ETHANOL OXIDATION BY COMPONENTS OF RAT LIVER MICROSOMES

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Summary

A new method was employed for the purification of cytochrome P-450 from rat liver microsomes. The purified cytochrome was essentially free from possible contaminants and the recovery and degree of purification were high. Although 15% of the original P-450 was recovered through the purification procedure used, only 0.8% of the total original microsomal ethanol oxidation activity was associated with this fraction. Addition of this purified fraction to other fractions isolated did not further stimulate ethanol oxidation. The component of rat liver microsomes that was found most efficient in the oxidation of ethanol was the mixture of catalase and NADPH - cytochrome c - reductase. It is concluded that highly purified cytochrome P-450 by itself does not oxidize ethanol to any appreciable degree.

Since Orme-Johnson and Ziegler (1) first demonstrated the involvement of liver microsomes in the oxidation of ethanol, several investigators have attempted to further characterize the system. Lieber and DeCarli (2) demonstrated that the system was inducible by feeding rats ethanol. These same authors (3) suggested that the oxidation of ethanol by the microsomes was catalyzed by the mixed function oxidase system because the system required NADPH and oxygen. More recently, Mezey et al. (4) reported that a component of liver microsomes which was rich in cytochrome P-450 catalyzed the NADPH-dependent oxidation of ethanol.

The present investigation was undertaken to determine whether purified cytochrome P-450, alone and in combination with other fractions, is involved in the oxidation of ethanol.

Methods

Male albino Sprague-Dawley rats weighing 100-200 gms were given intraperitoneal injections of phenobarbital (60 mgs/kg) for 5 days. The rats were

sacrificed 24 hours after the last injection by a blow on the head. Livers were suspended in 3 volumes of 0.25 M sucrose in 0.1 M Tris-HCl buffer, pH 7.5 and homogenized in a Waring blender at full speed for 60 seconds. The homogenate was centrifuged at 10,000 x g for 10 min., filtered through 4 layers of cheesecloth and centrifuged again at 10,000 x g for 10 min. The supernatant solution was then centrifuged at 105,000 x g for 1 hour.

The resulting microsomal pellet was solubilized in a solution which was a modification of that used by Lu et al. (5). The solubilization solution consisted of 110 mM sucrose, 20 mM KCl, 20 mM sodium citrate pH 7.5, 1.25 mM DTT, 20% glycerol and 0.5% deoxycholate. The microsomal pellet was stirred for 1 hour at 4°C in the solubilization buffer then centrifuged for 2 hours at 105,000 xg and the resultant pellet discarded.

The supernatant solution was applied to a DEAE-cellulose column (4.0 x 45 cms) which had been equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, containing 0.1 mM DTT and 5% glycerol. After application of the solubilized microsomal fraction, the column was washed with 0.1 M potassium phosphate buffer until the A_{280} of the eluate dropped to approximately 0.150. This step washed out all material that did not bind to the DEAE-cellulose as well as the catalase fraction (5). Cytochrome P-450 was then eluted by using a linear 0.1 M - 0.3 M potassium phosphate gradient. Material that was still absorbed to the DEAE-cellulose after the end of the gradient was eluted with 1.0 M potassium phosphate buffer.

Fractions with a high P-450 specific activity were pooled and concentrated through a PM-10 Amicon filtration membrane. The concentrate was applied to a Sephadex G-200 column (2.0 x 60 cms) and eluted with 0.4 M potassium phosphate buffer.

Assays for P-450 and P-420 were performed according to the method of Omura and Sato (6) using a Colemen 124 spectrophotometer. NADPH reductase was assayed by the method of Masters et at. (7), ethanol oxidation according to Lieber and DeCarli (3), catalase activity with a Clark oxygen electrode according to the procedure of Goldstein (8) and protein concentration by the method of Lowry et al. (9)

Results and Discussion

In order to establish whether or not cytochrome P-450 catalyzes the NADPH-

dependent oxidation of ethanol, it is essential that it is purified free of contaminants. The procedure that was followed in purifying cytochrome P-450 resulted in preparations that were essentially free from catalase or NADPH-cytochrome c - reductase as determined by the methods employed. The different steps in the preparation of cytochrome P-450 are shown in Table 1.

Table 1.

Steps in the purification of cytochrome P-450

Fraction	Total Protein	Total P-450	Specific Activity
	mgs	nmoles	nmole P-450/ mg protein
Microsomes	2184.0	2132	0.98
DEAE-Cellulose	167.0	325	1.90
Sephadex G-200	68.0	319	4.70

According to Lu et at. (5), catalase could be eluted from DEAE-cellulose with 0.1 M potassium phosphate buffer. Thus washing the column with this buffer after application of the solubilized microsomes eluted the materials that did not initially bind to DEAE-cellulose as well as the catalase fraction. This fraction had no detectable amount of cytochrome P-450.

