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STUDIES ON THE EFFECT OF CHRONIC CONSUMPTION OF MODERATE AMOUNTS OF ETHANOL ON MALE RAT HEPATIC MICROSOMAL DRUG-METABOLIZING ACTIVITY*

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Abstract—Weanling, male Sprague–Dawley rats given 10% ethanol in the drinking water and food *ad lib.* for up to 8 weeks consumed 17% of their calories as ethanol. The alanine aminotransferase (ALT), aspartate aminotransferase (AST), and liver histology by light microscopy were unaffected by this treatment. Similarly, hepatic microsomal NADPH-cytochrome *c* reductase, ethylmorphine *N*-demethylase and benzphetamine *N*-demethylase activities were also not affected by ethanol consumption. On the other hand, cytochrome P-450 content, aniline hydroxylase activity and acetaminophen metabolism as measured by both the cysteine conjugate and the [^{3}H]acetaminophen covalently-bound to microsomal protein were increased significantly by ethanol consumption. The maximal effect was seen by 6 weeks. The 2- to 3-fold increase in aniline and acetaminophen metabolism, the absence of liver damage, and the similarity in weight gains and caloric intakes for controls and treated animals suggest that the rat on 10% ethanol in the drinking water is a reasonable model for studies of the effect of moderate alcohol consumption on specific biochemical pathways.

Numerous studies in both human and animal models have indicated that the heavy consumption of ethanol significantly increases the activity of the drug-metabolizing enzymes [1–5]. Further, the heavy consumption of ethanol has been associated with an increase in the toxicity of those agents which, like acetaminophen (AAP)‡, become toxic only after metabolic activation [6–8]. This increase in drug metabolism after chronic alcohol consumption has been shown to be associated with the induction of a specific cytochrome P-450 as evidenced by the increased metabolism of specific substrates in ethanol-treated animals [1–5] and by the purification of an ethanol-induced cytochrome P-450 from rabbit hepatic microsomes [9–10].

It is estimated that nearly two-thirds of the U.S. population consume alcohol on a regular basis with the majority drinking only moderate amounts [11]. Although definitions vary, we will consider moderate drinkers those who chronically consume less than 20% of their daily caloric intake as ethanol [12]. Previous studies from our laboratory suggest that this pattern of drinking also elicits specific alterations of drug metabolism and toxicity [13].

In our current studies on the effects of chronic ethanol consumption on hepatic microsomal mixed-function oxidases, we wanted to use an animal model which would represent the moderate or social drinker. Previous efforts to develop an appropriate animal model for alcoholism have utilized several species of laboratory animals administered ethanol by various methods [14] including semi-defined liquid diets [1, 2, 4, 15], in the atmosphere [3, 16], and in the drinking water [13, 17]. Another consideration was the need to treat relatively large numbers of animals for the planned characterization and purification of the ethanol-induced enzyme(s). Thus, we have focused our attention on the ethanol drinking-water model as it is a less labor-intensive method than use of the more fully described liquid diet.

In contrast to numerous reports describing the nutritional and biochemical effects of a liquid diet [1, 2, 4, 14, 15] as a model for heavy chronic alcohol consumption, effects for the ethanol-in-drinking-water model have not yet been systematically described. Therefore, we have determined the effects of this treatment on the microsomal mixed-function oxidases and on the nutritional status and growth of the male rat. Our studies indicate that weanling rats fed 10% ethanol in the drinking water (*ad lib.*) consumed about 17% of their daily caloric intake as ethanol. This is similar to the quantity consumed by moderate drinkers [12]. Further, we have found no overt effect on the growth or liver function of these animals when compared to controls. On the other hand, there was an increase in the NADPH-dependent metabolism of specific cytochrome P-450 substrates.

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‡ Abbreviations: AAP, acetaminophen; CYS, cysteine; and CYS-AAP, cysteine-acetaminophen conjugate.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Harlan, Madison, WI), 4- to 5-weeks-old and weighing about 140 g, were fed standard rat chow and water *ad lib.* The animals were either housed individually or in groups of six. Treated rats received 10% ethanol (v/v) in their drinking water. Using individually caged rats, the food and water consumption of each animal was noted for 6 weeks. At the end of this time they were killed, and the serum and liver samples were collected for aminotransferase activity and histopathology respectively. The grouped rats were killed at the end of 3, 4, 6, and 8 weeks of ethanol exposure.

