H₂O₂ was being produced by both the reductase and the complex reconstituted system. In microsomal studies with an alcohol that does not serve as an effective substrate for the peroxidatic activity of catalase, e.g., 1-butanol, catalase inhibited 1-butanol oxidation (14). Catalase inhibited KTBA oxidation catalyzed by the reductase which is consistent with H₂O₂ being the precursor of 'OH (22).

**Effect of Iron and Iron-chelating Agents on Ethanol Oxidation**—The production of 'OH by the reductase system involves an iron-catalyzed reaction since oxidation of 'OH scavengers was inhibited by the presence of the iron-chelating agent desferrioxamine, whereas activity was stimulated by the external addition of EDTA and, especially, by iron-EDTA (22). Results in Table III show that ethanol oxidation by the reductase system responds to these variables in the same manner as did the classical 'OH scavengers. The oxidation of ethanol by EDTA and by iron-EDTA stimulated by EDTA and by iron-EDTA (Table III). It should be stressed that a striking stimulation of ethanol oxidation was found in the presence of as little as 5 μM iron-EDTA (Table III; cf. "Discussion"). In contrast to the strong inhibition by desfer-rioxamine of ethanol oxidation by the reductase system, ethanol oxidation by the complete reconstituted system was insensitive to desferrioxamine. The effect of desferrioxamine on ethanol oxidation was 0, -2, and -13% in 3 separate experiments.

**Organic Hydroperoxide Supported Oxidation of Ethanol**—During normal NADPH-dependent mixed function oxidation activity, cytochrome P-450 may be viewed as an oxygenase (Equation 1).

\[
\text{SH} + \text{NADPH} + \text{O}_2 \xrightarrow{\text{P-450}} \text{SOH} + \text{NADP} + \text{H}_2\text{O} \quad (1)
\]

On the other hand, several organic hydroperoxides have been shown to promote the oxidation of mixed function oxidation substrates even in the absence of NADPH and/or molecular oxygen (Equation 2).

\[
\text{SH} + \text{ROOH} \xrightarrow{\text{P-450}} \text{SOH} + \text{ROH} \quad (2)
\]

This type of activity reflects the ability of P-450 to act as a peroxygenase. Organic hydroperoxides have been shown to be capable of supporting the oxidation of ethanol by cytochrome P-450 (12, 24). Results in Fig. 3 show that both cumene and t-butyl hydroperoxide promoted cytochrome P-450-dependent oxidation of ethanol and 1-butanol in a manner analogous to the oxidation of aminopyrine. By contrast, KTBA, a classic 'OH scavenger, was not a substrate for this peroxygenase activity of cytochrome P-450 (Fig. 3). Moreover, whereas primary alcohols such as ethanol and 1-butanol were oxidized by this system, a tertiary alcohol, t-butyl alcohol, was not (Fig. 3). t-Butyl alcohol was oxidized by the reductase-dependent system (22). We have also observed in studies with isolated microsomes, that other 'OH scavengers such as Me₃S'O and [7-¹⁴C]benzoate (25) were not oxidized to formaldehyde or CO₂ when cumene or t-butyl hydroperoxide replaced the NADPH-generating system.

The above results suggest that the oxidation of ethanol by the organic peroxygenase-dependent system reflects a cytochrome P-450-mediated reaction only, with little or no role for 'OH.

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**Table II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Ethanol oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reductase-dependent activity</td>
</tr>
<tr>
<td></td>
<td>anol min⁻¹ anol min⁻¹</td>
</tr>
<tr>
<td>Note</td>
<td>5.4</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>2.8</td>
</tr>
<tr>
<td>Catalase</td>
<td>18.3</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>5.8</td>
</tr>
</tbody>
</table>

**Table III**

**Effects of desferrioxamine, EDTA, and iron EDTA on the NADPH-cytochrome P-450 reductase-dependent oxidation of ethanol**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Nanomoles of acetaldehyde min⁻¹</th>
<th>Effect of addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.8</td>
<td>-61</td>
</tr>
<tr>
<td>Desferrioxamine (330 μM)</td>
<td>0.7</td>
<td>-61</td>
</tr>
<tr>
<td>EDTA (50 μM)</td>
<td>2.7</td>
<td>+50</td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>6.8</td>
<td>+280</td>
</tr>
<tr>
<td>10 μM</td>
<td>11.4</td>
<td>+50</td>
</tr>
<tr>
<td>25 μM</td>
<td>27.0</td>
<td>+1400</td>
</tr>
</tbody>
</table>

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*Values represent the mean of 3 experiments performed in duplicate. The final reaction mixture contained 2500 units of NADPH-cytochrome P-450 reductase and 100 μg of diisouanyl phosphatidylcholine in a volume of 1.0 ml, as described under "Methods." Ethanol was present at a final concentration of 53 mM.**

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**TABLE IV**

Effects of Me$_3$SO and metyrapone on the organic hydroperoxide-supported oxidation of ethanol

