An ESR and HPLC-EC Assay for the Detection of Alkyl Radicals

Christo P. Novakov, Dennis Feierman, Arthur I. Cederbaum, and Detcho A. Stoyanovsky*

Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, New York 10029

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The correlation of lipid peroxidation with release of alkanes (RH) is considered a noninvasive method for the in vivo evaluation of oxidative stress. The formation of RH is believed to reflect a lipid hydroperoxide (LOOH)-dependent generation of alkoxyl radicals ($LO^•$) that undergo β-scission with release of alkyl radicals ($R^•$). Alternatively, $R^•$ could be spin-trapped with a nitrone before the formation of RH and analyzed by ESR. Extracts from the liver and lung of CCl_4- and asbestos-treated rats that were previously loaded with nitrones exhibited ESR spectra suggesting the formation of iso-propyl, *n*-butyl, ethyl, and pentyl radical-derived nitroxides. In biological systems, various nitroxides with indistinguishable ESR spectra could be formed. Hence, experiments with *N*-*tert*-butyl-α-phenylnitrone (PBN) for spin trapping of $R^•$ were carried out in which the nitroxides formed were separated and analyzed by HPLC with electrochemical detection (EC). The C_{1-5} homologous series of PBN nitroxides and hydroxylamines were synthesized, characterized by ESR, GC-MS, and HPLC-EC, and used as HPLC standards. For in vivo generation and spin trapping of $R^•$, rats were loaded with CCl_4 and PBN. The HPLC-EC chromatograms of liver extracts from CCl_4-treated rats demonstrated the formation of both the nitroxide and hydroxylamine forms of PBN/$^•CCl_3$, as well as the formation of a series of unidentified PBN nitroxides and hydroxylamines. However, formation of PBN adducts with retention times similar to these of the PBN/C_{2-5} derivatives was not observed. In conclusion, we could not correlate the production of PBN-detectable alkyl radicals with the reported CCl_4-dependent production of C_{1-5} alkanes. We speculate that the major reason for this is the low steady-state concentrations of $R^•$ produced because only a small fraction of $LO^•$ undergo β-scission to release $R^•$.

Introduction

The correlation of lipid peroxidation with release of alkanes (RH)[1] has attracted considerable interest as a noninvasive method for the in vivo evaluation of oxidative stress. It has been reported that patients with cystic fibrosis, alcohol liver disease, rheumatoid arthritis, asthma, and Alzheimer's disease have an increased, GC-detectable exhalation of ethane, propane, *n*-butane, isobutane, pentane, and isopentane (reviewed in ref *1*). Under conditions of oxidative stress, the formation of RH is believed to reflect a lipid hydroperoxide (LOOH)-dependent generation of alkoxyl radicals ($LO^•$) that undergo β-scission with release of alkyl radicals [$R^•$; Scheme 1, **4** → **5** → **7** (*2*−*5*)]. The latter are a highly reactive species that can trigger chain reactions within the cellular membranes, react with oxygen to form peroxyl radicals ($ROO^•$; Scheme 1, **7** → **9**) and/or to react with H-donors to form RH (Scheme 1, **7** → **8**).

The in vivo observation of RH, however, provides limited information about the sites of radical formation. Alternatively, $R^•$ could be spin-trapped with a nitrone before the formation of RH; the resulting alkyl-derived nitroxide may be extracted from tissues subjected to oxidative stress and analyzed by ESR. It has been reported that extracts from the liver and lung of CCl_4- and asbestos-treated rats that were previously loaded with nitrones exhibited ESR spectra with hyperfine structure which allows the assignment of the adducts as those formed by the addition of iso-propyl and *n*-butyl, and ethyl and pentyl radicals, respectively (*6*, *7*).

The detection and quantitation of alkyl radicals has proven to be difficult as they are species with half-lives within the microsecond scale; furthermore, $R^•$ reacts in a diffusion-controlled manner with oxygen to form secondary, oxygen-centered radicals ($ROO^•$). Hence, when experiments are carried out for spin trapping of $R^•$ in biological systems, a particular complication in the interpretation of the ESR data may be the formation of multiple nitroxides with similar ESR spectra. Recently, Iwahashi et al. have reported that pentyl radical could be spin-trapped with α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) in a complex reaction system; the resulting POBN/C_5H_{11} nitroxide was analyzed by HPLC with ESR and MS detection (*8*). Alternatively, characterization of alkyl-derived nitroxides could be carried out by HPLC analysis with electrochemical detection [EC (*9*−*12*)]. In this report, we have extended the HPLC-EC methodology for detection of alkyl radicals within the homologous series of methyl to pentyl radicals (C_{1-5}). PBN/C_{1-5}

* To whom correspondence should be addressed. Phone: (212) 241-6810, Fax: 212-996-7214, E-mail: stoyanovsky@hotmail.com.

[1] Abbreviations: ESR, electron spin resonance; PBN, *N*-*tert*-butyl-α-phenylnitrone; R_t, retention time; EC, electrochemical; LPO, lipid peroxidation.

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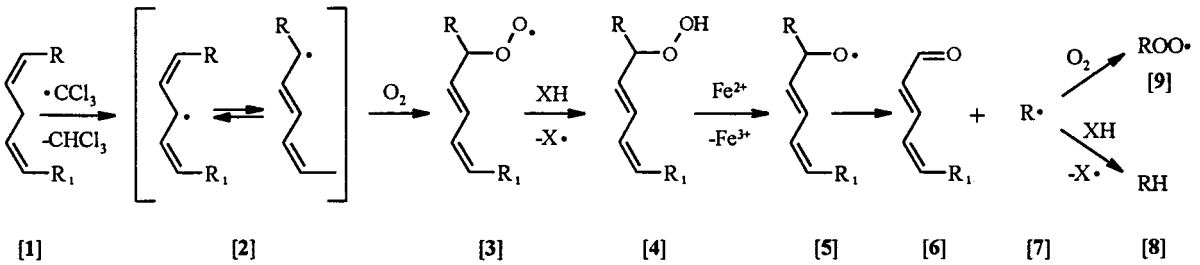
Scheme 1



nitroxides were prepared under the conditions of the Wurtz reaction (*13*) and/or via direct coupling of PBN with Grignard reagents; ESR, GC-MS, and HPLC-EC analysis were used for characterization of the corresponding adducts. Liver extracts from PBN plus CCl_4-treated rats were analyzed by HPLC-EC with the ultimate goal to evaluate whether the formation of PBN/C_{2-5} nitroxides could be used as an in vivo index of radical-dependent phospholipid alteration.

