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Chemistry and Biology of Spin-Trapping Radicals Associated with Halocarbon Metabolism *in Vitro* and *in Vivo*

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The spin-trapping method is introduced and discussed. Some chemistry of nitroxides and nitrones is reviewed. Pattern recognition of ESR spectra of nitroxides is outlined. Factors controlling the magnitude of hyperfine splitting constants are mentioned. Methods of assigning spin adducts are listed. Review articles in the literature are referenced.

Results in the electrochemical reduction of halocarbons are presented and some parallels with superoxide chemistry shown. Various speculative reactions are given. The *in vitro* and *in vivo* experiments where halocarbon radicals have been detected by spin trapping are reviewed and some new results reported. A comparison for different animals is added.

Introduction

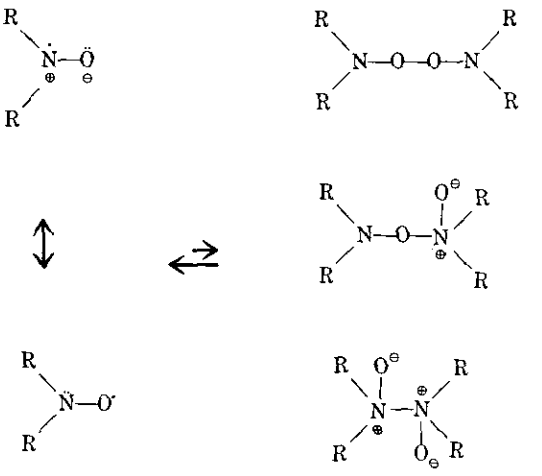
The possible involvement of free radicals in biochemical reactions has been suggested for a long time. However direct evidence for such intermediates has been lacking for many years. This is because typical concentrations of reactive free radicals in biochemical systems are usually too low for direct ESR detection. The method of spin-trapping was developed to help with this problem. Although the use of this technique is becoming quite popular it is also possible to make erroneous deductions with this method. Caution must be exercised and the literature consulted before conclusions are reached. Nevertheless, spin trapping appears to be the only approach to the detection of short-lived reactive free radicals at low concentrations in a biological environment and considerable promise is already evident for the use of this technique in *in vivo* experiments. In this application the method is unique.

The Spin-Trapping Method

Nitroxides

The spin-trapping reaction takes advantage of the well known stability of the nitroxyl free radical function:

Nitroxides* are "stable" free radicals because the unpaired electron is resonance stabilized in the monomer and strong bonds cannot be formed in the dimer:



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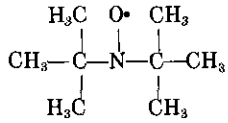
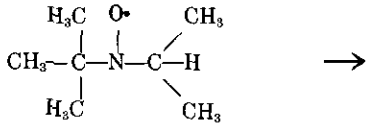
*The IUPAC recommended name is aminoxyl instead of nitroxide; this nomenclature was selected to facilitate naming of nitroxides on the basis of the secondary amine precursor, e.g., dimethylaminoxyl.

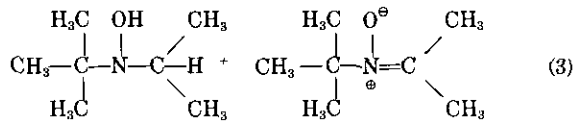
The most stable nitroxides have "inert" groups attached to the nitrogen atom, typically methylated carbon atoms:



(2)

The complete replacement of neighboring hydrogens with methyl groups serves two purposes: the bulky groups reduce what small tendency there might be for dimerization and the rate of disproportionation to the hydroxylamine and nitrone is reduced, e.g.,





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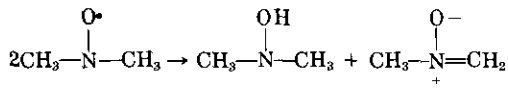
Unsubstituted nitroxides are actually very reactive and disproportionate rapidly, e.g.



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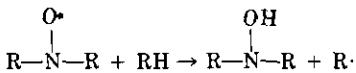
The most common reactions of stable nitroxides are reduction and oxidation:



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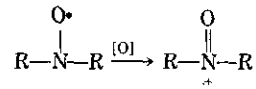
For example, ascorbic acid reduces nitroxides rapidly and sulfhydryl groups are capable of the same reaction in the presence of transition metal ions.

Oxidation produces the nitroxonium ion with strong oxidizing agents:



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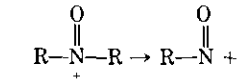
This species is not stable in aqueous solution, undoubtedly dissociating to the carbonium ion:



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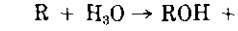

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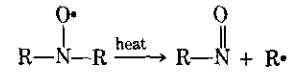
If nitroxides are warmed to above room temperature, e.g., during heating in GC equipment or while recrystallizing or distilling, dissociation to the nitroso compound can occur:



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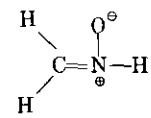
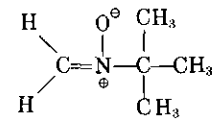
Nitrones

The nitrone function is the *N*-oxide of an imine. The simplest nitrone is not stable, apparently polymerizing rapidly to unknown products. The smallest isolable nitrone is *N-tert*-butyl nitrone but even

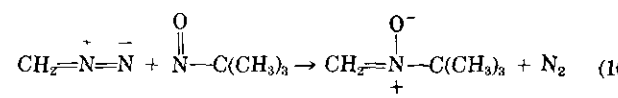


Nitrone
(unstable)



N-tert-butyl
nitrone

this compound has very poor shelf-life. The *tert*-butyl nitrone is made from diazomethane (explosive) and 2-methyl-2-nitrosopropane and is not commercially available:



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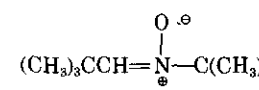
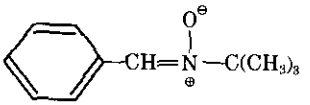
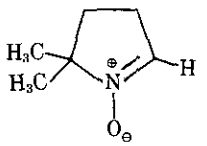
Replacing one of the methylene hydrogen with an alkyl or aryl group, or incorporating the nitronyl function into a ring produces compounds of reasonable stability (BBN, PBN, DMPO).



α-*tert*-butyl *N*-*tert*-butyl nitrone
BBN

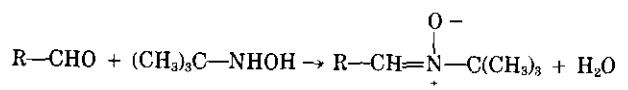


α-Phenyl *N-tert*-butyl nitrone
PBN



5,5-Dimethyl-1-pyrroline-*N*-oxide
DMPO

The general method of synthesis involves condensation of the aldehyde with the hydroxylamine [Eq. (11)], although for PBN the imine can be oxygenated with a

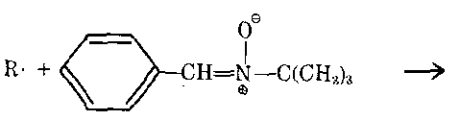


(11)

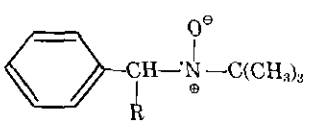
peracid, which after heating produces the nitrone. PBN and DMPO are commercially available. It should be noted that hydrolysis of nitrones does occur slowly at pH 7, and the rate increases at lower pH. Hydrolysis is the reverse of the above reaction and produces two reactive products, the aldehyde and the hydroxylamine, both of which can become involved in free radical reactions.

Spin-Trapping

The spin-trapping technique is an analytical method for the detection of reactive short-lived free radicals (which are not highly resonance-stabilized) by the addition reaction to chemical traps e.g. nitrones, which give relatively persistent radical addition products [Eq. (12)]:



PBN
a spin trap



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Substituted benzyl *tert*-butyl nitroxide
a spin adduct

When the spin trap reacts with radicals, the persistent spin adduct nitroxide accumulates until the detection limit of the ESR spectrometer is exceeded and a signal due to the nitroxide is recorded. If the spin trap concentration is high enough and the rate constant of spin trapping is fast, the detection of R· can be quantitative.

The major challenge in spin-trapping is assigning the structure of the spin adduct correctly because only in this way can the identity of R· be ascertained. Since until now such assignments most commonly have been made on the basis of the ESR spectra obtained some discussion on the ESR spectroscopy of nitroxides will be necessary.

ESR Spectroscopy of Nitroxides

The ESR spectrum of the unpaired electron alone consists of one peak. The interaction of the unpaired electron with a nucleus with no spin produces no change i.e. the spectrum is still one line. However, when the unpaired electron interacts with a nucleus which itself has spin (e.g., nuclear spin, I = 1/2, 1, 3/2, etc.), the single peak is replaced by a set of equally intense lines, where the number of lines in the set equals $2I + 1$ (see Fig. 1 for examples).

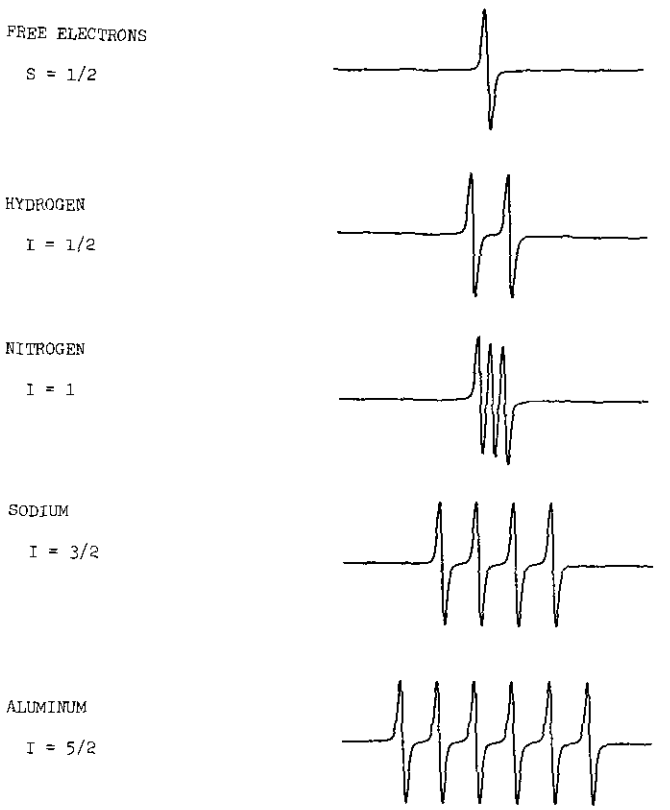
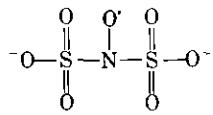
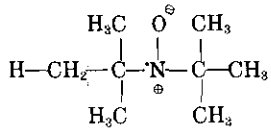


FIGURE 1. Computer-simulated ESR spectra of the unpaired electron with various atoms or nuclei. (Top) The first-derivative ESR signal anticipated for free electrons in solution. (Spectra below) Illustrative ESR spectra of chemical species (e.g., atoms, molecules, or ions) that possesses one unpaired electron as well as one nearby nucleus (i.e., H, N, Na, or Al) with spin.

