Alcohol, Vol. 2, pp. 17–22, 1985. © Ankho International Inc. Printed in the U.S.A. 0741-8329/85 $3.00 + .00

Effects of Chronic Ethanol Consumption on Male Syrian Hamster Hepatic, Microsomal Mixed-Function Oxidases

CHARLOTTE R. ELLIOTT, JAGARLAPUDI S. PRASAD, ALAN D. HUSBY, RANDY J. ELLINGSON, JORDAN L. HOLTZMAN AND DAUNE L. CRANKSHAW[1]

Departments of Pharmacology and Medicine, University of Minnesota, Minneapolis, MN 55455 and Research Service 151, Veterans Administration Medical Center, Minneapolis, MN 55417

ELLIOTT, C. R., J. S. PRASAD, A. D. HUSBY, R. J. ELLINGSON, J. L. HOLTZMAN AND D. L. CRANKSHAW. *Effects of chronic ethanol consumption on male Syrian hamster hepatic, microsomal mixed-function oxidases.* ALCOHOL 2(1) 17–22, 1985.—Chronic alcohol consumption significantly increases the risk of drug interactions. We have described its effects on hamster microsomal monooxygenases. Male Syrian hamsters (85 g) were given 10% ethanol in water and food ad lib for up to 6 weeks. Microsomal electron transport components and metabolism of ethylmorphine, benzphetamine, aniline, and acetaminophen were measured. At 4 weeks, SDS-PAGE of ethanol microsomes showed an induced band with an Mr of 53,900 daltons and there was a 2–3 fold stimulation of aniline and acetaminophen metabolism. Cytochrome P-450 increase was not significant. For the six week period, Caloric intake (3 weeks, $p<0.001$), liquid consumption (3 weeks, $p<0.05$) and body weights (6 weeks, $p<0.05$) of ethanol animals were significantly greater than controls; kidney weights were significantly less ($p<0.05$). Ethanol consumption increased from 20% of the daily caloric intake (week 1) to 31% (week 6). Induction of specific substrate metabolism without apparent deleterious physiological changes establishes hamsters fed 10% ethanol in drinking water as a biochemical model for the study of chronic alcohol consumption and specific drug interactions.

Ethanol Hamster Microsomal oxidase Alcohol consumption

NUMEROUS studies both in human and in animal models have indicated that the heavy consumption of ethanol significantly increases the activity of the drug metabolizing enzymes [14,25]. Further, studies have associated consumption of ethanol with increased toxicity of those agents, such as acetaminophen, which become toxic only after metabolic activation [3, 14, 18, 21, 25]. It has been reported that the increase of drug metabolism following chronic alcohol consumption is associated with the induction of a specific cytochrome P-450 as evidenced by the increased metabolism of specific substrates in ethanol-treated animals and the purification of an ethanol-induced cytochrome P-450 from rabbit hepatic microsomes [12].

In recent years, the effects of moderate alcohol consumption has been a growing concern. It is estimated that nearly two-thirds of the US population consume alcohol on a regular basis and the majority drink only moderate amounts [27]. Although definitions vary, moderate drinkers have been defined as those who chronically consume less than 20% of their daily caloric intake as ethanol [11]. Previous studies from this laboratory suggest that this pattern of drinking also elicits specific alterations of drug metabolism and toxicity [26].

In the current studies, we wished to investigate the effect of moderate ethanol consumption on hepatic microsomal mixed-function oxidases. Of the available species, we focused on the hamster, for it has become increasingly popular as a model for pathological and carcinogenic studies [8,9]. In comparative studies, it has also been shown that hamster liver microsomes have relatively higher basal drug metabolism activities and readily activate chemicals to hepatotoxic products [4]. Previous efforts to develop an alcohol model [1] have utilized various methods to administer ethanol, including in the atmosphere [20], semi-defined liquid diets [15], and in the drinking water [16]. Our projected studies include purification and characterization of the ethanol-induced microsomal enzymes, which require relatively large quantities of microsomes. We chose to further characterize ethanol in the drinking water as a model for the chronic consumption of moderate amounts of alcohol. It is less labor-intensive than liquid diet methods and large numbers of animals required for enzyme puification can be easily treated.