Materials and Methods

Reagents. All reagents used were purchased from Sigma Chem. Co. (St. Louis, MO). The solutions used in the experiments were prepared in deionized and Chelex-100 treated (batch method) water or 0.1 M potassium phosphate buffer (pH 7.4).

ESR Measurements. ESR measurements were performed on a Bruker ECS106 spectrometer with 50 kHz magnetic field modulation at room temperature (25 °C). ESR spectrometer settings were: modulation amplitude 0.7 G, scan time 41 s, time constant 0.164 s, microwave power 20 mW and receiver gain 1 $\times$ 10^5−1 $\times$ 10^6. ESR spectra simulations were made using a program created by Philip D. Morse II and Richard Reiter (EPR Simulation System 2.01, Scientific Software Services, IL). The hyperfine splitting constants used for ESR spectra simulation were taken from Table 1 and/or (*14*).

Preparation of Grignard Reagents. Alkylmagnesium bromides (RMgBr) were prepared by refluxing (1−1.5 h) the corresponding alkylbromide (3 M) with an equimolar amount of Mg in dry diethyl ether (30 mL). The reactions were carried out by a dropwise addition of the bromide at a rate that secured moderate boiling of the solvent; in some experiments, initiation of the reaction required an addition of a crystal of iodine. In dry atmosphere (20 °C), the solutions of alkylmagnesium bromides were stable for several months.

Preparation of PBN/C_{1-5} Adducts. Alkyl-derivatization of PBN was carried out via direct coupling of RMgBr (0.3 M) with PBN (0.08 M) in helium-deaerated, dry diethyl ether (10 mL). After an incubation of 10 min (20 °C), the excess of the Grignard reagent was carefully decomposed with 50% ethanol (v/v; 2 mL), the organic layer was separated, dried over Na_2SO_4 and rotor-evaporated (25 °C). The dry residue containing both the nitroxide and hydroxylamine forms of the corresponding PBN derivative was redissolved in ethanol and used as an HPLC-EC standard. When stored at −80 °C, the PBN/C_{1-5} adducts were stable for several months. Preparation of PBN/C_{1-5} adducts was also carried out under the condition of the Wurtz reaction (Supporting Information and ref *13*).

GC/MS Analysis. GC/MS analysis was performed with a Hewlett-Packard (HP) 5890 Series II gas chromatograph, equipped with a HP 7673 automatic injector, a DB-17 capillary column (15 m $\times$ 0.25 mm; 0.25 μm film thickness; J & W Scientific, Folson, CA). A HP 5972 mass selective detector (MSD) was employed in the scan mode. The underivatized samples (2 μL) were injected with the purge valve closed for the first minute (splitless mode). Helium was the carrier gas at a pressure of 72 kPa. Initial temperature settings for the oven, injector, and MSD were 100, 225, and 280 °C, respectively. The oven temperature was increased at a rate of 10 °C per min to 280 °C and was then held constant for the remaining time of the analysis.

Table 1. ESR Hyperfine Coupling Constants (in G) for PBN/C_{1-5} Nitroxides in 80% Methanol at 20 °C[a]

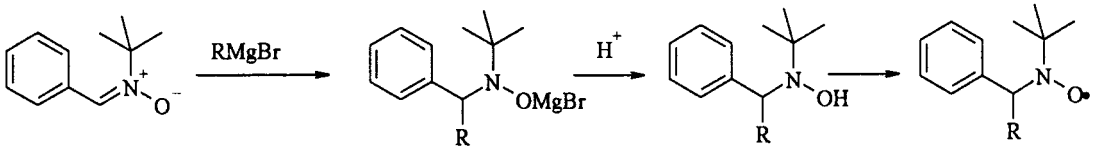
Compound	a_N	a_H
PBN	15.76	4.05
PBN	15.67	3.78
PBN	15.63	3.66
PBN	15.67	2.95 *
PBN	15.67	3.64
PBN	15.63	3.22
PBN	15.55	3.66
PBN	15.59	3.56
PBN	15.59	3.69
PBN	15.69	2.88 *

[a] Preparation and ESR analysis of PBN/C_{1-5} nitroxides was carried out as described in the Materials and Methods. (*) Nitroxides with ESR spectra that differ from the ESR spectra of PBN/C_2H_5.

HPLC Measurements. HPLC was performed with an ESA model 5102 liquid chromatograph (Milford, MA). Separation was achieved with either a 4.6 mm $\times$ 15 or 25 cm C-18 column (5 μm, 100 A; Microsorb, Rainin Instrument Company, Inc., Emeryville, CA). The mobile phase was saturated with helium and contained 20 mM lithium perchlorate and either 70 or 80% (v/v) methanol. All HPLC analyses were conducted at a flow rate of 1 mL/min. Electrochemical detection of PBN/C_{1-5} adducts was carried out at + 0.7 V with an ESA coulochem detector. A 0.020 mL injection loop was used for all experiments.

