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In Vivo Formation of a Free Radical Metabolite of Ethanol

KATHRYN T. KNECHT, BLAIR U. BRADFORD, RONALD P. MASON, and RONALD G. THURMAN

Curriculum in Toxicology (K.T.K.) and Laboratory of Hepatobiology and Toxicology, Department of Pharmacology (B.U.B., R.G.T.), University of North Carolina, Chapel Hill, North Carolina 27599-7365 and Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 (K.T.K., R.P.M.)

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SUMMARY

Free radical metabolism of ethanol has been suggested as a factor in its hepatotoxicity. Although evidence of lipid radical formation due to ethanol treatment *in vivo* has been reported, free radicals from ethanol itself have not been detected in living animals. However, by applying the EPR spectroscopy technique of spin trapping to the study of ethanol-treated alcohol dehydrogenase-deficient deermice (*Peromyscus maniculatus*), we have detected the α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN)/α-hydroxyethyl radical adduct in bile from animals administered [1-^{13}C]ethanol and the spin trap POBN. Hyperfine coupling constants were a^N = 15.48, a_β^H = 2.02, and a_β^{13C} = 4.61 G. In addition, an ethanol-dependent but ^{13}C-invariant radical adduct, presumably lipid derived, was detected. Hyperfine coupling constants were a^N = 15.38 and a_β^H = 2.5 G. This report demonstrates, for the first time, the *in vivo* formation of the α-hydroxyethyl free radical metabolite of ethanol.

Chronic ethanol abuse in humans produces widespread pathological changes, including marked hepatic toxicity. The initial fatty changes in the liver were first attributed to lipid peroxidation by Di Luzio in 1963 (1), and a number of subsequent studies have both supported and questioned the role of this process in toxicity (2–6). In 1972, Slater (7) suggested that the metabolism of ethanol to a free radical, which could then initiate lipid peroxidation, might be a factor in hepatic damage. Albano *et al.* (8, 9) and Reinke *et al.* (6) have used EPR spectroscopy and spin trapping techniques to detect cytochrome P-450-catalyzed formation of the α-hydroxyethyl radical in microsomal incubations.

Although oxidation of ethanol *in vivo* via cytochrome P-450 may be toxicologically important, it is unlikely to be a major pathway in the metabolic elimination of this compound from most normal biological systems. In both humans and experimental animals, ADH and catalase are responsible for the majority of ethanol oxidation (10). However, a mutant strain of deermouse (*Peromyscus maniculatus*) lacks ADH activity (11) but still metabolizes ethanol via catalase and cytochrome P-450 (12–16). Ethanol metabolism can be induced by chronic treatment with ethanol in this strain (17).

Free radical metabolites of ethanol have not been detected directly with EPR spectroscopy, due to their instability. However, radical adducts of ethanol, products of the spin trapping of the radicals, are more stable and have been detected *in vitro* using EPR spectroscopy (8, 9). *In vivo*, the same technique of spin trapping has been applied to the study of free radical metabolites from halocarbons, most recently in the detection of radical adducts in bile (18). Therefore, we have chronically pretreated ADH-deficient deermice with ethanol, administered bolus doses of ethanol and the spin trap POBN, and examined the bile for radical adducts.

Materials and Methods

Female ADH$^-$ deermice (*P. maniculatus*) were obtained from a colony maintained at the University of North Carolina at Chapel Hill in accordance with institutional guidelines. The colony was established from breeding stock kindly provided by Dr. M. R. Felder of the University of South Carolina. Ethanol-induced animals were given Lieber and DeCarli liquid diet (Dyets Inc., Bethlehem, PA) for at least 3 weeks. Corn oil and ethanol each supplied 30% of total calories. Control animals were fed the same diet with ethanol replaced isocalorically by maltose/dextrin or were given laboratory chow (Wayne Lab Blox), as described in the text.

Deermice were administered 0.35 ml of 20% ethanol (v/v in normal saline) by gavage and/or 0.1 ml of POBN (Sigma Chemical Co., St. Louis, MO) solution (50 mg/ml in deionized water). Doses for an estimated 15-g deermouse were 3.7 g/kg and 333 mg/kg for ethanol and spin trap, respectively. For ethanol-induced animals, food was removed at 8:00 a.m. and animals were Breathalyzed at 1:00 p.m. to insure that levels of [1-^{12}C]ethanol from the diet were minimal (corresponding blood alcohol level below 100 mg/dl).

