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EFFECTS OF ETHANOL ON HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYMES IN THE RAT*

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Abstract—The activities of several hepatic microsomal drug-metabolizing enzymes were determined in male and female rats after administration of 20% ethanol or an isocaloric amount of glucose in drinking water for 10-49 days. The aniline hydroxylase activity increased, whereas the activities of both pentobarbital hydroxylase and benzphetamine *N*-demethylase were decreased in male rats given ethanol and killed without ethanol withdrawal. Twenty-four hr after removal of ethanol, the aniline hydroxylase remained elevated but a striking increase of both pentobarbital hydroxylase and benzphetamine *N*-demethylase above control values occurred. Six days later, all three of these microsomal enzymes returned to normal control values. The reduction in pentobarbital hydroxylase and benzphetamine *N*-demethylase could not be attributed simply to high endogenous ethanol levels since: (1) addition of high concentrations of ethanol *in vitro* inhibited microsomal aniline hydroxylase and pentobarbital hydroxylase but did not reduce benzphetamine *N*-demethylase; and (2) acute administration of ethanol by gastric tube, which markedly elevated the blood ethanol level, did not result in a decline in the enzyme activities. These two findings suggest that the observed decrease in microsomal enzymes in male rats required persistent ethanol exposure. In contrast to male rats, female rats given ethanol for 28 days showed a significant increase in aniline hydroxylase activity, but the activities of pentobarbital hydroxylase and benzphetamine *N*-demethylase were not decreased. Moreover, administration of ethanol for 28 days to female rats did not reduce the response of these three enzyme activities to pentobarbital administration. It is concluded that the effects of chronic ethanol ingestion on the hepatic microsomal drug-metabolizing enzymes are complex and depend on sex, exposure to other agents and, most importantly, on the duration and proximity of ethanol intake.

Recent studies in man and in experimental animals have reported that chronic administration of ethanol increases the metabolism of ethanol [1, 2], pentobarbital [3, 4], meprobamate [4] and aniline [4–6], and accelerates the plasma disappearance rate for pentobarbital [3, 7], meprobamate [3, 7], tolbutamide [8] and zoxazolamine [9]. Evidence also suggests that ethanol shifts the cytochrome P-450 absorption spectra *in vitro* [10] and increases the microsomal cytochrome P-450 levels [7, 11] or the hepatic microsomal mixed-function oxidase system [4, 12, 13]. Because of these various effects, it has been suggested that ethanol alters the mixed-function oxidase system in the same manner as drugs such as phenobarbital [3, 10], a known inducer of hepatic drug metabolism [14]. However, others have observed that ethanol differs from barbiturates in affecting the drug-metabolizing enzyme system [15]. Furthermore, there is conflicting evidence regarding the effect of chronic ethanol administration on the metabolism of barbiturate. Kato and Chiesara [16] found no effect of ethanol administration on pentobarbital metabolism *in vivo* or *in vitro*, and Remmer [17] reported that ethanol administration to rats for 12 days had no effect on the microsomal hydroxylation of hexobarbital. Kalant *et al.* [18] reported a 33 per cent decrease in the rate of pentobarbital metabolism by liver slices from male rats fed ethanol chronically with a nutritionally adequate diet. These findings contrast with the reports of Rubin *et al.* [12] and Rubin and Lieber [4], who observed an increased activity of pentobarbital hydroxylase in rats fed ethanol with a protein- and choline-deficient diet. The rate of disappearance of pentobarbital from rat plasma has been reported to increase [3, 7] or not change [18].

A preliminary report from this laboratory showed a decrease in microsomal demethylase activity toward aminopyrine and benzphetamine after chronic ethanol feeding in male rats [19], although Joly *et al.* [20] reported an enhancement of aminopyrine and ethylmorphine demethylase activity after chronic ethanol feeding in female rats.

The discrepancies between these various reports could result from sex or strain differences in the experimental animals, the techniques of feeding and ethanol administration, or the enzymatic techniques used in assessment of drug-metabolizing enzymes.

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Therefore, the present studies were designed to investigate in more detail the effects of prolonged ethanol ingestion, with an adequate diet, on microsomal drug-metabolizing enzymes of both male and female rats. The influence of duration of ethanol intake and withdrawal of ethanol on these activities was also determined. The results indicate that the effect of chronic ethanol administration in the rat is sex dependent and more closely resembles changes reported after starvation than the induction produced by barbiturate administration.

MATERIALS AND METHODS

Animals and diets. Male and female rats of the Sprague–Dawley strain weighing 200–250 g were used in the chronic ethanol studies. Male rats of the same strain weighing about 300 g were used for the acute ethanol studies. Each rat received an intramuscular injection of 30,000 units of sterile benzathene penicillin G (Wyeth) 3 days before the chronic experiment began. Rats were housed in individual metabolic cages and were allowed approximately 1 week after arrival from the vendor to acclimate to their new environment under a light cycle of 8:00 a.m. to 8:00 p.m. Air-conditioning maintained the room temperature at 25°.