Preparation of microsomes. The rats were killed by decapitation, and the livers were perfused *in situ* with ice-cold 50 mM Tris–HCl (pH 7.5, 0–4°) and 150 mM KCl containing 20 ng each of leupeptin and pepstatin per ml. The livers were removed, weighed and minced. The minced livers were homogenized (1.0 g/3.0 ml Tris–KCl perfusion buffer) in a Brinkmann Polytron at 0–4°. Phenylmethylsulfonyl fluoride was added to a concentration of 0.5 mM during homogenization. The microsomal fraction was sedimented at 105,000 *g* from the 10,000 *g* supernatant fraction by the standard methods of differential centrifugation. The microsomal pellets were resuspended to a final protein concentration of 50–60 mg protein/ml in 50 mM Tris–HCl (pH 7.5, 0–4°), 0.01% butylated hydroxytoluene (BHT), 20 ng each of leupeptin and pepstatin per ml, and 50% glycerol and stored under N_2 at −15°. Before assaying enzymatic activities, the microsomes were carefully thawed and washed by resuspending (1–2 mg protein/ml) in 0.1 M sodium pyrophosphate and 1.0 mM EDTA, pH 7.5, and resedimenting at 105,000 *g*. Preliminary experiments indicated no loss of enzyme activity during storage for any of the enzymatic indices studied.

Enzyme assays. Assays of drug-metabolizing activity were run for 10 min at 37° using a final volume of 1.0 ml Tris (50 mM)–KCl (150 mM) buffer (pH 7.4 at 37°) containing 1.0 mg washed microsomal protein, 2.0 mM substrate, 0.43 mM $NADP^+$, 5.0 mM $MgCl_2$ and 5.90 mM glucose-6-phosphate. The reaction was initiated by the addition of 0.6 units of glucose-6-phosphate dehydrogenase. The N-demethylation of ethylmorphine and benzphetamine was determined by the rate of formaldehyde formation using the Nash reaction [18]. Aniline hydroxylase activity was measured by the amount of *p*-aminophenol formed according to Holtzman *et al.* [19].

The cysteine (CYS) conjugate of acetaminophen (AAP) was quantitated by high performance liquid chromatography (HPLC) using a reverse-phase HPLC column (ODS-C18, Regis) with methanol (7.0%), glacial acetic acid (0.75%) in 0.1 M KH_2PO_4 as the mobile phase [20]. In this method, acetaminophen (2.0 mM) was incubated in the presence of 2.0 mM cysteine. Covalent binding of [^{3}H]AAP to microsomal protein was measured as described earlier [13]. The trichloroacetic acid (TCA) precipitate, however, was washed thoroughly with 100% ethanol rather than methanol–water mixtures. The precipitate was solubilized in NCS tissue solubilizer (Amersham/Searle Co., Arlington Heights, IL) and counted in a Beckman LS-100 C liquid scintillation counter.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), washed microsomal samples were prepared and run (2 μg protein/lane) on 0.75 mm 8.0% resolving gels according to the method of Laemmli [21]. After fixation in isopropanol–acetic acid–deionized water (10:25:65), the gels were stained with the silver method of Oakley *et al.* [22] and photographed the same day.

Spectral analysis for cytochrome b_5 and P-450 content and rate assays for NADPH-cytochrome *c* reductase and NADH-ferricyanide reductase activities were performed according to standard procedures [23, 24]. Serum aminotransferase determinations, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured on a Multistat III using Spin Chem reagent kits (Smith Kline Instruments, Inc., Sunnyvale, CA). Protein concentrations of the samples were determined using the method of Lowry *et al.* [25] with bovine serum albumin (fraction V, Sigma) as the protein standard. Statistical analysis was done using Student's *t*-test. Experimental values are given as the mean ± the standard error of the mean (S.E.).

Chemicals. Ethylmorphine (Dionin) was purchased from Merck & Co. (Rahway, NJ). Cytochrome *c* (type VI), NADPH (type III), $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, Tris–HCl, Tris-base, acetaminophen and bovine serum albumin (fraction V) were purchased from the Sigma Chemical Co. (St. Louis, MO). Butylated hydroxytoluene was purchased from the Aldrich Chemical Co., Inc. (Milwaukee, WI).

