

Hepatic Microsomal Ethanol-oxidizing System

IN VITRO CHARACTERISTICS AND ADAPTIVE PROPERTIES IN VIVO*

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SUMMARY

A hepatic microsomal ethanol-oxidizing system is described both in men and rats. It is distinguished from alcohol dehydrogenase by its subcellular localization (cytosol for alcohol dehydrogenase, microsomes for this system), its pH optimum (physiological pH versus pH 10 to 11 for alcohol dehydrogenase), and its cofactor requirements (NADPH versus NAD⁺ for alcohol dehydrogenase). It also requires oxygen and is inhibited by CO, properties commonly found among microsomal drug-detoxifying enzymes. That catalase is probably not involved was revealed by the partial or complete failure of cyanide, pyrazole, azide, or 3-amino-1,2,4-triazole to inhibit the NADPH-dependent microsomal ethanol-oxidizing system under conditions which diminished catalase activity. Moreover, a combination of administration *in vivo* of pyrazole and addition *in vitro* of azide virtually blocked catalase activity and abolished 95% of a H₂O₂-dependent microsomal ethanol oxidation, whereas two-thirds of the activity of the NADPH-dependent ethanol oxidation persisted. Ethanol feeding resulted in a striking rise of hepatic NADPH-dependent microsomal ethanol-oxidizing activity, whereas under the same conditions, activities of alcohol dehydrogenase in the cytosol and of microsomal as well as of total hepatic catalase did not increase. Furthermore, blood ethanol clearance was accelerated, which suggests that microsomal ethanol oxidation may play a role *in vivo*. Pyrazole, which inhibits alcohol dehydrogenase strongly (affecting also other hepatic functions, including microsomal enzymes) markedly reduced but did not block ethanol metabolism *in vivo* or in liver slices. Even after pyrazole, ethanol clearance rates remained significantly higher in ethanol-pretreated rats. The existence of a microsomal ethanol-oxidizing system, especially its capacity to increase in activity adaptively after ethanol feeding, may explain various effects of ethanol, including proliferation of hepatic smooth endoplasmic reticulum, induction of other hepatic microsomal drug-detoxifying enzymes, and the metabolic tolerance to ethanol which develops in alcoholics.

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It was observed recently that ethanol ingestion produces proliferation of the hepatic smooth endoplasmic reticulum, both in man (2-4) and in rats (5-7). Proliferation of the smooth endoplasmic reticulum has also been described for a variety of other drugs and it probably represents the morphological counterpart of the induction of microsomal drug-detoxifying enzymes (8). Enzymes previously described to oxidize ethanol *in vitro* include catalase (EC 1.11.1.6) and alcohol dehydrogenase (EC 1.1.1.1), but it is generally accepted that catalase plays no major role *in vivo* (9) and that alcohol dehydrogenase is responsible for hepatic oxidation of ethanol to acetaldehyde (10). Alcohol dehydrogenase, however, is localized in the cytosol compartment of the hepatocyte (11); the finding that ethanol possesses the property to produce proliferation of smooth endoplasmic reticulum raised the possibility that the hepatic microsomes, which comprise the smooth endoplasmic reticulum, may also be involved in the oxidation of ethanol. The present study describes a hepatic microsomal system which, both in man and in rats, actively oxidizes ethanol to acetaldehyde. This microsomal ethanol-oxidizing system was also found to adaptively increase in activity after ethanol feeding under conditions which left alcohol dehydrogenase and catalase unchanged. This adaptive response was associated with enhanced ethanol disappearance from the blood (even when alcohol dehydrogenase was blocked), which suggests that this system is active *in vivo* as well as *in vitro*. Results of part of this study have appeared in preliminary reports (12, 13).

EXPERIMENTAL PROCEDURES AND METHODS

Experimental and Clinical Materials—Sprague-Dawley rats (Charles River, CD) 100 to 300 g, body wt, were used and they were fed Purina laboratory chow unless stated otherwise. Sixty-four rats (21 pairs of male and 11 pairs of female) were pair-fed liquid diets (with ethanol or isocaloric carbohydrates) as described previously (14) and were killed by decapitation (after 5, 16, 24, and 90 days) to determine hepatic ethanol-oxidizing activity (see below). Similar experiments were done in 30 pairs of rats (14 male, 16 female) fed the liquid diets for 24 days and given pyrazole (4.4 mmoles per kg) by gastric tube 23 hours prior to killing or given intraperitoneal injections (1 g per kg) of 3-amino-1,2,4-triazole (Aldrich) or isotonic 0.85% NaCl solution 3 hours prior to killing. The ethanol was withdrawn 5 to 12 hours prior to the drug administration.

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In 13 pairs of female rats fed the liquid diets for 24 days, blood ethanol clearance was determined after an overnight fast by administering intragastrically the ethanol-containing liquid diet at the dose of 3 g per kg and collecting blood from the tail vein every 30 min for 6½ hours. Similar measurements were carried out in a group of five pairs of female rats, treated for 2 weeks only.

In 11 pairs of female rats, treated for 24 days, pyrazole (4.4 mmoles per kg) was given intragastrically 3 hours after withdrawal of the liquid diet and 11 to 14 hours prior to administration of the test dose of ethanol; blood was collected from the tail vein every 2 to 3 hours over the following 24 hours.

Thirteen pairs of rats fed the Purina chow were given (by gastric tube) 1 dose of the control liquid diet described above (30 ml per kg), with or without 10 mg per ml of pyrazole (4.4 mmoles per kg), and were killed after 10 or 23 hours.

