Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 9223–9227, December 1987
Medical Sciences

Reactive free radical generation *in vivo* in heart and liver of ethanol-fed rats: Correlation with radical formation *in vitro*

(spin trapping/cytochrome P-450/dietary fat/lipid peroxidation/1-hydroxyethyl radicals)

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Communicated by Ralph T. Holman, August 10, 1987

ABSTRACT **Rats fed a high-fat ethanol-containing diet for 2 weeks were found to generate free radicals in liver and heart *in vivo*. The radicals are believed to be carbon-centered radicals, were detected by administering spin-trapping agents to the rats, and were characterized by electron paramagnetic resonance spectroscopy. The radicals in the liver were demonstrated to be localized in the endoplasmic reticulum. Rats fed ethanol in a low-fat diet showed significantly less free radical generation. Control animals given isocaloric diets without ethanol showed no evidence of free radicals in liver and heart. When liver microsomes prepared from rats fed the high-fat ethanol diet were incubated in a system containing ethanol, NADPH, and a spin-trapping agent, the generation of 1-hydroxyethyl radicals was observed. The latter was verified by using ^{13}C-substituted ethanol. Microsomes from animals fed the high-fat ethanol-containing diet had higher levels of cytochrome P-450 than microsomes from rats fed the low-fat ethanol-containing diet. The results suggest that the consumption of ethanol results in the production of free radicals in rat liver and heart *in vivo* that appear to initiate lipid peroxidation.**

Chronic excessive use of alcohol by humans results in liver disease (1, 2) characterized by fatty infiltration, which can lead to fibrotic degeneration and necrosis (1, 3, 4). However, the mechanisms leading to the development of alcoholic liver disease remain unclear. Lipid peroxidation was linked to ethanol consumption by Di Luzio and coworkers (5, 6), who reported that antioxidants prevented the development of fatty liver after a large acute dose of ethanol. Investigations by others have subsequently indicated that lipid peroxidation appears to occur in the livers of animals soon after the administration of an acute dose of alcohol. Much of the evidence for these claims has been based on the thiobarbituric acid assay for malondialdehyde in livers of animals treated with ethanol (7, 8), but increases in conjugated dienes and chemiluminescence as well as decreases in hepatic glutathione have also been reported (8). In addition, Müller and Sies (9) have reported an enhanced production of ethane and *n*-pentane, which are believed to be products of the peroxidative degradation of membrane lipids, during the metabolism of ethanol by perfused livers. These various findings have been interpreted as evidence that lipid peroxidation has occurred in the liver as a result of ethanol metabolism. Lipid peroxidation has also been proposed as a mechanism of ethanol-induced toxicity in the heart (10), gastric mucosa (11), and testes (12). However, the role of peroxidative events in the development of human alcohol-related disease is highly controversial.

Mechanisms that may initiate lipid peroxidation after ethanol exposure are also uncertain. Several laboratories have presented evidence that chronic ethanol feeding stimulates the production of hydroxyl radicals by liver microsomes (13, 14). If this type of radical production occurs in the hepatic endoplasmic reticulum *in vivo*, the possibility for initiating lipid peroxidation in that organelle would seem likely. In this report, we have used the spin-trapping procedure (15) to demonstrate the *in vivo* generation of carbon-centered lipid radicals in the heart and liver of rats being fed ethanol on a chronic basis. In addition, we have obtained evidence that liver microsomes generate 1-hydroxyethyl radicals during the *in vitro* metabolism of ethanol. Furthermore, the intensity of radical formation *in vivo* and *in vitro* was significantly greater in rats fed ethanol with a diet rich in fat. The results indicate that lipid peroxidation may be a continuing event in the heart and liver during and after ethanol intake and that dietary factors enhance the free radical generation in the liver by facilitating the induction of a form of cytochrome P-450 by ethanol consumption (16).

MATERIALS AND METHODS

α-Phenyl-*N*-*t*-butylnitrone (PBN) was obtained from Eastman Organic Chemicals. α-2,4,6-Trimethoxyphenyl-*N*-*t*-butylnitrone [$(MeO)_3$PBN] was synthesized in our laboratory as described by DuBose (17). ^{13}C-substituted ethanol was purchased from MSD Isotopes (Dorval, Quebec, Canada). All solvents and chemicals used were of reagent grade or best available quality.

