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Metabolism of Ethanol to 1-Hydroxyethyl Radicals *In Vivo*: Detection with Intravenous Administration of α-(4-Pyridyl-1-oxide)-*N*-*t*-butylnitrone

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SUMMARY

Intravenous administration of the spin-trapping agent α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN) to anesthetized but otherwise untreated rats was used to test for formation of 1-hydroxyethyl radicals *in vivo*. The only EPR signals observed in bile samples from rats that had received ethanol but no POBN could be attributed to low concentrations of ascorbyl radical. However, when POBN (700 mg/kg, intravenously) was also administered, a nitroxide with a six-line EPR spectrum was readily detected in bile. This spin adduct was proven to be the 1-hydroxyethyl radical adduct of POBN through injection of [1-^{13}C]ethanol to rats, which resulted in the presence of an adduct with a 12-line EPR spectrum. Comparable results were obtained in experiments with isolated perfused rat livers. 1-Hydroxyethyl radical spin adducts of POBN were readily detectable in bile in the presence of only moderate (10–15 mM) concentrations of alcohol. In these experiments, bile samples were collected into a mixture of dipyridyl and bathocuproine disulfonic acid, and the effectiveness of these chelators to prevent *ex vivo* signal formation was confirmed experimentally. No EPR signals for nitroxide spin adducts were observed in plasma or perfusate, even though high concentrations of POBN and alcohol were present. Taken together, these data indicate that 1-hydroxyethyl radicals are formed *in vivo* and can be readily detected in bile when high concentrations of POBN are achieved through intravenous injection.

Spin trapping and EPR spectroscopy studies have demonstrated that liver microsomes metabolize ethanol to a free radical metabolite, the carbon-centered 1-hydroxyethyl radical (1–7), but the mechanisms involved in this reaction are still unclear. It is generally agreed that iron has an important catalytic role, because rates of ethanol radical formation are increased when iron salts are added to the incubation systems, and iron-chelating compounds such as deferoxamine markedly inhibit the reaction (1, 3, 5–7). An important role of hydrogen peroxide was suggested from observations that catalase inhibitors such as azide stimulated ethanol radical formation in microsomes, whereas addition of catalase was inhibitory (1, 3, 7). In addition, Knecht *et al.* (5) have recently postulated that 1-hydroxyethyl radicals are formed in a reaction requiring superoxide and transition metals. Superoxide and hydrogen peroxide are formed primarily by microsomal cytochrome P-450 enzymes (8), and microsomes contain non-heme iron that can be released to participate in lipid peroxidation and other free radical reactions (9). Some evidence also implicates a direct role of cytochrome P-450 in microsomal metabolism of ethanol to the 1-hydroxyethyl radical (10).

Although 1-hydroxyethyl radicals can be readily detected by spin trapping in microsomal incubations, it has been considerably more difficult to demonstrate their formation *in vivo*. Knecht *et al.* (11) observed 1-hydroxyethyl radical adducts of POBN in bile of deer mice and indicated that the EPR signals were especially intense if the mice were also deficient in alcohol dehydrogenase and had been fed a high-fat, ethanol-containing diet. We found that acute alcohol administration to ethanol-fed (2) or fasted (12) rats caused formation of lipid radicals in the liver, and we detected extremely low concentrations of 1-hydroxyethyl radical adducts of α-phenyl-*N*-*t*-butylnitrone in liver extracts from glutathione-depleted rats (12).

There are several possible explanations for why ethanol radicals are more easily detected in microsomes than *in vivo*. Well washed microsomes are almost devoid of superoxide dismutase and catalase, and the presence of these enzymes in cytosol could impair superoxide- or H_2O_2-dependent radical formation (5). Concentrations of free transition metals in

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ABBREVIATION: POBN, α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone.

intact tissue may be insufficient to catalyze radical formation. Intact liver also contains high concentrations of glutathione and other antioxidants, which could compete with spin-trapping agents for radicals that might be formed. And, finally, spin adducts could be degraded to EPR-silent products by liver enzymes (13) or other biological reducing agents.

In the current report, we have used intravenous injection of POBN to achieve high blood concentrations of the spin-trapping agent. Under these conditions, it was possible to reproducibly demonstrate the presence of 1-hydroxyethyl radical spin adducts in bile from rats that had received no previous treatment.

Materials and Methods

Male Sprague-Dawley rats (200–250 g) were allowed free access to laboratory chow and water. The rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally), and small supplemental doses were administered if needed. The bile duct was cannulated with PE-10 tubing, and the bile was collected into tared 1.5-ml polyethylene centrifuge tubes, which also contained 30 μl of a solution of dipyridyl (30 mM) and bathocuproine disulfonic acid (300 mM) to prevent *ex vivo* radical formation (14). POBN was dissolved in water (100 mM) and was given by slow intravenous injection into the tail vein or directly into the inferior vena cava, which had been exposed during cannulation of the bile duct. The POBN and alcohol doses are indicated in the figure legends.

