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Ethylene Glycol Generates Free Radical Metabolites in Rats: An ESR in Vivo Spin Trapping Investigation

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Ethylene glycol, best known as antifreeze, is most often ingested accidentally or as a substitute for alcochol by chronic alcohol abusers. The toxicity of ethylene glycol poisoning is due to its toxic metabolites rather than to ethylene glycol itself. In this study, electron spin resonance (ESR) spectroscopy has been used to study free radical generation in rats by acute ethylene glycol poisoning. The radical spin trapping technique was applied where the spin trapping agent α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) reacted with free radical metabolites to form radical adducts in vivo. The radical adducts from ethylene glycol intoxication were detected in both the bile and urine samples of male Sprague-Dawley rats. The identification of the POBN−•[^{13}C]ethylene glycol radical adduct provides for the first time direct ESR evidence for the generation of the ethylene glycol-derived radicals during acute intoxication by ethylene glycol, suggesting a new metabolic pathway. Simultaneous administration of alcohol dehydrogenase inhibitor 4-methylpyrazole with ethylene glycol resulted in an enhanced free radical generation in the bile. This report is the first evidence of ethylene glycol free radical metabolism in rats with acute ethylene glycol intoxication.

Introduction

Although ethylene glycol, best known as antifreeze and/or coolant, has long been recognized as a potentially lethal poison, it remains readily available and continues to be fatally ingested (*1*). The most common sources of ethylene glycol are automotive antifreeze and windshield deicer fluids. It is also found in many industrial solvents, detergents, corrosives, paints, and pharmaceuticals (*2*). Literature reviewed for health effects in humans shows that the exposure route most commonly associated with its adverse effects is acute oral ingestion (*1*, *2*). It is most often ingested as a suicide attempt, accidentally, or as a substitute for alcohol by chronic alcohol abusers (*1*). Fatal ethylene glycol poisonings from accidental or intentional ingestion have been reported in many studies (*1*, *3*−*5*). Although the accepted opinion that a high number of intentional ethylene glycol poisonings observed during a short period of time is uncommon, mass epidemics of poisoning from ethylene glycol have been reported (*6*−*10*).

The toxicity of ethylene glycol is due to its toxic metabolites rather than to ethylene glycol itself. Once ingested, ethylene glycol itself is rapidly absorbed from the stomach and distributed throughout the body, with peak serum levels seen within 1−4 h (*1*, *2*). It is quickly converted in the liver and kidneys to toxic metabolites. Ethylene glycol is metabolized to glycolaldehyde, and then through glycolic acid to glyoxylate, which is then metabolized to formate and oxalate prior to its ultimate elimination as respiratory CO_2 (*1*, *2*, *11*−*17*). The formation of oxalate crystals is associated with renal toxicity encountered after exposure to ethylene glycol (*1*, *2*, *18*). Since formic acid is the major product of metabolism, the importance of the severe metabolic acidosis produced by ethylene glycol poisoning has been well documented (*2*, *14*, *18*).

Although rats are less susceptible than humans to ethylene glycol poisoning, ethylene glycol consumption has been reported to be fatal to laboratory animals when administered in their drinking water or as an additive in their diet (*19*, *20*). The specific toxicity has been attributed to the direct effect of ethylene glycol on the central nervous system or to the toxicity of its oxidation products glycolaldehyde, glycolate, glyoxalate, and oxalate (*6*, *18*, *20*−*23*). Experiments with oxidation of ethylene glycol to formaldehyde by rat liver microsomes suggested that ethylene glycol is oxidized to formaldehyde by an oxidant derived from H_2O_2 and non-heme iron (*16*). A number of recent investigations into the mechanism of ethylene glycol toxicity have demonstrated that ethylene glycol metabolites cause inhibition of the synthesis of RNA in the cell nucleus (*24*, *25*).

