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Effect of chronic ethanol feeding on the activities and submicrosomal distribution of reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase and the demethylases for aminopyrine and ethylmorphine*

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CHRONIC ETHANOL feeding has been shown to produce a proliferation of the endoplasmic reticulum[1–3] associated with increased activities of several drug-detoxifying enzymes.[3,4] Ariyoshi *et al.*[5] reported increased aminopyrine demethylase activity after chronic administration of ethanol in drinking water, but this finding has recently been questioned.[6] This prompted us to investigate the activities of aminopyrine and ethylmorphine demethylases after chronic ethanol feeding as well as that of their rate-limiting enzyme, NADPH-cytochrome P-450 reductase.[7,8] We also measured these enzyme activities in rough and smooth microsomes to elucidate the submicrosomal distribution of these enzymes and the site of the induction of their activity, since we have recently shown a preferential increase in cytochrome P-450, protein, phospholipid and microsomal ethanol-oxidizing system (MEOS) in smooth microsomes after ethanol feeding.[9]

Female Sprague–Dawley littermate rats were pair-fed Purina laboratory chow and tap water *ad libitum* until the start of the experiment. When they reached a weight of 120–150 g, they were pair-fed a nutritionally adequate liquid diet containing 18% of total calories as protein, 35% as fat, 11% as carbohydrate (Dextri-Maltose, purchased from Mead–Johnson, Evansville, Ind.) and 36% of the remaining calories either as additional carbohydrate (controls) or as ethanol[10] for a 5-week period. The diets were provided until the time of sacrifice, except for the subfractionation experiments in which the rats were fasted for 18 hr prior to sacrifice. The animals were killed by decapitation, the liver was quickly perfused with ice-cold 0·15 M KCl, excised and homogenized in 3 volumes of 0·25 M sucrose using a glass homogenizer with a Teflon pestle. The liver homogenate was centrifuged at 12,000 *g* for 20 min. The supernatant was then spun at 105,000 *g* for 60 min. The microsomes were washed once with 0·15 M KCl and resuspended in 0·05 M Tris-0·15 M KCl (pH 7·6). The microsomal recovery was 35–40 per cent and the yield was similar in ethanol and control rats (Table 1). For the preparation of rough and smooth microsomes, the livers were perfused with 0·25 M sucrose and homogenized as described. The homogenate was spun at 12,000 *g* for 20 min and the supernatant transferred and mixed with 1 M CsCl to a final concentration of 0·015 M. Rough and smooth microsomes were then prepared by the method of Bergstrand and Dallner.[11] With this method, the yield of total microsomal protein[12] was not significantly different in ethanol-fed (31·3 ± 1·7 mg/g liver) and control rats (29·4 ± 1·5 mg/g liver), but there was a significant increase of smooth microsomes in ethanol-treated rats (Table 2).

Activities of microsomal *N*-demethylation were measured according to the method of Holtzman *et al.*[13] using aminopyrine (8 mM) and ethylmorphine (1 mM) as substrates. The reaction was stopped after 7 min of incubation with 1 ml of 10% (w/v) trichloroacetic acid. Formaldehyde produced was determined according to Nash.[14] The demethylase-dependent formaldehyde production was calculated by subtraction of the amount of formaldehyde produced in the absence of substrate. NADPH-cytochrome P-450 reductase was assayed by the method of Schenkman and Cinti.[7] All measurements were done in triplicate. NADPH-cytochrome *c* reductase was measured as described by Masters *et al.*[15] Cytochrome P-450 was measured in homogenates by the method of Greim[16] and in microsomal suspensions by the method of Omura and Sato,[17] using an Aminco–Chance spectrophotometer in the split beam mode. Corrections were made for preparative losses of microsomes by comparing the content of cytochrome P-450 per g of liver in whole homogenates and microsomes according to Greim.[16] In all experiments, the values of ethanol-fed rats were compared to those of their pair-fed controls. The mean of individual differences was tested by the Student *t*-test for pairs.[18]

In the first experimental group (nine pairs) (Table 1), cytochrome P-450 was higher (by 40 per cent) after ethanol than in the controls, whether expressed per milligram of microsomal protein or per g of liver. Aminopyrine demethylase activity increased 48 per cent per milligram of microsomal protein and 77 per cent per 100 g of body weight. There was a 73 per cent rise in NADPH-cytochrome P-450 reductase when expressed per milligram of microsomal protein and a 109 per cent increase per 100 g of body weight. We also found a significant increase in NADPH-cytochrome c reductase activity (Table 1).

