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# EFFECTS OF ETHANOL ON GLUTATHIONE CONJUGATION IN RAT LIVER AND LUNG

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**Abstract**—The ability of ethanol to alter glutathione (GSH) conjugation and its dependence upon duration of administration were investigated in rats in correlation with lipid peroxidation and the induction of microsomal enzymes. Significant decreases in hepatic GSH and glutathione-*S*-transferase (GST) activity in both liver and lung were found in rats treated acutely with ethanol (4 g/kg body weight 6 hr prior to killing). These decreases were accompanied by an increased loss of both GSH and GST into the plasma and increased hepatic lipid peroxidation. On the other hand, there was a dose-dependent increase in hepatic GSH after chronic administration of ethanol in drinking water (5 and 10%) for 3 weeks. This increase in hepatic GSH may be due to increased synthesis of GSH in the liver. No significant induction of GST by chronic ethanol treatment was observed in either organ. Ethanol was compared with the well-known inducers phenobarbital and β-naphthoflavone. Although there was some evidence of increases in lipid peroxidation and/or microsomal enzyme activity with the inducers, no simple link between these increases and the induction of GST activity was identified.

The glutathione-*S*-transferases (EC 2.5.1.18) are a family of enzymes which catalyze the conjugation of glutathione (GSH) with a large number of electrophiles as an early step in their detoxification. Although they are generally cytosolic enzymes, the glutathione-*S*-transferases are inducible along with the microsomal drug-metabolizing enzymes. Glutathione-*S*-transferases have been reported to be induced in liver by phenobarbital (PB) [1, 2], 3-methylcholanthrene [1, 2] and β-naphthoflavone (BNF) [2], which are well-known inducers of cytochrome P450.

The influence of ethanol on hepatic GSH conjugation has been studied, but discrepancies exist which appear to depend on the level and the duration of ethanol consumption. Chronic administration of ethanol in a liquid diet for 2 weeks increases the activity of hepatic glutathione-*S*-transferases and GSH content in rats [3] and mice [4]. These results are in contrast to those observed with the administration of a single either oral [5] or intraperitoneal [6, 7] dose of ethanol which causes decreases in glutathione-*S*-transferase (GST) activities [5, 6] and GSH concentration [6, 7] in rat liver.

The mechanisms responsible for the ethanol-induced alterations in GSH conjugation are not understood completely. However, evidence has accumulated to suggest that increases in lipid peroxidation [5, 6, 8, 9], and cross-induction with microsomal enzymes [10, 11] may play important roles. Therefore, the possible correlation between the effects of ethanol on the GSH-conjugating system and lipid peroxidation and/or induction of microsomal enzymes was studied with respect to the dose and duration of ethanol administration.

## MATERIALS AND METHODS

*Animals*. Male, Sprague–Dawley rats (Harlan Sprague–Dawley Inc., Indianapolis, IN) weighing 200–300 g were used. Rats receiving i.p. treatments were housed in community stainless steel cages (six to seven rats per cage). Rats used in the ethanol-drinking studies were housed individually in stainless steel cages. Food (Purina Lab Chow, Ralston Purina Co., St. Louis, MO) and water were allowed *ad lib*. Lights were on a 12:12 hr light:dark cycle with the temperature maintained at 21°.

*Treatments*. Two experiments were conducted in order to study the effects of ethanol on GSH conjugation. The first experiment was aimed at determining the effect of a single dose of ethanol. In the ethanol treatment group (N = 6), the rats received 4 g ethanol/kg body weight, i.p., 6 hr before decapitation. Controls (N = 6) received saline. To study prolonged ethanol administration and to compare those effects with PB and BNF treatments, rats were assigned to five groups: controls (group I, N = 6) drank tap water, group II (N = 6) and group III rats (N = 6) received 5 and 10% ethanol (w/v) in drinking water for 3 weeks. Water replaced the ethanol solution 24 hr before the killing. Group IV rats (N = 4) were injected i.p. daily for 3 days with PB (80 mg/kg body weight), and group V rats were injected i.p. with BNF (40 mg/kg body weight) daily for 3 days. Animals were killed 24 hr after the last dose.

