Mechanisms of Hydroxyl Radical Formation and Ethanol Oxidation by Ethanol-inducible and Other Forms of Rabbit Liver Microsomal Cytochromes P-450*

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The hydroxyl radical-mediated oxidation of 5,5-dimethyl-1-pyrroline N-oxide, benzene, ketomethylbutyric acid, deoxyribose, and ethanol, as well as superoxide anion and hydrogen peroxide formation was quantitated in reconstituted membrane vesicle systems containing purified rabbit liver microsomal NADPH-cytochrome P-450 reductase and cytochromes P-450 LM2, P-450 LMeb, or P-450 LM4, and in vesicle system devoid of cytochrome P-450. The presence of cytochrome P-450 in the membranes resulted in 4-8-fold higher rates of O₂⁻, H₂O₂, and hydroxyl radical production, indicating that the oxyctytochrome P-450 complex constitutes the major source for superoxide anions liberated in the system, giving as a consequence hydrogen peroxide and also, subsequently, hydroxyl radicals formed in an iron-catalyzed Haber-Weiss reaction. Depletion of contaminating iron in the incubation systems resulted in small or negligible rates of cytochrome P-450-dependent ethanol oxidation. However, small amounts (1 μM) of chelated iron (e.g. Fe²⁺-EDTA) enhanced ethanol oxidation specifically when membranes containing the ethanol and benzene-inducible form of cytochrome P-450 (cytochrome P-450 LMeb) were used. Introduction of the Fe-EDTA complex into P-450 LMeb-containing incubation systems caused a decrease in hydrogen peroxide formation and a concomitant 6-fold increase in acetaldehyde production; consequently, the rate of NADPH consumption was not affected. In iron-depleted systems containing cytochrome P-450 LM2 or cytochrome P-450 LMeb, an appropriate stoichiometry was attained between the NADPH consumed and the sum of hydrogen peroxide and acetaldehyde produced. Horseradish peroxidase and scavengers of hydroxyl radicals inhibited the cytochrome P-450 LM4-dependent ethanol oxidation both in the presence and in the absence of Fe-EDTA. The results are not consistent with a specific mechanism for cytochrome P-450-dependent ethanol oxidation and indicate that hydroxyl radicals, formed in an iron-catalyzed Haber-Weiss reaction and in a Fenton reaction, constitute the active oxygen species. Cytochrome P-450-dependent ethanol oxidation under in vivo conditions would, according to this concept, require the presence of non-heme iron and endogenous iron chelators.

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1 Chronic ingestion of ethanol produces a proliferation of the smooth hepatic endoplasmic reticulum (1), and increased ability of microsomal fractions to metabolize ethanol (2, 3) as well as drugs (4, 5). Comai and Gaylor (6) first presented evidence for the presence of cytochrome P-450 in liver microsomes inducible by ethanol. Also Lieber and DeCarli (3) and Joly and co-workers (7, 8) provided evidence for the existence of an ethanol-inducible species of cytochrome P-450 in rat liver, effective in aniline (7) and ethanol (8) oxidation. These findings are in accordance with the observations of enhanced rates of aniline and ethanol metabolism in liver microsomes following ethanol treatment of the rats (9). Recently, Sato and collaborators (10, 11) found that a single intragastric dose of ethanol resulted in an enhanced metabolism of various aromatic (e.g. benzene) and chlorinated hydrocarbons in rat liver preparations. These changes were dose-dependent and disappeared almost completely after a 1-day ethanol withdrawal period (10). A similar fast disappearance of ethanol-induced elevation of cytochrome P-450 levels and enhanced rates of aniline hydroxylation in rat liver microsomes has been described (9). In addition, Petersen et al. (12) have shown that the rate of microsomal ethanol oxidation is enhanced already 2 h after intraperitoneal injection of ethanol to mice. These results thus indicate the existence of an ethanol-inducible microsomal species of cytochrome P-450 with a very rapid turnover that participates in the metabolism of ethanol and hydrocarbons.

The cytochrome P-450 LM2-dependent¹ oxidation of ethanol in reconstituted membrane vesicles was previously shown to involve an iron-catalyzed Haber-Weiss reaction whereby hydroxyl radicals, formed from superoxide anions liberated from the oxyctytochrome P-450 complex, and hydrogen peroxide, as the dismutation product of O₂⁻, constituted the active oxygenating species (13). The almost complete inhibition found by adding superoxide dimutase to the vesicular incubation system indicated that the reaction was not specific, but rather involved free diffusible superoxide anions and hydroxyl radicals. It was later shown that also the small aromatic compounds aniline (14) and benzene (15) underwent the same type of oxidative metabolism in liver microsomes and reconstituted vesicular systems. It was therefore hypothesized that administration of either benzene or ethanol to rabbits would result in the induction of cytochrome P-450

¹ Cytochrome P-450 LM2 and cytochrome P-450 LM4 are forms of rabbit liver microsomal cytochrome P-450 designated according to their electrophoretic mobilities. Cytochrome P-450 LMeb is the ethanol and benzene-inducible form of rabbit liver microsomal cytochrome P-450. This form of cytochrome P-450 is probably identical to cytochrome P-450 LM3a isolated in Prof. M. J. Coon's laboratory (18).
species participating in the radical-dependent oxidation of these compounds. Indeed, both benzene and ethanol induced apparent Michaelis-Menten parameters were homogeneous according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The preparations having a specific activity of 150 nmol/min, with an apparent molecular weight of 10,000 were pooled. From this pool, approxi-
mately to 3 g of microsomal protein, fraction V according to Koop et al. (24) was isolated using Renex 690 as detergent (DEAE-Sepharose was used instead of DEAE-cellulose). This fraction (300 nmol of P-450) was treated with calcium phosphate and applied on a CM-Sepharose column at pH 6.0 as described (24), except that 5 mm potassium phosphate, pH 6.0, containing 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM diethioerythritol and 2% Renex 690 (v/v) was used as buffer. The column (1 × 25 cm) was eluted with a linear gradient (250 + 250 ml) of KCl from 0 to 0.5 M. The ethanol- and benzene-inducible forms of cytochrome P-450 LM4 were identified electrochemically and spectrophotometrically (390–416 nm) and eluted approximately at 0.2 M of KCl. The fractions having an absorbance ratio of A390/ A450 greater than 1.1 were pooled. The preparation (80 nmol of P-450) was diluted twice with redistilled water and adsorbed on a hydroxylapatite column (1 × 5 cm), equilibrated in 10 mM phosphate, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, and 0.1% Renex. The column was washed with 10 volumes of the equilibration buffer and, subsequently, with 20 volumes of similar buffers based on 30 and 45 mM phosphate, respectively. Renex was removed by washing the column with 50 mM potassium phosphate buffer, pH 7.4, until the absorbance at 275 nm of the eluent was 0. The column was equilibrated with 500 mM phosphate, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, and 0.2% (w/v) cholate and finally dialyzed against 2 × 250 volumes of 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The preparations, having specific activities of 11–14 g mmoles/min/mg, were electrophoretically homogeneous and contained alanine as the NH2-terminal amino acid.