Studies using alcohol dehydrogenase inhibitors in animals and also in human poisonings have shown that inhibition of ethylene glycol metabolism will reverse the toxicity, thus confirming the role of metabolites (*26*−*29*). For example, in their studies on the effect of pyrazole on ethylene glycol toxicity, Chou and Richardson (*28*) demonstrated significant reduction in the mortalities observed in rats.

The data presented here utilize the ESR spin-trapping approach for the detection of free radical metabolites generated during acute ethylene glycol intoxication. We demonstrate that free radical formation in an animal model of acute poisoning by ethylene glycol occurs from both ethylene glycol and lipids. This is the first report of direct evidence for the in vivo induction of free radical

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generation by acute ethylene glycol poisoning.

Materials and Methods

α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), 2,2′-dipyridyl (DP), bathocuproinedisulfonic acid, disodium salt hydrate (BC), and 4-methylpyrazole were purchased from Sigma Chemical Co. (St. Louis, MO). Ethylene glycol was obtained from Aldrich Chemical Co. (St. Louis, MO). [$^{13}C_2$]Ethylene glycol (minimum of 99 at. % $^{13}C_2$) was from Icon Services Inc. (Summit, NJ).

Sprague-Dawley male rats (400−450 g, Charles River Breeding Laboratories, Raleigh, NC) fed a standard chow mix (NIH open formula, Zeigler Brothers Inc., Gardner, PA) were used in all experiments. Nonfasted rats were anesthetized (50 mg of Nembutal/kg of body weight) and bile ducts were cannulated with a segment of PE 10 tubing (Becton Dickinson & Co., Sparks, MD). All animals were given an ip injection of the spin trap POBN (1.5 g/kg) dissolved in deionized water. Experimental groups were given intragastric injections of ethylene glycol (4.5 g/kg, 1.5 or 0.5 g/kg ip). Where indicated, rats were treated with Desferal (50 mg/kg ip) 60 min prior to ethylene glycol treatment.

Bile samples (400 μL) were collected every 20 min for 2 h into plastic Eppendorf tubes containing 50 μL of a solution of DP (30 mM) and BC (30 mM) (*30*). Bile samples were frozen on dry ice immediately after collection and stored at −70 °C until ESR analysis was performed.

Urine samples (500 μL) were collected from the bladder into 50 μL of a solution of DP (30 mM) and BC (30 mM) 2 h after the injection of the alcohol and the spin-trapping agent. Urine samples were frozen on dry ice immediately after collection and stored at −70 °C until ESR analysis was performed.

ESR spectra were recorded on an EMX spectrometer equipped with a super high-Q cavity. The instrumental conditions are indicated in the figure legends. Spectra were recorded on an IBM-compatible computer interfaced with the spectrometer. Hyperfine coupling constants were determined with a spectral simulation program (*31*).

Serum enzyme activities of lactic dehydrogenase (LDH), alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), and 5′-nucleotidase (5′-NT) and the serum concentration of total bile acids (TBA) were measured at NIEHS in the Laboratory of Experimental Pathology.

For all the experiments in which the results of the experimental group were compared with controls, an analysis of variance was applied. P values of ≤ 0.05 were taken to indicate a significant difference.

Results

The generation of ESR spectra of POBN radical adducts in the bile of rats after acute intragastric ethylene glycol poisoning is shown in Figure 1. The spectra were detected in rat bile collected between 60 and 80 min after the administration of ethylene glycol and POBN (Figure 1A). When the experiment was repeated without the alcohol, only a residual signal of the POBN radical adduct was observed, thereby confirming the alcohol dependence of the radical formation (Figure 1B). Likewise, there was no ESR signal of a radical adduct without spin trap injection into the bile of the rat treated with ethylene glycol (Figure 1C). The collection of bile samples into solutions of DP and BC was used to prevent ex vivo free radical formation by trace transition metals (*30*). All spectra that are shown were from bile samples collected into an aqueous solution of DP and BC. We found that to achieve an ESR signal different from that of the control, the concentration of ethylene glycol needed to be at least 1.5 g/kg ig (Figure 2).

