Hydroxyl-radical production and ethanol oxidation by liver microsomes isolated from ethanol-treated rats

Gunilla EKSTRÖM, Tomas CRONHOLM and Magnus INGELMAN-SUNDBERG*

Department of Physiological Chemistry, Karolinska Institute, S-104 01 Stockholm, Sweden

In order to distinguish between the mechanism of microsomal ethanol oxidation and hydroxyl-radical formation, the rate of cytochrome *P*-450 (*P*-450)-dependent oxidation of dimethyl sulphoxide (Me_2SO) was determined in the presence and in the absence of iron-chelating compounds, in liver microsomes from control, ethanol- and phenobarbital-treated rats. Ethanol treatment resulted in a specific increase (3-fold) of the microsomal ethanol oxidation and NADPH consumption per nmol of *P*-450. A form of *P*-450 was purified to apparent homogeneity from the ethanol-treated rats and characterized with respect of amino acid composition and *N*-terminal amino acid sequence. Specific ethanol induction of a cytochrome *P*-450 species having a catalytic-centre activity of 20/min for ethanol and consuming 30 nmol of NADPH/min could account for the results observed with microsomes. Phenobarbital treatment caused 50% decrease in the rate of ethanol oxidation and NADPH oxidation per nmol of *P*-450. The rate of oxidation of the hydroxyl-radical scavenger Me_2SO was increased 3-fold by ethanol or phenobarbital treatment when expressed on a per-mg-of-microsomal-protein basis, but the rate of Me_2SO oxidation expressed on a per-nmol-of-*P*-450 basis was unchanged. Addition of iron-chelating agents to the three different types of microsomal preparations caused an 'uncoupling' of the electron-transport chain accompanied by a 4-fold increase of the rate of Me_2SO oxidation. It is concluded that ethanol treatment results in the induction of *P*-450 forms specifically effective in ethanol oxidation and NADPH oxidation, but not in hydroxyl-radical production, as detected by the oxidation of Me_2SO .

INTRODUCTION

Chronic treatment of rats with ethanol results in an increased ability of liver microsomal fractions to oxidize ethanol (Lieber & DeCarli, 1970; Ohnishi & Lieber, 1977) as well as drugs (Ariyoshi *et al.*, 1970; Powis, 1975). Many workers have shown that the enhanced ethanol-oxidizing capability of microsomes (microsomal fractions) isolated from ethanol-treated rats is accompanied by an increase in the specific content of cytochrome *P*-450 in the membranes (Ischii *et al.*, 1973; Liu *et al.*, 1975; Morgan *et al.*, 1981; Teschke *et al.*, 1981). However, with ethanol as a preferred substrate, no form of *P*-450 has hitherto been purified from rat liver microsomes, although Comai & Gaylor (1973), Joly and co-workers (Villeneuve *et al.*, 1976; Mungikar *et al.*, 1980) and others (Ullrich *et al.*, 1975; Morgan *et al.*, 1981) have provided evidence for the existence of specific forms of *P*-450 in microsomes from ethanol-treated rats with special properties such as increased affinity for Me_2SO , cyanide and tetrahydrofuran. Levin and co-workers have isolated two forms of *P*-450 from liver microsomes of ethanol-treated rats (Ryan *et al.*, 1984), one of which is active in the metabolism of retinoic acid (Leo *et al.*, 1984). A form of *P*-450 inducible by ethanol (Koop *et al.*, 1982; Ingelman-Sundberg & Hagbjörk, 1982), pyrazole (Ingelman-Sundberg & Jörnvall, 1984) and imidazole (Ingelman-Sundberg & Jörnvall, 1984; Koop & Coon, 1984) has been purified from rabbit liver microsomes in

the laboratory of Professor M. J. Coon (Koop *et al.*, 1982) and in ours (Ingelman-Sundberg & Hagbjörk, 1982; Ingelman-Sundberg & Jörnvall, 1984). This protein is a high-spin type of *P*-450 that upon interaction with primary aliphatic alcohols, Me_2SO and other compounds, is converted into the low-spin form (Morgan *et al.*, 1982; Ingelman-Sundberg & Johansson, 1984). When reconstituted with NADPH:cytochrome *P*-450 reductase, it metabolizes ethanol with a high catalytic-centre activity, provided that small amounts of chelated iron are present (Ingelman-Sundberg & Johansson, 1984) and at a lower rate in the absence of chelated iron (Morgan *et al.*, 1982; Ingelman-Sundberg & Johansson, 1984). Immunochemical studies have revealed that almost all *P*-450-dependent ethanol oxidation in liver microsomes from rabbits treated with ethanol, pyrazole or imidazole is catalysed by this form of *P*-450 (Koop *et al.*, 1984; Ingelman-Sundberg & Jörnvall, 1984).