In nitroxides the unpaired electron is located in a π-orbital on the nitrogen and oxygen atoms. The spin of the unpaired electron will interact with both nuclei, but since the oxygen nucleus has no spin, only the interaction with the nitrogen nucleus will be noticed (for ^{14}N, $I = 1$). Thus a spectrum of three equally spaced equally intense lines is expected for a nitroxide with groups attached consisting of atoms with no nuclear spin, e.g., Fremy's salt (for ^{16}O, and ^{32}S, $I = 0$):

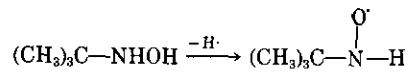


If the groups attached have atoms with nuclear spin but these are attached too far away for significant interaction, the spectrum will still be only three lines: e.g., di-*tert*-butyl nitroxide (since for ^{12}C, $I = 0$, but for ^{1}H, $I = 1/2$):



(where hydrogens are three bonds away). Nuclei with spin three bonds or more away from the nitroxyl function usually do not contribute extra lines to the ESR spectra of nitroxides, but a small unresolved interaction in *tert*-butyl nitroxides from the nine γ-hydrogens does increase the line-width of the peaks obtained.

It is to be expected that when a group or atom with nuclear spin is attached to the nitrogen such nitroxides would give spectra reflecting the interaction of the unpaired electron with this nucleus. An example is *tert*-butyl hydronitroxide, the radical obtained from hydrogen atom abstraction from *tert*-butyl hydroxylamine:



(13)

The hydrogen is one bond away from the nitroxyl function (α-position), and the unpaired electron interacts significantly with this atom. The three lines of the nitroxide spectrum are further "split" into two lines giving a six-line pattern in total (Fig. 2). The appearance of the splitting pattern will depend on the magnitude of the interaction of the extra nucleus. In this case the splitting of the hydrogen is approximately equal to that of the nitrogen and the spectrum consists of four lines with intensity 1:2:2:1, or depending on the solvent, six lines with intensity 1:1,1:1,1:1 where the intensities separated by commas may be only partially resolved (Fig. 2).

Atoms with nuclear spin located two bonds away also interact significantly with the unpaired electron on the nitroxyl function (β-position). This interaction is thought to be due to hyperconjugation [Eq. (14)]:

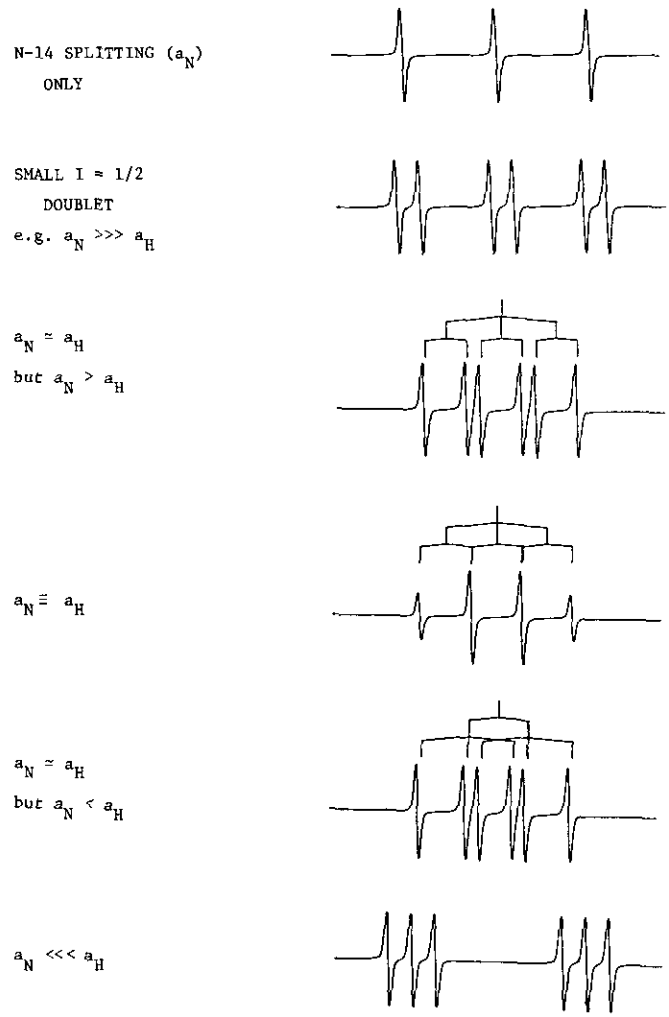
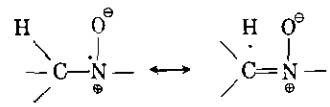
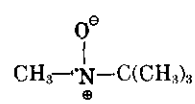


FIGURE 2. Computer-simulated ESR spectra of nitroxides with and without doublet splittings. (Top) ESR triplet spectrum due to a free radical such as Fremy's salt or di-*tert*-butyl nitroxide which exhibit hyperfine splitting only from the nitroxide nitrogen ($I = 1$). (Spectra below) Illustrative triplet of doublets spectra showing splittings of various magnitudes arising from the nitroxide nitrogen ($I = 1$) and β-hydrogen nuclei, respectively.



(14)

Since a number of examples of this kind are commonly encountered, these will have to be divided into two categories: those with freely rotating groups and others where free rotation is hindered. In methyl *tert*-butyl nitroxide both the methyl and *tert*-butyl groups can be considered freely rotating:



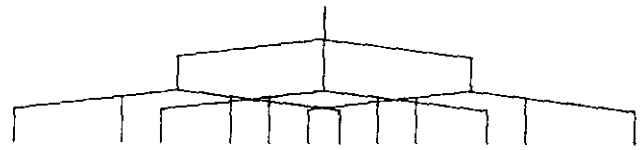
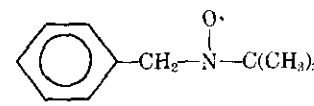


FIGURE 3. Splitting pattern for methyl *tert*-butyl nitroxide.

The methyl hydrogens are in the β-position and will interact with the unpaired electron on the nitroxyl function. All hydrogens will be equivalent. The splitting pattern (Fig. 3) can be predicted on the basis of this equivalence (see Fig. 4).

Also the number of lines expected can be deduced from the expression $2nI + 1$ where n = number of magnetically equivalent nuclei with spin. The spectrum of methyl *tert*-butyl nitroxide will consist of 12 lines, since each of the lines in the nitrogen triplet will be split into 1:3:3:1 quartets.

If one of the hydrogens in the methyl group is replaced by a group which has no nuclear spin in the β-position, say, phenyl,



two hydrogens are left in the β-position which are expected to be magnetically equivalent. Then the splitting pattern anticipated would be a triplet of 1:2:1 triplets according to the splitting diagram shown in Figure 5.

The ESR spectrum of benzyl *tert*-butyl nitroxide, in fact consists of nine lines with the nitrogen triplet split into

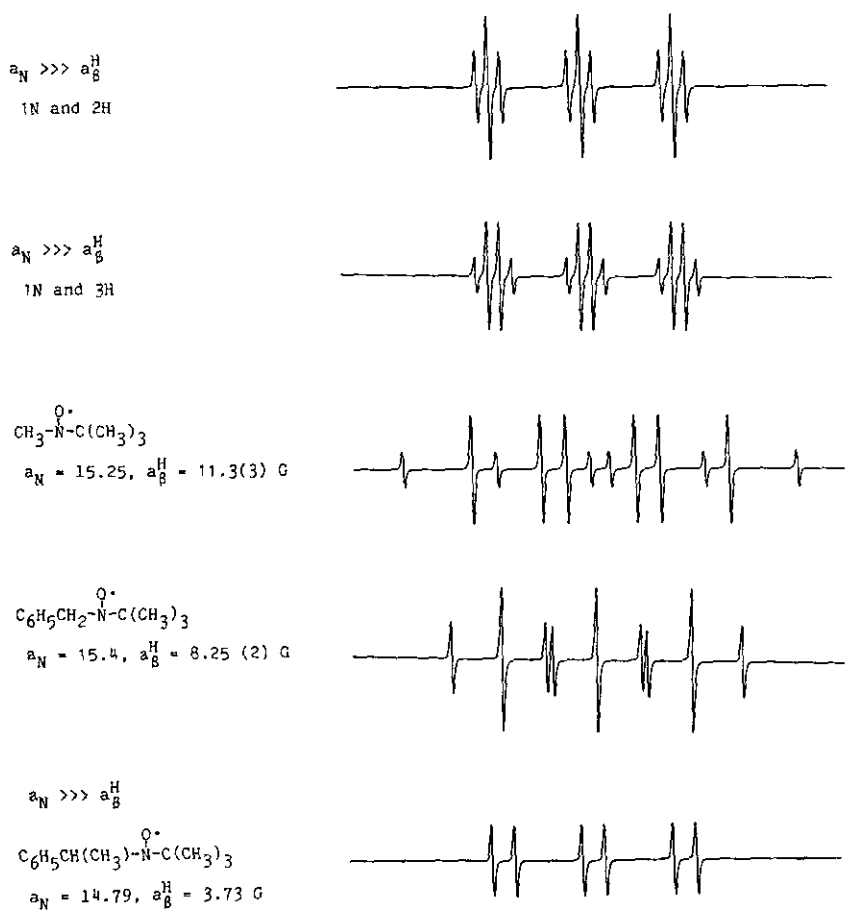


FIGURE 4. Computer-simulated ESR spectra of various substituted *N-tert*-butyl nitroxides. (Top two spectra) The first illustrative spectrum exhibits a relatively large nitroxide nitrogen triplet splitting with respect to the β-hydrogen triplet splitting. The second illustrative spectrum is a triplet of quartets again with a relatively large nitroxide nitrogen splitting with respect to the β-hydrogen splitting. (Bottom three spectra) In descending order are shown the anticipated ESR spectra for methyl, benzyl, and 1-phenylethyl) *tert*-butyl nitroxide, respectively. *It should be noted that the ESR patterns for these nitroxides are those expected in organic solvents (e.g., benzene). The ESR patterns may change appreciably in solvents of different polarity (e.g. water).*

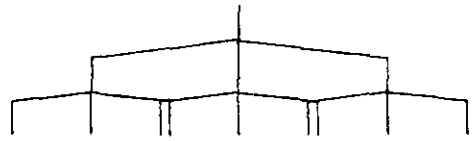
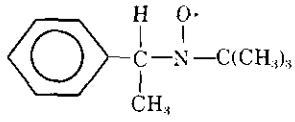


FIGURE 5. Splitting pattern for benzyl *tert*-butyl nitroxide.