Experiments were performed with ^{13}C-substituted ethylene glycol since isotope substitution techniques allow

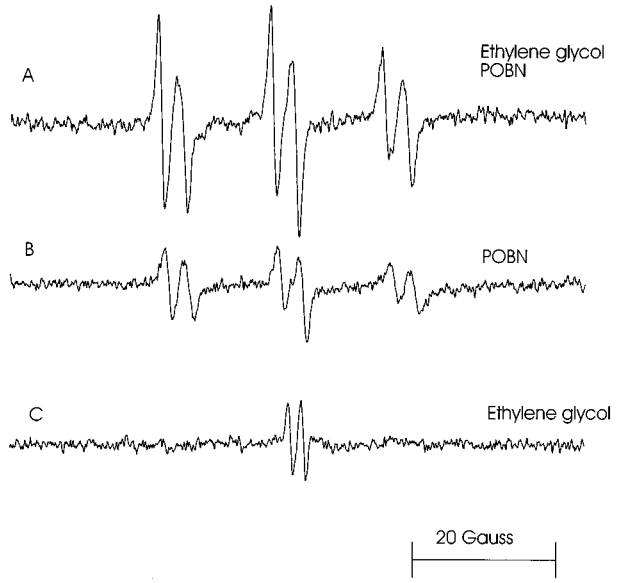

Figure 1. (A) ESR spectrum of radical adducts detected in the bile of rats 60−80 min after intragastric administration of ethylene glycol (4.5 g/kg) and POBN (1.5 g/kg). (B) Same as for panel A, but rats were not administered ethylene glycol. (C) Same as for panel A, but rats were not administered POBN. Instrumental settings of the Bruker EMX spectrometer were as follows: microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 0.6 s; and time constant, 1.3 s.

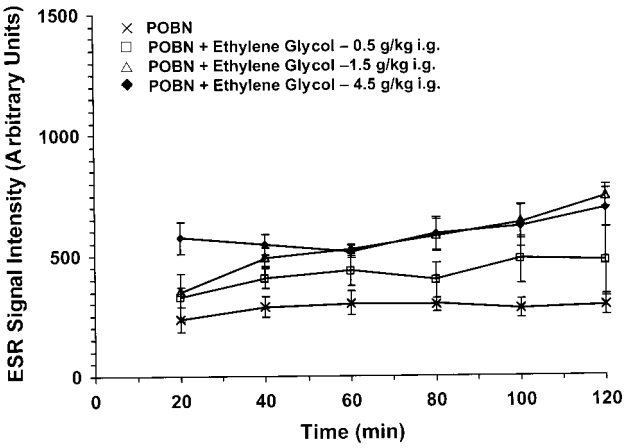

Figure 2. Time course for the formation of POBN/•CH(OH)-CH_2OH radical adduct in bile following the dosing of rats with POBN (1.5 g/kg i. p.) and different doses of ethylene glycol given intragastrically. The error bars represent the standard errors obtained using 6 rats/group.

unambiguous identification of free radicals trapped in complex biological systems. As shown in Figure 3A, when [^{13}C]ethylene glycol was used in place of isotopically normal ethylene glycol, a 12-line spectrum was detected instead of the 6-line spectrum of Figure 1A. The detection of a 12-line spectrum due to the ^{13}C hyperfine coupling constant proved that the trapped radical arises from the ^{13}C-labeled parent compound. The computer simulation of this spectrum showed that it clearly contains a signal from the POBN−•^{13}CH(OH)$^{13}CH_2OH$ adduct, which is the dominant species (Figure 3B). However, two radical species were used for the simulation of the spectrum shown in Figure 1A. The two radical species used to calculate the spectrum shown in Figure 2B are shown in panels C and D of Figure 3.