It has been found that the oxidation of ethanol by the ethanol-inducible cytochrome *P*-450 form can be carried out by hydroxyl radicals as the active oxygen species. They are formed in a Fenton-like reaction from H_2O_2 produced by the enzyme (Ingelman-Sundberg & Johansson, 1984). This finding is in agreement with the implication of hydroxyl radicals, or oxygen species with similar oxidizing power, in the microsomal oxidation of ethanol (Cederbaum *et al.*, 1978; Ohnishi & Lieber, 1978; Krikun *et al.*, 1984). However, when using the purified ethanol-inducible form of cytochrome *P*-450, it turned

Abbreviations used: *P*-450, cytochrome *P*-450; Me_2SO , dimethyl sulphoxide; SDS, sodium dodecyl sulphate.

* To whom correspondence and reprint requests should be sent.

out that hydroxyl-radical scavengers such as deoxyribose, oxomethiobutyric acid, or Me_2SO were not better substrates for this enzyme in the presence of chelate iron than for other forms of rabbit liver *P*-450 (Ingelman-Sundberg & Johansson, 1984). This work suggests the existence of different mechanisms for the microsomal metabolism of ethanol and other hydroxyl-radical scavengers.

On the other hand, Klein *et al.* (1983) showed that, after ethanol treatment of rats, the increased rate of microsomal ethanol oxidation was accompanied by an enhanced capability of these membranes to oxidize hydroxyl-radical scavengers. This could imply an increased microsomal production of oxy radicals as a reason for an oxidative membrane damage after ethanol consumption. It was therefore considered important to evaluate whether the increased rate of oxidation of hydroxyl-radical scavengers was specifically enhanced after ethanol treatment of the animals or only a function of the amount of the electron-transport components NADPH:cytochrome *P*-450 reductase and *P*-450 in the microsomal membrane. Furthermore, we found it important to evaluate to what extent the oxidation of hydroxyl-radical scavengers was affected by the presence of iron-chelating agents in the microsomes, since the hydroxyl radicals might be formed after redox cycling of iron chelate in the electron-transport chain and this should thus represent a more artificial oxidizing system. The rate of ethanol oxidation and Me_2SO oxidation was therefore determined in microsomal membranes from control, ethanol- and phenobarbital-treated rats in the presence or in the absence of chelating agents.

EXPERIMENTAL PROCEDURES

Materials

Sucrose and pyrophosphate were obtained from BDH, NaN_3 and Me_2SO from Merck, EDTA from Fluka AG, NADPH from Sigma and Chelex-100 was from Bio-Rad laboratories.

Animals

Male Sprague-Dawley rats were fed the liquid diets described by DeCarli & Lieber (1967). The alcohol diet, which provides 36% of the energy from ethanol and the control diet, with carbohydrate replacing ethanol, were obtained from Bioserv (Frenchtown, NJ, U.S.A.). The alcohol was introduced in a stepwise manner (DeCarli & Lieber, 1967). Three groups of animals were used. The first group ($n = 8$, group I) consisted of 140 g rats fed *ad libitum* for 20 days; the second group ($n = 5$, group II), was composed of 110 g rats that were pair-fed (DeCarli & Lieber, 1967) for 24 days; the third group of rats weighing 200 g ($n = 5$, group III) were fed *ad libitum* for 14 days. In addition, another group of rats (200 g) were given injections of phenobarbital (80 mg/kg) intraperitoneally once daily for 3 days; they received standard laboratory diet and water *ad libitum*.

Preparation of liver microsomes

The animals, which were not starved beforehand, were killed by a blow on the head and the livers were quickly removed. When the first group of animals were used, the livers were homogenized in 3 vol. of 1.14% (w/v) KCl

containing 10 mM-EDTA with a Potter-Elvehjem homogenizer. The resulting homogenate was centrifuged at 9000 *g* for 20 min and the supernatant obtained was thereafter centrifuged at 105000 *g* for 60 min. The microsomal pellet was washed once in the KCl solution and the pellet was homogenized in 50 mM-potassium phosphate buffer, pH 7.4. These microsomes will be referred to below as 'type I'. In case of the second group of rats, the livers were homogenized in 0.25 M-sucrose. The microsomal pellet obtained was washed once in 1.14% KCl and the final pellet was homogenized in 50 mM-potassium phosphate buffer, pH 7.4. These microsomes are designated 'type II'. Livers obtained from the third group of animals were homogenized in 0.25 M-sucrose. The microsomes were prepared and washed in 0.25 M-sucrose and the final microsomal pellet was suspended in 50 mM-potassium phosphate buffer, pH 7.4. These microsomes are designated 'type III'. Microsomes from phenobarbital-treated rats were prepared in a manner identical with that used for the type III fraction. All microsomal preparations were stored in small vials under N_2 at -80°C .

