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Nucleic Acid Alkylation by Free Radical Metabolites of Ethanol. Formation of 8-(1-Hydroxyethyl)guanine and 8-(2-Hydroxyethyl)guanine Adducts

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Alcohol consumption is associated with an increased risk of several types of malignancy by mechanisms that remain to be elucidated. Most of the ingested ethanol is converted to acetaldehyde but the formation of free radical metabolites such as the 1-hydroxyethyl radical has been also demonstrated to occur in vitro and in vivo. Here we tested the possibility of ethanol-derived free radicals alkylating nucleic acid and nucleic acid components. Ethanol oxidation by Fenton systems has been extensively used to mimic ethanol metabolism to free radical intermediates and it was also employed in our studies. Two adducts, 8-(1-hydroxyethyl)-guanine and 8-(2-hydroxyethyl)guanine, were isolated in incubations containing guanine/ethanol/hydrogen peroxide/iron(II) at pH 1 under anaerobic conditions. The adducts were produced in comparable yields and were characterized by ultraviolet absorption, mass spectrometry, and proton nuclear magnetic resonance spectroscopy. Both adducts were also produced in incubations containing DNA and RNA at pH 4 and 7. Under these conditions, the obtained yields of 8-(1-hydroxyethyl)guanine were about 10 times higher than those of 8-(2-hydroxyethyl)guanine. Higher yields of both adducts were obtained at pH 4 than at pH 7 and with RNA as compared with DNA. As expected, nucleic acid oxidation products such as 8-oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydroadenine were also produced under the employed experimental conditions. Their yields tended to increase in the presence of ethanol, particularly at pH 4, suggesting that ethanol can protect oxidized bases from further degradation. Parallel spin-trapping experiments with α-4-pyridyl-1-oxide *N-tert*-butylnitrone and 3,5-dibromo-4-nitrosobenzenesulfonic acid confirmed that ethanol was oxidized to both the 1-hydroxyethyl and 2-hydroxyethyl radicals by hydrogen peroxide/iron(II) at pH 4−7 in the presence and in the absence of nucleic acids. The results demonstrate that free radical metabolites of ethanol can alkylate nucleic acids in vitro. Both the 1-hydroxyethyl and 2-hydroxyethyl radicals may play a role in ethanol-mediated toxicity.

Introduction

Alcohol consumption is associated with an increased risk of several types of malignancy by mechanisms that remain to be elucidated. Most of the metabolized ethanol is converted to acetaldehyde by enzymatic processes. Metabolism of ethanol to free radical intermediates, however, has also been demonstrated to occur both in vitro and in vivo. As a consequence, toxic effects of ethanol have been attributed to acetaldehyde, reactive oxygen species, and the 1-hydroxyethyl radical (*1*−*10*). This species has been detected by EPR[1] spin-trapping experiments in vitro and in vivo (*6*−*10*). Relevantly, alkylation of proteins by the 1-hydroxyethyl radical has been shown to induce immunologic responses in alcoholic patients (*11*−*13*), although the structure of the adduct remains to be characterized.

A role for nucleic acid alkylation by the 1-hydroxyethyl radical in the genotoxic effects of ethanol has rarely been considered in the literature (*8*, *14*). However, DNA alkylation by 1-hydroxycarbon radicals produced from photochemical reactions of alcohols other than ethanol has been described since the early 1970s (see, for instance, refs *15*−*17*). These species, as is the case of other free radicals, attack purine bases by substituting at their C8 position. More recently, analogous C8-substituted guanine adducts have been demonstrated to be formed from DNA attack by carbon-centered radical metabolites of genotoxic compounds such as methylated hydrazines (*18*, *19*), *tert*-butylhydroperoxide (*20*), diazoquinones (*21*), and arenediazonium ions (*22*). These alkylated guanines have also been shown to be produced in vivo. 8-Methylguanine was isolated from the colon and liver DNA of rats treated with 1,2-dimethylhydrazine (*19*) and 8-(*p*-methylphenyl)guanine, 8-(*p*-methoxymethylphenyl)guanine, and 8-(*p*-hydroxymethylphenyl)guanine were isolated from DNA from keratinocytes and C50 cells treated with the corresponding aryldiazonium ions (*22*). Both 8-methylguanine (*23*) and 8-phenylguanine (*24*) have recently been incorporated into oligodeoxyribonucleotides and shown to be miscoding lesions capable of

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[1] Abbreviations: DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; 8-(1-HE)Gua, 8-(1-hydroxyethyl)guanine; 8-(2-HE)Gua, 8-(2-hydroxyethyl)guanine; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EPR, electron paramagnetic resonance; 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxoAde, 8-oxo-7,8-dihydroadenine; POBN, α-4-pyridyl-1-oxide *N-tert*-butylnitrone.

