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Original Contribution

THE STRUCTURE OF FREE RADICAL METABOLITES DETECTED BY EPR SPIN TRAPPING AND MASS SPECTROSCOPY FROM HALOCARBONS IN RAT LIVER MICROSOMES

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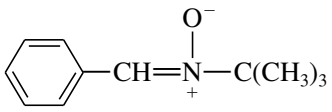
Abstract—Electron impact (EI) tandem mass spectrometry (MS/MS) combined with EPR spin trapping was used to detect and identify the free radical metabolites of various halocarbons in rat liver microsomal dispersions. EPR spectra of the spin adducts of radical metabolites derived from fluorine-containing halocarbons display fluorine hyperfine splitting, which can be used as proof for the identification of this kind of halocarbon-derived free radical spin adduct. For halocarbons without fluorine atoms, MS/MS was found to be a very useful and simple method for the detection and identification of the structures of halocarbon-derived spin adducts from radical metabolites. The molecular ions from spin adducts of these halocarbon-derived free radical intermediates were observed for the first time by scanning the precursor ion spectrum of *m*/*z* 57. These assignments were further confirmed by the use of perdeuterated *tert*-butyl PBN which provides the precursor ion spectrum of *m*/*z* 66. *Copyright © 1997 Elsevier Science Inc.*

Keywords—MS/MS, EPR spin trapping, Rat liver microsomes, PBN, Halocarbons, Free radical metabolites, Spin adducts, Precursor ion spectrum

INTRODUCTION

The possibility that free radicals might be associated with the toxicity of carbon tetrachloride (CCl_4) was first proposed by Butler,[1] Wirtschafter,[2] Rechnagel,[3,4] and Slater.[5,6] However, proof for the existence of this species as a discrete metabolite was lacking because direct electron paramagnetic resonance (EPR) detection in vitro in a biological system or in vivo in a model animal such as the rat was unsuccessful.[7] Early reports have described the attempted detection of this free radical in liver slices,[8,9] however, these studies have been criticized.[10,11] In fact, the steady-state level of $\cdot CCl_3$ in the tissue slices would be much below the limit of detection by EPR.[12]

With the advent of spin trapping, it was shown that free radicals could be detected by the use of C-phenyl N-*tert*-butyl nitrone (PBN, see structure **1**) in the reductive metabolism of CCl_4 in rat liver microsomal dispersions[13,14] as well as in vivo in the rat.[15]



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PBN

These free radical metabolites were subsequently shown to be trichloromethyl radicals by the use of ^{13}C ($I = \frac{1}{2}$) carbon tetrachloride.[16,17] The $^{13}CCl_3$ spin adduct of PBN gives a 12-line EPR spectrum that is composed of a triplet of double doublets distinctly different from the spectrum of any other carbon- or oxygen-centered spin adducts. This feature is a necessary and sufficient

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proof of structure for the spin adduct if a correct and unequivocal assignment is to be made. Many studies have been subsequently based on the assignment of this spin adduct.[18–23]

Later work showed that mass spectroscopy could also be useful in verifying the structure of the trichloromethyl spin adduct of PBN.[24–26] The two different isotopes of chlorine are very useful for identification because they give a specific mass pattern that is characteristic of three chlorine atoms. This mass pattern (100:97.5:31.7:3.4) for the $\bullet CCl_3$ adduct of PBN[27] is a necessary and sufficient proof of structure for the trichloromethyl group in the molecule under investigation.

A number of different workers went on to study other halocarbons and searched for PBN spin adducts from microsomal dispersions and/or in vivo systems. Among these halocarbons, halothane was studied most by several research groups. Halothane is a widely used halogenated anaesthetic in surgical and other clinical procedures, and it produces rare and unpredictable liver damage. It was found by the EPR-spin trapping technique that a halothane-dependent free radical was formed when halothane is metabolized by liver microsomes, or produced in the liver of intact rats which have been subjected to halothane anesthesia.[28] The observation of a free radical metabolite from halothane in microsomes and in vivo was later reported also by several other research groups.[29–32] This metabolite arises from the reductive debromination of halothane as confirmed by gas chromatography mass spectroscopy (GC/MS)[33] and also by the use of [1-^{13}C] and [2-^{13}C] halothane.[34]

Radical adducts derived from $CHCl_3$, $CHBr_3$, CHI_3, and $BrCHCl_2$ have been studied in isolated hepatocytes and in the rat in vivo.[35] These trihalogenated methanes are known to be strongly hepatotoxic when administered experimentally to animals.[36,37] They are ubiquitous contaminants of chlorinated water supplies.[38] Chloroform is the most widely employed of the trihalomethanes, being used extensively as an industrial solvent and as an intermediate for chemical processes.[39] The origin of the $CHCl_3$- and $CHBr_3$-derived radicals has been confirmed by using ^{13}C-labeled $CHCl_3$.[35] Free radicals formed during in vivo and in vitro metabolism of $CHBr_3$ were also confirmed by using $^{13}CHBr_3$.[40] The in vivo and in vitro metabolic formation of radicals was also observed for 1,1,1-trichloroethane (TCE),[41,42] which is a widely used industrial solvent for degreasing and dry cleaning.[43]

Although in many of the instances reviewed above EPR spectra were obtained consistent with spin adducts arising from halocarbon-derived radicals, no proof of these assignments was available. Only rarely was the luxury of having ^{13}C-labeled halocarbons experienced and thus essential verification was often lacking. This problem was aggravated by the fact that the nitrogen and β-hydrogen hyperfine splitting constants (N- and β-H HFSC's) of halocarbon radical spin adducts are very similar to those of alkoxyl radical spin adducts. Because the latter could conceivably also be present in the same metabolizing systems, no firm assignments could be made.