Purification of *P*-450

Liver microsomes obtained from ethanol-treated rats corresponding to 3 g of microsomal protein were solubilized with cholate, fractionated with poly(ethylene glycol) and chromatographed on DEAE-Sepharose as described for purification of the rabbit ethanol-inducible *P*-450 (Ingelman-Sundberg & Johansson, 1984). The protein fraction eluted with 10 mM- K_2SO_4 was adjusted to pH 6.0 with 6 M-HCl and applied to a CM (carboxymethyl)-Sepharose column (0.8 cm $\times$ 35 cm) equilibrated in 5 mM-potassium phosphate buffer, pH 6.0, containing 20% (v/v) glycerol, 2% (v/v) Renex 690 and 1 mM-EDTA. The column was washed with 3 column vol. of equilibration buffer and subsequently eluted with a linear 0–0.3 M-KCl gradient (400 ml) in the equilibration buffer. The fractions were analysed by SDS/polyacrylamide-gel electrophoresis. The *P*-450-containing fractions that were eluted at approx. 0.1 M-KCl and, in addition, were homogeneous on the evidence of electrophoresis, were pooled. Detergent was removed with hydroxyapatite (Ingelman-Sundberg & Johansson, 1984) and the final preparation (10 μM) was dialysed twice against 100 mM-potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.2 mM-EDTA, and stored at -20°C .

Incubation conditions

The microsomal oxidation of ethanol was determined as described by Ingelman-Sundberg & Johansson (1981). The procedure includes the measurement of the amount of acetaldehyde formed after diffusion into an inner tube containing semicarbazide. Calibration was done by using a standard curve of acetaldehyde prepared in buffer and the absorption of the semicarbazide solution at 224 nm (cf. Lieber & DeCarli, 1970). The incubations were carried out at 37°C for 5, 10 or 20 min in 25 ml stoppered tubes containing 5 ml tubes with 15 mM-semicarbazide. Two different types of incubation conditions were used: under the first condition the tubes contained microsomes corresponding to 1 mg of protein, 50 mM-ethanol and 0.5 mM-azide in Chelex-100-treated 50 mM-potassium phosphate buffer, pH 7.4, to give a final volume of 2 ml. In other cases the conditions as described by Klein *et al.*

(1983) were used. The tubes contained microsomes corresponding to 1 mg of microsomal protein, 10 mM-potassiumpyrophosphate, 10 mM-MgCl₂, 0.1 mM-EDTA, 1 mM-azide and 50 mM-ethanol in 50 mM-phosphate buffer, pH 7.4. All buffers and water solutions had previously been Chelex-100-treated. The incubations were started by addition of 0.5 mg of NADPH and stopped by adding 0.2 ml of 6 M-trichloroacetic acid. Control incubations were performed in the absence of ethanol or NADPH.

The oxidation of Me₂SO was detected as formation of formaldehyde, essentially as described by Nash (1953). Incubation conditions were as described above, except that 33 mM-Me₂SO replaced ethanol, microsomes corresponding to 2 mg of protein were used, the tubes were incubated for 10 min and contained 0.4 mg of NADPH; the final incubation volume was 1 ml. The incubations were stopped by the addition of 143 µl of 60 % ZnSO₄ and 560 µl of saturated Ba(OH)₂. After centrifugation at 5000 g for 10 min, 0.9 ml of the supernatant was mixed with 125 µl of the concentrated Nash reagent [7.5 g of ammonium acetate, 0.15 ml of acetic acid and 0.1 ml of acetylacetone (pentane-2,4-dione) in a total volume of 15 ml]. The samples were heated at 60 °C for 30 min and the A₄₁₅ was monitored. Oxidation of NADPH was determined by continuous spectrophotometric recording at 340 nm, an absorption coefficient of 6.2 mM⁻¹·cm⁻¹ being used. The activity of NADPH:cytochrome P-450 reductase was determined at 30 °C in 0.33 M-potassium phosphate buffer, pH 7.4, by monitoring the reduction of cytochrome *c* at 550 nm. One unit of activity is expressed as 1 nmol of cytochrome *c* reduced/min under the conditions used. Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

For statistical evaluation, Students *t*-test was used and *P* values less than 0.05 were considered significant.