Eight pairs of rats (4 male and 4 female) fed Purina chow were given a single dose of either 6 g per kg of ethanol or isocaloric glucose by gastric tube and were killed after 16 hours.

All of the animals were killed by decapitation and the liver (and, in some animals, kidneys, brain, heart, and stomach) was quickly excised.

Human liver tissue was obtained either at surgery or by needle biopsy. The surgical biopsies were performed in individuals in whom this procedure was indicated for a variety of clinical reasons. The percutaneous liver biopsies were performed with a Menghini needle on volunteers who participated in other studies for which this procedure was required. The specimens were used only if the appearance on histological examination was normal or near normal.

Assay Procedures—After homogenization of tissue in KCl (0.15 M), microsomes were obtained by centrifugation according to the procedure of Kato (15), except that the 9,000 × *g* centrifugation lasted 30 min. In some instances, the microsomes were washed by resuspending them in KCl, followed by a second centrifugation at 100,000 × *g*. When used, the supernatant (cytosol) was centrifuged a second time at 100,000 × *g*. Alcohol dehydrogenase activity was measured in the cytosol and other subcellular fractions of the liver according to the method of Bonnichsen and Brink (16) at pH 7 and 9.6 and expressed in units corresponding to the change in absorption, measured at 340 mμ (ΔE_{340}) per min per g of liver. This unit corresponds to 161 nmoles of acetaldehyde formed per min per g of liver. Catalase activity was measured in both total homogenates and in washed microsomes according to the method of Feinstein (17), and expressed in units corresponding to the milliequivalent of perborate destroyed per g of liver per min.

To measure ethanol oxidation in microsomes, the following technique was used: the subcellular fractions (corresponding to 250 mg of tissue, unless stated otherwise) were incubated in a phosphate buffer (80 mM) at pH 7.4, with 0.3 mM NADP⁺, 5 mM magnesium chloride, 20 mM nicotinamide, 8 mM sodium isocitrate, and 2 g per liter of isocitrate dehydrogenase (crude-type I; Sigma Chemical Company). Preliminary studies indicated that under the conditions of our assay, 20 mM nicotinamide had no inhibitory effect on the rate of the reaction, contrary to its reported inhibition of some other microsomal enzyme activities (18).

In some instances (specifically indicated) the NADPH generating system (NADP⁺, isocitrate, and isocitrate dehydrogenase) was replaced by a modification of one of the H₂O₂-generating

systems described by Keilin and Hartree (19), 3.3 mM hypoxanthine and 1 unit per ml of xanthine oxidase (purchased from Sigma Chemical Company). This H₂O₂-generating system was selected because, unlike commercially available glucose oxidase, we found it to be virtually catalase free.

Inhibitors *in vitro* studied included carbon monoxide (40%), sodium cyanide (0.1 mM), pyrazole (2 and 4 mM), sodium azide (0.1 mM), and 1 mM SKF 525 A (β -diethylaminoethyl diphenylpropylacetate).

The incubations were carried out in the main chambers of 50-ml Erlenmeyer flasks with center wells containing 0.6 ml of 0.015 M semicarbazide hydrochloride in 0.16 M potassium phosphate buffer (pH 7.0). After 10 min of preliminary incubation at 37°, ethanol was added to the incubation medium to achieve a final concentration of 50 mM and a total incubation volume of 3 ml. When used, xanthine oxidase was added at the same time as ethanol. The flasks were then stoppered with a serum cap and incubated for various periods of time in a shaking water bath at 37°. To stop the reaction, 0.5 ml of 70% trichloroacetic acid was injected into the main compartment. After an overnight diffusion period at room temperature, the flasks were opened, the contents of the center wells were harvested, and the concentration of acetaldehyde bound to the semicarbazide was determined as described by Gupta and Robinson (20).

With each set of incubations, flasks were incorporated to which known amounts of acetaldehyde were added to be used as standards. The absorption spectrum of the acetaldehyde-semicarbazone solution obtained with standard flasks was the same as the spectrum observed after incubation of microsomes with ethanol. In a group of experiments, the identity with acetaldehyde of the compound produced by the microsomes was also confirmed by measurement of the retention time on gas-liquid chromatography.¹

For each determination, at least six incubation flasks were used, with duplicate incubations for 0, 5, and 10 min, to verify linearity of the reaction. Activity of the microsomal ethanol-oxidizing system was expressed in units corresponding to the nanomoles of acetaldehyde produced per min during the initial linear phase of the reaction. No substantial amounts of acetaldehyde were produced in acidified reaction vessels or in the absence of any of the cofactors.

When used, 9000 × *g* supernatant fractions were obtained by centrifuging the liver homogenate for 30 min. Preliminary results indicated that ethanol-oxidizing activity in the 9,000 × *g* fraction (obtained from amounts of liver tissue varying from 10 to 50 mg) was comparable with that of the corresponding isolated microsomes.

Liver slices (from Purina chow-fed rats) were prepared and incubated for 3 hours as described previously (21) in the presence of 30 mM ethanol, with or without 2 mM pyrazole.

Ethanol concentration in incubation media and in plasma was determined according to the method of Bonnichsen (22), protein was measured according to the method of Lowry *et al.* (23), and total hepatic lipids were assessed as described before (14).

In all experiments, each individual result was compared with its corresponding control, and the mean of the individual differences was tested by the Student *t*-test.

¹ The gas-liquid chromatographic determinations were kindly performed by Dr. E. Truitt, Battelle Memorial Institute, Columbus, Ohio.

