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# DIFFERENTIAL EFFECT OF CHRONIC ETHANOL CONSUMPTION BY THE RAT ON MICROSOMAL OXIDATION OF HEPATOCARCINOGENS AND THEIR ACTIVATION TO MUTAGENS*

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**Abstract**—The effect of chronic ethanol consumption by rats on hepatic microsomal activation of the hepatocarcinogens dimethylnitrosamine (DMN) and 2-acetylaminofluorene (2-AAF) was investigated. There was a marked increase in the rate of the oxidative demethylation of DMN and its activation to a mutagen by microsomes following ethanol intake. N- and C-hydroxylation of 2-AAF were measured at substrate concentrations ranging from 2 to 70 $\mu$M. The ratio of formation of *N*-hydroxy-2-acetylaminofluorene to *C*-hydroxy-2-acetylaminofluorenes increased with decreasing substrate concentration, suggesting enhanced carcinogenic potential of 2-AAF with diminishing levels of carcinogen. Kinetic analysis indicated that N-hydroxylation as well as 7-, 5- and 3-hydroxylation of 2-AAF do not follow Michaelis–Menten kinetics. In contrast to the marked inductive effect of ethanol consumption on the metabolic activation of DMN, only a minimal random effect on the N-hydroxylation of 2-AAF was demonstrable in two separate experiments. Furthermore, N-hydroxylation of 2-AAF by microsomes from control and ethanol-treated rats followed similar kinetics. While ethanol consumption enhanced the mutagenic activation of DMN by hepatic microsomes, no such effect of ethanol consumption on the conversion of 2-AAF to a mutagen was observed. The data indicate that chronic ethanol consumption does not have a general inductive effect on the microsomal activation of hepatocarcinogens.

Damage to the human liver by excessive intake of alcohol is well documented [1], and a correlation between excessive alcohol consumption and hepatocellular carcinoma of the human liver has been suggested [2, 3]. In experimental animals, chronic ethanol administration increases the activity of several enzymes associated with the hepatic microsomal mixed-function oxidase [4–11]. These enzymes are responsible for detoxication of xenobiotics. Simultaneously, they oxidize procarcinogens to activated intermediates that may be converted to electrophiles which then react with cellular macromolecules, thereby initiating the carcinogenic process. Because of the inducibility of these enzymes by ethanol the balance between detoxication and activation, which is a critical factor in determining the carcinogenic potential of a compound [12], may conceivably be shifted by ethanol consumption.

Dimethylnitrosamine (DMN)‡ and 2-acetylaminofluorene (2-AAF), two potent hepatocarcinogens for the male rat, are compounds that require metabolic activation to exert their biological effects. The proposed first step in the activation of DMN is an oxidative demethylation via hydroxylation of the $\alpha$-carbon atom [13–15]. N-Hydroxylation of 2-AAF to an arylhydroxamic acid is the first step in its activation to mutagenic and carcinogenic species, while C-hydroxylation to phenolic derivatives is considered a detoxication reaction [16]. The participation of the microsomal mixed-function oxidase enzymes, in particular cytochrome P-450, in the activation of both compounds has been demonstrated [15, 17].

The induction of DMN demethylase by ethanol ingestion in rats has been reported [5, 18]. However, the effect of chronic ethanol consumption on the N-hydroxylation of 2-AAF has not been established even though there are numerous reports of the induction of N-hydroxylation, in animal systems, by a variety of chemicals [17, 19–25]. The purpose of this investigation is to compare the effects of ethanol consumption on the microsomal oxidation and mutagenic activation of two procarcinogens, DMN and 2-AAF, which differ widely in structure, solubility in aqueous media, and initial activation reaction (C- versus N-hydroxylation).

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‡ Abbreviations: DMN, dimethylnitrosamine; 2-AAF, 2-acetylaminofluorene; C-OH-2-AAF, *C*-hydroxy-2-acetylaminofluorene; *N*-OH-2-AAF, *N*-hydroxy-2-acetylaminofluorene; 7-OH-2-AAF, 7-hydroxy-2-acetylaminofluorene; 5-OH-2-AAF, 5-hydroxy-2-acetylaminofluorene; 3-OH-2-AAF, 3-hydroxy-2-acetylaminofluorene; 1-OH-2-AAF, 1-hydroxy-2-acetylaminofluorene; HPLC, high-pressure liquid chromatography; cyt. P-450, cytochrome P-450; EtOH, ethanol; and MeOH, methanol.

## MATERIALS AND METHODS

### *Reagents*

DMN was purchased from the Aldrich Chemical Co., Milwaukee, WI and di-[$^{14}$C]methylnitrosamine (sp. act. 15 mCi/mmole) from the Amersham Corp., Arlington Heights, IL. 2-AAF and 2-[$^{3}$H]acetylaminofluorene (2-[$^{3}$H]AAF) (sp. act. 250 mCi/mmole) were synthesized as described previously [26]. Ultraviolet spectra of these compounds were identical to that of authentic 2-AAF, and they gave a single peak by HPLC. *N*-OH-2-AAF, 7-OH-2-AAF, 5-OH-2-AAF and 3-OH-2-AAF were prepared as previously described [26] and shown to be pure by HPLC.

### *Preparation of hepatic microsomes*

Male Sprague–Dawley rats (110–130 g) were matched by weight and were pair-fed a nutritionally adequate liquid diet [27] containing 18% protein, 35% fat and 47% carbohydrate (BioServ, Inc., Frenchtown, NJ). Ethanol-treated rats received 36% of their total calories from ethanol, which replaced an isocaloric amount of carbohydrate. These rats were weaned on the ethanol diet for a period of 7 days as suggested by the manufacturer. During this period the controls were also fed the liquid diet. Feeding was continued for 28 additional days. In several other specifically designated experiments, rats were allowed unlimited access to Purina Rat Chow and water. Hepatic microsomes were prepared, and the protein and cytochrome P-450 contents were assayed as previously described [26]. Freshly prepared microsomes were used for metabolic and binding studies and for mutagenicity tests.