RESULTS

Ethanol oxidation by rat liver microsomes

Treatment of rats with ethanol for 2 weeks resulted in a 2.4-fold enhancement of the *P*-450 levels and a 30%

increase of NADPH:cytochrome *P*-450 reductase activity in isolated liver microsomes in all three groups studied (Table 1). Phenobarbital administration enhanced the *P*-450-levels 4-fold and the reductase activity almost 2-fold (Table 1). The increase of *P*-450 by ethanol feeding was reflected in 2.5–4.6-fold higher rates of ethanol oxidation per mg of protein in liver microsomes of these animals whether or not they were prepared in EDTA. Expressed on a per-nmol-of-*P*-450 basis, the increase was 1.5–2.5-fold (Table 2). Phenobarbital administration resulted in 2.6-fold higher rates of ethanol oxidation/min and per mg of microsomal protein, but a 40% decrease of acetaldehyde production, when related to the amount of *P*-450 in the membranes.

When the rate of ethanol oxidation per mg of protein was plotted against the *P*-450 content (Fig. 1), a correlation (*r* = 0.92) between the two variables was seen at a specific content of cytochrome *P*-450 greater than 0.45 nmol/mg of protein. The slope of the line above this point was 20 nmol of acetaldehyde formed/min per nmol of *P*-450. Thus the presence of a *P*-450 form in specifically the ethanol-treated-rat liver microsomes with a catalytic centre activity for ethanol of 20/min could explain the results. By contrast, the corresponding values obtained when using microsomes from phenobarbital-treated animals, about 2 nmol/mg and 5 nmol of acetaldehyde/min per mg, did not fit into this line (cf. Fig. 1). When the microsomes from control and ethanol-treated animals were analysed by SDS/polyacrylamide-gel electrophoresis, it was evident that ethanol treatment caused an apparent induction of two different microsomal proteins with apparent *M_r* values 51 500 and 50 000 (Fig. 2). We have purified two different species of cytochrome *P*-450 from ethanol-treated rats, one isolated as a high-spin protein with apparent *M_r* 50 500 and the other isolated as a low-spin protein with apparent *M_r* 50 000 (cf. Fig. 2). The low-spin form of *P*-450 (I in Fig. 2) was subjected to analysis of amino acid composition and *N*-terminal amino acid sequence (Table 3). The *N*-terminal sequence was apparently identical with that of *P*-450 h, purified in Dr. W. Levin's laboratory (Haniu *et al.*, 1984), whereas the amino acid composition was somewhat different. The preparations were inactive in

Table 1. Specific content of *P*-450 and specific activity of NADPH:cytochrome *P*-450 reductase in liver microsomes prepared from control, ethanol-treated and phenobarbital-treated rats

Microsomes from control and ethanol-treated rats were isolated in 1.14% KCl, containing 10 mM-EDTA (type I), in 0.25 M-sucrose and washed in 1.14% KCl (type II) or in 0.25 M-sucrose and washed in the same medium (type III) (see the Experimental procedures section). Microsomes from phenobarbital-treated rats were prepared and washed in sucrose (type III). Values are means ± s.d. Significance: **P* < 0.001; ***P* < 0.01; N.D., not determined.

Type of microsomes	<i>P</i> -450		NADPH:cytochrome <i>P</i> -450-reductase	
	(nmol/mg)	(% of control)	(units/mg)	(% of control)
Control				
I (<i>n</i> = 8)	0.47 ± 0.08		62 ± 7	
II (<i>n</i> = 4)	0.64 ± 0.12		N.D.	
III (<i>n</i> = 5)	0.46 ± 0.04		95 ± 11	
Ethanol-treated				
I (<i>n</i> = 8)	1.11 ± 0.15*	236	88 ± 10*	142
II (<i>n</i> = 5)	1.11 ± 0.10*	170	N.D.	
III (<i>n</i> = 5)	1.14 ± 0.14*	242	112 ± 15	117
Phenobarbital-treated				
III (<i>n</i> = 5)	2.00 ± 0.13*	434	180 ± 40**	189

Table 2. Oxidation of ethanol in liver microsomes from control, ethanol-treated and phenobarbital-treated rats

Three groups of control and ethanol-treated rats were used and liver microsomes were isolated as outlined in Table 1. The incubations contained liver microsomes corresponding to 1 mg of protein, 50 mM-ethanol and 0.5 mM-azide in 50 mM-potassium phosphate buffer, pH 7.4, and were performed as described in the Experimental procedures section. Values given are means $\pm$ S.D. Statistical significance is expressed as in Table 1.