1:2:1 triplets by the splitting from the methylenic hydrogens (Fig. 4).

If another hydrogen in methyl *tert*-butyl nitroxide is replaced with a group with no nuclear spin in the β-position, for example methyl,



the remaining β-hydrogen causes the nitrogen triplet to be split into 1:1 doublets and a total of six lines result (see Fig. 4).

The interesting feature of these three spectra is that the magnitude of the β-hydrogen splitting is markedly different and decreases with substitution in the β-position. Thus the β-hydrogen splitting is 11.3, 8.25, and 3.73 G respectively for methyl, benzyl, and 1-phenylethyl *tert*-butyl nitroxide (Fig. 4)! If the groups were all freely rotating the β-hydrogen splitting should be essentially the same. The conclusion of course is that these groups are not freely rotating and that the *tert*-butyl nitroxyl function presents substantial hindrance to free rotation of the other groups attached.

Thus the bulk of the R group in the spin adduct is expected to influence the rotation of all the groups attached to the nitroxyl function including the β-hydrogen so that a unique splitting should be realized for every different R- group encountered. This prediction is usually demonstrated by consideration of a Newman projection. Looking down the C-N bond it is obvious that the largest groups will be oriented so that the distance between them is maximized (Fig. 6).

Moreover the magnitude of the β-hydrogen splitting depends on the "dihedral" angle between the C-H bond and the p-orbital on nitrogen. When the angle is large the splitting is small; conversely when the dihedral angle is small the splitting is large. This relationship is accommodated by the expression

$$a_\beta^H = (B_0 + B \cos^2 \theta)\rho_N$$

where θ is the dihedral angle defined in Figure 7, ρ_N is the spin density on the nitrogen atom of the nitroxyl function, B is a proportionality constant for each radical, and B_0 is the same constant when $\theta = 90°$. For nitroxides $B_0 \approx 0$, $\rho_N \simeq 0.5$, and $B \simeq 52$ G.

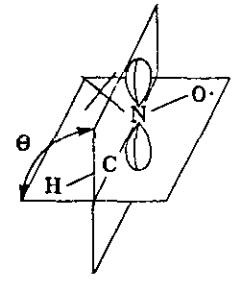
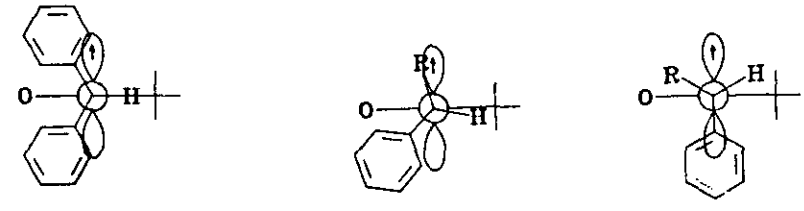


FIGURE 7. Projection defining the dihedral angle θ.

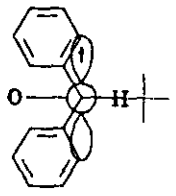
It follows that when the bulk of R is equal to that of phenyl in the above examples the β-H splitting should be small because $\theta \simeq 90°$ (actually the C-H bond rocks above and below the plane of the nitroxyl π-bond). This is found to be the case (Fig. 8).

For smaller R groups, the β-hydrogen splitting is



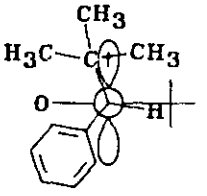
bulk of R:	equal to phenyl	smaller than phenyl	larger than phenyl

FIGURE 6. Newman projections of spin adduct nitroxides.



a_N = 14.41 G

a_β^H = 2.21 G



a_N = 14.60 G

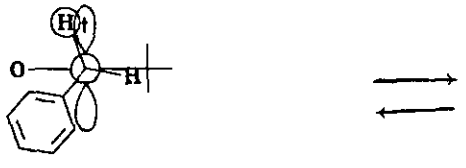
a_β^H = 2.60 G

FIGURE 8. Newman projections of phenyl and *tert*-butyl adducts of PBN.

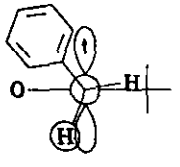
expected to increase; it does when R is hydrogen (Fig. 9). For R groups larger than phenyl, the β-hydrogen splitting also increases as expected (Fig. 10).

It would be nice to say that all other R groups fall into the same pattern, but this is not the case. In fact, almost no other examples can be predicted with confidence and the reason for this fact is still a subject of research. In general, polar groups with electron-withdrawing atoms cause a decrease in the nitrogen splitting and usually (but not always) the β-hydrogen splitting is smaller as well. However, predictions for unknown spectra based on splitting constants can be hazardous and other routes need to be used to make assignments of structure.

This is because changes in solvent polarity in general produce larger effects on the splitting of the nitroxyl nitrogen and the β-hydrogen than changes in structure. As expected from consideration of the two resonance structures for the nitroxyl function shown earlier, polar solvents lead to an increase in the nitrogen splitting since the "fraction" of the unpaired electron (spin density) increases on nitrogen when the solvent stabilizes the dipolar resonance form. Since the magnitude of the β-hydrogen splitting depends on the spin density on nitrogen, the increase in solvent polarity should also cause an increase in the β-hydrogen splitting. This is found to be true for some but not all cases. Thus it is advisable to test the spectrum of a nitroxide in a number of solvents of differing polarity before assignments can be considered firm.



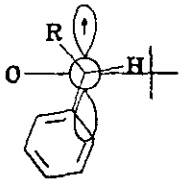
R = H, circled



a_N = 14.84 G

a_β^H = 7.42 G

FIGURE 9. Newman projection of benzyl *tert*-butyl nitroxide.



R = 2,4,6-trimethoxyphenyl

a_N = 14.52 G

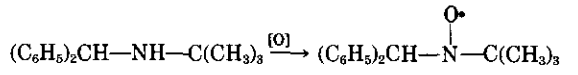
a_β^H = 5.81 G

FIGURE 10. Newman projection of phenyl adduct of MO_3PBN.

Methods for Assigning Spin Adduct Spectra

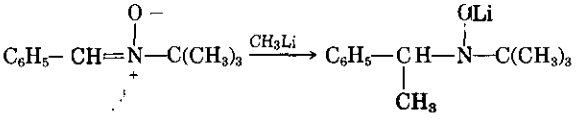
A number of methods have been used to assign spin adduct spectra.

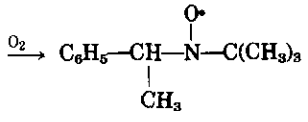
Oxygenation of the Appropriate Secondary Amine Precursor. This method is excellent but tedious and will usually not be suitable for functionalized R groups.



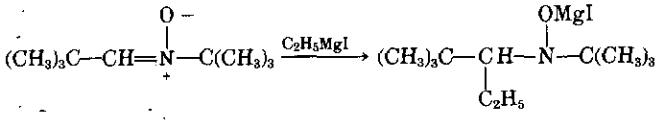
Phenyl radical spin adduct of PBN (15)

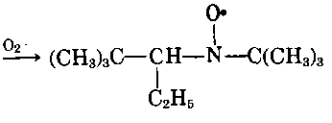
Oxidation of the Hydroxylamine or Hydroxylamine Anion of the Spin Adduct Nitroxide. A convenient way to make this intermediate is to add the organolithium or organo-Grignard to the nitrone being used as a spin trap:





(16)

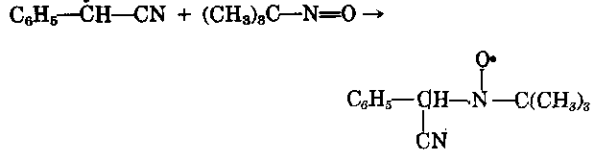




(17)

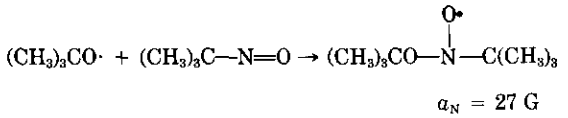
This approach is very useful where organolithium or organo-Grignard reagents are available.

Spin Trapping with 2-Methyl-2-Nitrosobutane. The trapping of appropriate radicals with 2-methyl-2-nitrosopropane can be very useful for confirming spin adduct spectral assignments.



