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# STRUCTURE IDENTIFICATION OF FREE RADICALS BY ESR AND GC/MS OF PBN SPIN ADDUCTS FROM THE *IN VITRO* AND *IN VIVO* RAT LIVER METABOLISM OF HALOTHANE

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Free radicals were detected from the *in vitro* metabolism of halothane (rat liver microsomes) by the PBN spin trapping method. The detected radical species include the 1-chloro-2,2,2-trifluoro-1-ethyl radical (**I**), as determined by mass spectral analysis, and lipid-type radicals assigned by high resolution ESR spectroscopy with the use of $d_{14}$-deuterated PBN. The lipid-derived radicals are a carbon-centred radical with the partially assigned structure $^{\cdot}CH_2R$ and an oxygen-centred radical of the $^{\cdot}OR'$ type. From the mass spectral analysis of the spin adduct mixture there is also evidence for a halocarbon double adduct of PBN of the type I-PBN-I.

KEY WORDS: ESR spin-trapping, halothane, GC/MS

## INTRODUCTION

The formation of free radicals from the metabolism of halothane by rat liver *in vivo*, rat hepatocytes and rat liver microsomes *in vitro* has been reported before[1-4] using the spin trap PBN (α-phenyl *tert*-butyl nitrone), with subsequent detection of the spin adducts by electron spin resonance spectroscopy (ESR). With the use of PBN and unmodified halothane the ESR spectrum of the halothanyl-PBN adduct gives a nitrogen and a β-hydrogen hyperfine splitting constant ($a_N$ and $a_\beta^H$) of 14.6 and 2.4–2.5 Gauss, respectively.[1] These values do not confirm the origin of the attached radical species nor do they differentiate between the loss of either the chlorine or bromine atom from the halothane molecule ($CF_3$–CHClBr).

$$CF_3\text{–}CHClBr \rightarrow \underset{\mathbf{I}}{CF_3\text{–}\dot{C}HCl} \text{ or } \underset{\mathbf{II}}{CF_3\text{–}\dot{C}HBr}$$

Unfortunately, C-13 enriched halothane is not readily available to confirm the assignment of the spin adduct structure, as was done in the case of carbon tetrachloride,[5]

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and therefore a combination of ESR, with deuterated PBN, and gas chromatography/mass spectrometry has been implemented to assign the structures of the spin adducts obtained from the metabolism of halothane in the presence of PBN. Deuterated PBN, particularly PBN-$d_{14}$ (deuterated on the phenyl and *tert*-butyl groups), offers the possibility of obtaining substantially better resolution for each ESR line (narrower line widths),[6] thus obtaining dramatically better separation of each component in a spectrum containing numerous components. We have previously shown with the use of PBN-$d_{14}$ that it was possible to resolve three components in the ESR spectrum from the *in vitro* rat liver metabolism of $^{13}CCl_4$.[7] The assignment of the detected spin adducts was determined to be PBN-$^{13}CCl_3$, PBN-OR and PBN-$CH_2R$, with the PBN-$CH_2R$ adduct giving a clearly resolved 1:2:1 triplet from the corresponding $\gamma$-hydrogen hyperfine splitting. The use of deuterated PBN can also aid in the mass spectral analysis of rat liver lipid extracts since the masses of the parent ion and/or fragment peaks increase by the amount of deuteration i.e. an increase of 9 for PBN-$d_9$ (*tert*-butyl deuteration) or 14 for PBN-$d_{14}$ (both phenyl and *tert*-butyl deuteration).[8]

## MATERIALS AND METHODS

### *(1) In vitro experiments*

Wistar rats (one month old, male, 100–150 g) were killed by $CO_2$ asphyxiation and their livers removed. Microsomal preparations were obtained as previously described.[7] The reaction mixture which contained rat liver microsomes (RLM) (30 mg/ml of protein) in a 0.15 M phosphate buffer (pH 7.4), 0.14 M PBN (either PBN, PBN-$d_9$ or PBN-$d_{14}$) 0.20 M halothane and 0.3 mM each of NADPH and NADH, were incubated under $N_2$ gas for 15 min. Experiments were also done using 2 mM sodium dithionite ($Na_2S_2O_4$) instead of NADPH/NADH. PBN was obtained from Aldrich and further purified by vacuum sublimation. PBN-$d_9$ and PBN-$d_{14}$ were synthesized in this laboratory.[9]

