Ethanol Oxidation by a Component of Liver Microsomes Rich in Cytochrome P-450*

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**SUMMARY**

A cytochrome P-450-rich fraction free of alcohol dehydrogenase activity and containing only small amounts of catalase was prepared from rat liver microsomes and shown to catalyze the NADPH-dependent oxidation of ethanol. The recoveries of cytochrome P-450 and the activity of the NADPH-dependent ethanol oxidation were 15.6% and 14.0%, respectively. This fraction oxidized ethanol only in the presence of a system generating H2O2. It is concluded that ethanol oxidation can be catalyzed by a microsomal component rich in cytochrome P-450 and separate from containing alcohol dehydrogenase and catalase.

**EXPERIMENTAL PROCEDURE**

**Materials**—Cholic acid (sodium salt), dithiothreitol, horse heart cytochrome c, hypoxanthine, xanthine oxidase (Grade II), O-dianisidine, horseradish peroxidase (type I), isocitric acid, isocitric dehydrogenase (type IV), NAD+, NADP+, 3-aceetylpyridine adenine dinucleotide, and DEAE-cellulose were purchased from Sigma. Sephadex G-25 was obtained from Pharmacia, sodium azide and sodium cyanide from Fisher, pyrazole from Aldrich, and aminopyrine and N,N-dimethyl-n-aminoditylamine from K & K Laboratories (Plainview, N. Y.). Carbon monoxide was purchased from Matheson. SKF 525-A was a gift of Smith, Kline and French Laboratories (Philadelphia). The DEAE-cellulose was washed prior to use by the method of Peterson and Sober (8).

**Methods**—Male albino Wistar rats weighing between 150 and 200 g were fed Purina Laboratory Chow ad libitum and administered daily intraperitoneal injections of phenobarbital (100 mg per kg) for 3 days. The rats were killed by decapitation 48 hours after the last injection of phenobarbital. The livers were excised, rinsed repeatedly with 1.15% KCl, weighed, minced, and then homogenized in a Potter-Elvehjem homogenizer with 3 volumes of 0.25 M sucrose in 0.1 M Tris-HCl buffer, pH 7.4. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C. The resulting precipitate was discarded and the supernatant fraction centrifuged at 106,000 × g for 60 min at 4°C. The resulting microsomal pellet was washed once with 8 ml of the above buffer and then centrifuged again. The washed microsomes were then solubilized by the method used by Lu et al. for the preparation of their cytochrome P-450 fractions (9). Solid ammonium sulfate was then added to 50% saturation. The resulting precipitate was dissolved with 0.01 M Tris-HCl buffer, pH 7.7, containing 10⁻⁴ M dithiothreitol, 10⁻⁴ M EDTA, 20% glycerol, and 0.2% sodium cholate, and dialyzed against the same buffer for 2 hours. The dialyzed preparation could then be stored at −20°C for up to 1 month without any significant loss of cytochrome P-450. Subsequently, 10 to 15 ml of the dialyzed preparations were dialyzed against a Sephadex G-25 column (2.5 × 45 cm) eluted with the above buffer. The desalted fraction containing the cytochrome P-450 was then placed on a DEAE-cellulose column (2.5 × 45 cm) previously equilibrated with the same buffer, and eluted by adding 0.1 M KCl to the buffer and increasing the KCl concentration stepwise by 0.1 M increments up to 0.5 M (10). Fractions of 7 ml were collected at
a flow rate of about 30 ml per hour. In a different experiment, a lipid fraction was separated directly from solubilized microsomes by DEAE-cellulose chromatography (11).

Assays were performed as follows: cytochrome P-450, P-420, and b3 by the methods of Omura and Sato (12) with a Cary model 15 recording spectrophotometer; NADPH-cytochrome c reductase by the method of Masters, Williams, and Kamin (13); the NADPH ethanol-oxidizing activity as described by Lieber and DeCarli (3); alcohol dehydrogenase as described previously (14), except that NADP+ was substituted by 3-acetylpyridine adenine dinucleotide in the reaction mixture (6); catalase activity by the method of Feinstein (15), and when not detectable also with a Clark O2 electrode according to Goldstein (16); N-demethylation of aminopyrine by the method of Lu, Strobel, and Coon (17); N,N-dimethyl-n-octylamine N-oxidase activity spectrophotometrically by measuring the substrate-dependent oxidation of NADPH (18), and also by measuring the amine oxidation product (19); protein concentration according to Lowry et al. (20); and lipid concentration by the method of Amenta (21).