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generating G-T and G-C transversions and deletions in vitro.

In the above context, we considered it important to examine the possibility of ethanol-derived free radicals alkylating nucleic acids. Ethanol metabolism to the 1-hydroxyethyl radical is considered to be dependent on both enzymatic and nonenzymatic processes (*6*−*10*). In the latter case, the involvement of hydrogen peroxide and traces of transition metal ions has been well established, leading to the widespread use of Fenton systems to mimic ethanol metabolism (see, for instance, refs *11*−*13*). Here we demonstrated that ethanol oxidation by iron(II)/hydrogen peroxide produces both the 1-hydroxyethyl and the 2-hydroxyethyl radicals which are able to alkylate guanine residues in DNA and RNA.

Experimental Procedures

Chemicals. Chelex-100, guanine, DNA (calf thymus, type I), RNA (calf liver), α-4-pyridyl-1-oxide *N*-*tert*-butylnitrone (POBN), DMSO-d_6, and [1-^{13}C]ethanol were obtained from Sigma Chemical Co. (St. Louis, MO). Ethanol, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), and ferrous sulfate were obtained from Merck (Darmstadt, Germany). Hydrogen peroxide was obtained from Fisher Scientific (Pittsburgh, PA). Deuterium oxide and 8-oxoGua were from Aldrich (Milwaukee, WI) and 8-oxoAde was provided by Professor J. Cadet (Service de Chemie Inorganique et Biologique, CEA, Grenoble, France). 3,5-Dibromo-4-nitrosobenzenesulfonic acid (DBNBS) was synthesized as previously described (*25*). Stock solutions of ferrous sulfate were prepared in sulfuric acid to minimize autoxidation (*26*). DNA and RNA concentrations were determined by UV spectroscopy at 260 nm, using the relations $A = 1$ for 50 μg/mL and 40 μg/mL for DNA and RNA, respectively (*27*). All solutions were prepared in distilled water treated in a Millipore Milli-Q system; when necessary, the pH was adjusted by the addition of HCl or NaOH.

Guanine Alkylation. The standard incubation mixture (250 mL, final volume) contained guanine (1.5 mM), ethanol (75 mM), ferrous sulfate (6.0 mM), and hydrogen peroxide (4.5 mM) at pH 1 under a nitrogen flux. After 30 min of incubation at room temperature, the pH was increased to 11−12 with NaOH to precipitate excess iron ions that were removed by filtration (*19*, *28*). The obtained supernatant was acidified to pH 4−5 and submitted to treatment with Chelex-100 for 10−12 h to completely remove iron ions. After filtration the sample was analyzed by analytical HPLC and the products were purified in a two-step semipreparative chromatography. In this case, the HPLC system consisted of a Waters Associates model 501 equipped with a Rheodyne injector, a UV detector (Waters 486), and a C18 μBondapak (19 × 150 mm) column. The first separation was performed at 40 °C with water/methanol (99:1 v/v) at a flow rate of 5 mL/min as the mobile phase. Fractions corresponding to the products were collected from several injections, pooled together, concentrated, and resubmitted to chromatographic separation by changing the mobile phase to diluted formic acid, pH 3.2, at a flow rate of 5 mL/min. Each product was collected from several injections of the enriched fractions. The pooled samples were concentrated under reduced pressure and lyophilized to dryness. The white solids were further dried under vacuum and submitted to spectroscopic analysis.

Analytical chromatography was performed with a Waters system model 625 LC equipped with a Rheodyne injector, a photodiode array detector (Waters 991), and an electrochemical detector (Waters 460).

Nucleic Acid Alkylation and Oxidation. The standard incubation mixture (3 mL final volume) contained calf thymus DNA (1.3 mg/mL) or liver RNA (1.3 mg/mL), ethanol (100 mM), ferrous iron (1 mM), and hydrogen peroxide (4 mM) in water at pH 4 or 7 under a nitrogen flux; the pH was adjusted by the addition of HCl or NaOH. After 15 min of incubation at room temperature, the reaction was stopped by the addition of 1 mL of NaCl (5 M) and 8 mL of cold ethanol (−20 °C), and the nucleic acids were allowed to precipitate at −20 °C. The supernatant was then removed and the pellet was washed with cold 70% (v/v) ethanol. The pellet was dried under vacuum and submitted to acid hydrolysis. DNA (1.5 mg/mL) was resuspended in 0.1 M HCl and heated at 70 °C for 60 min (*29*). RNA (1.5 mg/mL) was resuspended in 1.0 M HCl and was heated at 95 °C for 60 min (*30*). The acid hydrolysates were analyzed by analytical HPLC with the above-described system by the use of both UV (254 nm) and electrochemical (0.81 V) detectors. Chromatographic separation was carried out with two C18 Resolve columns (3.9 × 150 mm) in series. The mobile phase was constituted of 50 mM KH_2PO_4 (pH 5)/methanol (99:1 v/v), at a flow rate of 0.5 mL/min.