RESULTS

Characterization and Localization of Microsomal Ethanol-oxidizing System

Subcellular Localization and Organ Specificity—In the liver, substantial NADPH-dependent ethanol oxidation at physiological pH was found in the microsomal fraction only, as illustrated in Fig. 1. This contrasted with alcohol dehydrogenase activity, which was localized in the cytosol. Fig. 1 shows alcohol dehydrogenase activity of the cytosol measured at pH 9.6; when determined at pH 7, the activity was 5 to 8 times lower. Activity of the NADPH-dependent microsomal ethanol-oxidizing system

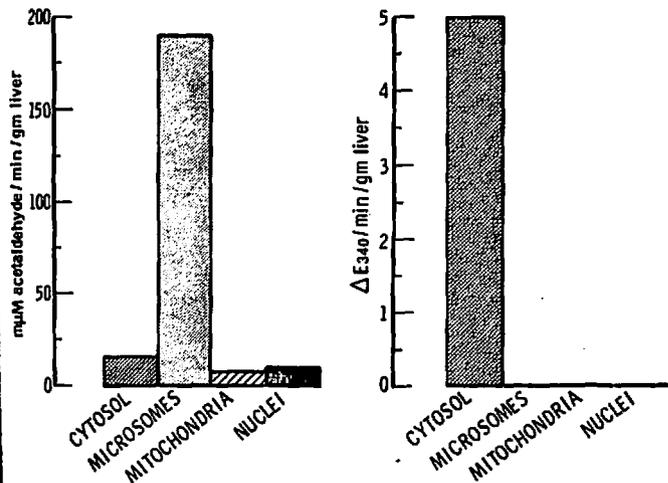


Fig. 1. Comparison of the subcellular localization of NADPH-dependent ethanol oxidation and alcohol dehydrogenase activity in the hepatocyte. Each bar represents the average result of four incubations. Left, activity of ethanol-oxidizing system at pH 7.0 to 7.4; right, activity of alcohol dehydrogenase at pH 9.6.

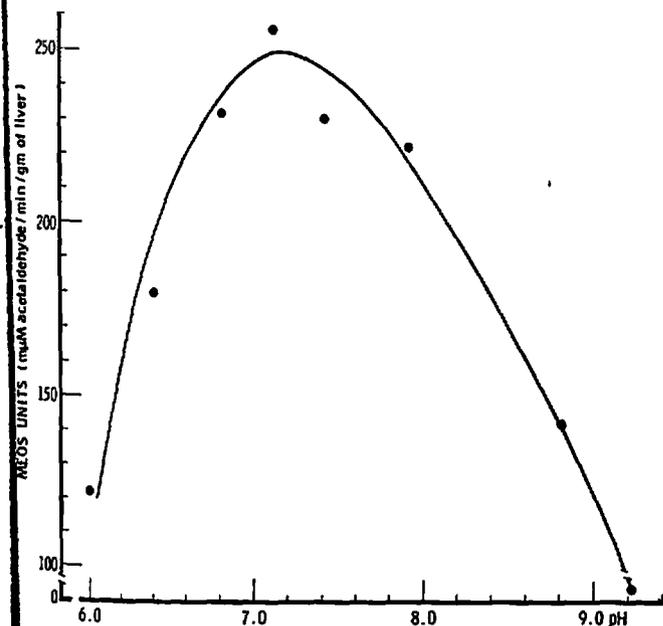


Fig. 2. Effect of pH on the activity of the hepatic microsomal ethanol-oxidizing system (MEOS). Maximal activity was observed in the 6.8 to 7.4 pH range. Each point (●) represents the average of four incubations.

in kidney, brain, heart, and stomach was negligible. In the livers of 10 male rats (averaging 250 g, body wt) fed the Purina chow diet, the activity measured 8.6 ± 0.72 units per mg of microsomal protein.

Proportionality of Activity of Amount of Microsomes and Time of Incubation—Under the standard conditions of the assay, the activity was found to be proportional to the amount of microsomes used, in a range corresponding to 20 to 300 mg of liver tissue. The activity was also linear during the initial period of the reaction lasting for at least 10 min. Linearity extended sometimes for additional 10 or 20 min, depending on the activity of the preparation used.

Effect of pH and Substrate Concentration—Optimum activity coincided with a pH range of 6.8 to 7.4 (Fig. 2). The effect of ethanol concentration is illustrated in Fig. 3. Near maximum rates of activity were observed with concentrations of ethanol of 50 mM and above.

Cofactor and Oxygen Requirements—As indicated in Fig. 4, substantial activity was observed only in the presence of either NADPH- or a NADPH-generating system. The preparation had low activity with NADP⁺, NAD⁺, or NADH. There was an almost absolute requirement for oxygen (Fig. 5). Replacement of air by nitrogen virtually abolished the activity and reduction of oxygen from 21% (air) to 4% (a mixture of 4% O₂ and 96% N₂) diminished the activity by 45% ($p < 0.01$).

Effects of Inhibitors—When microsomal preparations were incubated under an atmosphere containing 40% CO, the activity was reduced (on the average, by 40%) compared with control incubations ($p < 0.02$).

Sodium cyanide (0.1 mM) inhibited the activity by 12% (200.4 versus 228.2 units in the controls, with a mean difference of 27.8 ± 6.13 units; $p < 0.01$). SKF 525 A (β -diethylaminoethyl diphenylpropylacetate), 1 mM, had no significant effect.