Female Sprague–Dawley rats, weighing 140–150 g, were obtained from Sasco (Omaha, NE) and were normally housed in groups of three rats per cage. Ethanol-fed rats were offered liquid diets containing ethanol as 36% of total calories, and fat as either 35% (high-fat) or 12% (low-fat) of calories. The diets were offered at 4:00 p.m. daily, so that fresh diet was available to the rats during their nocturnal feeding periods. Groups of control rats were offered a volume of an isocaloric control diet equal to the volume that the corresponding group of ethanol-fed rats had consumed during the preceding day. In the control diet, dextrin/maltose was isocalorically substituted for ethanol. All diets were prepared essentially as described by Lieber and DeCarli (18), except that the 1977 American Institute of Nutrition recommendations for the amounts of vitamins and minerals (19) were followed. The diets were fed for at least 2 weeks before the experimental procedures were carried out.

In the *in vivo* experiments in which free radicals were trapped in the tissues of intact animals, rats from each dietary group were intraperitoneally injected with 1.0 ml of a 0.05 M solution of the spin-trapping agent [either $(MeO)_3$PBN or PBN]. Thirty minutes after administration of the spin trap, the animals were decapitated and the various organs were

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Abbreviations: PBN, α-phenyl-*N*-*t*-butylnitrone; $(MeO)_3$PBN, α-2,4,6-trimethoxyphenyl-*N*-*t*-butylnitrone; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

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removed, weighed, and homogenized in 15 vol of chloroform/methanol (2:1, vol/vol). One-fifth volume of 0.9% NaCl was added to bring about phase separation, and the chloroform layer was recovered and evaporated to dryness. Samples (0.5 ml) of the lipid extracts were placed in quartz tubes (2.8 mm diameter), deoxygenated with N_2, and assayed in either an IBM ER-300 or a Varian E-9 EPR spectrometer equipped with an X-band microwave bridge. The spectrometer settings are given in the figure legends.

The intensity of the EPR signals of the spin adducts obtained from the various experiments can be compared by dividing the height of the signal by the number of grams of tissue extracted. The signal intensity is expressed as signal height in millimeters per gram of liver tissue or per milligram of microsomal protein.

Localization of the source of the EPR signal in the livers of ethanol-treated rats was determined by fractionation of the subcellular components before extraction of the lipids. The fractionation was performed by differential centrifugation in 0.25 M sucrose.

Hepatic microsomes were prepared as described (20) and were resuspended in 0.15 M potassium phosphate buffer, pH 7.4, so that 0.1 ml of the suspension contained 1.0 mg of microsomal protein. The reaction systems contained 1.0–2.0 mg of microsomal protein, 0.1 M PBN, 50 μl of absolute ethanol, and 0.1 ml of an NADPH-generating system providing final concentrations of 5 mM glucose 6-phosphate, 0.3 mM NADP, and glucose-6-phosphate dehydrogenase at 0.5 Kornberg unit/ml. The volume was made up to 1.0 ml with the phosphate buffer and incubated for 30 min at 37°C. In experiments with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as the spin-trapping agent, all conditions were the same as for the PBN *in vitro* studies except that 0.13 M DMPO was substituted for PBN. At the end of the incubation period the reaction systems were extracted with benzene or chloroform/methanol (2:1), followed by deoxygenation and concentration of the extract. The concentrated samples were transferred into quartz tubes (2.8 mm) and assayed in an EPR spectrometer. Microsomal protein was measured by the method of Lowry *et al.* (21), and the microsomal cytochrome P-450 content was determined as described by Omura and Sato (22).