In this study, we have repeated much of this work, attempting in every case to verify the structure of the species producing the EPR spectrum of the spin adduct by EI tandem mass spectroscopy (MS/MS). We have shown previously that, with the use of deuterated PBN (PBN-d_{14}, perdeuterated phenyl and *tert*-butyl groups; PBN-d_9, perdeuterated on *tert*-butyl group only) that the MS/MS technique can be used successfully to identify the trichloromethyl adduct of PBN from in vitro rat liver microsomal metabolism of $BrCCl_3$ and CCl_4.[26] Because MS/MS reduces the need to perform chromatographic separation, it is expected to be especially useful for the analysis of radical spin adducts, which usually are present at low concentration and are not very stable for chromatography. Because PBN spin adducts produce *m*/*z* 57 (*tert*-butyl ion, $C_4H_9^+$) as a characteristic fragment, a precursor ion spectrum of *m*/*z* 57 is used in this study to search for PBN spin adducts produced in rat liver microsomal dispersions that have been incubated with halocarbons in the presence of an NADPH-generating system. Use of deuterated PBN (PBN-d_9 and PBN-d_{14}) further confirms the assignment of PBN spin adducts because the masses of the molecular ions of any PBN spin adducts in the precursor ion spectrum of *m*/*z* 66, which corresponds to the deuterated *tert*-butyl ion, $C_4D_9^+$, will increase 9 mass Da by using PBN-d_9 or 14 mass Da by using PBN-d_{14}.

MATERIALS AND METHODS

Chemicals

PBN, PBN-d_{14}, and PBN-d_9 were purchased from OMRF Spin Trap Source (Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, Oklahoma 73104, USA).

Sample preparation

Rat liver microsomal dispersions were prepared as reported previously.[21] The reaction mixture (a total volume of 15 ml) consisted of a rat liver microsomal dispersion (3 mg/ml protein) in a 0.05 M potassium phosphate buffer (pH 7.4); 0.01 M PBN (or 0.01 M PBN-d_9, or 0.01 M PBN-d_{14}); 0.025 M halocarbon, an NADPH-generating system (GS). The NADPH-generating sys-

tem consisted of 5.5 mM glucose-6-phosphate, 0.3 mM NADP, and 0.5 units/ml of glucose-6-phosphate dehydrogenase.

The system was incubated under nitrogen atmosphere at 37°C for 1 h. After incubation, spin adducts were extracted by the addition of 10 ml hexane, agitated by vortex, centrifuged (bench-top), and the hexane layer was separated and analyzed by EPR. More than 90% of the spin adducts was extracted by 10 ml hexane, a value estimated by measuring the EPR signal intensity of the hexane extracts. The concentrated extracts were used for MS measurements.

Mass spectrometric analysis

EI mass spectra were obtained with a VG-Fisons Quattro triple stage quadruple mass spectrometer. A direct insertion probe was used. Source temperature was 180°C. The electron energy employed was 15 eV. The probe temperature was about 40–60°C.

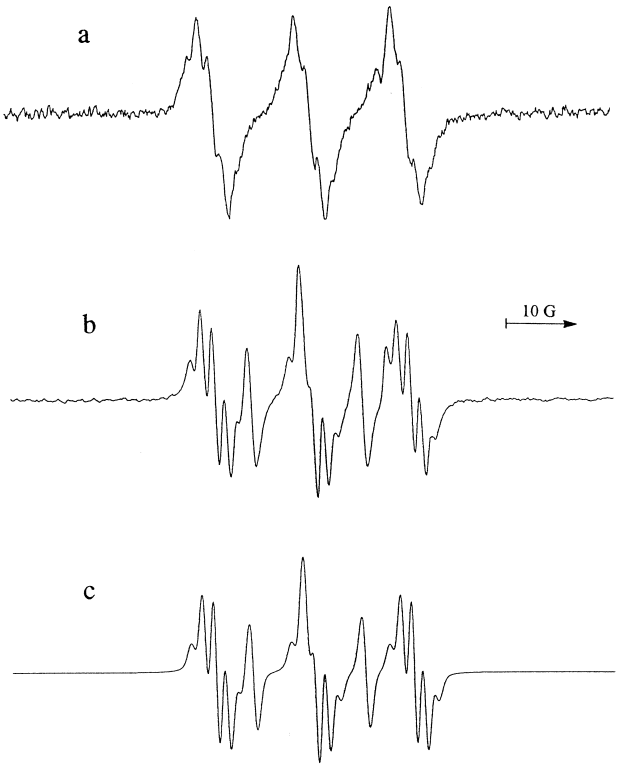


Fig. 1. (a) EPR spectrum recorded from a hexane extract of rat liver microsomes incubated with CF_3I in the presence of PBN. (b) EPR spectrum obtained by UV illumination of CF_3I in hexane in the presence of PBN-d_{14}. (c) Simulated spectrum of (b). The spectrum was simulated assuming the presence of two radical species: $\bullet CF_3$ spin adduct (I) and PBNOX (II). Parameters used for this simulation are: radical I, $a^N = 14.05$ G, $a^H_\beta = 1.55$ G, $a^F_\gamma = 1.55$ G (3F), LW (line width) = 1.2 G; radical II, $a^N = 8.00$ G, LW = 1.20 G, $\Delta G = -0.9$ G. The relative concentrations are 6:1.