### *(2) In vivo experiments*

Rats (200 g) were administered halothane (200 $\mu$l/200 g rat in a saline solution containing 5 % Emulphor) and PBN (either non-deuterated or deuterated) via an i.p. injection. Animals were killed (30 and 60 min after halothane and PBN administration) and their livers removed and homogenized.

### *(3) Extraction of spin adducts*

Spin adduct mixtures from either *in vivo* or *in vitro* experiments were extracted as follows. Absolute ethanol (4 ml) was added to 1 ml of liver homogenate followed by agitation on a vortex mixer to precipitate the proteins. The spin adducts were then extracted by the addition of 2 ml of hexane, mixed, centrifuged (bench-top) and the hexane layer separated.

### *(4) ESR analysis of PBN spin-adducts*

Rat liver hexane extracts obtained from *in vitro* or *in vivo* experiments were trans-

ferred to a round ESR cell and degassed under $N_2$ for 15 min prior to ESR analysis. ESR spectra were obtained from a Bruker EPR ER-200D X-band spectrometer. Spectral accumulation and averaging was done on a Bruker ER-140 (Aspect 2000) data system. Spectral assignments were confirmed with a computer simulation program.[10]

### *(5) GC/MS analysis*

The concentrated hexane samples were analyzed with a Hewlett-Packard series 5790 gas chromatograph interfaced with a VG model 12000 quadrupole mass spectrometer equipped with a VG model 11/250 data system. Chromatographic separation was carried out on a J & W 0.25 mm i.d. × 15 m DB-5 fused silica capillary column, temperature programmed from 100 °C to 320 °C at 10 °/minute, with helium as the carrier gas. The mode of operation was electron ionization (EI) at 70 eV.

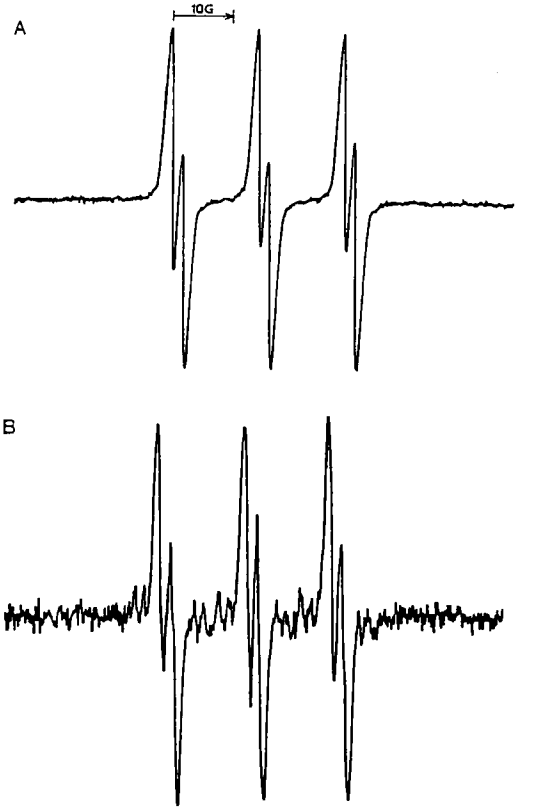



FIGURE 1 ESR spectra of hexane extracts after incubation (15 min) of rat liver microsomes (37 °C) with either (a) halothane, PBN and NADPH/NADH or (b) halothane, PBN and sodium dithionite. Prior to ESR analysis, the hexane lipid extracts were flushed with nitrogen (10–15 min) to remove oxygen. $a_N$ = 14.6 and $a_\beta^H$ = 2.3 G.