Type of liver microsomes	Acetaldehyde production			
	(nmol/min per mg)	(% of control)	(nmol/min per nmol of <i>P</i> -450)	(% of control)
Control				
I (<i>n</i> = 8)	2.8 $\pm$ 0.45		6.1 $\pm$ 1.9	
II (<i>n</i> = 4)	4.5 $\pm$ 0.8		4.0 $\pm$ 0.8	
III (<i>n</i> = 5)	2.1 $\pm$ 0.3		4.4 $\pm$ 0.9	
Ethanol-treated				
I (<i>n</i> = 8)	12.9 $\pm$ 2.6*	460	11.9 $\pm$ 2.0*	195
II (<i>n</i> = 5)	11.4 $\pm$ 3.4**	253	10.1 $\pm$ 1.9**	252
III (<i>n</i> = 5)	7.5 $\pm$ 0.8*	357	6.6 $\pm$ 0.3**	150
Phenobarbital-treated				
III (<i>n</i> = 5)	5.5 $\pm$ 0.3*	261	2.7 $\pm$ 0.2**	61

Fig. 1. Liver microsomal oxidation of ethanol as a function of the specific content of cytochrome *P*-450 in the membranes

Incubations with ethanol (50 mM) were carried out for 20 min at 37 °C with liver microsomes corresponding to 1 mg of protein in the presence of 0.5 mM-azide and 0.4 mM-NADPH as described in the Experimental procedures section. The results of experiments performed with microsomes prepared in EDTA (type I) and sucrose (type II) are shown. ●, Ethanol-treated rat; ○, control rat.

NADPH-dependent ethanol oxidation when reconstituted in membranes with purified NADPH:cytochrome *P*-450 reductase (data not shown).

NADPH consumption in the microsomal preparations

The rate of NADPH consumption in microsomes from ethanol-treated rats was about 3-fold higher, compared with control, on a per-mg-of-protein basis (Table 4). There was a relationship ($r = 0.89$) between the cytochrome *P*-450 content and the rate of NADPH oxidation in the membranes (Fig. 3). NADPH consumption was enhanced 2.4-fold on a per-mg-of-protein basis in microsomes from phenobarbital-treated animals, compared with control, but a 50% decrease in the rate of NADPH oxidation was observed per-nmol-of-*P*-450 basis (Table 4). The results

Fig. 2. SDS/polyacrylamide-gel electrophoresis of liver microsomes from control (C) and ethanol-treated rats (E) and of two types of cytochromes *P*-450 isolated from liver microsomes of ethanol-treated rats (I and II)

Electrophoresis was performed from top to bottom in the discontinuous buffer system described by Laemmli (1970) with 8.5% (w/v) acrylamide separating gel. A 3 μ g portion of each protein fraction and microsomes corresponding to 12 μ g of protein were applied on to the gel.

Table 3. Amino acid composition and *N*-terminal amino acid sequence of *P*-450 type I, isolated from liver microsomes of ethanol-treated rats

The analysis were performed as previously described (Ingelman-Sundberg & Jörnvall, 1984). *N*-Terminal analysis was carried out by reverse-phase h.p.l.c. of phenylthiohydantoin derivatives from 10 nmol of the protein. The initial yield was 70% and the repetitive yield was 95%. The values given are nmol of stable residues recovered. The values for amino acid composition are estimated total residues per protein chain.

<i>N</i> -Terminal sequence:														
1	5	10	15											
Met-Asp-Pro-Val-Leu-Val-Leu-Val-Leu-Thr-Leu-Ser-Ser-Leu-Leu-														
7.0	6.6	6.1	6.2	6.7	4.8	6.1	5.7	5.4						
Amino acid composition:														
Asx, 50; Thr, 26; Ser 28; Glx, 50; Pro, 32; Gly, 32; Ala, 21;														
Val, 31; Met, 12; Ile, 25; Leu, 56; Tyr, 15; Phe, 45; Lys, 28;														
His, 16; Arg, 26														

Table 4. Oxidation of NADPH in liver microsomes from control, ethanol-treated or phenobarbital-treated rats.

Assays were performed using microsomes prepared in 0.25 M-sucrose (type III) corresponding to 0.25 mg of protein at 37 °C. Incubations were carried out under two different conditions, A and B. Incubations under A contained 50 mM-ethanol, 0.5 mM-azide and 0.1 mM-NADPH in 50 mM-potassium phosphate buffer, pH 7.4, in a final volume of 2 ml, whereas incubations under B contained 10 mM-pyrophosphate, 10 mM-MgCl₂, 0.1 mM-EDTA, 1 mM-azide, 50 mM-ethanol and 0.5 mM-NADPH in a final volume of 2 ml adjusted with the phosphate buffer. The reaction was monitored continuously for 5 min. The results are expressed as mean $\pm$ S.D. ($n = 5$). Statistical significance: * $P < 0.001$; ** $P < 0.05$; *** $P < 0.01$.