The average biliary flow in these experiments was 2.1 ± 0.7 μl/g of liver/min, so that enough bile to fill the sensing area of a flat EPR cell could be collected in 5 or 6 min, but samples were normally collected for 10-min intervals. The bile samples were weighed immediately after collection and were frozen on dry ice. The bile volume was calculated from its weight, assuming a specific gravity of 1 g/ml.

At the end of the collection period, the animals were sacrificed by cutting the diaphragm, and blood samples were taken by cardiac puncture for determination of blood alcohol or preparation of plasma (15). Plasma and blood samples were immediately frozen on dry ice. Alcohol concentrations in the blood and bile were measured enzymatically (16). Biliary concentrations of POBN were estimated by diluting 10 μl of bile to 1.0 ml with water and measuring the intensity of the POBN absorption peak at 330 nm. Absorbances were measured with a Perkin-Elmer Lambda 4B UV/visible double-beam spectrophotometer, and bile collected from the same rat before POBN injection was similarly diluted to provide a reference sample. The POBN concentration was determined using an experimentally determined extinction coefficient of 36.8 mM^{-1} cm^{-1}. In some experiments, rat livers were perfused with Krebs-Henseleit bicarbonate buffer, pH 7.4, in a nonrecirculating perfusion system (17), and bile was tested for the presence of spin adducts as indicated for *in vivo* experiments.

To test for extraction of the 1-hydroxyethyl radical adduct of POBN by organic solvents, this adduct was prepared by photolysis (18). Briefly, POBN (100 mM), ethanol (7.5 M), and H_2O_2 (2.5 M) were subjected to 30 sec of intense UV light. The identity of the resulting spin adduct was proven by substituting [1-^{13}C]ethanol in the photolytic experiments.

Samples of bile and plasma were tested for the presence of spin adducts within a few hours of their collection and were thawed and warmed to room temperature immediately before EPR analyses. The samples were transferred into a quartz flat EPR cell and placed in the cavity of a Bruker 300E EPR spectrometer. Samples that were dissolved in organic solvents were thoroughly bubbled with nitrogen to remove dissolved oxygen. Typical EPR operating conditions were as follows: gain, 1×10^6; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; microwave power, 20 mW; conversion time, 92 msec; sweep time, 84 sec. In most experiments, it was necessary to accumulate multiple scans to obtain suitably resolved EPR spectra.

Results

Ethanol (5 g/kg) was administered by gastric lavage to a rat that was otherwise untreated. After 30 min, to allow for initial absorption of the alcohol, the rat was anesthetized with pentobarbital and the bile duct was cannulated. No EPR signals were observed in a bile sample taken before POBN injection (Fig. 1A). After this initial bile sample had been collected, POBN (700 mg/kg) was given by slow intravenous injection over a period of 2 min. After 5 min, to allow for redistribution of the POBN, sequential 10-min bile samples were obtained (Fig. 1, B–D). A six-line EPR spectrum was observed in all bile samples after injection of POBN, and the hyperfine splitting values (a_N = 15.6 G, a_H = 2.5 G) were similar to those reported for the 1-hydroxyethyl radical adduct of POBN in aqueous solutions (6, 11, 19). The EPR signal intensities increased as the experiment progressed, and the strongest signals were observed in the last sample, which was taken 55–65 min after POBN injection (Fig. 1D). Over this same time period, the biliary alcohol concentration decreased from 37 mM in the sample collected between 5 and 15 min after POBN injection to 28 mM in the last sample (Fig. 1, legend).

The same experiment was repeated with a rat that had not been treated with ethanol before POBN injection. A bile sample that was collected before POBN was injected contained only weak evidence of the ascorbyl radical (Fig. 2A), which was observed as a minor EPR signal in some experiments. After POBN was injected, an extremely weak nitroxide signal developed over time (Fig. 2). This adduct had hyperfine splitting values virtually identical to those of the

