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Free Radical Metabolism of Halothane *In Vivo:* Radical Adducts Detected in Bile

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SUMMARY

Two radical adduct species have been detected in the bile of living rats treated with halothane and phenyl-*N*-*t*-butylnitrone (PBN). The treatment of rats with 12% oxygen was required for radical adduct detection. Analysis of the corresponding EPR spectra obtained when deuterated PBN and deuterated halothane or [2-^{13}C]halothane was used shows that these two species result from the spin trapping of two halothane-derived free radicals. Coupling constants were a^N = 15.72 G, a_β^H = 2.09 G, a_γ^H = 0.79 G, and a^F = 0.63 G(3F) and a^N = 15.16 G, a_β^H = 4.14 G, a_γ^H = 0.48 G, and a^F = 0.3 G(3F) for the two species. Two radical adducts with similar coupling constants were detected when halothane was reduced by zinc dust in the presence of PBN, suggesting that the formation of these two distinct species from halothane can be attributed to the one-electron reduction of halothane and the formation of diastereomeric radical adducts. The identification of both radical adducts as halothane-derived species indicates that there is no *in vivo* EPR evidence for lipid radical formation during halothane intoxication, as had previously been reported.

The volatile anesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) has been implicated in metabolism-dependent human hepatotoxicity. Oxidative metabolism, haptenation, and an idiosyncratic immune response are considered responsible for the more severe but less common form of this toxicity. However, as many as 20% of patients suffer a mild hepatotoxicity, which has been related to reductive metabolism (1).

Animal studies have shown that hepatotoxicity develops in phenobarbital-pretreated rats administered halothane under hypoxic conditions (2). Hypoxia in rats enhances reductive metabolism by cytochrome P-450. Both phenobarbital treatment and hypoxia are necessary for the development of toxicity, implicating a reduced metabolite in the toxic action of halothane. It should be noted, however, that hypoxia itself is hepatotoxic (3) and that the interaction may, therefore, be merely additive.

A free radical metabolite from halothane was detected in microsomes, in hepatocyte systems, and *in vivo* by the EPR spectroscopy technique of spin trapping (4–8). This metabolite arises from the reductive debromination of halothane, as confirmed by mass spectrometry of *in vitro* incubations (7).

Reactive free radicals arising from halothane could bind covalently to critical cellular proteins or to lipids, in which case lipid peroxidation might ensue. These destructive processes have been considered to play a role in the development of carbon tetrachloride toxicity. A number of studies have reported increases in lipid peroxidation after treatment with halothane, although it has also been suggested that lipid peroxidation is a consequence and not a precursor of cell death (9). Janzen *et al.* (7) have detected two lipid-derived radicals formed from the action of halothane *in vitro* and *in vivo*. When partially deuterated PBN was used as a spin trap in halothane-containing microsomal incubations, the resolution of the EPR spectrum was greatly increased. With one species, two identical γ-hydrogen atoms could be discerned, suggesting that the trapped carbon-centered radical was an alkyl ($\cdot CH_2R$) fragment of a polyunsaturated fatty acid. The second species was assigned as a radical adduct of LO·.

Recently, radical adducts derived from carbon tetrachloride, bromotrichloromethane, bromoform, and ethanol (10–12) have been measured in the bile of living rodents. Radical adducts have also been detected but not identified in the bile of rats treated with halothane (8). The same technique was used here to detect radical adducts from halothane and the spin trap PBN in the bile of anesthetized rats, with the deuterated form of PBN being used in order to improve spectral resolution. Deuterated or ^{13}C-substituted halothane was used in order to determine the origin of the two radical adduct species that were detected. Two PBN radical adducts of halothane were also

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ABBREVIATION: PBN, phenyl-*N*-*t*-butylnitrone.

detected when halothane was reduced in a purely chemical system, indicating that these radical adducts are diastereomers.

Materials and Methods

Adult male CD rats (350–400 g) were used for this study. Some animals were pretreated with sodium phenobarbital (80 mg/kg, intraperitoneally) for 3 days. Surgery and collection, treatment, and analysis of samples were as described earlier (10, 12). Animals received 936 μg/kg halothane (Sigma Chemical Co., St. Louis, MO) or an equal volume of [1-^{13}C]- or [2-^{13}C]halothane (Cambridge Isotopes, Woburn MA) or of [2-^{2}H]halothane (MSD Isotopes, St. Louis, MO) intraduodenally and 50 mg/kg PBN (Sigma) or PBN-d_{14} (all positions deuterated except the nitrone hydrogen; MSD Isotopes, St. Louis, MO), in deionized water, by intraperitoneal injection. Some animals were administered halothane via a fluothane vaporizer, with O_2, compressed air, or an air/N_2 mixture as the carrier gas. The same radical adducts were detected with this system as with intraduodenal administration, so the simpler method of intraduodenal administration was used. For initial studies, hypoxia was accomplished by using a plastic rebreathing bag filled with nitrogen. In later studies, a 3 liter/min flow of a compressed air/N_2 mixture at 12% O_2 concentration was used, with essentially the same results.