Some animals (Fig. 3B) were depleted of ethanol as described above, dosed with a 0.35-ml oral bolus of [1-^{13}C]ethanol (MSD Isotopes, St. Louis, MO) solution, and depleted of ethanol a second time before

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ABBREVIATIONS: ADH, alcohol dehydrogenase; POBN, α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone.

being given POBN. This procedure ensured that signals produced by endogenous lipids could be distinguished from those produced by traces of ethanol remaining in the animal.

One and one half hours after dosing, all deermice were given an intraperitoneal overdose (0.12 ml) of Nembutal. The peritoneal and thoracic cavities were opened, and the bile duct distal to the gall bladder was clamped with a hemostat. The surface area was rinsed with a 100 mM solution of Desferal (Ciba, Summit, NJ) in deionized water. Liver tissue around the gall bladder was removed, and the intact gall bladder was harvested using a loop of thread. The gall bladder was rinsed with Desferal solution, cleaned of remaining tissue, and then submerged immediately in 150 μl of 100 mM Desferal solution in a 1.5-ml capped plastic centrifuge tube. The samples were frozen on dry ice and stored at −70°.

Samples were thawed and a plastic pipette tip was used to break the gall bladders and mix their contents with the Desferal solution. The resulting solution was pipetted into a quartz flat cell for ESR analysis. A Varian E-109 spectrometer equipped with a TM_{110} cavity was used. Instrument conditions were 20-mW microwave power, 0.53-G modulation amplitude, 80-G scan width, 0.5-hr scan time, and 2-sec time constant. After EPR analysis, samples were bubbled with oxygen for 10 min to reoxidize any radical adduct that may have been reduced to its EPR-invisible hydroxylamine form, bubbled with nitrogen for 10 min to remove the line-broadening effects of the oxygen, and examined again. No significant changes in results were noted as a result of the procedure. Simulations of EPR spectra were performed on a Hewlett-Packard HP 9835B computer equipped with a Varian data acquisition system. All experiments were performed at least twice, with similar results in each case.

Results

Fig. 1A shows the radical adduct signal of bile from six deermice that were chronically treated with an ethanol-containing high fat diet and given an acute dose of ethanol (3.7 g/kg) and of POBN (100 mg/kg). The central doublet (Fig. 1; one line superimposed on the six-line species) represents the ascorbyl semidione radical and is seen in samples from most animals. The six-line spectrum is characteristic of many free radicals trapped by POBN. The hyperfine coupling constants of the radical adduct in Fig. 1A are not definitive and could be those of the α-hydroxyethyl radical adduct or of some other radical adduct, such as an ethanol-induced lipid-derived radical adduct. Coupling constants of POBN radical adducts are relatively independent of the structure of the trapped free radical, and solvent effects can change coupling constants by more than 1 G (19).

^{13}C substitution on the α-carbon of a spin-trapped species creates an additional hyperfine coupling due to the magnetic interaction of the ^{13}C nucleus with the free electron. The subsequent production of a 12-line spectrum proves that the trapped radical arises from the labeled parent compound. Thus, in order to identify the radical adduct of ethanol unequivocally, ethanol labeled at the α-carbon with ^{13}C was employed. Fig. 1B shows the spectrum of bile from six animals treated as in Fig. 1A except that [1-^{13}C]ethanol was used. A 12-line spectrum was detected instead of the six-line spectrum of Fig. 1A. Thus, the radical detected in Fig. 1A was derived from ethanol, and the unpaired electron density was centered at the α-carbon of ethanol.

Hyperfine coupling constants for the 12-line species (POBN/$^{\cdot 13}CHOHCH_3$) are similar to those reported in the literature (20, 21), although some variation results from the solvent effects of the bile (18).

