ETHANOL OXIDATION BY LIVER MICROSOMES: EVIDENCE AGAINST A SEPARATE AND DISTINCT ENZYME SYSTEM*

Kurt J. Isselbacher and Edward A. Carter

Department of Medicine, Harvard Medical School

and Massachusetts General Hospital, Boston, Mass. 02114

Received April 1, 1970

Summary

The TPNH dependent oxidation of ethanol by rat liver microsomes has been studied. Microsomes isolated by the usual centrifugation techniques contain TPNH oxidase, catalase and alcohol dehydrogenase (ADH). The latter two enzymes, known to be involved in ethanol metabolism, appear to be present largely as contaminants and their activity can be removed or decreased by washing. No evidence was obtained for the presence of a separate and distinct membrane bound ethanol oxidase. It is concluded that the TPNH and 0_2 dependent metabolism of ethanol by microsomes can readily be explained by the coupled and combined activities of TPNH oxidase, catalase and ADH.

Several recent reports[1-4] have suggested that the microsomal fraction of the liver catalyzes the oxidation of ethanol to acetaldehyde. This so-called microsomal ethanol oxidizing system ("MEOS") requires TPNH and 0_2 and has been considered distinct and separate from both alcohol dehydrogenase (ADH) and catalase. Lieber and De Carli[3,4] have considered this system to be also of significance *in vivo* and to show an increase in activity following ethanol administration. The present studies were carried out to better define and elucidate the mechanism involved in the microsomal oxidation of ethanol *in vitro*. The results obtained provide no evidence for the existence of a separate and distinct membrane-bound ethanol oxidase.

Experimental Procedure

Female Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were killed by decapitation and 20% liver homogenates prepared using 0.25 M

*This work was supported by grants from the John A. Hartford Foundation, Inc. and the National Institutes of Health (MH-16892).

sucrose and a Potter-Elvehjem homogenizer. Microsomes were prepared by centrifugation at 105,000 x g as described previously[5] and resuspended in an equal volume of 1.15% KCl. "MEOS" was determined according to Lieber and De Carli[3] by measuring the production of acetaldehyde semicarbazone (E_{224} = 9.41 cm^2 $micromole^{-1}$, pH 7.0). ADH activity in the liver supernatant was measured using previously described methods[6,7]. In experiments with 3-acetylpyridine DPN (3-AP-DPN), the rate of 3-AP-DPNH formation was measured according to Mezey et al[6]. Assays for catalase[8] and TPNH oxidase[9] activities were carried out using 10% microsomal suspensions. In experiments with 3-amino-1,2,4-triazole rats were injected intraperitoneally (1g/Kg) and sacrificed 3 hours later[10].

Results

Although "MEOS" as described by Lieber and De Carli[4] has been reported to have a specific TPNH requirement, the findings in Table 1 indicate that 3-AP-DPN is very effective as a cofactor in the microsomal oxidation of ethanol; in fact 3-AP-DPN yielded an activity of 83% compared to TPNH. Consistent with previous reports on the binding of pyridine nucleotides to liver ADH[11], when 3-AP-DPN was substituted for DPN, the ADH activity of the rat liver supernatant increased 4.5 fold.

The observations on the effective oxidation of ethanol by microsomes when 3-AP-DPN was added suggested that ADH was present in this fraction. Studies were therefore carried out with a variety of agents known to inhibit ADH and to examine their effect on "MEOS". It will be noted in Table 2, that the in vitro addition of EDTA, pyrazole and ethanolamine resulted in 92 to 100% inhibition of ADH activity and an approximately 50% inhibition of "MEOS". The effect of dimethyl sulfoxide and sodium sulfide on ADH was less pronounced (i.e. 50% inhibition) but these agents still produced a decrease in microsomal ethanol oxidation. These findings are at variance with those of Lieber and De Carli[4], who reported that pyrazole, at concentrations which completely inhibited ADH in vitro, had no effect on "MEOS".