EPR spectra were obtained with a quartz flat cell and a Varian E-109 EPR spectrometer fitted with a TM_{110} cavity. Data were collected and computer simulations were performed with either a Hewlett-Packard or an IBM-type computer interfaced to the spectrometer. The fit of simulations to experimental data was quantitated by Spearman's nonparametric rank correlation coefficient.

For *in vitro* studies, halothane or [2-^{2}H]halothane was degassed with nitrogen for at least 12 min, and 0.5 ml was added to zinc dust (1 M) and PBN-d_{14} (0.14 M), in a cylindrical quartz EPR tube (3-mm inner diameter, 4-mm outer diameter) containing a spin bar. Samples were bubbled with N_2, with stirring, for 30 min or less. The magnet was removed, and samples were degassed again and kept under a gentle stream of N_2 during EPR analysis. EPR measurements were made as described above.

Results

The administration of halothane and PBN to rats under hypoxic conditions resulted in an intense EPR spectrum of two radical adduct species in bile (Fig. 1A). Hyperfine coupling constants for the two species were $a^N = 15.78$ and $a^H_\beta = 2.30$ for species I and $a^N = 15.22$ and $a^H_\beta = 4.33$ for species II. The two species (Fig. 1, C and D) in the composite spectrum (Fig. 1B) saturated similarly with microwave power, decayed at the same rate, and did not vary significantly, in ratio, from animal to animal. Without 12% oxygen treatment of the rats, no radical adduct spectrum was detected (Fig. 1E). Broadening of the high-field lines was probably due to the partial immobilization of the radical adduct in the biliary micelles. This effect can be seen more strikingly in Figs. 3–6.

Pretreatment of animals with phenobarbital did not alter the intensity of the radical adduct spectrum (data not shown), although phenobarbital pretreatment did increase the radical adduct detected in the bile of rats treated with CCl_4 (12). These results suggest that some factor other than cytochrome P-450 was rate-limiting for the detection of halothane-derived radical adducts in bile. LaCagnin *et al.* (13) suggested that NADPH might be rate-limiting in CCl_4 and $CBrCl_3$ metabolism in the perfused liver. The results shown here (Fig. 1, A and E) suggest that oxygen tension is the most important factor in halothane reduction.

The use of partially deuterated PBN (7) (Fig. 2) provided

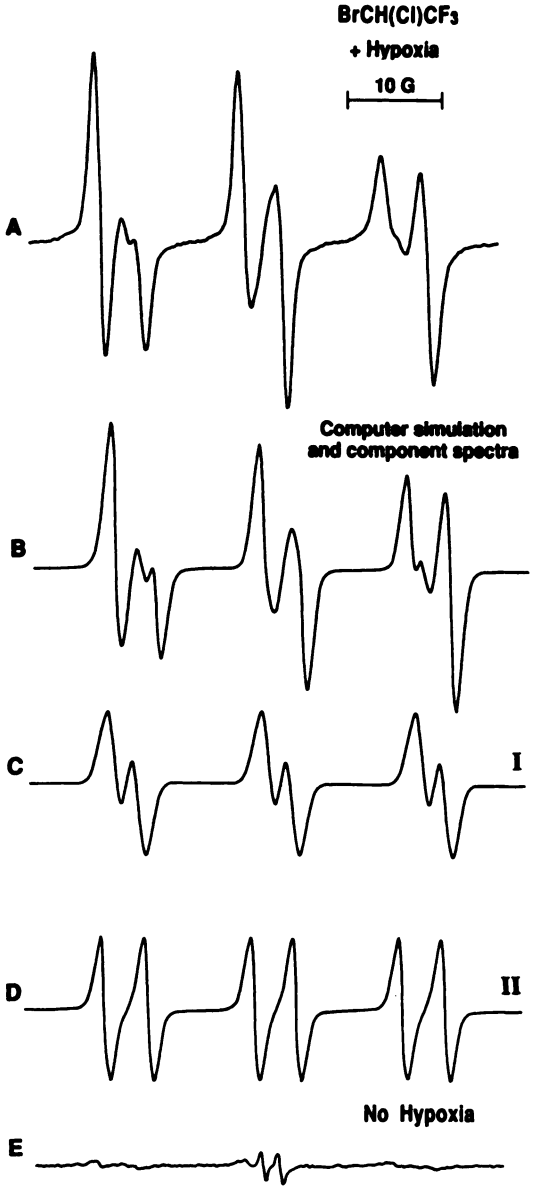

Fig. 1. EPR spectra of bile from rats administered PBN and halothane. Bile was collected 30–45 min after treatment of rats with PBN (50 mg/kg, intraperitoneally) and halothane (936 μg/kg, intragastrically). Rats were made hypoxic at 15–30 min after administration of PBN and halothane. EPR parameters: 20-mW power, 100-kHz modulation frequency, 0.53-G modulation amplitude, 80 G/1-hr scan, 4-sec time constant. A, EPR spectrum from bile of rat treated as described above. B, Composite computer simulation of spectrum in A. C, Unidentified first component of composite computer simulation of spectrum in B (species I). D, Unidentified second component of composite computer simulation of spectrum in B (species II). E, EPR spectrum from bile of rat treated as described above but with no hypoxia.