Type of liver microsomes	NADPH oxidized					
	Per mg of protein		Per nmol of <i>P</i> -450		Per unit of reductase	
	(nmol/min)	(% of control)	(nmol/min)	(% of control)	(nmol/min)	(% of control)
Condition A						
Control	13.0 $\pm$ 1.2		28.6 $\pm$ 3.2		0.138 $\pm$ 0.022	
Ethanol	34.7 $\pm$ 2.8*	266	30.5 $\pm$ 3.7	106	0.310 $\pm$ 0.031*	224
Phenobarbital	31.4 $\pm$ 1.1*	241	15.5 $\pm$ 0.8*	54	0.144 $\pm$ 0.032	104
Condition B						
Control	43.8 $\pm$ 8.9		97 $\pm$ 24		0.450 $\pm$ 0.044	
Ethanol	69.9 $\pm$ 6.9**	159	62 $\pm$ 10***	64	0.630 $\pm$ 0.116***	140
Phenobarbital	122.9 $\pm$ 9*	278	61 $\pm$ 6***	63	0.707 $\pm$ 0.144**	157

Fig. 3. Rate of liver microsomal NADPH consumption as a function of the specific content of cytochrome *P*-450 in the membranes

The NADPH oxidase activity was determined spectrophotometrically at 340 nm using liver microsomes corres-

ponding to 0.25 mg of protein in the presence of 50 mM-ethanol and Chelex-100-treated 50 mM-potassium phosphate buffer, pH 7.4, at 37 °C. Liver microsomes from animal groups II and III were used. ●, Ethanol-treated rat; ○, control rat.

ponding to 0.25 mg of protein in the presence of 50 mM-ethanol and Chelex-100-treated 50 mM-potassium phosphate buffer, pH 7.4, at 37 °C. Liver microsomes from animal groups II and III were used. ●, Ethanol-treated rat; ○, control rat.

Table 5 Oxidation of Me₂SO in liver microsomes from control, ethanol-treated and phenobarbital-treated rats

The microsomes were prepared in 0.25 M-sucrose and homogenized in 50 mM-potassium phosphate buffer, pH 7.4, as described in the Experimental procedures section (type III). Two different sets of incubation conditions were used (A, without pyrophosphate and EDTA; B, with EDTA and pyrophosphate) as described in Table 4. All incubations contained microsomes corresponding to 2 mg of protein, 1 mM-azide, 0.4 mg of NADPH and 33 mM-Me₂SO and were performed for 10 min. Formaldehyde was quantified as described in the Experimental procedures section. Control incubations were performed by adding NADPH after ZnSO₄. The values are means $\pm$ s.d., $n = 5$. Statistical significance: * $P < 0.001$; ** $P < 0.05$.

Type of liver microsomes	Formaldehyde production			
	Per mg of protein		Per nmol of <i>P</i> -450	
	(nmol/min)	(% of control)	(nmol/min)	(% of control)
Condition A				
Control	0.15 $\pm$ 0.07		0.33 $\pm$ 0.15	
Ethanol	0.43 $\pm$ 0.09*	286	0.38 $\pm$ 0.10	115
Phenobarbital	0.50 $\pm$ 0.03*	333	0.25 $\pm$ 0.01	76
Condition B				
Control	0.40 $\pm$ 0.08		0.87 $\pm$ 0.16	
Ethanol	1.62 $\pm$ 0.26*	405	1.44 $\pm$ 0.32**	165
Phenobarbital	1.58 $\pm$ 0.07*	395	0.79 $\pm$ 0.05	91

When the rate of NADPH consumption was related to the specific activity of NADPH:cytochrome *P*-450 reductase, it was evident that microsomes from ethanol-treated animals exhibited a 2.2-fold higher rate than did control microsomes and consumed 1.8-fold more NADPH than did microsomes from phenobarbital-treated animals (Table 4).