## RESULTS AND ASSIGNMENTS

The ESR spectrum obtained from the *in vitro* metabolism of halothane by rat liver microsomes incubated with PBN and NADPH/NADH or sodium dithionite are shown in Figure 1. The ESR spectra from either *in vitro* or *in vivo* experiments are shown in Figure 2. The best computer simulated fit obtained by summing three components with different hyperfine splitting constants, g-values and relative amounts is shown in Figure 3 with assignments indicated in Table I. Structure **III** can be either **I** or **II** since hyperfine splitting from chlorine or bromine cannot be resolved in the $\gamma$-position. Structures **IV** and **V** are present as overlapping spectra thus making the analysis of the spectrum very complex without the aid of computer simulation.

GC/MS analysis was done on a similar hexane extract of an *in vitro* microsomal incubation sample using PBN-$d_9$ instead of PBN-$d_{14}$. The GC peak numbered 99 in Figure 4a with a mass of 186 represents the parent PBN-$d_9$ molecule (Figure 4b). The GC peak numbered 182 in Figure 4a gives the mass spectrum shown in Figure 4c with the highest ion mass of 303 which is assigned to the spin adduct **VI** derived from **I**, not **VII** from **II**. Since a mass 66 fragment ion is detected, it is possible to assign this

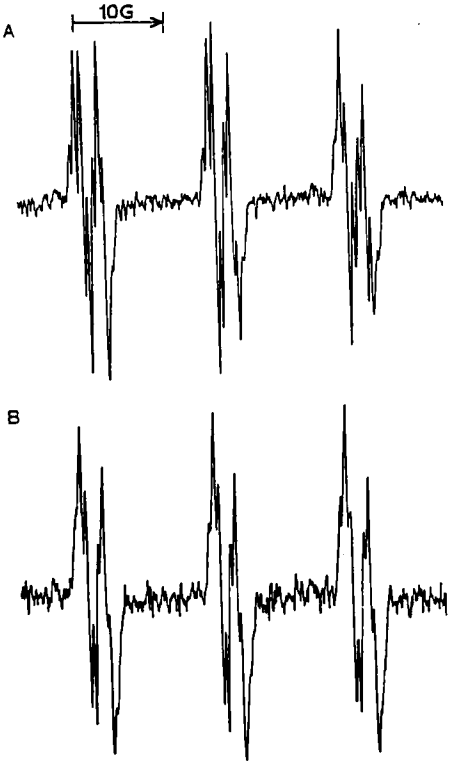



FIGURE 2 *In vitro* ESR spectrum of a microsomal lipid hexane extract after incubation (15 min) of rat liver microsomes (37 °C) with halothane, PBN-$d_{14}$ and NADPH/NADH. Treatment of the lipid extract was similar to that described in Figure 1. (b) *In vivo* ESR spectrum of a hexane extract from a liver homogenate 30 min after administration of halothane and PBN-$d_{14}$. Treatment of the liver extract was similar to that described in Figure 1.