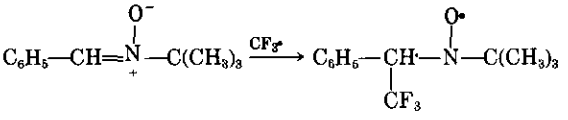
Cyanyl radical spin adduct of PBN (18)

Di-*tert*-butyl peroxalate, an excellent thermal source of *tert*-butoxyl radicals, can often be used to generate the radicals needed to add to 2-methyl-2-nitrosopropane to produce a PBN spin adduct since the addition of *tert*-butoxyl radicals to the nitroso compounds gives a nitroxide with quite a different nitrogen splitting.



a_N = 27 G (19)

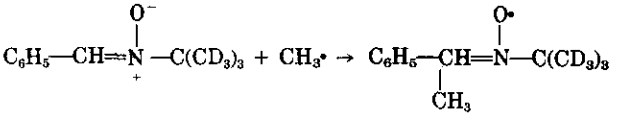
Resolution of γ-Splitting in the R Group Added. In some cases, long range splitting can identify the radical trapped.



a_N = 15.29 G

$a_\beta^H = a_\gamma^F$ = 2.84 G (20)

Resolution of long-range splitting can be improved by deuteration of the *tert*-butyl group:

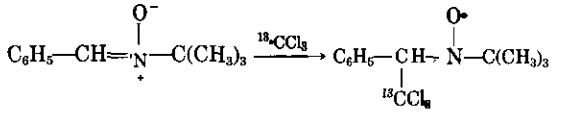


a_N = 14.83

a_β^H = 3.63

a_γ^H = 0.48 G (21)

Replacement of a Nucleus in the Radical with an Isotope of Different Nuclear Spin. This is exemplified by a reaction of the type:

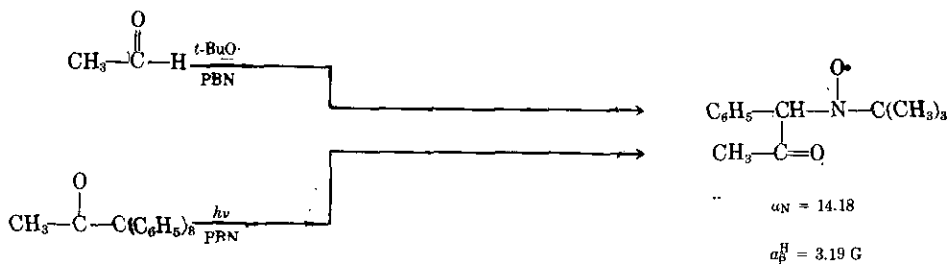


a_N = 14.10

a_β^H = 1.74

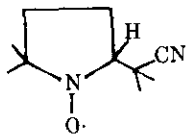
$a_\beta^{C^{13}}$ = 9.68 G (22)

Trapping of the Same Radical from Different Sources. This method is frequently used when all previous methods are not suitable or other attempts have failed. If reactions are carefully selected this approach is quite dependable.

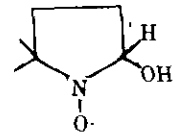


(23)

Consideration of g-Value Differences in Similar Adducts. Nitroxide g values can vary according to the radical center trapped (e.g., C vs. O), although the relationship is not always straightforward and is susceptible to solvent effects.



a_N = 14.6, a_β^H = 20.4 G
g value = 2.0044



a_N = 15.3, a_β^H = 15.3 G
g value = 2.0060

Spin-Trapping Literature

The spin-trapping literature includes numerous reviews on the subject. The first few written by Perkins (*1*), Lagercrantz (*2*), and Janzen (*3*) appeared around 1970. A number of specialized articles on this topic by Janzen appeared later (*4,5*). Additional reviews covering the physical organic aspects of spin trapping were published by Perkins (*6*) and Evans (*7*). More recently, spin trapping applications in biological chemistry have been extensively reviewed and these references should be consulted: Janzen (*8*), Finkelstein, Rosen, and Rauckman (*9*), Kalyanaraman (*10*), Buettner (*11*) and Mason (*12*). Some useful chapters have been written on spin trapping by a number of authors in two recently published books (*13,14*) and a special issue of the *Canadian Journal of Chemistry* is devoted to papers in spin trapping and nitroxyl radical chemistry (*15*). Tabulations of spectroscopic parameters for spin adducts (up to 1978) are available, listed among all other nitroxide radicals (*16*) and an updated set of tables where entries are listed in increasing order of empirical formula of the radical trapped will be available shortly (*17*). A chapter is being prepared with emphasis on pitfalls and artifacts in spin-trapping (*18*).

Chemistry of Halocarbon Radical Formation

Electrochemical Reduction

When interest developed in the possibility that the spin trapping technique could be used to detect trichloromethyl radicals in biological systems from carbon tetrachloride, and halocarbon radicals from other halocarbons, a method needed to be developed to provide authentic spin adduct spectra for assignment purposes. The electrochemical generation of halocarbon radicals in the presence of the spin trap, PBN, was found to be successful in most cases where three (and sometimes two) halogens are attached to the same carbon atom:

$$CX_4 + e^- \rightarrow \cdot CX_3 + X^- \quad (24)$$

$$CHX_3 + e^- \rightarrow \cdot CHX_2 + X^- \quad (25)$$

where X = Cl, Br.

However, HPLC methods were needed to ascertain the structure of the radical produced when both chlorine and bromine were present in the same molecule. Because reasonably polar solvents are needed for electrochemical studies, all investigations had to be made in acetonitrile (MeCN) or *N*,*N*-dimethylformamide (DMF).

The first question of interest was whether free radicals could be detected in the electrochemical reduction of halocarbons using the spin trapping method. These results could be important in comparison to biological systems, because the reduction of carbon tetrachloride, for example, by the reduced form of cytochrome P_{-450}, could conceivably proceed by a one-electron or two-electron process.

One-electron process:

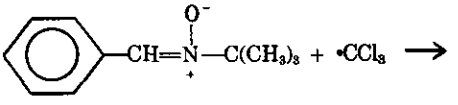
$$CCl_4 + e^- \rightarrow \cdot CCl_3 + Cl^- \quad (26)$$

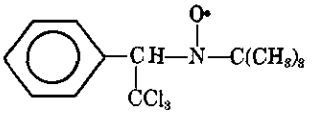
Two-electron processes:

$$CCl_4 + (2e^-) \rightarrow {}^-CCl_3 + Cl^- \quad (27)$$

$${}^-CCl_3 \rightarrow :CCl_2 + Cl^- \quad (28)$$

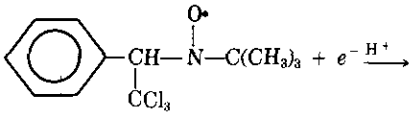
In the electrochemical experiment, the trichloromethyl radical would have to diffuse away from the electrode surface into the body of the solution and be detected by the spin trap by addition.

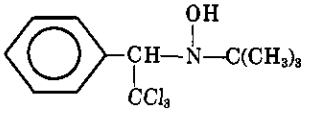




(29)

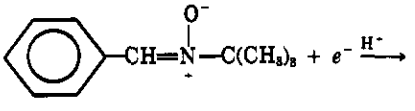
In an analogous fashion the trichloromethyl radical would have to diffuse away from the enzyme active site after formation and find the spin trap before further reduction to the anion could occur. Moreover, in both the electrochemical and biochemical systems the additional possibility that the spin adduct might be reduced to the hydroxylamine anion also existed.

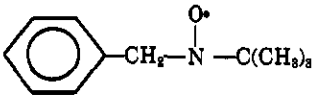




(30)

Finally it was of interest to investigate which halocarbons would reduce before reduction of the spin trap itself takes place e.g., CCl_4, $CHCl_3$, CH_2Cl_2,CH_3Cl. The reduction of the spin trap gives a benzyl *tert*-butyl nitroxide in the presence of protons:





(31)

Recently completed studies have shown that the electrochemical reduction of the following halocarbons gives the corresponding radicals by loss of chloride or bromide ion at potentials before reduction of PBN (*19*): $\cdot CCl_3$ from CCl_4, $\cdot CBr_3$ from CBr_4, $\cdot CHBr_2$ from $CHBr_3$, $\cdot CHCl_2$ from $CHCl_3$, $\cdot CH_2Br$ from CH_2Br_2. These radicals could be trapped by PBN and ESR spectra assigned either by inspection or by HPLC methods. The presence of the halide ion as a product of reduction could be determined electrochemically. Methylene chloride (CH_2Cl_2), methyl chloride (CH_3Cl), and methyl bromide (CH_3Br), could not be reduced before reduction of spin trap or solvent. Thus we conclude that a biochemical reduction pathway is quite feasible for production of halocarbon radicals from certain halocarbons. Whether a given reductive process is likely will depend on the redox potentials available.

The next question of interest was concerned with which halide would be lost in the reduction if both chlorine and bromine exist in the halocarbon. It was known that the toxic activity of the halocarbon increases in the order Br > Cl > F, but no information was available as to which halide bond was broken first in the metabolism of the halocarbon. The electrochemical reduction showed consistently that the bromine is lost first (as bromide) in all cases containing bromine, chlorine, and fluorine: $\cdot CCl_3$ from $BrCCl_3$, $\cdot CHCl_2$ from $BrCHCl_2$, $\cdot CHBrCl$ from Br_2CHCl, $\cdot CHCl\text{-}CF_3$ from $CHClBr\text{-}CF_3$. Moreover, the ease of reduction increases with the replacement of chlorine with bromine. Thus, a reductive dissociation can accommodate the observed increase in toxicity of the chloro and bromo halocarbons. Also it appears quite feasible that the various chloro- and bromo-substituted radicals could be trapped by PBN in the biological sample before further reduction or decomposition, since the lipid environment is not likely as hostile to the radicals, at least with respect to reduction as the region around the electrode in the electrochemical experiment.