Catalase activity of the washed microsomal fraction represented only about 2% of the catalase activity of the total liver

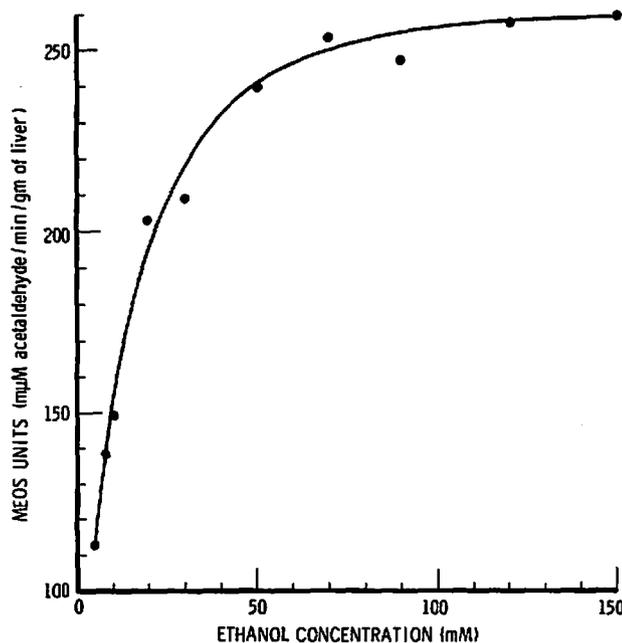


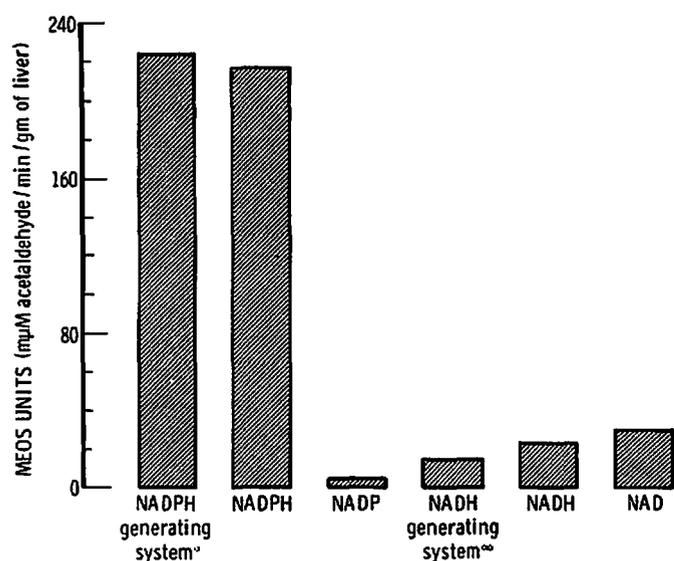
Fig. 3. Effect of ethanol concentration on the activity of the hepatic microsomal ethanol-oxidizing system (MEOS). Each point (●) represents the average result of four incubations.

homogenate. After 3-amino-1,2,4-triazole administration, both were reduced by 90 to 95% (10.5 ± 1.42 catalase units in total liver homogenates and 0.21 ± 0.02 in microsomes *versus* 102.6 ± 5.02 and 2.25 ± 0.14 , respectively, in the controls; $p < 0.001$). 3-Amino-1,2,4-triazole had a much less striking effect on NADPH-dependent microsomal ethanol oxidation. In male rats, activity of this system was 5.2 ± 0.46 units per mg of microsomal protein *versus* 11.1 ± 0.51 in 0.85% NaCl-treated controls ($p < 0.001$). In female rats, the inhibition by 3-amino-1,2,4-triazole was somewhat less striking, 5.1 ± 0.65 units per mg of protein *versus* 8.6 ± 0.20 in controls ($p < 0.01$). Moreover, 3-amino-1,2,4-triazole did not abolish the difference in microsomal ethanol-oxidizing activity between five male rats fed ethanol for 24 days and their pair-fed controls, 7.7 ± 0.84 units per mg of protein *versus* 5.2 ± 0.46 ($p < 0.02$), respectively.

The dissociation between catalase and microsomal ethanol oxidation was also striking after pyrazole. Ten hours after administration of pyrazole, the inhibition of catalase activity in

liver homogenates and microsomes was 66 and 60%, respectively ($p < 0.001$), whereas microsomal ethanol oxidation by the NADPH-dependent system was not significantly changed. Twenty-three hours after a single dose of pyrazole (4.4 mmoles per kg), catalase activity was decreased 90% in washed liver microsomes ($p < 0.001$) and ethanol oxidation by a H_2O_2 -generating system was reduced by 80% ($p < 0.001$), whereas ethanol oxidation with a NADPH system was not affected (Table I). In unwashed microsomes, a small (16%; $p < 0.02$) reduction of microsomal ethanol oxidation with the NADPH-generating system was observed 23 hours after pyrazole administration.

Addition of azide (a catalase inhibitor) to the control preparation *in vitro* markedly reduced activities of both microsomal catalase and that of ethanol oxidation with the H_2O_2 -generating system by 81% and 92%, respectively ($p < 0.001$) (Table I).



^o NADP + isocitrate & isocitric dehydrogenase
^o NAD + glucose & glucose dehydrogenase

FIG. 4. Cofactor requirements of the microsomal ethanol-oxidizing system (MEOS). Each bar represents the average result of four incubations.