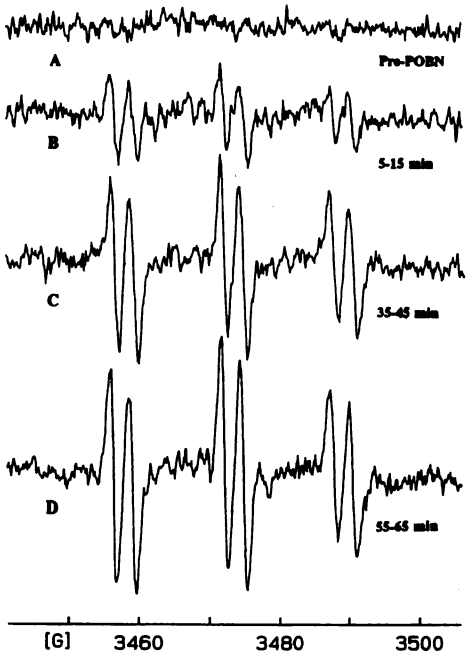

Fig. 1. Detection of spin adducts in bile from an alcohol-treated rat. A rat was given ethanol (5 g/kg) by gavage, and after 30 min it was anesthetized with pentobarbital and a cannula was inserted into the bile duct. After one bile sample (A) was taken, the rat received POBN (700 mg/kg, intravenously) and sequential 10-min bile samples were taken for 1 hr, beginning 5 min after POBN injection. Selected EPR spectra are shown (B–D), and the collection times, relative to POBN injection, are indicated. Biliary alcohol concentrations were 34 mM for sample A, 37 mM for sample B, 34 mM for sample C, and 28 mM for sample D.

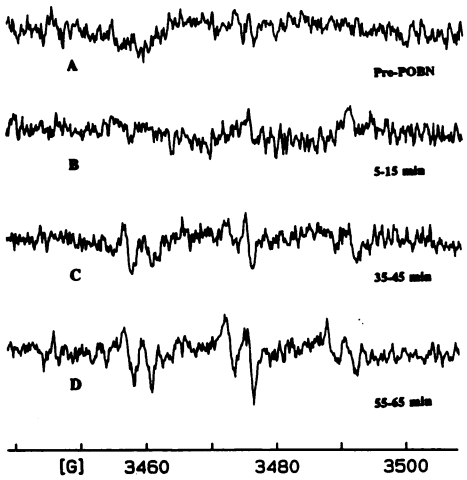

Fig. 2. EPR spectra of bile samples from a control rat. All conditions were identical to those indicated in Fig. 1, except that no ethanol was given. Biliary concentrations of POBN were estimated from absorbance spectra of bile and were 5.3 mM for sample B, 4.6 mM for sample C, and 4.3 mM for sample D. The weak midfield doublets in A are the EPR signals for the ascorbyl radical.

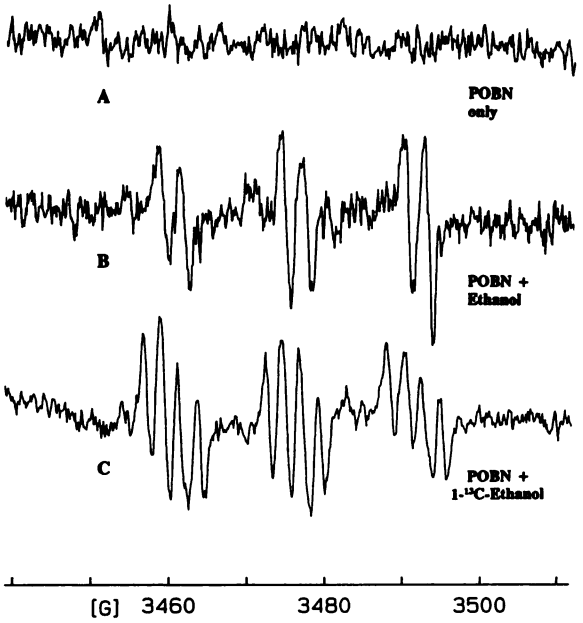

Fig. 3. Biliary spin adducts detected after intravenous injection of both POBN and ethanol. Rats were anesthetized with pentobarbital and were then given POBN (700 mg/kg, intravenously). The first two bile samples collected after POBN injection typically contained no EPR signals for spin adducts, and spectrum A is from the second bile sample. Spectrum B shows the next bile sample from the same rat and was collected between 5 and 15 min after injection of ethanol (1.06 g/kg, intravenously). Spectrum C is a sample from a different POBN-treated rat, which was collected between 25 and 35 min after injection of [1-^{13}C]ethanol (1.06 g/kg, intravenously). Biliary alcohol concentrations for samples B and C were 28 mM and 27 mM, respectively.

adduct detected after ethanol administration (Fig. 1). However, the signal appeared much later and was weaker at all time points, compared with EPR signals observed when ethanol was also administered (compare Figs. 1 and 2). POBN was rapidly excreted into the bile (Fig. 2, legend). The highest POBN concentrations were typically observed in the first or second sample collected after POBN injection but remained in excess of 4 mM for periods of at least 1 hr.