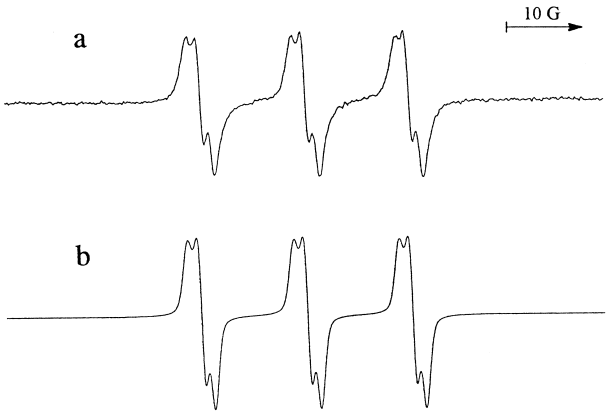


Fig. 2. (a) EPR spectrum recorded from a hexane extract of rat liver microsomes incubated with CBr_2ClF in the presence of PBN. (b) Simulated spectrum of (a). The spectrum was simulated assuming the presence of two radical isomers. Parameters used for this simulation are: isomer I, $a^N = 14.05$ G, $a^H_\beta = 1.50$ G, $a^F_\gamma = 1.23$ G, LW = 1.15 G; radical II, $a^N = 14.05$ G, $a^H_\beta = 1.23$ G, $a^F_\gamma = 1.10$ G, LW = 1.15 G, $\Delta G = 0.4$ G. The relative concentrations are 1:1.

Argon gas was used for the collision-induced dissociation (CID). The collision energy was 25 ev.

GC-MS spectra were recorded with a Hewlett-Packard 5890 series II GC interfaced directly with the VG-Fisons Quattro triple stage quadruple mass spectrometer. Chromatographic separation was performed with a Quadrex Corporation 0.25-mm × 30-m methyl 5% phenyl fused silica column with temperature programmed from 55°C to 180°C at 10°C/min. Injector temperature was 150°C. Helium was the carrier gas.

EPR measurement

EPR spectra were measured by a Bruker ER300 spectrometer with 100-KHz field modulation. Round quartz sample cells with 3.5-mm i.d. were employed.

RESULTS AND DISCUSSION

EPR spin trapping study of free radical metabolites from halocarbons in RLM in the presence of PBN

Halocarbons having fluorine atoms: CF_3I, CBr_2ClF, CBr_2F_2, and CCl_3CClF_2. The EPR spectra obtained from the in vitro metabolism of CF_3I, CBr_2ClF, CBr_2F_2, and CCl_3CClF_2 in rat liver microsomes (RLM) in the presence of PBN and NADPH-generating system are shown in Figs. 1–4. In addition to N- and β-H hyperfine splitting, all spectra display hyperfine splitting from the fluorine atom. These fluorine hyperfine splittings can be used as a proof to identify these halocarbon-derived radical spin adducts. The hyperfine splitting constants (hfsc's) of the corresponding PBN spin adducts listed in Table 1 were obtained by computer

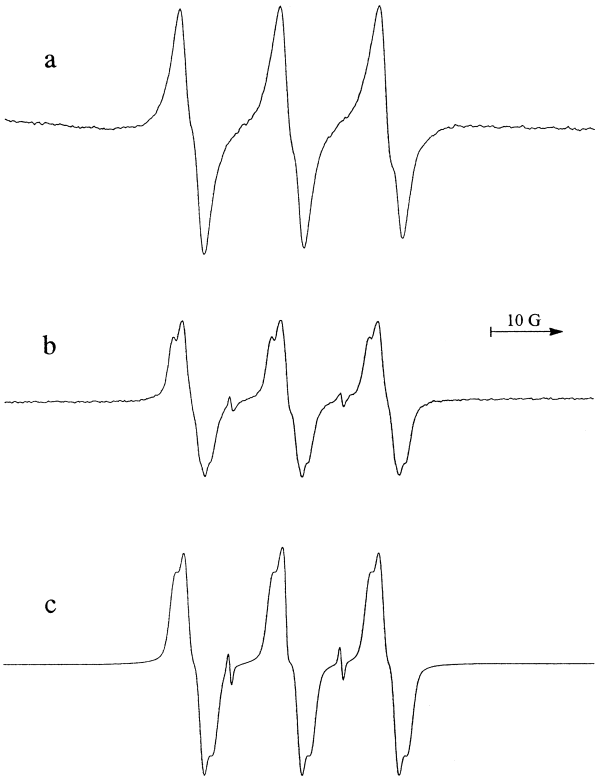


Fig. 3. (a) EPR spectrum recorded from a hexane extract of rat liver microsomes incubated with CBr_2F_2 in the presence of PBN. (b) EPR spectrum obtained by UV illumination of CBr_2F_2 in hexane in the presence of PBN-d_{14}. (c) Simulated spectrum of (b). The spectrum was simulated assuming the presence of two radical species: $\cdot CBrF_2$ spin adduct (I) and PBNOX (II). Parameters used for this simulation are: radical I, a^N = 13.80 G, a^H_β = 2.00 G, a^F_γ = 1.30 G (2F), LW = 1.45 G; radical II, a^N = 7.90 G, LW = 0.5 G, $\Delta G = -0.8$ G. The relative concentrations are 100:0.5.

simulation and/or by comparison with the spectra produced by UV irradiation of the corresponding halocarbons in hexane.

Fig. 1a was recorded from the hexane extract of an incubation system consisting of RLM, CF_3I, NADPH-GS, and PBN. The spectrum shows additional splitting probably caused by the fluorine atom (5 lines due to equivalent splitting from β-H and γ-F),[44,45] which is a positive indication that the radical species trapped is indeed a radical derived from CF_3I. A very similar but better resolved EPR spectrum (Fig. 1b) was observed when a hexane solution of CF_3I was illuminated by UV in the presence of PBN-d_{14}; also, a small amount of benzoyl aminoxyl radical (PBNOX) was formed. This radical is assigned to the $\cdot CF_3$ adduct of PBN. The computer simulation (Fig. 1c) using the hyperfine splitting from three fluorine atoms agrees with the experimental result.