### *Effect of* in vitro *addition of ethanol on demethylation of DMN and hydroxylation of 2-AAF by hepatic microsomes*

The effects of ethanol addition to DMN demethylase reaction mixtures *in vitro* were tested by replacing 50 $\mu$l of buffer with 50 $\mu$l of appropriate concentrations of ethanol to give final ethanol concentrations of 0.41, 0.85 and 1.71 M. The reactions were carried out under conditions identical to those described below.

Reaction mixtures for the hydroxylation of 2-AAF contained 50 $\mu$l MeOH (0.41 M) which was required to solubilize the substrate (2-AAF). To test the effect of ethanol on the hydroxylation reactions *in vitro*, MeOH was omitted and amounts of ethanol were added to give the concentrations indicated in Table 3. The reactions were carried out and the metabolites were assayed as described below.

### *Quantitative determination of microsomal metabolites of DMN and 2-AAF*

*DMN demethylation by microsomes from controls and ethanol-treated rats.* Reaction mixtures containing 0.7 to 5.0 mM di-[$^{14}$C]methylnitrosamine were incubated for 25 min at 37°. DMN demethylase activity was assayed by measuring the formation of [$^{14}$C]formaldehyde [28] under the conditions of Garro *et al.* [5].

*2-AAF hydroxyiation by microsomes from controls and ethanol-treated rats.* Reaction mixtures (3.0 ml) contained 50 mM phosphate buffer (pH 7.4), 25 mM KCl, 1.5 mM NADPH, 1 nmole cytochrome P-450 and 2-[$^{3}$H]AAF, dissolved in 50 $\mu$l MeOH, to give final concentrations of 1.7 to 66.7 $\mu$M. Formation of *N*-OH-2-AAF and 7-OH-2-AAF was linear for 15 min with cytochrome P-450 concentrations up to 2 nmoles/3 ml incubation mixture. The mixtures were incubated for 12 min at 37°. The reaction was terminated by addition of 1 M sodium acetate, pH 4.5. Unlabeled *N*-OH-2-AAF and 7-OH-2-AAF were added as carrier compounds and the mixtures were stored at −20° until they were analyzed. The metabolites under study were extracted from the reaction mixtures with diethyl ether [26] or benzene and separated by HPLC. C-hydroxy metabolites (7-OH-2-AAF, 5-OH-2-AAF, 3-OH-2-AAF, 1-OH-2-AAF) and *N*-OH-2-AAF were eluted from a Permaphase ODS column, maintained at 45°, with 20% and 100% MeOH respectively. C-hydroxy metabolites were then separated on a Corasil II column [26], and the peak corresponding to 7-OH-2-AAF was collected for analysis. The amounts of labeled *N*-OH-2-AAF and 7-OH-2-AAF formed were determined by the method of inverse isotope dilution. The radioactivity of all samples was counted to 1.5% efficiency. Only samples with radioactivity greater than two times background radioactivity were analyzed.

### *Measurement of the kinetics of N- and C-hydroxylation of 2-AAF*

The apparent kinetic constants of the hydroxylation of 2-AAF were determined at 2–70 $\mu$M 2-AAF. A different microsomal preparation was used to measure the kinetics of N-hydroxylation at a concentration range of 0.01 to 1.67 $\mu$M 2-AAF. Ratios of the formation of *N*-OH-2-AAF to 7-OH-2-AAF at 0.02, 0.17 and 1.67 $\mu$M 2-AAF were obtained in a separate experiment. Reaction mixtures were identical to those described above except that microsomes from rats fed Purina chow *ad lib.* were used as a source of microsomal cytochrome P-450, and the incubation period was 10 min. Following their extraction from the incubation mixtures, the metabolites were isolated by HPLC on a Permaphase ODS column, maintained at 40°. Elution with distilled water yielded the C-hydroxylated metabolites as a single peak [26]. 2-[$^{3}$H]AAF and *N*-OH-2-AAF were separated by elution with 15% and 40% MeOH respectively. Following elution of *N*-OH-2-AAF, the column was washed, sequentially, with MeOH, water, MeOH and water before injection of the next sample. Control experiments showed that neither the C-hydroxylated metabolites nor *N*-OH-2-AAF were contaminated by substrate radioactivity. The C-hydroxylated metabolites were subsequently separated on a Corasil II column [26] and quantified as described above. Apparent kinetic constants were computed and statistically analyzed on a Hewlett–Packard 9830A calculator using the weighted least squares method.

### *Binding spectra*

Difference spectra for the interaction of 2-AAF with hepatic microsomal cytochrome P-450 were

determined essentially as published previously [26]. Microsomes containing 8 nmoles of cytochrome P-450 were added to reference and sample cuvettes. 2-AAF, dissolved in MeOH, was added to the sample cuvette in 5-$\mu$l (80 nmoles) increments. Compensatory amounts of MeOH were added to the reference cuvette. Difference spectra were recorded at concentrations of 2-AAF ranging from 27 to 262 $\mu$M. Values for the apparent $K_s$ (spectral dissociation constant) were obtained on a Hewlett–Packard 9830A calculator with the use of the weighted least squares method.

*Mutagenicity tests*

Hepatic microsomes from controls and ethanol-treated rats were tested for their capacity to activate DMN and 2-AAF to mutagenic compounds [29]. *Salmonella typhimurium* tester strains TA1538, TA1535, TA100 and TA92 were provided by Dr. Bruce Ames, University of California at Berkeley, CA. The DMN reaction mixture (0.65 ml) was essentially that of Garro *et al.* [5] except that *S. typhimurium* strain TA92 was used. In agreement with Glatt *et al.* [30], we found strain TA92 to be more sensitive to mutagenic activation of DMN than strains TA1535 or TA100. The 2-AAF reaction mixture (0.61 ml) contained 0.1 M phosphate buffer (pH 7.4), 8 mM $MgCl_2$, 33 mM KCl, 4 mM NADPH, 1.2 nmoles cytochrome P-450, 0.1 ml of an overnight culture of TA1538 cells and 2-AAF at concentrations designated in Fig. 4. All reaction mixtures were incubated on a rotary shaker at 37° for 25 min (DMN) or 12 min (2-AAF). They were then added to 2 ml top agar and overlaid on minimal glucose plates [29]. Triplicate plates were overlaid for each concentration tested. After incubation at 37° for 48–60 hr, the plates were scored for the number of histidine prototrophs.