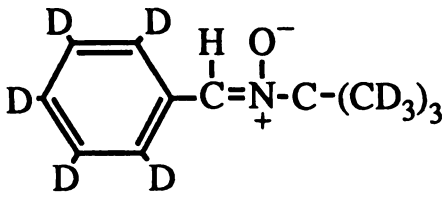

Fig. 2. Structure of partially deuterated PBN.

increased spectral resolution, due to decreased linewidths of the spectra (Fig. 3), and allowed additional coupling constants to be determined (Table 1). The composite computer simulation of the spectrum obtained in bile when partially deuterated PBN was used is shown overlaying the experimental spectrum in Fig. 3, with its component spectra (species I and II) displayed below. Coupling constants are reported in Table 1.

When partially deuterated PBN was used, the spectrum of species I contained splittings from four additional nuclei of spin 1/2, three of which were equivalent. These nuclei are most likely the hydrogen and the three fluorines from the halothane molecule. Janzen *et al.* (7) also resolved three identical fluorine couplings from the spectrum of apparently the same halothane-derived radical adduct.

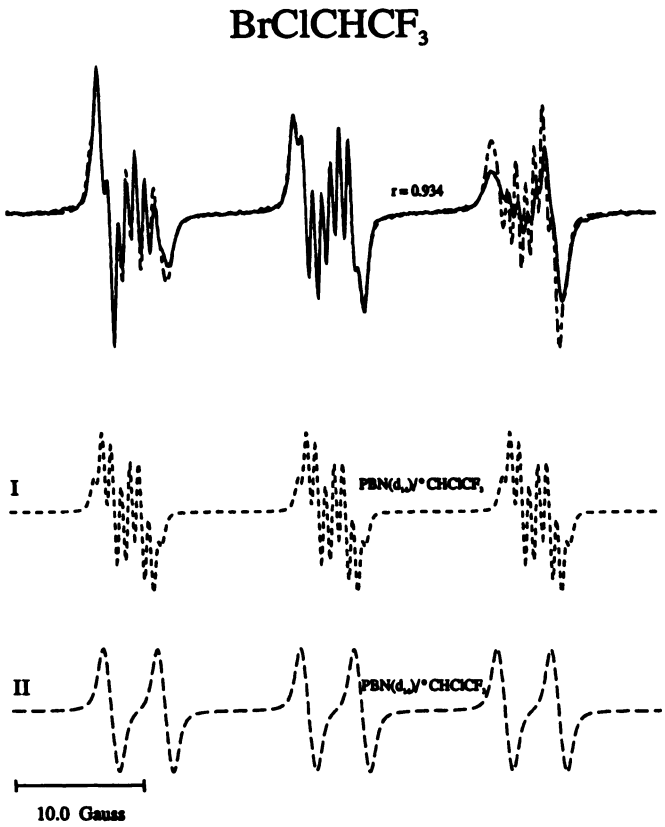

Fig. 3. EPR spectrum of bile from rats administered partially deuterated PBN and halothane, with computer simulation. Animal treatments and EPR instrument settings were as in Fig. 1, except that deuterated PBN and 0.17-G modulation amplitude were used. ——, EPR spectrum from bile of rat treated as described above. – – –, Composite computer simulation of the spectrum composed of species I (24 resolved lines) and species II (six lines).

Similarly, use of the partially deuterated PBN allowed the determination of four additional hyperfine couplings for species II. These additional hyperfine couplings were not resolved but caused changes in line shape that affected the positioning of other peaks. The omission of any of these couplings caused a detectable decrease in the quality of the simulation.

An excellent fit to the experimental spectrum could be obtained when an entirely different set of parameters was used in computer simulation, as had been reported earlier in a preliminary communication (14). The experimental spectrum of Fig. 3 is repeated in Fig. 4, with the composite simulation and its components shown as in Fig. 3. Coupling constants for species II were the same for this simulation as in Fig. 3, but species I had 18 resolved lines, instead of 24 (Table 1). For this species, $a_\gamma^H = 0.69$ (2H), as would be expected for an alkyl β-scission fragment, as had been proposed by Janzen *et al.* (7). However, these parameters could not be used or adapted to make a good simulation of the spectrum generated with deuterated halothane.