When RLM were incubated with CBr_2ClF in the presence of PBN, a spectrum exhibiting incompletely

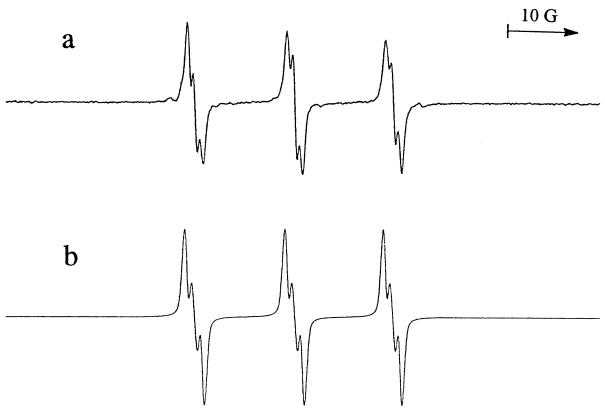
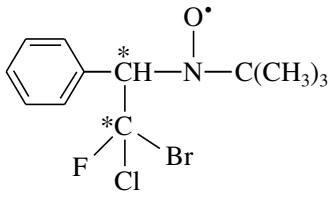


Fig. 4. (a) EPR spectrum recorded from a hexane extract of rat liver microsomes incubated with CCl_3CClF_2 in the presence of PBN-d_{14}. (b) Simulated spectrum of (a). Parameters used for this simulation are: a^N = 14.15 G, a^H_β = 1.40 G, a^F_δ = 0.80 G (1F), LW = 0.80 G.

resolved hyperfine splitting was obtained (shown in Fig. 2a). This spectrum is not centrosymmetric, probably due to the existence of two chiral centers in the proposed PBN spin adduct of $\cdot CBrClF$ (see structure **2;** the chiral carbons are labeled with a star).



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Computer simulation (Fig. 2b) using parameters for the two possible spin adducts provided good agreement with the experimental spectrum.

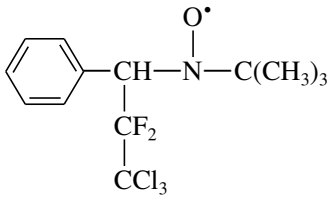
The metabolism of CBr_2F_2 in RLM in the presence of PBN gave a poorly resolved EPR spectrum (Fig. 3a). It has been reported that PBN-d_{14} can greatly improve the sensitivity and especially the resolution within an EPR spectrum.[46] Indeed, the resolution of most of EPR spectra can be improved by using PBN-d_{14}. However, for these halocarbons, PBN-d_{14} did not improve reso-

Table 1. The Hyperfine Splitting Constants of PBN Spin Adducts

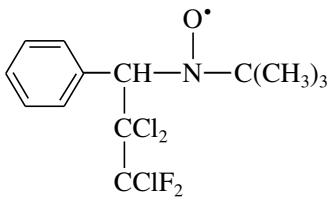
Source	Radical	a^N (G)	a^H_β (G)	a^F_γ (G)
CF_3I	$\cdot CF_3$	14.05	1.55	1.55 (3F)
CBr_2ClF	$\cdot CBrClF$	14.05	1.50	1.23 (1F)
		14.05	1.23	1.10 (1F)
CBr_2F_2	$\cdot CBrF_2$	13.80	2.00	1.30 (2F)
CCl_3CClF_2	$\cdot CF_2CCl_3$ or $\cdot CCl_2CClF_2$	14.15	1.40	0.80 (1F)

lution very much. When the hexane solution of CBr_2F_2 was illuminated by UV in the presence of PBN-d_{14}, a better-resolved spectrum was obtained (Fig. 3b). Based on the EPR results and computer simulation (Fig. 3c), this radical is assigned to $\cdot CBrF_2$ adduct of PBN.

Fig. 4a is the EPR spectrum recorded from a hexane extract of RLM incubated with CCl_3CClF_2 in the presence of PBN-d_{14}. The splitting pattern consists of a nitrogen triplet with two small doublet splittings: a^N = 14.5G, $a^H_\beta \cong 1.40$G, $a^F_\gamma = 0.80$G (1F). Of the two possible radical metabolites, the spin adduct most likely to give this pattern is 3a. The fluorine hyperfine splitting from the δ-position in 3b is not expected to be detectable. This assignment proposes that only one of the two γ-fluorines provides detectable hyperfine splitting at this level of resolution. This situation is possible if hindered rotation of the added group exists. A small asymmetry in the spectrum indicates the presence of probably another spin adduct at a lower concentration.



3a



3b

Based on the above EPR results for these types of compounds, it is clear that the fluorine hyperfine splitting can be used as a proof for the identification of these halocarbon-derived spin adducts if the resolution of the EPR spectrum is high enough to display the fluorine hyperfine splitting.

Halocarbons which do not contain fluorine atoms: $CHBrCl_2$, $CHBr_2Cl$, $CHBr_3$, $CHCl_3$, and CCl_3CH_3. The EPR spectra of all of the spin adducts from these halocarbon free radical metabolites from RLM show triplets of doublets with relatively minor differences in hyperfine splitting constants and g factor values. The hyperfine splitting constants obtained by the analysis of the EPR spectra are listed in Table 2. These values are very close to those of alkoxyl PBN spin adducts. If the EPR spectra would display the γ-H hyperfine splitting, which is the splitting caused by the proton from the halocarbons like $CHBrCl_2$, $CHBr_2Cl$, $CHCl_3$, $CHBr_3$, one should be able to assign the radical spin adduct by EPR. Unfortunately, the resolution of the EPR spectra of most halocarbon-derived radicals is generally very poor because of the existence of the halogens (Br, Cl), which broaden the EPR lines. Even PBN-d_{14} could not resolve the splitting from the γ-hydrogen. Therefore, one cannot assign these spectra based only on these EPR data if the ^{13}C isotope-labeled substitute is not available.