Animals and Animal Treatments. For induction of P450 2E1, Sprague−Dawley rats (n = 9) weighing 120−150 g were injected i.p. with pyrazole (200 mg/kg body wt/day, 3 days). The rats were starved overnight prior to being killed and experiments with CCl_4 were conducted as described previously (*6*, *15*). Briefly, the animals were loaded with PBN (5−200 mg/kg body weight) dissolved in olive oil (2 mL); 15 min later, CCl_4 was injected i.p. as a 20% solution in olive oil at a dose of 5 mL solution per kg body weight (1 mL of pure CCl_4/kg). In a typical experiment, the animal was sacrificed 60 min after the administration of CCl_4, its liver was homogenized in 20 mL of PBS

Scheme 2



and the homogenate subjected to extraction with $CHCl_3$ (40 mL) and methanol (20 mL). The resulting emulsion was centrifuged (15 min × 10000*g*), the chloroform phase was collected and the solvent rotor-evaporated at room temperature. The residue was re-suspended in 5 mL of ethanol (80%) and centrifuged (30 min × 10000*g*). The supernatant was used for ESR and HPLC-EC analysis, while the bottom layer (0.1−0.5 mL of fats) was discarded.

Results

Preparation of PBN/C_{1-5} Adducts. Nucleophilic reagents in which the nucleophilic center is located on a carbon atom, such as alkylmagnesium bromides (Grignard reagents), CH_3NO_2, RNa, and CN^- react with PBN via a 1,3-addition mechanism to form the corresponding PBN-derived hydroxylamines (Scheme 2; reviewed in ref *16*). The latter readily undergo oxidation to nitroxides that could be analyzed by direct ESR spectrometry. Both nitroxide and hydroxylamine forms of PBN can be quantified by HPLC-EC (*9*, *11*, *12*).

According to the Scheme 2, alkyl-derived PBN nitroxides were prepared for the C_{1-5} homologous series (Table 1). When analyzed by HPLC-EC, the PBN/C_{1-5} adducts exhibited increased retention times on a C18 matrix with increasing length of the carbon chain of the alkyl group. In Figure 1, panel A depicts the HPLC-EC profile of a reaction solution consisting of PBN plus pentylmagnesium bromide. The fraction defined by peak 1 exhibited an ESR spectra that could be assigned to the PBN/C_5H_{11} nitroxide (Figure 1A, ESR spectrum 1; in gauss (Table 1) $a_N = 15.59$ and $a_H = 3.56$). The fraction collected under peak 2 did not exhibit any ESR activity (Figure 1A, ESR spectrum 2). An addition of potassium ferricyanide (0.02 mM) to this fraction, however, resulted in the appearance of an ESR spectrum that was identical to ESR spectrum 1 (Figure 1A, ESR spectrum 3; $a_N = 15.59$ G; $a_H = 3.56$ G), suggesting that peak 2 reflects the elution of the hydroxylamine form of the adduct. A brief pretreatment of the PBN/C_5H_{11} solution with either potassium ferricyanide or ascorbate in 0.1 M phosphate buffer (pH 7.4) resulted in an interconversion of peak 2 into peak 1 (oxidation of the hydroxylamine form) and vice versa (reduction of the nitroxide form; data not shown). The PBN/C_5H_{11} structure was further supported by GS-MS analysis (Figure 1B). The GC profile of the reaction solution exhibited one major peak (Inset to Figure 1B, peak 1). The same GC peak was obtained after injection of HPLC fraction 2 (Figure 1A, peak 2) that supposedly reflected the elution of the hydroxylamine form of PBN/C_5H_{11} (data not shown). The mass profile of the latter included a species with a mass-to-charge ratio (*m*/*z*) of 249 that could be assigned to the protonated PBN/C_5H_{11} nitroxide and molecular fragments with *m*/*z* values of 161 and 192 that most likely reflect the fragmentation of PBN/C_5H_{11} to $PhC_5H_{11}+$ and $PhC_5H_{11}NHO^+$, respectively. A more detailed assignment of the molecular fragments shown in Figure 1B is proposed in Table 2.

The PBN/C_{1-5} hydroxylamines autoxidize in phosphate buffer back to their nitroxide forms. The oxidation of the hydroxylamines can also be carried out with potassium ferricyanide (Figure 2A, first arrow); however, excess of

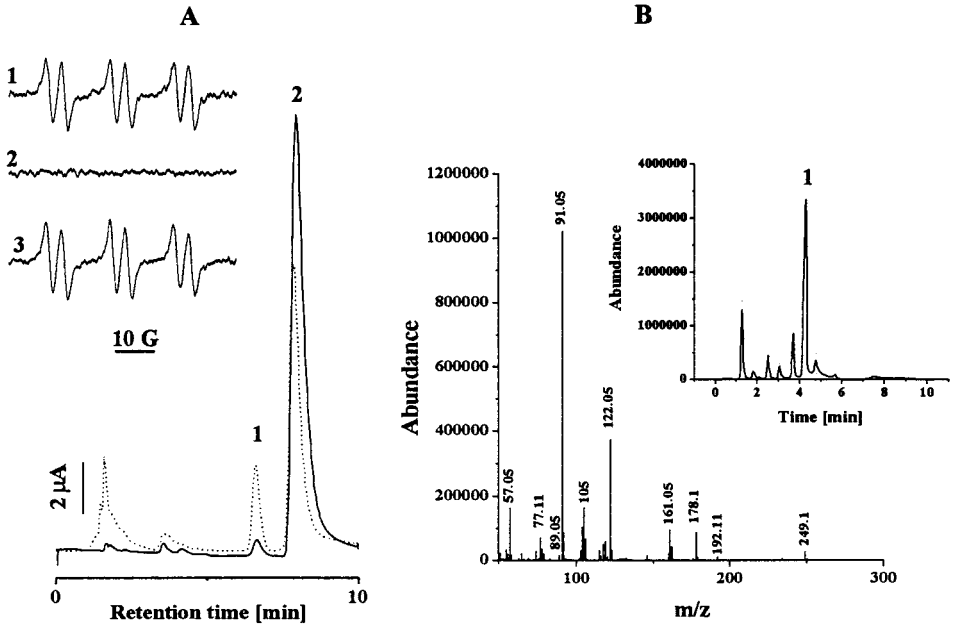
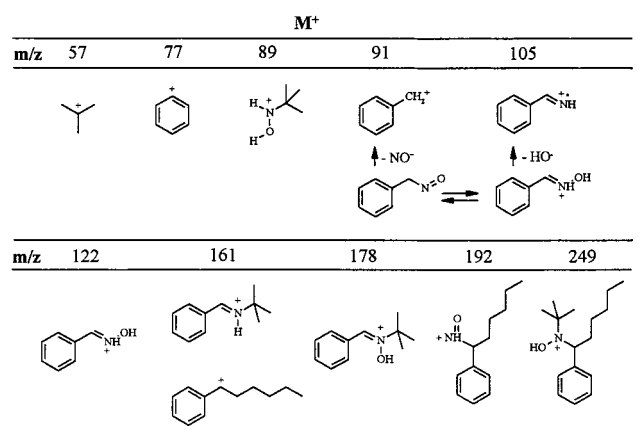