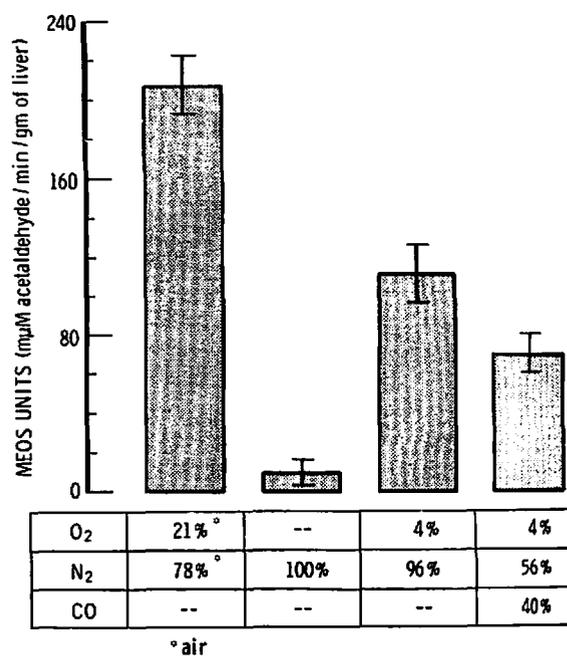


FIG. 5. Effect of O₂ and CO on the activity of the hepatic microsomal ethanol-oxidizing system (MEOS). Each bar represents the mean (\pm standard error) of six studies, each performed in duplicate.

TABLE I

Catalase activity and oxidation of ethanol to acetaldehyde in hepatic microsomes

Washed microsomes (of control rats or animals given pyrazole intragastrically 23 hours prior to killing) were incubated with 50 mM ethanol, with or without azide, in the presence of either a NADPH-generating system (NADP⁺, isocitrate, and isocitrate dehydrogenase) or a H_2O_2 -generating system (hypoxanthine and xanthine oxidase).

Pyrazole 4.4 mmoles/kg	Azide 10 ⁻⁴ M	Incubation system	Catalase activity		Ethanol Oxidation	
			mq perborate/flask	% of control	nmoles/flask	% of control
-	-	NADPH generated	0.931 \pm 0.196	100 (control)	49.1 \pm 4.5	100 (control)
-	-	H ₂ O ₂ generated	0.810 \pm 0.161	100 (control)	96.0 \pm 12.8	100 (control)
-	+	NADPH generated	0.220 \pm 0.036	23.6	31.0 \pm 3.7	63.1
-	+	H ₂ O ₂ generated	0.156 \pm 0.021	19.2	7.2 \pm 2.8	7.5
+	-	NADPH generated	0.097 \pm 0.020	10.4	47.6 \pm 6.0	96.9
+	-	H ₂ O ₂ generated	0.083 \pm 0.017	10.2	18.9 \pm 4.2	19.7
+	+	NADPH generated	0.016 \pm 0.005	1.7	32.9 \pm 2.2	66.9
+	+	H ₂ O ₂ generated	0.009 \pm 0.004	1.1	4.9 \pm 1.5	5.1

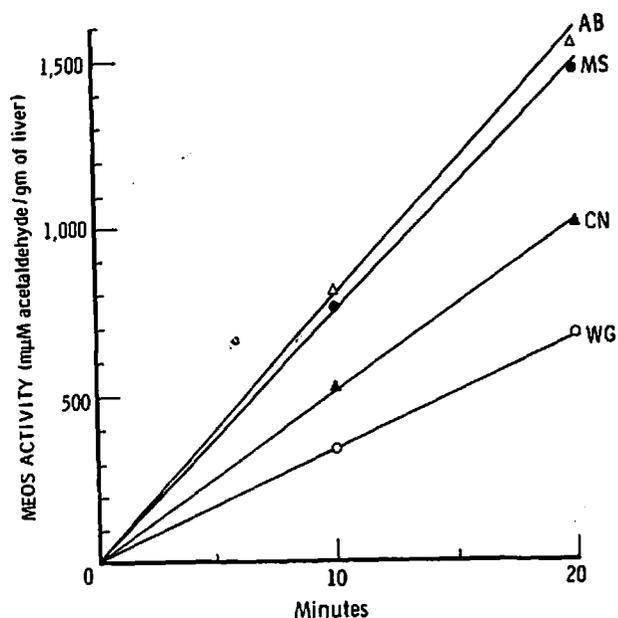


Fig. 6. Activity of a NADPH-dependent microsomal ethanol-oxidizing system (MEOS) in four human surgical liver biopsies. Each point (●) represents the average of two incubations.

By contrast, ethanol oxidation with the NADPH-dependent system decreased by only 37%.

The most striking dissociation between the activities of catalase and ethanol oxidation by a H_2O_2 -generating system on the one hand and the ethanol oxidation by the NADPH-linked system on the other hand was found when both inhibitors (pyrazole *in vivo*, azide *in vitro*) were combined. Addition of azide to the microsomal preparation of pyrazole-treated animals virtually abolished both microsomal catalase activity and H_2O_2 -linked ethanol oxidation, whereas two-thirds of the activity of the NADPH-linked system remained (Table I). Although in the control preparations acetaldehyde production from ethanol by the microsomal NADPH-dependent system was only half that with microsomes and the H_2O_2 -generating system, the difference, was dramatically reversed either by addition of azide to the incubation media ($p < 0.01$), or pyrazole pretreatment ($p < 0.01$), and especially the combination of both ($p < 0.001$); in the latter experiments, acetaldehyde production with the NADPH system was 7-fold that of the H_2O_2 system (Table I).

Despite the dilution inherent in the assay technique, liver samples of the animals given pyrazole had initial alcohol dehydrogenase activity 75 to 90% lower than that of untreated animals. A comparable inhibition was found in the animals fed the ethanol-containing or control liquid diets and then given pyrazole. *In vitro*, at concentrations of 2 and 4 mM, pyrazole had no effect on catalase activity but it reduced microsomal ethanol oxidation by 11 and 22%, respectively, and it also completely inhibited alcohol dehydrogenase activity. In liver slices, addition of 2 mM pyrazole reduced ethanol metabolism 76% from 6.0 ± 0.30 mg of ethanol per g of liver tissue per 3 hours to 1.4 ± 0.18 ($p < 0.001$).