*Tissue preparations*. Animals were killed between 10:00 and 11:00 a.m. Subcellular fractions were prepared by homogenizing the liver or lung in an all glass Potter–Elvehjem tissue homogenizer or in a Brinkmann polytron, respectively, in 0.1 M Tris buffer containing 1.15% KCl (pH 7.5). The homogenate was centrifuged at 10,000 *g* for 20 min to remove nuclei, debris and mitochondria. The supernatant was further centrifuged at 105,000 *g* for 60 min. The resulting soluble supernatant (cytosolic) fraction was collected, and the microsomal pellet

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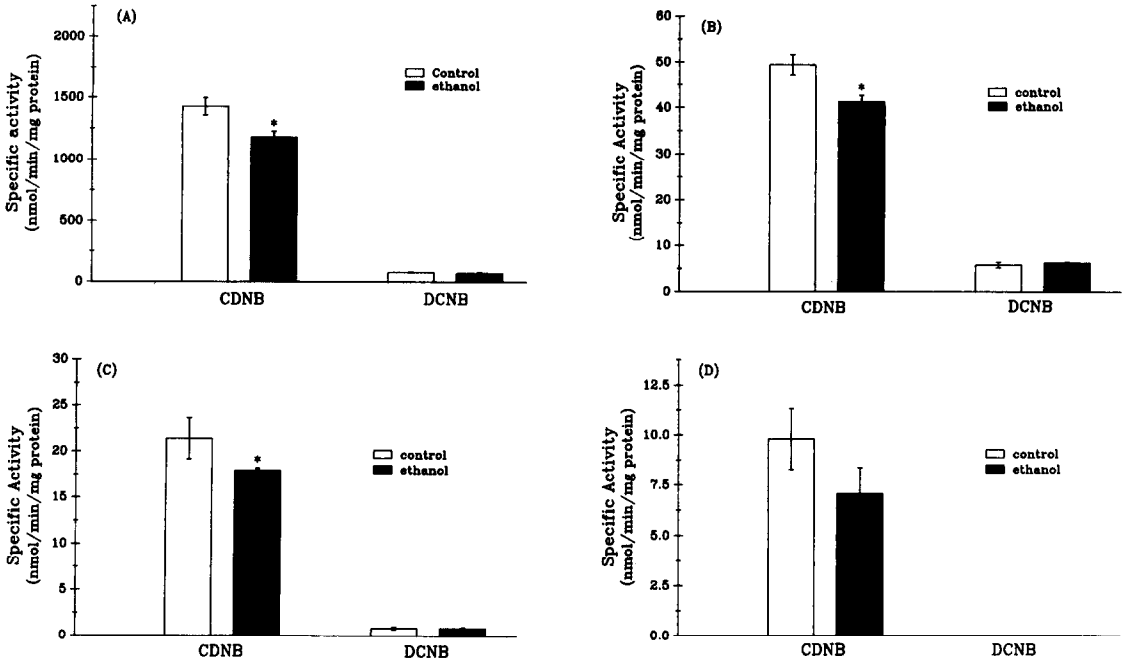


Fig. 1. Effect of acute ethanol administration on GST activity. In the ethanol treatment group (N = 6), the rats received 4 g ethanol/kg body weight, i.p., 6 hr before decapitation. Controls (N = 6) received saline. The GST activities are (A) hepatic cytosol, (B) hepatic microsomes, (C) pulmonary cytosol and (D) pulmonary microsomes. The vertical lines indicate the SEM. Key: (*) significantly different from control ($P < 0.05$).

was resuspended in 1 M phosphate buffer (pH 7.25) containing 10 mM EDTA (pH 7.5), 20% glycerine, 0.25 mM phenylmethylsulfonyl fluoride and 0.1 mM dithiothreitol. Microsomes from four to six animals were pooled. The preparations were stored at −70° until assayed.