In a similar set of experiments, untreated rats were anesthetized before intravenous injection of both POBN and ethanol. When POBN was injected intravenously, no EPR signals were observed in the first bile samples collected (Fig. 3A). However, when ethanol (1.06 g/kg) was subsequently administered by intravenous injection, the next bile sample again contained a six-line EPR signal (Fig. 3B), which was similar to those observed after ethanol was given orally (Fig. 1). In other experiments, [1-^{13}C]ethanol (1.06 g/kg, intravenously) was administered to rats that had already received POBN, and a 12-line EPR spectrum was detected in the bile of these animals (Fig. 3C). The additional splitting is due to the presence of ^{13}C in the spin adduct (a^{13}_{C} = 4.5 G) and identifies the major radical that was trapped as the 1-hydroxyethyl radical. The more intense central peaks of each quartet of the spectrum in Fig. 3C can be explained by the presence of low concentrations of a ^{13}C-invariate signal, which was usually observed when POBN was injected in the absence of ethanol (Fig. 2).

Experiments were conducted to determine whether the EPR signal intensity in the bile was influenced by the ethanol concentration. An anesthetized rat was given POBN by intravenous injection, and the initial bile sample again contained no EPR signal (Fig. 4A). Ethanol was then given in four incremental intravenous doses of 0.35 g/kg each. After each dose, 5 min were allowed for redistribution of the alcohol, and then one bile sample was collected over a 10-min period before another ethanol dose was given. The bile sample collected after the first ethanol dose contained a barely perceptible EPR signal for a nitroxide, but the signal intensity increased with each subsequent ethanol dose (Fig. 4, B–E). The results of this experiment must be interpreted with caution, because signal intensity normally increased with time of the experiment (Fig. 1). Nevertheless, these data strongly suggest that 1-hydroxyethyl radical formation can be rapidly initiated by only modest concentrations of alcohol (e.g., 10–15 mM).

Experiments by other investigators have clearly shown that spin adducts can be formed through reactions among biliary trace metals, spin-trapping agents, and alcohol or dimethylsulfoxide (11, 14). This problem can be illustrated in an experiment in which a rat was given POBN (700 mg/kg) by intraperitoneal injection. When an intravenous dose of [1-^{13}C]ethanol was subsequently given, bile collected into the chelator mixture contained a weak signal often observed with POBN alone, but the 12-line spectrum of the 1-[^{13}C]hydroxyethyl adduct was not observed (Fig. 5A). The absence of the 1-hydroxyethyl radical adduct might be explained by less efficient spin trapping after intraperitoneal administration, due to lower available concentrations of POBN. For example, biliary POBN concentrations in this experiment ranged between 2.5 and 3.7 mM, whereas higher concentrations were routinely observed after intravenous administration of the same POBN dose (Fig. 2, legend). However, when the next bile sample was collected without addition of chelators and was otherwise treated identically, the 1-[^{13}C]hydroxyethyl adduct could be readily detected (Fig. 5B). Because the only significant difference between samples shown in Fig. 5, A and B, was the presence of iron and copper chelators, these data demonstrate that metal-catalyzed reactions can produce artifactual signals in the bile. However, the absence of 1-hy-

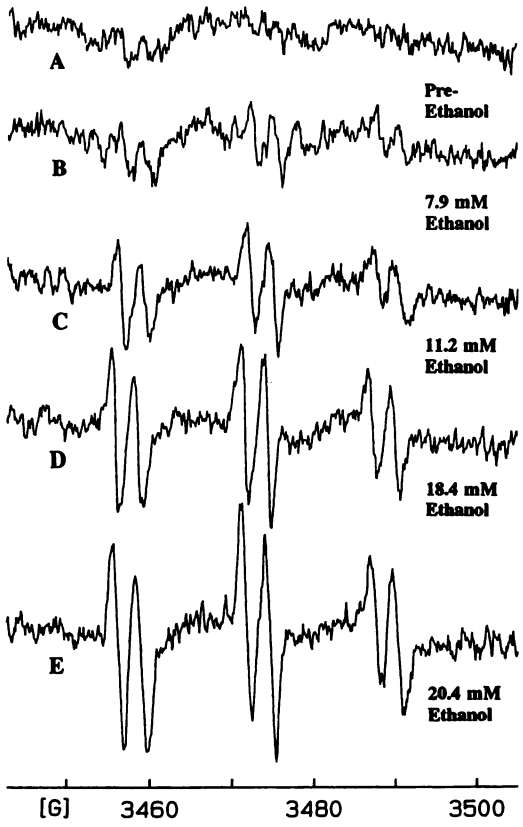

Fig. 4. Influence of ethanol dose on EPR signal intensity in bile samples. A rat was anesthetized and given POBN (700 mg/kg, intravenously), and one bile sample (A) was collected. The rat was then given four sequential intravenous doses of ethanol (0.35 g/kg per dose) (B–E), which were separated by 5 min to allow for redistribution of alcohol and 10 min for collection of one bile sample for EPR analyses. Biliary alcohol concentrations are indicated.