Microsomal Ethanol Oxidation in Human Liver Microsomes—Human microsomes (obtained from surgical liver biopsy specimens) were found to be capable of actively oxidizing ethanol in the presence of an NADPH-generating system, as illustrated in Fig. 6. The activity was linear over the time period studied but

somewhat lower than the activity observed in rats. This may be because the specimens were taken from the surface of the liver and contained a large amount of fibrous capsular tissue, which most likely resulted in a reduction of activity through a decrease in the actual number of hepatocytes present per g of liver. This interpretation is substantiated by the fact that when ethanol-oxidizing activity was determined in $9000 \times g$ supernatant fractions of needle biopsies obtained in five normal volunteers, the activity was comparable to that of the rat; it averaged 229.3 ± 31.64 units per g of liver. As in the rats, when human microsomes were incubated under anaerobic conditions, ethanol oxidation was negligible.

Sex Difference—Expressed per g of liver, NADPH-linked microsomal ethanol-oxidizing activity was lower in female (190.0 ± 12.7 units) than in male (248.1 ± 15.4 units) rats fed the control diet for 24 days ($p < 0.02$).

Effect of Ethanol Feeding

Effect of Ethanol Feeding on Activity of Microsomal Ethanol-oxidizing System, Alcohol Dehydrogenase, and Catalase—After 5 days of ethanol feeding, microsomal ethanol-oxidizing activity was comparable with that of the rats fed the control diet. In a group of rats treated with ethanol for 2 weeks, the activity was already increased compared with the controls (but not as markedly as in the 24-day groups) and on statistical analysis, the difference was not significant as yet. After 24 days of ethanol feeding, NADPH-linked ethanol oxidation increased and there was a greater increase in females (451.9 ± 33.4 units per g of liver after ethanol *versus* 190.0 ± 12.7 units in the controls; $p < 0.001$) than in males (368.2 ± 31.4 *versus* 248.1 ± 15.4 units per g of liver; $p < 0.001$). This was accompanied by a significant increase of microsomal protein in the male rats (26.0 ± 0.95 mg per g of liver after ethanol *versus* 22.6 ± 1.29 in the controls; $p < 0.01$); no significant change was seen in the females (24.5 ± 1.1 *versus* 24.9 ± 0.86 mg per g). Twenty-four days of ethanol feeding affected liver weights as follows. In the males, the values were 3.7 ± 0.11 g per 100 g, body wt, after ethanol *versus* 3.4 ± 0.13 in the controls ($p < 0.01$) and in the females, the corresponding values were 4.1 ± 0.12 *versus* 3.8 ± 0.12 g per 100 g, body wt ($p < 0.01$). Fat-free liver weights (expressed in grams per 100 g, body wt) were 3.4 ± 0.09 after ethanol (*versus* 3.2 ± 0.13 in the controls; not significant) in the males and 3.8 ± 0.11 (*versus* 3.6 ± 0.11 ; not significant) in the females. When ethanol-oxidizing activity was expressed per mg of microsomal protein, the values were as follows. In the males, the values were 14.1 ± 1.08 units (after 24 days of ethanol) *versus* 11.1 ± 0.50 units in the controls ($p < 0.02$); in the females, the values were 18.7 ± 1.71 after ethanol *versus* 7.6 ± 0.38 in the controls ($p < 0.001$).

In a group of six female rats fed the ethanol diet for 3 months, NADPH-dependent microsomal ethanol-oxidizing activity was 383.6 ± 22.3 units per g of liver compared with 201.3 ± 14.14 in the controls.

Unlike the chronic feeding of ethanol, 1 acute dose of 6 g per kg of ethanol given 16 hours prior to killing was without effect on microsomal ethanol-oxidizing activity.

Apparent K_m of microsomal ethanol-oxidizing activity was determined in three pairs of male rats fed ethanol (or control diets) for 24 days, and the average results have been represented in Fig. 7. Whereas maximal activity increased with ethanol feeding, the apparent K_m remained similar at about 8 mM.

Contrasting with microsomal ethanol oxidation, alcohol dehy-

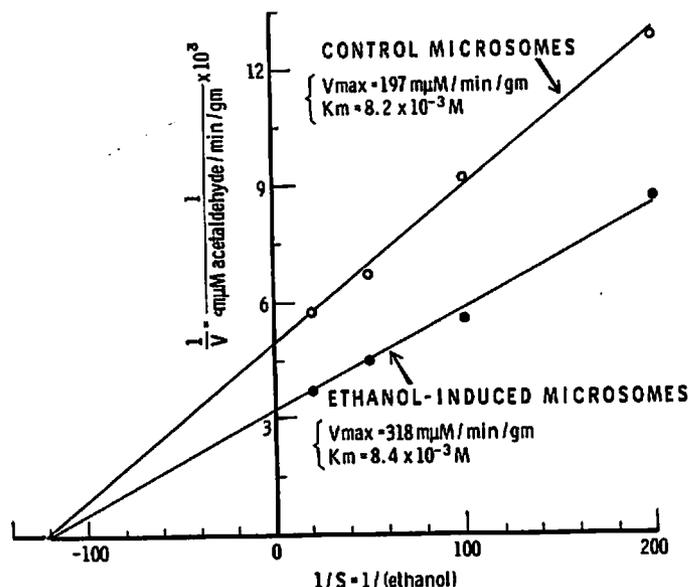


FIG. 7. Lineweaver-Burk plot of microsomal ethanol-oxidizing activity. Each point (●) represents the average of at least four incubations.