Superoxide Anion Reduction

We have recently initiated studies concerned with the reaction chemistry of superoxide and halocarbons (*19,20*). When fresh commercial potassium superoxide is exposed to CCl_4 containing PBN (powdered KO_2 is insoluble in CCl_4 but a surface reaction seems to occur), the spin adduct of the trichloromethyl radical is produced. This reaction is quite slow. However when the same method is used with $BrCCl_3$, the reaction is violent with fresh potassium superoxide! We assume the reaction is simple electron transfer:

$$O_2^{\cdot-} + CCl_4 \rightarrow \cdot CCl_3 + O_2 + Cl^- \quad (32)$$

$$O_2^{\cdot-} + BrCCl_3 \rightarrow \cdot CCl_3 + O_2 + Br^- \quad (33)$$

The chemistry of trichloromethyl radicals with oxygen is not known. It is assumed that the combination of trichloromethyl radicals with oxygen is very fast and diffusion-controlled:

$$\cdot CCl_3 + O_2 \rightarrow Cl_3COO\cdot \quad (34)$$

What the rate and products of the reaction of trichloromethyl radicals with superoxide might be is not known:

$$\cdot CCl_3 + O_2^- \rightleftharpoons {}^-CCl_3 + O_2 \quad (35)$$
$$\rightarrow Cl_3COO^-$$

The chemistry of the trichloromethylperoxyl radical or the trichloromethyl hydroperoxide or its anion is also a puzzle. In the fast reaction between $BrCCl_3$ and potassium superoxide, and in the reaction of trichloromethyl radicals (produced in chloroform from chloroform and *tert*-butoxyl radicals) with oxygen in the presence of PBN, the spin adduct resulting from trapping chlorine atoms was detected along with the trichloromethyl adduct. Other signals were detected, perhaps due to the spin adduct of trichloromethylperoxyl or trichloromethoxyl radicals (*20,21*). We suggest the reactions mechanism [Eqs. (36) and (37)] to account for these results:

$$2Cl_3COO\cdot \rightarrow Cl_3COOOOCCl_3 \rightarrow 2Cl_3CO\cdot + O_2 \quad (36)$$

$$Cl_3CO\cdot \rightarrow Cl\cdot + Cl{-}\overset{\overset{O}{\|}}{C}{-}Cl \quad (37)$$

The proposal is completely analogous to known reactions of *tert*-butylperoxyl radicals which are believed to combine to the tetroxide before dissociating to *tert*-butoxyl radicals (*22–24*).

$$2(CH_3)_3COO\cdot \leftrightarrow (CH_3)_3COOOOC(CH_3)_3 \quad (38)$$

$$(CH_3)_3COOOOC(CH_3)_3 \rightarrow 2(CH_3)_3CO\cdot + O_2 \quad (39)$$

$$(CH_3)_3CO\cdot \rightarrow CH_3\cdot + CH_3{-}\overset{\overset{O}{\|}}{C}{-}CH_3 \quad (40)$$

Superoxide can be made electrochemically by reduction of oxygen and subsequently reacted with the halocarbon (*19,25,26*). Sawyer and co-workers (*5,25,26*) have published some studies on compounds with the trichloromethyl group performed in this manner. End products and the stoichiometry of the reaction were given. In the case of carbon tetrachloride in DMF, the products detected were chloride, bicarbonate, oxygen, and hydrogen peroxide. However, since water was added to the final mixture on work-up, peroxyformates or percarbonates could have decomposed to the bicarbonate. No mechanism for the formation of these products has been offered other than that the first step is assumed to be electron transfer through the "chlorine atom complex" followed by a concerted replacement of chloride by superoxide or dissociation to the trichloromethyl radical which combines rapidly with oxygen in the solvent cage to produce the trichlormethylperoxyl radical [Eq. (41)].

$$CCl_4 + O_2^- \leftrightarrow \left[\begin{matrix} Cl & & ClOO \\ & C & \\ Cl & & Cl \end{matrix}\right]^- \rightarrow Cl_3COO\cdot + Cl^- \quad (41)$$

Since we have found that the trichloromethyl radical is easily detected by PBN we prefer a simple electron-transfer step.

It is of interest to speculate on a mechanism which predicts the stoichiometry and products reported by Sawyer. The scheme shown in Eqs. (42)–(53) presents one possibility.

$$2CCl_4 + 2O_2^- \rightarrow 2Cl_3C\cdot + 2O_2 + 2Cl^- \quad (42)$$

$$2Cl_3C\cdot + 2O_2 \rightarrow 2Cl_3COO\cdot \quad (43)$$

$$2Cl_3COO\cdot \rightarrow Cl_3COOOOCCl_3 \rightarrow 2Cl_3CO\cdot + O_2 \quad (44)$$

$$2Cl_3CO\cdot \rightarrow 2Cl{-}\overset{\overset{O}{\|}}{C}{-}Cl + 2Cl\cdot \quad (45)$$

$$2Cl\cdot + 2O_2^- \rightarrow 2Cl^- + 2O_2 \quad (46)$$

$$Cl{-}\overset{\overset{O}{\|}}{C}{-}Cl + O_2^- \rightarrow Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O\cdot + Cl^- \quad (47)$$

$$Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O\cdot + O_2^- \rightarrow Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O^- + O_2 \quad (48)$$

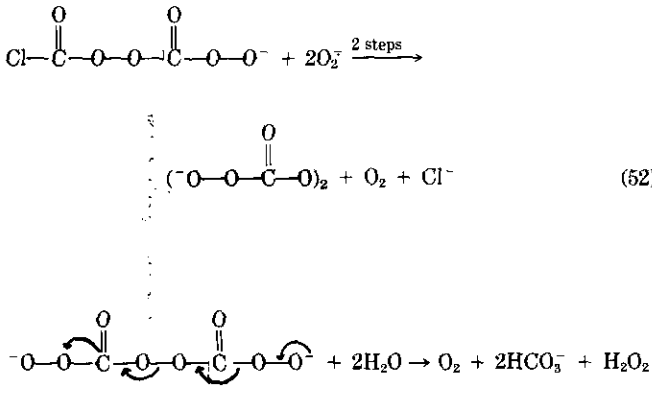
$$Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O^- + Cl{-}\overset{\overset{O}{\|}}{C}{-}Cl \rightarrow Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O{-}\overset{\overset{O}{\|}}{C}{-}Cl + Cl^- \quad (49)$$

$$Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O{-}\overset{\overset{O}{\|}}{C}{-}Cl + O_2^- \rightarrow$$

$$Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O{-}\overset{\overset{O}{\|}}{C}{-}O{-}O\cdot + Cl^- \quad (50)$$

$$Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O{-}\overset{\overset{O}{\|}}{C}{-}O{-}O\cdot + O_2^- \rightarrow$$

$$Cl{-}\overset{\overset{O}{\|}}{C}{-}O{-}O{-}\overset{\overset{O}{\|}}{C}{-}O{-}O{-}\overset{\overset{O}{\|}}{C}{-}O{-}O^- + O_2 \quad (51)$$



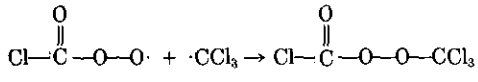
(52)

Sawyer's stoichiometry is: (53)

$$CCl_4 + 5O_2^- + H_2O \rightarrow 4Cl^- + 7/2O_2 + HCO_3^- + 1/2H_2O_2$$
$$1 \quad 5.0\pm0.5 \quad 1 \quad \rightarrow 4.0\pm0.2 \quad 3.3\pm0.2 \quad 1.0\pm0.2 \quad 0.5$$

Other possibilities are obvious, but the chemistry of the products expected is unknown; for example the peroxide of trichloromethane, and the trichloromethyl perester of chloroformate:

$$CCl_3OO\cdot + \cdot CCl_3 \rightarrow Cl_3COOCCl_3 \quad (54)$$



(55)

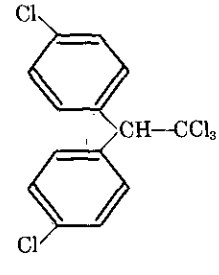
Of biological relevance might be the suggestion that *trichloromethylperoxyl radicals could produce chlorine* atoms either by the route shown earlier or through reaction with lipid peroxyl radicals, (LOO·) probably present in the system:

$$Cl_3COO\cdot + LOO\cdot \rightarrow Cl_3COOOOL \quad (56)$$

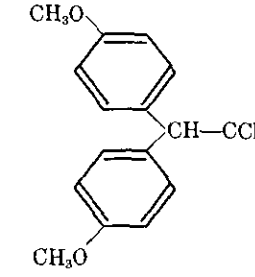
$$Cl_3COOOOL \rightarrow Cl_3CO\cdot + LO\cdot + O_2 \quad (57)$$

Indeed, this may be the method whereby lipidoxyl radicals (LO·) are produced which are detected by spin trapping when carbon tetrachloride is metabolized (*27*).

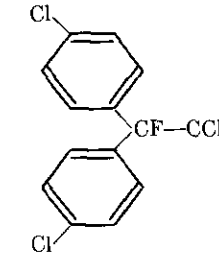
The reaction of superoxide with a variety of chlorinated ethanes and ethylenes (DDT, methoxychlor, F-DDT) was also studied by Sawyer and co-workers (*25,26,28*).



DDT



Methoxychlor



F-DDT

All of these compounds react with superoxide to produce the appropriate ethylene:

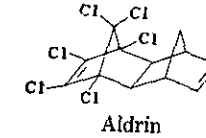
$$Aryl_2CH{-}CCl_3 \xrightarrow{O_2^-} Aryl_2C{=}CCl_2 + HCl \quad (58)$$

The ethylene reacts further when the aryl group is *p*-chlorophenyl to produce the appropriate benzophenone (which is isolated) and presumably phosgene. Sawyer's stoichiometry (*25,26*) is given as by Eq. (59).

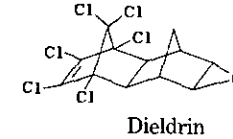
$$(p\text{-}ClC_6H_4)_2C{=}CCl_2 + 3O_2^- + H_2O \rightarrow (p\text{-}ClC_6H_4)_2C{=}O + HCO_3^- + 2Cl^- + 1/2H_2O_2 + O_2 \quad (59)$$

The reaction between superoxide and simple chloroethylenes is relatively slow and *trans*-1,2-dichloroethylene, aldrin and dieldrin are completely unreactive.

trans-1,2-Dichloroethylene

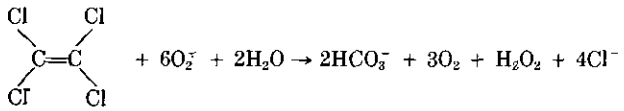


Aldrin



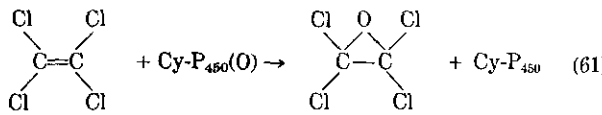
Dieldrin

However *cis*-1,2-dichloroethylene, 1,2-dichloroethylene, trichloroethylene and tetrachloroethylene all react with superoxide to give varying amounts of bicarbonate as the carbon-containing product,



(60)

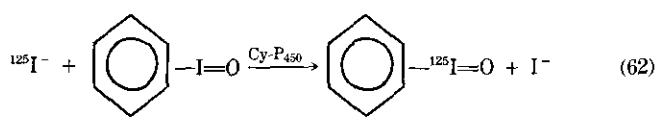
It should be emphasized that these reactions are slow, at least 100 × slower than for carbon tetrachloride and not likely to be important in the biological system in comparison to direct oxygenation by cytochrome P-450.