RESULTS

In Vivo Experiments with $(MeO)_3PBN$

Effect of Dietary Fat. In these experiments, rats that had been fed the different diet regimens for 2 weeks were given the spin trap $(MeO)_3PBN$ intraperitoneally 30 min prior to removal of the liver for extraction and assays. In Fig. 1*A*, the EPR spectrum observed is typical of those observed in liver lipid extracts of rats that had been fed the high-fat diet containing ethanol. The nitrogen and β-hydrogen splitting constants of the signal (a_N = 15.0 G, a_β^H = 2.0 G) are consistent with those of $(MeO)_3PBN$ spin adducts of carbon-centered radicals that have undergone ortho demethylation and are similar to the adducts observed during the hepatic metabolism of carbon tetrachloride (20), which is known to initiate lipid peroxidation. Liver lipid extracts of rats that were fed the control isocaloric high-fat diet containing no ethanol showed no evidence of radical adducts (Fig. 1*B*). An additional control was performed to determine if the radical adducts might have been formed during the extraction process and, hence, might be artifacts: the livers of ethanol-fed animals were homogenized in the chloroform/methanol solvent containing 0.05 M $(MeO)_3PBN$, followed by recovery of the total extracted lipids, which were then scanned in the EPR spectrometer. None of these control extractions of livers from ethanol-fed animals exhibited an EPR signal,

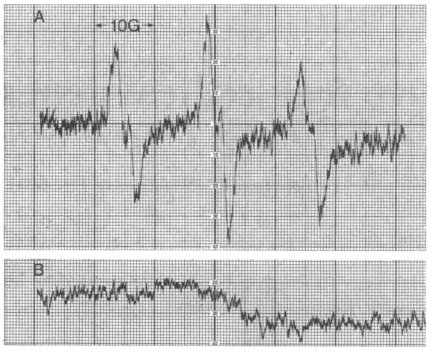

FIG. 1. (*A*) EPR signal of a lipid extract of liver from a rat fed the ethanol-containing high-fat diet for 2 weeks and given $(MeO)_3PBN$ 30 min before sample preparation (a_N = 15.0 G and a_β^H = 2.0 G; 1 G = 0.1 mT). The spectrometer settings were as follows: microwave power, 25 mW; modulation amplitude, 2.5 G; gain, 8×10^4; time constant, 3 sec; scan range, 100 G; scan time, 30 min; temperature, 25°C. (*B*) Same as above except that the rat was given the ethanol-free high-fat control diet.

demonstrating that the radicals observed in the liver extracts of ethanol-fed animals given the spin trap intraperitoneally must have been generated and trapped *in vivo*. Examination of brain, spleen, and kidney extracts of the ethanol-fed rats given $(MeO)_3PBN$ showed no evidence of free radical formation in those organs (data not shown).

Rats fed the low-fat diet containing an equivalent amount of ethanol also showed the same type of carbon-centered radical generation (Fig. 2*A*), but the intensity of the signal was always considerably less than that observed in animals fed the high-fat diet containing ethanol. The control animals that received no ethanol showed no evidence of an EPR signal (Fig. 2*B*).

Table 1 shows the relative intensities of lipid radical generation in the livers of animals fed the different diets. The intensity of radical generation in the livers of animals given ethanol in the low-fat diet was consistently less than half of that observed in animals fed ethanol in the high-fat diet. These results indicated that a higher level of fat consumption *per se* appears to augment free radical formation in the liver, since the caloric densities of the high- and the low-fat diets were the same (18). It should be noted that a comparable intake of ethanol (14–16 g/kg body weight per day) was maintained in the feeding of both the high-fat and low-fat diets containing ethanol.

Intracellular Site of the Radical Generation. In our earlier spin-trapping experiments with animals exposed to carbon tetrachloride or subjected to high-energy radiation it was possible to determine the intracellular site where the radicals were spin-trapped in membranous organelles (20, 23). This

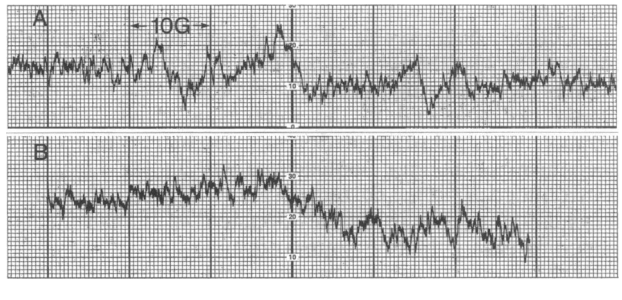

FIG. 2. (*A*) EPR signal of a lipid extract of liver from a rat fed the ethanol-containing low-fat diet for 2 weeks and given $(MeO)_3PBN$ 30 min before sample preparation. (*B*) Same as above except that the rat was given no ethanol in the low-fat diet. Spectrometer settings as in Fig. 1 except modulation amplitude was 2.0 G; gain, 6.3×10^4; and scan time, 16 min.