## RESULTS

*Food and ethanol consumption by experimental animals*

All animals gained weight throughout the period of feeding. The nutritional intake of three different groups of rats was very consistent (Table 1). Ethanol-fed rats consumed 3.4 to 3.5 g ethanol/day, which corresponds to an average daily intake of approximately 18 g ethanol/kg body weight. The consumption of ethanol was greater than that reported by several other investigators [4, 31–33] who used the same or alternative feeding regimens. There was no significant difference ($P > 0.1$) in average daily caloric intake between controls and ethanol-fed rats or between rats of the three experimental groups. In general, untreated rats gained more weight than ethanol-fed rats even though their caloric intake was the same. This observation is in agreement with results of other workers [4, 31, 33]. All rats in the same group gained weight at approximately the same rate. The apparent lower total weight gain of rats in experiment 3 was due to the shorter feeding period (28 days vs 35 days in experiments 1 and 2). These data indicated that the recommended weaning period is not necessary to ensure that animals will adapt to the ethanol-containing diet. Chronic ethanol consumption by the animals did not cause an increased level of hepatic microsomal cytochrome P-450/mg protein. Typical values obtained in this laboratory for hepatic microsomal cytochrome P-450/mg protein were 1.34, 1.17 and 1.28 nmoles for microsomes from controls and, correspondingly, 1.36, 1.31 and 0.91 nmoles for microsomes from ethanol-treated rats.

*Effect of ethanol in vitro on the demethylation of DMN and the hydroxylation of 2-AAF by hepatic microsomes*

Addition of ethanol to rat liver microsomes strongly inhibited the demethylation of DMN, assayed at 0.7 and 5.0 mM (Table 2). This result was in line with findings of other investigators who reported that ethanol is a potent inhibitor of the demethylation of DMN and other nitrosamines *in vitro* [18, 34]. The extent of inhibition in the present study (70–90%) was significantly greater than that observed previously (40–80%) [18, 34]. The difference is likely due to the higher concentrations of ethanol used in the current study. Use of high concentrations of ethanol was mandatory because the purpose of this investigation was to assess the action of ethanol *in vitro* on the microsomal activation of DMN and 2-AAF under comparable conditions. Since solubilization of 2-AAF in aqueous media required

Table 1. Food and ethanol consumption of pair-fed rats*

| Expt. | Rats | Starting weight (g) | Average intake | | Average weight gain | |
|---|---|---|---|---|---|---|
| | | | kcal/day | g EtOH/day | g/day | Total (%) |
| 1 | Controls | 123 ± 8 | 76.0 ± 0.8 | | 5.6 | 159 |
| | Ethanol-treated | | 74.9 ± 0.5 | 3.4 ± 0.1 | 4.3 | 121 |
| 2 | Controls | 115 ± 3 | 81.4 ± 5.8 | | 5.7 | 173 |
| | Ethanol-treated | | 79.3 ± 5.4 | 3.5 ± 0.3 | 4.3 | 131 |
| 3 | Controls | 117 ± 6 | 70.6 ± 7.9 | | 5.2 | 124 |
| | Ethanol-treated | | 68.7 ± 8.4 | 3.4 ± 0.4 | 4.3 | 103 |

* Rats (three or four animals/groups) were pair-fed a nutritionally adequate liquid diet [27] containing isocaloric amounts of ethanol and/or carbohydrate. In two cases (experiments 1 and 2), the animals were weaned for 7 days in order to condition the group receiving the ethanol-containing diet. They were then fed for a period of 28 days. In one case (experiment 3), the animals were fed for 28 days without prior weaning. Body weight of the animals was measured at the start and finish of the feeding period; diet consumption was recorded daily. The liquid diets contained 1 kcal/ml.

Table 2. Effect of the addition of ethanol on the demethylation of DMN *in vitro* by rat liver microsomes*

| DMN (mM) | Ethanol in incubation mixture | HCHO formed (pmoles/min/mg protein) | % Inhibition† |
|---|---|---|---|
| 0.7 | None | 50.3 ± 2.0 | |
| | 0.41 M | 9.0 ± 5.0 | 82 |
| | 0.85 M | 7.4 ± 3.5 | 85 |
| | 1.71 M | 4.1 ± 2.0 | 92 |
| 5.0 | None | 164.5 ± 14.1 | |
| | 0.41 M | 54.5 ± 14.1 | 67 |
| | 0.85 M | 29.5 ± 14.1 | 82 |
| | 1.71 M | ND‡ | |

* The effect of ethanol on the formation of HCHO was determined by varying the concentration of ethanol in the DMN demethylase reaction mixtures. Conditions of the reactions and analysis of metabolite formation are described in Methods. Microsomes, from rats fed Purina chow *ad lib.*, contained 0.73 nmole cyt. P-450/mg protein.

† Values indicate the percent change in metabolite formation relative to the reaction mixture containing no ethanol.

‡ ND = not determined.

minimally 0.41 M methanol, the same concentration of ethanol was adopted as limiting concentration in the analysis of the action of ethanol on DMN demethylase.