To analyze 8-oxoGua, parallel HPLC runs were performed in a system constituted by a Shimadzu pump (model LC 10AD), a Waters UV detector (model 484), and a Shimadzu electrochemical detector (L-ECD 6A) working at 0.60 V. Separation was achieved with a C18 μBondapak (3.9 × 300 mm) column by using as the mobile phase 50 mM KH_2PO_4 (pH 5)/methanol (99:1 v/v), at a flow rate of 0.5 mL/min.

Quantitation of all detected adducts was performed by integration of the corresponding HPLC peaks and comparison of the areas obtained with those obtained for the standards under the same HPLC conditions.

The hydrodynamic voltammograms were obtained by injecting 30 ng of the adduct (or 45 μg of DNA when analyzing nucleic acid samples) in the analytical HPLC system at different oxidation potentials.

Spectroscopic Measurements. ^{1}H NMR spectra were recorded on Bruker AC 200 and DPX 300 spectrometers. Electrospray mass spectra were run on a Micromass Quattro II tandem mass spectrometer, operating in the positive ionization mode; the compounds were dissolved in acetonitrile/water (90:10 v/v) to about 10 ng/μL and formic acid was added to a final concentration of 0.1% (v/v). UV absorption spectra were recorded with a Hitachi 2000U spectrophotometer. To determine the extinction coefficients, solution concentrations were calculated by integration of the ^{1}H NMR spectra in D_2O using DSS as quantitative standard. EPR spectra were recorded at room temperature on a Bruker ER 200 D-SRC or a Bruker EMX spectrometer. The concentration of radical adducts was estimated by using 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy radical as standard.

Results

Guanine Alkylation. To examine nucleic acid alkylation by ethanol-derived free radicals it was necessary to synthesize standards. The reported preferential attack of guanine residues by free radical metabolites (*14*−*17*, *30*) led us to adapt previously reported procedures to alkylate guanine by free radicals generated in Fenton systems at acidic pHs (*20*, *28*). Incubations of guanine (1.5 mM) with ethanol (75 mM), hydrogen peroxide (4.5 mM), and iron(II) (6 mM) at pH 1 under nitrogen led to the formation of two main products as attested by HPLC analysis (Figure 1). These compounds were purified (see Experimental Procedures) and fully characterized as 8-(2-hydroxyethyl)guanine (20.3 min retention time) and 8-(1-hydroxyethyl)guanine (25.6 min retention time) (Figure 1; see structures in Figure 2). Indeed, all features of the ^{1}H NMR spectra of the purified adducts in DMSO-d_6 (Table 1) and in D_2O (not shown) are consistent with the inferred structures, including the absence of the H8 guanine absorption at ca. 7.8 ppm (*30*) that demonstrates substitution at the C8 position of guanine. The obtained electrospray mass spectra confirmed the adduct struc-

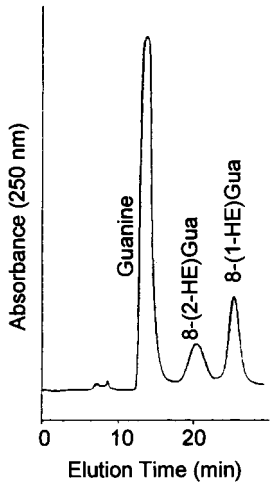


Figure 1. HPLC profile of the spent reaction mixture of guanine treated with iron(II), hydrogen peroxide, and ethanol. Guanine (1.5 mM) was incubated with ferrous sulfate (6 mM), hydrogen peroxide (4.5 mM), and ethanol (75 mM) in water at pH 1 for 15 min at room temperature under a nitrogen flow. The chromatographic separation was carried out with a μBondapak C18 column eluted with water/methanol (99:1 v/v) at 40 °C. The flow rate was 5 mL/min from 0 to 15.5 min and 2.5 mL/min from 15.6 min to the end of the run.