Microsomal oxidation of Me₂SO

As described by Klein *et al.* (1981), microsomal production of formaldehyde from Me₂SO can be used as a tool for measuring hydroxyl-radical formation by the membranes. In order to distinguish between the oxidation of hydroxyl-radical scavengers and ethanol oxidation by the microsomes, it was considered important to determine the rate of Me₂SO oxidation in all types of microsomes prepared in the absence of EDTA under conditions with or without addition of excess pyrophosphate and iron-chelating agents. Ethanol- or phenobarbital-treatment of rats resulted in a 3-fold increase of hydroxyl-radical production when expressed on a per-mg-of-protein basis when assayed in the absence of EDTA, pyrophosphate and MgCl₂. By contrast, no significant differences were observed between the three types of microsomes when the Me₂SO oxidation was expressed in relation to the content of *P*-450 in the membranes (Table 5). Applying conditions with added EDTA, pyrophosphate and MgCl₂ resulted in a 4-fold increase, compared with control, of hydroxyl-radical production per mg of protein, both when liver microsomes prepared from ethanol-treated and phenobarbital-treated rats were used. Expressed per nmol of *P*-450, only small differences between the types of liver microsomes were noted (Table 5).

DISCUSSION

The results presented indicate that ethanol-feeding of rats results in the induction of *P*-450 form(s) in liver microsomes with apparent M_r 50000 that is (are) effective in ethanol oxidation and consume an unusually high

amount of NADPH. A form of *P*-450 oxidizing ethanol at a rate of 20 nmol/min per nmol and consuming about 30 nmol of NADPH/min per nmol in the microsomes could explain the inducibility of both the acetaldehyde formation, the NADPH consumption and the *P*-450 content in the membranes (Figs. 1 and 3).

Significant increases in the rate of ethanol oxidation by ethanol feeding were reached whether or not the microsomes had been isolated in the presence of EDTA (Table 2). By contrast, phenobarbital treatment caused a decrease in the specific ethanol oxidation when related to the amount of cytochrome *P*-450. Oxidation of the hydroxyl-radical scavenger Me₂SO proceeded at similar rates in control, ethanol- and phenobarbital-treated-rat microsomes when related to the *P*-450 content (Table 5). This oxidation was markedly enhanced in the presence of EDTA and pyrophosphate. The addition of these compounds to the microsomal preparations also caused a similar increase in the rate of NADPH oxidation (Table 4). This indicates that the electron-transport chain is 'uncoupled' under these conditions. Interaction of chelated iron with NADPH:cytochrome *P*-450 reductase and cytochrome *P*-450 will cause a reduction of Fe(III)-EDTA to Fe(II)-EDTA. Thereby the redox equilibrium will be influenced causing an enhanced rate of NADPH oxidation (see Morehouse *et al.*, 1984). Part of the electrons will reduce dioxygen to superoxide anions that spontaneously dismutate to H₂O₂ (cf. Ingelman-Sundberg & Johansson, 1984). The interaction of Fe(II)-EDTA with H₂O₂ will generate hydroxyl radicals in the Fenton reaction:

Subsequently, the hydroxyl radical (OH[·]) formed will oxidize the hydroxyl-radical scavenger. According to this concept the rate of oxidation of hydroxyl-radical scavenger will be proportional to the amount of NADPH:cytochrome *P*-450 reductase and *P*-450 in the membrane and not be specifically dependent upon the type of *P*-450 present. This is in accordance with the results reached here, where only small differences in the

rate of Me_2SO oxidation were seen between the three types of microsomes studied (Table 5). By contrast, the rate of ethanol oxidation was specifically increased in relation to the *P*-450 content in the membranes obtained from ethanol-treated rats (Table 2). These results thus indicate that ethanol does not induce any specific *P*-450-dependent hydroxyl-radical formation in liver microsomes other than is observed by the general induction of the *P*-450 level.

Comparison of the rate of NADPH oxidation between the three types of liver microsomes examined revealed that the cytochrome *P*-450 forms induced by ethanol had about double the specific activity of those induced by phenobarbital (Table 4). This is in accordance with previous results using the ethanol-inducible *P*-450 from rabbit liver microsomes that was found to exhibit a three-fold higher NADPH consumption than other types of *P*-450 (Morgan *et al.*, 1982; Ingelman-Sundberg & Johansson, 1984). One might speculate that the enhanced rate of NADPH oxidation in the liver microsomes after ethanol treatment might influence NADPH-dependent redox equilibria in the cell and thus be of importance for the susceptibility of the tissue towards oxidative damage.

In conclusion, the results presented indicate that ethanol treatment of rats causes the induction of *P*-450-forms in liver microsomes consuming an unusual amount of NADPH and participating in the NADPH-dependent oxidation of ethanol. By contrast, the NADPH-dependent and hydroxyl-radical-mediated oxidation of the hydroxyl-radical scavenger Me_2SO appears to be a more unspecific reaction, which is to a great extent dependent on the presence of iron-chelating agents in the system and not specifically induced by ethanol treatment.