**Assay Methods**—Protein was determined according to Lowry et al. (25) using bovine serum albumin as standard. Cytochrome b5 was determined using the absorption coefficient 100 mM-1 cm-1 for the absorption difference between the reduced and the oxidized form of the protein at 424 nm (27), NADPH-oxidase activity was determined spectrophotometrically at 340 nm using the absorption coefficient 6.2 mM-1 cm-1. Oxidation of ethanol was quantitated essentially according to Leib and DeCarli (2) as previously described (13). Cytochrome P-450 was determined spectrophotometrically using the absorption coefficient 91 mM-1 cm-1 for the 450–490 nm absorption difference of the ferrous carboxyl complex. Hydroxyl radical-mediated damage of deoxyribose was detected fluorimetrically using thiobarbituric acid as described by Halliwell and Gutteridge (26) and previously presented (16). Hydroxyl radical-dependent production of ethylene from KMBA was determined as previously described (16). Formaldehyde was detected according to Nash (29). Superoxide anions were quantitated using succinylated cytochrome c in part as previously described (13). Cytochrome c was treated with succinic anhydride according to Takeuchi et al. (30). The preparation was passed through a Sephadex G-25 column equilibrated in 100 mM potassium phosphate buffer, pH 7.4, and the resulting protein fraction was adsorbed on a column of DEAE-Sepharose (Pharmacia) equilibrated in 10 mM phosphate buffer. The column was eluted with equilibration buffer containing 0.1 M KCl and subsequently with 0.5 M KCl. The latter fraction was used for the experiments. The preparation (1.5 mM) was tested for reduction by NADPH-cytochrome P-450 reductase by incubations performed in the absence or in the presence of excess superoxide dismutase. The ability of the modified enzyme to be reduced by 1 mM dihydroxyfumarate, known to generate superoxide anions in water solution (31), was tested against native cytochrome c and was found to be the same. The superoxide anions produced in membrane vesicles were quantitated from the linear phase of reduction of succinylated cytochrome c followed spectrophotometrically at 550 nm. Hydrogen peroxide was determined by the ferrous thiocyanate method (32). Benzene oxidation was quantitated as previously described (15).

**EPR Measurements**—The EPR experiments were performed on a Varian V-450 X-band (9.5 GHz) EPR spectrometer equipped with a 100-kHz modulation, a 4-inch magnet, and a V-4531 multipurpose cavity. The EPR-spectra were recorded at room temperature using a microwave frequency of 9.229 GHz, modulation frequency of 100 kHz, modulation intensity of 2 G, field set of 3510 G, and a scan range of 100 G.

Preparation of membrane vesicles was performed according to the cholate gel filtration technique (21). In some experiments, 10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl and 0.2 mM EDTA was used as eluant, whereas in experiments performed under "iron-free"
conditions, chelexed 15 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl was used during the Sephadex chromatography. In the latter type of experiments, all buffers were passed through a 3 × 25 cm column of Chelex 100 (Bio-Rad Laboratories). The gel filtration column was extensively equilibrated in the chelexed buffer and all glassware used was rinsed in chelexed water.

The numbers given in this paper constitute the mean values from two to eight different experiments performed with at least two different enzyme preparations, except in the case of cytochrome P-450 LM4, where only one batch of enzyme was used.

Materials—Microsomal phospholipids were extracted from liver microsomes of phenobarbital-treated rabbits according to Bligh and Dyer (35) and stored in sealed tubes under nitrogen at –20 °C. Horse liver peroxidase (grade I) was obtained from Boehringer Mannheim. Superoxide dismutase (specific activity, 2410 units/mg), cytochrome c, Type II A, DETAPAC, DMPO and bathophenantroline were purchased from Sigma. Desferoxamine was from CIBA.

RESULTS

Cytochrome P-450 and NADPH-Cytochrome P-450 Reductase—Dependent Hydroxyl Radical Formation As Detected by EPR—DMPO is a spin trap used for the detection of a lot of different types of radicals (cf. Ref. 34); also, superoxide anions and hydroxyl radicals will react with the compound, giving characteristic EPR spectra (35). One drawback using this spin trap for the differential detection of these two types of oxygen radicals is that the hydroperoxy adduct will decompose to the hydroxynitroxide which is formed upon interactions between the spin trap and hydroxyl radicals (35, 36). This can partly be overcome by adding a hydroxyl radical scavenger such as ethanol to the system; in such a case an a-hydroxynitroxide radical will be produced giving an EPR spectrum with DMPO distinguishable from that produced by the hydroxyl adduct (35). Membrane vesicles were prepared under “iron-free” conditions, as described under “Experimental Procedures,” having a molar ratio of cytochrome P-450: NADPH-cytochrome P-450 reductase: microsomal phospholipids of about 1:0.3:1300 and incubated with 100 mM DMPO in the presence of 1.5 mM NADPH. As seen from Fig. 1, the characteristic EPR spectrum of the hydroxyl radical adduct (35) was produced in the vesicular system. The amplitude of the spectrum formed was detected in systems containing cytochrome P-450 LM2, P-450 LMb and/or comparable amounts of NADPH-cytochrome P-450 reductase alone (Fig. 1). Approximately 3.5-fold higher amplitudes were reached in the P-450-containing vesicles compared to reductase vesicles, whereas no significant difference was noted between vesicular systems containing either of the two cytochromes. Inclusion of 200 mM ethanol in the vesicular systems converted approximately 50% of the EPR spectra into the spectra produced by the a-hydroxynitroxide adduct of DMPO (Ref. 35, not shown in figure) indicating that a substantial portion of the spectra detected were attributable to interactions of hydroxyl radicals with the spin trap.

Cytochrome P-450-dependent Hydroxyl Radical-mediated Destruction of Deoxyribose and Production of Ethylene from KMBAs—As described in a previous paper (16), hydroxyl radicals produced by the action of the P-450 system will destroy deoxyribose and induce the formation of ethylene from KMBAs. Evaluation of the capacities among various enzymes to destroy deoxyribose (Table I) revealed that vesicles containing P-450 LM2 were most effective and vesicles with P-450 LM4 least effective, whereas membranes containing comparable amounts of the reductase alone were rather inefficient. The ethanol- and benzoquinone-inducible form of rabbit liver microsomal cytochrome P-450 was moderately effective in deoxyribose destruction, but not significantly more efficient than P-450 LM2 in generating ethylene from KMBAs (Table I).