The replacement of halothane with deuterated halothane (Fig. 5) resulted in spectral changes consistent with the replacement of a hydrogen with a deuterium atom in both radical adduct species (Table 1). Deuterium has a spin of 1 and, thus, splits each line of the spectrum into three rather than two lines. However, the associated hyperfine coupling constant would be approximately one-sixth that of an identically placed hydrogen atom, making the resulting value of the deuterium hyperfine coupling constant too far below the linewidth to be realistically included in the computer simulation. The effect of deuterium substitution on species II was difficult to detect when the spectrum of this species was plotted by itself (Fig. 5), but the fit of the composite spectrum that resulted when the hydrogen nucleus remained in the computer simulation was noticeably poorer. The detection of a deuterium effect in both radical adduct spectra demonstrated that both radical adducts are derived from halothane and that both retain the hydrogen atom of the parent compound.

Use of [1-^{13}C]halothane resulted in no detectable difference in the radical adduct spectrum (data not shown), indicating that neither radical adduct was centered at the 1-position of halothane. The use of [2-^{13}C]halothane, however, resulted in an additional hyperfine coupling from a nucleus with spin 1/2, for both radical adduct species (Fig. 6; Table 1), proving that

TABLE 1

Hyperfine coupling constants (in Gauss) of halothane-derived radical adduct spectra detected in bile

Treatment of animals, collection of samples, EPR spectroscopy, and computer simulation of spectra were as described in Materials and Methods. Sets of hyperfine coupling constants labeled Halothane and False solution derived from different computer simulations of the same EPR spectrum.

	Halothane (Fig. 3)		False solution (Fig. 4)		[2-^{2}H]Halothane (Fig. 5)		[2-^{13}C]Halothane (Fig. 6)	
	Species I	Species II	Species I	Species II	Species I	Species II	Species I	Species II
a^N	15.72	15.16	15.74	15.21	15.74	15.18	15.77	15.29
a^H (PBN)	2.09	4.14	2.10	4.13	2.11	4.18	2.09	4.17
a^H	0.79	0.48	0.69	0.52			0.84	0.48
a^{13C}							4.18	3.37
a^F (3F)	0.63	0.30			0.70	0.30	0.63	0.30
ΔG (Gauss)	0.058			0.026	0.050		0.047	
Species amount (%)	55.2	44.8	47.1	52.9	55.2	44.8	41.6	58.4
Linewidth (Gauss)	0.320	0.465	0.398	0.541	0.340	0.419	0.320	0.498
Lorentzian (%)	56	96	100	80	22.5	99	60.5	100

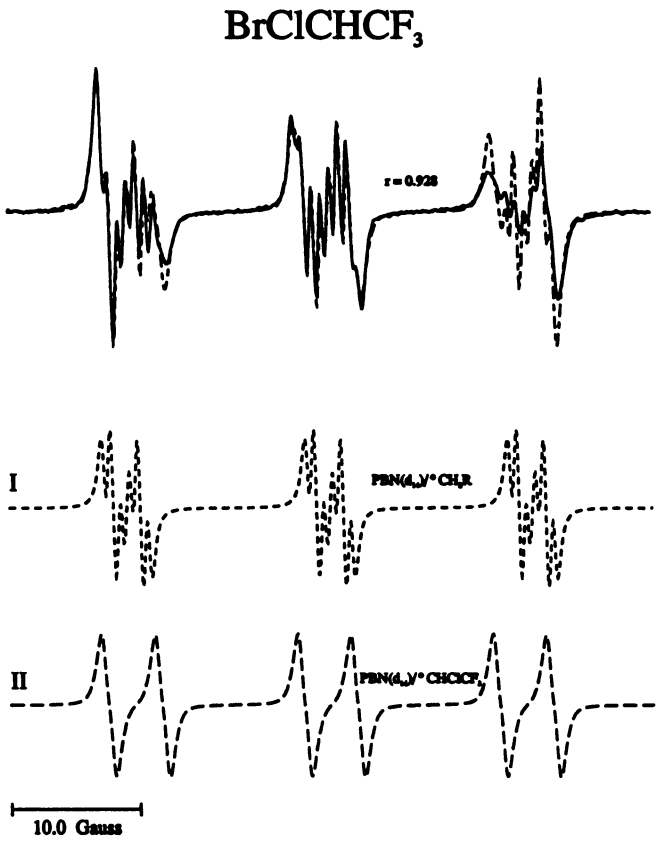

Fig. 4. EPR spectrum of bile from rats administered partially deuterated PBN and halothane, with alternative computer simulation. Same experimental spectrum as Fig. 3, but different hyperfine coupling constants were used in the computer simulation. ——, EPR spectrum from bile of rat treated as described above. – – –, Composite computer simulation of the spectrum composed of species I (18 lines) and species II (six lines).