(61)

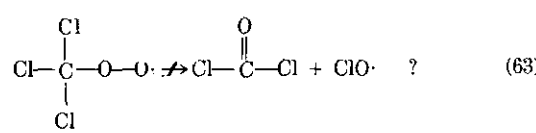
Oxygenation Reactions

It is quite commonly accepted that cytochrome P-450 are able to oxygenate a wide variety of compounds, including the iodine atom in iodobenzene (*29*).



(62)

We have considered the possibility that such a mechanism pertains in the case of carbon tetrachloride metabolism but no evidence is available to support this notion at this time (Sawyer found all of the chlorine in carbon tetrachloride converted to chloride by superoxide):



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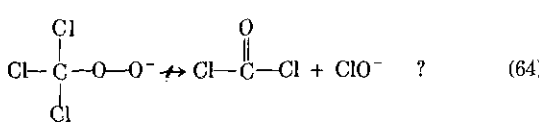
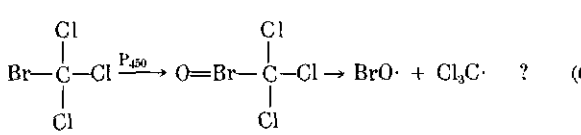


(64)

However with bromo and iodo halocarbons, either a direct reaction with cytochrome P-450 or an intramolecular reaction of the peroxyl radical or hydroperoxide anion are real possibilities.



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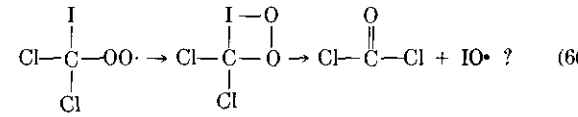

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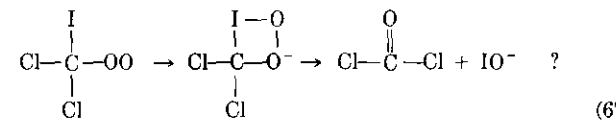


(67)

Biochemistry and Halocarbon Radical Formation

In Vitro Investigation with Spin-Trapping Agents

Although the method of spin-trapping has been used for some time in organic chemistry to investigate mechanism of radical reactions, the use of spin-trapping agents to investigate free-radical processes in biological systems is relatively new and has been pursued in only a few laboratories. The methods developed have focused primarily on identifying the radicals trapped when the biological system is exposed to foreign chemicals, e.g., carbon tetrachloride.

Quantitation of radicals produced is still a problem, because the number of radicals trapped is almost certainly less than the number of radicals actually formed. Many competing reactions are assumed to occur in the biological milieu, e.g., methylenic hydrogens and double bonds which are present in the membrane lipids can react with free radicals by abstraction and addition reactions, respectively. It is this feature of biological membranes which allows the process of lipid peroxidation to be initiated and propagated so easily.

However, notwithstanding the problem of quantitation valuable information about free radical reactions in biological systems can be obtained with the use of spin-trapping agents. Since the integrated ESR signal intensity is directly proportional to the concentration of spin adduct obtained (*30*), the spin-trapping assay can be a useful and sensitive tool to probe not only the existence of radicals *per se*, but also determine whether a given manipulation of the system producing the radicals causes an increase or a decrease in radical production. In addition, the duration of radical production in a living system can be assessed by timed introduction of the spin-trapping agent into the system. Thus, if treatment of an animal with a certain substance initiates free radical formation in a target organ (carbon tetrachloride metabolism in the liver, for instance), the du-

ration of radical production following the treatment can be estimated by administering the spin-trapping agent to a series of animals at different times following the treatment. Finally, the identity of radicals produced in biological systems can often be determined by the spin-trapping procedure.

Thus far, the results which have been reported from the laboratories where spin-trapping agents are being used as probes for detecting radicals in biological systems have been encouraging (*8*, *31*). An early report of the detection of a radical in a biological system by spin-trapping was made by Harbour and Bolton, who were investigating radical production in spinach chloroplasts exposed to light (*32*). In 1977, Saprin and Piette trapped a radical in a system composed of liver microsomes, iron, pyrophosphate (*33*). This radical appears to have been derived from the buffer, Tris, through a reaction of a reactive species (presumably a radical) with the Tris molecule (*8*). Nevertheless it demonstrated the feasibility of radical trapping in a system containing particulate biological material as well as the likely identification of the radical that was trapped. The following year, Lai and Piette reported that they were able to trap hydroxyl radicals (HO·) in a system containing microsomes, NADPH, and an EDTA-Fe^2 chelate, in which the microsomal membrane was undergoing lipid peroxidation (*34*). Although the spin-trapping agent used, DMPO, inhibited lipid peroxidation in this system, the relationship of the production of the hydroxyl radicals to the process of lipid peroxidation in this reaction system was not clear. During this period, at least two different free radicals were demonstrated to be generated when a nitrosamine was incubated with liver microsomes (*35*).

The first ESR data demonstrating that trichloromethyl radicals are produced in liver microsomes during carbon tetrachloride metabolism was reported in 1978 by Poyer et al. (*36*) who used the PBN. The radical adduct obtained exhibited a spectrum of a triplet of doublets (a_N = 14.1, a_β^H = 1.8 G) (*36*). Although these results were questioned at first (*37*), the radicals were later unequivocally identified as trichloromethyl adducts of PBN by use of ^{13}C-labeled carbon tetrachloride (*38*). The strategy for using $^{13}CCl_4$ lay in the fact that in the ESR spectrum of the spin adduct obtained with $^{13}CCl_3$ radicals a characteristic doubling of each line occurs because the nuclear spin of ^{13}C- is 1/2. Since the line doubling does not occur when $^{12}CCl_4$ is being metabolized (Fig. 11), it is clear that the radical responsible for this effect is trichloromethyl.

These results have now been confirmed by other laboratories (*39*,*40*). It was also demonstrated that the trichloromethyl radicals apparently are generated in rat liver microsomes by only one form of cytochrome P-450 (*41*,*42*). This form of cytochrome P-450 has a minimum molecular weight of 52,000 daltons and is inducible by phenobarbital. This particular cytochrome is the first polypeptide component of rat liver microsomes to show alternation following exposure of rats to carbon tetrachloride (*41*). Following treatment of rats with CCl_4, the amount of 52,000-dalton cytochrome decreases very

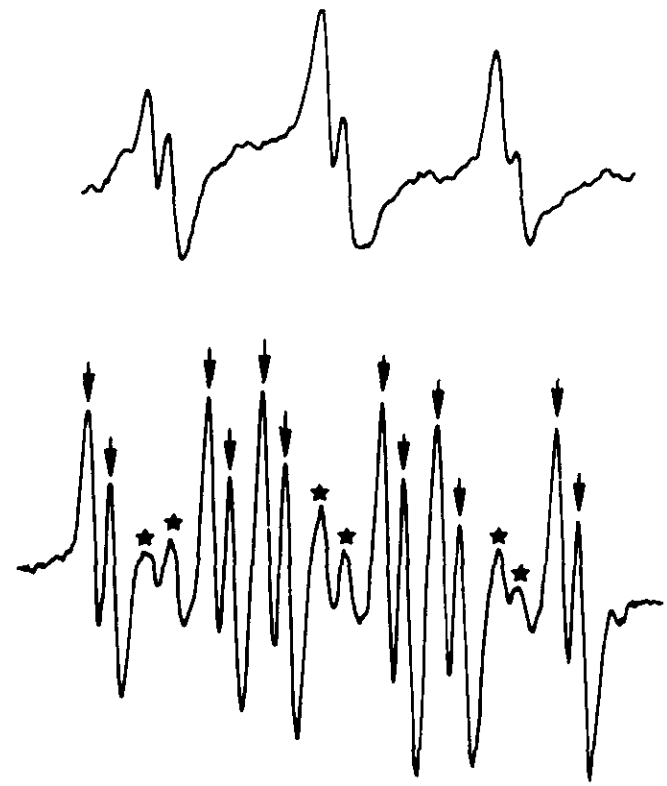


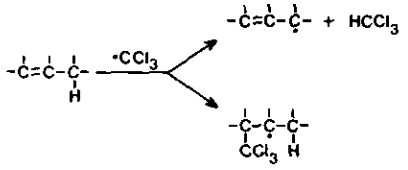
FIGURE 11. ESR spectra of the unlabeled and ^{13}C-labeled trichloromethyl spin adducts of PBN produced in rat liver microsomes. (Top) ESR spectrum of the trichloromethyl ($\cdot CCl_3$) spin adduct of phenyl-*N*-*tert* butyl nitrone (PBN), a_N = 14.1, a_β^H = 1.8 G). Note that this spectrum consists of six lines (i.e. a triplet of doublets). (Bottom) ESR spectrum of the ^{13}C-labeled trichloromethyl ($\cdot^{13}CCl_3$) spin adduct of PBN, a_N = 13.9, a_β^H = 1.5, $a_\beta^{C\text{-}13}$ = 9.5, $a_\gamma^{Cl\text{-}35}$ = 0.23 G. The above six-line spectrum is split into a twelve-line spectrum because the nuclear spin of ^{13}C is one-half. This verifies that in both cases that spin-trapped radical is indeed trichloromethyl ($\cdot^{13}CCl_3$).

rapidly and essentially disappears within 1 hr. Eventually, other cytochromes decrease also, but this appears to be due to secondary damage to the microsomal membrane caused by lipid peroxidation which is initiated by the formation of the trichloromethyl radicals. It has been well established that prior induction of rats with phenobarbital markedly enhances the toxicity of a given dose of carbon tetrachloride. In theory, at least, increasing the level of this cytochrome should facilitate the production of a greater number of $\cdot CCl_3$ radicals during carbon tetrachloride metabolism and may account for the increased toxicity of this halocarbon in the induced state.