In contrast to numerous reports which have described the nutritional and biochemical effects of a liquid diet as a model for heavy chronic alcohol consumption [14,15], the ethanol-in-the-drinking-water model has not yet been similarly described. In light of this, we have determined the effects that chronic 10% ethanol-water treatment has on the nutritional

[1]Requests for reprints should be addressed to Dr. Daune L. Crankshaw, Research Service (151), VA Medical Center, 4801 East 54th Street, Minneapolis, MN 55147.

status, growth and hepatic microsomal mixed-function oxidases of the male hamster.

METHOD

Male Syrian hamsters (N.C.I., Fredrick Cancer Research Facility, Fredrick, MD), weighing approximately 85 g and male Sprague Dawley rats (100 g; Harlan, Madison, WI) were given standard rat chow and water ad lib. They were housed in groups of six and maintained under normal lighting conditions (06:00–18:00, light). Animals were treated with 10% ethanol (v/v) for up to 6 weeks, 1.0% phenobarbital for 10 days, or 1.0% acetone for 7 days in their drinking water. Imidizole (200 mg/kg in 0.1 M KPO_4, pH 7.6), pyrazole (100 mg/kg in 0.1 M KPO_4, pH 7.6) and β-naphthoflavone (β-NF) (80 mg/kg in corn oil) was injected IP once daily at midmorning for 4 days. Animals were killed the morning after receiving the last injection. Liquid was replaced every 2 days and fresh food was given every week.

Animals were killed by decapitation and the livers immediately perfused with 20 ml of ice-cold 50 mM Tris-HCl (pH 7.4, 0–4°C) and 150 mM KCl, removed and weighed. Liver, kidney, adrenal glands, heart, and spleen weights were recorded. Livers were pooled (2 per group), homogenized in 3 parts of perfusion media to one part liver (wet weight) with a Brinkman polytron (0–4°C) and centrifuged at 10,000 ×g for 20 min (0–4°C). Microsomes were isolated at 105,000 ×g (70 min) in a Beckman Ti 60 rotor (0–4°C) from the 10,000 ×g supernatant that had been filtered through glass wool. The isolated microsomes were resuspended in 50 mM Tris-HCl (pH 7.4, 0–4°C) and 50% glycerol, 0.01% butylated hydroxytoluene, 20 ng leupeptin and pepstatin per ml at a final protein concentration of about 50.0 mg/ml. They were stored under nitrogen at −17°C. Before assaying enzymatic activities, the frozen suspensions were carefully thawed and washed by resuspending (1–2 mg protein/ml) in 0.1 M Na pyrophosphate and 1.0 mM EDTA (pH 7.5) and then resedimenting at 105,000 ×g. Preliminary experiments showed no apparent loss of enzymatic activity during storage.

Individual drug metabolizing assays were run for 30 min in a shaking water bath at 37°C in a final volume of 1.0 ml Tris (50 mM)-KCl (150 mM) buffer (pH 7.4 at 37°C). The reaction mixture contained 1–2 mg of washed microsomal protein, 2.0 mM of substrate, 5.0 mM $MgCl_2$, 0.43 mM $NADP^+$, and 5.90 mM glucose-6-phosphate. After preincubating for 5 min, the reaction was initiated by the addition of 0.6 units of glucose-6-phosphate dehydrogenase. Under these conditions the following assays were performed. The N-demethylation of ethylmorphine and benzphetamine was determined by the rate of formaldehyde formation as detected by the Nash reaction [22]. Aniline hydroxylase activity was measured by the amount of p-aminophenol formed according to Holtzman *et al.* [7]. The cysteine conjugate of acetaminophen (CYS-AAP) was quantitated by 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 [29]. The CYS-APP standard and benzphetamine-HCl were donated by McNeil Laboratories (Dr. Lam) and UpJohn Co. (Dr. P. W. O'Connell).

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 [13]. After fixation in isopropanol:acetic acid:deionized water (10:25:65), the gels were stained with the silver method of Oakley [23] and photographed the same day.