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cumene hydroperoxide-supported activity (nmol/min/nmol of cytochrome P-450)</th>
<th>t-Butyl hydroperoxide-supported activity (nmol/min/nmol of cytochrome P-450)</th>
<th>% change</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.4</td>
<td>3.0</td>
<td>-6</td>
<td>3.4</td>
</tr>
<tr>
<td>Me$_3$SO (30 mM)</td>
<td>3.2</td>
<td>3.4</td>
<td>-6</td>
<td>3.4</td>
</tr>
<tr>
<td>Metyrapone (1 mM)</td>
<td>1.6</td>
<td>1.8</td>
<td>-53</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Activity expressed as nanomoles/min/nmol of P-450. Values represent the mean of two experiments in duplicate.*

It should therefore follow that this peroxynitrite-supported oxidation of ethanol would be sensitive to inhibitors of the mixed function oxidase activity but insensitive to competing hydroxyl radical scavengers. Table IV shows that indeed this was the case. The mixed function oxidase inhibitor, metyrapone, produced 53 and 40% inhibition of the cumene- and t-butyl hydroperoxide-supported oxidation of ethanol, respectively. In contrast, 30 mM Me$_3$SO, a dose sufficient to inhibit several hydroxyl radical-mediated oxidations by >70% (25), showed essentially no ability to inhibit the peroxynitrite-dependent oxidation of ethanol by cytochrome P-450. In studies with isolated microsomes, desferrioxamine did not inhibit organic hydroperoxide-dependent oxidation of ethanol or 1-butanol, further attesting to the absence of a role for 'OH in ethanol oxidation by this system.

**DISCUSSION**

The results presented herein with highly purified, reconstituted components of the mixed function oxidase system strongly suggest that a duality exists for the mechanism of the NADPH-dependent oxidation of ethanol by microsomes. It was observed that the ethanol-oxidizing system had properties which were similar to as well as different from, the oxidizing system which catalyzes the metabolism of KTBA, Me$_3$SO, or t-butyl alcohol. Similar to the latter substrates, ethanol could be oxidized by the reductase alone. This pathway of ethanol oxidation was sensitive to inhibition by competitive 'OH scavengers, superoxide dismutase, and desferrioxamine, but was stimulated by either EDTA or by iron. Similar characteristics have been described in the preceding paper with regard to the oxidation of typical 'OH scavengers (22).

The present results suggest that the reductase-dependent pathway of ethanol oxidation can be attributed to interaction of ethanol with 'OH generated by the reductase via an iron-catalyzed Haber-Weiss reaction. As described in detail in our microsomal studies (14-16), 'OH also plays a role in the oxidation of ethanol by purified microsomal components.

On the other hand, interaction with 'OH does not appear to be the sole mechanism by which ethanol is oxidized by microsomes or reconstituted systems. In the previous paper, it was observed that cytochrome P-450 was not a rate-limiting component in the oxidation of any of the typical hydroxyl radical scavenging agents used as substrates. With regard to ethanol oxidation, it is clear that cytochrome P-450 is indeed a rate-limiting component. Titration curves demonstrate a dependence of ethanol oxidation on both the reductase and on the cytochrome P-450 (Fig. 1). That the cytochrome P-450-dependent ethanol oxidation system does not appear to involve 'OH can be seen from the weak extent of inhibition produced by competitive 'OH scavengers, superoxide dismutase and desferrioxamine. In fact, the inhibition of ethanol oxidation by all of these agents can be ascribed to inhibition of the reductase-dependent, i.e. 'OH-dependent, oxidation of ethanol. In other studies, it was observed that KTBA, Me$_3$SO, and benzoate do not produce binding spectra when added to microsomes, whereas ethanol produced the typical reverse type I cytochrome P-450 binding spectrum as has been observed by others (28). An important finding is that ethanol and 1-butanol, but not KTBA and t-butyl alcohol (Fig. 3), serve as substrates for the organic hydroperoxide-supported peroxidase activity of cytochrome P-450. The oxidation of ethanol in this system is inhibited by metyrapone, but not by Me$_3$SO (Table IV). These results clearly indicate a mechanism of ethanol oxidation which involves an oxygenated cytochrome P-450 intermediate that is independent of 'OH and does not support the oxidation of 'OH scavengers.