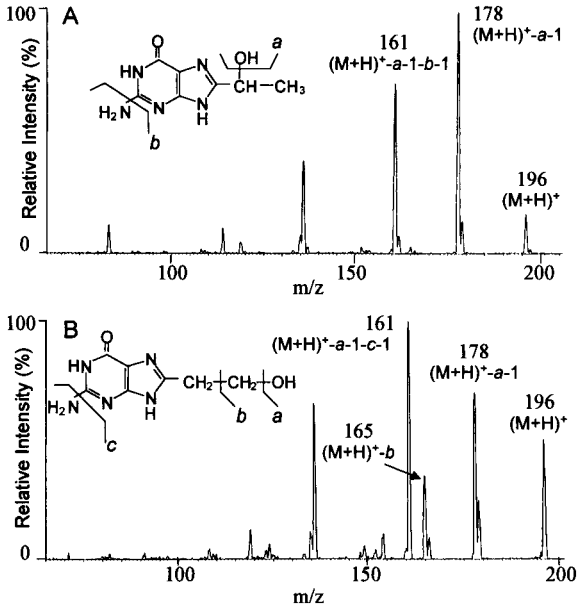


Figure 2. Electrospray mass spectra of 8-(1-hydroxyethyl)-guanine (A) and 8-(2-hydroxyethyl)guanine (B). Spectra were obtained from the compounds purified from incubations of guanine, iron(II), hydrogen peroxide, and ethanol. Instrumental conditions were as described under Experimental Procedures.

tures (Figure 2). Both compounds have the same molecular weight (195) and a similar fragmentation pattern. This includes the sequential loss of H_2O and NH_2 to produce the 161 *m/z* fragment (Figure 2) as proved by the presence of the latter in the MS-MS of the 178 *m/z* fragment (not shown). Sequential fragmentation of an ethenoguanine adduct during electrospray ionization has been described before (*31*). The UV spectra of the adducts demonstrated that guanine aromaticity is preserved in the adducts (Figure 3, inset), and the calculated extinction coefficients (Table 2) were in the range of those previously determined for other 8-alkyl-substituted guanine adducts (*30*).

Table 1. ^{1}H NMR Chemical Shifts of 8-(1-Hydroxyethyl)guanine and 8-(2-Hydroxyethyl)guanine[a]

compound	assigned protons[b]	δ (ppm)	multiplicity[c]	J (Hz)
8-(1-HE)Gua	CH_3-	1.34	d	($-CH-CH_3$) 6.6
	$CH-$	4.62	q	
	$2\text{-}NH_2$	6.48	s	
8-(2-HE)Gua	CH_2-	2.74	t	($-CH_2-CH_2$) 7.0
	$-CH_2OH$	3.70	t	
	$2\text{-}NH_2$	6.33	s	

[a] All spectra were obtained in DMSO-d_6 (δ = 2.49 ppm), which was used as internal standard. [b] Low resolution precluded detection of protons on N1 and N9. [c] Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet.

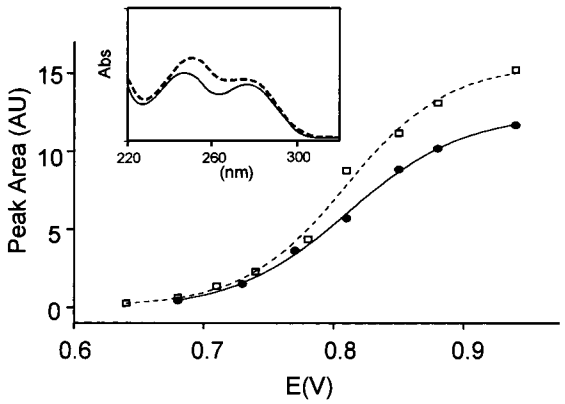


Figure 3. Hydrodynamic voltammogram of 8-(1-hydroxyethyl)-guanine (□) and 8-(2-hydroxyethyl)guanine (●) obtained under analytical HPLC conditions as described in Experimental Procedures. The mobile phase consisted of 50 mM KH_2PO_4 (pH 5)/methanol (99:1 v/v) at a flow rate of 0.5 mL/min. Peak area values were normalized to account for the quantity of injected sample. The inset shows the UV absorption spectra of 8-(1-hydroxyethyl)guanine (- - -) and 8-(2-hydroxyethyl)guanine (—) in phosphate buffer at pH 7.

Table 2. UV Absorption Spectra of 8-(1-Hydroxyethyl)guanine and 8-(2-Hydroxyethyl)guanine[a]

adduct	pH	λ_{max} (nm)	ϵ ($M^{-1}\cdot cm^{-1}$)
8-(1-HE)Gua	1	250	10 320
	7	249	9734
8-(2-HE)Gua	1	250	10 630
	7	247	8945

[a] The spectra were obtained in diluted HCl, pH 1, and 10 mM phosphate buffer, pH 7.