In the acute studies, ethanol was given orally (4 g/kg) in a volume of 20 ml/kg by gastric tube. One group was given only a single dose, while another received two doses of 4 g/kg at 6-hr intervals. Control rats were given glucose solution isocaloric to that of ethanol. Rats were killed and liver microsomes prepared (described later) 90 min after the single administration of ethanol or glucose and 24 hr after the initial dose in the group give ethanol twice.

In the chronic experiments, rats were fed 20% (v/v) ethanol as the only source of drinking water in Richter feeding tubes for periods of 10, 28 or 49 days. The ethanol solution was offered until the time of sacrifice. Purina lab chow was fed *ad lib*. The chow contained 23% protein, 4·34% fat, 0·36% choline and 59% mixed starches by weight. The control rats, paired according to weight before starting the experiment, were fed an amount of chow equal to that consumed by the paired rats given ethanol and a volume of 30% (w/v) glucose which was isocaloric to the ethanol ingested by the experimental rats. The intake of ethanol was about 12 g/kg of body wt/day. Ethanol or glucose accounted for approximately 33 per cent of the total caloric intake. At the end of the experimental period, each pair of rats had consumed an identical amount of chow and isocaloric amounts of either ethanol or glucose.

In another experiment, 12 pairs of male rats were pair-fed 20% ethanol or isocaloric glucose for a period of 28 days and then tap water was substituted for the ethanol or glucose. Six rats of each group were killed 1 and 6 days after discontinuation of the ethanol or glucose solution.

Twenty-four female rats were pair-fed for 28 days with either ethanol or glucose as described for male rats. Four days before the rats were killed, six rats of each group were injected intraperitoneally with sodium pentobarbital (25 mg/kg in a volume of 2 ml/kg) once daily for 4 consecutive days. Matched groups of six rats fed ethanol or glucose were given saline in place of pentobarbital.

Preparation of microsomes. Rats were anesthetized with ethyl ether and killed without fasting by exsanguination from the abdominal aorta. Samples of blood were placed into heparinized tubes for determination of blood ethanol levels. The livers were removed promptly, washed in ice-cold 1·15% KCl–0·05 M Tris buffer solution (pH 7·5), weighed, minced with scissors and then homogenized with 4 vol. (w/v) of the same buffer, using an electric hand drill and a Teflon glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min in a Sorval refrigerated centrifuge. The resulting supernatant was centrifuged at 10,000 *g* for 20 min and the pellet discarded. Microsomal fractions were prepared from this supernatant by centrifugation at 105,000 *g* at 4° for 60 min in a Beckman ultracentrifuge using a No. 40 rotor. The surface of the microsomal pellet was washed three times with 2 ml of ice-cold 0·05 M Tris buffer, pH 7·5. The final microsomal pellet was resuspended in 4 ml of this buffer. Aliquots of microsomal suspensions from each rat liver were used immediately for the determination of enzyme activities or the microsomal pellet was covered by 1 ml of the buffer and stored at −40° until the following day. Control and experimental preparations were assayed simultaneously.

Drug-metabolizing reactions. The activity of aniline hydroxylase was measured by formation of *p*-aminophenol according to the method of Imai and Sato [21]. The reaction mixture for pentobarbital hydroxylation contained 1·5 mM glucose 6-phosphate, 0·12 mM NADP, 1·5 unit of glucose 6-phosphate dehydrogenase (type XV, Sigma Chemical Co.), 0·5 mM ATP, 50 mM KCl, 2·5 mM $MgCl_2$, 30 mM nicotinamide, 50 mM Tris buffer (pH 7·5), 0·35 mM sodium pentobarbital containing 0·2 μCi ^{14}C-pentobarbital sodium and 0·3 ml of the microsomal enzyme preparation equivalent to 5 mg microsomal protein in a final volume of 4·0 ml. Incubations were conducted at 37° for 15 min in an atmosphere of air. The activity of pentobarbital hydroxylase was measured by the rate of total pentobarbital metabolites formed as described by Kuntzman *et al.* [22].

The *N*-demethylation of benzphetamine was measured at 37° in an assay mixture containing 0·33 mM NADP, 3·3 mM $MgCl_2$, 8 mM sodium isocitrate, 1·7 mM semicarbazide, 0·5 unit of isocitric dehydrogenase (Sigma Chemical Co.), 1·8 mM benzphetamine, 0·2 ml of the microsomal suspension equivalent to 3 mg microsomal protein and 43 mM Tris buffer (pH 7·5) in a final volume of 1·5 ml. The mixture was incubated for 12 min under air. A blank without benzphetamine was included in each assay. The reaction was terminated by the addition of 0·5 ml of 20% zinc sulfate and 0·5 ml of saturated barium hydroxide. For-