drogenase activity was higher in females than in males and in neither sex was alcohol dehydrogenase activity significantly increased by the ethanol feeding; in the males the control alcohol dehydrogenase values, measured at pH 9.6, were 10.8 ± 1.35 compared with 11.7 ± 0.75 after 24 days of ethanol; in the females there was even a significant decrease, 14.7 ± 1.30 in the controls versus 11.5 ± 0.69 after ethanol ($p < 0.01$). A similar reduction of alcohol dehydrogenase activity was found in female rats fed ethanol for 3 months. No significant changes in alcohol dehydrogenase activity appeared in male or female rats given 1 dose of ethanol.

In 10 pairs of rats (5 female, 5 male) fed ethanol (or the control diet) for 24 days, microsomal and total hepatic catalase activity was found to be unaffected by ethanol feeding.

Effect of Ethanol Feeding on Blood Ethanol Disappearance; Average Blood Alcohol Concentration—Peak blood ethanol levels were achieved 150 to 180 min after ethanol administration and were comparable in ethanol-treated and in control rats. Ethanol disappearance rates, however (measured over the 210- to 390-min time period after the gastric intubation, when gastric absorption can no longer be expected to occur), increased with ethanol feeding. After 2 weeks, there was already a significant acceleration of ethanol clearance (38.2 ± 6.8 mg per 100 ml per hour versus 27.2 ± 5.1 in the controls; $p < 0.02$). After 24 days the values were 38.8 ± 2.7 versus 25.5 ± 1.3 , respectively ($p < 0.001$).

After 1 dose of pyrazole, blood ethanol clearance was decreased to 5.2 ± 0.26 mg per 100 ml per hour in control animals and to 11.2 ± 0.93 in littermates that had been fed alcohol for 24 days. Although blood ethanol clearance was markedly reduced by pyrazole, it was not completely blocked and a significant difference persisted between ethanol and control rats ($p < 0.001$).

After 24 days of ethanol feeding, serum ethanol concentration was 69.0 ± 18.1 mg per 100 ml in animals which had free access to the ethanol diet at the time of killing.

DISCUSSION

The present study revealed the existence, both in man and in rats, of a hepatic microsomal system (Fig. 1) which actively oxidizes ethanol to acetaldehyde at a physiological pH (Fig. 2) and is capable of an adaptive increase in activity upon ethanol feeding. This microsomal ethanol-oxidizing system has properties comparable to those of a variety of other microsomal drug-detoxifying enzymes; it is NADPH dependent (Fig. 4), requires oxygen, and can be partially inhibited by CO (Fig. 5). The latter property distinguishes this system from a microsomal system previously described by Orme-Johnson and Ziegler (24), who reported results indicating that mammalian microsomes can oxidize methanol and who also mention that the same preparation oxidizes ethanol. The latter system, however, was insensitive to CO; moreover, its level of activity was only $\frac{1}{4}$ that of the present system. Furthermore, whereas Orme-Johnson and Ziegler's system oxidized ethanol at a rate one-half that of methanol, our system (in untreated animals) has a rate of ethanol oxidation twice that of methanol.²

The activity we observed was lower in females than in males, whereas the capacity for adaptation was greater in the females, a sex difference common for microsomal drug-detoxifying enzymes (8). Another sex difference was also found in the response of the microsomal protein, which increased more in the male than in the female rats after ethanol feeding. Unlike many, but not all (8, 25) microsomal drug-metabolizing enzymes, the microsomal ethanol-oxidizing system was insensitive to 1 mM SKF 525 A (β -diethylaminoethyl diphenylpropylacetate).

The demonstration that hepatic microsomes can oxidize ethanol at a physiological pH raises the question of the role of this system for the oxidation of ethanol *in vivo*. Hitherto, alcohol dehydrogenase was generally thought to be the only enzyme responsible for the oxidation of ethanol *in vivo* (9, 10), although catalase is also active *in vitro* (19). That catalase is not likely to be responsible for the microsomal oxidation is indicated by stimulation and inhibition studies; the significant increase of ethanol oxidation in the microsomal fraction produced by ethanol feeding was not accompanied by a corresponding change of either microsomal or total hepatic catalase activity. Furthermore, cyanide inhibited microsomal ethanol-oxidizing activity only slightly (12%) at a concentration (0.1 mM) which almost completely abolishes catalase activity (26). Moreover, almost complete inhibition of microsomal and total hepatic catalase by aminotriazole, a known catalase inhibitor (27), produced a much smaller change in microsomal ethanol-oxidizing activity and did not abolish the increase in activity produced by ethanol feeding. Though microsomal ethanol-oxidizing activity was much less affected by aminotriazole than catalase, it nevertheless decreased. While the present paper was being written, another study appeared which also revealed partial inhibition of microsomal ethanol oxidation (28). This partial depression may not reflect a specific inhibition of catalase, but rather a nonspecific action on microsomal function. Aminotriazole does indeed markedly depress the activity of a variety of microsomal drug-metabolizing enzymes and slows rates of drug metabolism 2 hours after its administration (29); it also inhibits the stimulation of microsomal enzyme activity and P450 synthesis by the administration of drugs (29, 30). The most striking dissociation between the activities of the NADPH-dependent microsomal

² C. S. Lieber and L. M. DeCarli, unpublished observations.