Another studied characteristic of 8-(1-hydroxyethyl)-guanine and 8-(2-hydroxyethyl)guanine was the hydrodynamic voltammogram (Figure 3), because of its potential utility in the characterization of the adducts in biological samples. Their oxidation potentials at half-wave were close to 0.81 V (Figure 3), a value similar to the one previously reported for 8-methylguanine (0.93 V) (*32*). Electrochemical detection of these C-8 alkyl-substituted adducts was determined to be 3 orders of magnitude more sensitive than UV absorbance (not shown). However, the high potentials required impose a limit on selectivity because guanine and several other guanine adducts that may be present in biological samples are oxidized at such potentials (*33*).

Formation of 8-(1-hydroxyethyl)guanine and 8-(2-hydroxyethyl)guanine in incubations of guanine/ethanol/iron(II)/hydrogen peroxide in similar yields (15% and 8%, respectively) (Figure 1) was a surprising result because

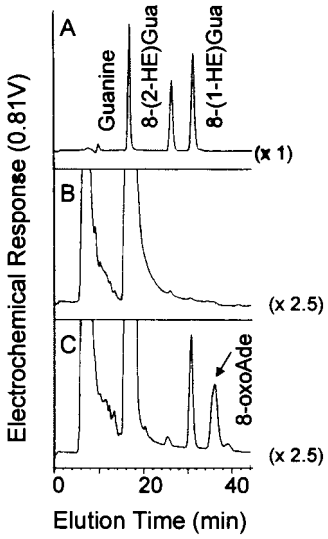


Figure 4. Representative chromatograms obtained by electrochemical detection of (A) the standard mixture of guanine (56 ng), 8-(1-hydroxyethyl)guanine (51 ng) and 8-(2-hydroxyethyl)-guanine (50 ng); (B) the acid hydrolysates of 1.3 mg/mL DNA treated with 1 mM ferrous sulfate at pH 4; and (C) the acid hydrolysates of 1.3 mg/mL DNA treated with 1 mM ferrous sulfate, 4 mM hydrogen peroxide, and 100 mM ethanol at pH 4. The HPLC injections of (B) and (C) correspond to 90 μg of initial nonhydrolyzed DNA. Conditions are as described under Experimental Procedures.

there are few reports on the formation of the 2-hydroxyethyl radical in Fenton systems (*34*−*36*) (see, also, below).

Nucleic Acid Alkylation. Incubations of calf thymus DNA (1.3 mg/mL) or liver RNA (1.3 mg/mL) with ethanol (100 mM), iron(II) (1 mM), and hydrogen peroxide (4 mM) under reduced oxygen tensions led to the formation of guanine adducts that are detectable by HPLC analysis of DNA and RNA acid hydrolysates (see, for instance, Figure 4). Both 8-(1-hydroxyethyl)guanine and 8-(2-hydroxyethyl)guanine were produced at pH 4 (Figure 4), whereas only C8-(1-hydroxyethyl)guanine was easily detectable at pH 7 (Table 3). In these cases, the adducts present in nucleic acid hydrolysates (Figure 4) were identified by comparison of their retention times, UV spectra, and/or hydrodynamic voltammograms with those of the purified standards. The relatively low quantities of 8-(2-hydroxyethyl)guanine present in hydrolysates of DNA treated at pH 7 precluded unambiguous quantification and identification of the adduct in a one-step chromatographic separation (Table 3). Under these conditions, however, the hydrolysates presented a peak that had the same retention time as authentic 8-(2-hydroxyethyl)guanine and coeluted with it.

Detection of both 8-oxoGua and 8-oxoAde under our experimental conditions (see, for instance, Figure 4 and Table 3) demonstrated that DNA and RNA were also oxidized as expected from their incubation with Fenton systems (*37*−*40*). 8-oxoAde was detectable under the chromatographic conditions of Figure 4 but parallel HPLC runs were used to detect 8-oxoGua (see Experimental Procedures). The oxidized bases present in treated nucleic acids were identified by their retention times and UV spectra. Their quantification in hydrolysates of DNA treated with iron(II)/hydrogen peroxide in the absence and in the presence of ethanol demonstrated a tendency to higher yields under the latter conditions (Table 3). These results indicate that ethanol can protect the oxidized bases from further degradation, particularly at pH 4. This is not surprising because 8-oxopurines have lower redox potentials than the parent purines (*33*) and are expected to be more susceptible to some oxidants. In agreement, 8-oxoGua has been demonstrated to be a better substrate than guanine in peroxynitrite-mediated oxidations (*41*).