Cytochrome P-450 and Reductase-dependent Formaldehyde Production from Me3SO—Klein et al. (37) have described that formaldehyde is produced from Me3SO as a consequence of interactions between hydroxyl radicals and the solvent. Membrane vesicles containing cytochrome P-450 LMb and cytochrome P-450 LM4 were about twice as effective in the Me3SO-dependent formaldehyde production as vesicles containing P-450 LM2, whereas vesicles containing only the reductase were quite ineffective (Table II). The formaldehyde production was in all cases inhibited by thiourea, catalase, and superoxide dismutase to extents between 60 and 90%.
**Table II**

**Cytochrome P-450- and NADPH-cytochrome P-450 reductase-dependent production of formaldehyde from MeSO in reconstituted membrane vesicles**

<table>
<thead>
<tr>
<th>Addition</th>
<th>LM2</th>
<th>LMeb</th>
<th>LM4</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.6</td>
<td>4.1</td>
<td>4.3</td>
<td>0.11</td>
</tr>
<tr>
<td>Thiourea</td>
<td>1.1</td>
<td>58</td>
<td>1.3</td>
<td>69</td>
</tr>
<tr>
<td>Catalase, 10 mM</td>
<td>0.42</td>
<td>84</td>
<td>0.37</td>
<td>91</td>
</tr>
<tr>
<td>Superoxide dismutase, 50 μg/ml</td>
<td>0.37</td>
<td>86</td>
<td>0.23</td>
<td>94</td>
</tr>
</tbody>
</table>

*The rate of formaldehyde production in the absence of MeSO was approximately 0.6 nmol/nmol of P-450, minute, using all types of P-450. Reductase-dependent formaldehyde production is expressed as nanomoles of HCHO formed per 0.3 nmol of reductase, minute.*

*Boiled (15 min) preparations of catalase were without effect.*

*Boiled (15 min) preparations of superoxide dismutase inhibited the formaldehyde production by 10-20%.*

(Table II), indicating the participation of hydroxyl radicals, hydrogen peroxide, and superoxide anions in the process. Boiled preparations of catalase did not affect the rate of formaldehyde production, whereas boiled superoxide dismutase inhibited the process slightly, by 10-20%.

**Superoxide Anion and Hydrogen Peroxide Production as a Function of the Amount of Cytochrome P-450 LM2 in the Vesicles**—Hydroxyl radicals produced in cytochrome P-450-containing membrane systems were previously proposed to be generated in an iron-catalyzed Haber-Weiss reaction between superoxide anions and hydrogen peroxide (13, 16). In order to indirectly estimate the relative contribution of cytochrome P-450 for the hydroxyl radical formation, superoxide anions released and hydrogen peroxide produced were quantitated in vesicles containing increasing amounts of cytochrome P-450 LM2. As shown in Fig. 2, hydrogen peroxide was produced at a rate of about 3 nmol/nmol of reductase/min in vesicles containing only NADPH-cytochrome P-450 reductase; inclusion of P-450 LM2 into the vesicles resulted in a maximally 5-fold stimulation of the H2O2-production. The superoxide anion formation increased in a concomitant manner in response to P-450 incorporation and saturation was observed at a 1:1 molar ratio of P-450 reductase to P-450 (Fig. 2).

**Cytochrome P-450 LM2 and Cytochrome P-450 LMeb-dependent Ethanol Oxidation**—Membrane vesicles were prepared according to the standard gel filtration technique (21) from cytochrome P-450 LM2 or P-450 LMeb, NADPH-cytochrome P-450 reductase, and microsomal phospholipids at a molar ratio of about 1:0.3:1300. The vesicles were incubated with 50 mM ethanol in conventional 50 mM potassium phosphate buffer, pH 7.4. The production of acetaldehyde in the presence of 0.4 mM NADPH was linear for at least 60 min under the incubation conditions used (Fig. 3A) using both types of vesicles. From the substrate curves shown in Fig. 3B, apparent \( K_m \) values for ethanol of 14 and 8 mM could be calculated for P-450 LMeb and P-450 LM2, respectively. In other experiments, vesicles were prepared from microsomal phospholipids, NADPH-cytochrome P-450 reductase, and variable amounts of cytochromes P-450 LM2 and P-450 LM2 and the rate of NADPH-dependent ethanol oxidation was subsequently determined. In the absence of cytochrome P-450, 13% (P-450 LMeb) or 5% (P-450 LMeb) of the maximal rate of acetaldehyde production was detected (Fig. 4). Saturation of the activity was reached at approximately a 1:1 molar ratio between the hemoprotein and the flavoprotein.

**Ethanol Oxidation under “Iron-free” Conditions**—The rates of cytochrome P-450-dependent ethanol oxidation in conventional incubation systems were compared with those obtained under “iron-free” conditions (see “Experimental Procedures”). It was found that the cytochrome P-450- and NADPH-cytochrome P-450 reductase-dependent ethanol oxidations were inhibited by between 65% (P-450 LMeb) and 83% (P-450 LM2) (Table III) in the system where chelexed buffers and enzyme solutions were used in comparison to the conventional systems containing iron and residual EDTA from the gel filtration column (13, 21). Superoxide dismutase further inhibited the cytochrome P-450 LM2- and reductase-dependent but not the P-450 LMeb-dependent ethanol oxidation in the “iron-free” system (Table III).

The abilities of three different iron chelators to inhibit the P-450-dependent ethanol oxidation in the “iron-free” system were tested (Table IV). Neither desferroxamine, nor bathophenanthroline or DETA at 100 μM concentration did significantly inhibit the process. A slight inhibition by desferroxamine was observed using both P-450 LM2 and P-450 LMeb-containing membranes, but this compound was also found to inhibit the rate of NADPH oxidation in P-450 LM2-
Cytochrome P-450-dependent Hydroxyl Radical Formation

Cytochrome P-450-dependent and NADPH-cytochrome P-450 reductase-dependent oxidation of ethanol in reconstituted phospholipid vesicles using conventional and "iron-free" incubation systems

Incubations were performed with membrane vesicles corresponding to 130 μM microsomal phospholipids, 0.1 μM cytochrome P-450, and/or 0.03 μM NADPH-cytochrome P-450 reductase in 2 ml of 50 mM potassium phosphate buffer at 37 °C for 20 min using 0.4 mM NADPH. The “iron-free” system was generated using chelaxed buffers for incubations and for preparation of vesicles (see “Experimental Procedures”). Vesicles used in the conventional incubation system were prepared as described in Table I. ND, not determined.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Acetaldehyde produced*</th>
<th>“Iron-free” system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional system</td>
<td>Without superoxide dismutase</td>
</tr>
<tr>
<td>P-450 LM2</td>
<td>5.5</td>
<td>0.9</td>
</tr>
<tr>
<td>P-450 LMeb</td>
<td>12.8</td>
<td>4.7</td>
</tr>
<tr>
<td>P-450 LM4</td>
<td>4.0</td>
<td>0.8</td>
</tr>
<tr>
<td>P-450 reductase</td>
<td>1.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*The values are given as nanomoles of acetaldehyde formed per nmol of P-450 and minute. In case of vesicles only containing reductase, the turnover is given as nanomoles of acetaldehyde formed per 0.3 nmol of reductase and minute.