We are grateful to Mrs Ann-Louise Hagbjörk for skilful technical assistance and to Professor Hans Jörnvall for amino acid analysis. This work was supported by grants from the Swedish Council for Planning and Co-ordination of Research and from the Swedish Medical Research Council.

REFERENCES

- Ariyoshi, T., Takasbatake, E. & Remmer, H. (1970) *Life Sci.* **9**, 361–369
- Cederbaum, A. I., Dicker, E. & Cohen, G. (1978) *Biochemistry* **17**, 3059–3064
- Comai, K. & Gaylor, J. L. (1973) *J. Biol. Chem.* **248**, 4974–4955
- DeCarli, L. M. & Lieber, C. S. (1967) *J. Nutr.* **91**, 331–336
- Haniu, M., Ryan, D. E., Iida, S., Lieber, C. S., Levin, W. & Shively, J. E. (1984) *Arch. Biochem. Biophys.* **235**, 304–311
- Ingelman-Sundberg, M. & Hagbjörk, A.-L. (1982) *Xenobiotica* **11**, 673–686
- Ingelman-Sundberg, M. & Johansson, I. (1981) *J. Biol. Chem.* **256**, 6321–6326
- Ingelman-Sundberg, M. & Johansson, I. (1984) *J. Biol. Chem.* **259**, 6447–6458
- Ingelman-Sundberg, M. & Jörnvall, H. (1984) *Biochem. Biophys. Res. Commun.* **124**, 375–382
- Ischii, H., Joly, J.-G. & Lieber, C. S. (1973) *Biochim. Biophys. Acta* **291**, 411–420
- Klein, S. M., Cohen, G. & Cederbaum, A. I. (1981) *Biochemistry* **20**, 6006–6012
- Klein, S. M., Cohen, G., Lieber, C. S. & Cederbaum, A. I. (1983) *Arch. Biochem. Biophys.* **223**, 425–432
- Koop, D. R. & Coon, M. J. (1984) *Mol. Pharmacol.* **25**, 494–501
- Koop, D. R., Morgan, E. T., Tarr, G. & Coon, M. J. (1982) *J. Biol. Chem.* **257**, 8472–8480
- Koop, D. R., Nordblom, G. D. & Coon, M. J. (1984) *Arch. Biochem. Biophys.* **235**, 228–238
- Krikun, G., Lieber, C. S. & Cederbaum, A. I. (1984) *Biochem. Pharmacol.* **33**, 3306–3309
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leo, A. M., Iida, S. & Lieber, C. S. (1984) *Arch. Biochem. Biophys.* **234**, 305–312
- Lieber, C. S. & DeCarli, L. M. (1970) *J. Biol. Chem.* **245**, 2505–2512
- Liu, S., Ramsey, R. K. & Ramsey, R. K. (1975) *Biochem. Pharmacol.* **24**, 369–378
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Morehouse, L. A., Thomas, C. E. & Aust, S. D. (1984) *Arch. Biochem. Biophys.* **232**, 366–377
- Morgan, E. T., Devine, M. & Skett, P. (1981) *Biochem. Pharmacol.* **30**, 595–600
- Morgan, E. T., Koop, D. R. & Coon, M. J. (1982) *J. Biol. Chem.* **257**, 13951–13957
- Mungikar, A. M., Hetu, C. & Joly, J.-G. (1980) *Adv. Exp. Med. Biol.* **132**, 51–56
- Nash, T. (1953) *Biochem. J.* **55**, 416–421
- Ohnishi, K. & Lieber, C. S. (1977) *J. Biol. Chem.* **252**, 7124–7131
- Ohnishi, K. & Lieber, C. S. (1978) *Arch. Biochem. Biophys.* **191**, 798–803
- Powis, G. (1975) *Biochem. J.* **148**, 269–277
- Ryan, D. E., Iida, S., Wood, A. W., Thomas, P. E., Lieber, C. S. & Levin, W. (1984) *J. Biol. Chem.* **259**, 1239–1250
- Teschke, R., Moreno, F. & Petrides, A. S. (1981) *Biochem. Pharmacol.* **30**, 1745–1751
- Ullrich, V., Weber, P. & Hollenberg, P. (1975) *Biochem. Biophys. Res. Commun.* **64**, 808–813
- Villeneuve, J.-P., Mavie, P. & Joly, J.-G. (1976) *Biochem. Biophys. Res. Commun.* **70**, 723–728

Received 9 July 1985/16 September 1985; accepted 8 October 1985