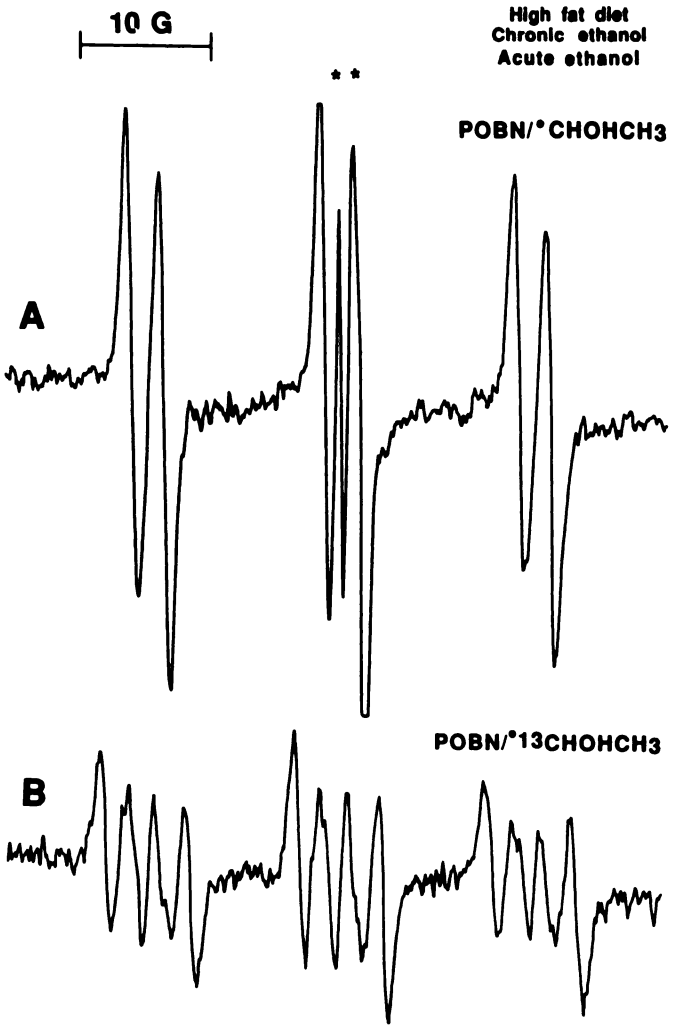

Fig. 1. EPR spectra of dilute bile from ADH$^-$ deermice. Deermouse treatments described in Materials and Methods. EPR conditions: microwave frequency, 9.4 GHz; microwave power, 20 mW; scan range, 80 G; scan time, 30 min; time constant, 2 sec; modulation amplitude, 0.53 G. A, EPR spectrum of bile from six ethanol-induced deermice treated with acute ethanol and POBN. B, As in A, except acute [1-^{13}C]ethanol.

Fig. 2A shows a spectrum from the bile of two animals treated as in Fig. 1A, except that a 3.3-fold higher POBN dose was used. Fig. 2B shows the corresponding spectrum when [1-^{13}C] ethanol was used. In this figure, a six-line spectrum overlays the 12 lines of the POBN/$^{\cdot 13}CHOHCH_3$. Fig. 2C shows the composite computer simulation of this spectrum, and Fig. 2, D and E, shows the component spectra. Coupling constants of these radical adducts are $a^N = 15.48$, $a_\beta^H = 2.02$, and $a_\beta^{^{13}C} = 4.61$ G for the 12-line species and $a^N = 15.38$ and $a_\beta^H = 2.5$ G for the six-line species.

Fig. 2B is duplicated in Fig. 3A for ease of comparison. In Fig. 3B, animals were allowed to metabolize most of their body content of ethanol before treatment with an oral bolus of [1-^{13}C]ethanol and were then allowed to metabolize most of this second dose before receiving an intraperitoneal dose of POBN. The residual ethanol in these deermice was thus ^{13}C-labeled, and any radical adduct formed from this residual ethanol would produce a 12-line spectrum. Only a six-line spectrum was detected in the bile of these animals, indicating that residual ethanol did not make a significant contribution to radical adduct formation.