*Assays.* GST activities were measured kinetically with 1-chloro-2,4-dinitrobenzene (CDNB) and 3,4-dichloro-1-nitrobenzene (DCNB) according to Habig *et al.* [12]. GSH content was assayed in plasma and tissue using 5,5′-dithiobis-(2-nitrobenzoic acid) according to the method of Speisky *et al.* [7]. Cytochrome P450 in liver was determined by the method of Omura and Sato [13], using an extinction coefficient of 91 $mM^{-1}$ for the 490 − 450 nm wavelength pair. To rule out interference from hemoglobin and/or cytochrome oxidase, cytochrome P450 in the lung was determined by the method of Johannesen and DePierre [14], which utilizes the difference between oxidized-CO-bound and reduced-CO-bound cytochrome, based upon an extinction coefficient of 105 $mM^{-1}$ for 475 − 450 nm. Hepatic lipid peroxidation was determined as the formation of thiobarbituric acid-reactive products according to the method of Uchiyama and Mihara [15]. Microsomal monooxygenase activities were determined by dealkylation of ethoxyresorufin and pentoxyresorufin as described by Prough *et al.* [16]. The activities of dealkylases were quantified using resorufin standards. Protein concentrations were determined according to the method of Lowry *et al.* [17] using bovine serum albumin as the standard.

*Statistical analysis.* Data are expressed as means ± SEM. Statistical comparison of the treatment group with its control was made using Student's *t*-test. When there was more than one treatment group, a one-way ANOVA was used to compare the treatment groups to control, and Duncan's new multiple range finding test [18] was used to evaluate differences among the group means.

## RESULTS

A single dose of ethanol (4 g/kg) caused a significant ($P < 0.05$) decrease in hepatic GSH from 7.00 ± 0.24 μmol/g tissue to 4.17 ± 0.13 μmol/g tissue, whereas no changes were observed in the lung (control: 1.56 ± 0.04 μmol/g tissue and ethanol-treated: 1.60 ± 0.06 μmol/g tissue) after 4 hr. Hepatic GSH has an especially important relationship with lipid peroxidation because of its ability to bind with free radicals that may initiate peroxidation [19, 20]. Hepatic lipid peroxidation in ethanol-treated rats showed a 44% increase in absorbance (535 − 600 nm) from 0.09 to 0.14, although the difference was not statistically significant because of the large variation among animals.

The activity of GST with CDNB as the substrate was decreased in both cytosol and microsomes of liver and lung from acutely treated rats (Fig. 1), but hepatic enzyme activity with DCNB was not altered. Microsomal GST activity with DCNB in the lung was not detectable.

Hepatocyte GSH easily passes into the plasma

Table 1. Effect of acute ethanol administration on cytochrome P450 content and ethoxyresorufin and pentoxyresorufin O-dealkylations in the rat

| Group | | Cytochrome P450 (pmol P450/mg protein) | EROD (pmol/min/mg protein) | PROD | EROD (pmol/min/nmol P450) | PROD |
|---|---|---|---|---|---|---|
| (A) | Liver | | | | | |
| | Control | 556 ± 44 | 121 ± 7 | 54 ± 3 | 219 ± 22 | 98 ± 9 |
| | Ethanol | 543 ± 69 | 169 ± 1* | 58 ± 9 | 327 ± 66 | 112 ± 28 |
| (B) | Lung | | | | | |
| | Control | 45 ± 4 | 10 ± 2 | 43 ± 7 | 212 ± 7 | 1167 ± 218 |
| | Ethanol | 34 ± 2 | 15 ± 0* | 52 ± 6 | 362 ± 80 | 1269 ± 298 |

In the ethanol treatment group (N = 6), the rats received 4 g ethanol/kg body weight, i.p., 6 hr before decapitation. Controls (N = 6) received saline. Six samples were pooled. Data are means ± SEM of three pools.
* Significantly different from control ($P < 0.05$).

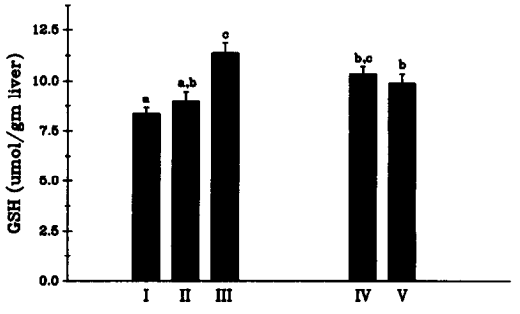


Fig. 2. Efect of chronic ethanol administration on hepatic GSH concentration. Controls (group I, N = 6) drank tap water. Group II (N = 6) and group III rats (N = 6) received 5 and 10% ethanol (w/v) in drinking water for 3 weeks, respectively; group IV rats (N = 4) were injected i.p. with PB (80 mg/kg body weight) and group V rats (N = 4) were injected i.p. with BNF (40 mg/kg body weight) daily for 3 days. The vertical lines indicate the SEM. Key: ($^{a-c}$) values with different superscripts are significantly different ($P < 0.05$).