RESULTS

Effect of ethanol treatment on growth. The consumption of 10% ethanol in drinking water had no significant effect on the weight gain or caloric intake when compared to that of control animals (Fig. 1). The individual, day-to-day total caloric intake in treated animals for 6 weeks ranged between a low of 46.2 ± 3.50 and a high of 268.4 ± 10.09 kcal/day over the period of 6 weeks, and was not different from that of 47.8 ± 5.50 and 334.1 ± 6.50 kcal/day observed in the controls. Except for an initial weight loss in the ethanol-treated animals, the rate of weight gain during the treatment period was similar in control and treated rats as indicated by the slopes of the curves (Fig. 1). The final weight of the ethanol-fed animals of 306.3 ± 9.50 g was not statistically different from that of control animals (317.8 ± 13.87). In these studies, the percentage of calories consumed as ethanol showed little variation, ranging between 13.7 ± 0.30 and 19.9 ± 1.45% for the 6-week period.

The activities of AST (154.6 ± 9.5 vs 147.4 ± 16.8 I.U./l) and ALT (54.4 ± 2.6 vs 48.1 ± 4.2 I.U./l) in control and treated animals were not significantly different. The animals were routinely killed at mid-morning several hours after feeding, and there was no detectable blood ethanol.

Effect of ethanol treatment on microsomal mixed-

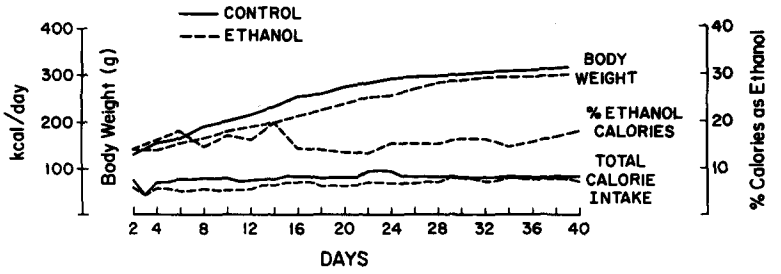

Fig. 1. Effect of 10% ethanol in the drinking water on body weight and caloric intake and percent of calories consumed as ethanol. Rats were randomized and housed in individual cages for 12 days before starting treatment with ethanol, and the drinking tubes contained a ball bearing to minimize the evaporate loss of ethanol. Measurements of food and water consumption were taken every other day.

function oxidases. Ethanol treatment did not alter the activities of ethylmorphine *N*-demethylase, benzphetamine *N*-demethylase, NADPH-cytochrome *c* reductase and NADH-ferricyanide reductase activities, or the specific content of cytochrome b_5 (Table 1). However, rats consuming drinking water containing 10% ethanol showed at 6 weeks an increase in cytochrome P-450 specific content from 0.59 ± 0.039 in controls to 0.73 ± 0.047 in treated rats (Table 1). The difference is statistically significant at $P < 0.05$. Aniline hydroxylase activity was also increased significantly in treated rats, 0.69 ± 0.023 nmoles *p*-aminophenol/min/mg protein, over that of 0.45 ± 0.030 in controls ($P < 0.01$) (Table 2). The increase in CYS-AAP conjugate formation (Fig. 2), from 4.09 ± 0.365 to 5.85 ± 0.453 nmoles/min/mg protein, and the increase of the V_{max} of covalent protein binding (Fig. 3, representative values) to 4.16 ± 0.455 from 2.12 ± 0.238 pmoles bound/min/mg protein in ethanol-treated versus control rats were both statistically significant ($P < 0.05$). The Michaelis–Menten analysis of AAP-covalent binding to protein showed the K_m for binding was, however, not different between the groups (Fig. 3). Thus, the increase in enzyme activities was solely the result of an increased V_{max}.

The results obtained with rats housed as groups of six are shown in Fig. 2. The mode of housing did not alter the apparent ethanol intake as the results obtained with these animals were not different from those obtained for rats housed individually. For these studies, the rate of protein binding was measured at a single substrate concentration of 2 mM acetaminophen and found to be increased significantly in the microsomes of ethanol-fed rats ($P < 0.01$). SDS-PAGE analysis of microsomes of control and ethanol pretreated animals showed that a band of M_r of 52,000 daltons in the controls was intensified in microsomes from rats fed ethanol for 6 weeks.