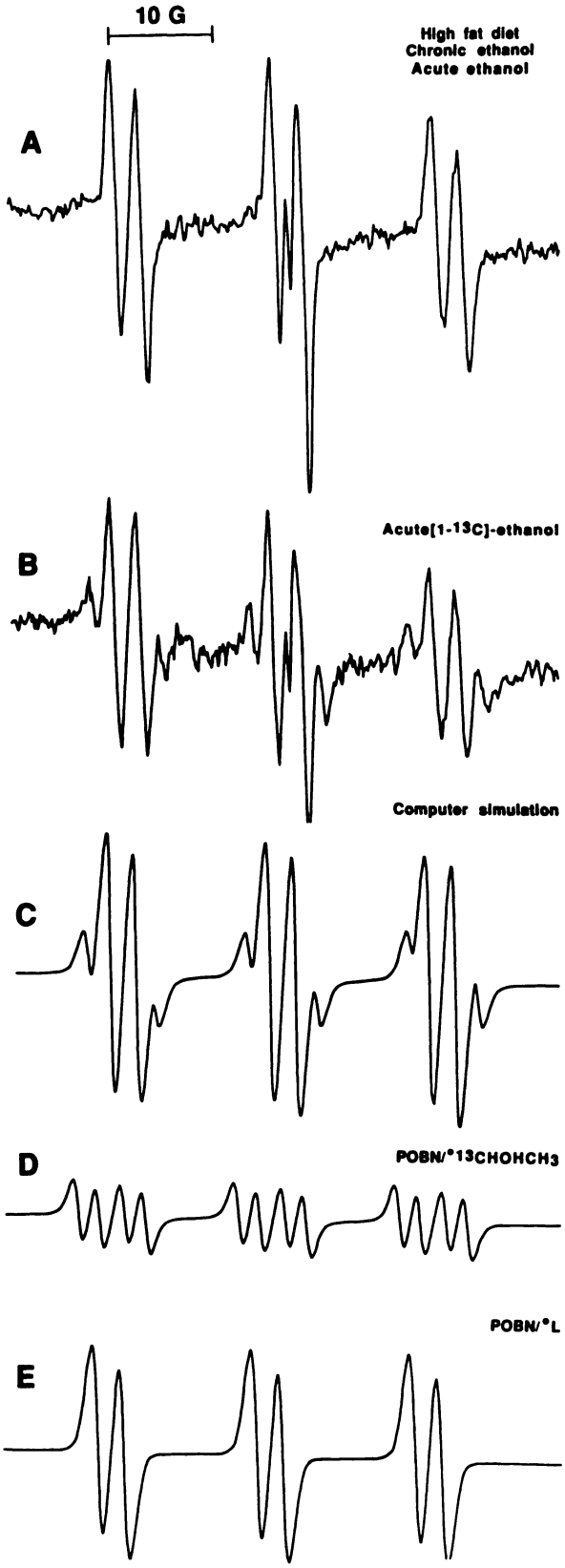

Fig. 2. Computer simulation of EPR spectra of dilute bile from ADH$^-$ deermice. Deermouse treatment described in Materials and Methods. EPR conditions as in Fig. 1. A, EPR spectrum of bile from two ethanol-induced deermice treated with acute ethanol and POBN. B, As in A, except acute [1-^{13}C]ethanol. C, Computer simulation of spectrum in A. Components shown in D and E. Simulations of EPR spectra performed on a Hewlett-Packard HP 9835B computer equipped with a Varian data acquisition system. D, POBN/$\dot{C}$HOHCH$_3$. Coupling constants as in text. Linewidth, 0.8 G; 75% Lorentzian, 25% Gaussian. E, Six-line species (POBN/$\dot{L}$). Coupling constants as in text. Linewidth, 0.7 G; 30% Lorentzian, 70% Gaussian.

Fig. 3C shows a spectrum from the bile of animals treated as in Fig. 3A but administered a 15 mg/kg dose of the iron chelator Desferal intraperitoneally 1 hr before treatment with POBN and acute ethanol. Fig. 3, D and E, shows spectra from animals fed high fat (no ethanol) diets but otherwise treated as in Fig. 3, A and B, respectively. For Fig. 3F, animals were fed a low fat diet without ethanol before treatment as in Fig. 3A.