Table 2. The Hyperfine Splitting Constants of PBN Spin Adducts

Source	Radical	a^N (G)	a^H_β (G)	a^C_γ (G)
$CHBrCl_2$	$\cdot CHCl_2$	14.23	2.03	5.63
$CHBrClCF_3$	$\cdot CHClCF_3$	14.60	2.30	
$CHBr_2Cl$	$\cdot CHBrCl$	14.30	2.19	
$CHBr_3$	$\cdot CHBr_2$	14.23	1.98	
$CHCl_3$	$\cdot CHCl_2$	14.20	2.04	
CCl_3CH_3	$\cdot CCl_2CH_3$	14.29	1.85	

Although EPR spin trapping is a powerful technique of great value in the detection of free radical intermediates in the metabolic activation of haloalkanes, the unequivocal identification of the structures of free radical metabolites of halocarbons by EPR spin trapping cannot be accomplished without the aid of ^{13}C-labeled substrates or fluorine-substitution. In addition, EPR only reports on the existence of the halocarbon-derived free radical adduct; the EPR spectra can not differentiate the loss of either the chlorine, bromine, or iodine atom from the precursor halocarbons during the reductive process. This is because the resulting EPR spectra are very similar and show relatively minor differences in splitting constants and g-values.

MS/MS detection of the free radical metabolites of halocarbons produced from RLM in the presence of PBN

A combination of the mass spectrometric technique and spin trapping was found to assign unambiguously the structures of halocarbon-derived free radical adducts. Because PBN and its spin adducts usually produce an abundant fragment by EI, namely, *m*/*z* 57 ($C_4H_9^+$ *tert*-butyl ion), a scan of precursors of *m*/*z* 57 was recorded during tandem mass spectrometry analysis to find ions that are potentially due to PBN spin adducts. Compared with conventional mass spectrometry, precursor ion spectra can exclude most of the interfering ions derived from the mixture. It greatly reduces the number of peaks observed in the spectrum because only ions that produce *m*/*z* 57 are detected. These halocarbon-derived radical adducts are not dif-

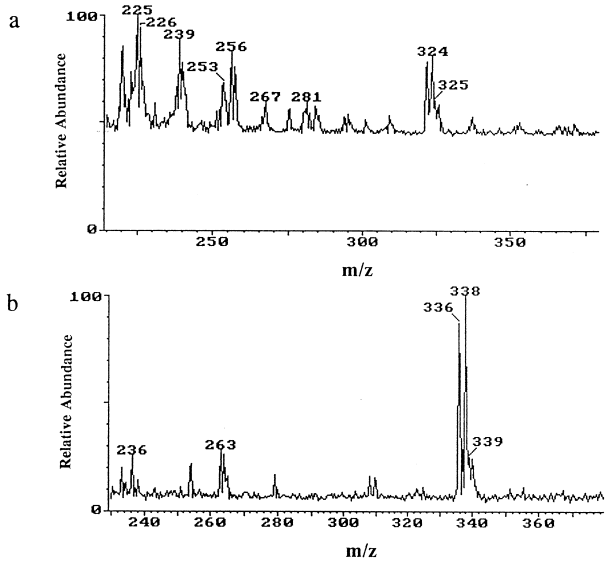


Fig. 5. (a) The precursor ion spectrum of *m*/*z* 57 recorded during the EI (15 ev) MS/MS analysis of a hexane extract of rat liver microsomes incubated with CBr_2ClF in the presence of PBN. (b) The precursor ion spectrum of *m*/*z* 66 recorded from the same system as (a) but using PBN-d_{14} as a spin trap.

ficult to recognize because most of them (except CF_3I) display isotopic peaks from bromine or chlorine atoms.

Free radical metabolites formed by the debromination of halocarbons

Fig. 5a is the precursor ion spectrum of *m*/*z* 57 recorded from a hexane extract of RLM incubated with CBr_2ClF in the presence of PBN. The isotopic peaks at *m*/*z* 322, 324, and 326 are the appropriate molecular ions of the PBN adduct of •CBrClF, which is produced by the reductive debromination of CBr_2ClF. The relative intensity of these isotopic peaks indicates this species contains one chlorine and one bromine (77:100:24). The characteristic patterns resulting from combinations of the chlorine, bromine isotopes can be calculated from the binomial expansion $(a + b)^n = a^n + na^{n-1}b + n(n-1)a^{n-2}b^2/2! + n(n-1)(n-2)a^{n-3}b^3/(3!) + \cdots$.[27]

A precursor ion spectrum of *m*/*z* 66 (from $C_4D_9^+$, deuterated *tert*-butyl ion) was used to confirm this assignment by using PBN-d_{14} instead of PBN. Unlike PBN, *m*/*z* 66 is not a common fragment ion, so it is very characteristic. If, in the molecular ion of a spin adduct, which is observed in the precursor ion spectrum of *m*/*z* 57, a shift of 14 Da in the precursor ion spectrum of *m*/*z* 66 is observed after using PBN-d_{14} instead of PBN, then these ions are confirmed to be due to the PBN spin adducts.

It can be seen from the Fig. 5b that the peaks at *m*/*z* 322, 324, and 326 shift by 14 Da to *m*/*z* 336, 338, and 340 when PBN-d_{14} is used, which confirms the above assignment.