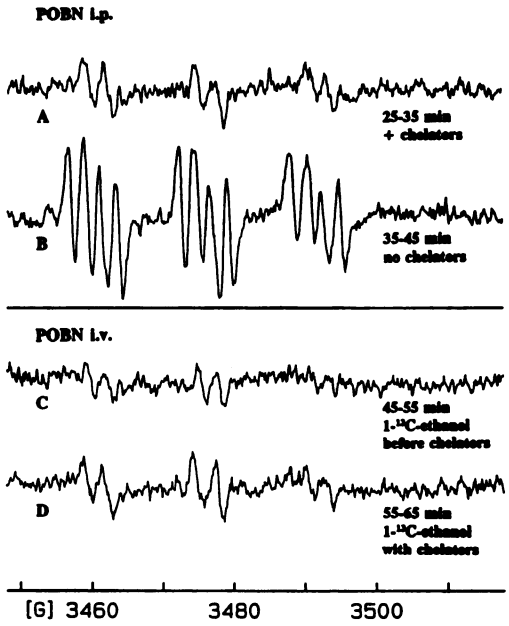

Fig. 5. Evaluation of chemical reactions between ethanol, POBN, and trace metals in bile. A rat received POBN (700 mg/kg) by intraperitoneal injection. After 30 min, the bile duct was cannulated, and [1-^{13}C]ethanol (0.8 g/kg, intravenously) was injected. Bile samples were collected during the time intervals indicated, either in the presence (A) or in the absence (B) of the usual mixture of dipyridyl and bathocuproine. Another rat received POBN (700 mg/kg) by intravenous injection, and bile samples were collected into empty tubes. Then [1-^{13}C]ethanol (30 mM final concentration) was added either 4 min before (C) or concurrently with (D) the mixture of chelators.

droxyethyl radical adducts when chelators were present suggests that such reactions may not form detectable levels of radicals during the bile collection interval.

The question of *ex vivo* spin adduct formation during collection of bile was addressed more directly in experiments designed to estimate the time for transport of ethanol and POBN from blood to the collection tube. Sequential bile samples were collected in 2-min intervals from two rats. One rat received ethanol (1 g/kg, intravenously) by slow injection over the 2-min collection period for the first bile sample. The resulting biliary alcohol concentrations were 31.6 mM in the sample during which ethanol was injected and 38 mM and 35 mM in the next two samples. Biliary alcohol concentrations subsequently decreased slowly, as in Fig. 1. Because ethanol is miscible with both water and lipids, this rapid distribution into bile was expected. This experiment was then repeated with a second rat, except that POBN (700 mg/kg) was given by slow injection during collection of the first 2-min bile sample. The resulting POBN concentrations in the first three bile samples were 4.1 mM while POBN was being injected and 5.5 mM and 3.8 mM for the second and third samples, respectively. Biliary POBN concentrations then decreased slowly in the remaining samples. These data show that POBN, like ethanol, is rapidly distributed from blood into bile. Furthermore, because peak concentrations of both POBN and ethanol were achieved within 2 min after their injections were completed, the time interval during which artifactual EPR signals could be produced during bile collection must also be <2 min.

These data were used to design additional experiments to test for artifactual EPR signal formation during the interval before the bile is mixed with chelators. POBN (700 mg/kg, intravenously) was injected, and after collection of bile samples [1-^{13}C]ethanol was added to the tubes at a final concentration of 30 mM, which is comparable to the alcohol concentrations found in other experiments (Figs. 1 and 3). After 4 min (twice the probable time period between bile formation and its mixing with chelators, as determined above), the dipyridyl/bathocuproine chelator solution was added (Fig. 5C). In Fig. 5D, the chelators and [1-^{13}C]ethanol were added concurrently. Both samples contained only a six-line, ^{13}C-invariate signal normally observed after injection of POBN alone (Fig. 2), with no evidence of the 1-[^{13}C]hydroxyethyl radical adduct. In other experiments, ethanol was given by intravenous injection and POBN (5 mM final concentration) was added to bile samples in the same design, but no EPR signals were observed (data not shown). Collectively, the data shown in Fig. 5 provide strong evidence that insignificant concentrations of 1-hydroxyethyl radical adducts are formed as a result of chemical reactions in bile during the interval of bile collection.