The POBN−•^{13}CH(OH)CH_2OH radical adduct is characterized by the following hyperfine coupling constants:

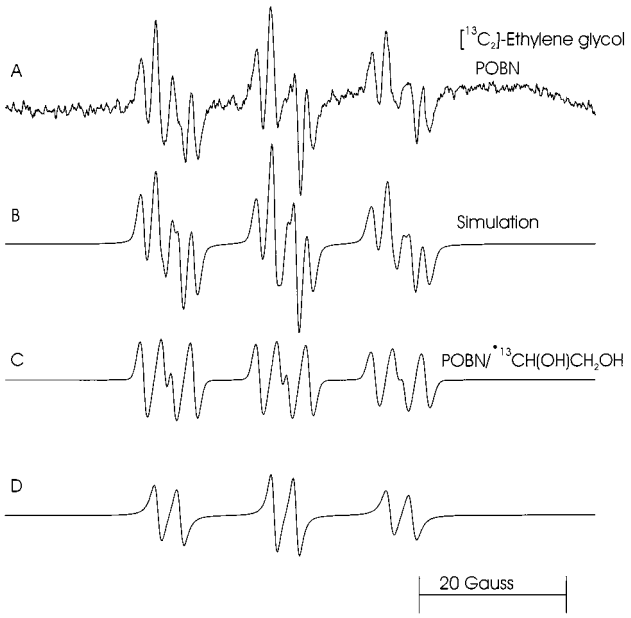

Figure 3. (A) ESR spectrum of radical adducts detected in bile of rats 1 h after administration of [$^{13}C_2$]ethylene glycol (5 g/kg ig) and POBN (1.5 g/kg ip). (B) Computer simulation of the spectrum shown in panel A. (C) Simulation of the relative contribution of the POBN−•^{13}CH(OH)CH$_2$OH radical adduct to the spectrum shown in panel A. (D) Simulation of the relative contribution of the second POBN radical adduct to the spectrum shown in panel A. Instrumental settings of the Bruker EMX spectrometer were as follows: microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 0.6 s; and time constant, 1.3 s.

$a^N = 15.65 \pm 0.08$ G, $a_\beta^H = 2.97 \pm 0.04$ G, and $a_\beta^{13C} = 3.96 \pm 0.03$ G ($n = 4$). The computer simulation of the second radical species (Figure 3D) present in the bile from ^{13}C-labeled ethylene glycol and POBN-treated rat showed the following hyperfine coupling constants: $a^N = 15.69 \pm 0.04$ G and $a_\beta^H = 2.89 \pm 0.09$ G ($n = 4$). The hyperfine coupling constants for this last radical species were virtually identical to the hyperfine coupling constants of the adducts detected in the bile of rats treated with POBN alone (Figure 1B). Radical adducts with these coupling constants are formed by lipid peroxidation and may be the pentyl or ethyl radical formed from the decomposition of lipid hydroperoxides (*32*).

It is possible that POBN radical adducts could be produced in vitro during bile collection and sample handling. To investigate this possibility, further control experiments were carried out. When [$^{13}C_2$]ethylene glycol was administered in vivo, but POBN (20 mM) was placed in the collection tube with DP and BC, no 12-line spectrum could be detected (Figure 4A). When POBN was administered in vivo and [$^{13}C_2$]ethylene glycol (1 mM) was added ex vivo to the collection tube instead of being administered in vivo, no 12-line spectrum was detected (Figure 4B). In addition, when [$^{13}C_2$]ethylene glycol was added in vitro to bile from nontreated rats containing POBN (20 mM), DP (30 mM), and BC (30 mM), no 6- or 12-line ESR signal could be detected (Figure 4C). These findings demonstrate that the signal detected in the bile of rats acutely intoxicated with ethylene glycol must be produced in vivo before sample collection. All these control experiments demonstrate that the trapping of free radicals by POBN occurred in vivo and not during sample collection.