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The submicrosomal distribution of aminopyrine demethylase activity was studied in seven pairs of rats. In controls, the activity was not significantly different in rough and smooth microsome. Compared to controls, after ethanol there was a greater increase of activity in smooth microsomes than in the rough fraction (a 100 per cent vs a 38 per cent rise, respectively) (Table 2). The difference in activity between rough and smooth microsomes was significant ($P < 0.01$) in ethanol-fed rats. In twelve other pairs, the submicrosomal localization of ethylmorphine demethylase activity was also studied and found to be equally distributed between rough and smooth microsomes of control animals (Table 2). Ethanol feeding again increased the activity in the smooth microsomes by 108 per cent, whereas in rough microsomes there was only a 56 per cent rise. The difference in activity between the rough and smooth microsomes of ethanol-fed rats was again significant ($P < 0.01$). The degree of induction in both subfractions was strikingly similar for both demethylase activities. In thirteen pairs of animals, NADPH-cytochrome *c* reductase distribution was studied. In controls, no difference was observed between the rough and smooth microsomes. After ethanol, a significant increase in activity was observed in rough microsomes (29 per cent) and smooth microsomes (51 per cent) (Table 2). In a group of five pairs of rats, the distribution of cytochrome P-450 and NADPH-cytochrome P-450 reductase was measured in the microsomal subfractions. Both parameters showed a similar increase in smooth microsomes after ethanol feeding (Table 2). In contrast to another report,[19] we found no significant difference in aminopyrine demethylase activity between rough and smooth microsomes of control animals but our data on the submicrosomal distribution of demethylase and cytochrome *c* reductase activities are in agreement with previous studies.[20–22]

Ethanol feeding has been shown to increase the microsomal mass[9] and the microsomal content of mixed function oxidases components.[3,23] The present study shows that chronic ethanol administration increases NADPH-cytochrome P-450 reductase activity, thought to be rate limiting[7,8] for aminopyrine and ethylmorphine demethylation which requires a three-component system.[24] The increase of NADPH-cytochrome P-450 reductase activity, as well as that of demethylase and *c* reductase activities, occurred preferentially in the smooth fraction of the microsomes.

Our findings are at variance with the decrease in aminopyrine demethylation activity reported by Ramsey and Fallon[6] after ethanol feeding. The latter, however, used a chow diet, which is low in fat,

TABLE 1. EFFECT OF CHRONIC ETHANOL FEEDING ON MICROSOMAL CYTOCHROME P-450 AND THE ACTIVITIES OF NADPH-CYTOCHROME P-450 REDUCTASE, NADPH-CYTOCHROME *c* REDUCTASE AND AMINOPYRINE DEMETHYLASE OF THE RAT LIVER*

		Control	Ethanol	P†
Microsomal protein (mg/g of liver)‡		36·6 ± 1·2	37·0 ± 1·1	NS
Cytochrome P-450 (nmoles/g of liver)		28·8 ± 1·3	40·8 ± 1·2	<0·01
Cytochrome P-450 (nmoles/mg protein)		0·788 ± 0·038	1·110 ± 0·042	<0·01
NADPH-cytochrome P-450 reductase ($\Delta O.D._{450-465}$ $sec^{-1} \times 10^{-3}$)	per mg protein	4·31 ± 0·52	7·45 ± 0·99	<0·02
	per g of liver	163 ± 20	283 ± 47	<0·05
	per 100 g body wt	576 ± 56	1215 ± 218	<0·02
NADPH-cytochrome *c* reductase (nmoles cytochrome *c* reduced min^{-1})	per mg protein	116 ± 5	146 ± 11	<0·01
	per g liver	4393 ± 251	5491 ± 514	<0·02
	per 100 g body wt	15,753 ± 822	23,544 ± 2511	<0·01
Aminopyrine demethylase (nmoles HCHO formed min^{-1})	per mg protein	4·91 ± 0·39	7·24 ± 0·44	<0·001
	per g liver	187 ± 17	272 ± 21	<0·001
	per 100 g body wt	657 ± 43	1160 ± 103	<0·001

* Mean ± S.E.M.; NS = not significant.
† Difference between ethanol and control.
‡ Corrected for preparative losses.