Table 1. Effect of high- and low-fat diets on intensity of carbon-centered lipid radicals generated in liver

Diet	EPR signal intensity, mm peak height/g liver
High-fat + ethanol	14.3 ± 3.5
High-fat control (no ethanol)	0.0
Low-fat + ethanol	6.3 ± 2.2
Low-fat control (no ethanol)	0.0

Results are mean ± SD of three experiments.

was accomplished by subcellular fractionation of the liver immediately after removal from the animal and then extraction of the total lipids from each fraction. A similar set of experiments was performed with ethanol-fed rats. Rats were fed the high-fat diet containing ethanol for 2 weeks and then were given the spin-trapping agent $(MeO)_3PBN$. After 30 min, the livers were removed, homogenized, and fractionated by differential centrifugation in 0.25 M sucrose. Each subcellular fraction was then extracted with chloroform/methanol and the total lipids were recovered. EPR scans of each of the lipid extracts were made and it was found that all of the radical adduct signal was in the microsomal fraction (data not shown). Neither the mitochondrial, the plasma membrane/nuclear, nor the cytosolic fractions had a detectable EPR signal.

In Vitro Experiments

Generation of 1-Hydroxyethyl Radicals from Ethanol by Liver Microsomes. Because the carbon-centered free radical spin adduct signal was detected only in the microsomal fraction of the liver, spin-trapping studies were conducted with this subcellular fraction to investigate the mechanism leading to the generation of these radicals. When rat liver microsomes were incubated with PBN, ethanol, and an NADPH-generating system, an EPR spectrum characteristic of the 1-hydroxyethyl radical was obtained (Fig. 3*A*). The adduct was not observed when any one of the components was omitted from the system. An identical radical adduct was obtained when ethanol was subjected to UV radiation in the presence of PBN and the photosensitizer benzophenone (Fig. 4*A*) (24). The benzophenone triplet produced in this system

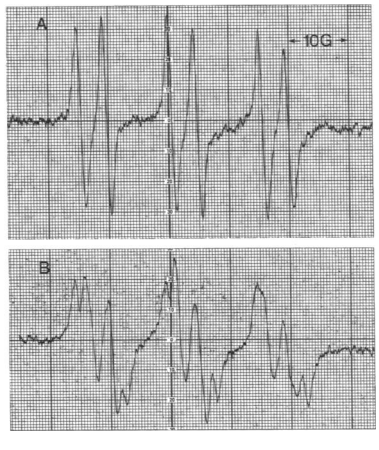

FIG. 3. (*A*) The 1-hydroxyethyl radical adduct signal observed in a hepatic microsomal system metabolizing ethanol in the presence of PBN. Incubation system composition and conditions are described in *Materials and Methods* (a_N = 15.1 G and a_β^H = 4.2 G in benzene). The microsomes were from rats fed the high-fat ethanol-containing diet for 2 weeks. (*B*) Same as above except that (1-^{13}C)ethanol (99 atom % ^{13}C) was the substrate. Spectrometer settings were as in Fig. 2 except the gain was 2.5×10^4.

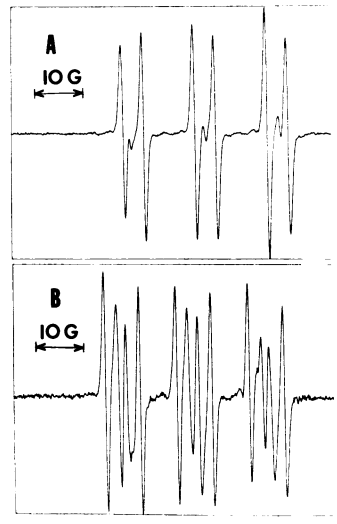

FIG. 4. (*A*) EPR signal of the 1-hydroxyethyl radical adduct of PBN formed by the photochemical action of UV light and benzophenone on ethanol (24) in benzene (a_N = 15.08 G and a_β^H = 4.37 G). An additional weak signal is due to a small amount of di-*t*-butyl nitroxide. (*B*) The 1-hydroxy(1-^{13}C)ethyl radical adduct of PBN formed by the same reaction using (1-^{13}C)ethanol (a_N = 15.08 G, a_β^H = 4.47 G, and $a_{^{13}C}$ = 3.0 G).