Further evidence for the involvement of this particular cytochrome P-450 (52 k dalton) component in the generation of $\cdot CCl_3$ radicals is the observation that metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) inhibits the amount of trapped radicals up to 90% when the animals are treated with this pyridine derivative (*43*). Metyrapone is known to inhibit phenobarbital-induced drug metabolism involving the same form of cytochrome c (*44*). The radicals which are trapped are all located

within the microsomal membrane. This was shown as follows. Rat liver microsomes were allowed to metabolize carbon tetrachloride in the presence of a spin trapping agent (PBN). The reaction system was then subjected to centrifugation at 100,000 *g* to form a firm pellet. The supernatant layer was separated from the pellet and both fractions were assayed for the trichloromethyl radical adduct signal. All of the detectable signal was contained in the pellet. None has been observed in the soluble fraction. Since the radicals are formed in the microsomes, they are apparently trapped there and remain in the membrane, perhaps at or near the site of their formation.

It has now become apparent that when carbon tetrachloride is being metabolized by rat liver microsomes, a sequence of different radicals are trapped as a function of time (27). This sequence is shown in Figure 12. The spectra shown in Figure 12 clearly indicate that a progression of radical trapping has occurred during a 2-hr. period. Initially, the majority of the radicals being trapped are probably lipid oxy radicals (LO·). The factors responsible for this observation are believed to be as follows. When carbon tetrachloride enters the microsomes, it is reductively cleaved by the mixed function oxidase system to trichloromethyl radicals and chloride anions. These promptly react with available oxygen in the membrane at an essentially diffusion-limited rate to form trichloromethyl peroxy radicals. Some of the $\cdot CCl_3$ radicals as well as some of the peroxy radicals will react with membrane lipids. In this event, the radicals may either abstract methylenic hydrogens from polyunsaturated fatty acid groups in the phospholipids, or they may add directly to double bonds to form covalently-linked derivatives.



(68)

In either case, the reaction results in the formation of a lipid radical (L·) which also reacts with oxygen resulting eventually in the formation of lipid oxy radicals.

$$L \cdot + O_2 \rightarrow \rightarrow \rightarrow LO\cdot \qquad (69)$$

The reaction rate for $\cdot CCl_3$ with unsaturated lipid structures is approximately 3×10^6/M-sec. The net result is that initially, essentially all the $\cdot CCl_3$ radicals are converted to $\cdot OOCCl_3$. These can react both with membrane lipids and with the spin trap (PBN). If the latter forms an adduct, however, it is unstable and does not persist long enough for detection by ESR spectroscopy. The LO· radicals also react with PBN, and in this case relatively stable adducts are formed (the reaction rate has been estimated to be approximately 5.5×10^6/M-sec). Thus, the spectrum obtained during the initial minutes of the reaction is primarily that of the spin-trapped LO· radicals (see the 6-min. line of Fig. 12). When oxygen is depleted, L· radicals can partake significantly in the slower reaction with PBN:

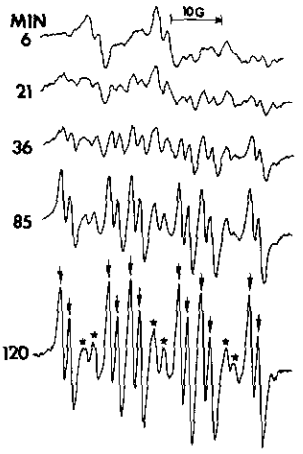


FIGURE 12. Evidence for lipid oxyl (LO·) and ^{13}C-labeled trichloromethyl ($\cdot^{13}CCl_3$) of radicals during the metabolism of ^{13}C-labeled carbon tetrachloride ($^{13}CCl_4$) by rat liver microsomes. The time sequence of radical generation observed by ESR during metabolism in a system composed of rat liver microsomes, an NADPH-generating system, phenyl-N-*tert*-butyl nitrone (PBN), and ^{13}C-labeled carbon tetrachloride ($^{13}CCl_4$) is shown. Incubation was carried out at 25°C under an air atmosphere for the times indicated. The hyperfine splitting constants (a_N = 13.8, a_β^H = 2.26 G), generally broad appearance, as well as the diminished intensity of the high field (right) wing of the ESR spectrum at the six-minute incubation time are consistent with the spin-trapping of a relatively large lipid oxyl radical (LO·) such as one derived from a phospholipid. At later time intervals (e.g., 120 min) the ^{13}C-labeled trichloromethyl ($\cdot^{13}CCl_3$) spin adduct (a_N = 13.9, a_β^H = 1.5, $a_\beta^{C\text{-}13}$ = 9.5, $a_\gamma^{Cl\text{-}35}$ = 0.23 G) becomes the dominant species (indicated by vertical arrows) while the lipid oxyl (LO·) spin adduct (indicated by stars) becomes a minor component.

$$PBN + L\cdot \rightarrow \cdot PBN\text{-}L \qquad (70)$$

After 21 min, the signal intensity of the lipid oxy radical (LO·) begins to diminish because the PBN adduct of that radical, while relatively stable in a chloroform extract, is not stable in the milieu of the microsomal membrane. The signal of the PBN adduct of $\cdot CCl_3$ becomes the dominant feature of the spectrum within 36 min (see Figure 12). If this reaction is performed under conditions where most of the oxygen in the system has been removed, the formation of LO· radicals becomes limited and most of the radicals which are trapped in the first 15 min of the reaction are L· (Fig. 13). Note that the third peak in the spectrum has a distinct shoulder on the right side indicated by the inverted delta. The splitting constants of the peaks marked with the inverted delta throughout the spectrum are: a_N = 13.5, a_β^H = 2.0 G. These values, together with computer simulation analyses, indicate that this portion of the spectrum is due to an oxygen-centered lipid radical. The spectral peaks marked with daggers have the following splitting constants: a_N = 14.5,

a_β^H = 3.25 G and have also been shown by similar analyses to be due to a carbon-centered lipid radical. Thus, both LO· and L· spin adducts are clearly present at this time as well as the ·CCl_3 spin adduct. The latter quickly becomes the dominant feature of the spectrum. Presumably, this is due to reduction of the LO· and L· radical adducts while formation of the much more stable ·CCl_3 adducts continue to exceed the rate of reduction for at least another 30 min under the conditions of these experiments.

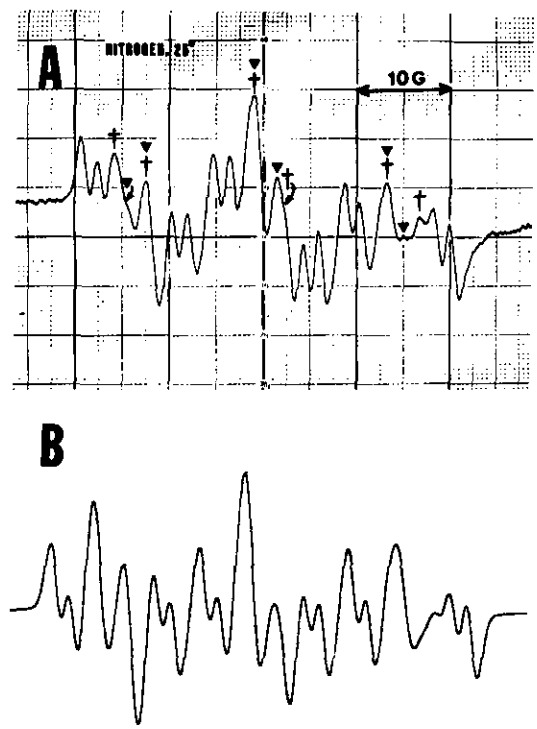


FIGURE 13. Formation of lipid (L·), lipid oxyl (LO·), and ^{13}C-labeled trichloromethyl (·$^{13}CCl_3$) spin adducts of PBN under low oxygen tension. (*A*) ESR signal observed during metabolism in a system composed of rat liver microsomes, an NADPH-generating system, phenyl *N-tert*-butyl nitrone (PBN), and ^{13}C-labeled carbon tetrachloride ($^{13}CCl_4$). The reaction system was subjected to a nitrogen atmosphere (containing 25 ppm of oxygen) and incubated for 15 min. The lipid (L·) spin adduct of PBN (a_N = 14.4, a_β^H = 3.25 G) is indicated by daggers, the lipid oxyl (LO·) spin adduct (a_N = 13.8, a_β^H = 2.0 G) is indicated by inverted triangles, whereas the ^{13}C-labeled trichloromethyl (·$^{13}CCl_3$) spin adduct (a_N = 13.9, a_β^H = 1.5, $a_\beta^{C\text{-}13}$ = 9.5, $a_\gamma^{Cl\text{-}35}$ = 0.23 G) is unmarked. The diminished intensity of high field wing of the ESR signal again suggests the spin-trapping of relative large lipid (L·) as well as lipid oxyl (LO·) radicals. (*B*) Computer simulation of the three spin adduct mixture (L·, LO·, ·$^{13}CCl_3$) using the hyperfine splitting constants reported in Figure 13*A*.

From these experiments, and from what is known from the work of others, several deductions are supported. In any system where ·CCl_3 radicals are being formed, they will react preferentially with O_2 as long as the latter is available to form Cl_3COO·. Both of these radicals will attack lipids and proteins in the vicinity of the enzymic production of the trichloromethyl radicals resulting in the loss of cytochrome P-450 activity and initiation of lipid peroxidation. The latter process also causes destruction of cytochrome P-450 which potentiates the overall loss of the total microsomal cytochrome content. Significant trapping of the ·CCl_3 radicals by PBN will only begin when the availability of both oxygen and membrane lipids becomes limiting in the domain of radical production. Trapping of the ·CCl_3 radicals then appears to continue until the process of lipid peroxidation and direct radical attack on the 52 k dalton cytochrome destroys remaining activity.

The oxygen tension in biological systems may be critical in determining the degree of toxicity resulting from exposure to halogenated hydrocarbons. *In vitro* studies have shown that the partial pressure of oxygen in the system can determine the extent of radical damage to membranous structures in which the halogenated hydrocarbon is being metabolized (*45*). When liver microsomes were incubated with NADPH and halothane, membrane damage, (determined by the extent of membrane lipid peroxidation in the system, measured by malondialdehyde formation) was observed to increase with the partial pressure of O_2. Malondialdehyde formation increased until the partial pressure reached about 3 mm Hg. But when the pressure was increased above 3 mm Hg, the extent of peroxidation decreased (*45*). The authors interpreted the results as indicating that the oxygen tension must be low enough to permit reductive metabolism of the halocarbon, but not so low as to make the rate of lipid peroxidation insignificant.