Figure 1. HPLC-EC and GC-MS analysis of the reaction products of pentylmagnesium bromide and PBN. $C_5H_{11}MgBr$-dependent derivatization of PBN and subsequent HPLC-EC and GC-MC measurements were carried out as described in the Materials and Methods. HPLC separation of the electroactive analytes was carried out with a C18 Microsorb column (4.6 mm × 15 cm; 5 μm) after a 10-fold dilution of the reaction solution in ethanol. ESR spectra 1 and 2 refer to the spectrum of peaks 1 and 2, respectively, while spectrum 3 refers to peak 2 treated with $K_3[Fe(CN)_6]$. HPLC profile: solid lines, minus $K_3[Fe(CN)_6]$; dashed lines, plus $K_3[Fe(CN)_6]$.

Table 2. Mass Spectral Data of PBN/C_5H_{11} Hydroxylamine[a]



[a] Preparation and GC-MS analysis of PBN/C_5H_{11} hydroxylamine was carried out as described in the Materials and Methods.

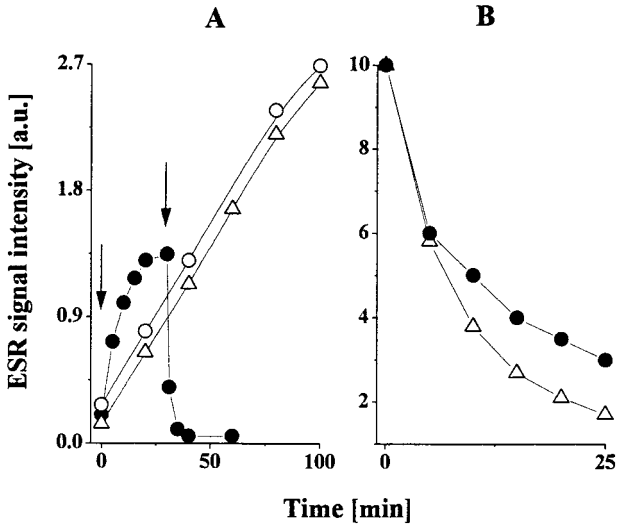


Figure 2. ESR-monitored kinetics of redox interconversion of PBN/C_5H_{11} and PBN/CH_3 adducts. All experiments were carried out in 0.1 M phosphate buffer (pH = 7.4; 25 °C). The nitroxide and hydroxylamine derivatives of PBN were prepared as described in the Materials and Methods. (A) Autoxidation of PBN/CH_3 (△) and PBN/C_5H_{11} (○) hydroxylamines in buffered aqueous solutions, and PBN/C_5H_{11} hydroxylamine (●) in the presence of $K_3[Fe(CN)_6]$ (1 mM). The addition of $K_3[Fe(CN)_6]$ is indicated with an arrow. (B) ESR signal intensity of PBN/C_5H_{11} (●) and PBN/CH_3 (△) nitroxides in the presence of ascorbic acid (5 mM).

$K_3[Fe(CN)_6]$ also reacts with the nitroxide form of the adducts and causes loss of the ESR signal (Figure 2A, second arrow). Reduction of radical-derived ESR active nitroxides in spin trapping experiments is an unavoidable occurrence in most in vivo systems (*17*). Therefore, when biological samples are analyzed, any procedure that leads to dilution of the reaction mixture could lower the reductive potential of the system and may trigger the autooxidation of the reduced nitroxide(s) (*12*). Although both the nitroxide and hydroxylamine derivatives can readily be detected by HPLC-EC (Figure 3A), to avoid fluctuations in the ratio of these forms, the HPLC-EC analysis can be preceded by incubation of the analyzed solutions for 10−15 min with ascorbate. Figure 2B shows the effectiveness of 5 mM ascorbate in reducing the PBN/C_5H_{11} and PBN/CH_3 nitroxides. The latter procedure would result in the detection of the total amount of the C_{1-5} spin-trapped radicals as the hydroxylamine form of their nitroxides. Since the separation of the hydroxylamine derivatives could be carried out with a mobile phase with lower polarity, the time of the analysis could be considerably decreased (Figure 3B). Moreover, the nitroxide and hydroxylamine forms of the alkyl adducts can be further distinguished from each other by I/V curves of their voltage dependence as shown in Figure 4 for the ethyl and pentyl PBN nitroxides and hydroxylamines. The HPLC peaks obtained in analyses of complex systems could be positively identified via their retention time, co-injected of preformed standards (Figure 3), as well as via redox interconversion (Figures 1 and 2), ESR spectra (Figure 1A and Table 1), and I/V curves (Figure 4).