Effect of iron chelators (100 μM) on the rate of cytochrome P-450 LM2 and P-450 LMeb-dependent oxidation of ethanol in reconstituted membrane vesicles under “iron-free” conditions

Preparation of vesicles and incubations were performed as described in Table III.

<table>
<thead>
<tr>
<th>Chelator used</th>
<th>Acetaldehyde formed (nmol/nmol, min)</th>
<th>NADPH-oxidase (LM2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.9</td>
<td>5.5</td>
</tr>
<tr>
<td>Desferoxamine</td>
<td>0.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Bathophenantroline</td>
<td>2.1</td>
<td>7.5</td>
</tr>
<tr>
<td>DETAPAC</td>
<td>2.6</td>
<td>10.0</td>
</tr>
</tbody>
</table>

However, unexpectedly, the cytochrome P-450 LMeb-dependent reaction showed saturation-like kinetics with respect to the amount of the Fe-EDTA complex. Half-maximal activation was observed at 1 μM concentration; maximal activity was 30 nmol of acetaldehyde produced per nmol of P-450 and minute. By optimizing the conditions with respect to the amount of reductase in the vesicles, a turnover for ethanol of over 100 could be reached. Insertion of data for the Fe-EDTA activation into a double-reciprocal plot gave a straight line (Fig. 5); extrapolation of the values to the rate of ethanol oxidation seen in the absence of the complex (5 nmol/nmol of P-450, minute) yielded an “endogenous” complex concentration of 0.25 μM, i.e. a value rather similar to the cytochrome P-450 concentration used in these experiments (0.15 μM). In separate experiments, it was found that iron(III) chloride alone did not enhance the rate of cytochrome P-450 LMeb-dependent ethanol oxidation; a turnover of about 5 was reached, despite the presence of 1, 3, 6, 15 or 30 μM FeCl₃ in the incubation buffer.

Apparent Stoichiometry of the Cytochrome P-450 LMeb- and P-450 LM2-dependent Ethanol Oxidation—In order to get information about the mechanism of cytochrome P-450 LMeb-dependent ethanol oxidation, the rate of NADPH-consumption, and acetaldehyde and hydrogen peroxide production was quantitated upon addition of increasing amounts of the Fe-EDTA complex to incubation systems containing...
Addition of the Fe-EDTA complex resulted in further de-ethanol to membrane vesicles containing cytochrome P-450 decreased coupling only 15%. The NADPH oxidase activity diminished in response to addition of complex. Addition of potassium phosphate buffer, pH 7.4, for chromatography. Incubations were performed in chelexed 50 mM potassium phosphate buffer, pH 7.4, for 26 min at 37 °C using 50 mM ethanol and 0.4 mM NADPH with vesicles corresponding to 0.2 nmol of P-450. Inset, double reciprocal plot for the Fe-EDTA activation of cytochrome P-450 LMeb-dependent ethanol oxidation.

Cytochrome P-450-dependent Hydroxyl Radical Formation

FIG. 5. Cytochrome P-450-dependent ethanol oxidation under “iron-free” conditions as a function of the amount of Fe(III)-EDTA complex present in the incubation mixtures. Membrane vesicles were prepared from cytochrome P-450, NADPH-cytochrome P-450 reductase, and microsomal phospholipids at a molar ratio of about 15000:0.3:1 using chelexed 15 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl during the Sephadex chromatography. Incubations were performed in chelexed 50 mM potassium phosphate buffer, pH 7.4, for 26 min at 37 °C using 50 mM ethanol and 0.4 mM NADPH with vesicles corresponding to 0.2 nmol of P-450. Inset, double reciprocal plot for the Fe-EDTA activation of cytochrome P-450 LMeb-dependent ethanol oxidation.

P-450 LMeb and compared to the results reached using cytochrome P-450 LMeb-containing membrane vesicles. In the absence of ethanol, membrane vesicles composed of microsomal phospholipids, NADPH-cytochrome P-450 reductase, and cytochrome P-450 LMeb exhibited poor coupling between NADPH consumption and hydrogen peroxide production (Fig. 6A). The NADPH oxidase activity was about 63 nmol/nmol of P-450, minute, compared to about 28 nmol of H₂O₂ produced/nmol minute, i.e. a coupling efficiency of 44%. Addition of the Fe-EDTA complex resulted in further decreased coupling—only 15%. The NADPH oxidase activity was constant, whereas the hydrogen peroxide production diminished in response to addition of complex. Addition of ethanol to membrane vesicles containing cytochrome P-450 LMeb resulted in a diminished rate of NADPH-consumption. This decrease was about 30% both in the presence and the absence of the Fe-EDTA complex and was half-maximal when the ethanol concentration was 15 μM (not shown). As a consequence, the coupling effectiveness was considerably enhanced, also taking the rate of acetaldehyde production into account, to about 83%. Upon addition of the Fe-EDTA complex, the hydrogen peroxide production decreased whereas there was a concomitant increase in the rate of acetaldehyde formation and as a result the coupling effectiveness was not changed by addition of complex. It may thus be concluded that two electrons are required for the oxidation of one molecule of ethanol by cytochrome P-450 LMeb in this system.

Similar measurements using cytochrome P-450 LMb-containing vesicles revealed a better coupling efficiency in the presence of ethanol in the “iron-free” system (92%) than obtained previously in a conventional incubation medium (13). Here, addition of the Fe-EDTA complex resulted in a prompt reduction of the hydrogen peroxide produced (Fig. 7B). By contrast, this decrease was not correlated to enhanced acetaldehyde production; the two processes were apparently not related to each other. A similar decrease in hydrogen peroxide production upon addition of complex was also seen in the absence of substrate (Fig. 7A).

