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CHANGES IN THE RAT HEPATIC MIXED FUNCTION OXIDASE SYSTEM ASSOCIATED WITH CHRONIC ETHANOL VAPOR INHALATION*

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Abstract—Chronic ethanol vapor inhalation by rats increased hepatic microsomal aniline hydroxylase activity, increasing the turnover number and decreasing the K_m. Activity of ethanol-induced microsomes toward other substrates was also examined. The increase in aniline hydroxylase activity as a result of ethanol treatment is attributed to an increase in a form of cytochrome P-450 with a high specific activity toward aniline. Since the ethanol effect on aniline hydroxylation had disappeared 24 hr after treatment was discontinued, a high rate of turnover of this enzyme was deduced. Dimethylsulfoxide (56 mM) produced a reverse type I spectral change in ethanol-induced, but not in control, microsomes. This was interpreted as being due to a change in the spin state of the cytochrome P-450 in these microsomes. Acetone added to the incubation produced an increased rate of aniline hydroxylation by microsomes from control and ethanol-induced rats. The difference between the rate of aniline hydroxylation by control microsomes and the rate by ethanol-induced microsomes was, however, abolished at higher acetone concentrations.

The heterogeneity of hepatic microsomal cytochrome P-450§ is well documented. Following early observations that microsomes from animals treated with various inducers exhibited different spectral properties and catalytic activities with different substrates, distinct forms of P-450 were first purified to electrophoretic homogeneity by Haugen and Coon [1] using rabbit liver. The corresponding major forms of cytochrome induced by PB, 3-MC, or BNF in rat liver have been purified and characterized [2]. Other forms of P-450 have been separated and purified to varying degrees, as reviewed elsewhere [3, 4], including isoenzymes from ethanol-treated [5–8] animals.

Since the initial report by Comai and Gaylor [5] of a form of P-450 induced specifically by ethanol, evidence has accumulated that chronic ethanol administration can induce a form that is distinct from those induced by PB, 3-MC, or BNF. This species of P-450 has a high affinity and high catalytic activity for aniline [6, 7], and a high affinity for tetrahydrofurane, a specific inhibitor of 7-ethoxycoumarin metabolism in ethanol-induced microsomes [7, 9]. The latter studies, however, did not confirm early reports [5, 10] of a high affinity of the ethanol-induced P-450 (hereafter designated P-450Et) for cyanide. SDS-polyacrylamide gel electrophoresis of microsomes from ethanol-treated rats revealed a unique induction pattern [11], and the same treatment of a partially purified P-450 preparation from ethanol-induced rats showed the P-450 to be different from forms induced by PB or 3-MC [8]. The ethanol-induced P-450 had an increased ability to metabolize ethanol, and the ferrous-carbonyl complex had a Soret maximum at 451 nm. Although there is some contention as to the significance of P-450 in the metabolism of ethanol in naive animals [12, 13], there is considerable support for involvement of P-450Et in ethanol metabolism in animals treated previously with ethanol [8, 14].

Studies of the chronic effects of ethanol on P-450 have generally involved administration of the compound in the drinking water or as part of a liquid or semi-liquid diet, over a period of 14–28 days [5–10]. Physical dependence on ethanol has been demonstrated after administration of ethanol vapor to rats over a 10-day period [15]. The present study used this method to induce P-450Et in the rat liver and to study the effects of this induction on the catalytic and spectral properties of the microsomes.

EXPERIMENTAL PROCEDURES

Ethanol vapor administration. Male Wistar rats weighing between 250 and 300 g were used. The rats were housed in a Perspex box (1 m × 1.5 m × 0.3 m) with a removable lid, in which they had free access to food and water. Air entered the box at a rate of 8 liters/min through a heated flask in which ethanol was evaporated. Ethanol was pumped into the flask

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§ Abbreviations: P-450, hepatic microsomal cytochrome P-450; P-450Et, ethanol-induced P-450; PB, phenobarbital; 3-MC, 3-methylcholanthrene; DMSO, dimethylsulfoxide; SDS, sodium dodecylsulfate; BNF, β-naphthoflavone; and fp_T, NADPH–cytochrome P-450 reductase.

at a controlled rate to give the desired nominal ethanol vapor concentration in the air.