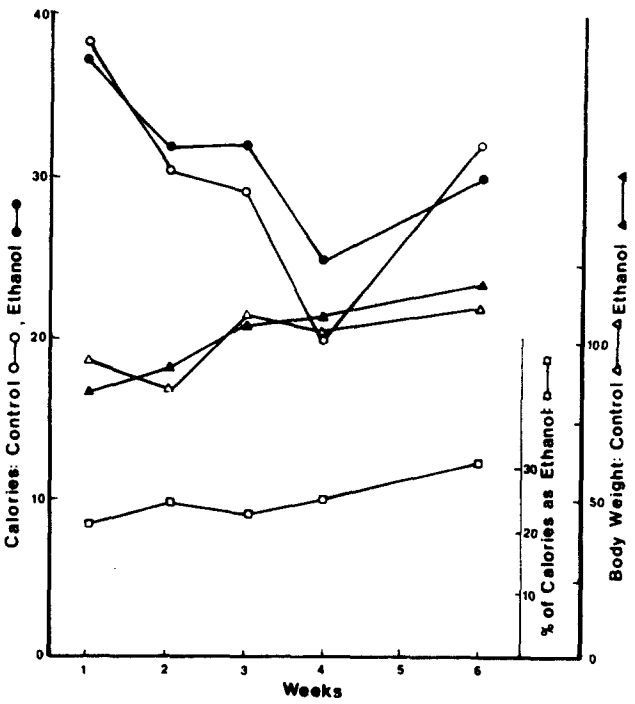

FIG. 1. The effect of chronic 10% ethanol in the water on body weight and caloric intake and percent of calories consumed as ethanol. Results were averaged from 3 separate experiments. When analyzed by the Student's *t*-test, caloric intake ($p<0.001$) and body weights ($p<0.05$) of the treated animals were significantly different from the controls.

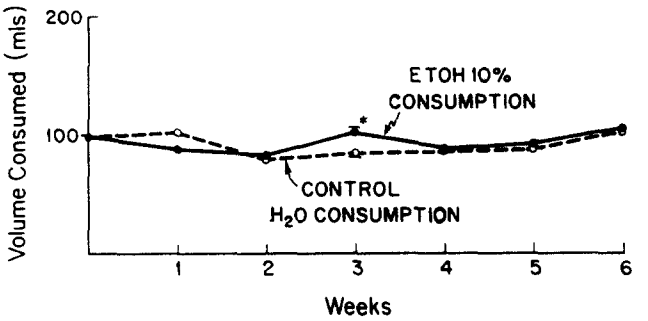

FIG. 2. Liquid consumption of male Syrian hamsters fed water or 10% ethanol in the drinking water ad lib. The water bottle drinking tubes contained a ball bearing to minimize the evaporation of ethanol. Liquid consumption of the ethanol group was significantly different ($p<0.05$) from that of the controls during the third week of treatment.

Spectral analyses for cytochrome b_5 and P-450 content [24] and rate assays for NADPH-cytochrome c reductase and NADH-ferricyanide reductase activities [6] were performed according to standard procedures. Protein concentrations of the samples were determined using the method of Lowry *et al.* [17] with bovine serum albumin (fraction V, Sigma) as the protein standard. Statistical analysis utilized the Student's *t*-test. Experimental values are given as the mean±the standard error of the mean (SE).