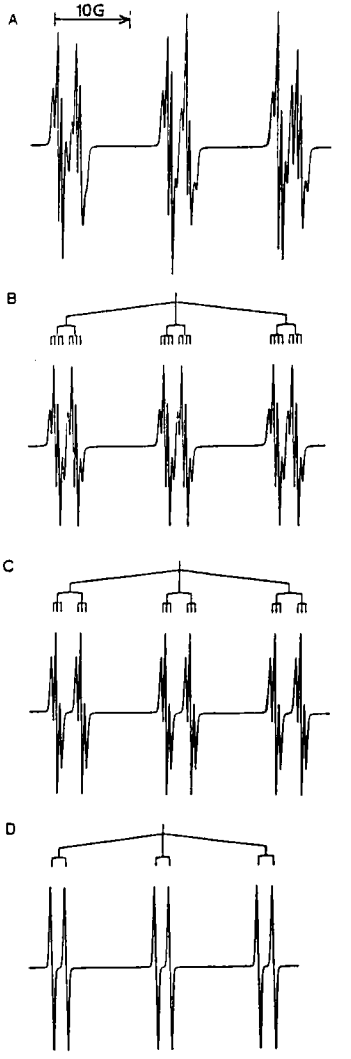



FIGURE 3 (a) Best computer simulation fit of the ESR spectrum in Figure 2a using the parameters listed in Table 1. (b) Computer simulated spectrum and splitting pattern of the PBN-CHClCF$_3$ adduct; $a_N$ = 14.53, $a_\beta^H$ = 2.43 (1*H*) and $a_\delta^F$ = 0.5 (3*F*). (c) Computer simulated spectrum and splitting pattern of the PBN-CH$_2$R adduct; $a_N$ = 14.52, $a_\beta^H$ = 3.33 and $a_\gamma^H$ = 0.55 (2*H*). (d) Computer simulated spectrum and splitting pattern of the PBN-OR adduct; $a_N$ = 13.80 and $a_\beta^H$ = 1.90 (see Table I for relative amounts).

TABLE I
Computer simulated ESR spectral assignments.

| | $CF_3$-$\dot{C}$H-$R_1R_2$ III | $R_3$-$CH_2$· IV | $R_4$-O· V |
|---|---|---|---|
| $a_N$ = | 14.53 | 14.52 | 13.80 |
| $a_H^\beta$ = | 2.43 (1*H*) | 3.33 | 1.90 |
| $a_H^\gamma$ = | – | 0.55 (2*H*) | – |
| $a_F^\delta$ = | 0.5 (3*F*) | – | – |
| *g*-value = | 2.00621 | 2.00612 | 2.00631 |
| relative amount = | 1.00 | 0.26 | 0.07 |

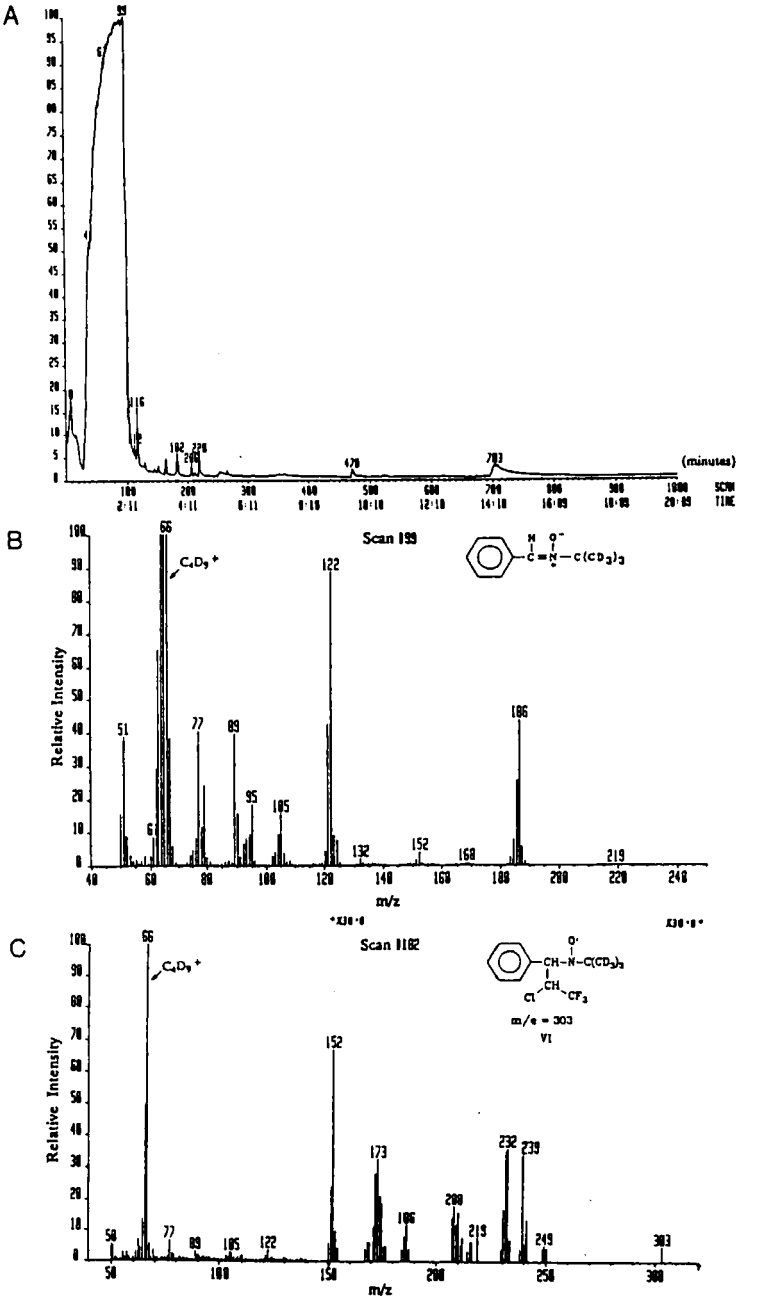
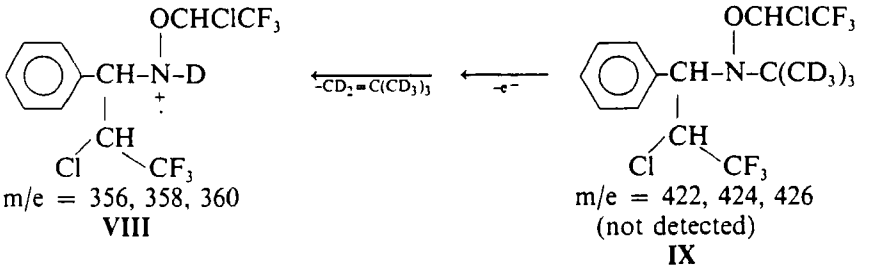