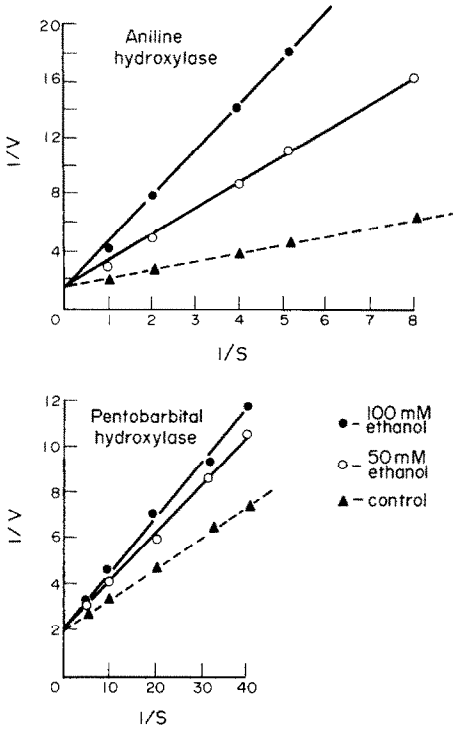

Fig. 1. Effects of ethanol addition on the activities of aniline hydroxylase (upper panel) and pentobarbital hydroxylase (lower panel) in hepatic microsomes prepared from normal male rats. V indicates nmoles *p*-aminophenol and nmoles total pentobarbital metabolites formed per mg of microsomal protein per min for aniline hydroxylase and pentobarbital hydroxylase respectively. S is the concentration (mM) of aniline or pentobarbital. Each point is the mean of three determinations.

maldehyde formed was measured according to the method of Nash [23]. Duplicate determinations were performed for each rat for all enzyme assays.

Cytochrome P-450 content of microsomes was determined by the difference spectrum of the carbon monoxide complex of P-450 according to the method of Omura and Sato [24].*

Microsomal protein concentration was determined in duplicate by the method of Lowry *et al.* [25] with crystalline bovine serum albumin used as a standard. Blood ethanol levels were determined according to the automated fluorometric procedure of Ellis and Hill [26].

RESULTS

Effects of ethanol addition on liver microsomal drug-metabolizing enzymes in vitro. Ethanol was added to the incubation systems at several different concentrations. A double-reciprocal plot of the data (Lineweaver–Burk method) revealed the inhibition by ethanol (50 and 100 mM) to be of the competitive type for both aniline hydroxylation (Fig. 1, upper-panel) and pentobarbital hydroxylation (Fig. 1, lower panel). In contrast, the activity of benzphetamine *N*-demethylase was not inhibited by the addition of ethanol at concentrations as high as 100 mM (460 mg/100 ml), as shown in Fig. 2. This lack of ethanol inhibition was noted using microsomes from rats fed chow, those given ethanol or glucose, and rats previously treated with phenobarbital. In addition, ethanol did not inhibit the *N*-demethylation of ethylmorphine when this drug was used as the substrate in the same incubation mixture. Because 2-diethylaminoethyl-2,2-diphenylvalerate HCl (SKF 525A) is well known to inhibit the metabolism of several drugs by hepatic mixed-function oxidase [27], its effect on benzphetamine *N*-demethylase was measured. Figure 2 also shows that SKF 525A, at the low concentration of 0·1 mM, inhibited about 80 per cent of the activity of this enzyme in the presence of 0·2 mM substrate. Similarly, SKF 525A was shown to inhibit both aniline and pentobarbital hydroxylase *in vitro*. Therefore, the assay techniques used are reliable for detection of substances which inhibit *N*-demethylase activity.

Glucose 6-phosphate dehydrogenase was used to generate NADPH in both the aniline and pentobarbital assays, while isocitric dehydrogenase was used to generate NADPH in the benzphetamine *N*-demethylation assay. It was possible that ethanol could reduce aniline and pentobarbital hydroxylase activities by inhibiting glucose 6-phosphate dehydrogenase in the NADPH-generating system. However, when isocitric dehydrogenase was used to generate NADPH in the aniline hydroxylase assay, it was found that the inhibition by ethanol (100 mM) was unchanged. Furthermore, ethanol had no inhibitory effect on benzphetamine *N*-demethylation when glucose 6-phosphate dehydrogenase was used in place of isocitric dehydrogenase in generating the NADPH for this assay. Therefore, ethanol inhibits both aniline and pentobarbital

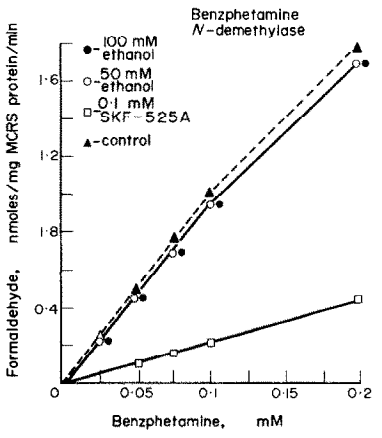

Fig. 2. Effect of the addition of ethanol and SKF 525A on benzphetamine *N*-demethylation in hepatic microsomes prepared from normal male rats. Each point is the mean of three determinations.

* Performed in the laboratories of Dr. James Fouts.