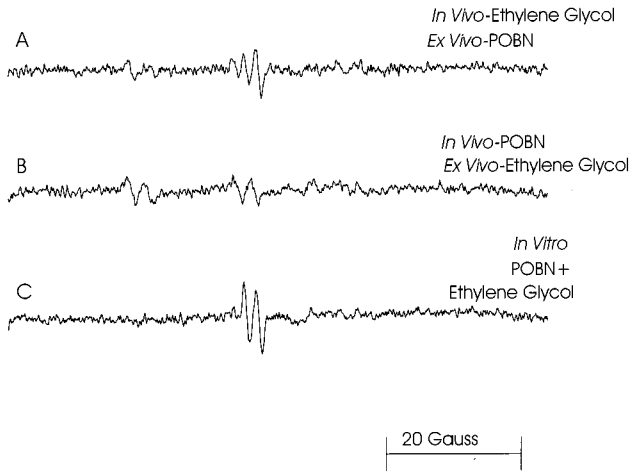

Figure 4. (A) ESR spectrum of radical adducts detected in the bile from a rat when [$^{13}C_2$]ethylene glycol was administered in vivo (5 g/kg ig), but POBN (20 mM) was added to the collection tube containing DP (30 mM) and BC (30 mM). (B) Same as for panel A, but POBN (1.5 g/kg) was injected in vivo and [$^{13}C_2$]-ethylene glycol was added to the collection tube (1 mM). (C) ESR spectrum detected in the bile of a nontreated control rat containing POBN (20 mM), [$^{13}C_2$]ethylene glycol (1 mM), DP (30 mM), and BC (30 mM). Instrumental settings of the Bruker EMX spectrometer were as follows: microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 0.6 s; and time constant, 1.3 s.

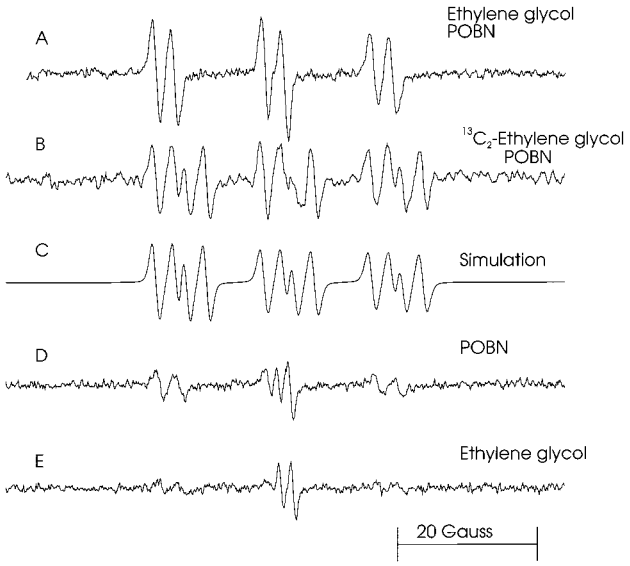

Figure 5. (A) ESR spectrum of radical adducts detected in the urine samples from rats 2 h after administration of ethylene glycol (4.5 g/kg ig) and POBN (1.5 g/kg ip). (B) Same as for panel A, but rats were administered [$^{13}C_2$]ethylene glycol (5 g/kg ig). (C) Computer simulation of the spectrum shown in panel B. (D) Same as for panel A, but rats were not administered ethylene glycol. (E) Same as for panel A, but rats were not administered POBN. Instrumental settings of the Bruker EMX spectrometer were as follows: microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 0.6 s; and time constant, 1.3 s.

When ESR measurements were performed on the urine samples collected from the same rats that had been treated with ethylene glycol, 6-line ESR signals of POBN radical adducts were detected (Figure 5A). If similar experiments were carried out with [$^{13}C_2$]ethylene glycol, additional hyperfine splittings of the spectra were obtained from the ^{13}C in the urine samples, and the 12-line spectra that were detected are shown in Figure 5B. The hyperfine coupling constants for the simulated