Effect of Active Oxygen Scavengers on the Rate of Cytochrome P-450 LMeb-dependent Ethanol Oxidation—The cytochrome P-450 LMb-dependent ethanol oxidation was previously shown to be mediated by hydroxyl radicals formed in an iron-catalyzed Haber-Weiss reaction (13) and it was therefore considered of importance to evaluate whether a similar reaction mechanism was operating in membrane systems containing the ethanol and benzene-inducible form of cytochrome P-450 in the absence or in the presence of the Fe-EDTA complex. Superoxide dismutase, when present at 50 μg/ml, did not inhibit the cytochrome P-450 LMb-dependent ethanol oxidation in the absence of complex. In the presence of 10 μM Fe-EDTA, about 30% inhibition was reached (Table V). The hydroxyl radical scavengers formate and mannitol inhibited the P-450 LMb-dependent ethanol oxidation by 42–90% both in the presence and in the absence of complex (Table V).

An important question concerns whether cytochrome P-450 LMb-dependent ethanol oxidation in the absence of

FIG. 6. NADPH-oxidase activity, hydrogen peroxide production, and acetaldehyde formation in “iron-free” incubation systems containing cytochrome P-450 LMeb as a function of the amount of Fe(III)-EDTA complex. Conditions are as described in the legend to Fig. 5. A, without substrate. B, with 50 mM ethanol.

FIG. 7. NADPH-oxidase activity, hydrogen peroxide production, and acetaldehyde formation in “iron-free” incubation systems containing cytochrome P-450 LMb as a function of the amount of the Fe(III)-EDTA complex. Conditions are as described in the legend to Fig. 5. A, without substrate. B, with 50 mM ethanol.
Cytochrome P-450-dependent Hydroxyl Radical Formation

Effect of superoxide dismutase and hydroxyl radical scavengers on the rate of cytochrome P-450 LMeb-dependent ethanol oxidation in the presence or the absence of the Fe-EDTA complex (10 μM)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-Fe-EDTA</th>
<th>+Fe-EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.5</td>
<td>28.0</td>
</tr>
<tr>
<td>Superoxide dismutase, 50 μg/ml</td>
<td>4.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Formate, 50 mM</td>
<td>1.6</td>
<td>64.5</td>
</tr>
<tr>
<td>Mannitol, 200 mM</td>
<td>1.5</td>
<td>67.0</td>
</tr>
</tbody>
</table>

Fe(III)-EDTA is mediated by hydroxyl radicals. In order to answer this, the effect of different hydroxy radical scavengers on the rate of P-450 LMeb-dependent acetdehyde formation was evaluated and the concentration of these scavengers giving 50% inhibition of the reaction in the presence of 50 mM ethanol was determined. As seen from Table VI, half-maximal inhibition was registered in the concentration range 0.1-26 mM. Under these conditions, the hydroxyl radicals would react at equal rate with the scavenger as with ethanol itself, i.e. the following relationship would occur when assuming a second order reaction mechanism.

\[ k_{scavenger} \cdot [\cdot OH] = k_\text{ethanol} \cdot [\cdot OH] \]

\[ \frac{1}{k_c} = \frac{[\text{scavenger}]}{k_c} \cdot [\text{ethanol}] \]

where \( k_c \) is the rate constant for reaction between hydroxyl radical and the scavenger and \( k_c \) is the similar rate constant for reaction with ethanol. When \( 1/k_c \) was plotted against [scavenger], i.e. the scavenger concentration giving half-maximal inhibition, a straight line was obtained (Fig. 8). The slope, with a value of 16 s, thus represents \( 1/(k_c \cdot [\text{ethanol}]) \).

Since the ethanol concentration used was 50 mM, we could calculate the rate constant for reaction between the active oxygen species mediating the P-450 LMeb-dependent ethanol oxidation and the substrate. The rate constant obtained in this way was \( 1.05 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \), a value very similar to the literature data of \( 1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \) for reaction between hydroxyl radical and ethanol (38). Based upon these data one may suggest that hydroxy radicals mediate the P-450 LMeb-dependent ethanol oxidation even in the absence of Fe-EDTA.

### Table V

Effect of superoxide dismutase and hydroxyl radical scavengers on the rate of cytochrome P-450 LMeb-dependent ethanol oxidation in the presence or the absence of the Fe-EDTA complex (10 μM)

<table>
<thead>
<tr>
<th>Condition</th>
<th>-Fe-EDTA</th>
<th>+Fe-EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.5</td>
<td>28.0</td>
</tr>
<tr>
<td>Superoxide dismutase, 50 μg/ml</td>
<td>4.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Formate, 50 mM</td>
<td>1.6</td>
<td>64.5</td>
</tr>
<tr>
<td>Mannitol, 200 mM</td>
<td>1.5</td>
<td>67.0</td>
</tr>
</tbody>
</table>

### Table VI

Properties of the hydroxyl radical scavengers used

<table>
<thead>
<tr>
<th>Compound</th>
<th>Apparent ( k_c )</th>
<th>( k_{50} )</th>
<th>( k )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>&gt;20</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>Deoxyribose</td>
<td>&gt;20</td>
<td>26</td>
<td>1.9</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>40</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Formate</td>
<td>&gt;200</td>
<td>21</td>
<td>2.5</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>&gt;200</td>
<td>0.1</td>
<td>12</td>
</tr>
<tr>
<td>KMBA</td>
<td>&gt;200</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Mannitol</td>
<td>&gt;800</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Thiourea</td>
<td>&gt;200</td>
<td>7</td>
<td>4.7</td>
</tr>
<tr>
<td>Ethanol</td>
<td>220</td>
<td>1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*Concentration of hydroxyl radical scavenger giving 50% inhibition of cytochrome P-450 LMeb-dependent ethanol oxidation; ND, not detectable.
should be a more efficient catalyst in this reaction. However, this was found not to be the case (Table IX). Using a benzene concentration of 84 μM, cytochrome P-450 LMB-containing membrane vesicle systems oxidized benzene only at a rate approximately 20% higher than cytochrome P-450 LM2-containing incubation systems. Furthermore, the Fe-EDTA-complex (10 μM) was found to enhance product formation by a factor of about 4 both in cytochrome P-450 LMB and in P-450 LM2-containing incubation mixtures. Superoxide dismutase (50 μg/ml) inhibited benzene oxidation in the presence of the Fe-EDTA complex by 40% (P-450 LMB) or 54% (P-450 LM2) i.e. to a greater extent than when ethanol was used as substrate.