The results shown in Table 3 provide yet another example that the interpretation of the effect of scavengers upon oxidative damage is not straightforward (*36*, *42*). Ethanol has been extensively used as a hydroxyl radical scavenger, i.e., to protect biomolecules from hydroxyl radical attack. However, the possibility of ethanol derived free radicals attacking the target under study, such as nucleic acids (Table 3) and proteins (*11*−*13*), has hardly been considered in analyzing the effects of ethanol as scavenger. Our results (Table 3) emphasize yet another aspect that should be taken into account, i.e., the possibility of ethanol protecting detectable oxidized products such as 8-oxoGua from further oxidation and decay to unknown products. Consequently, a scavenger of oxidizing intermediates may modify the product profile of biomolecule oxidation by various mechanisms. However, only a few studies have considered this scavenger property in the literature. The effects of ethanol in changing DNA oxidation by Fenton systems have been studied in detail by Linn and co-workers (*39*, *40*); the formation of DNA alkylation products under their experimental conditions remains to be examined.

Spin-Trapping Experiments. The formation of both 8-(1-hydroxyethyl)guanine and 8-(2-hydroxyethyl)guanine in acidic incubations of Fenton systems with guanine, DNA, and RNA was an unanticipated result. There are few reports on the formation of the 2-hydroxyethyl radical in Fenton systems (*34*−*36*) and none on its formation during ethanol metabolism (*6*−*10*). However, this was probably due to the use of spin-traps such as DMPO, PBN, and POBN, whose adducts of the 1-hydroxyethyl and 2-hydroxyethyl radical are difficult to discriminate by EPR spectroscopy. As shown in Figure 5, the radicals detected during ethanol oxidation by Fenton systems changed with the spin trap used. In the presence of DBNBS, the main detected radical is the DBNBS−2-hydroxyethyl radical adduct (a_N = 1.42 mT, a_H = 1.13 mT, and $a_{H(m)}$ = 0.075 mT) (Figure 5A) (*34*−*36*). In the presence of POBN, the main detected radical is the POBN−1-hydroxyethyl radical adduct (a_N = 1.58 mT and a_H = 0.26 mT) (Figure 5B), as confirmed by experiments with [^{13}C]ethanol labeled at C1 (Figure 5C) (*8*−*10*). In this case, the six-line EPR spectrum of the POBN−1-hydroxyethyl radical adduct was substituted by a 12-line spectrum (a_N = 1.55 mT and a_H = 0.26 mT; a_{13C} = 0.42 mT) due to the contribution of the ^{13}C atom (I = $^1/_2$) (Figure 5C). The EPR spectra of the POBN adducts, however, showed unequal peak heights (Figure 5B,C) suggesting that low quantities of another radical, most probably the POBN−2-hydroxyethyl radical adduct, also contribute to the final spectra.

It is important to note that the total yield of radicals trapped with both DBNBS and POBN was affected less than twice by the pH under our experimental conditions in the presence (Figure 5) or in the absence of DNA (not shown). The yield of the DBNBS−2-hydroxyethyl radical adduct was estimated to be 15 and 9 μM at pH 4 (Figure 5A) and pH 7, respectively. In the case of the POBN−1-hydroxyethyl radical adduct, the yields were 135 and 180 μM at pH 4 (Figure 5B) and pH 7, respectively.

Table 3. Yields of Alkylated Guanines and Oxidized Bases in Hydrolysates of Nucleic Acids Treated with Ethanol in the Presence of Iron and Hydrogen Peroxide[a]

	adducts[b]			
system	8-(1-HE)Gua (pmol/mg)	8-(2-HE)Gua (pmol/mg)	8-oxoGua (pmol/mg)	8-oxoAde (pmol/mg)
DNA/Fe(II), pH 4	nd[c]	nd	2410	nd
DNA/Fe(II)/H_2O_2, pH 4	nd	nd	2180	600
DNA/Fe(II)/H_2O_2/EtOH, pH 4	1350	190	7290	1560
DNA/Fe(II), pH 7	nd	nd	1130	nd
DNA/Fe(II)/H_2O_2, pH 7	nd	nd	3670	520
DNA/Fe(II)/H_2O_2/EtOH, pH 4	21	<5[d]	3040	649
RNA/Fe(II), pH 4	nd	nd		
RNA/Fe(II)/H_2O_2/EtOH, pH 4	3015	289		
RNA/Fe(II), pH 7	nd	nd		
RNA/Fe(II)/H_2O_2/EtOH, pH 7	226	31		

[a] Calf thymus DNA (1.3 mg/mL) or liver RNA (1.3 mg/mL) was incubated with ferrous sulfate (1 mM), hydrogen peroxide (4 mM), and ethanol (100 mM) in water for 15 min at room temperature under a nitrogen flow. Nucleic acid hydrolysis and analysis are described under Experimental Procedures. [b] Yields expressed in relation to total nucleic acid present in acid hydrolysates; average of two independent determinations. [c] Nondetectable. [d] Unambiguous quantitation and identification was not possible in one chromatographic separation as described in the text.