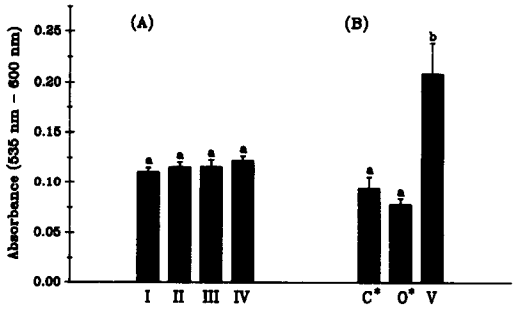


Fig. 3. Effect of chronic ethanol administration on hepatic lipid peroxidation. (A) Controls (group I, N = 6) drank tap water. Group II (N = 6) and group III rats (N = 6) received 5 and 10% ethanol (w/v) in drinking water for 3 weeks, respectively; group IV rats (N = 4) were injected i.p. with PB (80 mg/kg body weight) daily for 3 days. (B) Controls either drank tap water (group C*, N = 6) or were injected i.p. with corn oil (group O*, N = 5). Group V rats (N = 5) were injected i.p. with BNF (40 mg/kg body weight) daily for 3 days. The vertical lines indicate the SEM. Key: ($^{a,b}$) Values with different superscripts are significantly different ($P < 0.05$).

following injury [21], and plasma GSH is increased by acute ethanol administration in rats [22]. Therefore, plasma GST activities as well as GSH concentrations were measured to evaluate translocation processes from liver into plasma. The acute treatment with ethanol resulted in a 34% increase in plasma GSH from 4.05 ± 0.73 nmol/mL to 5.44 ± 1.15 nmol/mL and a 39% increase in CDNB-conjugating activity from 58.3 ± 3.22 nmol/min/mL to 80.8 ± 6.87 nmol/min/mL.

The effect of a single dose of ethanol on the mixed-function oxidase (MFO) system was studied by measuring total cytochrome P450 content as well as dealkylation of ethoxyresorufin and pentoxyresorufin (Table 1). Ethanol had no effect on either hepatic or pulmonary cytochrome P450 content. In both tissues ethoxyresorufin *O*-deethylase (EROD) was approximately 40% higher than that in controls, whereas pentoxyresorufin *O*-depentylase (PROD) was not altered.

The effects of chronic ethanol were studied in rats receiving ethanol in drinking water at concentrations of 5 and 10% for 3 weeks. Average daily doses were 4.98 and 8.45 mL ethanol/kg/day. Hepatic GSH in rats drinking ethanol increased in a dose-dependent manner (Fig. 2). PB and BNF also increased hepatic GSH, but the induction was less than in rats treated with 10% ethanol. Only with BNF did ethanol cause hepatic lipid peroxidation (Fig. 3).

The effects of chronic ethanol, PB and BNF on GST activity were measured in cytosolic and microsomal fractions of liver and lung (Table 2). In the liver, microsomal GST activity with both substrates was approximately 10% of the cytosolic GST activity. In the lung, it was approximately 30% of cytosolic GST with CDNB. With DCNB it was essentially undetectable.