The dosage regimen employed was a modification of that used by Ferko and Bobyock [15] and involved increasing the concentration of ethanol vapor in the air over a 10-day period. At the end of the treatment period, the rats were killed immediately after removal from the box, except for one experiment in which the animals were kept in a normal environment for 24 hr before being killed. Control animals were housed in wire cages, and no attempt was made to modify their caloric intake to match that of the ethanol-treated rats. All animals were allowed free access to food (Oxoid 41B, Asschem, Falkirk, U.K.) and water at all times.

Injection of PB and 3-MC. Phenobarbitone sodium was administered intraperitoneally for 3 days at a daily dose of 90 mg/kg. 3-MC was administered by the same route in corn oil at a daily dose of 20 mg/kg for 3 days. Rats were killed 24 hr after the last injection.

Preparation of microsomes. Rats were killed by stunning and exsanguination, and the livers were excised and flushed retrogradely with 50 ml of 0.9% NaCl at 4°. The microsomal fraction was prepared in 0.25 M sucrose containing 0.05 M Tris–HCl, pH 7.4, according to Ernster *et al.* [16]. Microsomes were stored at a protein concentration of about 8 mg/ml at 4° for up to 6 hr. All assays were performed within this period.

Enzymatic assays. The metabolism of aniline, aminopyrine, ethylmorphine and benzphetamine by the microsomal fraction was measured for 10 min at 37° in a reaction mixture containing 4 mg of microsomal protein, 112 μmoles Tris–HCl buffer, 25 μmoles glucose-6-phosphate, 2 units glucose-6-phosphate-NADP$^+$ oxidoreductase, 1 μmole $NADP^+$, 10 μmoles $MgCl_2$, and 50 μmoles nicotinamide, in a final incubation volume of 2 ml. Unless otherwise stated, the concentrations of aniline and aminopyrine were 2.5 and 5 mM respectively. Incubation with aniline was stopped by the addition of 1.0 ml of ice-cold 20% trichloroacetic acid, and *p*-aminophenol was determined by the phenol–indophenol reaction method [17]. Incubation with aminopyrine, ethylmorphine, or benzphetamine was stopped by the addition of 0.5 ml of ice-cold 25% $ZnSO_4$ and, after 5 min at 4°, 0.5 ml of saturated $Ba(OH)_2$ was added. Formaldehyde formed during these incubations was trapped with semicarbazide (10 μmoles) and determined according to Nash [18].

The microsomal metabolism of 7-ethoxyresorufin was measured at ambient temperature (23°) in a reaction mixture containing 2 mg protein, 200 μmoles Tris–HCl buffer, 0.5 μmole $NADP^+$, 5 μmoles isocitrate, 0.2 units isocitrate dehydrogenase, and 10 μmoles $MgCl_2$, in a final incubation volume of 2 ml. The appearance of resorufin was followed on an Aminco–Bowman spectrophotofluorimeter as described by Prough *et al.* [19]. The linear change in fluorescence was monitored for 5 min. Incubations with microsomes from 3-MC-treated rats contained only 200 μg of protein. The rate of reaction was linear with regard to protein concentration in the range 0.1 to 1.0 mg/ml.

NADPH–cytochrome *c* reductase activity was determined according to Mazel [20], using an extinction coefficient for cytochrome *c* of 19.6 mM^{-1} cm^{-1} at 550 nm. NADPH–cytochrome P-450 reductase activity was measured as described by Powis [21], using an extinction coefficient for cytochrome P-450 of 91 mM^{-1} cm^{-1} at 450 nm.

Assay of microsomal proteins. Total microsomal protein content was determined by the method of Lowry *et al.* [22], using crystalline bovine serum albumin as standard. Microsomal cytochrome b_5 and P-450 were measured by the method of Dallner [23], using an extinction coefficient for cytochrome b_5 at 423 nm of 171 mM^{-1} cm^{-1}.

Spectral determinations. The difference spectra caused by the addition of substrates to the microsomal fraction were measured at 25° on a Pye–Unicam SP-8000 spectrophotometer, as described by Schenkman *et al.* [17], at a protein concentration of 4 mg/ml.

Measurement of ethanol concentrations. Blood ethanol levels in the rats were measured enzymatically [24] in blood taken from the neck vessels at death. Ethanol concentrations in air from the outlet pipe of the ethanol vapor box were determined by the same method in a sealed Erlenmeyer flask with a silicone stopper through which the sample was injected.