Similarly, the radical adducts of •CHBrCl, •$CHBr_2$, and •$CHClCF_3$ produced by the debromination of $CHBr_2Cl$, $CHBr_3$ and $CHBrClCF_3$, respectively, are also observed by the precursor ion spectra of *m*/*z* 57, and confirmed by the precursor ion spectra of *m*/*z* 66 (spectra are shown in Fig. 6). The isotopic peaks at *m*/*z* 318, 320, and 322 in Fig. 6a are appropriate molecular ions of the CHBrCl-PBN-d_{14} spin adduct. The relative intensity of the isotopic peaks indicate that this ion contains one chlorine and one bromine atom (77:100:24). The molecular ions of $CHBr_2$-PBN-d_{14} were observed at *m*/*z* 362, 364, and 366 in Fig. 6b, with the relative peak intensity of 1:2:1, which indicates the presence of two bromine species. The observation of the isotopic peaks at *m*/*z* 303 and 305 with relative intensity of a one-chlorine species (3:1) in Fig. 6c indicates the formation of $CHClCF_3$-PBN-d_9.

The molecular ions of PBN adducts of •$CBrF_2$ and •$CHCl_2$ also produced by the debromination of their

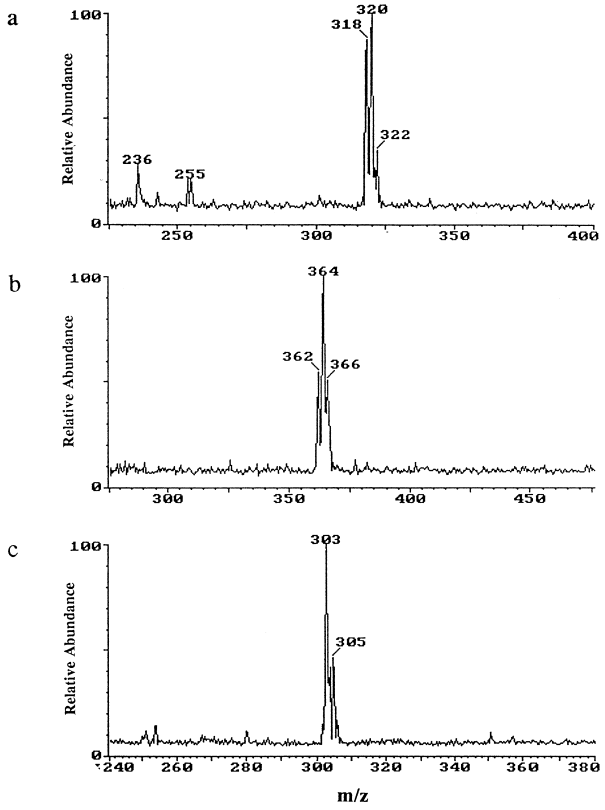


Fig. 6. The precursor ion spectra of *m*/*z* 66 recorded during the EI (15 ev) MS/MS analysis of a hexane extract of rat liver microsomes incubated with (a) $CHBr_2Cl$ in the presence of PBN-d_{14}, (b) $CHBr_3$ in the presence of PBN-d_{14}, (c) $CHBrClCF_3$ in the presence of PBN-d_9.

precursor halocarbons CBr_2F_2 and $CHBrCl_2$, respectively, were observed in the precursor ion spectra of *m*/*z* 57. All the molecular ions show isotopic peaks with expected relative intensity. Their assignments have been further confirmed by using PBN-d_{14} or PBN-d_9 and the precursor ion spectrum of *m*/*z* 66; the results are summarized in Table 3.

Thus, we have shown that the reductive metabolism of a range of halogenated methanes and ethanes, which contain bromine and/or chlorine atoms or fluorine atoms, produce free radical intermediates with RLM. The first step in the metabolism of such bromine containing-halocarbons in RLM is the one-electron reductive debromination by liver microsomal cytochrome P450, which results in the formation of the corresponding radical adducts. The C-Br bond is the weakest in the halocarbons, and so the primary free radical product results from the debromination.

All the incubations in this work have been done under nitrogen atmosphere. A similar incubation of RLM in the presence of air resulted in an EPR signal and MS peaks that were less intense than those seen under anaerobic conditions.

MS/MS of the free radical metabolites formed by the dechlorination of halocarbons

Fig. 7a is the precursor ion spectrum of *m*/*z* 66 recorded from a hexane extract of RLM incubated with CCl_3CH_3 in the presence of PBN-d_9 and NADPH-GS. Peaks at *m*/*z* 283, 285, and 287 with relative intensity of a two-chlorine ion (100:64:10) are attributed to the PBN-d_9 spin adduct of $\cdot CCl_2CH_3$ which is produced by the reductive dechlorination of CCl_3CH_3.

A chloride ion elimination from CCl_3CClF_2 could result in the formation of the radical adduct of $\cdot CCl_2CClF_2$ or $\cdot CF_2CCl_3$. This ion is also observed at *m*/*z* 344, 346, and 348 in the precursor ion spectrum of *m*/*z* 57 (see Fig. 7b). Earlier we assigned the EPR spectrum to the latter possibility.

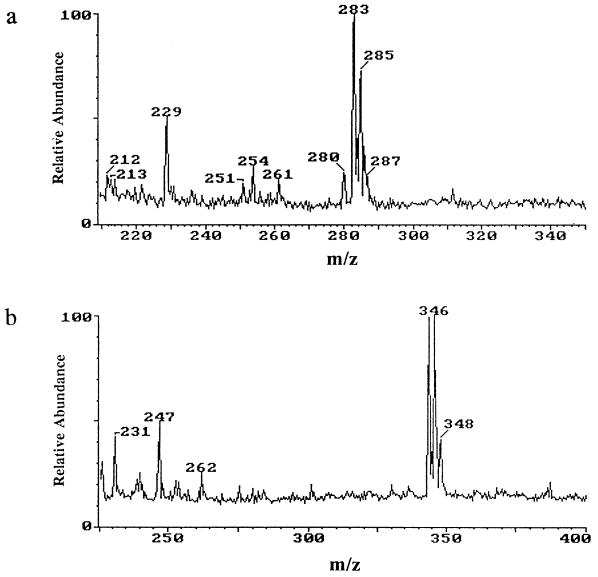


Fig. 7. (a) The precursor ion spectrum of *m*/*z* 66 recorded during the EI (15 ev) MS/MS analysis of a hexane extract of rat liver microsomes incubated with CCl_3CH_3 in the presence of PBN-d_9. (b) The precursor ion spectrum of *m*/*z* 57 recorded during the EI (15 ev) MS/MS analysis of a hexane extract of rat liver microsomes incubated with CCl_3CClF_2 in the presence of PBN.