The use of a gradient to elute cytochrome P-450 from the DEAE-cellulose column (Fig. 1) resulted in a more homogeneous preparation. This is evident from the fact that with increasing A_{280} there is a concomitant and proportional increase in the P-450 content, i.e. there is a relatively constant specific activity in the fractions collected. Inclusion of the Sephadex G-200 column (Fig. 2), purified cytochrome P-450 from other contaminants that might have been carried over from the DEAE-cellulose column. Cytochrome P-450 recovered from the G-200 step showed no detectable activity of either catalase or NADPH-cytochrome c- reductase. Moreover, all of the P-450 applied to the G-200 was recovered (Table 1) which makes this an efficient purification step. The recovery of 15% of the total P-450 accom-

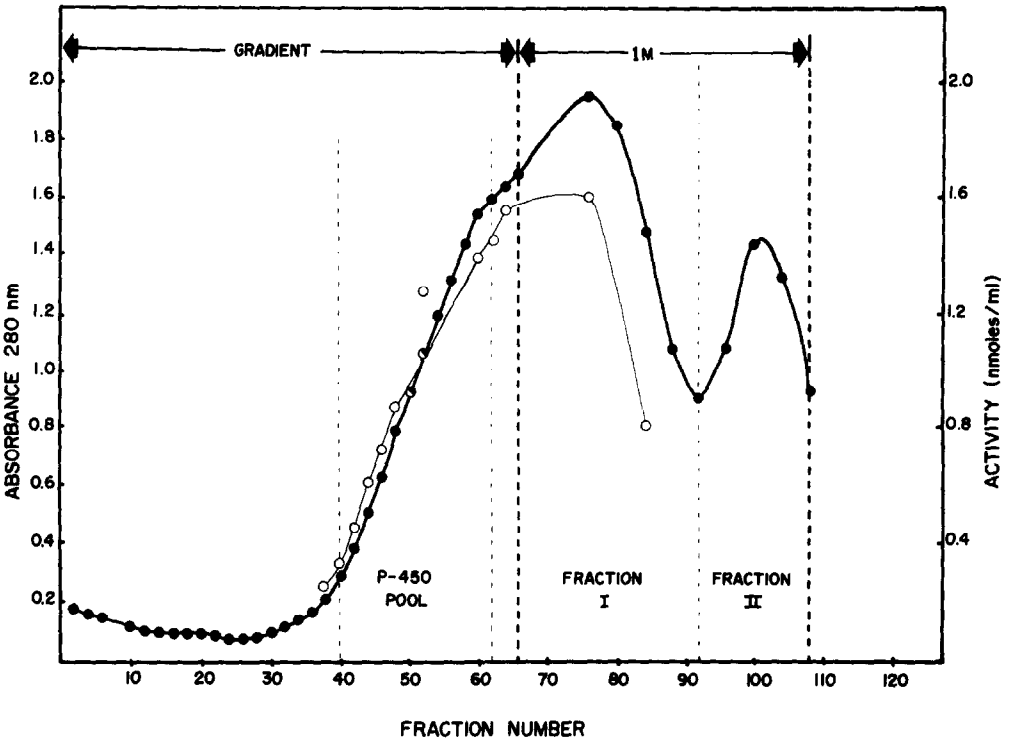

Fig. 1. Elution of cytochrome P-450 and other proteins from the DEAE-cellulose column (4.5 x 45). Cytochrome P-450 was eluted with 0.1 - 0.3 M potassium phosphate linear gradient and 12 mls fractions were collected. Other proteins were eluted with 1.0 M potassium phosphate. Pools were made as shown in the figure. ●—● A_{280}; O—O cytochrome P-450 concentration (nmoles/ml).

panied with a 5 fold purification demonstrates the efficiency of this procedure.

The material that remained absorbed to the DEAE-cellulose column after the end of the gradient was eluted with 1.0 M potassium phosphate buffer and saved (Fraction I and II, Fig. 1). No attempt was made to determine the concentration or the constituents of these fractions other than assaying for NADPH-cytochrome c reductase. It was assumed that both the NADPH-cytochrome c - reductase and the lipid fraction of the microsomes would be eluted with 1.0 M potassium phosphate since it is reported that 0.3 M potassium phosphate elutes the reductase and 0.5 M elutes the phospholipid (4, 5). Fraction I did not contain any detectable amounts of the reductase but had low concentration of P-450 and was not pooled with the other fractions of P-450. Fraction II contained NADPH-cytochrome c - reductase activity and traces of cytochrome P-450 (0.3 nmoles/ml).