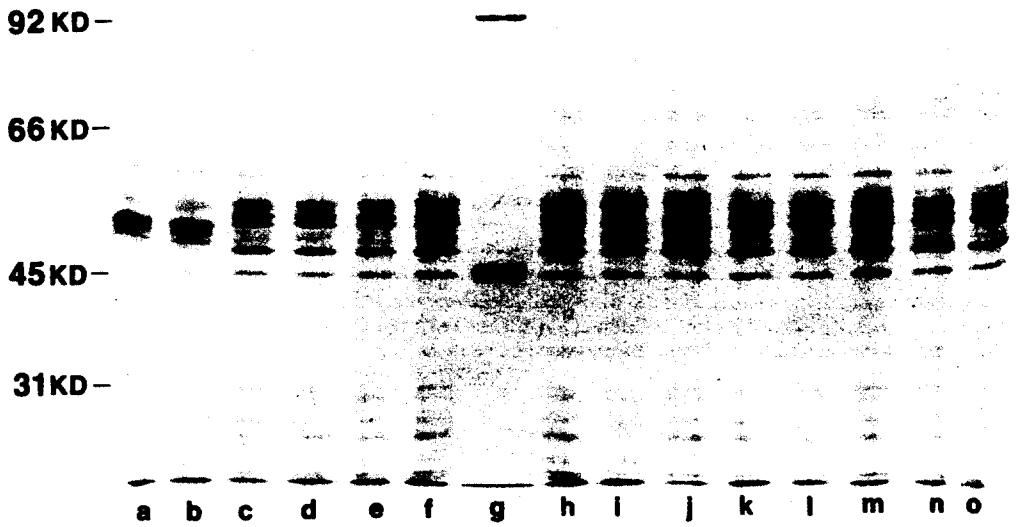

FIG. 3. SDS-PAGE silver stained profile of washed liver microsomes from Syrian hamsters of Sprague Dawley rats pretreated with ethanol (E), phenobarbital (PB), β-naphthaflavone (β-NF), acetone, imidizole or pyrazole. Animals were pretreated as described in the Method section. Except for lane g (standards), each lane was loaded with 2.0 μg of washed microsomal protein from control (a) and E rat (b), 3 week E (d), 4 week E (f), β-NF (i), PB (j), acetone (l), imidizole (m), and pyrazole (o) pre-treated hamsters. Appropriate controls are lanes c, e, h, k and n. The 0.75 mm 8.0% acrylamide gels were run in 0.1% SDS and silver stained.

TABLE 1

EFFECT OF FEEDING 10% ETHANOL IN THE DRINKING WATER TO MALE SYRIAN HAMSTERS FOR 4 WEEKS: BODY WEIGHT, ORGAN WEIGHTS AND CALORIC INTAKE

	Body weight (g)	Liver (g/100 g Body Wt)	Organ Weights (mg/100 g Body Wt)				
			Spleen	Kidneys	Heart	Adrenals	Calories
Control	103.1 ± 2.3	4.4 ± 0.02	120.0 ± 2.8	820.0 ± 2.3	358.0 ± 7.5	23.0 ± 0.96	19.2 ± 2.50
Ethanol	108.1 ± 2.5	4.1 ± 0.02	110.0 ± 3.8	730.0 ± 1.0	333.0 ± 5.5	19.8 ± 1.10	25.3 ± 2.09
P	NS	NS	NS	<0.05	NS	NS	NS

The results are compiled from 2 experiments and are expressed as the mean ± S.E.M. Experimental values were analyzed for significance by the Student's *t*-test (n=21 for both groups; NS means not significant). At 4 weeks, % cal consumed as ethanol was 25.9.

RESULTS

Effect of Ethanol Treatment on Growth

Body weights, caloric intake and percent of calories consumed as ethanol during the 6 week treatment period are shown in Fig. 1. Using change in body weight as an indicator of growth, both control and treated animals showed increases in weight from 93.5±3.7 g and 84±4.1 g (Week 1) to 111.3±4.0 g and 120.3±1.9 g, (6 weeks), respectively. The weight of the hamsters on ethanol-water was significantly greater than that of the controls at 6 weeks ($p<0.05$). During the first 4 weeks of treatment the caloric intake of both groups declined and then increased at the sixth week to the levels measured for weeks 2 and 3. The caloric intake of the ethanol animals during the third week was significantly greater than that of the controls ($p<0.001$). The percent of calories consumed as ethanol by the treated hamsters was 20.7% the first week and increased gradually to 31.4% by the sixth week. For both groups the volume of liquid consumed was essentially the same except for week 3 when ethanol animals drank significantly more than the controls ($p<0.05$). Chronic exposure to ethanol for 4 weeks did not cause any significant change in weights of liver, spleen, adrenal glands or heart whereas kidney weighed significantly less than controls, 0.85±0.24 versus 0.79±0.017 mg/100 g, respectively ($p<0.05$).

Effect of Ethanol Treatment on Microsomal SDS-Polyacrylamide Gel Electrophoretic Profiles

Washed microsomes (2.0 μg/lane) from controls and those pre-treated with ethanol, phenobarbital and β-naphthaflavone were electrophoresed in the presence of 0.1% SDS and stained with silver. The profiles of the

TABLE 2

LEVELS OF HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASE ELECTRON TRANSPORT COMPONENTS IN MALE SYRIAN HAMSTERS FED WATER OR 10% ETOH IN THE DRINKING WATER

	Reductases		Cytochomes	
	NADPH-cytochome c (μmole/min/mg)	NADH-ferricyanide (μmole/min/mg)	b_5 (nmole/mg)	P-450 (nmole/mg)
Control	0.247 ± 0.012	4.47 ± 0.86	0.550 ± 0.05	1.00 ± 0.42
Ethanol	0.289 ± 0.015	4.96 ± 1.23	0.489 ± 0.05	1.17 ± 0.27

Assays were conducted as described in the Method section and results from 2 experiments are expressed as the mean ± S.E.M. According to the Student's *t*-test, none of the values obtained with ethanol pretreated microsomes were significantly different from those of controls.