Table 1. Cofactor requirement for "MEOS" and ADH activity of rat liver

Cofactor	"MEOS" activity[a]	ADH activity[b]
	%	%
TPNH	100	0
TPN	16	1
DPNH	26	0
DPN	30	100
3-AP-DPN	83	450

[a]Incubation system of 1 ml contained microsomal protein (3 mg), 43 mM ethanol, 0.3 mM TPNH, 5 mM $MgCl_2$, and 80 mM Na_2HPO_4 at pH 7.4. Incubations were carried out at 37° for 10 min. in sealed center well flasks previously gassed with O_2. Center wells contained 15 mM semicarbizide. "MEOS" was measured by the production of acetaldehyde semicarbazone[3]. Where indicated, TPNH was replaced by equimolar amounts of the other nucleotides.

[b]ADH activity was measured on the 105,000 x g supernatant. Incubation system of 3.2 ml contained 0.5 mg supernatant protein, 16 mM ethanol, 0.5 mM DPN and 10 mM sodium pyrophosphate, at pH 10.3. Rate of DPNH formation at 340 mμ was measured at 30° in a Gilford recording spectrophotometer. Where indicated, DPN was replaced by equimolar amounts of other nucleotides.

Table 2. Effect of addition of ADH inhibitors on "MEOS" activity[a]

Inhibitor	Concentration	Enzyme activity		p
		ADH	"MEOS"	
	M	% inhibition		
EDTA	10^{-2}	100	56	< 0.001
Pyrazole	10^{-3}	93	48	< 0.025
Ethanolamine	5×10^{-1}	92	53	< 0.005
Dimethyl sulfoxide	1.4×10^{-2}	50	21	< 0.01
Sodium sulfide	10^{-3}	49	28	< 0.01

[a]ADH and "MEOS" incubation systems and assays were as described in Table 1. TPNH was not added but generated from 0.3 mM TPN, 8 mM isocitrate and isocitric dehydrogenase (2 mg; crude Type I - pig heart).

Catalase is known to be inhibited by sodium cyanide and azide when added in vitro and by 3-amino-1,2,4-triazole following its administration in vivo[10]. In the present study cyanide and azide (at 10^{-3}M) produced, respectively, 100 and 75% inhibition of catalase; under these conditions "MEOS" activity was inhibited 80 and 33%. Following the administration of aminotriazole in vivo there was a 90% decrease in hepatic catalase activity and a 54% inhibition of "MEOS".

The reported requirement of TPNH and O_2 for microsomal oxidation of ethanol[4] suggested the possibility that TPNH oxidase might be involved in this system. We therefore studied the effects of known inhibitors of TPNH oxidase[9]. It was found that the in vitro addition of potassium cholate (1.2×10^{-2}M) to the microsomal system led to an approximately 80% inhibition of both TPNH oxidase and "MEOS". It is noteworthy that under these conditions, potassium cholate had no effect on either ADH or catalase activities. The addition of $HgCl_2$ or $CuCl_2$ (2×10^{-4}M) to microsomal suspensions resulted in complete inhibition of the ADH, catalase, and TPNH oxidase activity, and "MEOS" activity was also completely abolished.

Since it seemed possible that the ADH and catalase activities associated with the microsomes represented varying degrees of adsorption of these enzymes or their trapping within the microsomal pellet, microsomes were washed with 0.05 M

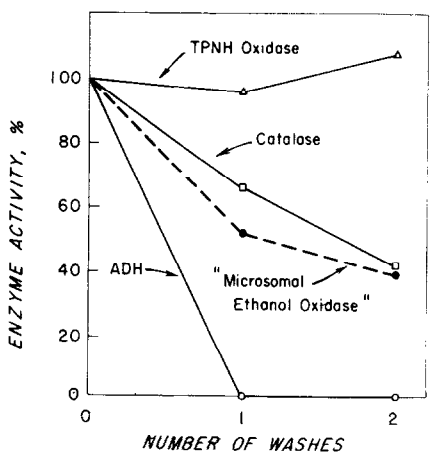

Figure 1. Effect of washing with 0.05 M Tris-acetate buffer, pH 7.4, on microsomal enzyme activity.