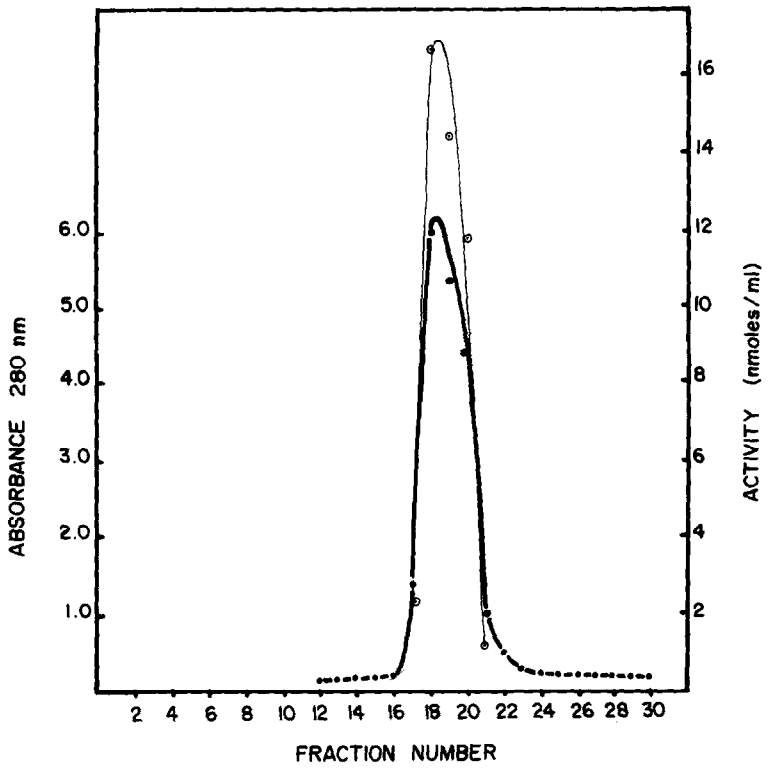

Fig. 2. Elution profile of cytochrome P-450 from Sephadex G-200 column (2.0 x 60). The cytochrome was eluted with 0.4 M potassium phosphate buffer and 3 mls were collected in each fraction. ●—● A_{280}; O—O P-450 concentration (nmoles/ml).

Each of the four fractions obtained, i.e. the Wash, purified cytochrome P-450, and Fractions I and II, was assayed for its ethanol-oxidizing activity separately and in various combinations as is shown in Table 2.

The individual specific activities of the Wash, P-450 and Fraction I were approximately 25% of that of the solubilized microsomes whereas that of Fraction II was approximately half. Total acetaldehyde producing capacity was lowest in the purified cytochrome P-450 and less than 1% of the original activity was recovered. Although 15% of the total P-450 content of the solubilized microsomes was recovered after the G-200 step, only 0.8% of the original ethanol oxidation activity was associated with this fraction. This is in contrast to Mezey et al. (4) who found that the recoveries of cytochrome P-450 and the activity of the NADPH-dependent ethanol oxidation were 15.6% and 14.0% respectively.

Table 2.

Ethanol oxidation by different fractions from rat liver microsomes

The incubations were done in center-well flasks according to the procedure of Lieber and DeCarli (3). Each flask contained 0.36 µmoles NADPH; 0.30 mmoles ethanol, sample (1-3 mgs protein) and 0.10 M potassium phosphate buffer pH 7.4 to make a final volume of 3.0 mls. The center well contained 0.6 ml of 0.15 M semicarbazide hydrochloride in 0.10 M potassium phosphate buffer pH 7.0

	µgs Acetaldehyde Produced/ mg protein	Total Acetaldehyde produced (µgs)	Percent stimulation (over individual) sums
Microsomes	1.250	2725	
Wash	0.375	255	
P-450	0.310	21	
Fraction I	0.350	79	
Fraction II	0.610	45	
Wash + P-450	0.590	449	57
Fraction II + P-450	0.275	39	2
Fraction II + Wash	1.620	1221	220
Fraction II + Wash + P-450	0.890	732	175
Fraction II + Catalase	1.425		300

When combinations of the various fractions were assayed for ethanol oxidation, a pattern became evident. Fraction I in combination with any other fraction showed no significant stimulatory effect; neither did mixing cytochrome P-450 and Fraction II. Mixing cytochrome P-450 with the Wash, however, showed an almost 60% stimulation in ethanol oxidation over the sums of the oxidizing capacity of either fraction alone. When Fraction II and Wash were mixed and assayed for ethanol oxidation, there was 220% stimulation. Furthermore, the specific activity of this mixture was higher than that of the microsomal fraction, thus denoting a more purified (or activated) ethanol oxidizing system. Including cytochrome P-450 with the latter mixture lowered the specific activity because there was

an increase in the protein content without a proportional increase in the ethanol oxidation activity. Finally, commercial catalase, in combination with Fraction II gave a specific activity and a stimulatory effect that was comparable to the combination of Wash and Fraction II.

These data indicate that all three fractions are necessary for maximal oxidation of ethanol by the microsomal system. No one component alone, in terms of total activity, could oxidize ethanol to any appreciable extent, although each fraction retained some oxidative capacity. This probably indicates that each of the fractions obtained may have been contaminated with the other components of the oxidizing system. Evidence of the involvement of the three fractions is obtained from the degree of stimulation of the ethanol oxidizing capacity of the mixtures as compared to the sums of the individual fractions. It is possible that Fraction II contained enough P-450 for ethanol oxidation and hence, addition of more P-450 did not enhance this activity.

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