RESULTS

Preliminary experiments with the components obtained by DEAE-cellulose chromatography of soluble microsomes using the first method described by Lu and Coon (11) indicated that the NADPH-dependent ethanol-oxidizing activity was associated with the cytochrome P-450 component and that neither cytochrome c reductase nor lipid was required. However, since the recovery of the cytochrome P-450 was inadequate, improved methods for the preparation of cytochrome P-450 were then undertaken (9). The steps in the preparation are shown in Table I. There was a similar recovery of cytochrome P-450 and the NADPH-dependent ethanol-oxidizing activity at each step. By DEAE-cellulose chromatography (Fig. 1) there was a partial separation of cytochrome P-450 from catalase and cytochrome c reductase. Catalase activity determined by the method of Feinstein (15) was detected in the early fractions containing cytochrome P-450, but not in the peak fractions, while assay with an oxygen electrode revealed an activity

![Table I](http://www.jbc.org/)  
Steps in preparation of cytochrome P-450 fraction and corresponding activities of NADPH-dependent ethanol-oxidizing system

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cytochrome P-450</th>
<th>NADPH-dependent ethanol-oxidizing activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein/ mg</td>
<td>Total activity/ nmol/ min</td>
</tr>
<tr>
<td>Microsomes</td>
<td>550.4</td>
<td>880.3</td>
</tr>
<tr>
<td>50% saturated (NH4)2SO4</td>
<td>338.3</td>
<td>412.9</td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td>215.3</td>
<td>331.9</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>84.5</td>
<td>137.5</td>
</tr>
</tbody>
</table>

![Fig. 1](http://www.jbc.org/)  
DEAE-cellulose chromatography of a solubilized microsomal preparation from rat liver. The column (2.5 X 40 cm) was eluted by stepwise increases in KCl concentration from 0.1 to 0.5 M in buffer, and fractions of 7 ml were collected at a flow rate of about 30 ml per hour. The methods for the various assays shown are listed in the text.
of 0.03 mmole of O₂ evolved per min per ml in the peak cytochrome P-450 fraction. This activity is 1/6 of the 1.6 mmoles of O₂ evolved per min per ml found in the peak catalase fraction by the same method. The NADPH-dependent ethanol-oxidizing activity clearly paralleled the concentration of cytochrome P-450, and was not present in the fractions rich in catalase.

Small amounts of cytochrome P-420 were found in the cytochrome P-450 fractions. The concentration of cytochrome P-420 in the peak cytochrome P-450 fraction (No. 43) was 0.18 nmole per ml which is 1/4 of the 0.06 nmoles per ml of cytochrome P-450 present in that fraction. No alcohol dehydrogenase activity, cytochrome b₅₄₈ or N,N-dimethyl-N-ethylamine N-oxidase activity were detected in any of the fractions eluted.

Reconstitution experiments indicated that the addition of separated cytochrome c reductase to the cytochrome P-450 fraction resulted in an increase in NADPH-dependent oxidation of ethanol (Table II), while the addition of microsomal lipid had a mild inhibitory effect on the activity exhibited by the combined cytochrome P-450 and cytochrome c reductase fractions. Addition to the cytochrome P-450 fraction of excess amounts of cytochrome c reductase, 17.5 nmols per min or more, both in the presence and absence of microsomal lipid resulted in inhibition of NADPH-dependent oxidation of ethanol. The significant oxidation of ethanol by the cytochrome P-450 fraction alone and the mild inhibitory effect of microsomal lipid on the oxidation is in contrast to the requirement of a combination of cytochrome P-450, cytochrome c reductase, and lipid for optimal aminopyrine demethylation (Table III). The cytochrome P-450 fraction exhibited typical type I and II binding spectra in the presence of 3.3 mm hexobarbital and 3.2 mm aniline, respectively. Addition of ethanol resulted in a spectral change with a trough at 387 and a peak at 415 nm. The Kₘ for the NADPH-dependent oxidation of ethanol by the cytochrome P-450-rich fraction was determined to be 10.3 mM.