DISCUSSION

The present studies were designed to evaluate rats on 10% ethanol in the drinking water as a model for the chronic consumption of moderate amounts of ethanol. The model has many advantages in that the rats do not show any of the acute effects on food or liquid consumption of high intakes of ethanol. The rats grew well with 10% ethanol in drinking water, with weight gains and total caloric intake similar to those of the control group. In contrast to higher doses of ethanol which require pair-fed controls, such as 20% in the drinking water [17] or 36% of the total calories as ethanol [1, 2, 4, 15], all of the animals readily consumed 10% ethanol in drinking water after the initial day or two. This eliminated the necessity for expensive and labor-intensive pair-feeding experiments.

We found that in this model the animals consistently consumed 17% of their total caloric intake as ethanol. For man, ethanol consumption contributing more than 20% of calories is considered excessive [12]. In this respect, these animals may be considered to be consuming only moderate amounts of alcohol.

For the 6 weeks of treatment, there was no evidence of these levels of ethanol causing hepato-

Table 1. Effect on male rat hepatic microsomal mixed-function oxidases (cytochrome content and catalytic activity) of 6 weeks of 10% ethanol in the drinking water*

	Cytochrome		Reductase		*N*-Demethylase	
	P-450	b_5	NADPH	NADH	Ethylmorphine	Benzphetamine
Control	0.59 ± 0.04	0.52 ± 0.04	0.20 ± 0.03	3.17 ± 0.09	8.08 ± 0.79	6.02 ± 0.63
Ethanol	0.73 ± 0.05†	0.59 ± 0.02	0.17 ± 0.01	3.11 ± 0.36	9.03 ± 0.98	7.16 ± 0.82

* Enzyme units are nmoles of cytochrome/mg protein for cytochrome P-450 and b_5, μmoles electron acceptor reduced/min × mg protein^{-1} for NADPH-cytochrome *c* reductase and NADH-ferricyanide reductase activities, and nmoles HCHO/min × mg protein^{-1} for ethylmorphine and benzphetamine *N*-demethylase activities. Experimental details for the enzyme assays are described in Materials and Methods. Each value is the mean ± S.E.M. of six rats.

† Significantly different from control levels (Student's *t*-test): $P < 0.05$.

Table 2. Effect on rat hepatic microsomal drug metabolism of 6 weeks of 10% ethanol in the drinking water*

	Aniline hydroxylase (nmoles *p*-aminophenol/ min × mg protein^{-1})	CYS-AAP-conjugate formation (nmoles/min × mg^{-1})	Covalent protein binding V_{max} (pmoles/min × mg^{-1})	K_m (mM)
Control	0.45 ± 0.030 (7)	2.09 ± 0.365 (6)	2.12 ± 0.238 (6)	0.24 ± 0.021 (6)
Ethanol	0.69 ± 0.023 (7)	5.24 ± 0.337 (7)	4.16 ± 0.729 (6)	0.27 ± 0.021 (6)
P	< 0.01	< 0.05	< 0.01	< 0.01

* Assays were conducted as described in Materials and Methods, and the numbers in parentheses indicate the number of animals used for the reported value. Values are expressed as the mean ± S.E.M., and significance (P) was determined by Student's *t*-test.

cellular damage as indicated by the normal range of values for serum aminotransferase activities and normal histology in ethanol-treated rats. Yet, though the consumption of ethanol was relatively moderate, there were significant increases in the rate of aniline hydroxylation, CYS-AAP-conjugate formation and covalent protein binding of the acetaminophen metabolite. These increases in metabolic activity were associated with small but significant increases in the specific content of cytochrome P-450. The treated animals, however, failed to show any significant change in NADPH-cytochrome *c* reductase or benzphetamine *N*-demethylase activities as the decrease observed in studies using higher concentrations of ethanol [16, 17]. The increase in both the rate of aniline hydroxylation and acetaminophen metabolism is in agreement with earlier reports for animals fed high dosages of ethanol [1, 2, 16, 17]. Thus, the effect of moderate and chronic feeding of alcohol differs in part from those observed with higher dosages and would appear to affect only specific enzymatic activities. Another difference between the effects of ethanol administered in the water (10%) and in liquid diets (36%) is a more rapid induction of enzyme activity and cytochrome P-450 content observed with the liquid diets [26].