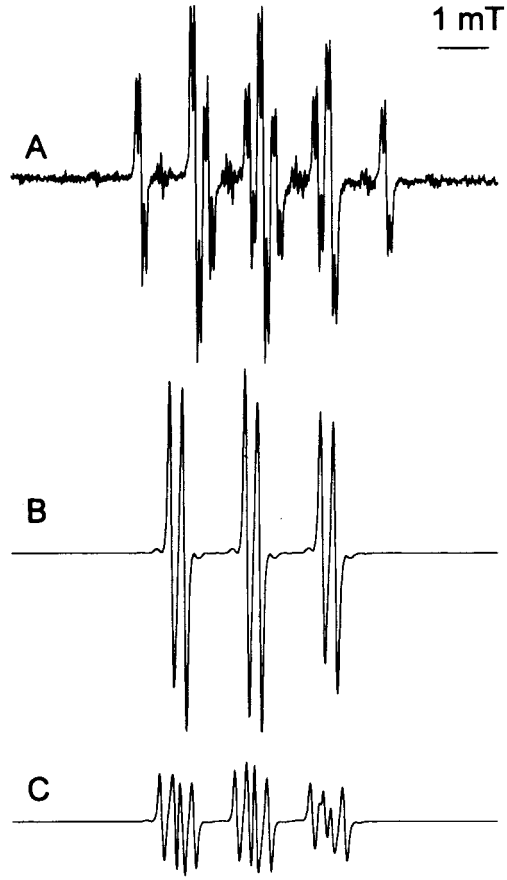


Figure 5. EPR spectra of spin trap adducts obtained during incubations of ethanol with iron(II) and hydrogen peroxide. The spectra were obtained after 1 min of incubation of 100 mM ethanol, 1 mM ferrous sulfate, 4 mM hydrogen peroxide, and 1.3 mg/mL DNA at pH 4.0 in the presence of (A) 20 mM DBNBS; (B) 100 mM POBN; and (C) 100 mM POBN after substitution of ethanol by labeled ethanol (H_3C-$^{13}CH_2OH$). Instrumental conditions: microwave power 20 mW; time constant 327.7 ms; scan rate 0.0298 mT/s; modulation amplitude 0.05 mT; gain 2.52 $\times$ 10^6 and 2.00 $\times$ 10^4 for (A) and for (B) and (C), respectively.

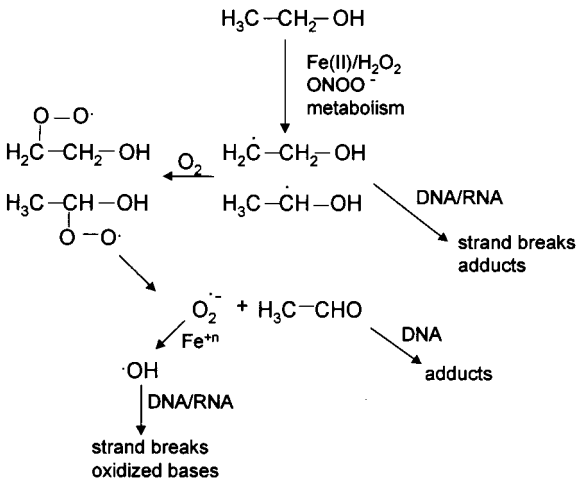
Consequently, the 10−60 times higher nucleic acid alkylation observed at pH 4 as compared to pH 7.0 (Table 3) cannot be attributed to higher yields of radicals in the bulk solution (spin-trappable radicals). Spin-trapping experiments cannot be used to estimate either total concentration of produced free radicals or local free radical concentrations (*43*, *44*) such as those produced at sites of iron chelation to nucleic acid bases (*14*, *20*, *39*, *40*, *45*).