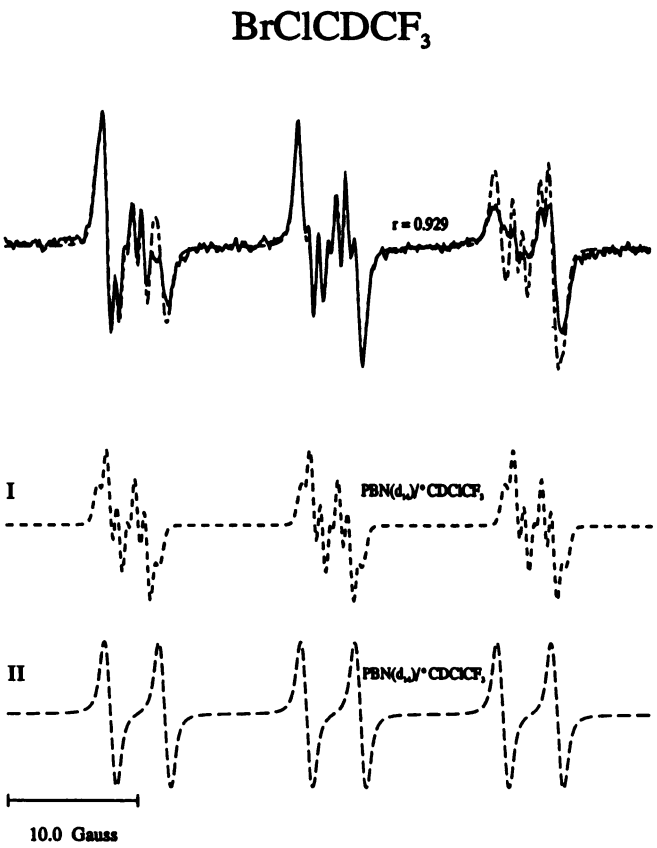

Fig. 5. EPR spectrum of bile from rats administered partially deuterated PBN and deuterated halothane, with computer simulation. Animal treatments and EPR instrument settings were as in Fig. 3, except that deuterated halothane was used. ——, EPR spectrum from bile of rat treated as described above. – – –, Composite computer simulation of the spectrum composed of species I (21 resolved lines) and species II (six lines).

both radical adduct species are produced from carbon 2-centered halothane-derived radicals.

Two radical adducts were also obtained from the reduction of halothane by zinc dust, in a purely chemical system. Halothane, PBN, and zinc dust, the reducing agent, were mixed under nitrogen, as described in Materials and Methods. The most likely chemical reaction is the reductive debromination of halothane, giving the carbon-centered free radical and bromide. The resulting EPR spectrum and its computer simulation are shown in Fig. 7. The spectrum was stable for hours at room temperature, and the relative ratio of the two species did not change with time. Hyperfine coupling constants (Table 2) are similar in number, kind, and relative intensity to those of the radical adducts detected in bile (Table 1), suggesting that the radical adducts detected in bile are the same as those detected *in vitro*. Differences in absolute values of these coupling constants are most likely due to the difference in solvent, which can cause dramatic changes in the hyperfine coupling constants of radical adducts of PBN (15). The change in spectra for both species as a result of deuteration (Fig. 8; Table 2) is further evidence that both are derived from halothane and that the computer simulations of the spectra are valid.

The detection of nearly equal amounts of each species and the stability of both radical adducts over time suggest that both radical adducts form at the same time and that one radical or radical adduct is not formed from another. Indeed, it is not necessary to postulate such reactions in order to account for the formation of two radical adducts. A simpler explanation is that these two radical adduct species are diastereomers.

In the radical adduct formed by the trapping of $\cdot CHClCF_3$ by PBN, carbon 2 from halothane and the carbon α to the phenyl ring are both chiral. Thus, four distinct stereochemical isomers (*RR*, *SS*, *SR*, and *RS*) of this radical adduct are possible (Fig. 9). Because the *RR* isomer is the mirror image of the *SS* isomer, they are enantiomers and have identical hyperfine coupling constants and, thus, identical EPR spectra. The same is true for the *RS* and the *SR* isomers. However, because the *RR*/*SS* isomers are not mirror images of the *RS*/*SR* isomers, the hyperfine coupling constants may differ and give rise to nonidentical spectra. Thus, the two β-H coupling constants of *RR*/*SS* and *RS*/*SR* depend on their respective dihedral angles (θ), in the normal manner (16). That is, the hyperfine coupling constant of the β-H is related to the dihedral angle (i.e., the angle that the β-H subtends with the N-2p_z orbital) (Fig. 10) by the following relationship:

$$a_\beta^H = B \cos^2\theta \quad (1)$$

The dihedral angle θ is influenced not only by the nature and positioning of the trapped radical species but also by the solvent in which the radical adduct is dissolved. The fact that diastereomeric pairs of radical adducts can have markedly different dihedral angles and, hence, different hyperfine cou-