abstracts a hydrogen atom from the carbon α to the hydroxyl group in ethanol to produce the 1-hydroxyethyl radical. Further confirmation of the formation of the 1-hydroxyethyl adduct was obtained by using (1-^{13}C)ethanol as a substrate for both the microsomal system and the benzophenone reaction. Both spectra possess the expected 12 lines that are the result of additional splittings due to the nuclear spin of the ^{13}C atom in the 1-hydroxyethyl radical (Figs. 3*B* and 4*B*, respectively), and confirm the assignment of the 1-hydroxyethyl radical to the signal in both the biological and chemical systems. The slight discrepancy between the values of the coupling constants for the 1-hydroxyethyl PBN adducts in Figs. 3*A* and 4*A* is likely due to a difference in the polarity of the solvent systems because of the abundance of microsomal lipids in the sample giving the spectrum shown in Fig. 3*A*.

The effects of ethanol feeding on the formation of 1-hydroxyethyl radicals by liver microsomes are demonstrated in Table 2. Ethanol feeding consistently increased the intensity of radical generation, particularly when the ethanol was fed in combination with a high-fat diet. This may reflect a greater induction of cytochrome P-450 in the high-fat + ethanol-fed animals (0.66 nmol/mg of protein) as compared to the low-fat + ethanol-fed animals (0.56 nmol/mg of protein). Most of this increase may be due to a form of the cytochrome that metabolizes ethanol (16).

Hydroxyl radicals form persistent adducts with DMPO and

Table 2. Correlation between intensity of the 1-hydroxyethyl PBN adduct signal and microsomal cytochrome P-450 content

Diet	EPR signal intensity, mm peak height/mg microsomal protein	Cytochrome P-450 content, nmol/mg microsomal protein
Low-fat + ethanol	7.3 ± 3.3*	0.56 ± 0.06*
Low-fat control	5.0 ± 2.4	0.41 ± 0.03
High-fat + ethanol	21.8 ± 11.5*	0.66 ± 0.04*
High-fat control	4.9 ± 2.7	0.42 ± 0.04

Liver microsomes were prepared from rats fed either the high- or low-fat diet, with or without ethanol supplementation, for 2 weeks. Values are mean ± SD. The difference in the signal intensity ratio, ethanol-fed/control, was significant between the low-fat and the high-fat dietary groups ($P < 0.01$).
*The difference between these values is significant ($P < 0.02$).

have a characteristic EPR spectrum. Therefore, this spin trap was utilized to test for ·OH formation in a microsomal system containing an NADPH-generating system. No EPR signal was observed in the absence of alcohol. However, when ^{13}C-substituted ethanol was added to this microsomal system, the 12-line spectrum of the 1-hydroxyethyl radical was observed (data not shown), confirming the results obtained with PBN and providing further confirmation that this radical is produced when ethanol is incubated with the microsomal system.

***In Vivo* Experiments with PBN: Demonstration of Ethanol-Dependent Free Radical Formation in the Heart and Liver.** As described above for the *in vitro* experiments with microsomes, the 1-hydroxyethyl radical was trapped by using PBN. We had shown earlier that this spin-trapping agent penetrated into the various organs of the intact rat (23). Therefore, a series of experiments was done in which PBN was administered *in vivo* to rats that had been fed the high-fat ethanol-containing diet to determine if the 1-hydroxyethyl radical could be trapped and detected in organs of the intact animal. PBN, together with an acute dose of ^{13}C-substituted ethanol (5.0 g/kg body weight), was administered intraperitoneally. The acute dose was given to raise the tissue concentration of ethanol and enhance radical production during the period in which trapping would take place. In this case the ^{13}C-substituted ethanol was used to establish whether any carbon-centered radicals that might be trapped were 1-hydroxyethyl radicals. After 30 min, the different organs of these rats were extracted and examined by EPR spectroscopy. The results demonstrated that carbon-centered lipid free radicals are generated in the hearts and livers of the ethanol-fed rats given the acute dose of ^{13}C-substituted ethanol but are not seen in control animals (rats fed the high-fat diet and not given ethanol) (Fig. 5). The 6-line spectra observed demonstrate that the free radical species was not the 1-hydroxyethyl radical adduct (the latter would have shown a 12-line spectrum). The radical adducts that were observed (Fig. 5) are most likely carbon-centered radical adducts of membrane lipids. These spectral scans also provided indications that a small contribution due to a second radical (possibly the 1-hydroxyethyl radical) might be present, but no assignment for this minor component of the signal can be made at this time.