spectrum of POBN radical adduct in a urine sample from an ethylene glycol-poisoned rat, reflecting a small solvent effect relative to bile, were as follows: $a^N = 15.44$ G, $a_\beta^H = 2.84$ G, and $a_\beta^{13C} = 4.37$ G (Figure 5C). In contrast to the results with bile, no second species was necessary to obtain a satisfactory simulation of the spectrum detected in the urine. Only a weak background ESR signal from POBN could be detected in the urine sample from the control rats with the injection of the spin trap alone (Figure 5D). The formation of radical adducts was totally dependent on the presence of the spin-trapping agent POBN (Figure 5E). The results demonstrate that the radical species trapped by POBN in vivo in the urine from ethylene glycol-challenged rats were unambiguously derived from the corresponding glycol.

We investigated the effects of Desferal on free radical generation induced by ethylene glycol intoxication. One hour after pretreatment with Desferal and 2 h after treatment with ethylene glycol ($n = 6$), the intensity of ESR signals detected in the bile following the administration of Desferal (440 ± 91 arbitrary units) was comparable to that detected following the administration of ethylene glycol alone (692 ± 83 arbitrary units). Additional experiments with [^{13}C]ethylene glycol after pretreatment with Desferal confirmed this result (spectrum not shown).

4-Methylpyrazole (3 mmol/kg) administered to rats as a single dose simultaneously with ethylene glycol (4.5 g/kg) monotonically increased the bile ESR signal intensity with time (Figure 6). The administration of 4-methylpyrazole to ethylene glycol-treated rats did not result in statistically significant enhanced radical adduct generation in the urine samples (data not shown) probably because the ESR signal intensity in the urine samples varied tremendously from animal to animal.

To determine whether liver damage occurred during ethylene glycol poisoning, the activities of hepatic enzymes in blood serum were measured. There were no statistically significant differences in the enzyme activities studied between the control group and the rats poisoned with ethylene glycol (data not shown).

Discussion

This is the first study to demonstrate free radical formation during ethylene glycol intoxication. The experiments described above provide evidence that the POBN radical adduct detected in bile and urine is derived from ethylene glycol.

It is well documented that the toxicity of ethylene glycol results from its metabolism to more toxic metabolites (*1*, *2*, *18*) which may affect the ultrastructure of hepatocytes and the endoplasmic reticulum (*24*, *25*). As already mentioned in the Introduction, the generally accepted pathway of ethylene glycol metabolism follows initial oxidation catalyzed by alcohol dehydrogenase to glycolaldehyde and oxidation of glycolate to glyoxalate catalyzed by glycolic acid oxidase (*28*). Thus, ethylene glycol and glycolate are excreted in the urine because their concentration exceeds the capacity of the enzymes that are available for their oxidation. Glycolaldehyde and glyoxylate fail to accumulate in the plasma because they are readily metabolized (*28*).

According to the ESR spin trapping experiments presented in this paper, it is suggested that a new free radical metabolic pathway exists in addition to that already known for the metabolism of ethylene glycol. The ESR evidence for ethylene glycol-derived radical metabolites in vivo during acute ethylene glycol intoxication is provided by the spin trapping of the radical adduct from the metabolized ^{13}C-labeled ethylene glycol. The isotope substitution of ethylene glycol with ^{13}C allowed the unique and unambiguous assignment of the POBN$-^{\bullet 13}$-CH(OH)CH$_2$OH radical adduct in the bile of rats during acute ethylene glycol intoxication. Pulse radiolysis studies have determined that this 1,2-dihydroxylethyl radical reacts with molecular oxygen to form the corresponding peroxyl radical at a nearly diffusion-limited rate of 4.9×10^9 M^{-1} s^{-1} (*33*).