Materials. All enzymes and cofactors were obtained from the Boehringer Corp., Lewes, England. Ethanol (99.5%) was purchased from James Burrough Ltd., London, England. Aniline HCl was from B.D.H., Poole, England, aminopyrine from Ralph N. Emanuel Ltd., Wembley, England, and ethylmorphine from MacFarlan Smith Ltd., Edin-

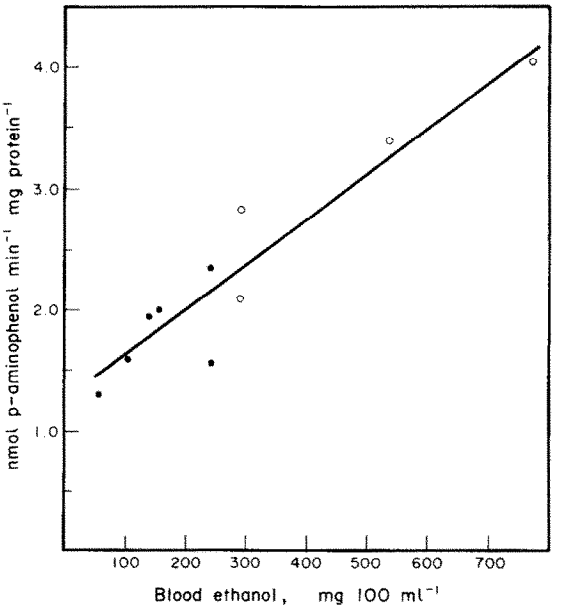

Fig. 1. Aniline hydroxylase activities of microsomes from individual rats with various blood ethanol concentrations after 10 days of ethanol vapor inhalation. Values shown are taken from two separate experiments, in which the final ethanol vapor concentrations were 20 mg/l (●) and 28 mg/l (○). Linear regression analysis of the data yielded a correlation coefficient of 0.938.

Table 1. Effects of ethanol vapor inhalation and withdrawal on hepatic microsomal drug-metabolizing activity and mixed-function oxidase components*

	Control	Ethanol (10 days)	After withdrawal† (24 hr)
Cytochrome P-450 (nmoles/mg protein)	0.41 ± 0.02	0.69 ± 0.06‡	0.44 ± 0.04
Cytochrome b_5 (nmoles/mg protein)	0.34 ± 0.02	0.25 ± 0.03§	0.37 ± 0.04
Aniline hydroxylation [nmoles·min^{-1}·(nmole P-450)$^{-1}$]	1.61 ± 0.09	2.87 ± 0.16‡	1.70 ± 0.14
Aminopyrine demethylation [nmoles·min^{-1}·(nmole P-450)$^{-1}$]	21.8 ± 1.1	11.7 ± 1.2‡	18.1 ± 1.9
NADPH–cytochrome P-450 reductase [nmoles·min^{-1}·(mg protein)$^{-1}$]	0.53 ± 0.12	1.71 ± 0.23‡	ND‖
NADPH–cytochrome *c* reductase [nmoles·min^{-1}·(mg protein)$^{-1}$]	154 ± 8	92 ± 22§	110 ± 9§
Blood ethanol (mg/100 ml)	ND	140 ± 31	1 ± 1

* Values are the means ± S.E.M. of six observations. Mean values were compared by Student's *t*-test.

† Rats were housed in normal cages after the 10-day treatment, and then killed.

‡ Value is significantly different from control, $P < 0.01$.

§ Value is significantly different from control, $P < 0.05$.

‖ Not determined.

burgh, Scotland; DMSO and 3-MC were from Sigma London Ltd., Poole, England. Benzphetamine and 7-ethoxyresorufin were donations from Dr. D. McKillop, University of Surrey, and Dr. M. D. Burke, University of Aberdeen, respectively.

Statistical treatment of results. Groups of data were compared by Student's *t*-test [25] to determine whether a significant difference existed.

RESULTS

Chronic administration of ethanol by vapor inhalation. The mean blood ethanol level in rats after 10 days of ethanol inhalation at ethanol concentrations increasing from 14 mg/l on the first day to 20 mg/l on the final day was 169 ± 12 mg/100 ml (100 observations). The final nominal ethanol vapor concentration in the air was 20 mg/l, but measurement of the concentration in the outlet pipe revealed a true concentration of 15.2 ± 0.4 mg/l (three observations). The discrepancy was attributed to uptake of ethanol into food, water and bedding and to its metabolism by the rats. The treatment caused a 3 per cent decrease in body weight over the 10-day period as opposed to a 9 per cent gain in body weight by untreated control rats (100 observations).