Tris-acetate buffer, pH 7.4 (containing 2 mM glutathione and 30 mM nicotinamide)[12]. As seen in Figure 1, after 2 washings, "MEOS" fell to 40% of the original activity together with a 60% decrease of catalase and complete loss of ADH activity. As expected, TPNH oxidase, a known membrane-bound enzyme, showed no significant change in activity as a result of these washing procedures.

To extend these observations we examined the effect of the addition of ADH and catalase to microsomes and the incubation system containing TPNH and O_2. As

Table 3. Effect of ADH and catalase on "MEOS" activity of native, washed and cholate treated microsomes

Microsome preparation	"MEOS"
	% activity
1. Native (unwashed)[a]	100
+ ADH[b]	169
+ Catalase[c]	275
+ ADH + catalase	435
2. Washed (x2 with Tris-acetate 0.1 M, pH 7.4)	37
+ ADH	93
+ Catalase	260
+ ADH + catalase	358
3. Potassium cholate[d] (1.4×10^{-2}M)	18
+ ADH	75
+ Catalase	158
+ ADH + catalase	262

[a]Incubation system (as in Table 1) contained 3 mg microsomal protein.

[b]1 mg Crystalline horse liver ADH (S.A.=2μmoles DPNH formed/min.).

[c]1 mg Lyophylized beef liver catalase (S.A.=40 mEq perborate oxidized/min.).

[d]Cholate added to incubation system. Under these conditions TPNH oxidase was inhibited 76%, while ADH and catalase were unaffected

seen in Table 3, the addition of either crystalline ADH, or catalase or both, resulted in increased ethanol oxidation even though no other pyridine nucleotides or H_2O_2 were added to the incubation system. When these crystalline enzymes were added to microsomes whose "MEOS" activity had been reduced by washing, ethanol oxidation could be restored to the level obtained with unwashed microsomes and increased 3.5 times above normal when both ADH and catalase were added. Similarly, when TPNH oxidase was inhibited with potassium cholate, the decrease in ethanol oxidation was reversed by the addition of ADH or catalase.

Discussion

Both Orme-Johnson and Ziegler[1] and Lieber and De Carli[2,4] have considered the microsomal ethanol oxidizing system ("MEOS") reported by them to be a separate enzyme system, requiring TPNH and O_2 and distinct from the two enzymes known to oxidize ethanol, namely ADH and catalase. These authors based their conclusions on the following observations: (1) the system required TPNH and O_2 instead of DPN[1]; (2) H_2O_2, when added to the system could not substitute for TPNH[1]; (3) ADH activity could not be detected in microsomes[3]; (4) at pH 7.4 ADH favors the conversion of acetaldehyde to ethanol; and (5) the apparent finding that the *in vitro* addition of pyrazole, a potent ADH inhibitor, did not effect "MEOS"[4]. The present results are not consistent with these conclusions nor with some of the assumptions on which they were based.

While it appears correct that H_2O_2 when added directly to the microsomal system, cannot substitute for TPNH, we have confirmed (unpublished observations) the findings of Roach *et al*[13] that an H_2O_2 generating system (such as glucose plus glucose oxidase) can effectively replace TPNH. This observation suggested the possibility that TPNH oxidase, a microsomal enzyme, might be a component of the system and serve to produce H_2O_2 from TPNH and O_2. Subsequently, if catalase were present, that enzyme together with H_2O_2 might then oxidize ethanol to acetaldehyde.

That catalase appears to be involved in the *in vitro* oxidation of ethanol by microsomes is supported by our observations that the addition of catalase

to the microsomal system led to increased ethanol oxidation, while inhibitors of the enzyme produced significant inhibition of "MEOS". Roach et al[13] also recently concluded that catalase must be a component of "MEOS". In addition to the requirement of TPNH and O_2, the likelihood that TPNH oxidase is involved in "MEOS" was strengthened by the fact that when TPNH oxidase was inhibited by potassium cholate, ethanol oxidation by microsomes was inhibited to a comparable degree.