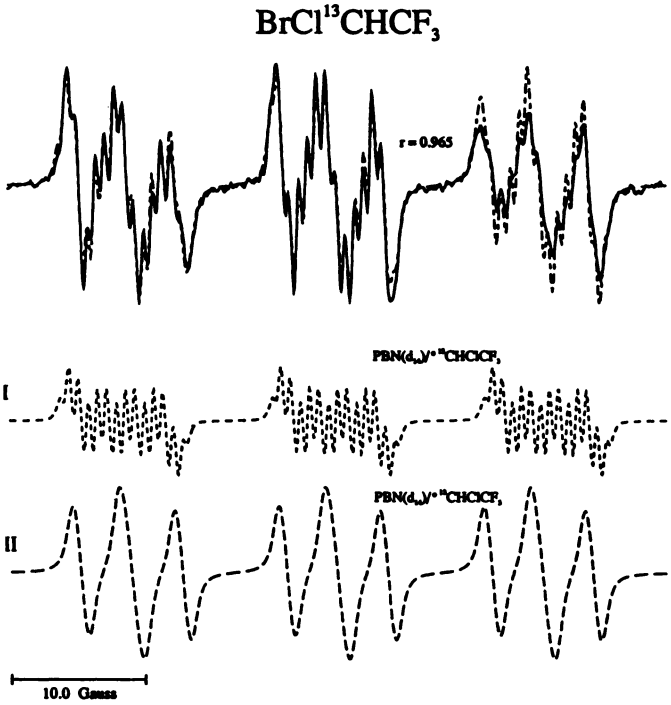

Fig. 6. EPR spectrum of bile from rats administered partially deuterated PBN and [2-^{13}C]halothane. Animal treatments and EPR instrument settings were as in Fig. 3, except that [2-^{13}C]halothane was used. ——, EPR spectrum from bile of rat treated as described above. – – –, Composite computer simulation of the spectrum composed of species I (42 resolved lines) and species II (nine lines).

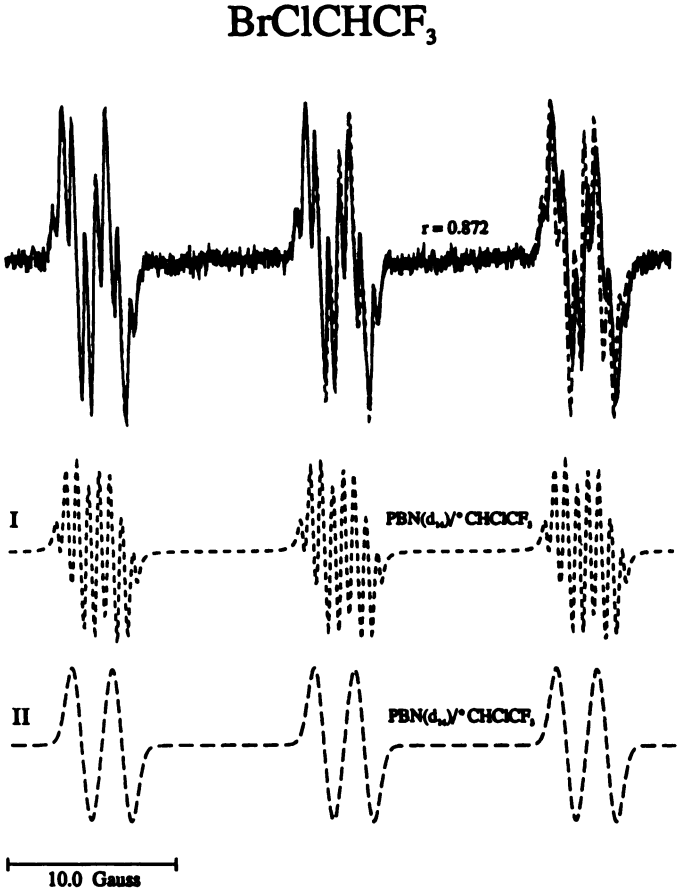

Fig. 7. EPR spectrum of a mixture of partially deuterated PBN, halothane, and zinc dust. Incubation was performed as described in Materials and Methods. EPR parameters: 20-mW power, 0.21-G modulation amplitude, 40-G/1-hr scan, 0.5-sec time constant. ——, Experimental spectrum obtained as described above. – – –, Composite computer simulation of the spectrum composed of species I (24 resolved lines) and species II (six lines).

pling constants was demonstrated by Kotake *et al.* (17), who trapped radicals from a series of alcohols with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide to form radical adducts with chiral centers. These researchers found that each radical adduct produced a pair of radical adduct spectra with widely differing a_β^H values and even slightly differing a^N values. Similar results were later obtained when radicals from ethers, amines (18), and alcohols (19) were trapped with PBN. The importance of the solvent system used was shown in the former study (17) by the disappearance of differences in hyperfine coupling constants when the radical adducts were dissolved in toluene rather than in a corresponding alcohol. Thus, differences between the coupling constants for species I and species II, whether this pair of radical adducts were detected in bile or *in vitro*, can be accounted for by the chirality of these radical adducts. The fact that coupling constants for either species in bile are different from those obtained in an *in vitro* system can be accounted for by the effects of the different environments. This effect is largest on a percentage basis for a^H (PBN) of species II.