Therefore, halocarbons CCl_3CH_3, CCl_3CClF_2, and $CHCl_3$, which do not contain bromine atoms, are reductively dechlorinated to form the corresponding radicals by cytochrome P450. The radical metabolites $\cdot CCl_2CH_3$, $\cdot CF_2CCl_3$, and $\cdot CHCl_2$ were detected by the precursor ion spectra of 57 and have been confirmed by the precursor ion spectrum of *m*/*z* 66. The MS results are summarized in Table 3.

MS/MS of the free radical metabolite formed by the loss of an iodide ion

For CF_3I, which does not contain bromine and chlorine, MS results show that it is metabolized reductively

Table 3. The Molecular Ions of Halocarbon-Derived Radical Spin Adducts Observed in the Precursor Ion Spectrum of *m*/*z* 57 (PBN as a Spin Trap) and *m*/*z* 66 (PBN-d_9 or PBN-d_{14} as a Spin Trap)

Source	Radical	$M^{\bullet +}$ (PBN)	$M^{\bullet +}$ (PBN-d_9)	$M^{\bullet +}$ (PBN-d_{14})
$CHBrCl_2$	$^{\bullet}CHCl_2$	260, 262, 264		274, 276, 278
$CHBr_2Cl$	$^{\bullet}CHBrCl$	304, 306, 308	313, 315, 317	318, 320, 322
CBr_2ClF	$^{\bullet}CBrClF$	322, 324, 326	331, 333, 335	336, 338, 340
CBr_2F_2	$^{\bullet}CBrF_2$	306, 308	320, 322	
$CHBr_3$	$^{\bullet}CHBr_2$	348, 350, 352	357, 359, 361	362, 364, 366
$CHBrClCF_3$	$^{\bullet}CHClCF_3$	294, 296	303, 305	
$CHCl_3$	$^{\bullet}CHCl_2$	260, 262, 264		274, 276, 278
CCl_3CH_3	$^{\bullet}CCl_2CH_3$	274, 276, 278	283, 285, 287	
CCl_3CClF_2	$^{\bullet}CF_2CCl_3$ or $^{\bullet}CCl_2CClF_2$	344, 346, 348	358, 360, 362	
CF_3I	$^{\bullet}CF_3$	246		260

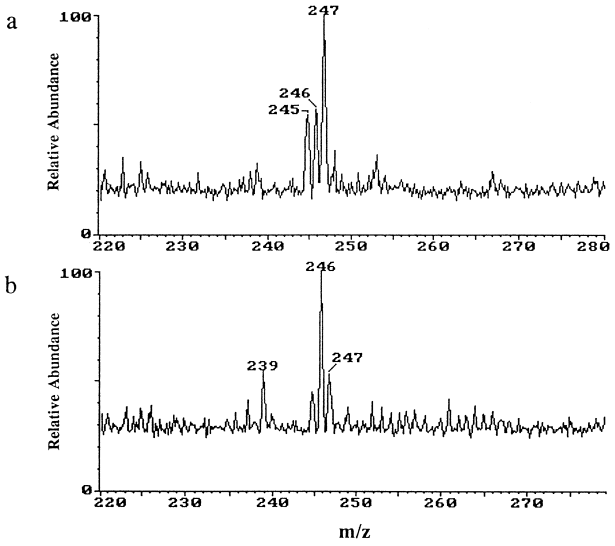
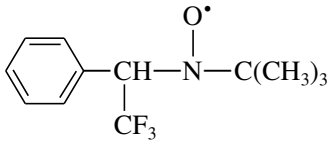
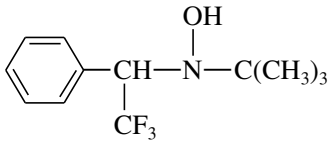


Fig. 8. The precursor ion spectrum of *m/z* 57 obtained from (a) a hexane extract of rat liver microsomes incubated with CF_3I in the presence of PBN, (b) a hexane solution of CF_3I illuminated by UV in the presence of PBN.

to form $\cdot CF_3$ radical by the elimination of an iodide. The peak at *m/z* 246 in Fig. 8a is due to the $\cdot CF_3$ spin adduct (see structure **4**) produced by the loss of an iodide from CF_3I. The peak at *m/z* 247 is considered to be the hydroxylamine (see structure **5**), which is 1 Da higher than the radical adduct in the mass spectrum.



4



5

We found that RLM metabolism of CF_3I results in the formation of a larger amount of hydroxylamine than of the free radical spin adduct. However, the UV irradiation generates more free radicals than hydroxylamine, as shown in Fig. 8b. It appears that this free radical adduct produced in RLM is easily reduced by NADPH to the hydroxylamine.