Because of the low solubility of 2-AAF in aqueous media, microsomal metabolism of 2-AAF has been investigated in media containing MeOH at a final concentration of 0.4 to 0.5 M [20, 35–38]. At these concentrations, MeOH appears to have no effect on 2-AAF hydroxylation [23, 38]. In preliminary experiments, it was noted that ethanol appeared to have differential effects on N- and 7-hydroxylation of 2-AAF. At saturating substrate concentrations N-hydroxylation was not affected while formation of 7-OH-2-AAF was inhibited significantly. These observations led us to examine the effect of ethanol within a range of substrate concentrations at which 2-AAF is completely soluble (2–70 $\mu$M). Because of the insolubility of 2-AAF in the medium at concentrations exceeding 70 $\mu$M, this concentration was the upper limit in all experiments. Under these conditions the effect of ethanol on hydroxylation of 2-AAF was a function of ethanol as well as substrate concentration (Table 3). At 1.67 $\mu$M 2-AAF, increasing ethanol concentration stimulated N-hydroxylation but inhibited 7-hydroxylation. At 66.67 $\mu$M 2-AAF, N-hydroxylation was independent of ethanol concentration while 7-hydroxylation was inhibited with increasing ethanol concentrations. At both substrate concentrations the formation of *N*-OH-2-AAF relative to that of 7-OH-2-AAF increased with increasing ethanol concentration. These results suggested that ethanol shifts the microsomal hydroxylation of 2-AAF in favor of N-hydroxylation and thus may promote activation of 2-AAF. The differential action of ethanol on activation of DMN and 2-AAF *in vitro* prompted us to compare the effect of chronic ethanol consumption on the microsomal metabolism of the two carcinogens.

Table 3. Effect of the addition of ethanol on the hydroxylation of 2-AAF *in vitro* by rat liver microsomes*

| 2-AAF ($\mu$M) | Ethanol in incubation mixture | *N*-OH-2-AAF formed† | % Change‡ | 7-OH-2-AAF formed | % Change | *N*-OH-2-AAF/7-OH-2-AAF§ |
|---|---|---|---|---|---|---|
| 1.67 | 0.41 M | 0.23 ± 0.01 | | 0.11 ± 0.00 | | 2.09 |
| | 0.85 M | 0.31 ± 0.02 | 35 | 0.08 ± 0.01 | −27 | 3.88 |
| | 1.71 M | 0.28 ± 0.01 | 22 | 0.08 ± 0.00 | −27 | 3.50 |
| 66.67 | 0.41 M | 0.46 ± 0.12 | | 1.37 ± 0.00 | | 0.34 |
| | 0.85 M | 0.49 ± 0.06 | NC¶ | 0.73 ± 0.06 | −46 | 0.67 |
| | 1.71 M | 0.44 ± 0.00 | NC | 0.29 ± 0.05 | −78 | 1.52 |

* The effect of ethanol on the formation of *N*-OH-2-AAF and 7-OH-2-AAF was determined by varying the concentration of ethanol in the reaction mixture. Analysis of metabolite formation is described in Methods.

† Expressed in nmoles/12 min/nmole cyt. P-450.

‡ Values indicate the percent change in metabolite formation relative to the lowest ethanol concentration (0.41 M).

§ Ratio of nmoles of metabolites formed at a given ethanol concentration.

¶ NC = No change; differences are not statistically significant ($P > 0.05$).

Table 4. Effect of chronic ethanol consumption by the rat on oxidative demethylation of DMN by hepatic microsomes*

| DMN (mM) | HCHO formed (nmoles/min/mg protein) | | % | HCHO formed (nmoles/min/nmole cyt. P-450) | | % |
|---|---|---|---|---|---|---|
| | Controls† | Ethanol‡ | Increase§ | Controls | Ethanol | Increase§ |
| 0.70 | 0.28 ± 0.03 | 0.60 ± 0.05 | 114 | 0.21 ± 0.02 | 0.49 ± 0.04 | 133 |
| 1.00 | 0.40 ± 0.03 | 0.77 ± 0.01 | 93 | 0.30 ± 0.02 | 0.62 ± 0.01 | 107 |
| 1.67 | 0.49 ± 0.01 | 0.97 ± 0.00 | 98 | 0.37 ± 0.01 | 0.76 ± 0.00 | 105 |
| 2.50 | 0.62 ± 0.05 | 1.18 ± 0.01 | 90 | 0.46 ± 0.04 | 0.96 ± 0.01 | 109 |
| 5.00 | 0.73 ± 0.02 | 1.26 ± 0.07 | 73 | 0.54 ± 0.01 | 1.02 ± 0.06 | 89 |

* Rats (three animals/group) were pair-fed a nutritionally adequate liquid diet [27], containing isocaloric amounts of ethanol or carbohydrate, for 28 days. The microsomal fraction was isolated from the pooled livers of each group and was used as an enzyme source. Microsomes contained 1.34 and 1.23 nmoles cyt. P-450/mg protein for untreated and ethanol-treated rats respectively. The formation of [$^{14}$C]formaldehyde from di-[$^{14}$C]methylnitrosamine was measured following a 25-min incubation at 37°, and resultant values were related to (a) protein and (b) cyt. P-450 concentrations in the reaction mixtures. Each value is the average of duplicate determinations, ± S.D.

† Microsomes for these determinations were obtained from the livers of rats maintained on the control liquid diet.

‡ Microsomes for these determinations were obtained from the livers of rats maintained on the liquid diet containing ethanol.

§ Change in activity due to ethanol effect.

*Metabolic activation of DMN and 2-AAF by hepatic microsomes from control and ethanol-treated rats*

*Demethylation of DMN.* The results of these experiments (Table 4) indicated a significant increase in DMN demethylase activity of hepatic microsomes from ethanol-treated rats. Double-reciprocal plots (not shown) of the data gave the same kinetic constants whether velocities were expressed in terms of protein or cytochrome P-450 concentration. There was a marked increase in the maximal velocity ($V_{max}$) of the demethylation reaction by hepatic microsomes of ethanol-treated rats, confirming an earlier report [5]. Apparent differences in the $K_m$ values of the microsomal demethylation by controls (1.5 ± 0.2 μM) and of ethanol-treated rats (1.0 ± 0.2 μM) were not statistically significant.