The pronounced inhibition of "MEOS" by ADH inhibitors, especially pyrazole, also supports the participation of ADH in the overall oxidation of ethanol by the microsomal system. Lieber and De Carli[4] discounted this possibility because of their failure to detect ADH in the microsomal fraction. These authors used DPN in their assay for ADH. However when the assay is carried out with 3-AP-DPN, which has a much greater affinity for liver ADH than DPN[11], ADH activity is readily detectable (Table 1). It is also important to recall that while the activity of ADH is greatest with DPN or its analogues, Dalziel and Dickinson[14] have shown that when chromatographically pure nucleotides are used, TPN can also be shown to function as a cofactor for this enzyme.

While ADH as well as catalase might be intrinsic components of the microsomes per se, it seems more likely that to a large extent these enzymes are either trapped in the microsomes or adsorbed to them as a result of the centrifugation procedures. As seen in Figure 1, ADH is almost completely removed from the microsomal fraction after one washing with Tris-acetate buffer, and over 60% of the catalase is removed with two washings. In other experiments we have found that when known amounts of crystalline ADH or catalase are added to previously washed microsomes, 4 to 7% of the added enzymes can reproducibly be recovered and detected in the microsomal pellet.

The present findings therefore suggest that the TPNH-dependent oxidation of ethanol by microsomes is not due to a distinct and separate membrane-bound enzyme but the result of at least 3 separate enzymatic reactions:

1. $TPNH + O_2 + H^+ \xrightarrow{\text{TPNH oxidase}} TPN^+ + H_2O_2$

2. $TPN^+ + C_2H_5OH \xrightarrow{\text{ADH}} TPNH + H^+ + C_2H_4O$

3. $H_2O_2 + C_2H_5OH \xrightarrow{\text{Catalase}} 2H_2O + C_2H_4O$

Whether the microsomal oxidation of ethanol mediated by these reactions is of any quantitative and pharmacologic significance in the metabolism of ethanol is still debatable. In this regard it is noteworthy that recently Tephly et al[15] and Klaasen[16], using a variety of drugs to stimulate and inhibit microsomal enzyme reactions, could find no evidence to support a role for microsomal ethanol oxidation in vivo.

References

1. Orme-Johnson, W.H. and Ziegler, D.M. Biochem. Biophys. Res. Commun., 21, 78 (1965).
2. Lieber, C.S. and De Carli, L.M. J. Clin. Invest., 47, 629 (1968).
3. Lieber, C.S. and De Carli, L.M. Science, 162, 917 (1968).
4. Lieber, C.S. and De Carli, L.M. Clinical Res., 27, 306 (1969).
5. Isselbacher, K.J., Chrabas, M.F. and Quinn, R.C. J. Biol. Chem., 237, 3033 (1962).
6. Mezey, E., Cherrick, G.R. and Holt, P.R. J. Lab. Clin. Med., 71, 798 (1968).
7. Raskin, N.H. and Sokoloff, L. Science, 162, 131 (1968).
8. Feinstein, R.N. J. Biol. Chem., 180, 1197 (1949).
9. Gillette, J.R., Brodie, B.B. and La Du, B.N. J. Pharm. Expl. Therap., 119, 532 (1957).
10. Kinard, F.W., Nelson, G.H. and Hay, M.G. Proc. Soc. Exp. Biol. Med., 92, 772 (1956).
11. Kaplan, N.O., Ciotti, M.M. and Stolzenbach, F.E. J. Biol. Chem., 221, 833 (1956).
12. Moir, N.J., Miller, W.L. and Gaylor, J.L. Biochem. Biophys. Res. Commun., 33, 416 (1968).
13. Roach, M.K., Reese, W.N. and Creaven, P.J. Biochem. Biophys. Res. Commun., 36, 596 (1969).
14. Dalziel, K. and Dickinson, F.M. Biochem. J., 95: 311 (1965).
15. Tephly, T.R., Tinelli, F. and Watkins, W.D. Science, 166, 627 (1969).
16. Klaasen, C.D. Proc. Soc. Exp. Biol. and Med., 132, 1099 (1969).