### Table II

**Ethanol oxidation by reconstituted rat liver microsomal enzyme systems**

The volume of the reaction mixture was 3 ml. Its composition was as follows: K₂HPO₄-Na₂HPO₄ buffer (pH 7.4), 0.1 mol; magnesium chloride, 5.0 mm; nicotinamide, 5.0 mm; NADPH, 0.3 mm; sodium isocitrate, 8 mm; isocitric dehydrogenase (Sigma type IV), 100 µL; ethanol, 0.1 mg; and any of the following microsomal fractions: cytochrome P-450, 0.5 nmole (containing 1.2 nmoles per min of cytochrome c reductase); cytochrome c reductase, 5.0 nmols per min; lipid, 0.1 mg; and catalase, 0.3 meq of NaB₃O₃ destroyed per min. The amount of ethanol oxidized was determined from the acetaldehyde formed as described by Lieber and DeCarli (3).

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Acetaldehyde formed</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg/min</td>
<td>%</td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td>3.7</td>
<td>50.9</td>
</tr>
<tr>
<td>Cytochrome P-450 + cytochrome c reductase</td>
<td>6.5</td>
<td>100.0</td>
</tr>
<tr>
<td>Cytochrome P-450 + cytochrome c reductase + lipid</td>
<td>6.1</td>
<td>93.8</td>
</tr>
<tr>
<td>Cytochrome c reductase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome c reductase + lipid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Catalase</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The effect of inhibitors on the NADPH-dependent ethanol oxidation by the cytochrome P-450-rich fraction is shown in Table IV. Neither sodium cyanide nor sodium azide at 0.1 mm concentrations inhibited the activity. Incense of the sodium azide concentration to 1 mm resulted in marked inhibition. Pyrazole, 2 mm, was only a slight inhibitor, and SKF 525-A, 1 mm, resulted in 34.1% inhibition. Carbon monoxide decreased the activity markedly, while replacement of air by nitrogen abolished it completely.

The cytochrome P-450-rich fraction also oxidized ethanol in the presence of an H₂O₂-generating system, although at a slightly slower rate than with the NADPH-generating system (Table V). The catalase-rich fraction oxidized ethanol only with an H₂O₂-generating system. Ethanol oxidation activity was not increased by the addition of the catalase fraction to the cytochrome P-450 fraction in the presence of either the NADPH- or the H₂O₂-generating system. The addition of cytochrome c reductase to the catalase fraction failed to result in activity in the presence of the NADPH-generating system and did not enhance the activity with the H₂O₂-generating system. NADPH-dependent ethanol oxidation by the cytochrome P-450 fraction was not inhibited by sodium formate, 150 mm, a per...
Comparison of the NADPH-generating system were the same as those listed in Table II. Hydrogen peroxide was generated with 3.3 mM hypoxanthine and 1 unit per ml of reaction mixture of xanthine oxidase (Sigma type II). The microsomal fractions were present in the following amounts: cytochrome P-450, 0.6 nmole (containing 5.4 nmodes of cytochrome c reductase per min); catalase, 1.52 meq of NaB03 destroyed per min.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acetaldehyde formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADPH-generating</td>
</tr>
<tr>
<td></td>
<td>H2O2-generating</td>
</tr>
<tr>
<td></td>
<td>system</td>
</tr>
<tr>
<td></td>
<td>system</td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td>6.9</td>
</tr>
<tr>
<td>Catalase</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome P-450 + catalase</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Discussion