TABLE 2

Hyperfine coupling constants (in Gauss) of halothane-derived radical adduct spectra produced by the chemical reduction of halothane

Preparation of samples, EPR spectroscopy, and computer simulation of spectra were as described in Materials and Methods.

	Halothane (Fig. 7)		[2-^{2}H]Halothane (Fig. 8)	
	Species I	Species II	Species I	Species II
a^N	14.71	14.59	14.80	14.72
a^H (PBN)	1.98	2.42	1.98	2.38
a^H (Hal)	0.78	0.38		
a^F (3F)	0.61	0.30	0.71	0.30
ΔG (Gauss)	0.046		0.046	
Species amount (%)	50.2	49.8	52.5	47.5
Linewidth (Gauss)	0.240	0.428	0.261	0.428
Lorentzian (%)	100	14	38	0

Discussion

Free radical metabolism of halothane has been detected *in vitro* and *in vivo* and has been credited with a role in experimental hepatotoxicity due to this compound. We have detected two radical adduct species in the bile of living rats given halothane and the spin trap PBN.

Other workers have reported that hypoxia is a necessary factor in the production of hepatotoxicity in halothane-treated rats (2, 20). In a similar fashion, treatment with 12% O_2 was necessary for radical adduct detection in our studies, as would be expected if free radical metabolism were important in halothane hepatotoxicity. However, phenobarbital treatment was not required for radical adduct detection in bile, although it is required for toxicity (2, 20). Furthermore, the amplitudes of the radical adduct spectra in bile were not affected by phenobarbital treatment. These results are consistent with the findings of Plummer *et al.* (5), who found no increase in the radical adduct content of liver upon phenobarbital pretreatment. It is possible that the free radical metabolites of halothane are unrelated to its toxicity, that phenobarbital induction alone

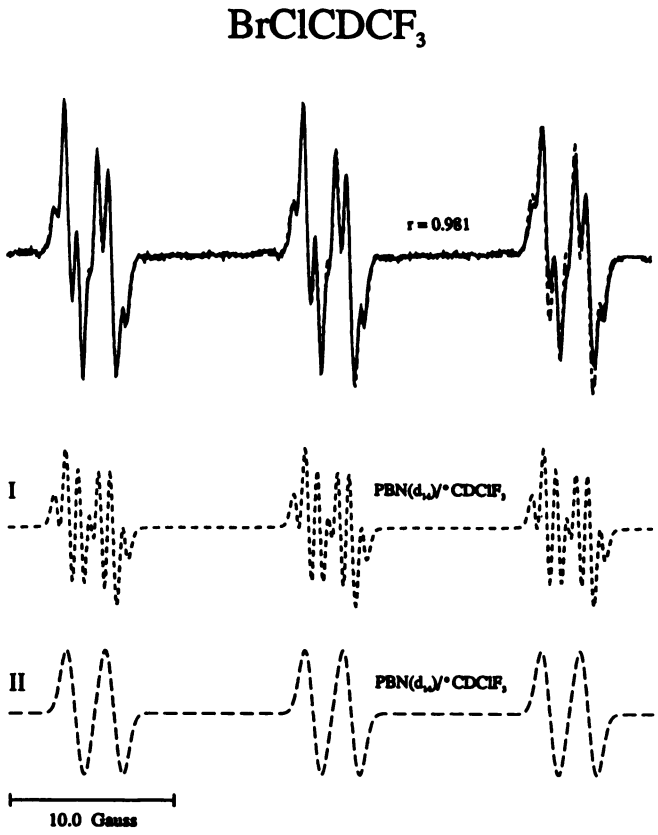

Fig. 8. EPR spectrum of a mixture of partially deuterated PBN, deuterated halothane, and zinc dust. Incubation conditions and EPR parameters were as in Fig. 7, except that [2-^{2}H]halothane was used in the incubation, with 40-G/16-min scan and 0.128-sec time constant. ——, Experimental spectrum obtained as described above. – – –, Composite computer simulation of the spectrum composed of species I (21 resolved lines) and species II (six lines).

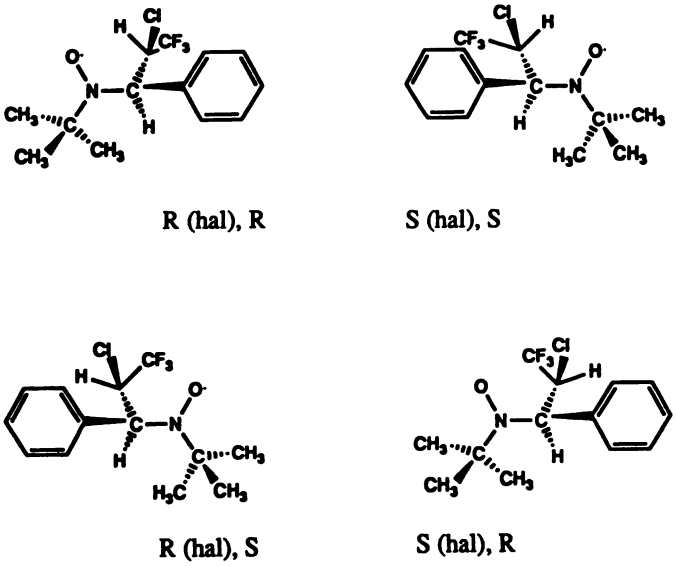

Fig. 9. Stereochemical isomers of halothane radical adduct.