*Hydroxylation of 2-AAF.* Since chronic ethanol consumption by the rat results in the induction of a large number of cytochrome P-450-dependent drug-metabolizing enzymes and since addition of ethanol to reaction mixtures *in vitro* had a differential effect on N- and 7-hydroxylation of 2-AAF, we investigated whether chronic ethanol consumption by the rat affects microsomal N-hydroxylation of 2-AAF and/or the balance of activation to detoxication (N-hydroxylation/C-hydroxylation). Formation of 7-OH-2-AAF was used as a measure of C-hydroxylation since preliminary experiments showed that 7-OH-2-AAF represented 70–85% of the major phenolic metabolites formed by hepatic microsomes from ethanol-treated or control rats within the substrate concentration-range tested. This observation has also been made by other workers [23, 35, 39, 40]. Only minor random changes in the rate of N-hydroxylation of 2-AAF were observed. In one experiment (Table 5, Expt. 1), there was a small increase at the highest substrate concentration tested; in a second experiment (Table 5, Expt. 2), a minimal increase was observed at the lowest substrate concentration. In experiment 1, formation of 7-OH-2-AAF was elevated slightly at all substrate concentrations tested. There appeared to be a uniformly slight increase in the rate of 7-hydroxylation of 2-AAF in experiment 2; however, the changes were not statistically significant. In no case was the increase in activity as marked as that of the hepatic DMN demethylase following chronic ethanol consumption. N- and 7-hydroxylation by microsomes from untreated and ethanol-treated rats were inhibited by 30–50% in an atmosphere of $CO:O_2$ (90:10), indicating that hydroxylation of 2-AAF in the liver of untreated as well as ethanol-treated rats is catalyzed by cytochrome P-450. The difference in the effect of ethanol consumption on N- and 7-hydroxylation of 2-AAF supports the view that these reactions are catalyzed by separate isozymes of cytochrome P-450 [26]. The data of Table 5 show also that the ratio of *N*-OH-2-AAF to 7-OH-2-AAF formed by microsomes of ethanol-treated and control rats increased as the substrate concentration was lowered (Table 5, columns 8 and 9), an observation in agreement with the findings of McManus *et al.* [40]. In a separate experiment, an increase in this ratio was also observed (Table 6) at substrate concentrations 10- and 100-fold lower than the lowest concentration shown in Table 5. However, ethanol consumption did not shift the ratio in favor of the activation reaction (Table 5, columns 8 or 9). Both experiments led us to conclude that chronic ethanol consumption does not affect microsomal N-hydroxylation of 2-AAF. Formation of 7-OH-2-AAF, if affected at all, appears to be stimulated to a minor extent. Most importantly, the balance between activation and detoxication of the hepatocarcinogen 2-AAF appears not to be affected by chronic ethanol consumption.

This conclusion was substantiated by double-reciprocal plots of the data of Table 5, which demonstrated similar kinetics for N- and 7-hydroxylation of 2-AAF in microsomes from both control and ethanol-treated rats. In addition, the plots suggested that more than one enzyme system is involved in N- as well as C-hydroxylation. Analysis of the kinetics of formation of *N*- and 7-OH-2-AAF by hepatic microsomes from preliminary experiments also gave consistent evidence for the nonlinearity of the kinetics of N- and 7-hydroxylation of 2-AAF. This finding was

Table 5. Effect of chronic ethanol consumption by the rat on 2-AAF hydroxylation by hepatic microsomes*

| 2-AAF ($\mu$M) | *N*-OH-2-AAF formed | | % Change§ | 7-OH-2-AAF formed | | % Change | *N*-OH-2-AAF/7-OH-2-AAF | |
|---|---|---|---|---|---|---|---|---|
| | Controls† | Ethanol‡ | | Controls | Ethanol | | Controls | Ethanol |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Expt. 1 | | | | | | | | |
| 1.67 | 0.12 ± 0.01 | 0.12 ± 0.01 | NC‖ | 0.12 ± 0.01 | 0.16 ± 0.02 | 33 | 1.00 | 0.75 |
| 3.33 | 0.12 ± 0.00 | 0.13 ± 0.01 | NC | 0.21 ± 0.01 | 0.31 ± 0.01 | 48 | 0.62 | 0.42 |
| 13.33 | 0.16 ± 0.03 | 0.16 ± 0.00 | NC | 0.61 ± 0.01 | 0.82 ± 0.12 | 34 | 0.26 | 0.20 |
| 33.33 | 0.21 ± 0.02 | 0.23 ± 0.03 | NC | 1.29 ± 0.04 | 1.72 ± 0.08 | 33 | 0.16 | 0.13 |
| 66.67 | 0.29 ± 0.03 | 0.38 ± 0.01 | 31 | 1.77 ± 0.07 | 2.38 ± 0.19 | 34 | 0.16 | 0.16 |
| Expt. 2 | | | | | | | | |
| 1.67 | 0.08 ± 0.01 | 0.09 ± 0.00 | 13 | 0.09 ± 0.00 | 0.10 ± 0.01 | NC | 0.89 | 0.90 |
| 3.33 | 0.10 ± 0.02 | 0.11 ± 0.01 | NC | 0.15 ± 0.01 | 0.16 ± 0.01 | NC | 0.67 | 0.69 |
| 13.33 | 0.17 ± 0.02 | 0.17 ± 0.02 | NC | 0.42 ± 0.03 | 0.48 ± 0.06 | NC | 0.40 | 0.35 |
| 33.33 | 0.30 ± 0.08 | 0.32 ± 0.10 | NC | 0.92 ± 0.10 | 1.08 ± 0.15 | NC | 0.33 | 0.30 |
| 66.67 | 0.47 ± 0.05 | 0.49 ± 0.06 | NC | 1.40 ± 0.15 | 1.60 ± 0.29 | NC | 0.29 | 0.31 |

* Rats (four animals/group) were pair-fed a nutritionally adequate liquid diet [27], containing isocaloric amounts of ethanol and/or carbohydrate, for 28 days, following a 7-day period of weaning. The microsomes were isolated from the pooled livers of each group and used as enzyme source. The formation of *N*-OH-2-AAF and 7-OH-2-AAF, determined by HPLC and calculated by the method of inverse isotope dilution, is expressed as nmoles/12 min/nmole cyt. P-450. Each specified 2-AAF concentration was assayed in triplicate; the values shown are the averages of these determinations, ± S.D.

† Microsomes for these determinations were obtained from the livers of rats maintained on the control liquid diet.

‡ Microsomes for these determinations were obtained from the livers of rats maintained on the liquid diet containing ethanol.

§ Change in activity due to ethanol; changes shown are statistically significant ($P < 0.05$).