Direct EPR analyses of plasma or blood samples revealed signals from only the ascorbyl radical (Fig. 6). In the experiment shown, bile samples contained clear evidence of the 1-hydroxyethyl radical, indicating that the radical had been formed in this animal. It is possible that spin adducts in biological fluids could be reduced to EPR-silent hydroxy-

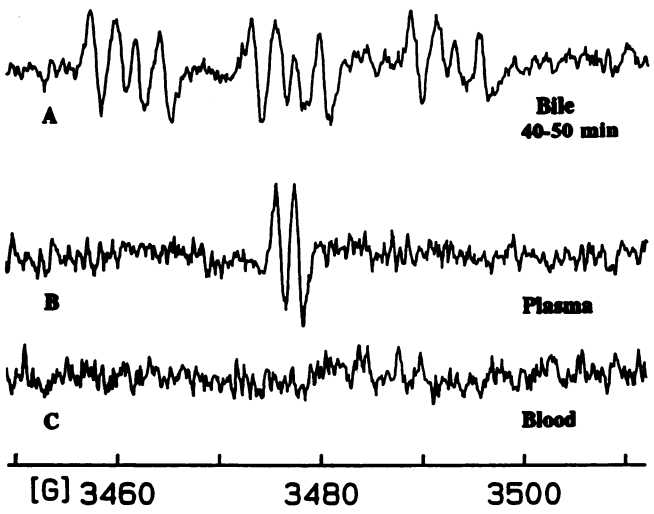

Fig. 6. EPR spectra of plasma and blood samples from an ethanol-intoxicated rat. POBN (700 mg/kg) and [1-^{13}C]ethanol (1 g/kg) were given by intravenous injection. An EPR spectrum of bile collected into the chelator solution 40–50 min later was obtained (A). Immediately after this bile sample was collected, the experiment was terminated, and heparinized blood was collected for EPR analyses of plasma (B) or whole blood (C). Plasma samples routinely contained the ascorbyl radical signal, which was often undetectable in whole blood. Removal of red blood cells during preparation of plasma has the effect of concentrating components found in serum. For this reason, EPR signal intensities from highly water-soluble radicals are often more intense in plasma than in blood samples (15).

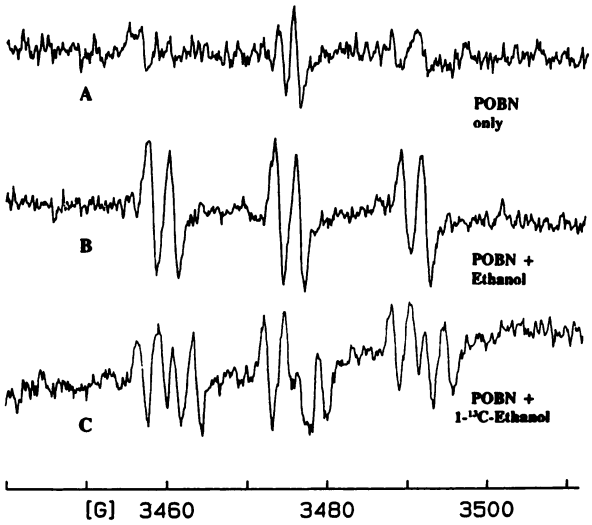

Fig. 7. EPR spectra of bile collected from perfused rat livers. Livers were removed under pentobarbital anesthesia and were perfused with Krebs-Henseleit bicarbonate buffer, pH 7.4, in a nonrecirculating system (17). In sample A, POBN (5 mM) was added to the influent perfusion buffer. In sample B, ethanol (50 mM) and POBN (5 mM) were infused. In sample C, [1-^{13}C]ethanol (50 mM) and POBN (5 mM) were infused.

lamines (13), and this possibility was tested by adding the mild oxidant potassium ferricyanide (20) in concentrations of 0.05–0.5 mM. However, ferricyanide addition did not result in detection of additional signals in plasma or blood and did not appreciably change the signal intensity of spin adducts in the bile (data not shown). When the photolytically prepared 1-hydroxyethyl radical adduct of POBN was added to plasma, no line broadening was observed, indicating that the adduct did not associate with proteins or other components in plasma (15). However, approximately 15% of the EPR signal intensity was lost after 20 min of incubation with plasma and was not restored by subsequent addition of 0.05 mM ferricyanide. These data suggest that spin adducts that may circulate in the blood could be degraded to EPR-silent products by undetermined mechanisms before the samples were analyzed.