It is possible that α-hydroxyethyl radical adduct could be produced *in vitro* by metal-catalyzed air oxidation of ethanol in bile, with subsequent trapping by POBN also secreted into bile. Thus, gall bladders from deermice were routinely harvested into a vial containing an aqueous solution of Desferal. To test whether free radical formation occurred *in vitro*, ethanol (final concentration, 100 mM) was added to the sample vial for gall bladders from ethanol-induced, ethanol-depleted, POBN-treated deermice (Fig. 3G). Conversely, POBN (final concentration, 10 mM) was added to the sample vial for gall bladders from ethanol-induced [1-^{13}C]ethanol-treated deermice (Fig. 3H). As shown, neither system produced the 12-line α-hydroxyethyl radical adduct seen in Figs. 1B and 2A, indicating that the signal detected in the bile of ethanol-pretreated ethanol- and POBN-treated deermice must be produced *in vivo* before sample collection. The six-line spectrum seen in Fig. 3G is probably the radical adduct that was dependent on chronic ethanol treatment and was described in Fig. 3B.

Discussion

A six-line spectrum has been detected in the bile of deermice chronically fed a high fat, ethanol-containing diet and treated with bolus doses of ethanol and POBN. The use of [1-^{13}C] ethanol and computer simulation of spectra identifies the subsequent 12-line signal as arising from the POBN/α-hydroxyethyl radical adduct (Figs. 1 and 2).

When higher concentrations of POBN were used, a ^{13}C-invariant (six-line) spectrum was also detected. This species appeared even when no bolus dose of ethanol was given (Fig. 3B) and has several possible origins. It could be due to a POBN nitroxide degradation product. This is not likely, because such a radical adduct signal would be present in all the spectra of Fig. 3. It could be POBN/$\dot{C}$HOHCH$_3$ formed from [1-^{12}C] ethanol still present in the deermice at the time of [1-^{13}C] ethanol dosing. This is also not likely, because that six-line spectrum would then be present in Fig. 1B as well. Furthermore, from Fig. 3B, residual [1-^{13}C]ethanol was found to have no effect on the radical adduct signal in the bile.

Finally, the six-line spectrum of Fig. 3A could be a radical adduct derived from an endogenous source such as lipids. This last possibility is quite likely, based on the already mentioned association between ethanol administration and lipid peroxidation. In particular, these results are similar to those of Reinke *et al.* (6), who reported a different lipid-derived radical adduct in the liver of ethanol-treated rats. The six-line species was detectable even in the presence of minimal levels of ethanol, although the signal was stronger when a bolus dose of ethanol had been administered (compare the six-line spectra of Fig. 3, A and B). The absence of this signal in Fig. 1B was due to the