‖ NC = no change; differences are not statistically significant ($P > 0.05$).

Table 6. Dependence of the ratio of *N*-OH-2-AAF to 7-OH-2-AAF formation on 2-AAF concentration*

| 2-AAF (μM) | *N*-OH-2-AAF/7-OH-2-AAF determined at | |
|---|---|---|
| | 0.3 nmole cyt. P-450 | 1.0 nmole cyt. P-450 |
| 1.67 | 0.9 | 0.9 |
| 0.17 | 2.9 | 2.7 |
| 0.02 | 4.9 | 4.2 |

* Microsomes isolated from the homogenate of pooled livers from five rats maintained on Purina chow and water were used as a source of 2-AAF hydroxylation enzymes. The reaction mixtures were incubated for 10 min under conditions outlined in Methods. The amounts of *N*-OH-2-AAF and 7-OH-2-AAF produced were determined as described in Methods.

independent of whether the rats had been fed Purina chow *ad lib.* or were maintained on the liquid diets described under Methods. These observations were confirmed by a detailed analysis of the kinetics of the hydroxylation reactions described below.

*Measurements of the kinetics of N- and C-hydroxylation of 2-AAF*

The kinetics of the formation of *N*-OH-2-AAF and 7-, 5-, 3- and 1-OH-2-AAF from 2-[$^3$H]AAF was analyzed at substrate concentrations ranging from 2 to 70 μM. Since the radioactivity associated with the eluted 1-OH-2-AAF was in all instances less than twice the background radioactivity, apparent kinetic constants for the formation of this metabolite were not calculated. Double-reciprocal plots (Fig. 1) indicated that the formation of N-, 7-, 5- and 3-OH-2-AAF followed biphasic kinetics, verifying the initial observations. Eadie–Scatchard plots, which give a better indication than double-reciprocal plots that more than one enzyme is present [41], confirmed the biphasic kinetics of the 7-hydroxylation of 2-AAF (Fig. 1b). N- as well as C-hydroxylation of 2-AAF appears to be catalyzed by enzymes with high and low substrate affinities. In agreement with a recent report [40], the data showed that 7-OH-2-AAF is the major product (80–90%) of the microsomal C-hydroxylation of 2-AAF followed, in descending order, by 5- and 3-OH-2-AAF. It was noted above

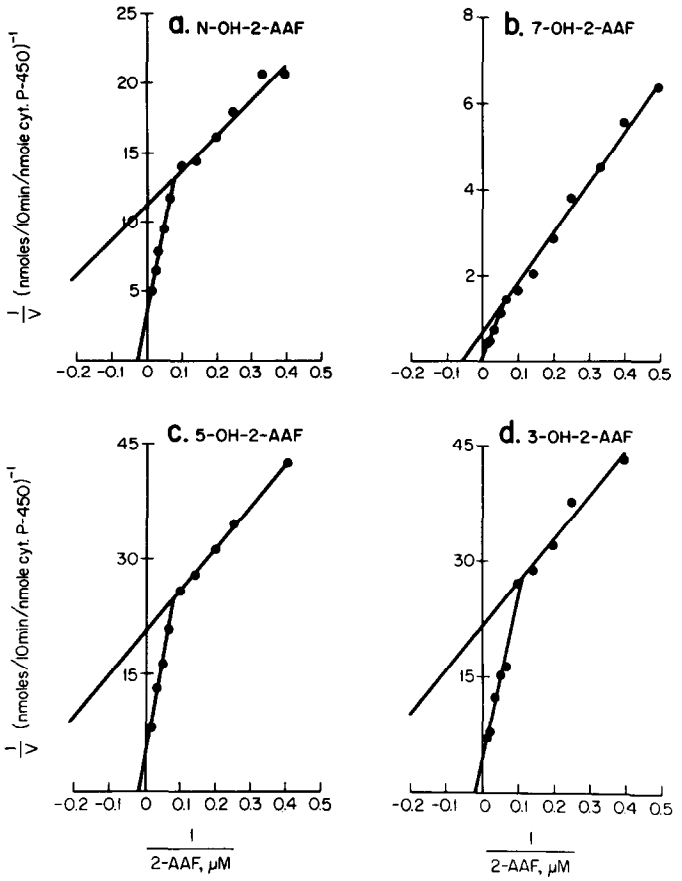


Fig. 1. Double-reciprocal plots of N- and C-hydroxylation of 2-AAF by microsomes from untreated rats, at 2–70 μM 2-AAF. The reaction mixtures and assays are described under Methods. (a) N-hydroxylation; (b) 7-hydroxylation; (c) 5-hydroxylation; and (d) 3-hydroxylation. The following values were obtained for apparent $K_m$ (μM) and $V_{max}$ (nmoles/10 min/nmole cyt. P-450): $K_m$ (N-hydroxylation) = 2.26 ± 0.32, 35.01 ± 7.80, $V_{max}$ (N-hydroxylation) = 0.09 ± 0.00, 0.28 ± 0.03; $K_m$ (7-hydroxylation) = 16.70 ± 3.54, 100.01 ± 29.91, $V_{max}$ (7-hydroxylation) = 1.52 ± 0.21, 5.69 ± 1.15; $K_m$ (5-hydroxylation) = 2.81 ± 0.13, 92.75 ± 36.62, $V_{max}$ (5-hydroxylation) = 0.05 ± 0.00, 0.34 ± 0.10; $K_m$ (3-hydroxylation) = 2.80 ± 0.44, 55.33 ± 12.05, $V_{max}$ (3-hydroxylation) = 0.05 ± 0.00, 0.25 ± 0.03.

that the ratio of the formation of *N*-OH-2-AAF to that of 7-OH-2-AAF increased as the substrate concentration was lowered. A similar shift in the balance of activation to detoxication was observed when the ratio of *N*-OH-2-AAF to total *C*-OH-2-AAF was calculated. Thus, the values of *N*-OH-2-AAF/(7- + 5- + 3-OH-2-AAF) at 50, 20, 5 and 2 $\mu$M 2-AAF were 0.07, 0.10, 0.15 and 0.25 respectively.