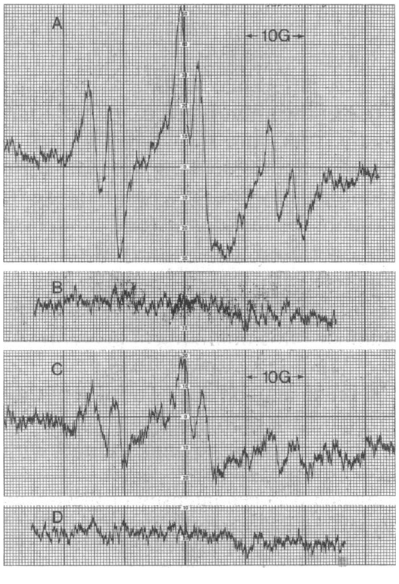

FIG. 5. EPR signals of PBN radical adducts formed in the heart and liver *in vivo*. A rat had been fed the high-fat ethanol-containing diet, following which an acute dose of ^{13}C-substituted ethanol (5.0 g/kg of body weight) was given along with PBN 30 min before preparation of heart and liver for extraction of lipids. Control rats were fed the high-fat diet but were not fed ethanol or given an acute dose of ethanol at the time of PBN administration. (*A*) EPR signal observed in heart extract of an ethanol-treated rat. (*B*) EPR signal in heart extract of a rat not given ethanol. (*C*) EPR signal observed in lipid extract of the liver of an ethanol-treated rat. (*D*) Same as *C* except no ethanol was given. For the EPR signal in *A* and *C* a_N = 14.4 G, a_β^H = 3.3 G (20). Spectrometer settings were the same as for Fig. 2.

DISCUSSION

The results of this study provide two important insights into the problem of alcoholic liver disease. First, the detection of carbon-centered radicals in livers of ethanol-treated rats provides direct evidence that a free radical process has been initiated by chronic ethanol exposure. Second, because radicals of the type observed may be associated with the process of lipid peroxidation, these data provide support for the hypothesis that lipid peroxidation occurs in liver as a result of ethanol administration (1, 5, 8, 25–29).

It is also significant that high levels of dietary fat enhance the intensity of lipid radical formation *in vivo* (Table 1). It is well established that ethanol-induced steatosis is enhanced when the diet contains a high amount of fat (30). Recently, Tsukamoto *et al.* have demonstrated that the continuous infusion of ethanol with fat as 25% of total calories led to liver fibrosis (31), whereas only steatosis and focal necrosis were observed when fat represented just 5% of total calories (32). Thus, the greater intensity of lipid radical formation when ethanol was fed in combination with a high-fat diet may explain, at least in part, the potentiation of alcoholic liver injury by dietary fat.

A second significant observation from these studies is that 1-hydroxyethyl radicals are trapped by PBN during the metabolism of ethanol by liver microsomes. This is consistent with recent findings by Albano *et al.* (33), who observed the same radical by using α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone as the trapping agent during hepatic microsomal metabolism of ethanol. Desferrioxamine has been shown to inhibit the intensity of radical trapping, suggesting that iron or copper could be involved in the production of radicals, but, as indicated by Albano *et al.* (33), desferrioxamine itself may act as a free radical scavenger (34). However, the involvement of 1-hydroxyethyl radical formation in microsomal ethanol oxidation has not been systematically studied. It is important to note that prior feeding with ethanol induced the rates of 1-hydroxyethyl radical formation, particularly in the model that also received high levels of dietary fat (Table 2). In general, 1-hydroxyethyl radical formation correlated with the microsomal cytochrome P-450 content (Table 2). These observations may be explained by the "permissive" effect of dietary fat on the induction of certain cytochrome P-450-dependent oxidations (35, 36). This permissive effect may facilitate the induction of cytochrome P-450 by ethanol. Dietary fat has been shown to enhance the induction of several drug-metabolizing enzyme activities during ethanol feeding experiments (37).