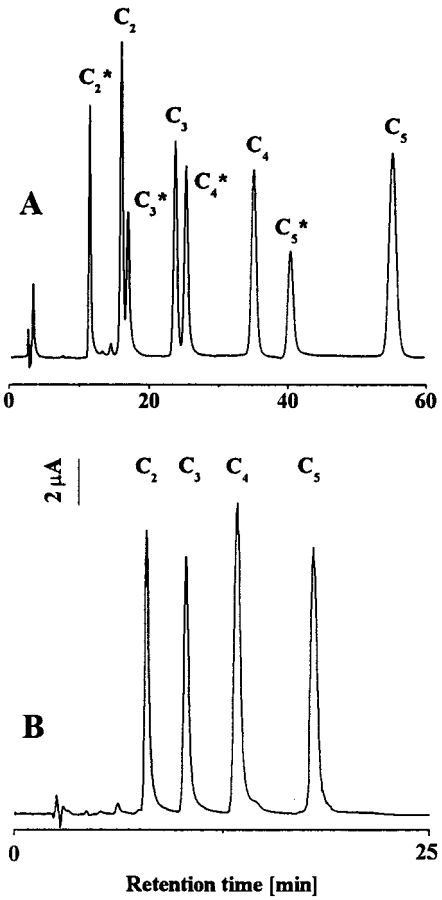


Figure 3. HPLC-EC chromatograms of PBN/C_{2-5} nitroxides and hydroxylamines. (A) HPLC-EC profile of PBN/C_{2-5} nitroxides (C_{2-5}*) and hydroxylamines (C_{2-5}). (B) HPLC-EC profile of PBN/C_{2-5} hydroxylamines after reduction of their nitroxide forms with ascorbic acid (15 mM) for 15 min in 0.1 M phosphate buffer (pH 7.4; at 25 °C). PBN/C_{2-5} nitroxides were prepared as described in the Materials and Methods and diluted 10 times in 0.1 M phosphate buffer (pH 7.4). The HPLC separation was achieved with a C18 Microsorb column (4.6 mm × 25 cm; 5 μ) and a mobile phase consisting of either 70% (A) or 80% (B) methanol, and 20 mM $LiClO_4$.

Alternatively, PBN/C_{1-5} nitroxides and hydroxylamines could be prepared under the conditions of the reaction of Wurtz [RBr + Na → R• (*13*); Supporting Information]. Figure 5 A, curve 1, depicts the ESR spectrum of a solution of C_3H_7Br and $C_5H_{11}Br$ in hexane containing PBN and Na. The PBN/C_3H_7 and PBN/C_5H_{11} nitroxides have similar hyperfine splitting constants (Table 1). The computer simulation of their ESR spectra presented in Figure 5A, curve 2, illustrates that they are ESR indistinguishable. The HPLC-EC analysis of the reaction

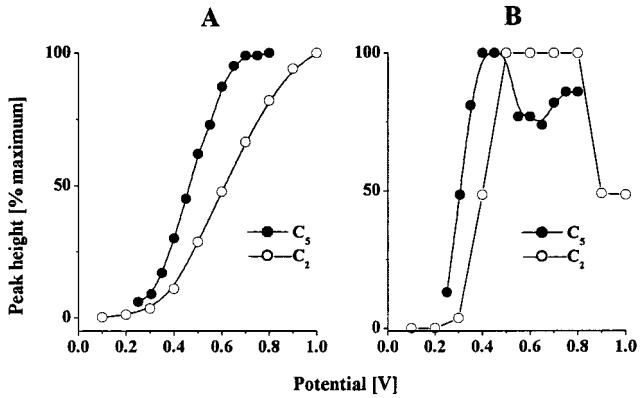


Figure 4. Voltage dependence of HPLC-EC signals of PBN/C_2H_5 and PBN/C_5H_{11}. Both hydroxylamine (A) and nitroxide (B) forms of the adducts were subjected to EC−HPLC analysis at different holding potential. The nitroxide and their hydroxylamine derivatives were prepared as described in the Materials and Methods and diluted 10 times in ethanol prior to HPLC separation.

solution, however, revealed that both the nitroxide and hydroxylamine forms of PBN/C_3H_7 and PBN/C_5H_{11} were produced (Figure 5B, spectrum I, peaks 1, 2 and 3, 4, respectively). The chromatographic resolution was also satisfactory in the analysis of the iso-pentyl- and pentyl-derived PBN nitroxides that were obtained from 1-bromo-2-methylbutane and 1-bromopentane, Na and PBN (Figure 5B, spectrum II, peaks 1 and 2, respectively). The kinetics of formation of the isopentyl and the pentyl PBN adducts is shown in Figure 5C.

ESR and HPLC-EC Analysis of the in Vivo Metabolism of CCl_4. The hepatotoxicity of CCl_4 is believed to result largely from the metabolic activation of the halocarbon molecule by P450 to $^{\bullet}CCl_3$ radical. The $^{\bullet}CCl_3$ radical has been shown to form adducts with hepatic molecules, to oxidize glutathione and protein thiols, and to induce lipid peroxidation (LPO) (reviewed in ref *18*). The metabolism of CCl_4 is also paralleled by a release of alkanes, presumably formed via the intermediate generation of alkyl radicals (*19*). Hence, we have carried out spin-trapping experiments with HPLC-EC detection to attempt to identify alkyl radicals formed in the liver of CCl_4-treated rats.