It has been reported that only a single enzyme is involved in the microsomal N-hydroxylation of 2-AAF [40]. The concentrations of 2-AAF used in these experiments (~0.02–~0.6 $\mu$M) were considerably lower than those employed in the present study (2–70 $\mu$M). Accordingly, we investigated the kinetics of the formation of *N*-OH-2-AAF at substrate concentrations from 0.01 to 1.67 $\mu$M, which covers the same range as that of the previous study [40]. Even under these conditions, N-hydroxylation of 2-AAF did not appear to follow Michaelis–Menten kinetics (Fig. 2). The biphasic kinetics of the N-hydroxylation reaction was confirmed with the use of Eadie–Scatchard plots ($V/S$ vs. $V$). The values of the apparent kinetic constants ($K_m = 0.063$ $\mu$M, $V_{max} = 4.30$ pmoles/min/nmole cyt. P-450) for the monophasic portion of the kinetic curve obtained at the five lowest substrate concentrations (Fig. 2) were in relatively close agreement with those reported for the single N-hydroxylase of 2-AAF ($K_m = 0.033$ $\mu$M, $V_{max}$ 3.63 pmoles/min/mg protein) [40]. However, our data suggest that multiple enzymes are involved in N-hydroxylation, as well as C-hydroxylation, of the carcinogen. The values of the apparent kinetic constants obtained at the two substrate concentration ranges are listed in the appropriate figure legends.

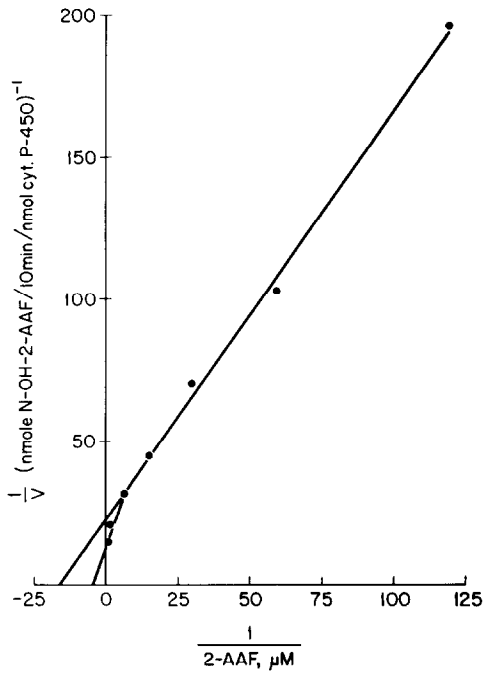


Fig. 2. Double-reciprocal plot of N-hydroxylation of 2-AAF by microsomes from untreated rats. The substrate range was 0.01 to 1.67 $\mu$M 2-AAF. The reaction mixtures and assays are described under Methods. The following values were obtained for the apparent kinetic constants: $K_m$ ($\mu$M) = 0.063 ± 0.005, 0.211 ± 0.039; $V_{max}$ (nmole/10 min/nmole cyt. P-450) = 0.043 ± 0.002, 0.075 ± 0.004.

*Determination of binding spectra of 2-AAF*

Addition of 2-AAF to hepatic microsomes from control or ethanol-fed rats gave characteristic type II [42] binding spectra ($\lambda_{max}$ 423–426 nm; $\lambda_{min}$ 394–397 nm). Similarly, the rate of increase in $\Delta A$ as a function of substrate concentration was the same whether hepatic microsomes were obtained from control or ethanol-treated rats (Fig. 3). The apparent $K_s$ values calculated from these data were identical (56.5 and 59.3 $\mu$M, respectively) within experimental error. Since 2-AAF is not completely soluble at the higher concentrations tested, the apparent $K_s$ values are subject to an indeterminate error. Nevertheless, the spectral data of Fig. 3 indicate that ethanol consumption did not induce a new cytochrome P-450 specific for hydroxylation of 2-AAF.

*Activation of DMN and 2-AAF to mutagens using microsomes from control and ethanol-treated rats*

These experiments compared the capacity of hepatic microsomes from untreated and ethanol-treated rats to activate DMN and 2-AAF to mutagens. Consumption of ethanol by the rat enhanced the ability of hepatic microsomes to activate DMN to a mutagen (Fig. 4A). These results are in agreement with those reported [5] and correlate with the finding that DMN demethylase activity was increased in microsomes from ethanol-treated rats (Table 4).

In contrast to results with DMN, microsomes from ethanol-treated rats were slightly less efficient ($P < 0.05$) in activating 2-AAF to a mutagen than were microsomes from controls (Fig. 4B). In a second experiment, no significant differences ($P > 0.1$) between the capacity of microsomes from ethanol-

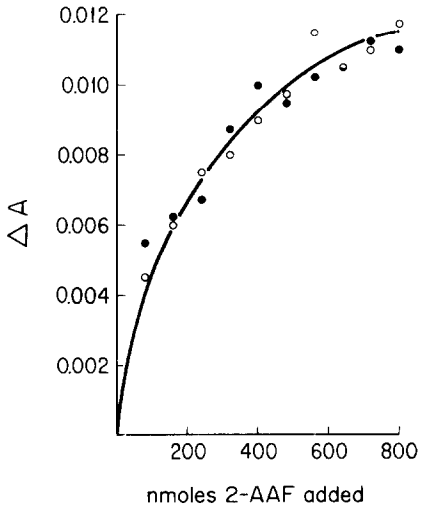


Fig. 3. Interaction of 2-AAF with hepatic microsomal cytochrome P-450 from untreated (○) and ethanol-treated (●) rats. 2-AAF, dissolved in 5 $\mu$l MeOH, was added to sample cuvettes in 80-nmole increments. Equal increments of MeOH were added to reference cuvettes. Spectra (460–370 nm) were recorded following each addition.