Table 1. Effect of ethanol, added *in vitro* on the activity of pentobarbital hydroxylase and benzphetamine *N*-demethylase in liver microsomes of various species*

Enzyme source	Pentobarbital hydroxylase (nmoles metabolites/mg microsomal protein/min)			Benzphetamine *N*-demethylase (nmoles formaldehyde/mg microsomal protein/min)		
	Control	Ethanol	P	Control	Ethanol	P
Rat	0·34 ± 0·01 (4)†	0·26 ± 0·01 (4)	<0·01	3·54 ± 0·04 (4)	3·42 ± 0·18 (4)	>0·1
Mouse	1·55 ± 0·05 (3)	0·76 ± 0·03 (3)	<0·01	3·02 ± 0·15 (3)	2·77 ± 0·17 (3)	>0·1
Rabbit	0·59 ± 0·04 (3)	0·43 ± 0·02 (3)	<0·05	3·49 ± 0·26 (3)	3·15 ± 0·15 (3)	>0·1
Guinea-pig	0·33 ± 0·02 (3)	0·11 ± 0·02 (3)	<0·01	4·19 ± 0·21 (3)	4·18 ± 0·08 (3)	>0·1

* Microsomes were prepared from the pooled livers of three rats, five mice, two guinea-pigs and several portions of one rabbit liver. Pentobarbital and benzphetamine, 2×10^{-4} M, were incubated with liver microsomes with or without the addition of 1×10^{-1} M ethanol for 15 and 12 min respectively. Values are means ± standard errors of mean.

† Numbers of parentheses refer to numbers of determinations.

hydroxylase directly and not by reducing the activity of the NADPH-generating system.

Species differences in the effects of inducers and inhibitors of drug metabolism have been reported for many compounds [28]. Therefore, the effect of ethanol addition on the activity of pentobarbital hydroxylase and benzphetamine *N*-demethylase from liver microsomes of mouse, rabbit and guinea pig was compared to that of the rat. The results are given in Table 1. A 2- to 3-fold difference in the activity of pentobarbital hydroxylase between species was observed and there was no correlation between the inhibition by ethanol and the activity of the reaction. However, benzphetamine *N*-demethylase activity was not substantially inhibited by ethanol at a concentration of 1×10^{-1} M in any of the species studied.

Effect of acute ethanol administration on liver microsomal enzyme activities. The effect of ethanol, given orally in a dose of 4·0 g/kg, 90 min or 18 and 24 hr before preparation of the microsomes is shown in Table 2. The blood ethanol concentration was 205 ± 14 mg/100 ml in rats killed 90 min after ethanol administration and was undetectable 18 hr after ethanol. Microsomal enzyme activities in rats killed 90 min after a single dose of ethanol were not significantly different from those of glucose-treated controls, as shown in Table 2. This result indicated that the presence of high concentrations of ethanol in the circulation or in tissues did not interfere with or inhibit the metabolism of drugs by isolated liver microsomes. To determine whether acute administration of ethanol would have any metabolic effect on the metabolism of drugs *in vitro* after ethanol elimination, the microsomal enzyme activities were determined after two doses of ethanol, given 18 and 24 hr previously. The results presented in Table 2 indicate that the activities of pen-

Table 2. Effect of acute ethanol administration on microsomal enzyme activities of rats*

Determination	Time of pretreatment			
	90 min		24 hr	
	Ethanol	Glucose	Ethanol	Glucose
Blood ethanol at death (mg/100 ml)	205 ± 14 (6)†		0	
Aniline hydroxylase	0·507 ± 0·020 (6)	0·517 ± 0·027 (6)	0·839 ± 0·075 (8)‡	0·533 ± 0·048 (8)
Pentobarbital hydroxylase	0·477 ± 0·066 (6)	0·455 ± 0·047 (6)	0·417 ± 0·052 (8)	0·513 ± 0·061 (8)
Benzphetamine *N*-demethylase	3·83 ± 0·450 (6)	4·00 ± 0·400 (6)	3·21 ± 0·327 (8)	3·73 ± 0·439 (8)

* Ethanol was given orally by gastric tube in a dose of 4·0 g/kg and glucose was given in isocaloric quantities in the same manner. In the first experiment, rats received only one treatment 90 min before the experiment; in the second experiment, rats received two treatments, 24 hr and 18 hr earlier. Microsomes prepared from livers were incubated as indicated under Methods. Enzyme activity was expressed as nmoles *p*-aminophenol, nmoles total pentobarbital metabolites and nmoles formaldehyde per mg microsomal protein per min for aniline hydroxylase, pentobarbital hydroxylase and benzphetamine *N*-demethylase respectively. Values are expressed as mean ± standard error of mean.

† Numbers in parentheses refer to number of rats.

‡ Significant difference from glucose-treated ($P < 0·01$).