Since CYP2E1 is known to be especially reactive in catalyzing the metabolism of CCl_4 (reviewed in ref *20*), Sprague−Dawley rats were treated for several days with pyrazole, which is known to cause a 2−4-fold increase of the hepatic CYP2E1 (*21*). Subsequently, the animals were treated with CCl_4 and PBN, and liver extracts were analyzed by ESR and HPLC-EC for detection of hepatic radical adducts. Figure 6 (ESR spectrum 1) depicts the ESR spectrum of an ethanolic extract from the liver of a CCl_4 plus PBN-treated rat. The hyperfine structure (in gauss) of the ESR spectrum presented in trace 1 suggests the presence of the PBN/$^{\bullet}CCl_3$ nitroxide (in 80% ethanol, a_N = 14 G; a_H = 1.9 G). However, similar ESR spectra may be exhibited by lipid and alkyl radical-derived PBN nitroxides. The intensity of the ESR spectrum of the liver extract was constant for a time period of 60 min. After an addition of $K_3[Fe(CN)_6]$ (1 mM), the intensity of the observed ESR spectrum increased with a shift in the hyperfine structure of the initial ESR spectrum to a_N = 14.3 G and a_H = 2.7, suggesting the presence of multiple PBN-derived nitroxides (Figure 6, ESR spectrum 2). In a detailed analysis of the liver extract from CCl_4-treated rats, Janzen et al. have identified PBN-derived nitroxides of $^{\bullet}CCl_3$ and carbon- and oxygen-centered radicals (*22*, *23*).

Within the time interval of PBN/C_{2-5} elution (Figure 7A, dashed rectangle, retention times of 7−18.5 min), the HPLC-EC profile of the liver extracts exhibited three major peaks. Peaks 1 and 2 had close but distinct retention times as compared to those of the nitroxide and hydroxylamine forms of PBN/C_2H_5 (R_t = 8.2 and 10.6 min compare to 8.4 and 11 min, respectively); their I/V curves also differed from those of the authentic PBN/C_2H_5

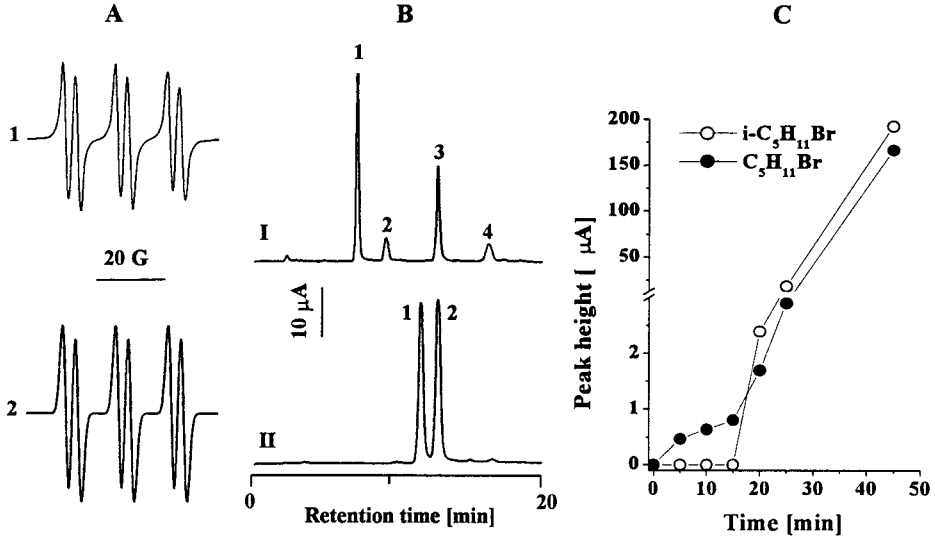


Figure 5. ESR spectra and HPLC-EC chromatograms of alkyl derivatives of PBN obtained under the condition of the Wurtz reaction. All reactions were carried out in dry hexane for 1 h at room temperature. The HPLC separations were carried out with a C18 Microsorb column (4.6 mm × 25 cm; 5 μm). (A) Trace 1: ESR spectrum of a solution of C_3H_7Br (0.08 M) and $C_5H_{11}Br$ (0.08 M) in hexane containing Na (0.01 g). Trace 2: overlapped, PC simulated ESR spectra of PBN/C_3H_7 and PBN/C_5H_{11} nitroxides. (B) HPLC-EC profiles of solutions of C_3H_7Br and $C_5H_{11}Br$ (trace I) and $C_5H_{11}Br$ and iso-$C_5H_{11}Br$ (trace II) in hexane containing Na. In chromatogram I, Peaks 1, 2 and 3, 4 reflect the elution of nitroxide and hydroxylamine forms of PBN/C_3H_7 and PBN/C_5H_{11}, respectively. In chromatogram II, peaks 1 and 2 reflect the elution of the nitroxide forms of PBN/iso-C_5H_{11} and PBN/C_5H_{11}, respectively. (C) HPLC-EC-monitored kinetics of formation of PBN/iso-C_5H_{11} and PBN/C_5H_{11} nitroxides in a solution of hexane containing $C_5H_{11}Br$ (0.08 M), iso-$C_5H_{11}Br$ (0.08 M), and Na (0.01 g).

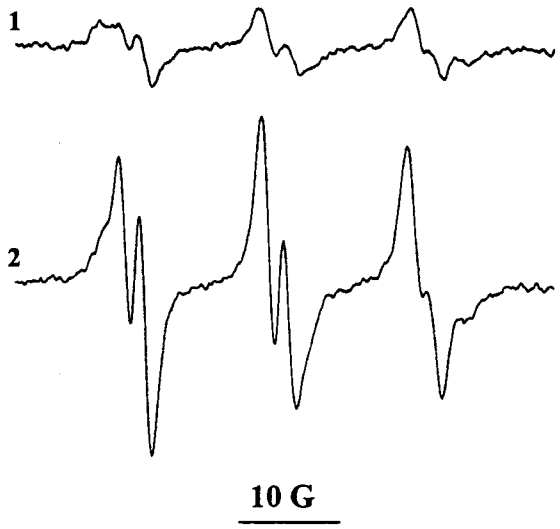


Figure 6. Effects of potassium ferricyanide on the ESR spectrum of a liver extract from a CCl_4-treated rat. The experiments were carried out in ethanol at 25 °C. Liver extract was prepared as described in the Materials and Methods. ESR spectra of liver extract from a CCl_4-treated rat in the absence (trace 1) or presence (trace 2) of $K_3[Fe(CN)_6]$ (1 mM) were recorded after an incubation of 20 min.