TABLE 2. ACTIVITY AND DISTRIBUTION OF MICROSOMAL CYTOCHROME P-450, NADPH-CYTOCHROME P-450 REDUCTASE, NADPH-CYTOCHROME *c* REDUCTASE AND AMINOPYRINE AND ETHYLMORPHINE DEMETHYLASES AFTER CHRONIC ETHANOL FEEDING*

	Treatment	No. of animals	Rough microsomes	Smooth microsomes
Microsomal protein (mg/g liver)	Control	9	17·3 ± 0·5	13·4 ± 0·7
			P < 0·05	P < 0·05
	Ethanol	9	14·8 ± 0·5	16·1 ± 1·1
Cytochrome P-450 (nmoles/mg protein)	Control	5	0·54 ± 0·08	0·38 ± 0·04
			NS	P < 0·05
	Ethanol	5	0·59 ± 0·11	0·61 ± 0·09
NADPH-cytochrome P-450 reductase ($\Delta O.D._{450-465}$ $sec^{-1} \times 10^{-3}$/mg protein)	Control	5	3·02 ± 0·63	2·66 ± 0·33
			NS	P < 0·05
	Ethanol	5	3·21 ± 0·67	4·01 ± 0·53
NADPH-cytochrome *c* reductase (nmoles cytochrome *c* reduced min^{-1}/mg protein)	Control	13	59·2 ± 12·1	63·1 ± 12·8
			P < 0·01	P < 0·001
	Ethanol	13	76·5 ± 16·5	95·3 ± 19·4
Aminopyrine demethylase (nmoles HCHO formed min^{-1}/mg protein)	Control	7	3·15 ± 0·26	2·77 ± 0·28
			P < 0·05	P < 0·001
	Ethanol	7	4·34 ± 0·45	5·40 ± 0·41
Ethylmorphine demethylase (nmoles HCHO formed min^{-1}/mg protein)	Control	12	1·62 ± 0·16	1·51 ± 0·32
			P < 0·001	P < 0·001
	Ethanol	12	2·20 ± 0·48	3·14 ± 0·67

* Mean ± S.E.M.; NS = not significant.

and dietary lipids are known to participate in enzyme induction.[25–27] Although such factors may play a role in our model, this is not likely to be the only cause for the difference in results, since Ariyoshi *et al.*[5] have also shown an increase in aminopyrine demethylase with a diet similar to that used by Ramsey and Fallon.[6] Moreover our laboratory has recently shown an enhancement in aminopyrine and benzphetamine demethylase activities after the feeding of ethanol in a diet containing only 5% of total calories as fat,[28] a composition comparable to that of chow diet. The amount of ethanol ingested, however, is important and might explain why the increase in activity we observed is greater than that of Ariyoshi *et al.*[5]

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Drug interaction—Effect of chlorpromazine on the disposition of 8-^{14}C-mescaline in fetal and maternal brain and liver in pregnant mouse

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Recent studies by us on the placental transfer of mescaline in pregnant mice have shown that the hallucinogen crossed the placenta and accumulated in readily measurable amounts in the whole fetuses and fetal tissues.[1] Wurtman and Axelrod[2] have shown that pretreatment of rats with chlorpromazine, 20 mg/kg, caused marked accumulation of ^{3}H-melatonin by all the organs studied. More recently, Lemberger *et al.*[3] reported marked elevation of ^{3}H-amphetamine in the brain of rats pretreated with 15 mg/kg of chlorpromazine. Observations from our laboratory have shown that pretreatment of adult male mice with chlorpromazine, 15 mg/kg, 30 min prior to or 45 min after administration of ^{14}C-mescaline resulted in marked prolongation of the disappearance of mescaline from the brain and several other tissues.[4] Interestingly, chlorpromazine and other phenothiazines have been employed to treat the toxicity induced by amphetamine, lysergic acid diethylamide (LSD), mescaline and other hallucinogens.[5–7] The aim of this study is to report the effects of different doses