Effects of ethanol vapor and its withdrawal on the mixed-function oxidases. Ethanol vapor inhalation caused increased aniline hydroxylase activity in microsomes, the activity being greater the higher the blood ethanol concentration was at the time of death (Fig. 1). Considerable variation existed in the final blood ethanol concentrations of rats in a single experiment, although, as observed in Fig. 1, rats subjected to higher ethanol vapor concentrations had higher blood ethanol concentrations. Because of an unacceptably high mortality rate in rats breathing a final ethanol vapor concentration of 28 mg/l, subsequent experiments employed a final concentration of 20 mg/l. This concentration had no significant effect on relative liver weight or microsomal protein yield (results not shown).

The effects of ethanol vapor inhalation and withdrawal on the microsomal drug-metabolizing system are shown in Table 1. Blood ethanol levels had returned almost to zero after the withdrawal period. The treatment caused a small increase in the P-450 content of the microsomes which was not apparent 24 hr later (Table 1). This was concomitant with an increase in the rate of aniline hydroxylation, which had also disappeared in 24 hr. Conversely, the rate of aminopyrine demethylation was decreased by ethanol, returning to control levels 24 hr later.

NADPH–cytochrome P-450 reductase activity was increased by ethanol vapor inhalation. Both cytochrome b_5 content and NADPH–cytochrome *c* reductase activities in the microsomes were decreased by the treatment, and the latter was still reduced 24 hr later.

Substrate specificity of ethanol-induced microsomes. The effect of ethanol vapor inhalation on the substrate specificity of microsomal drug-hydroxylating activity was studied. Table 2 shows the effects of ethanol on K_m and V_{max} for the metabolism of aniline, aminopyrine, ethylmorphine, benzphetamine and 7-ethoxyresorufin. The applicability of Michaelis–Menten kinetics, derived for a purified enzyme, to a multi-component system such as microsomes has been reviewed by Lenk [26]. Thus, although the measured values of K_m and V_{max} may not reflect the actual kinetic behavior of a particular enzyme, they may still serve as useful indicators of changes in enzyme activity. The apparent values of K_m and V_{max} obtained from double-reciprocal plots are referred to simply as K_m and V_{max} in this paper.

Ethanol stimulated the metabolism of aniline, causing a decrease in K_m and an increase in V_{max} expressed per nmole P-450, i.e. turnover number. In contrast, the turnover number for aminopyrine was decreased by ethanol, and the K_m increased.

Table 2. Effects of ethanol vapor inhalation on enzyme kinetic parameters of drug hydroxylations in rat liver microsomes*

	K_m†		V_{max}†	
	Control	Ethanol	Control	Ethanol
Aniline	0.25 ± 0.03	0.12 ± 0.01‡	1.01 ± 0.01	2.30 ± 0.27§
Aminopyrine	0.64 ± 0.15	1.53 ± 0.33‖	14.1 ± 1.9	8.2 ± 1.3‖
Ethylmorphine	0.37 ± 0.05	0.34 ± 0.03	11.2 ± 1.2	5.9 ± 1.0‡
Benzphetamine	0.15 ± 0.01	0.12 ± 0.01	21.6 ± 1.9	10.0 ± 1.1‡
7-Ethoxyresorufin	0.80 ± 0.10	0.27 ± 0.03‡	2.3 ± 0.5	1.1 ± 0.1

* Parameters were obtained from Lineweaver–Burk plots. Values are the means ± S.E.M. of at least three observations, each obtained using pooled microsomes from three rat livers.

† V_{max} values are expressed as nmoles substrate metabolized per min per nmole P-450. Units for K_m are mM.

‡ $P < 0.01$, compared to control.

§ $P < 0.001$, compared to control.

‖ $P < 0.05$, compared to control.

Ethanol vapor inhalation decreased V_{max} for both ethylmorphine and benzphetamine without affecting the respective K_m values. Conversely, the K_m for 7-ethoxyresorufin was decreased by the treatment, with no significant effect on V_{max} (although the mean value for the latter was decreased by 50 per cent).