Because CF_3I does not contain any bromine or chlorine atoms, no isotopic peaks can be used to aid in the recognition of its molecular ions; thus, the assignment of its molecular ions is not unambiguous. In addition to the precursor ion spectrum of *m/z* 66, the product ion spectra of *m/z* 246 and *m/z* 247 were also recorded to confirm this assignment (spectra not shown). Peaks at *m/z* 190 $[M-56]^+$, 159 $[PhCHCF_3]^+$, and 57 $[C_4H_9]^+$ observed in the product ion spectrum of *m/z* 246 support the assignment for $\cdot CF_3$ spin adduct, and peaks at *m/z* 191 $[M-56]^+$, 159 $[PhCHCF_3]^+$, 122 $[PhCHNHOH]^+$, and 57 $[C4H9]^+$, observed in the product ion spectrum of *m/z* 247, confirm the assignment for the hydroxylamine of $\cdot CF_3$ adduct.

GC/MS detection of a stable radical adduct: $CHCl_2$-PBN-d_{14}

The radical adduct $\cdot CHCl_2$-PBN-d_{14} produced from the metabolism of $BrCHCl_2$ in RLM was found to be fairly stable and survives during GC/MC analysis. Fig. 9a is the total ion current (TIC) GC chromatogram recorded from a hexane extract of RLM incubated with $CHBrCl_2$ and PBN-d_{14}. Fig. 9b is the reconstructed ion chromatogram of *m/z* 274, which is the first isotopic peak of molecular ions of $\cdot CHCl_2$–PBN-d_{14}. The peak

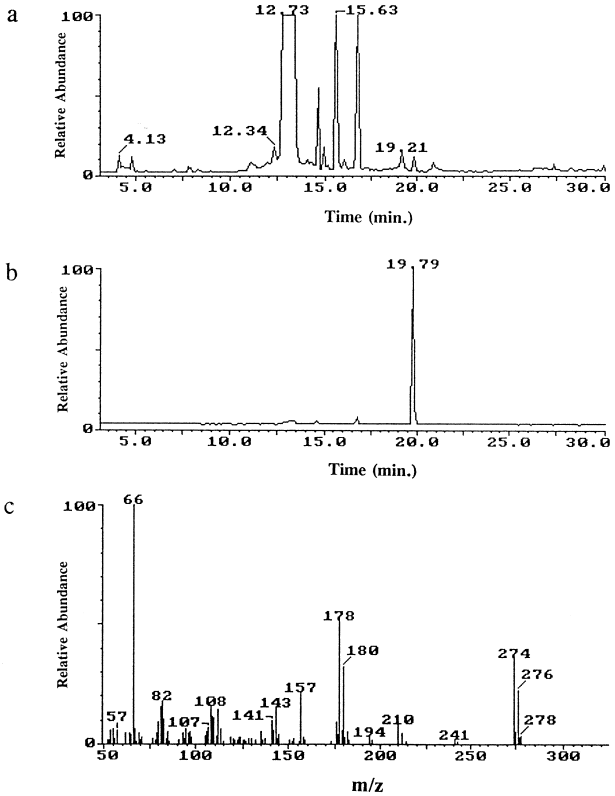


Fig. 9. (a) TIC gas chromatogram recorded from a hexane extract of rat liver microsomes incubated with $CHBrCl_2$ in the presence of PBN-d_{14}. (b) Reconstructed mass chromatogram of *m/z* 274 of spectrum (a). (c) EI (70 ev) MS spectrum at the retention time of 19.79 min.

at retention time 19.79 min is due to $\cdot CHCl_2-PBN\text{-}d_{14}$. The corresponding MS spectrum was shown in Fig. 9c. The isotopic peaks at *m/z* 274, 276, and 278 are due to the molecular ions of $\cdot CHCl_2\text{-}PBN\text{-}d_{14}$. The assignment for its fragments ions are: *m/z* 241, 243, 245 $[M\text{-}CD_3\text{-}O+H]^+$, *m/z* 210, 212, 214 $[M\text{-}C_4D_8]^+$, *m/z* 178, 180, 182 $[C_6D_5CHCHCl_2]^+$, *m/z* 157 $[C_6D_5CHNC(CD_3)_2]^+$, *m/z* 66 $[C_4D_9]^+$.

Thus, GC/MS can be used to assign unequivocally the structure of the above free radical intermediate. However, GC/MS is only suitable for those thermally stable spin adducts.

CONCLUSION

EPR spin trapping is an extremely sensitive technique for the detection of free radical intermediates in the metabolic activation of halocarbons. For the purpose of identification of the radical species so trapped, however, it has to have the aid of other information, i.e., utilization of ^{13}C-labeled halocarbons or fluorine-containing halocarbons. Alternatively, MS/MS spin trapping provides a simple and fast way to detect and identify those free radical intermediates because most of these free radical adducts (except CF_3I) display the isotopic peaks that are very characteristic and distinct. By the use of deuterated PBN, the assignments of these radical species based on the precursor ion spectrum of *m/z* 57 can be further confirmed by the precursor ion spectrum of *m/z* 66.

During the reductive metabolism of halocarbons, the formation of free radical intermediates can result from the breaking of the C-Br, C-Cl, C-I, C-H bond. This process depends upon the corresponding bond disassociation energies and the resonance stability of the resulting radicals. A variety of radicals have been detected in RLM under nitrogen. The intensity of the EPR signal or the MS peaks obtained with the various halocarbons increase in the order: $CHCl_3 < CHBrCl_2 < CHBr_3$, and $CH_2Cl_2 < CHCl_3 < CCl_4$ apparently in accordance with the ease of reductive breaking of the carbon-halogen bond, and correlating with the variations in the electrochemical potentials.

The MS/MS method presented in this paper has been found very useful for the detection of carbon-centered radical spin adducts of PBN and 2-phenyl-5,5,-dimethyl-1-pyrroline N-oxide (2-Ph DMPO), and also for the detection of some oxygen-centered radical spin adducts of PBN and 2-Ph DMPO.[47] The sensitivity of this method is slightly lower than that of EPR.

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