Table 3. Effect of chronic ethanol feeding on body weight gained, liver microsomal protein and liver weight of male rats*

Experimental group	Blood ethanol at death (mg/100 ml)	Body wt gained (g)	Wet liver wt (g/100 g body wt)	Microsomal protein (mg/g wet liver)
Ethanol 10 days	68·0 ± 17·0	35 ± 7·3	4·00 ± 0·152	
Glucose-pair		42 ± 8·5	3·80 ± 0·096	
Ethanol 28 days	89·6 ± 15·2	107 ± 10·7	3·62 ± 0·095	26·3 ± 1·37
Glucose-pair		87 ± 6·6	3·47 ± 0·055	23·3 ± 0·92
Ethanol 49 days	63·2 ± 24·1	150 ± 23·0	3·51 ± 0·080	22·8 ± 1·65†
Glucose-pair		181 ± 12·1	3·53 ± 0·041	17·3 ± 1·45

* Rats were given 20% (v/v) ethanol or isocaloric glucose in drinking water for different periods of time and killed without discontinuation of ethanol at the end of feeding. Blood samples were collected from abdominal aorta. Values are means ± standard errors from six to ten rats.

† Significant difference from glucose-pair controls ($P < 0·05$).

tobarbital hydroxylase and benzphetamine *N*-demethylase after this ethanol treatment were not significantly different from those of glucose-treated controls. However, aniline hydroxylase activity was significantly increased.

Effect of chronic ethanol feeding on body weight gained, liver microsomal protein concentration and liver weight in the male rat. The blood ethanol levels, body weight gained, wet liver weight and the microsomal protein concentrations of male rats after different periods of ethanol or glucose feeding are shown in Table 3. The animals were killed without withdrawal of ethanol or prior fasting. Mean blood ethanol levels were 63 to 90 mg/100 ml, with a range of 0 to 150 mg/100 ml. The rats fed ethanol and those given glucose received the same calories, and weight gain was approximately the same at the end of the experiment. However, this weight gain was approximately 40 per cent less than that of rats fed chow *ad lib.* Liver microsomal protein per g of wet liver weight was increased significantly only in rats fed ethanol for 49 days.

The microsomal cytochrome P-450, which was measured only in the group fed for 28 days, was increased by ethanol whether expressed per g of liver or per mg of microsomal protein, as shown in Table 4.

Effect of chronic ethanol feeding and subsequent withdrawal on hepatic microsomal enzyme activities in the male rat. Table 5 shows the effect of chronic ethanol feeding on hepatic microsomal enzyme activities measured during ethanol ingestion. Aniline hydroxylase activity was increased 20–72 per cent by ethanol treatment for 10–49 days. The activity of this reaction continued to increase over the time course of these experiments. In contrast to the effects on aniline hydroxylase, chronic ethanol feeding produced significant decreases in the activities of both pentobarbital hydroxylase and benzphetamine *N*-demethylase. A maximum decrease in the activity of these reactions was reached by day 28. The decrease in both benzphetamine *N*-demethylase and pentobarbital hydroxylase also was observed when rats fed ethanol for 28 days were compared with rats fed chow *ad lib.*

The effect of ethanol withdrawal on aniline hydroxylase activity is shown in Fig. 3. The increased level of aniline hydroxylase noted at the end of 28 days of ethanol feeding was maintained for 1 day after ethanol withdrawal, but by 6 days after withdrawal of ethanol, the level had returned to normal. In contrast, pentobarbital hydroxylase and benzphetamine *N*-demethylase (Fig. 4) were increased significantly above normal control levels 1 day after withdrawal of ethanol. These activities also returned to normal levels 6 days after ethanol withdrawal.

Effect of chronic ethanol feeding, pentobarbital treatment, or both, on microsomal protein concentrations and hepatic microsomal enzyme activities in the female rat. The effects of chronic ethanol treatment and combined ethanol and pentobarbital administration on female rats is shown in Table 6. The liver microsomal protein concentration was increased significantly by either ethanol feeding or pentobarbital treatment. Treatment with a combination of ethanol and pentobarbital

Table 4. Effect of chronic ethanol feeding on microsomal P-450 of the male rat liver*

Rats	Microsomal P-450 (nmoles/g liver)	Microsomal P-450 (nmoles/mg microsomal protein)
Control	34·7 ± 3·72	1·14 ± 0·07
Ethanol	45·2 ± 0·97	1·45 ± 0·08
P†	<0·01	<0·01

* Rats were given 20% (v/v) ethanol or isocaloric glucose in drinking water for 28 days and killed without discontinuation of ethanol at the end of feeding. Values are means ± standard errors from six rats.

† Significant difference between ethanol-treated and glucose control groups.