adducts (data not shown). The fraction defined by peak 1 did not exhibit any ESR activity in the absence or presence of $K_3[Fe(CN)_6]$, suggesting that the analyzed solution did not contain either a nitroxide or hydroxylamine form of PBN. The fraction under peak 2 had a well-resolved ESR spectrum ($a_N = 14.39$ G and $a_H = 2.22$ G; data not shown) that differed considerably from the ESR spectra of the PBN/C_{2-5} nitroxides (Table 1). Peaks 3 and 4 reflected the elution of the nitroxide and hydroxylamine forms of PBN/$^{\bullet}CCl_3$ (chromatograms I−III compared to chromatogram IV). Chromatogram IV represents the HPLC-EC profile of a standard solution of PBN/$^{\bullet}CCl_3$ nitroxide and hydroxylamine, respectively (*15*). At lower doses of PBN, peaks 3 and 4 considerably decreased (Figure 7A, traces I−III), suggesting that an increased $^{\bullet}CCl_3$-dependent formation of R$^{\bullet}$ could have occurred. However, our initial expectations to detect the formation of ethyl, isopropyl, *n*-butyl, pentyl and isopentyl derivatives of PBN were not confirmed as HPLC-EC evidence for the presence of these adducts could not be observed. When the HPLC separation was carried out with a mobile phase with lower polarity, two additional nitroxides and their hydroxylamine forms that previously eluted with the front of the higher polarity eluent were observed (Figure 7B, peaks 1−4). Upon treatment of the liver extract with ascorbic acid, a decrease of peaks 1 and 2 was observed that was paralleled by an increase of peaks 3 and 4 (Figure 7B, dashed lines), suggesting the formation of the corresponding hydroxylamines. In support of the latter assumption, the fractions defined by peaks 1 and 2 exhibited well resolved ESR spectra which were missing in peaks 3 and 4 (Figure 7C, ESR spectra 1−4); the appearance of the same ESR spectra in fractions 3 and 4 required an addition of potassium ferricyanide (Figure 7C, spectra 5 and 6). The structure of

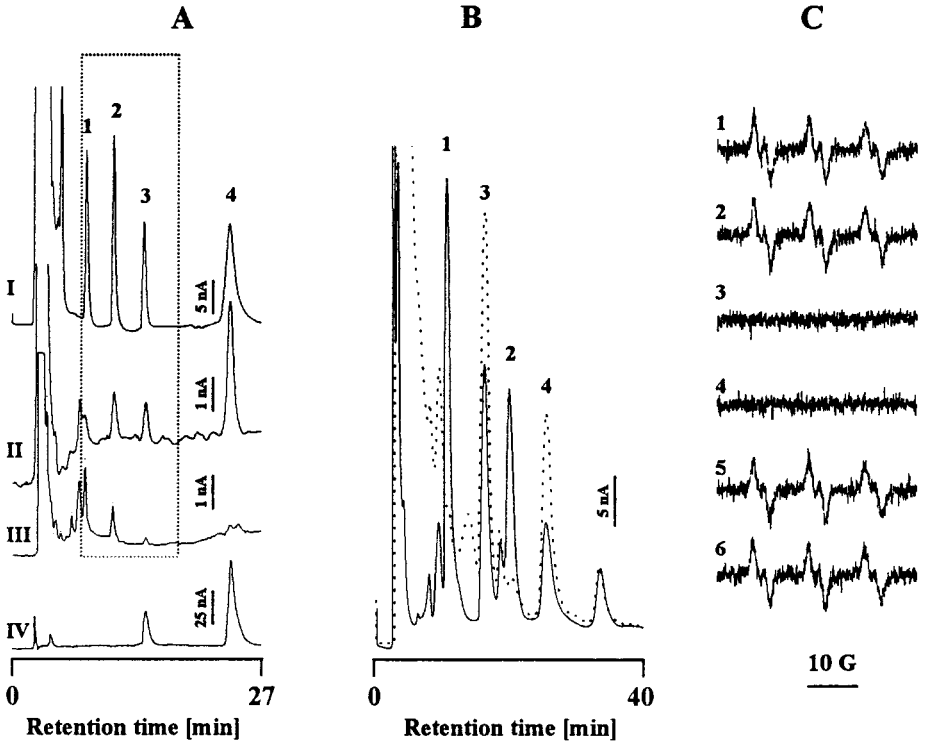


Figure 7. HPLC-EC analysis of liver extracts from CCl_4-treated rats. Liver extracts were prepared as described in the Materials and Methods. The HPLC separations were achieved with a C18 Microsorb column (4.6 mm × 25 cm; 5 μm). The HPLC-EC profiles of the liver extracts were obtained via an elution with either 80% (panel A) or 30% (panel B) methanol. Chromatograms I−III were obtained from experiments with rats loaded with 200, 100, and 5 mg of PBN/kg of body weight, respectively. Chromatogram IV reflects the elution of a standard solution of the nitroxide (peak 3) and hydroxylamine (peak 4) form of PBN/CCl_3 (prepared as described in ref *15*). Panel B depicts HPLC-EC profiles of a liver extract from a rat loaded with 200 mg of PBN/kg body weight. A treatment of the extract for 10 min with ascorbic acid (0.1 M phosphate buffer, pH 7.4) resulted in a decrease of peaks 1 and 2 that was paralleled by an increase of peaks 3 and 4 (dashed lines), respectively. In panel C are presented the ESR spectra of the fractions defined by peaks 1−4 (panel B). The numeration of the ESR spectra corresponds to the numeration of the HPLC peaks in panel B; ESR spectra 5 and 6 were obtained via treatment of fractions 3 and 4 for 15 min with $K_3[Fe(CN)_6]$ (1 mM).

these adducts, however, remains unknown, but they are not reflective of the C_{1-5} homologous series of alkyl PBN adducts.