Prolonged ethanol administration caused no significant induction in GST activity in either organ. In liver, PB induced cytosolic GST activity with CDNB and DCNB and microsomal GST with CDNB, whereas BNF induced microsomal GST with CDNB. Microsomal pulmonary GST activity with CDNB increased only with BNF. In contrast to the

Table 2. Effects of ethanol, phenobarbital (PB) and β-naphthoflavone (BNF) on cytosolic and microsomal glutathione-S-transferase activities in the rat

| Group | CDNB | | DCNB | |
|---|---|---|---|---|
| | Cytosol | Microsome | Cytosol | Microsome |
| (A) Liver | | | | |
| I. Control | 1375 ± 66* | 146 ± 1* | 65 ± 2*† | 6.4 ± 0.8* |
| II. Ethanol (5%) | 1382 ± 94* | 152 ± 8*† | 65 ± 3*† | 6.6 ± 0.8* |
| III. Ethanol (10%) | 1581 ± 90* | 148 ± 10*† | 71 ± 4† | 6.1 ± 0.4* |
| IV. PB | 1907 ± 78† | 172 ± 2†‡ | 89 ± 4‡ | 6.5 ± 1.0* |
| V. BNF | 1630 ± 64* | 177 ± 3‡ | 59 ± 4* | 6.8 ± 0.5* |
| (B) Lung | | | | |
| I. Control | 32 ± 1.3* | 11 ± 0.2*† | 1.2 ± 0.1* | ND§ |
| II. Ethanol (5%) | 34 ± 1.5* | 10 ± 0.3* | 1.1 ± 0.1* | ND |
| III. Ethanol (10%) | 35 ± 1.2* | 13 ± 1.1†‡ | 1.3 ± 0.2* | ND |
| IV. PB | 30 ± 1.4* | 11 ± 0.6*† | 1.1 ± 0.1* | ND |
| V. BNF | 33 ± 4.6* | 14 ± 0.3‡ | 1.1 ± 0.1* | ND |

Controls (group I, N = 6) drank tap water. Group II (N = 6) and group III rats (N = 6) received 5 and 10% ethanol (w/v) in drinking water for 3 weeks, respectively; group IV rats (N = 4) were injected i.p. with PB (80 mg/kg body weight) and group V rats were injected i.p. with BNF (40 mg/kg body weight) daily for 3 days. Six samples were pooled. Data are means ± SEM of two or three pools.

*–‡ Values within the same column with different symbols are significantly different ($P < 0.05$).

§ Not detectable.

effects of acute ethanol administration, chronic ethanol administration (10%) diminished plasma GST activity (Fig. 4). Neither PB nor BNF affected plasma GST activity.

There was no increase in cytochrome P450 in either organ following chronic ethanol administration (Table 3). PB and BNF caused 178 and 68% increases. In the liver, PB and BNF produced 381- and 7.6-fold increases in PROD activity and increased EROD activity by 73% and 304-fold. In controls, the specific activity of EROD in the lung was 20% that of liver, whereas PROD was higher. PROD in the lung was not responsive to PB treatment, but EROD showed an 18-fold increase following BNF treatment. When ethanol was administered as either a 5 or 10% solution in drinking water for 3 weeks, no significant increases in hepatic microsomal PROD or EROD were observed. In the lung, there was a 4.3-fold increase in ethoxyresorufin deethylation in rats receiving 10% ethanol for 3 weeks.

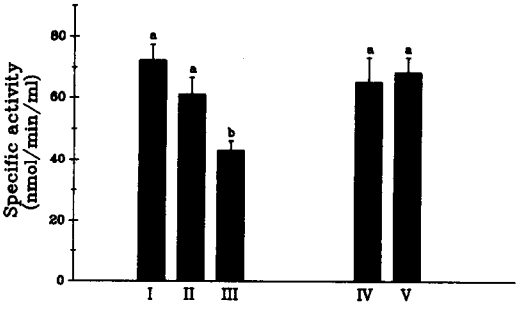


Fig. 4. Effect of chronic ethanol administration on plasma GST activity. Controls (group I, N = 6) drank tap water. Group II (N = 6) and group III rats (N = 6) received 5 and 10% ethanol (w/v) in drinking water for 3 weeks, respectively; group IV rats (N = 4) were injected i.p. with PB (80 mg/kg body weight) daily for 3 days and group V rats (N = 4) were injected i.p. with BNF (40 mg/kg body weight) daily for 3 days. The vertical lines indicate the SEM. Key: ($^{a,b}$) values with different superscripts are significantly different ($P < 0.05$).