The finding that 1-hydroxyethyl radicals are formed during ethanol metabolism by hepatic microsomes and could be trapped by PBN suggested that it might be possible to use PBN to trap these radicals *in vivo*. However, 1-hydroxyethyl radicals were not observed in the *in vivo* experiments. Instead, other carbon-centered radicals were the major components trapped by PBN in both the liver and the heart of ethanol-treated rats. This dissimilarity between the *in vitro* and *in vivo* results may be due to a difference in the concentrations of the spin-trapping agent, ethanol, or both at the site of radical generation *in vivo* as compared to *in vitro*. It is possible that the PBN concentration at the site of radical production *in vivo* is considerably lower than *in vitro*. If that

were the case, lipids might compete efficiently with PBN for reaction with 1-hydroxyethyl radicals *in vivo*, resulting in the initiation of lipid peroxidation and, ultimately, trapping of lipid radicals. In addition, the PBN adducts of 1-hydroxyethyl radicals were observed to decay within 30 min, so that any of these radicals that were trapped *in vivo* could have disappeared during the preparation of the tissue extract. In contrast, lipid radical adducts are much more persistent.

An alternative explanation to 1-hydroxyethyl radicals in the initiation of lipid peroxidation is the possible formation of hydroxyl radicals. Considerable evidence has been presented that hydroxyl radicals may participate in the metabolism of ethanol by liver microsomes (38–40). If ethanol feeding induces an enzyme system in the endoplasmic reticulum that increases the rates of hydroxyl radical generation, lipid peroxidation and tissue damage would also be expected to increase. Several studies have provided indirect evidence that ethanol feeding does, in fact, increase microsomal generation of hydroxyl radicals (13, 27), although Shaw *et al.* (14) were unable to detect enhanced lipid peroxidation under these conditions. On the other hand, Ekstrom *et al.* (41) recently suggested that the form of cytochrome P-450 induced by ethanol in rats is especially effective in ethanol oxidation but not hydroxyl radical formation. Similar conclusions have been reached in experiments with purified cytochrome P-450 isozyme LM 3a, the ethanol-inducible isozyme isolated from rabbit liver (42). Thus, these data, together with the inability to detect hydroxyl radicals with DMPO in experiments described in this report, cast some doubt on the role of hydroxyl radicals in initiating ethanol-induced hepatotoxicity.

The observation that lipid radicals were also trapped *in vivo* in hearts of ethanol-fed rats (Fig. 5*A*) supports previous suggestions that lipid peroxidation occurs in cardiac tissue as a result of ethanol abuse (10). However, the mechanisms for radical formation in the heart are unclear. Because the heart contains very low levels of cytochrome P-450, generation of 1-hydroxyethyl or hydroxyl radicals by this system seems unlikely. It has been suggested that acute and chronic ethanol exposure results in conversion of cardiac xanthine dehydrogenase to its oxidase form (43, 44), which could increase formation of superoxide radicals. Nevertheless, additional experiments are necessary to understand the mechanisms for ethanol-induced lipid peroxidation in the heart.

The results indicate that lipid peroxidation is initiated in the hepatic endoplasmic reticulum *in vivo* during the metabolism of ethanol. Also, diets containing fat at a level similar to that of the average diet in the United States significantly enhance the intensity of radical production. The next step is to examine the effects of antioxidants and of different types of fat on the production of the lipid free radicals *in vivo*.

The authors thank Edward G. Janzen for helpful discussions. We also thank Gemma Brueggemann for able technical assistance and Diana McLeod and Joanne McDonald for aid in the preparation of this manuscript. This work was supported in part by National Institutes of Health Grants ES-02812 and CA-30137.

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