### DISCUSSION

The results presented indicate that exogenous iron and, in addition, exogenous iron chelators are of great importance for cytochrome P-450-dependent hydroxyl radical formation and ethanol oxidation. Small or negligible amounts of acetaldehyde were produced in iron- and iron chelator-depleted incubation systems. In addition to the cytochrome P-450 LM2-dependent ethanol oxidation (13), the cytochrome P-450 LMB-dependent acetaldehyde production was also inhibited by scavengers of hydroxyl radicals, horseradish peroxidase and, in the presence of the Fe-EDTA complex, also by superoxide dismutase. The capability of superoxide dismutase to only partially inhibit especially the cytochrome P-450 LMB-dependent ethanol oxidation reactions, even in the presence of Fe-EDTA, might be explained by the inability of the enzyme to influence the steady state concentration of the superoxide anions near their site of formation due to a very rapid superoxide anion-dependent reduction of the Fe(III)-EDTA complex or more plausibly, that cytochrome P-450 LMB to a great extent directly reduces chelated iron, giving as a consequence a Fenton reaction for the generation of hydroxyl radicals.

The cytochrome P-450 LMB-dependent ethanol oxidation was markedly dependent upon the presence of the Fe-EDTA complex in the incubation medium. In contrast to the other cytochromes P-450, a very pronounced activation of the

### TABLE VII

**Effect of hydroxyl radical scavengers on the rate of cytochrome P-450 LMB-dependent NADPH-oxidation and hydrogen peroxide production**

<table>
<thead>
<tr>
<th>Scavenger used</th>
<th>NADPH*</th>
<th>Hydrogen peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- EtOH + EtOH</td>
<td>nmol/nmol P-450, min</td>
</tr>
<tr>
<td>None</td>
<td>62</td>
<td>45</td>
</tr>
<tr>
<td>Thiourea, 5 mM</td>
<td>63</td>
<td>46</td>
</tr>
<tr>
<td>Formate, 50 mM</td>
<td>55</td>
<td>44</td>
</tr>
<tr>
<td>MeSO, 200 mM</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td>Deoxyribose, 50 mM</td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>Mannitol, 200 mM</td>
<td>62</td>
<td>42</td>
</tr>
<tr>
<td>KMB, 2 mM</td>
<td>60</td>
<td>44</td>
</tr>
</tbody>
</table>

* 200 μM hydroquinone was used in these experiments.

### TABLE VIII

**Effect of horseradish peroxidase on the rate of cytochrome P-450 LMB-dependent ethanol oxidation in the presence or in the absence of the Fe-EDTA complex**

<table>
<thead>
<tr>
<th>Conditions are described in Table III.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Additions</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Hydroquinone, 20 μM</td>
</tr>
<tr>
<td>Hydroquinone, 20 μM + horseradish peroxidase, 1 unit</td>
</tr>
</tbody>
</table>

* The control values in the absence of NADPH (subtracted) were raised in the presence of hydroquinone (see text).

When using 0.1 units of horseradish peroxidase, 9.5 nmol of acetaldehyde was produced/nmol of P-450, minute.

reductant for horseradish peroxidase, hydroquinone, in conjunction with cytochrome P-450 LMB in the absence of NADPH, produced high blank values, a phenomenon not registered using cytochrome P-450 LM2 (113). Conditions were therefore sought in order to minimize this problem using low amounts of hydroquinone. The hydrogen peroxide production in P-450 LMB-containing incubation systems in the presence of 20 μM hydroquinone and 1 unit of horseradish peroxidase was found to be undetectable. Using a molar ratio of NADPH-cytochrome P-450 reductase:cytochrome P-450 LMB of 4:1 in the membranes, conditions which were found to give minimal effects of hydroquinone per se in the incubation system, the introduction of 1 unit of horseradish peroxidase resulted in 60% inhibition of the ethanol oxidation (Table VIII). Using the standard ratio between the protein components in the membrane, horseradish peroxidase inhibited the rate of ethanol oxidation by 75% both in the presence and in the absence of the Fe-EDTA complex (Table VIII). In the absence of the reductant hydroquinone, no inhibition of the hydrogen peroxide production as well as no inhibition of the cytochrome P-450 LMB-dependent ethanol oxidation was registered (not shown).

**Benzen Oxidation by Cytochromes P-450 LMB and P-450 LM2**—The cytochrome P-450 LM2-dependent oxygenation of benzene was previously found to involve an iron-catalyzed Haber-Weiss reaction (115) and it was hypothesized that the benzene and ethanol-inducible form of cytochrome P-450

### TABLE IX

**Effect of the Fe-EDTA complex (10 μM) in combination with superoxide dismutase (50 μg/ml) on the rate of cytochrome P-450 LM2 and P-450 LMB-dependent oxidation of benzene**

<table>
<thead>
<tr>
<th>System used</th>
<th>Hydroquinone</th>
<th>Catechol</th>
<th>Phenol</th>
<th>Total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450 LM2</td>
<td>3.4</td>
<td>20</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>P-450 LM2 + Fe-EDTA</td>
<td>14.5</td>
<td>6.6</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>P-450 LM2 + Fe-EDTA + superoxide dismutase</td>
<td>4.7</td>
<td>2.4</td>
<td>29</td>
<td>43</td>
</tr>
<tr>
<td>P-450 LMB</td>
<td>6.6</td>
<td>0.3</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>P-450 LMB + Fe-EDTA</td>
<td>12.3</td>
<td>2.8</td>
<td>54</td>
<td>100</td>
</tr>
<tr>
<td>P-450 LMB + Fe-EDTA + superoxide dismutase</td>
<td>8.3</td>
<td>3.0</td>
<td>42</td>
<td>60</td>
</tr>
</tbody>
</table>

* Total conversion includes three unidentified metabolites.
ethanol oxidation by the complex was attained (Fig. 5). Half-maximal activation was evident at only 1 μM concentration of the complex. As little as 0.25 μM endogenous iron complex could explain the rate of ethanol oxidation seen in the absence of added Fe-EDTA. This raises the question whether the enzyme might bind non-heme iron in a specific way. Control experiments in which the enzyme preparations were depleted from EDTA before gel filtration did not show other results. It may be suggested that the redox potential of iron, when chelated, is more appropriate for being reduced by the superoxide anions released from cytochrome P-450 or by P-450 itself. In this context it should be stressed that nonchelated iron did not activate the cytochrome P-450 LMB-dependent ethanol oxidation, even at a concentration of 30 μM. Adding the chelators bathophenanthroline or DETAPAC to “iron-free” incubation systems containing cytochrome P-450 LMB or cytochrome P-450 LM2 resulted in enhanced rates of ethanol oxidation, probably due to residual iron in the systems. These results indicate that the EDTA effects on the cytochrome P-450-dependent ethanol oxidation are caused by the chelating properties of the agent and not allosteric modifications of the enzymes. The necessity of chelated iron for hydroxyl radical production in other systems has previously been established (28, 39, 49).