Discussion

Our results demonstrated that oxidation of ethanol by iron(II) and hydrogen peroxide produces both the 1-hydroxyethyl and the 2-hydroxyethyl radicals (Figure 5), which are able to substitute at the C8 position of guanine (Figures 1−3; Tables 1 and 2) and guanine residues in RNA and DNA (Figure 4; Table 3) to produce the corresponding 8-(1-hydroxyethyl)guanine and 8-(2-hydroxyethyl)guanine adducts. Under all tested conditions, the extent of alkylation was higher for RNA than for DNA (Table 3), suggesting that the accessibility of guanine residues is an important factor in controlling the alkylation process. This is also supported by the increased alkylation observed at pH 4 as compared with pH 7 (Table 3). Indeed, the yields of free radicals as measured by spin-trapping experiments were not markedly affected by pH (Figure 5; see, also, Results), whereas acidic pHs are expected to favor guanine exposure to the solvent by increasing random coil regions in the DNA structure (*46*).

In the incubations containing DNA and RNA, the yield of 8-(2-hydroxyethyl)guanine was about 10% that of 8-(1-hydroxyethyl)guanine (Table 3). This is in reasonable agreement with the yields of the corresponding radicals expected to be produced by hydroxyl radical attack on ethanol. Indeed, pulse radiolysis studies have shown that the hydroxyl radical attacks ethanol, producing the 1-hydroxyethyl, 2-hydroxyethyl, and ethoxyl radicals in yields of 84.3%, 13.2%, and 2.5%, respectively (*47*). The formation of the hydroxyl radical in Fenton systems has been challenged (*39*, *40*, *48*). Still, most spin-trapping studies of ethanol oxidation by Fenton systems have described detection only of the 1-hydroxyethyl radical (*6*−*10*), indicating that it is formed at much higher yields than the 2-hydroxyethyl radical. Here, by the parallel use of two spin-traps, it was possible to demonstrate that both radicals are formed in Fenton systems (Figure 5). The relative yields of the trapped radicals were estimated and also found to be on the order of 10% (see Results). These comparisons may only be a coincidence because the adducts whose yields are being compared are different and the reaction rates of their formation (1-hydroxyethyl radical plus POBN or guanine residue; 2-hydroxyethyl radical plus DBNBS or guanine residues) are not

Scheme 1



known (see, also, Results). It should be noted that in incubations containing guanine at pH 1 the relative yield of the adducts was around 50% (Figure 1; see, also, Results). A possible explanation for the difference was the excess of iron(II) in relation to hydrogen peroxide in these incubations. This could increase the probability of the 1-hydroxyethyl radical reacting with iron(III) (eq 1) rather than with guanine. Reaction with iron(III) may not be as relevant in the case of the 2-hydroxyethyl radical (eq 2) because this radical is expected to react much more slowly than the 1-hydroxyethyl radical (*34*, *49*):

$$CH_3{-}C^{\bullet}HOH + Fe^{3+} \rightarrow CH_3{-}C^{+}HOH + Fe^{2+} \quad (1)$$

$$C^{\bullet}H_2{-}CH_2OH + Fe^{3+} \rightarrow C^{+}H_2{-}CH_2OH + Fe^{2+} \quad (2)$$

It is also important to emphasize that iron ion reactivity can be modulated by their chelation to nucleic acids (*20*, *39*, *40*, *45*). At this point, a thorough analysis of guanine alkylation is precluded because the rate constants and reaction rates of all concomitant processes are not known. However, the demonstration that ethanol oxidation by Fenton systems and peroxynitrite (*36*) produces both the 1-hydroxyethyl and the 2-hydroxyethyl radicals suggests these species may be produced during ethanol metabolism and play a role in ethanol-mediated genotoxicity by alkylating nucleic acids (Scheme 1) and/or other biomolecules (*11*−*13*).

Ethanol is considered to be a cocarcinogen and/or tumor promoter rather than a carcinogen by itself (*1*, *2*). Nevertheless, ethanol is teratogenic and all of its known metabolites can damage DNA and RNA (Scheme 1). The hydroxyl radical that is likely to be produced from the reaction of the 1-hydroxyethyl radical with oxygen followed by Fenton chemistry (Scheme 1) has been extensively shown to cleave nucleic acids and to oxidize their bases (*1*−*4*, *37*−*39*). Acetaldehyde is known to add to nucleosides in vitro (*50*); recently, one of these adducts, N^2-ethyl-2′-deoxyguanosine 3′-monophosphate, has been isolated from granulocyte and lymphocyte DNA from alcoholic patients (*51*). Here we demonstrated nucleic acid alkylation by ethanol-derived free radicals. These species are also expected to cleave DNA, as has been demonstrated in the case of other carbon-centered free radicals (for a review, see ref *14*). Reactions of ethanol-derived free radicals with DNA may outcompete their reactions with oxygen because the eukaryote nucleus is considered to be a poorly oxygenated compartment (*52*). Consequently, the relative importance of these (Scheme 1) and other routes (*11*−*13*) in the genotoxic effects of ethanol remains to be established.

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