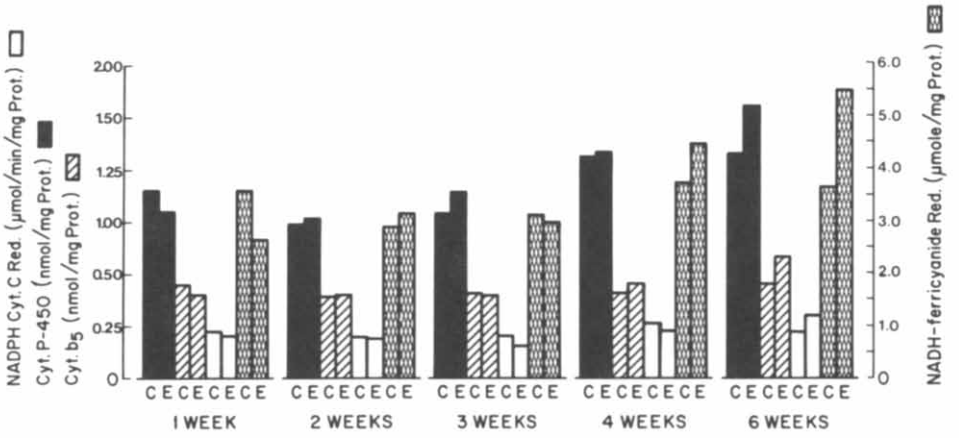

FIG. 4. The levels of hepatic microsomal mixed-function oxidase electron transport components in male Syrian hamsters fed water (C) or 10% ethanol in the drinking water (E). Microsomal fractions were isolated from perfused livers of fed hamsters and assayed as described in the Method section. None of the values obtained for E-treated microsomes were significantly different from the controls (Student's *t*-test).

phenobarbital and B-NF microsomes, when compared to the controls (Fig. 3, lanes c, h, i, j), have major bands induced at Mr of 47,300 and 49,000 daltons (PB) and at 49,000 and 53,000 daltons (β-NF). It was not until weeks 4 and 6 that bands at Mr of 53,700 and 54,200 daltons were apparent in ethanol pre-treated microsomes (Fig. 3, lanes c, d, e, f). With the appearance of the induced band at week 4, we proceeded to compare the effect of 4 weeks exposure to 10% ethanol in the drinking water on the microsomal mixed-function oxidases.

Effect of Ethanol Treatment on Microsomal Mixed-Function Oxidases

The levels of the microsomal electron transport components, over the course of six weeks, is shown in Fig. 4. For control and treated microsomes, NADPH-cytochrome c reductase activities were significantly different ($p<0.01$), at 2 weeks, with values of 0.255±0.001 and 0.38±0.010 μmol reduced/min × mg protein^{-1}, respectively. At 3 weeks, cytochrome P-450 content was 1.17±0.08 (control) versus 1.51±0.08 nmol/mg protein (ethanol) with a significant difference of $p<0.05$.

Tables 1 and 2 show the several parameters measured at 4 weeks of treatment. Body weight, liver weight, caloric intake, and percent of calories consumed as ethanol were not significantly different between control and ethanol-treated groups (Table 1). Neither were values for NADPH-cytochrome c and NADH-ferricyanide reductase activities or for cytochrome P-450 and b_5 specific content. Assays of drug metabolizing activity as represented by the N-demethylation of ethylmorphine and benzphetamine measured no significant difference between control and treated animals. Aniline hydroxylase activity was significantly increased 2.7-fold from 1.51±0.05 in controls to 4.01±1.02 nmol p-aminophenol/min × mg protein^{-1} and CYS-AAP conjugate formation increased 2.3-fold from 92.7 to 211.5 pmol/min × mg protein^{-1}.