The presence of an endogenous iron chelator in cytochrome P-450 LMB is further suggested by the finding that the hydroxyl radical scavengers inhibited the ethanol oxidation in the absence of Fe-EDTA in a way that might be predicted for competition with ethanol for hydroxyl radicals (see Table VI and Fig. 8). Furthermore, the rate constant calculated for the reaction between hydroxyl radicals, generated by the enzyme system and ethanol, was very close to the value obtained by other chemical methods as described in the literature. The fact that some scavengers, e.g. hydroquinone, thiourea, and KMBA did not perfectly fit the line might be explained by a higher affinity of these compounds for structures on the enzyme in the neighborhood of the hydroxyl radical production. The finding that microsomes prepared from ethanol-treated animals in sucrose oxidize ethanol at a 3-fold higher rate than microsomes prepared in the same manner from control animals also indicates that there exists an endogenous microsomal iron chelator.

In the Haber-Weiss reaction-dependent ethanol oxidation mechanism previously proposed for the cytochrome P-450 LM2-dependent ethanol oxidation (13, 16), it was suggested that heme iron also might participate in the cleavage of hydrogen peroxide to hydroxyl radicals and hydroxyl ions. The results presented concerning cytochrome P-450 LM2 and cytochrome P-450 LM4 in the present paper strongly suggest that this is not the case.

When using “iron-free” conditions, the expected stoichiometry was attained in the case of cytochrome P-450 LM2-dependent ethanol oxidation reactions. In the absence of the Fe-EDTA complex, the hydrogen peroxide and acetaldehyde formed accounted for more than 92% of the electrons utilized (Fig. 7). Introduction of the Fe-EDTA complex into the incubation system caused a sudden decrease in the amount of hydrogen peroxide produced, both in the presence and in the absence of ethanol. This indicates that the chelated iron participates in the cleavage of hydrogen peroxide even in the absence of ethanol and it is thus plausible that a substantial amount of the electrons end up in water. The Fe-EDTA complex-activated ethanol oxidation was independent of the hydrogen peroxide produced since the rate of ethanol oxidation was linearly increased in response to added complex. By contrast, using cytochrome P-450 LMB-containing membranes, complex-dependent increase in ethanol oxidation was a mirror of the decrease in hydrogen peroxide production (Fig. 6). This indicates a utilization of two electrons in the acetaldehyde formation upon addition of complex, since the rate of NADPH-utilization was not affected. The decrease in hydrogen peroxide production in the absence of ethanol following addition of Fe-EDTA complex exhibited a very similar shape (Fig. 6A). This indicates the presence of unidentified acceptors for hydrogen peroxide cleavage products in or at the neighborhood of the enzyme.

Also in incubation systems containing cytochrome P-450 LMB, the acetaldehyde and hydrogen peroxide formed accounted for a majority of the electrons utilized, in this case 83%, both in the presence and in the absence of the Fe-EDTA complex. The high rate of NADPH-utilization by this type of cytochrome P-450 is remarkable, a finding in agreement with that of Morgan et al. for cytochrome P-450 LM3a (19). By optimizing the amount of reductase in the membranes, the enzyme system consumed more than 200 nmol of NADPH/nmol of P-450 and minute.

The results presented are consistent with the reaction mechanism proposed in Fig. 9. Upon reduction, cytochrome P-450 rapidly binds oxygen and the oxyhemochrome P-450 complex thus formed autoxidizes to liberate superoxide anions. These will reduce ferric-EDTA to ferrous-EDTA and in addition also dismutate to hydrogen peroxide. In addition, the enzyme by itself seems to have the capability to directly reduce the chelated iron. Hydroxyl radicals are generated by an Fe-EDTA-mediated cleavage of the hydrogen peroxide formed. These will abstract a hydrogen atom from ethanol resulting in the formation of an α-hydroxethyl radical (40) which, upon interaction with oxygen, will yield superoxide anion and acetaldehyde. In addition, also the Fe(III)-EDTA complex might abstract the unpaired electron of the hydroxethyl radical. This scheme will fit the observed stoichiometry both regarding cytochrome P-450 LM2 and cytochrome P-450 LM3a-dependent ethanol oxidation reactions even in the absence of the Fe-EDTA complex if another, possibly endogenous type of chelator, replaces EDTA. When using membranes containing cytochrome P-450 LMB, a certain amount of hydroxyl radicals are formed in a Fenton reaction by the direct reduction of Fe(III)-EDTA by ferrous P-450 and the subsequent cleavage of hydrogen peroxide.

When examining ethanol oxidation dependent upon P-450 LM3a (proposed to be identical with or very similar to P-450 LMB), Morgan et al. (19) did not register any inhibition of the process by superoxide dismutase and therefore excluded a Haber-Weiss reaction for generation of hydroxyl radicals acting as the active oxygen species for ethanol oxidation. This finding is in agreement with ours, but, however, they also ruled out the participation of free hydroxyl radicals in the process, a statement which is contradictory to our results. It seems that their conclusion emerged because of the use of Me₃SO as the only hydroxyl radical scavenger, a compound that, in agreement with our findings, also binds to the enzyme.

Only small rates of hydroxyl radical-mediated destruction of deoxyribose, hydroxyl radical-dependent formation of the DMPO·-OH adduct and hydroxyl radical-dependent formaldehyde production from Me₃SO were attained in vesicles only containing NADPH-cytochrome P-450 reductase compared to vesicles having also cytochrome P-450 incorporated. Titration of NADPH-cytochrome P-450 reductase-containing vesicles with cytochrome P-450 LM2 resulted in a 7-fold increase in the rate of superoxide anion production, a 5-fold enhancement.