Formation of 1-hydroxyethyl radical adducts of POBN could also be documented in experiments with isolated perfused rat livers. When POBN (5 mM) was infused, bile samples sometimes contained weak EPR signals from the ascorbyl radical and unidentified nitroxides (Fig. 7A). If ethanol (50 mM) was also infused, bile typically contained six-line EPR signals (Fig. 7B). When [1-^{13}C]ethanol was used, the additional spectral splitting (Fig. 7C) again proved that the major nitroxide present was the 1-hydroxyethyl radical adduct of POBN. EPR analysis of perfusate indicated no detectable spin adducts (data not shown), even though high concentrations of POBN and ethanol were present. These data provide additional evidence for hepatic formation of 1-hydroxyethyl radicals, as opposed to nonspecific chemical reactions that may occur in biological fluids.

Attempts were made to detect the 1-hydroxyethyl spin adduct of POBN in organic extracts of blood, plasma, or liver homogenates. First, several organic solvents were tested for their ability to extract the photolytically prepared spin adduct. Virtually no EPR signal could be observed in the organic phase when aqueous solutions of the adduct were extracted with equal volumes of toluene, benzene, or hexane. Both chloroform and methylene chloride decreased the EPR signal of the aqueous phase by about 10%, and a weak 1-hydroxyethyl radical adduct could be detected in the organic phase. However, attempts to extract plasma or liver homogenates (12) from POBN- and [1-^{13}C]ethanol-treated rats with chloroform or methylene chloride resulted in only weak six-line signals in the extracts (data not shown). Detection of the 1-hydroxyethyl radical adduct of POBN in tissue extracts may not be feasible, because of the high water solubility of this spin adduct.

Biliary alcohol concentrations were routinely measured, and the data were used to investigate the relationship between biliary and blood alcohol concentrations. The ratio between the biliary alcohol concentration in the last bile sample collected and the corresponding blood alcohol concentration at the end of the experiment was 1.32 ± 0.21 (mean ± standard deviation of 12 experiments, with blood alcohol concentrations ranging from 2 to 32 mM). The 30% higher values in bile were unexpected and might be explained by continued alcohol catabolism during the biliary collection period and disruption of normal enterohepatic circulation of alcohol. Nevertheless, the data indicate that biliary alcohol concentrations (Figs. 1, 3, and 4) can be used to provide a reasonable estimate of the corresponding blood alcohol concentrations.

The high doses of POBN used in these experiments raise the concern that the spin-trapping agent may itself cause metabolic abnormalities in the animals. For these reasons, some experiments were conducted to assess the acute effects of POBN, given at an intravenous dose of 700 mg/kg. Rates of hepatic bile flow were measured as an indicator of overall hepatic function. The average biliary flow over the first 30 min after POBN injection (2.2 ± 0.4 μl/g of liver) was similar to that of controls (2.4 ± 0.1 μl/g of liver) in three pairs of

rats. When POBN was injected into two anesthetized rats, transient tachycardia, dilation of pupils, and respiratory depression were observed in both animals, but these parameters returned to base-line values within 20 min.[1]

Discussion

In spin-trapping experiments, it is necessary to provide a high concentration of spin-trapping agent at the site of radical production to successfully compete with endogenous compounds for reaction with the radicals. This objective is easily met in chemical or microsomal systems, where high concentrations of spin-trapping agent can be introduced into the reaction vessel. However, when experiments are performed *in vivo*, the spin trap concentration is influenced by rates of absorption from the site of administration, distribution into various compartments, metabolism, and excretion. In many experiments involving *in vivo* spin trapping, the spin trap has been administered by intraperitoneal administration (e.g., Refs. 2, 12, and 14). Although many drugs are rapidly absorbed after intraperitoneal injection, a sufficiently high concentration of spin-trapping agent may not be attained. For example, in an experiment in which POBN was given by intraperitoneal injection, the 1-hydroxyethyl radical adduct was not detected in bile (Fig. 5A), even though sufficient concentrations of POBN and ethanol were present to chemically generate radical adducts in the absence of metal chelators (Fig. 5B).

The results of these experiments demonstrate that 1-hydroxyethyl radical adducts of POBN can be reproducibly detected in bile of ethanol-intoxicated rats if the spin trap is given in a high dose by intravenous injection. Unlike previous studies involving alcohol dehydrogenase-deficient deer mice fed alcohol-containing, high-fat diets (11) or fasted, glutathione-depleted rats (12), the animals used in these experiments received no prior treatment. The identity of the 1-hydroxyethyl radical of POBN was proven with [1-^{13}C]ethanol, either given by intravenous injection (Figs. 3C and 5A) or added to the perfusion buffer (Fig. 7C). The adducts could be detected within 10 min of alcohol dosing (Figs. 3, 4, and 7), and rates of their formation appeared to increase over a period of 1 hr (Fig. 1). Spin adducts formed in the liver may be quickly excreted into bile through the same mechanisms that are responsible for rapid biliary excretion of both POBN and ethanol. Although there were no clear relationships between blood or biliary alcohol concentrations and EPR signal intensities in these studies, it should be noted that the radicals could be detected in the presence of rather modest (10–15 mM) concentrations of alcohol (Fig. 4). These concentrations are <0.1 g of alcohol/dl (0.1%) and are easily reached in the blood of social drinkers.