Spectral changes upon addition of substrate to the microsomal fraction from control and ethanol-treated rats were also studied. Aminopyrine (1 mM) ethylmorphine (1 mM), and benzphetamine (0.3 mM) all gave typical type I difference spectra [24] when added to microsomes from either source, whereas aniline (1 mM) produced a type II spectral change in both groups. Addition of DMSO (56 mM) to ethanol-induced microsomes gave a reverse type I spectral change, but no clear spectral change was observed in control microsomes. Furthermore, we were unable to observe a spectral change on addition of 56 mM DMSO to microsomes from rats treated with either PB or 3-MC.

No attempt was made to present values of K_s and A_{max} obtained from spectral binding data, because the complex nature of the Lineweaver–Burk plots did not allow such a straightforward interpretation.

CO-induced spectral change. The absorption maxima of the CO-binding spectra of reduced P-450 after treatment with different inducers are known to vary [27–29]. Absorption maxima were obtained in microsomes from rats treated with ethanol, PB, or 3-MC. Recorded on the same day, values were as follows: control, 450 nm; ethanol, 451 nm; 3-MC, 448 nm; and PB, 450–451 nm.

Acetone enhancement. The effects of ethanol vapor treatment on acetone enhancement of aniline hydroxylation were studied.

Rates of aniline hydroxylation by microsomes from control and ethanol-treated rats in the presence of various concentrations of acetone in the incubation are shown in Fig. 2. As the acetone concentration was increased, the difference in rates of aniline hydroxylation by control and ethanol-induced microsomes diminished, and at the highest concentrations it disappeared.

NADH synergism in aniline hydroxylation was observed in the presence of acetone, the effect increasing with increased acetone concentration (results not shown). No such effect was observed in ethanol-induced microsomes.

DISCUSSION

The nominal final, ethanol vapor concentration used was considerably lower than that employed by Ferko and Bobyock [15], and the measured concentration was almost half that used by these authors. As already stated, our laboratory has found that higher final ethanol vapor concentrations are fatal to rats.

The weight loss caused by the ethanol treatment is attributed to a decrease in food intake. Many studies involving chronic alcohol administration to rats have involved adjustment of the caloric intake of control rats with carbohydrate to match that of the ethanol-treated group [8, 30–34]. One of the disadvantages of administering ethanol by vapor inhalation is that the caloric intake of the treated animals cannot be measured conveniently, whereas oral administration is ideal for this purpose. However, changes in diet, such as increased carbohydrate

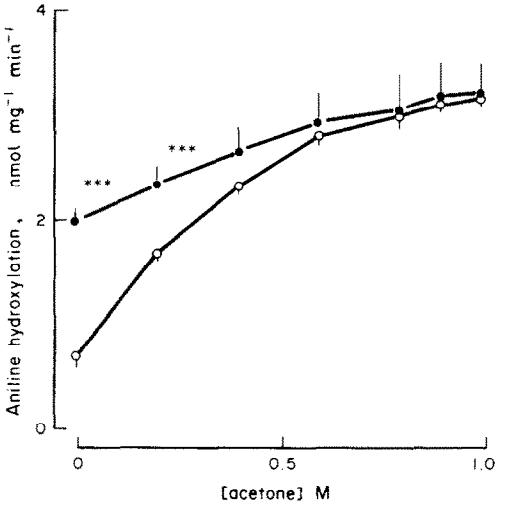

Fig. 2. Acetone enhancement of aniline hydroxylation in control and ethanol-induced microsomes. Aniline hydroxylation in the presence of 0 to 1.0 M acetone by microsomes from control (○) and ethanol-treated rats (●) was determined as described in the text. Values are the means ± S.E.M. of six observations. Asterisks indicate that $P < 0.001$, compared to control value.

content or altered fat or protein intake, are themselves known to affect drug-metabolizing activity [35–39]. Thus, the validity of a simple caloric adjustment as a control diet may be questionable.

The increase in aniline hydroxylase activity after ethanol vapor treatment and the disappearance of this effect 24 hr after withdrawal of the treatment have been reported previously [6, 7, 40] with oral administration, although in the latter study [40] an elevated aminopyrine demethylation rate occurred after withdrawal. In the present experiment no such effect on aniline hydroxylase activity occurred, although a decrease in aminopyrine demethylation due to the ethanol treatment had disappeared after 24 hr.

The concomitant small increase in P-450 content of the microsomes and in aniline hydroxylation after ethanol vapor administration, and the subsequent return of both variables to control levels after 24 hr, indicate that the ethanol-induced P-450 underwent a high rate of turnover in the microsomes. In addition, the temporal correlation of the effects on the two variables indicates that the extra P-450 present in the ethanol-induced microsomes may have been exclusively of the type with a high activity toward aniline. If this were true, then in ethanol-induced microsomes at least 40 per cent of the total P-450 present would be P-450Et.