Table 5. Effect of chronic ethanol feeding on liver microsomal enzyme activities of male rats*

Experimental groups	Aniline hydroxylase	Pentobarbital hydroxylase	Benzphetamine *N*-demethylase
Ethanol 10 days	0·923 ± 0·048†	0·511 ± 0·081	2·93 ± 0·181†
Glucose-pair fed	0·717 ± 0·041	0·538 ± 0·062	3·70 ± 0·201
Ethanol 28 days	0·897 ± 0·071†	0·406 ± 0·039†	2·14 ± 0·123†
Glucose-pair fed	0·620 ± 0·065	0·600 ± 0·029	3·22 ± 0·135
Ethanol 49 days	1·126 ± 0·055†	0·490 ± 0·024†	2·62 ± 0·130†
Glucose-pair fed	0·653 ± 0·050	0·619 ± 0·032	3·51 ± 0·138

* Rats were given 20% (v/v) ethanol or isocaloric glucose in drinking water for different periods of time and killed without discontinuation of ethanol at the end of feeding. Microsomes prepared from livers were incubated as indicated under Methods. Enzyme activity was expressed as nmole *p*-aminophenol, nmoles pentobarbital metabolites and nmoles formaldehyde formed per mg microsomal protein per min for aniline hydroxylase, pentobarbital hydroxylase and benzphetamine *N*-demethylase respectively. Values are means ± standard errors of mean from six to ten rats.

† Significant difference from glucose-pair controls ($P < 0·01$).

together did not result in a greater increase in the microsomal protein when compared with either ethanol or pentobarbital alone.

Aniline hydroxylase activity was increased by prolonged ethanol feeding but, in contrast to the findings in male rats, the activities of pentobarbital hydroxylase and benzphetamine *N*-demethylase were not significantly reduced by prolonged ethanol feeding.

As expected, pentobarbital treatment (25 mg/kg) for a 4-day period in control rats fed glucose resulted in a significant increase in the activity of pentobarbital hydroxylase. Aniline hydroxylase was not changed by treatment with pentobarbital, but benzphetamine *N*-demethylase activity was increased 97 per cent. When pentobarbital was given for 4 days to rats fed ethanol for 28 days, a substantial increase in all three of the drug-metabolizing enzymes studied was noted. Apparently, the induction of microsomal drug-metabolizing enzymes by this dose of pentobarbital was potentiated by chronic ethanol feeding in female rats.

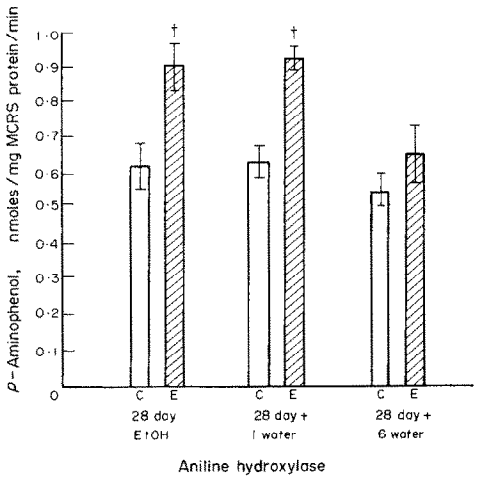

Fig. 3. Effect of ethanol feeding for 28 days and withdrawal of ethanol on microsomal aniline hydroxylase activity in male rats. Male rats were fed with 20% (v/v) ethanol or isocaloric glucose for a period of 28 days. The rats were killed at the completion of ethanol feeding or 1 and 6 days after withdrawal of ethanol. Microsomes were prepared from livers and incubated as indicated under Methods. Aniline hydroxylase activity was expressed as nmoles *p*-aminophenol formed per mg of microsomal protein per min. Values are means ± standard errors of means from six to ten rats. Withdrawal of ethanol for 1 day is indicated as "1 water" and for 6 days by "6 water". C = glucose-pair controls (open bars); E = ethanol-fed rats (striped bars). The symbol (†) indicates a significant difference from glucose-pair controls ($P < 0·01$).

DISCUSSION

The present studies were designed to evaluate the effects of chronic ethanol intake in rats of both sexes on the metabolism *in vitro* of drugs representing the two general classes of compounds interacting with the hepatic cytochrome P-450 system [29]. Pentobarbital and benzphetamine are examples of type I drugs, a group including other barbiturates and morphine. Aniline is a typical type II compound [30], and cortisol, alcohol and acetanilide are also included in this group.

The results demonstrate that chronic ethanol intake in male rats increases liver microsomal P-450 levels but causes discrepant effects on the microsomal metabolism of the two classes of drugs. A prompt and persistent increase in the enzyme activity of aniline hydroxylation was accompanied by a lesser decline in the activities of enzymes responsible for the metabolism of the type I compounds, pentobarbital and benzphetamine. In contrast, only the increase in the aniline hydroxylase was statistically significant in the female rats fed ethanol for prolonged periods. The activity of pentobarbital hydroxylase in the female rats also tended to increase, but the rise was not statistically different from that of glucose-pair controls. Several investigators have reported that chronic ethanol feeding of rats results in increases in microsomal P-450 levels [6, 11, 20] and in the activity of aniline hydroxylase [3, 5, 6, 12, 13]. However, our results apparently conflict with some earlier reports indicating an increase in pentobarbital hydroxylation [3, 4, 7, 12] and aminopyrine *N*-demethylation [20, 32] after chronic ethanol