Discussion

In biological systems, the identification of spin trapped radical species is difficult as usually various radical-derived nitroxides with similar ESR spectra are formed. Furthermore, the redox potential of the intracellular milieu is in the range of −0.26 to −0.28 V (*24*). The reducing environment in the cellular cytosol is maintained by reductants such as the thioredoxin and glutaredoxin systems, ascorbate and glutathione, and protein thiols. Recently, the redox potentials of a series of nitroxides have been reported to be in the range of 0.5−0.9V (*25*), suggesting that in spin trapping experiments in biological systems a major reaction pathway can be the reduction of the nitroxides to their ESR "silent" hydroxylamine derivatives. The latter process would considerably decrease the sensitivity of the ESR spin trapping technique. To overcome these experimental difficulties, we have conducted experiments for the in vivo spin trapping of alkyl radicals in which the identification of the PBN adducts was carried out by HPLC-EC. For positive identification of alkyl metabolites, PBN/C_{1-5} nitroxides were prepared and used as HPLC-EC standards. HPLC separation with EC detection proved to be reliable in distinguishing both the nitroxide and hydroxylamine forms of the various PBN/C_{1-5} adducts.

In vivo generation of carbon-centered radicals was carried out via administration of CCl_4 to rats. The hepatotoxicity of CCl_4 is believed to result largely from its metabolic activation by P450, especially P450 2E1, to $^{\bullet}CCl_3$ radical. The metabolism of CCl_4 was also correlated with the production of alkanes (*18*, *26*, *27*), presumably formed via the intermediate generation of alkyl radicals. According to Scheme 1, however, two experimental problems for spin trapping of R$^{\bullet}$ could be envisaged: (i) at high concentrations of PBN, species 2, 3, and 5 (including $^{\bullet}CCl_3$) may be spin trapped, therefore, the production of R$^{\bullet}$ (*7*) could be inhibited, because of the trapping of its radical precursors, and ii) at relatively low concentrations of PBN, the reduction of R$^{\bullet}$ to RH or/and its recombination with O_2 (Scheme 1, **7** → **8** and **7** → **9,** respectively) may be faster than its interaction with PBN. Hence, we have treated rats with CCl_4 and doses of PBN ranging from those indicated in (*6*) (10 mg/kg of body weight) to up to 200 mg/kg of body weight to attempt to have an effective concentration of PBN which would allow some formation of R$^{\bullet}$ to occur and allow trapping of the formed R$^{\bullet}$. One hour after the treatment, the animals were sacrificed, and liver extracts were prepared and analyzed by ESR and HPLC-EC. The ethanolic extracts from the liver of CCl_4 plus PBN-treated rats exhibited ESR spectra with hyperfine structure suggesting the formation of the PBN/CCl_3 nitroxide. However, upon addition of $K_3[Fe(CN)_6]$ the intensity of the ESR spectra increased with a small but noticeable shift of the hyperfine structure, suggesting the presence of multiple PBN-derived hydroxylamines in the liver extract. The latter observation should be taken into consideration when attempts for reoxidation of the hydroxylamine derivatives of spin trapped adducts are made.

Within the time interval of PBN/C_{1-5} elution, an unidentified nitroxide (Figure 7A, peak 2) and the nitroxide and hydroxylamine forms of PBN/CCl_3 (Figure 7A, peaks 3 and 4, respectively) were observed. When the liver samples were eluted with a mobile phase with lower polarity, the nitroxide and hydroxylamine forms of two additional PBN adducts were observed (Figure 7, panels B and C). Since $^{\bullet}CCl_3$ and R$^{\bullet}$ are equally reactive (*28*), the detection of PBN/$^{\bullet}CCl_3$ adducts attests that the spin trapping efficiency of PBN should be sufficient for intercepting alkyl radicals. Figure 7A illustrates that the HPLC-EC peaks reflecting the elution of the PBN/$^{\bullet}CCl_3$ adducts considerably decreased when lower doses of PBN were injected. It could be speculated that the decreased PBN/$^{\bullet}CCl_3$ concentrations reflect less effective trapping of $^{\bullet}CCl_3$ by the lower concentrations of PBN which could result in an increased amount of "free" $^{\bullet}CCl_3$ to interact with the hepatic phospholipids; this should ultimately lead to an increased production of R$^{\bullet}$. Under these experimental conditions, however, we could not detect the formation of C_{1-5} alkyl radicals. We could not also detect PBN/C_{1-5} adducts in model systems that are routinely used for induction of lipid peroxidation, such as liver microsomes, NADPH, and CCl_4 (independently of the presence or absence of O_2), or liver microsomes plus iron and ascorbate (data not shown), despite the evidence for lipid peroxidation by assays for TBARS.

In conclusion, HPLC-EC protocols for the analysis of PBN-spin trapped radical intermediates were established. The HPLC-EC peaks reflecting the elution of the PBN derivatives, in contrast to their ESR spectra, were well resolved. The use of PBN as a spin-trapping agent allowed a simultaneous detection of multiple radical intermediates. The sensitivity of the electrochemical detection of nitroxides was within the picomolar range. Although an unsuccessful attempt was made for the in vivo spin trapping of alkyl radicals, it is expected that the HPLC-EC analysis of alkyl-derived PBN adducts may be helpful for identification of spin-trapped radical species in biological systems. The lack of PBN-detectable alkyl radicals is likely due to the low steady-state concentrations of R$^{\bullet}$, as only a small fraction of LO$^{\bullet}$ go to produce R$^{\bullet}$ (29). Thus, the unstable nature of the lipid-derived radicals and the multiple reaction pathways of their decomposition reduces the efficient spin trapping of R$^{\bullet}$, which remains a severe limitation of such indirect detection techniques. However, the presented data do not exclude the possibility that other nitrones may prove more efficient scavengers of alkyl radicals.

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Supporting Information Available: Preparation of PBN/C_{1-5} nitroxides under the conditions of the Wurtz reaction. This Material is available free of charge via the Internet at http://pubs.acs.org.

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