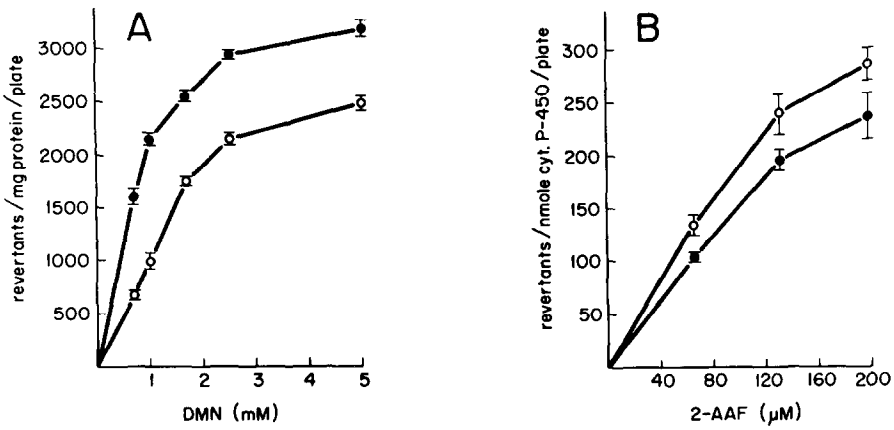


Fig. 4. Activation of DMN and 2-AAF to mutagens by hepatic microsomes from untreated (○) and ethanol-treated (●) rats. The reaction mixtures and incubation conditions are detailed under Methods. (A) activation of DMN to a mutagen; (B) activation of 2-AAF to a mutagen. Each point is the average number, ± S.D. (bars), of revertants from triplicate plates, corrected for the background number of revertants present when no test chemical was added to the reaction mixture.

treated and untreated rats to convert 2-AAF to mutagen were observed. It appears that microsomal activation of 2-AAF to a mutagen by rat liver is not enhanced by ethanol consumption.

## DISCUSSION

The demethylation of DMN and the N-hydroxylation of 2-AAF are prerequisite first steps in the activation of these compounds to carcinogenic and mutagenic derivatives. The present study confirms previous observations that ethanol consumption causes a marked increase in DMN demethylase activity as well as activation of DMN to a mutagen [5]. The ethanol-induced increase in the demethylase reaction was related to either protein or cytochrome P-450 concentrations. The latter mode of expression seems preferable since DMN demethylation is catalyzed by cytochrome P-450 [43]. Contrary to the findings of Garro *et al.* [5], we observed no increase in the amount of cytochrome P-450/mg protein concurrent with the stimulation of DMN demethylation by ethanol. However, an increase in mixed-function oxidase activity is not necessarily associated with an increased level of microsomal cytochrome P-450/mg protein [6, 8].

In contrast to the inductive effects of ethanol on microsomal DMN demethylase activity and mutagenic activation, the results of this study demonstrated that ethanol consumption by the rat does not stimulate microsomal N-hydroxylation of 2-AAF. The minor increases observed in the formation of 7-OH-2-AAF in microsomes from ethanol-treated rats with no apparent quantitative change in cytochrome P-450 may be the result of a redistribution in the relative proportions of isozymes [8]. This view is supported by data showing that a variety of compounds induce specific cytochrome P-450 isozymes and at the same time lower the levels of others [44]. Most importantly, the balance between activation and detoxication of 2-AAF was not affected by ethanol consumption, and there was no stimulatory effect on the capacity of hepatic microsomes from ethanol-treated rats to activate 2-AAF to a mutagen. We conclude that ethanol does not induce a novel species of microsomal cytochrome P-450 specific for metabolic activation of 2-AAF. This conclusion is supported by the identical spectral characteristics due to the interaction of 2-AAF with hepatic microsomes of ethanol-treated rats and of controls. If ethanol were to induce a novel microsomal cytochrome P-450 specific for N-hydroxylation, induction would likely be evident by a binding spectrum of 2-AAF demonstrably different from the type II spectrum resulting from the interaction of 2-AAF with cytochrome P-450 from control rats [45]. Moreover, binding of 2-AAF to microsomes of ethanol-treated rats would be expected to show a quantitative change. Neither of these changes was observed.

At least three different enzyme activities have been documented for the demethylation of DMN [46]. The present study provides evidence that more than one enzyme is involved in N-hydroxylation, as well as C-hydroxylation, of 2-AAF. Multiplicity of enzymes for C-hydroxylation of 2-AAF has already been reported [40]. In the same report it was concluded that N-hydroxylation is carried out by a single enzyme system. Our data indicate that 2-AAF, at a concentration range of 2–70 μM, is N-hydroxylated by at least two enzymes, with high and low affinities for 2-AAF. Investigation of the reaction at lower substrate concentrations, including the concentration range used in the previous work [40], suggested biphasic kinetics for the N-hydroxylation of 2-AAF by double-reciprocal and Eadie–Scatchard plots.

In addition to relating biphasic kinetics to multiple enzymes there are alternative interpretations [47, 48]. Accordingly, the data of this study do not prove that N-hydroxylation as well as C-hydroxylation of 2-AAF is carried out by more than one enzyme system. This does not affect the conclusions that these reactions are best described by biphasic kinetics or that ethanol consumption does not alter the kinetics of these reactions.

One observation of this study of possible biological significance is that the balance of microsomal activa-

tion and detoxication of 2-AAF is shifted as the substrate concentration is lowered. If the potency of a carcinogen depends on the balance of activated metabolites and detoxication products [49–51], our observations suggest that the hepatocarcinogenic potential of 2-AAF increases with decreasing concentration at the site of its microsomal oxidation.

In summary, the results of this study demonstrate that ethanol consumption does not have a uniform, predictable effect on the hepatic microsomal activation of xenobiotics and, in particular, on hepatocarcinogens. The effects of ethanol on demethylation of DMN and N-hydroxylation of 2-AAF *in vitro* and *in vivo* indicate that the two reactions take place on different forms of cytochrome P-450, not equally susceptible to ethanol. The differential effect may be mediated through an ethanol-induced alteration in the lipid composition and fluidity [52, 53] of the membranes with which these enzymes are associated. Since the properties of the monooxygenase system are determined by the properties of the membrane lipids and proteins [54], differential ethanol effects may be due to specific changes in the membrane microenvironment of the two enzymes.

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