Table 6. Effect of chronic ethanol feeding, pentobarbital treatment, or both, on weight gain, liver weight, liver microsomal protein and microsomal enzyme activities of female rats*

Experimental groups	Body wt gained (g)	Wet liver wt (g/100 g body wt)	Microsomal protein (mg/g wet liver)	Microsomal enzyme activity (nmoles/mg protein/min)		
				Aniline hydroxylase	Pentobarbital hydroxylase	Benzphetamine *N*-demethylase
Ethanol	73 ± 5·3	3·7 ± 0·20	16·4 ± 0·55†	1·03 ± 0·063†	0·307 ± 0·028	2·25 ± 0·143
Glucose	69 ± 4·9	4·1 ± 0·18	12·1 ± 1·15	0·675 ± 0·060	0·273 ± 0·027	2·58 ± 0·143
Ethanol + pentobarb.	71 ± 4·9	4·1 ± 0·15	14·4 ± 0·66	1·30 ± 0·089†‡	0·497 ± 0·039†‡	6·36 ± 0·590†
Glucose + pentobarb.	58 ± 3·1	3·9 ± 0·12	15·3 ± 0·92†	0·789 ± 0·101	0·390 ± 0·030†	5·07 ± 0·568†

* Rats were fed with 20% (v/v) ethanol or isocaloric glucose for 28 days. Rats of groups 3 and 4 were given pentobarbital (25 mg/kg, i.p., once daily) during the last 4 days. Microsomes prepared from livers were incubated as indicated under Methods. Enzyme activity was expressed as nmoles *p*-aminophenol, nmoles total pentobarbital metabolites and nmoles formaldehyde formed per mg microsomal protein per min for aniline hydroxylase, pentobarbital hydroxylase and benzphetamine *N*-demethylase respectively. Values are means ± standard errors of mean for five rats in each group.

† Significant difference from glucose-pair controls ($P < 0.01$).

‡ Significant difference from glucose plus pentobarbital group ($P < 0.01$).

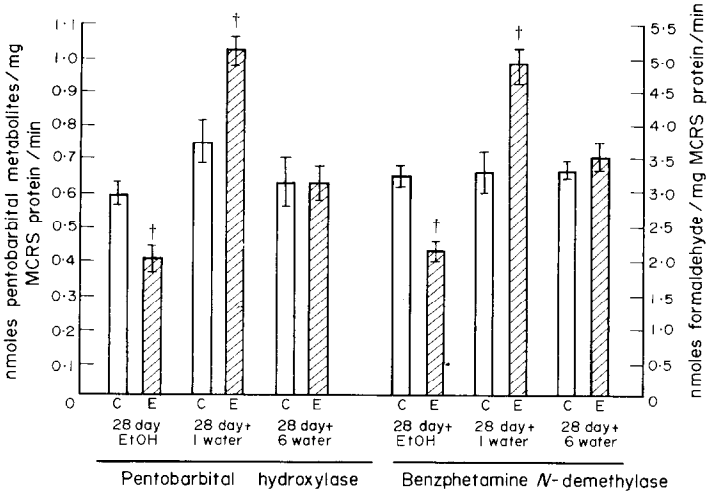

Fig. 4. Effect of ethanol feeding for 28 days and withdrawal of ethanol on the microsomal activities of pentobarbital hydroxylase and benzphetamine *N*-demethylase in male rats. Liver microsomes (see the legend for Fig. 3) were incubated as indicated under Methods. Enzyme activity was expressed as nmoles of total pentobarbital metabolites and nmoles formaldehyde formed per mg of microsomal protein per min for pentobarbital hydroxylase and benzphetamine *N*-demethylase respectively. Values are means ± standard errors of mean from six to ten rats. Abbreviations are as indicated in Fig. 3. The symbol (†) indicates a significant difference from glucose-pair controls ($P < 0.01$).

ingestion in rats. The discrepancy may be explained in part by a difference in experimental design. In the present experiments rats were killed without prior removal of ethanol, whereas in most earlier studies rats were fasted overnight before studying microsomal enzyme activities. The finding, shown in Fig. 4, demonstrates that 24 hr after withdrawing ethanol from chronically fed male rats, the levels of pentobarbital hydroxylase and benzphetamine *N*-demethylase were significantly increased compared to those of the paired controls, findings which are comparable with those of earlier studies [3, 4, 7, 12]. The latter studies report that chronic administration of ethanol increased the activity of pentobarbital hydroxylase [3, 4, 7, 12] and aminopyrine *N*-demethylase [32] when these hepatic drug-metabolizing enzymes were determined approximately 18 hr after withdrawal of food and ethanol.

In addition to this difference in the experimental design, most of the earlier studies were conducted exclusively in female rats [3, 4, 11, 20] or the sex of rats was not indicated [12, 32]. It is well known that the microsomal metabolism of type I drugs differs in male and female rats [33–35]. However, there is no sex difference in the hydroxylation of aniline [34, 35]. The results shown in this report demonstrate a marked sex difference in the response of pentobarbital and benzphetamine metabolism to chronic ethanol consumption in the rat.