POBN has several distinct advantages over other spin-trapping agents for studies of 1-hydroxyethyl radical formation. For example, POBN reacts rapidly with 1-hydroxyethyl radicals, and the resulting spin adducts are unusually stable to reduction and other reactions that transform spin adducts to EPR-silent products (21). POBN is highly water soluble, and concentrated aqueous solutions can be prepared for intravenous injection. Experiments comparing effects of POBN and α-phenyl-*N*-*t*-butylnitrone in hepatocytes and microsomes have shown that POBN has fewer effects on hepatocyte integrity and causes less inhibition of microsomal aminopyrine and ethoxycoumarin metabolism (22), even at concentrations of 25 mM. Although exhaustive pharmacological evaluations of POBN have not been performed, initial data indicate that the dose of 700 mg/kg intravenously, which results in biliary POBN concentrations of 4–6 mM (Fig. 2, legend), causes only transient abnormalities in heart and respiratory rates, as well as pupil dilation, in anesthetized rats.

Several potential disadvantages of POBN for studies of mechanisms of alcohol radical formation *in vivo* should also be acknowledged. One is that many carbon-centered spin adducts of POBN have similar hyperfine splitting constants, so that tentative identification of spin adducts from hyperfine splitting constants alone is not possible (11, 23). For example, low concentrations of POBN spin adducts with hyperfine splitting values similar to those of the 1-hydroxyethyl radical adduct were observed in some bile samples from rats that had not received ethanol (Figs. 2 and 5). Another difficulty is that POBN can reduce iron(III) in aqueous solution and can lead to formation of artifactual spin adducts under conditions where ferrous reoxidation is promoted (24). Finally, the high water solubility of the 1-hydroxyethyl radical adduct of POBN makes its extraction from biological samples quite ineffective.

Several lines of evidence strongly suggest that ethanol radical adducts of POBN detected in this report were formed in the liver and not by nonspecific reactions involving POBN, ethanol, and transition metals. Firstly, bile samples were collected into solutions of dipyridyl and bathocuproine, and the effectiveness of these agents for prevention of *ex vivo* radical formation in bile (14) was confirmed in the present studies (Fig. 5). Furthermore, the time during which POBN, ethanol, and transition metals may interact before collection of bile into the chelator solution is probably only about 2 min, but when bile containing appropriate concentrations of POBN and ethanol was incubated for 4 min no 1-hydroxyethyl radical adducts were detected (Fig. 5C). Secondly, POBN spin adducts were not observed in plasma, blood (Fig. 6), or perfusate (see Results), even though high concentrations of POBN and ethanol were present, along with trace concentrations of transition metals, in these fluids. Finally, artifactual signals might be expected to vary directly with the concentrations of the reactants. But in these studies, the 1-hydroxyethyl radical adducts increased with time (Fig. 1), even though concentrations of POBN and ethanol in the bile (Figs. 1 and 2, legends) were relatively constant or decreasing. The delayed appearance of the spin adducts in bile might be consistent with delayed secretion from hepatocytes, but this seems unlikely because of the rapid appearance of both POBN and ethanol (see Results). It is conceivable that ethanol initiates a sequence of events in hepatocytes that accelerates radical formation over a period of time, but this possibility requires additional study.

As stated previously, studies with liver microsomes have indicated that hydrogen peroxide, superoxide, and transition metals may be important catalytic intermediates for 1-hydroxyethyl radical formation. These intermediates also have been postulated to have a role in ethanol-induced oxidative stress *in vivo*. However, it has been suggested that the pres-

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ence of cytosolic enzymes, antioxidants, and iron-chelating agents might make such reactions unlikely *in vivo* (5), and this concept is supported by the difficulty of detecting 1-hydroxyethyl radicals using previous approaches. This problem has been overcome through the use of improved methods for spin trapping described in this report. It is noteworthy that the rats were not previously fed alcohol to increase the activity of microsomal enzymes (11), depleted of glutathione (12), or given a high dose of a transition metal (14) before 1-hydroxyethyl radicals could be detected, suggesting that ethanol-induced oxidative processes may be initiated routinely. Although the toxicological significance of the 1-hydroxyethyl radical has not been established, it has been shown to form immunoreactive protein adducts in *in vitro* studies (25). Because this radical has now been shown to form readily *in vivo*, the corresponding formation of radical-initiated antigens becomes a more likely possibility and should be evaluated further.

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