DISCUSSION

Due to the broad range of effects that chronic alcohol consumption has on man, no single animal model is adequate. The present studies were designed to evaluate the effect on hamsters of 10% ethanol in the drinking water as a model for the chronic administration of moderate amounts of

TABLE 3

EFFECT OF 4 WEEKS TREATMENT WITH 10% ETHANOL IN DRINKING WATER ON HEPATIC MICROSOMAL DRUG METABOLISM IN MALE HAMSTER

	N-demethylation		Aniline hydroxylation	Cys-AAP conjugate
	Ethylmorphine (nmoles of HCHO/mg/min)	Benzphetamine (nmoles of HCHO/mg/min)	(nmoles of p-aminophenol/ mg/min)	(pmoles of Cys-AAP/mg/min)
Control	6.40 ± 0.09	6.20 ± 0.14	1.51 ± 0.05	290.5 ± 16.5
Ethanol	6.55 ± 0.15	4.85 ± 0.32	4.01 ± 1.02	598.0 ± 13.0
P	NS	NS	<0.05	<0.01

Assays were conducted as described in the Method section. Data was obtained from 2 experiments (n=21) and reported as the mean ± S.E.M.

ethanol. The model has many advantages for such studies since the hamsters do not show any of the deleterious effects on food or liquid consumption as observed with methods using higher amounts of ethanol. The caloric intake of the treated groups, with 10% ethanol in drinking water, paralleled that of the controls and for weeks 2, 3, and 4 ($p<0.05$) was actually greater. Weight gains were higher than the controls for weeks 4 and 6 ($p<0.05$) and were similar to reported values [2].

In contrast to results published for higher dosages of ethanol, such as 20% in the drinking water [16] or 36% of the total calories as ethanol [15], all of the animals readily consumed 10% ethanol in drinking water after the first week. In fact, by the second week, hamsters on ethanol were consuming more liquid than the controls. This pattern of weight gain, caloric intake and liquid consumption in the treated animals eliminated the necessity for the expensive and labor-intensive pair-feeding experiments.

We have found in previous studies that the rat on 10% ethanol in the drinking water consistently consumed 17% of the total caloric intake as ethanol (J. S. Prasad, *et al.*, submitted for publication). Under similar conditions with hamsters, however, ethanol calories increased steadily from 20% for the first week to 31% by the sixth week. For man, ethanol consumption contributing more than 20% of calories is considered excessive and heavy [11]. In this respect the rat and hamster may be considered to be consuming moderate and heavy amounts of alcohol, respectively.

As we found in previous studies with the rat (J. S. Prasad, *et al.*, submitted for publication) there were significant increases in the rate of aniline hydroxylation and CYS-AAP-conjugate formation. The increased metabolic activities were associated with small but significant increases in the specific content of rat Cytochrome P-450, but not for the hamster. In both species, however, the treated animals failed to show any significant increase in NADPH-cytochrome c reductase activity or in N-demethylase activity with either ethylmorphine or benzphetamine.

This pattern of microsomal enzyme induction differs from results published for studies using higher percentages of ethanol. NADPH-cytochrome c reductase, ethylmorphine N-demethylase and aminopyrene N-demethylase activities were increased significantly in microsomes from rats fed a liquid diet containing 36% of the calories as ethanol [10]. Chronic exposure of rats to ethanol vapor significantly increased microsomal NADPH-cytochrome c reductase and aminopyrene N-demethylase activities [20]. In rats fed 20% ethanol-water, a significant decrease in microsomal benzphetamine hydroxylase activity was observed [16]. The increase in the aniline hydroxylation and acetaminophen metabolism are in agreement with earlier reports for animals fed higher dosages of ethanol [5, 16, 20]. Thus the effect of the chronic feeding of 10% ethanol in drinking water would appear to elicit a specific pattern of enhanced enzymatic activities which differs from that obtained with methods using higher ethanol concentrations.

Finally, epidemiological studies have suggested that chronic ethanol consumption is etiologically linked with gastrointestinal cancer and other malignancies [5]. Further, it is currently felt that many carcinogens must first be enzymatically activated to the ultimate carcinogen. This activation is also thought to be initiated by the microsomal mixed-function oxidases [28]. The mechanism(s) by which ethanol activates carcinogenesis is not known but may be related to a variety of effects on the cell including its ability to induce the microsomal mixed-function oxidases [14, 19, 25].

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