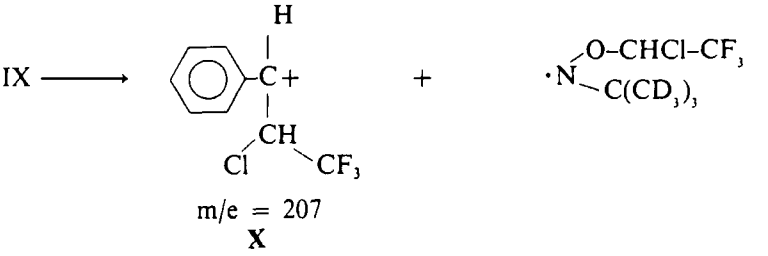

FIGURE 4 GC/MS analysis of a rat liver microsomal hexane extract after incubation (15 min) of rat liver microsomes (37 °C) with halothane, $d_9$-PBN and NADPH/NADH. (a) Total ion current gas chromatogram. (b) Mass spectrum (EI, 70 ev) for peak # 99, $d_9$-PBN, MW = 186. (c) Mass spectrum for peak # 182, $d_9$-PBN-halothanyl adduct (PBN-CHClCF$_3$), MW = 303.

GC peak to a spin adduct of deuterated PBN (PBN-$d_9$). The mass 66 ion is characteristic since it comes from $C_4D_9^+$ (the deuterated *tert*-butyl group) and is not a common fragment for any other compound.[8] This assignment is supported by the important fragment ion $m/z$ = 239, 240, 241, coresponding to $C_6H_5CH(CHClCF_3)NOD^+$, a result of the loss of isobutylene from the parent ion by internal deuterium transfer.