## DISCUSSION

The effect of ethanol on GSH conjugation and the possible correlations with lipid peroxidation and induction of microsomal enzymes were studied with respect to the dose and duration of ethanol administration. Significant decreases in GSH content in the liver and GST activity in both liver and lung from rats treated acutely with ethanol were found, but there was a dose-dependent increase in hepatic GSH after administration of ethanol in drinking water (5 and 10%) for 3 weeks. Chronic ethanol administration did not induce GST activity.

Several explanations can be proposed for this biphasic effect of ethanol. The decreases in hepatic GSH and GST upon acute ethanol administration could be due primarily to an increased loss of both GSH and GST from the liver into plasma. Speisky *et al.* [7] suggested that the suppression of hepatic GSH content by a large dose of ethanol might be mediated by hormonal responses which are independent of ethanol metabolism. Ethanol can cause hormonal responses including the release of corticosteroids, glucagon and epinephrine [7, 23], and these hormones increase the sinusoidal efflux of GSH from the perfused liver [24].

Table 3. Effects of chronic ethanol, phenobarbital (PB) and β-naphthoflavone (BNF) on cytochrome P450 content and ethoxyresorufin and pentoxyresorufin O-dealkylations in the rat

| Group | Cytochrome P450 (pmol P450/mg protein) | EROD (pmol/min/mg protein) | PROD (pmol/min/mg protein) | EROD (pmol/min/nmol P450) | PROD (pmol/min/nmol P450) |
|---|---|---|---|---|---|
| (A) Liver | | | | | |
| I. Cotrol | 683 ± 88* | 48 ± 5* | 7 ± 0* | 72 ± 2* | 11 ± 2* |
| II. Ethanol (5%) | 897 ± 107*† | 52 ± 14* | 12 ± 2* | 60 ± 17* | 14 ± 3* |
| III. Ethanol (10%) | 800 ± 54*† | 67 ± 10* | 16 ± 4* | 83 ± 9* | 20 ± 4* |
| IV. PB | 1904 ± 320‡ | 83 ± 6* | 676 ± 230† | 46 ± 11* | 1426 ± 119† |
| V. BNF | 1150 ± 77† | 14,628 ± 1284† | 60 ± 12* | 1285 ± 197† | 53 ± 14* |
| (B) Lung | | | | | |
| I. Control | 41 ± 4* | 9 ± 2* | 75 ± 17* | 163 ± 19* | 1350 ± 151* |
| II. Ethanol (5%) | 47 ± 2* | 30 ± 8*† | 64 ± 11* | 635 ± 190*† | 1347 ± 177* |
| III. Ethanol (10%) | 46 ± 4* | 48 ± 9† | 63 ± 6* | 1070 ± 233† | 1386 ± 183* |
| IV. PB | 48 ± 10* | 8 ± 2* | 53 ± 13* | 176 ± 5* | 1098 ± 39*† |
| V. BNF | 82 ± 9† | 167 ± 23‡ | 44 ± 7* | 2045 ± 63‡ | 534 ± 28† |

Controls (group I, N = 6) drank tap water. Group II (N = 6) and group III rats (N = 6) received 5 and 10% ethanol (w/v) in drinking water for 3 weeks, respectively; group IV rats (N = 4) were injected i.p. with PB (80 mg/kg body weight) and group V rats were injected i.p. with BNF (40 mg/kg body weight) daily for 3 days. Six samples were pooled. Data are means ± SEM of two or three pools.

*–‡ Values within the same column with different symbols are significantly different ($P < 0.05$).

GST activity with CDNB decreased in both liver and lung cytosols and microsomes upon acute ethanol treatment. Kocak-Toker *et al.* [5] reported that hepatic GST activity with CDNB decreased following 3 or 5 g/kg ethanol, although the plasma GST activity was increased only by the latter dose. Our finding of the lack of inhibition in GST activity with DCNB in both liver and lung suggests that the effect may be substrate dependent.

Evidence suggests a relationship between hepatic GSH content and lipid peroxidation after acute ethanol administration [22, 25]. In the present study, depletion of hepatic GSH was accompanied by increased lipid peroxidation. Since GSH provides several lines of defense against peroxidative damage associated with the GSH-peroxidases [3], increased lipid peroxidation may deplete hepatic GSH by increasing its utilization.