has its own effects on hepatotoxicity, which are additive with those produced by halothane, or that phenobarbital induction enhances radical adduct destruction as well as free radical production, thus masking a phenobarbital-induced increase in free radical metabolism (11).

Both radical adduct species detected in the bile derive from

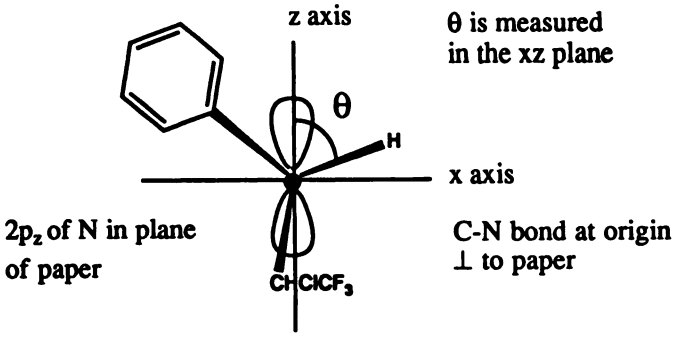

Fig. 10. Dihedral angle in radical adduct.

carbon 2-centered halothane radicals. Species I has four hyperfine coupling constants from nuclei of halothane with spin 1/2. Three of these nuclei are equivalent and must, therefore, be the three fluorine atoms at carbon 1 of halothane. The fourth nucleus must then be the hydrogen on carbon 2. Deuteration of halothane replaced this hydrogen nucleus of spin $I = 1/2$ with a deuterium nucleus. A ^{13}C coupling constant was detected when [2-^{13}C]halothane but not [1-^{13}C]halothane was used, proving that both radical adducts are due to carbon 2-centered radicals from halothane.

For species II, hyperfine coupling constants of hydrogen and fluorine were similarly detected, although distinct lines were not resolved and assignments are thus far less definitive than with species I. The hydrogen coupling also appeared to be replaced by a deuterium coupling when deuterated halothane was used, although, again, this assignment is less clear. ^{13}C substitution reveals that this radical adduct species also derives from a carbon 2-centered halothane radical.

A good simulation of the EPR spectrum can also be obtained when two identical hydrogens are substituted for the hydrogen and fluorines of species I. A radical adduct with this structure could result from the trapping of a $\cdot CH_2R$ fragment, such as would arise from lipid peroxidation, but experiments with [^{13}C] halothane show that both radical adducts are derived from halothane and not from lipid. Thus, even a good computer simulation of a radical adduct spectrum is not sufficient as proof of structure, emphasizing the need for supplemental techniques such as mass spectroscopy or isotopic substitution.

Both of the radical adducts detected in bile arise from the reductive debromination of halothane. In organic extracts of microsomal incubations and *in vivo*, Janzen *et al.* (7) have detected a radical adduct with three equivalent fluorine atoms. The structure of this radical adduct has been confirmed by mass spectrometry as that due to the trapping of the debrominated, carbon-centered, halothane-derived radical. Presumably, this radical adduct is the same as our radical adduct I, where fluorine hyperfine coupling is resolved. Similarly, when free radicals formed from halothane by UV irradiation (21) or by a reconstituted cytochrome P-450 system (22) were trapped by oleic acid, mass spectrometry of the purified stable products showed that debromination rather than dechlorination had taken place.

The present studies with a chemical system containing only halothane, PBN, and zinc dust have shown that two diastereomeric radical adducts are produced. The two radical adducts are present in roughly equal concentrations and their ratio does not change with time, so a conversion of one radical or radical adduct into another is unlikely. The detection of two radical

adduct species in a purely chemical system shows that biological processes such as lipid peroxidation, resulting in the formation of radical adducts of L· and LO·, need not be proposed in the interpretation of these spectra, as has been done in related work (7). In contrast to results with ethanol (11, 23), lipid-derived radicals have not been detected after halothane exposure *in vivo*.

In conclusion, two radical adducts derived from halothane have been detected in the bile of rats treated with halothane and spin trap. Treatment with 12% oxygen but not with phenobarbital was necessary for the detection of signal, in contrast to toxicity studies, which show that both of these factors are required for the development of hepatotoxicity. The appearance of two distinct radical adduct species resulted from the chirality of the debrominated halothane-derived radical adducts and is not due to the trapping of lipid-derived radicals.

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