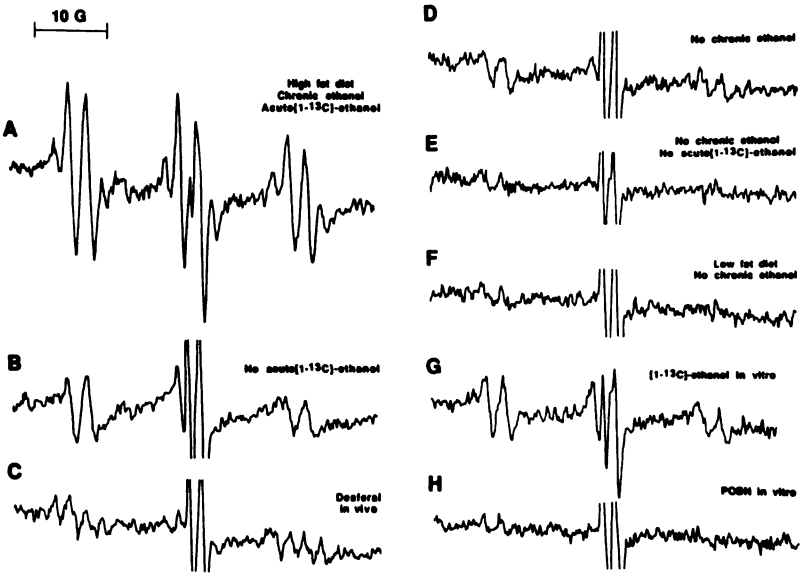

Fig. 3. EPR spectra of dilute bile from ADR⁻ deermice: controls. Deermouse treatments described in Materials and Methods. EPR conditions as in Fig. 1. A, EPR spectrum of bile from two ethanol-induced deermice treated with acute [1-^{13}C]ethanol and POBN. B, EPR spectrum of bile from two ethanol-induced deermice pretreated with acute [1-^{13}C]ethanol, kept several hours to eliminate acute ethanol, and then treated with POBN. C, As in A, except pretreated with 15 mg/kg Desferal (intraperitoneally) 1 hr before treatment with acute [1-^{13}C]ethanol and POBN. D, As in A, except ethanol in diet replaced isocalorically with maltose/dextrin. E, As in D, except no acute [1-^{13}C]ethanol. F, As in A, except animals fed laboratory chow. G, As in A, except no acute [1-^{13}C]ethanol. [1-^{13}C]ethanol (10 mM final concentration) added to the collection vial. H, As in A, except no POBN. POBN (10 mM final concentration) added to the collection vial.

lower concentration of POBN. Further, the coupling constants are similar to those reported in the literature for lipid-derived carbon-centered radical adducts of POBN (22, 23). However, the definitive identification of this radical adduct is difficult, due to the intrinsic similarity of most POBN radical adduct coupling constants.

Bile from two ethanol-pretreated deermice given Desferal before they received [1-^{13}C]ethanol and POBN (Fig. 3C) contained as much POBN/$^{\cdot}{}^{13}$CHOHCH$_3$ as did corresponding bile from similarly treated animals not receiving Desferal (Fig. 3A). In groups of six animals given 100 mg/kg POBN, the POBN/$^{\cdot}{}^{13}$CHOHCH$_3$ radical adduct signal was also unaffected when the signal amplitude was corrected for the reduced volume of bile collected from these animals (data not shown). This dose of Desferal approximates the maximal recommended daily human dose. A concomitant disappearance of the POBN/$^{\cdot}$L species indicated that sufficient quantities of the chelator were absorbed to produce a pharmacological effect. The lack of effect of Desferal on the spectrum of POBN/$^{\cdot}{}^{13}$CHOHCH$_3$ indicated that the formation of the radical adduct was not dependent on free iron. Furthermore, Handler *et al.* (17) could not detect any effect of Desferal on the rate of ethanol metabolism in the perfused liver. It should be noted, however, that the effect of Desferal on the six-line species may not reflect the role of free iron in lipid peroxidation but rather the chain-breaking antioxidant character of Desferal (24–26).

Animals administered a high fat diet containing no ethanol still showed faint traces of ethanol-dependent radical adduct in their bile after treatment with bolus doses of ethanol and spin trap, as is shown in Fig. 3, D and E. The bile from animals fed a low fat diet, however, did not contain detectable quantities of this radical adduct (Fig. 3F). Apparently, the metabolism of ethanol to the α-hydroxyethyl radical was increased in the high fat-treated animals. Hiramatsu *et al.* (27) have reported that the feeding of lipids induces microsomal enzymes.

In vitro manipulations of bile samples (Fig. 3, G and H) indicate that the POBN/α-hydroxyethyl radical adduct was formed *in vivo* rather than *in vitro*, as described in Results.

Definitive proof of the formation of a reactive free radical metabolite of ethanol *in vivo* has been obtained using spin trapping in ADH⁻ deermice, although we have subsequently found that ADH⁺ deermice also formed the α-hydroxyethyl free radical (data not shown). Ethanol pretreatment and/or a high fat diet were required to produce detectable radical adduct in the bile of these animals.

In summary, the α-hydroxyethyl free radical forms *in vivo*. This free radical is capable of undergoing a number of biochemical reactions *in vitro*, including the rapid reduction of heme-containing and other proteins (28) and reaction with glutathione (9) [possibly related to *in vivo* glutathione depletion (29)]. The alkylation of DNA bases by α-hydroxyethyl radical, a reaction reported for the chemically similar α-hydroxyisopropyl radical (30), is another way in which this free radical could affect cells. Whether the α-hydroxyethyl free radical is actually involved in the initiation of lipid peroxidation is still unknown. In short, the detection of this reactive free radical metabolite *in vivo* dictates further study to determine its biochemical consequences *in vivo* and its possible contributory role in the multifaceted toxicity of ethanol.

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Send reprint requests to: Kathryn T. Knecht, Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.