The decreased activity of type I drug-metabolizing enzymes in male rats after chronic ethanol ingestion could not be attributed to a direct inhibition by ethanol. Despite a blood ethanol level as high as 205 ± 14 mg/100 ml 90 min after administration of 4 g/kg of ethanol by gastric tube, there was no inhibition of enzyme activity. Eighteen hr after two successive doses of ethanol, a significant elevation in aniline hydroxylase had appeared, but a decline in the other two enzyme activities had not yet occurred. These results suggest that the decrease in pentobarbital hydroxylase and benzphetamine *N*-demethylase in male rats requires persistent ethanol exposure and is not a direct result of simple acute elevation of ethanol concentration in blood or tissue. These observations are supported by the effects of ethanol *in vitro* on the activity of these reactions. Ethanol (50 and 100 mM) significantly inhibited pentobarbital hydroxylase and aniline hydroxylase, but had no effect on benzphetamine or ethylmorphine *N*-demethylase. Since both pentobarbital hydroxylation and benzphetamine *N*-demethylation were suppressed in male rats given ethanol chronically, it seems unlikely that direct inhibition by this agent could account for such findings. The failure of ethanol to inhibit benzphetamine *N*-demethylase was noted in microsomal preparations from male rats fed glucose or chow and given ethanol or phenobarbital. Moreover, no inhibition of this reaction occurred in microsomes prepared from several other species.

The levels of blood methanol [36] and acetone [37] have been shown to increase during the period of ethanol intake. Our unpublished observations indicate that at levels as high as 50 mM these metabolites of ethanol had no inhibitory effect on the metabolism of pentobarbital and benzphetamine when added directly to the microsomal incubation mixture. The con-

centrations of methanol and acetone used were much higher than the maximum concentrations found during the period of chronic ethanol ingestion [36, 37].

The observed increase of the activities of drug-metabolizing enzymes after 24 hr of withdrawal of ethanol was relatively small, considering the high daily concentration of ethanol fed for long periods of time. None of the enzyme activities reached a doubling of the activity of controls; hence, they are not comparable with the marked enzyme induction observed with other neurotropic drugs. The increase in aniline hydroxylase activity reported by Rubin and Lieber [4] was much greater than our finding. However, a protein- and choline-deficient and high fat diet was used in their studies in female rats. Laboratory chow was used as the major source of calories in our study. The same authors [5] have noted that a deficient diet combined with ethanol led to greater increases in enzyme activity than did the same amount of ethanol given together with an adequate diet. Several other investigators [6, 31, 32] have also shown a maximum 2-fold increase in aniline hydroxylase when ethanol was given chronically with an adequate diet. Furthermore, the studies of Mezey [6] have shown a return of this activity to baseline 7 days after withdrawal of ethanol.

When pentobarbital, a known enzyme inducer [38], was given along with ethanol to female rats chronically fed ethanol, a potentiation of the increase in the drug-metabolizing enzyme activities was observed. An additive effect of ethanol and other microsomal enzyme inducers such as phenobarbital [32], DDT or chlorcyclizine [39] has been reported. Tobon and Mezey [15] suggested that ethanol differs from both pentobarbital and polycyclic hydrocarbons in that it is a relatively weak inducer and fails to stimulate liver growth and only slightly increases microsomal protein after a longer period of administration. As shown in this study, ethanol produced no changes in liver weight and only small increases in the microsomal protein concentration. Studies of the effect of ethanol administration on the microsomal protein content have yielded varying results: increases [5, 6], no change [11, 20, 31, 32], and even a decrease [39] have been reported. Our result with respect to the liver weight after chronic administration of ethanol is similar to that of other investigators [6, 32].

The markedly different responses of liver microsomal drug-metabolizing enzymes to ethanol in the male and female rats have not been reported previously. However, this is a well described response to starvation [34, 35, 40], administration of 3-methylcholanthrene [41, 42] and morphine [40]. All these treatments caused a marked decrease in the activities of type I drug-metabolizing enzymes of liver microsomes in male but not female rats. The activities of enzymes for type II agents such as aniline were increased by the same treatment in both sexes. The mechanism of these differences in drug metabolism is not yet clear. However, recent investigations with 3-methylcholanthrene [43, 44] have suggested that administration of this agent to rats causes the formation of a microsomal hemoprotein (cytochrome P_1-450 or P-448) which is qualitatively different from that found in untreated animals or in animals after administration of phenobarbital. It is possible that some similar mechanism accounts for the observations following chronic ethanol administration.

The present results imply that the effect of chronic ethanol ingestion on hepatic drug metabolism is more complex than has been previously indicated. The effects may depend on sex, exposure to other agents and, most importantly, the class of drug and recent intake of ethanol. Thus, barbiturates and chlordiazepoxide, both type I agents, may be metabolized more slowly during ethanol ingestion and more rapidly shortly after withdrawal. However, it is not yet possible to extrapolate these findings to the intact animal or man.

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