It is recognized that some caution should be expressed when interpreting such results. In view of the fact that the cytochrome P-450 used here represents predominantly the phenobarbital-inducible isozyme, the possibility that the 'OH scavengers might be substrates for other isozymes of cytochrome P-450 remains to be examined. As with the scavengers, the alcohols used in this study also might be rendered better or poorer substrates for other putative isozymes of P-450. As was mentioned in the introduction, cytochrome P-450 from chronic ethanol-fed rats displayed higher turnover numbers for ethanol oxidation than did control cytochrome P-450 (9-11). Since the same preparation and amount of reductase was used for the two cytochrome P-450 preparations, the higher turnover number for ethanol oxidation clearly resides at the level of the cytochrome P-450 isozyme. Recently, we have observed that microsomes from chronic ethanol-fed rats oxidized KTBA and Me$_3$SO at rates 2- to 3-fold greater than the rates found with microsomes from pair-fed controls. This increase in 'OH generation probably reflects an increase in H$_2$O$_2$ generation as a result of increased activity of the NADPH-cytochrome P-450 reductase (22, 30). Actually, the increase in H$_2$O$_2$ generation by microsomes from ethanol-fed rats originally thought to be sufficient with a role for catalase in the increased activity of microsomal ethanol oxidation (30). In view of recent results (14-18), a mechanism involving 'OH is more likely. It therefore appears that the induction of ethanol oxidation by microsomes which occurs after chronic ethanol consumption involves both the 'OH-dependent (reductase) as well as the cytochrome P-450-dependent ('OH-independent) ethanol-oxidizing systems present in the microsomes.

The above results suggest that ethanol is oxidized by two primary pathways in isolated microsomes, one which involves interactions of ethanol with 'OH, the other which appears to be independent of oxy radicals but rather involves cytochrome P-450. What is the relative contribution of each pathway? In studies with noninduced microsomes, desferrioxamine was found to inhibit the oxidation of 'OH scavengers by greater than 90%, while the oxidation of ethanol or 1-butanol was inhibited by only 40 to 60% (23). Microsomal oxidation of ethanol was inhibited by 35 to 60% by competitive 'OH scavengers (14-18). In the studies reported here, the addition of cytochrome P-450 from phenobarbital-treated rats increased the rate of ethanol oxidation by 2- to 3-fold. It therefore appears that both pathways contribute about equally to the overall metabolism of ethanol by microsomes. Clearly, the contribution made by each pathway can be dramatically al-

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3. G. W. Winston and A. I. Cederbaum, unpublished observations.

Hydroxyl radical-mediated phenomenon characterized by Haber-Weiss and/or Fenton chemistry following the generation of superoxide by NADPH-cytochrome P-450 reductase in the presence of iron.

In view of the above, it seems appropriate to stress the importance of monitoring iron in these systems lest mendacious interpretations be drawn. Recently it has been suggested that the entire mechanism of ethanol oxidation could be explained by an iron-catalyzed Haber-Weiss reaction (20). Indeed, we find also that a significant portion of the observed ethanol oxidation by these reconstituted systems was consistent with such a mechanism. However, it should be stressed that these investigators did not use chelexed buffers. It can be seen that 2500 units of reductase in our typical incubation systems that have been stringently chelexed, approximately 2 μmol of P-450 in their reconstituted system. Recalculating their data as turnover numbers for ethanol oxidation was added at levels which correspond to amounts of iron that would be equivalent to about 9 to 15 nmol/min/2500 units of reductase which would be equivalent to approximately 2 μmol iron present as adventitious iron in the assay mix as evidenced by the stimulation observed when EDTA was added to the incubation mixture. It has been calculated that at a concentration of 50 mM, non-Chelex-treated phosphate buffer may have as much as 5 to 10 μmol iron present (33, 34). We observed rates of ethanol oxidation of about 7 to 12 nmol/min/2500 units of reductase which would be equivalent to about 9 to 15 nmol/min of reductase when iron-EDTA was added at levels which corresponded to amounts of iron that could represent levels of contamination in 50 mM phosphate buffer (5 to 10 μmol). Ingelmann-Sunberg and Johansson have reported a value of ethanol oxidation of 6.5 nmol/min/nmol of P-450 in their reconstituted system. Recalculating their data presented as turnover numbers for ethanol oxidation per nmol of P-450 to per amol of reductase would have yielded a value of 13 nmol/min/nmol of reductase. This represents a value very similar to what we observed when we added levels of iron to our reaction mix that represent the amount present as contaminating iron present in the phosphate buffers. Levels of contaminating iron are very significant in evaluating the role of oxygen radicals in a biological system. In fact, some investigators use the contaminating iron in phosphate buffer as a source of catalytic iron (34, 35). As a consequence, at these levels of contaminating iron, it is possible to establish an assay condition in which a hydroxyl radical-mediated phenomenon might be grossly exaggerated and, in fact, mask the cytochrome P-450-dependent pathway of ethanol oxidation. This possibility may explain why those authors concluded that ethanol oxidation was mediated solely by a ‘OH-dependent mechanism.

In summary, the studies presented herein with reconstituted, purified components of the microsomal mixed function oxidase system are consistent with the idea that two independent pathways are operating simultaneously in oxidizing ethanol. One pathway appears to be typical of many xenobiotics which are metabolized via cytochrome P-450-mediated mixed function oxidations. The other pathway represents a hydroxyl radical-mediated phenomenon characterized by Haber-Weiss and/or Fenton chemistry following the generation of superoxide by NADPH-cytochrome P-450 reductase in the presence of iron.

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