Ethanol oxidation by liver microsomes was first reported by Orme-Johnson and Ziegler (2). Lieber and DeCarli (4) studied the characteristics of this enzyme system and demonstrated its inducibility by feeding ethanol. The oxidation of ethanol by the microsomes was considered to be mediated by the mixed function oxidase system since ethanol was shown to bind to cytochrome P-450 producing a modified type II spectrum (22), the reaction required NADPH and oxygen and was inhibited by carbon monoxide. Sodium cyanide, sodium azide, and the administration of 3-amino-1,2,4-triarsol (all inhibitors of catalase) and pyrazole (an inhibitor of alcohol dehydrogenase) produced only slight inhibition of the NADPH-dependent ethanol-oxidizing activity by the microsomes, suggesting that neither of these contaminating enzymes was responsible for the observed ethanol-oxidizing activity (4). Other investigators (5, 6), however, observed that substitutions of the NADPH-generating system by one generating H2O2 resulted in comparable oxidation of ethanol by the microsomes. In view of this and of their finding of more significant inhibition of the reaction by catalase inhibitors than found by Lieber and DeCarli, they postulated that ethanol was oxidized by catalase in the presence of H2O2 generated from NADPH and oxygen by microsomal NADPH oxidase. Subsequently, Thurman et al. (7) provided further support for this mechanism by demonstrating sufficient H2O2 formation from NADPH by microsomes, the same Michaelis-Menten constants for ethanol oxidation with both the NADPH- and H2O2-generating systems, as well as similar inhibition by catalase inhibitors, by a peroxidatic substrate of catalase, and by a H2O2-utilizing process in the presence of both generating systems. However, while these studies provided new evidence for the ability of catalase present in the microsomes to participate in the oxidation of ethanol, they did not resolve the question as to whether or not catalase is indeed the principal and only mediator of the NADPH-dependent ethanol oxidation by the microsomes. The present study indeed shows that a component of the microsomes rich in cytochrome P-450, free of alcohol dehydrogenase, and containing only small amounts of catalase, is capable of the oxidation of ethanol. The recovery of the NADPH-dependent ethanol-oxidizing activity in this fraction was 14.0% and approximated a 15.6% recovery of cytochrome P-450. The addition of cytochrome c reductase to the cytochrome P-450 fraction enhanced, while the further addition of lipid resulted in mild inhibition of the ethanol-oxidizing activity. Previous studies had shown that all three components were necessary for optimal hydroxylation and demethylation of a number of drugs (9, 17) and this was confirmed for the demethylation of amphetamine with the fractions obtained in this study. The mechanism by which the cytochrome P-450 fraction catalyzes the oxidation of ethanol remains unknown. In support of the involvement of cytochrome P-450 in the oxidation of ethanol are the demonstration of binding of ethanol to the hemoprotein and the inhibition of the reaction by carbon monoxide. NADPH may be able to reduce cytochrome P-450 directly (23) or by a flavoprotein other than NADPH cytochrome c reductase. The small amount of cytochrome c reductase remaining in the cytochrome P-450 fraction may mediate NADPH-linked cytochrome P-450 reduction which is sufficient to account for the NADPH-dependent ethanol oxidation in this fraction. The enhanced ethanol-oxidizing activity obtained after addition of cytochrome c reductase would be in support of this mechanism. Cytochrome P-450 has peroxidatic activity (24) and has been shown to support the hydroxylation of benzphetamine and ethylmorphine in the presence of a superoxide-generating system (xanthine and xanthine oxidase) (25). A similar mechanism may explain the oxidation of ethanol in the presence of xanthine and xanthine oxidase observed here. The NADPH-dependent oxidation of ethanol could also be mediated by superoxide or hydrogen peroxide generated from NADPH and O2 by an enzyme system present in the cytochrome P-450-rich fraction; however, failure to inhibit the reaction by a hydrogen peroxide-utilizing system makes this less likely. However, it is clear that the reaction which oxidizes ethanol is far from purified cytochrome P-450 and it may well be that it contains an enzyme system which oxidizes ethanol and does not require cytochrome P-450 as has been shown to be the case for the mixed function amine oxidase (18).

References

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