We previously found no alteration in pulmonary GSH despite a decrease in hepatic GSH when rabbits were treated with either ethanol [26] or buthionine sulfoximine [27]. In the present study, ethanol did not alter pulmonary GSH. Lung shows a lower turnover rate of GSH than liver [28], making GSH levels in lung less susceptible to alteration. This may account for the differential tissue effect. In contrast, chronic ethanol administration increased hepatic GSH content. This has been explained by increased GSH synthesis [3, 6, 29]. GSH synthesis is thought to be regulated by end-product feedback inhibition [30]. Since both the efflux of GSH to plasma and its oxidation result in decreases in hepatic GSH, either mechanism could be expected to cause a compensatory increase in synthesis.

Although there was no increase in hepatic lipid peroxidation in rats administered 10% ethanol in the drinking water, it does not rule out the possibility of chronic ethanol-induced lipid peroxidation under other conditions. Since the ethanol-inducible P450IIE1 has an especially high oxidase activity [31, 32], it is likely to contribute to microsomal NADPH-dependent lipid peroxidation [33, 34]. The increased GSH levels may reflect an adaptational change in response to chronic ethanol-induced lipid peroxidation as suggested by Hetu *et al.* [3].

Increases in GST activity after chronic ethanol treatment have been demonstrated [3, 6], and it has been suggested that this is an adaptational change in response to ethanol-induced lipid peroxidation. However, BNF, which caused a 1.3-fold increase in hepatic lipid peroxidation, did not induce GST activity to any greater extent than did ethanol. This argues against a simple link between lipid peroxidation and GST induction.

It is not clear whether microsomal GST reflects cytosolic contamination, selective adsorption and/or a functional interaction with endoplasmic reticulum *in vivo* [35–37]. If the microsomal GST localized on the endoplasmic reticulum is truly different from the cytosolic and is involved in xenobiotic metabolism, the microsomal GST protects the membranes of the endoplasmic reticulum from lipid peroxidation [37].

Chronic ethanol induces proliferation of hepatic microsomal membranes [10, 11] and hepatic MFO activities [38, 39]. Younes *et al.* [11] suggested that cytosolic GST activity is subject to similar factors which influence microsomal enzymes, but the response of GST to inducers differs from that of P450. In the lung, BNF increased cytochrome P450 content and EROD activity and chronic ethanol treatment increased EROD activity, but GST activity remained unchanged in both cases.

As expected, hepatic microsomal dealkylations of ethoxyresorufin and pentoxyresorufin were increased preferentially by BNF and PB [40–42], demonstrating that they are preferred substrates of the major BNF (P450IA1 and P450IA2) and PB-induced cytochrome P450 isozymes (P450IIB1 and P450IIB2). In the lung, BNF but not PB induced its corresponding

substrate. Omiecinski [43] suggested that P450IIB1 is expressed at very low levels in liver and is highly inducible by PB, while in the lung a constitutive level is expressed but no induction occurs.

The present study and previous work [44] showed induction of EROD in the lung and liver upon repetitive administration of ethanol. The ethanol-inducible form of cytochrome P450 (P450IIE1) is present in liver and lung [10, 31]. Ethoxyresorufin deethylation is primarily related to P450IA1. It is unknown if it is also a substrate for P450IIE1 as acetone, $CCl_4$ and butanol are [31, 45]. Microsomal metabolism of butanol was not altered in the liver and was decreased rather than increased in the lung by low doses of ethanol (0.5 and 1.0 mL/kg) given i.p. for 7 days [45]. Since ethoxyresorufin shows a high but not absolute substrate specificity for P450IA1 [40–42], it is difficult to speculate on which P450 isozyme(s) is involved in EROD in the lung.

In summary, the effects of ethanol on GSH conjugation depend on the dosing. Acute administration induced both a loss of GSH from the liver and an increased GSH utilization, whereas chronic treatment increased hepatic GSH presumably due to an increased synthesis. GST activity was decreased in both liver and lung and increased in plasma by acute ethanol treatment. However, no significant induction of GST by chronic treatment (5 and 10% ethanol in drinking water for 3 weeks) was observed in either organ. There was no simple link between lipid peroxidation and/or induction of microsomal enzymes and alterations in GST activity.

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