There were disparate effects of the treatment on NADPH–cytochrome *c* reductase and NADPH–cytochrome P-450 reductase activities, for while the amount of fp_T in ethanol-induced microsomes was decreased (NADPH–cytochrome *c* reductase activity), its ability to reduce cytochrome P-450 was increased.

The hypothesis that ethanol induces a form of P-450 that has a high affinity and catalytic activity for aniline [6, 7] is supported by the kinetic data presented here. There has been some debate over the effects of chronic oral administration of ethanol on the microsomal metabolism of other substrates. Some workers have reported increases in the metabolism of drugs such as aminopyrine and ethylmorphine [30–32, 41], while others have found decreases in the metabolism of benzphetamine, aminopyrine, and ethylmorphine [40, 42]. The present study supports the latter observations. Ethanol also caused a change in the kinetics of 7-ethoxyresorufin metabolism, producing a decrease in K_m with no effect or a decrease in V_{max}. One possibility is that ethanol caused a decrease in a low affinity enzyme normally present in considerable amounts, thus allowing the characteristics of a form with a high affinity for 7-ethoxyresorufin to be observed.

The substrate specificity of ethanol-induced microsomes is, therefore, quite different from that of microsomes from animals treated with PB, 3-MC, or BNF [19, 29, 43–46]. PB stimulates the metabolism of benzphetamine, ethylmorphine, and aminopyrine, whereas 3-MC increases markedly the rate of 7-ethoxyresorufin *O*-deethylation.

The reverse type I difference spectrum, as produced by the addition of DMSO to ethanol-induced microsomes, is indicative of a high spin–low spin transition of the heme iron of P-450. As indicated by Kumaki *et al.* [47], the magnitude of the spectral change produced by such a ligand depends on the *in vivo* spin state of the cytochrome(s) present. Thus, the fact that the concentration of DMSO used produced such a change in ethanol-induced microsomes only indicates a difference in the spin state of the cytochromes P-450 in these microsomes. The increased wavelength of the Soret maximum of the CO complex of the reduced cytochrome, caused by ethanol administration, supports the observation of Ohnishi and Lieber [8] with partially purified P-450Et. Such a spectrum in microsomes, however, is the resultant of the spectra of all the P-450 species present in these microsomes, and is not necessarily indicative of the Soret maximum for P-450Et.

Acetone is one of a group of enhancing agents that, when added to the reaction mixture, stimulates the microsomal metabolism of aniline, but not that of compounds such as aminopyrine, ethylmorphine or benzphetamine [48]. The phenomenon of NADH synergism is not normally observed in the microsomal metabolism of aniline, but it may be observed in the presence of acetone [49, 50]. Recent evidence that the rate-limiting step in aniline hydroxylation occurs at a stage after those of electron transfer, viz. C—H bond cleavage [51, 52], and the observation of enhancement of the cumene hydroperoxide-supported reaction by acetone [53] suggests that acetone acts to stimulate this step. Thus, the rate of second electron transfer may become rate limiting, and the synergistic effect of NADH observed.

In view of this effect of acetone, the disappearance of the difference in rates of aniline hydroxylation by control and ethanol-induced microsomes may indicate either a difference in the rate-limiting step of the reaction catalyzed by P-450Et or a decreased sensitivity of the ethanol-induced cytochrome to acetone. The possibility exists that ethanol may act *in vivo* to cause an enhancement of aniline hydroxylation similar to that observed *in vitro* with acetone. This might account for the observed withdrawal effect and the lack of an additive effect with acetone. This explanation cannot be excluded, but it is unlikely due to the lack of a synergistic effect of NADH on aniline hydroxylation in ethanol-induced microsomes, either in the presence or absence of acetone.

In conclusion, ethanol vapor administration provides a way of maintaining high blood ethanol concentrations in rats. Such a constant intake of ethanol eliminates the fluctuations in blood ethanol levels that may be observed with oral administration [15], and thus it is a useful tool for the study of ethanol induction of P-450, especially in view of the observed high rate of its turnover in the liver. This method may be particularly useful for the study of ethanol induction in other species such as the rabbit, which will not drink as high quantities of ethanol as the rat (unpublished observation).

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