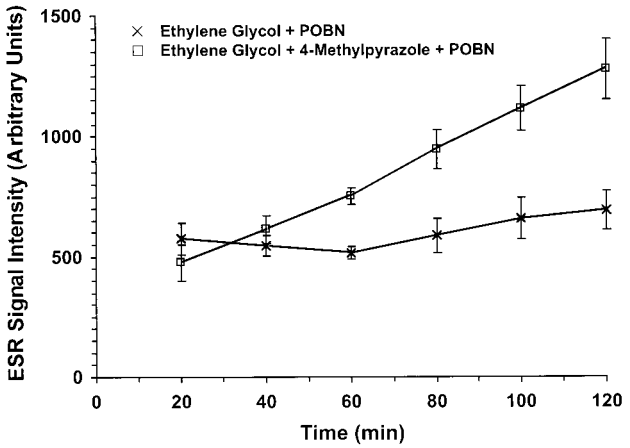

Figure 6. Time course for the formation of free radical adducts in bile following the administration to rats of POBN (1.5 g/kg ip) and either ethylene glycol (4.5 g/kg ig) or ethylene glycol (4.5 g/kg ip) and 4-methylpyrazole (3 mmol/kg ip). The error bars represent the standard errors obtained using six rats per group.

$$HOCH_2^{\bullet}CH(OH) + O_2 \rightarrow HOCH_2CH(OH)OO^{\bullet} \quad (1)$$

The peroxyl radical is known to eliminate protonated superoxide ($^{\bullet}HO_2$) to form an aldehyde (*34*).

$$HOCH_2CH(OH)OO^{\bullet} \rightarrow {}^{\bullet}HO_2 + HOCH_2CH(O) \quad (2)$$

Because the concentration of glutathione and protein sulfhydryl groups in vivo is much greater than the oxygen concentration, hydrogen atom abstraction to regenerate the ethylene glycol and form a thiyl radical may be the more important reaction (*35*, *36*).

$$HOCH_2^{\bullet}CH(OH) + GSH \rightarrow HOCH_2CH_2 + GS^{\bullet} \quad (3)$$

Since the glutathione thiyl radical (GS$^{\bullet}$) also ultimately forms oxygen-derived radicals, both reactions will generate reactive oxygen species.

Evidence for the generation of free radical metabolites by ethylene glycol has been provided by the control experiments, which demonstrated that the radical adduct formation was dependent on ethylene glycol intoxication and was not formed ex vivo during sample collection and handling. Further evidence for the generation of free radical metabolites during ethylene glycol poisoning has been provided by the direct ESR detection of radical adducts in the urine. Again, the results with [^{13}C]-ethylene glycol unambiguously demonstrated that the radical species trapped by POBN in vivo and detected in the urine from ethylene glycol-challenged rats was derived from the corresponding glycol.

It is known from other studies that ethylene glycol increases the level of lipid peroxidation products formed in rats during intoxication (*21*−*23*). Therefore, the suggested mechanism of ethylene glycol toxicity involves induction of lipid peroxidation since the generated peroxyl radical (reaction 1) can abstract hydrogen from lipid molecules. The 6-line radical adduct that was not derived from ethylene glycol may be that of a lipid hydroperoxide-derived radical, but no definitive assignment can be made with the available data.

There are numerous experimental and clinical case reports on the protective effect of pyrazole compounds against ethylene glycol toxicity (*26*−*29*). The enhanced free radical signal detected in the combination of 4-methylpyrazole and ethylene glycol simultaneously injected into the animal strongly suggests that ethylene glycol metabolism to a free radical competes with alcohol dehydrogenase, since 4-methylpyrazole would inhibit the activity of the non-radical-forming alcohol dehydrogenase so that more substrate would be available for the free radical metabolism. We suggest that free radical generation by ethylene glycol is an additional minor pathway in its total metabolism and is not responsible for its acute toxicity, which 4-methylpyrazole prevents.

We also investigated the activities of rat liver enzymes as indicators of liver damage. Two hours after acute ethylene glycol poisoning, the rat liver enzyme activities generally showed no change (data not shown). These results demonstrated that free radical generation occurred without liver damage in this model of acute ethylene glycol poisoning.

In summary, the findings demonstrate in vivo generation of ethylene glycol-derived free radical metabolites, which suggests a new metabolic pathway for ethylene glycol metabolism.

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