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4 Ekström, G., Cronholm, T., and Ingelman-Sundberg, M., unpublished results.
ment of hydrogen peroxide production (Fig. 2), and an 8-fold increase in the rate of cytochrome P-450 LM2-dependent ethanol oxidation (Fig. 4). These results indicate that cytochrome P-450 and not NADPH-cytochrome P-450 reductase is the major source for the superoxide anions liberated in the system, and thus also for the hydrogen peroxide and hydroxyl radicals formed. The results are well in agreement with those of Ullrich and Kuthan (41, 42). They have found that (i) the NADPH-dependent formation of superoxide anions in liver microsomes is as sensitive towards carbon monoxide as is the formation of hydrogen peroxide, (ii) maximal reversibility of the CO-inhibited production of superoxide anions and hydrogen peroxide is obtained by monochromatic light with a wavelength of 450 nm and (iii) a ratio of about 2:1 between superoxide anions and hydrogen peroxide is obtained in reconstituted cytochrome P-450-containing systems. Taken together, their results indicate that the major part of the hydrogen peroxide in liver microsomes is formed via dismutation of superoxide anions released from the oxyctochrome P-450 complex (41). In our previous paper about the mechanism of cytochrome P-450 LM2-dependent ethanol oxidation, it was described that substitution of cytochrome P-450 for cytochrome b5 resulted in only 14% of the rate of ethanol oxidation (13). Similar results were also obtained when examining cytochrome P-450-dependent hydroxyl radical-mediated destruction of deoxyribose (16), findings in agreement with the proposal of cytochrome P-450 as the major source for superoxide anions. Furthermore, we found that introduction of cytochrome b5 into membranes containing NADPH-cytochrome P-450 reductase and cytochrome P-450 LM2 resulted in increased ability of these vesicles to catalyze O-demethylation of paracetamol as well as a concomitant decrease in superoxide anion production and hydrogen peroxide formation by the membranes (23), which is in accordance with the oxyctochrome P-450 complex being the source for the superoxide anions in the system (43, 44). These results, however, are in disagreement with those of Winston and Cederbaum (45, 46). These authors did not find any role for cytochrome P-450 in the production of hydroxyl radicals and only a little role for cytochrome P-450 in ethanol oxidation in a reconstituted system consisting of cytochrome P-450 from phenobarbital-treated rats, dilauroylphosphatidylcholine and NADPH-cytochrome P-450 reductase. One plausible reason for this discrepancy might be the different incubation conditions used. Winston and Cederbaum have almost throughout their studies used a ratio of NADPH-cytochrome P-450 reductase to cytochrome P-450 of 10,000 units to 1 nmol, i.e. a molar ratio (32,000 units of reductase/mg (45)) for reductase:P-450 of 4.3:1 compared to 1:3.5 used in the present investigation. As is evident from Figs. 2 and 4, introduction of cytochrome P-450 into reductase-containing vesicles to yield their enzymic ratio will result in only a slight enhancement of superoxide anion production, hydrogen peroxide formation, and, thus, the concomitant hydroxyl radical generation. In this respect, one should keep in mind that the molar ratio of P-450 to reductase in microsomes is 20 (47).

When comparing the ethanol and benzene-inducible form of cytochrome P-450 with the other cytochromes P-450, it is evident that the response of this enzyme towards the Fe-EDTA complex during NADPH-dependent ethanol oxidation is the most striking difference between the enzymes. According to the results attained using either benzene, deoxyribose, or KMBA as substrates for the cytochrome P-450-dependent hydroxyl radicals, it is remarkable that membrane vesicles containing cytochrome P-450 LMeB do not liberate significantly more hydroxyl radicals into solution than do vesicles containing the other cytochromes. This is also true when examining the rates of benzene oxidation by P-450 LM2 and P-450 LMeB either in the presence or absence of the Fe-EDTA complex. The difference between P-450 LM2 and the other cytochromes P-450 is only evident when comparing the rate of cytochrome oxidation and also, in comparison to P-450 LM2, when taking MeSO3- into account. These two substrates are also the only ones that cause a decrease of the cytochrome P-450 LMeB-dependent NADPH-consumption (Table VII). One might therefore assume that these compounds affect the functional properties of the enzyme in some way. Addition of Fe-EDTA drastically decrease the rate of P-450 LMeB-dependent hydrogen peroxide production in the absence of ethanol (Fig. 6). This implies that the enhanced amounts of hydroxyl radicals thus formed are trapped by an endogenous scavenger since more hydroxyl radicals are not detected in solution. Under ordinary conditions, this proposed endogenous scavenger would have a higher rate constant for reaction with the hydroxyl radical. However, in the presence of ethanol, some functional property of the enzyme is changed (cf. above), and the exogenous scavenger is favored, possibly due to a conformational change of the enzyme. In such a case, other exogenous scavengers would compete with ethanol in a manner which is in agreement with the results presented in Fig. 8.

The results presented indicate the absence of a specific cytochrome P-450-catalyzed mechanism of ethanol oxidation.

\footnote{Interactions of hydroxyl radicals with MeSO3- will result in the production of methane and formaldehyde. Only the latter product was determined here.}
Significant ethanol metabolism is only observed in the presence of chelated iron. This raises the question of what significance the cytochrome P-450-dependent ethanol oxidation plays in vivo. Microsomal cytochrome P-450 might be important for generation of intracellular hydrogen peroxide (68). According to the results by us and by Ullrich and collaborators (see above), the cytochrome P-450-dependent hydrogen peroxide formation entirely occurs via dismutation of superoxide anions. The subsequent formation of hydroxyl radicals according to the Haber-Weiss scheme would require the presence of adventitious chelated iron in the endoplasmic reticulum, eventually near the active site of cytochrome P-450, as is suggested for cytochrome P-450 Lmub in the present paper. This concept is in part in accordance with the results of Cederbaum and Dicker (49) who showed that the effective iron-chelating agent desferoxamine inhibited NADPH-dependent microsomal hydroxyl radical generation.

There is presently evidence accumulating for a role of the iron-catalyzed Haber-Weiss reaction in vivo. Reoxidation of dialric acid, the reduced form of alloxan, will generate superoxide anions and the toxic effects of alloxan in the pancreatic islets have been found to be mediated by hydroxyl radicals (50, 51) subsequently formed in a Haber-Weiss reaction. Thus, the infusion of superoxide dismutase bound to polyethylene glycol to rats will prevent the toxic effects of alloxan in vivo (52); similarly, addition of superoxide dismutase, hydroxyl radical scavengers, or effective metal chelators will inhibit the alloxan-mediated destruction of isolated islets in cell culture and in vivo (51, 53-57). EDTA, which does not chelate iron in a manner that reduces its ability to allow production of hydroxyl radicals from hydrogen peroxide (58, see above) does not protect from alloxan (59). Cell damage caused by x-ray irradiation is thought to be mediated by hydroxyl radicals (60-63). Addition of superoxide dismutase will protect cells as well as whole animals from the ionizing radiation damage (64). These results imply the action of chelated iron in vivo for generation of hydroxyl radicals. Allylisopropylacetamide is known to decrease the hepatocyte concentration of glutathione (65) and will thus enhance microsomal lipid peroxidation. Administration of desferoxamine to rats in conjunction with allylisopropylacetamide will protect microsomal membranes from the enhanced lipid peroxidation activity (65), suggesting an in vivo role for adventitious iron in the microsomal lipid peroxidation process. Taken together, these results suggest a role for adventitious iron under in vivo conditions. It may therefore be suggested that under alcoholic liver disease conditions, ethanol-inducible forms of cytochrome P-450 in conjunction with the iron overload seen under these circumstances (66, 67) might contribute to enhanced rates of hepatic ethanol oxidation. The role of the proposed elevated hydroxyl radical formation by the ethanol-inducible form of cytochrome P-450 in causing cell damage remains to be established.

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Mechanisms of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochromes P-450.

M Ingelman-Sundberg and I Johansson


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