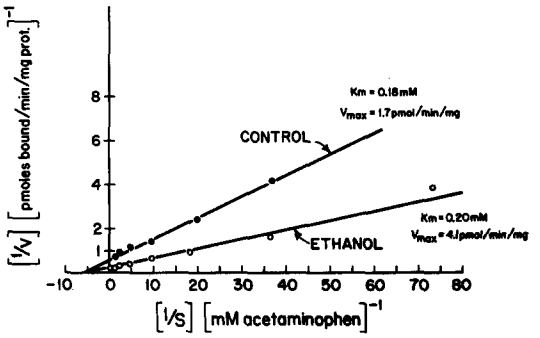

Fig. 3. Effect of 10% ethanol in the drinking water on the NADPH-dependent covalent binding of [^{3}H]acetaminophen to microsomal protein. The covalent binding assay was conducted as described in Materials and Methods using 0.015 to 5.0 mM acetaminophen.

We feel that the observation that chronic con-

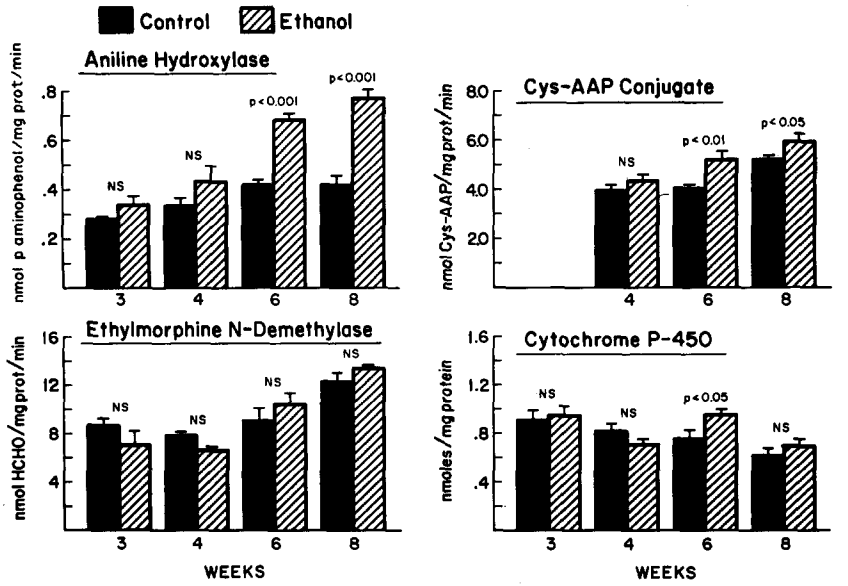

Fig. 2. Effect of 10% ethanol in the drinking water on hepatic microsomal mixed-function oxidases of male rats. Assays were conducted as described in Materials and Methods on microsomes isolated at the treatment time noted in weeks on the graph.

sumption of moderate amounts of alcohol enhances the metabolism of acetaminophen supports our previous suggestion that modest doses of ethanol may enhance the toxicity of acetaminophen. In these previous studies in mice, we had found that a similar level of ethanol consumption causes a parallel increase in toxicity *in vivo* and in protein binding *in vitro* [13].

It is of interest that the activation of acetaminophen as shown by the protein binding and CYS-AAP assays showed differences in sensitivity to the consumption of ethanol. Further studies in our laboratory indicate that these activities differ in other biochemical respects such as their sensitivity to inhibitors. These data suggest that they may, in part, be independent measures of the activation of acetaminophen.*

Finally, epidemiological studies have suggested that chronic ethanol consumption is etiologically linked with gastrointestinal cancer and other malignancies [27]. Further, it is currently felt that many carcinogens must first be activated enzymatically to an ultimate carcinogen [28, 29]. The mechanism of action of ethanol as a promotor of carcinogenesis is not known but may be related to a variety of effects on the cell including the ability of ethanol to induce the microsomal mixed-function oxidases [4, 5].

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