ethanol-oxidizing system and catalase was found after a combination of inhibitors, pyrazole administration *in vivo* and azide *in vitro* (Table I). This virtually abolished microsomal catalase activity, whereas two-thirds of the activity of the NADPH-dependent microsomal ethanol-oxidizing system remained. By contrast, the same combination of inhibitors blocked 95% of the H_2O_2 -dependent microsomal ethanol oxidation (Table I). Thus, when microsomal ethanol oxidation is dependent upon H_2O_2 generation, it is also sensitive to catalase inhibition. Conversely, the relative insensitivity of the NADPH-dependent microsomal ethanol-oxidizing system to catalase inhibition suggests that catalase is not implicated in the activity of this system, at least for its major fraction. For the same reason, H_2O_2 generation from NADPH oxidase is probably not involved in the NADPH-dependent microsomal ethanol oxidation, although the former system is capable of methanol oxidation when catalase is added to microsomes (31).

Hitherto, the opinion has prevailed that alcohol dehydrogenase is solely responsible for the oxidation *in vivo* of ethanol, but this concept is not fully satisfactory. The capacity of rat liver tissue to oxidize ethanol is twice that of man; yet its alcohol dehydrogenase activity is 3 to 4 times lower (32) and, when measured at pH 7, can account for only a fraction (10 to 20%) of the rates of ethanol oxidation *in vivo*. These discrepancies raise the possibility that in addition to alcohol dehydrogenase, other pathways may be involved in the oxidation of ethanol. The apparent K_m of 8 mM of the microsomal ethanol-oxidizing system (Fig. 7), if applicable to a situation *in vivo*, indicates that this system could indeed be near its maximal activity with blood alcohol concentrations commonly encountered after alcohol intoxication. In the rat, however, maximal microsomal oxidation *in vitro* is of the same order of magnitude as maximal alcohol dehydrogenase activity at pH 7, and, like alcohol dehydrogenase, it only accounts for a fraction (10 to 20%) of the ethanol oxidized *in vivo*. Rates of metabolism *in vitro*, however, are of doubtful significance since the degree and nature of the alteration of activity during the extraction procedures remain unknown.

Findings of the present study, as well as those reported by others (33) indicate that ethanol feeding results in enhanced blood clearance of alcohol in the rat and in man (34). Under our experimental conditions, concomitant measurement of hepatic alcohol dehydrogenase and catalase revealed no increase in the activity of these enzymes. Although reports by others concerning the possibility of alcohol dehydrogenase adaptation have been conflicting, as reviewed elsewhere (35), our observation of the lack of alcohol dehydrogenase adaptation is in accord with several previous publications (32, 36, 37). By contrast, activity of the NADPH-dependent microsomal ethanol-oxidizing system significantly rose in the animals given ethanol. This enhanced activity probably reflects a quantitative, rather than a qualitative change, because the apparent K_m of the enzyme system remained unchanged, though the V_{max} increased (Fig. 7). An increase in the clearance of ethanol by approximately 40 to 50% was accompanied by a rise of total microsomal ethanol-oxidizing activity of about 170%. Liver weight, when corrected for fat content, was not significantly affected by ethanol feeding and alcohol dehydrogenase and catalase activities did not increase. Since no marked hepatic blood flow changes are produced by low blood ethanol concentrations (38, 39) such as were encountered in the present study, one can formulate the hypothesis that the enhanced ethanol clearance was caused primarily by increased

microsomal ethanol-oxidizing activity. If this assumption is correct, it may indicate that, normally, this activity accounts for about one-third of the ethanol oxidized *in vivo*. These indirect quantitative assessments are also supported by the results obtained *in vitro*. In liver slices, the oxidation of ethanol was reduced 76% by 2 mM pyrazole, a concentration which, *in vitro*, inhibits alcohol dehydrogenase completely and microsomal ethanol oxidation 11%. If the results of enzyme assays are applicable to liver slice metabolism and if one can extrapolate from slices to conditions *in vivo*, these findings may indicate that normally, two-thirds of the ethanol is metabolized via alcohol dehydrogenase and one-third via an alternate system, probably microsomal, an estimate which concurs with the induction data discussed previously.

When administered *in vivo* at a dose of 4.4 mmoles per kg, pyrazole depressed ethanol clearance by 70 to 80%, an inhibition of the same order of magnitude as the effect previously reported by others (40, 41). This does not necessarily signify that alcohol dehydrogenase is responsible *in vivo* for a corresponding fraction of the clearance of blood ethanol. In addition to alcohol dehydrogenase inhibition, pyrazole exerts more general toxic effects upon the liver as witnessed by the striking ultrastructural changes it produces in the liver and the widespread disturbance of hepatic functions (including those of microsomes) (42). Even after pyrazole treatment, ethanol-fed rats had a more rapid clearance of ethanol than the controls (although alcohol dehydrogenase was similarly blocked), which again suggests that the increased rate of blood ethanol clearance after ethanol feeding involved mechanisms different from alcohol dehydrogenase. Since NADPH-dependent microsomal ethanol-oxidizing activity did increase by ethanol feeding, it is reasonable to postulate that it is responsible, at least in part, for the increase in the rate of ethanol clearance in rats fed ethanol. This may represent the experimental counterpart of the metabolic tolerance to ethanol known to develop in alcoholics (34, 43).

The rise in activity of the NADPH-dependent microsomal ethanol-oxidizing system following ethanol consumption is associated with an increased activity of a variety of other NADPH-dependent microsomal drug-detoxifying enzymes (7, 34, 44). This may explain the associated acceleration of drug metabolism (34) and, at least in part, the known resistance of alcoholics to the effects of various drugs (45).

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