The GC trace in Figure 5a is a repeat of Figure 4a except that only peaks containing mass 303 are shown (at 36, 40 and 47 seconds). From the mass spectra (Figure 5b shows only GC peak 36) it is clear that these are halothane related adducts from the presence of two chlorine atoms ($^{35}Cl/^{37}Cl \approx 2:1$). The suggested assignment is that mass peak 356 is due to ion structure **VIII** resulting from the fragmentation or thermolysis of **IX**, the double adduct of **I** and PBN.

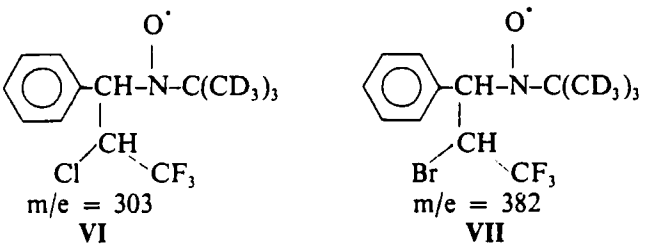



Compound **IX** is postulated to decompose on the column producing isobutylene (leading to mass 66) and the radical precursor to **VIII**. **IX** may also thermalize to give **VI** by loss of **I** to give a mass 303 peak which is present. However another cleavage route to give a stable benzylic ion with mass 207 is as follows:




The PBN-$CH_2R$ and PBN-OR radical adducts detected by ESR have not been found as yet by GC/MS, probably due to the instability and heat sensitive nature of these spin adducts.

In conclusion, structural identification of spin adducts obtained from the spin trapping of biologically generated free radical species requires not only the use of ESR spectroscopy with deuterated spin traps, but also the use of gas or liquid chromatography in conjunction with mass spectrometric analysis for further confirmation.



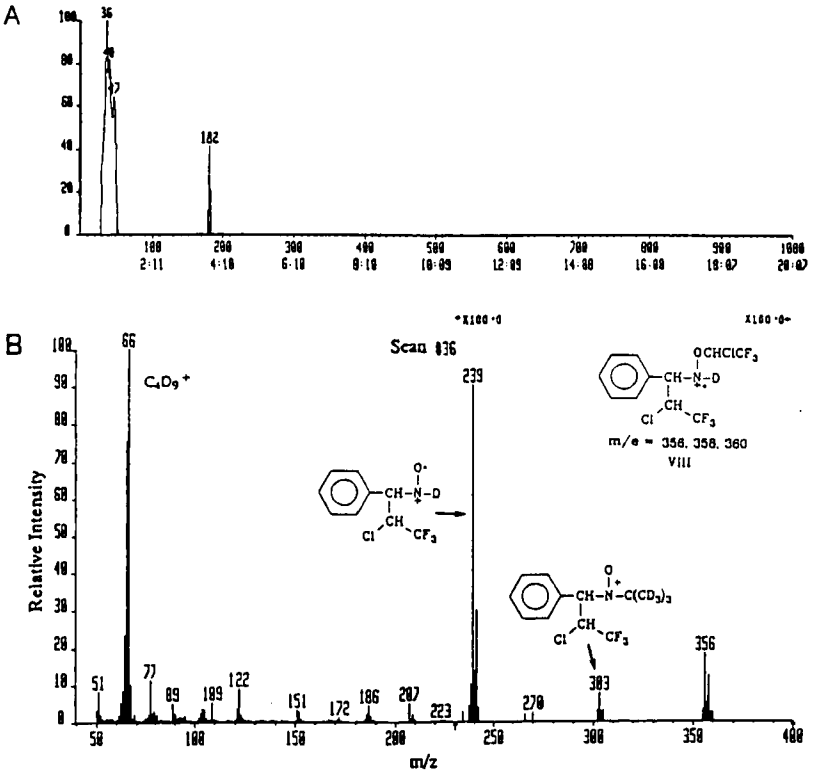




FIGURE 5 (a) Gas chromatogram obtained from Figure 4a outlining peaks containing a mass of 303. (b) Mass spectrum for peak # 36, $CF_3CHCl$-$d_9$-PBN-$CHClCF_3$ adduct, MW = 420 (parent ion not visible by EI).

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