In Vivo Investigations with Spin-Trapping Agents

Steady-state oxygen tension in liver tissue, a metabolically active tissue, is apparently low enough to permit lipid peroxidation to occur during the metabolism of carbon tetrachloride *in vivo*. That lipid peroxidation does occur *in vivo* as a consequence of CCl_4 metabolism has been considered likely for a number of years (*46*). Direct evidence has now been obtained for the formation of lipid radicals in intact animals exposed to CCl_4 through the use of spin-trapping agents (*27*). When ^{13}C-labeled CCl_4 was orally administered to rats along with PBN, detectable levels of trichloromethyl radicals were observed to form in the hepatic endoplasmic reticulum within 15 min (*47*). However, no other types of radicals were detected with that particular spin trap even though both lipid and lipid oxy radicals were trapped with PBN in the *in vivo* investigations described above. However, when a modified form of the spin trap (trimethoxy phenyl-*tert*-butyl nitrone (MO_3PBN) was administered to rats along with $^{13}CCl_4$, lipid extracts of livers removed from the rats displayed a strong electron spin resonance signal which was identified as a carbon-centered radical adduct of the spin trap (*27*). The adduct which was recovered was as a modified form of MO_3PBN, since one of the methoxy groups is demethylated during the events leading to the isolation of the spin adduct (*27*). The carbon-centered radical is lipid-soluble, and the char-

acteristics of its ESR spectrum indicate that the trapped radical is a large molecule, probably a phospholipid. At present there is no explanation why PBN should preferentially trap $\cdot CCl_3$ radicals while MO_3PBN appears to trap carbon-centered lipid radicals preferentially during CCl_4 metabolism *in vivo*. The most likely explanation is that the positioning of the particular spin trap in the lipid domain of the endoplasmic reticulum determines its accessibility to the site of $\cdot CCl_3$ formation at the cytochrome P-450 which generates these radicals. The structure, lipid solubility, and reaction rates with particular lipids of the various spin trapping agents must all interact to determine which radicals will be trapped during the sequence of events initiated by the reductive cleavage of carbon tetrachloride to the trichloromethyl radical. Thus MO_3PBN may penetrate the cytochrome P-450 domain less well than PBN, and, as a result, be unable to compete with lipids in that domain for $\cdot CCl_3$ radicals. The propagation of lipid peroxidation away from that domain may then provide ample opportunity for MO_3PBN to trap lipid radicals. Whatever the case of the selectivity, the results with MO_3PBN confirm that initiation of lipid peroxidation occurs in the hepatic endoplasmic reticulum as consequence of carbon tetrachloride metabolism. Since lipid peroxidation is thought to be involved in other types of diseases and toxic responses to environmental factors (*48,49*), this particular radical probe may be useful in determining the extent to which peroxidation may be involved.

Spin-Trapping Halothane Radicals

The halogenated hydrocarbon anesthetic, halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), used in surgical and other clinical procedures, might be expected to be metabolized in the endoplasmic reticulum of the liver to a free radical product as is CCl_4 (*38*). Numerous attempts have been made to determine a cause-and-effect relationship between halothane anesthesia and "halothane hepatitis," since reports of postoperative halothane-induced hepatitis began appearing in 1958, the year that the anesthetic was released for clinical use (*50*).

When a rat was given halothane in breathing air for 2 hr immediately following oral administration of PBN, a lipid extract of the liver presented a strong ESR signal as shown in Figure 14*A* (*51*). This spectrum was found to depend on the prior exposure of the animal to halothane. Administration of the spin-trapping agent alone yielded no signal in the lipid extract. When halothane was incubated with PBN, rat liver microsomes, and a NADPH-generating system, an EPR signal also was observed (Fig. 14*B*) which had the same characteristics as that obtained with the extract from the livers of animals administered halothane *in vivo*. The finding of the spin-trapping of a halothane radical *in vivo* (*52,53*) and *in vitro* (*54,55*) has been confirmed by others. The halothane free radical formed in the liver endoplasmic reticulum is probably the 1-chloro-2,2,2-trifluoroethyl radical. Trudell et al. have shown that, when halothane was incubated with vesicles of dioleoylphosphatidylcholine and egg phosphatidylethanolamine containing cytochrome P-450-HA-2, cytochrome P-450 reductase, cytochrome b_5, and NADPH, under argon, a dioleoylphosphatidylcholine product with a 1-chloro-2,2,2-trifluoroethyl group bound to either the 9- or 10-carbon atom of the fatty acid was formed (*56*). Gas chromatography-mass spectroscopy techniques were used to assign the 1-chloro-2,2,2-trifluoroethyl adduct (*56*).

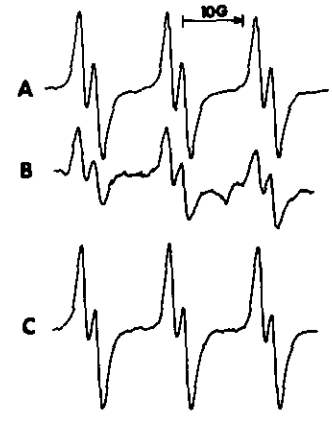


FIGURE 14. Spin trapping of a halothane-derived radical (probably 1-chloro-2,2,2-trifluoroethyl) with PBN *in vivo* and *in vitro*: (*A*) ESR spectrum of the halothane-derived radical spin adduct of PBN (a_N = 14.6, a_β^H = 2.5 G) obtained from a liver extract of a rat given halothane by inhalation and a PBN-phosphate buffer-corn oil emulsion orally prior to halothane administration; (*B*) ESR spectrum of the halothane-derived radical spin adduct of PBN obtained *in vitro* from liver microsomes, halothane, PBN, and an NADPH-generating system; (*C*) ESR spectrum of the halothane-derived radical spin adduct of PBN obtained from a concentrated lipid extract of the microsomal fraction of a liver of a rat given halothane and PBN.

The halothane radical which is formed *in vivo* appears to be identical to that formed by the metabolism of halothane by liver microsomes and is presumably a product of the drug-metabolizing system in the liver of the intact animal. This is consistent with the observation that like *in vivo* experiments done with CCl_4, nearly all of the spin-trapped radical is found in the microsomal fraction of the liver of animals administered halothane and PBN *in vivo* (Fig. 14C) (*51*). Also, as is the case for CCl_4 which forms $\cdot CCl_3$ radicals, metyrapone inhibited the formation of halothane radicals up to 90% when animals were treated with metyrapone prior to exposure to halothane (*43*). This indicates that the same phenobarbital-inducible cytochrome P-450 (*44*) also generates the halothane radicals from halothane. About 0.4 pmole of halothane radicals per gram of liver are trapped in the *in vivo* experiment (*51*), and this is about one-fourth the quantity of radicals compared to the amount trapped when the rats are administered an equivalent dose of CCl_4 (*57*). This lesser formation of halothane radicals, together with the fact that the reactivity of the 1-chloro-2,2,2-trifluoroperoxyl radical (formed from the reaction of the halothane radical with

oxygen) with fatty acids is only one-fifth that of the trichloromethylperoxyl radical (*58*) probably explains halothane being much less hepatotoxic than CCl_4.

Although no relationship between the formation of this radical and the etiology of halothane hepatitis has been established, the similarity of this reaction to that of CCl_4 in the liver suggests that, under some undefined conditions, a halothane radical associated with halothane metabolism may be toxic.

Differences between Rat, Chicken, and Turkey

When chicken liver microsomes were treated with CCl_4 in presence of PBN and an NADPH-generating system, only a weak, indistinct ESR signal was observed as compared to rat liver microsomes. Also, if the chicken was administered CCl_4 and PBN *in vivo* in the same manner as described for the *in vivo* rat studies, no appreciable ESR signal could be observed in a total lipid extract from the liver. These results are in accordance with the low levels of CCl_4-catalyzed lipid peroxidation observed in chicken liver microsomes (*59*), and the resistance of the chicken to CCl_4 liver damage (*60*). However, when turkey liver microsomes were incubated with PBN, CCl_4, and an NADPH-generating system, as was done for the chicken, a strong ESR signal was observed. This indicates a fundamental difference in the capability of the drug metabolizing system of these animals to metabolize halocarbons to highly reactive radicals.

Rat liver microsomes, when incubated with PBN, $CHBr_3$, and an NADPH-generating system as in the case of CCl_4, produced an ESR signal (a_N = 14.6, a_β^H = 2.2 G) which may be the PBN-trapped $\cdot CHBr_2$ radical. When chicken or turkey microsomes were incubated with PBN, $CHBr_3$ and an NADPH-generating system, ESR signals were observed for both cases. These results contrast markedly with the observation that the chicken does not metabolize CCl_4 to the $\cdot CCl_3$ radical, while the turkey does.

In Vitro Studies with Rat Liver Microsomes and Sodium Dithionite

Sodium dithionite ($Na_2S_2O_4$) has been used as a nonenzymic reducing agent in the study of the spectral binding characteristics of halogenated hydrocarbons to reduced cytochrome P-450 (*61*) and in the dehalogenation of several halocarbons by liver microsomes (*62,63*). During studies involving the formation and detection of trichloromethyl free radicals formed during the metabolism of CCl_4, it was found that a rat liver microsomal incubation system utilizing sodium dithionite as an electron donor for cytochrome P-450, in place of the enzymically active NADPH + NADPH cytochrome P-450 reductase, was very effective in producing $\cdot CCl_3$ radicals (*64*).

Trapped radicals were observed when CCl_4, $CHCl_3$, CH_2Cl_2, $CHBr_3$, CH_2Br_2, 1,1,1-trichloroethane, hexachloroethane, 1,1,1-trichloro-2,2,2-fluoroethane, the Freon CCl_3F, DDT, and the anesthetic halothane were used as substrates. Studies with $^{13}CCl_4$ and CCl_3F (Freon 11) indicate that the free-radical species trapped by the PBN are halocarbon radicals and not some other free radicals derived from the incubation system. Although only the phenobarbital-inducible "C" form of cytochrome P-450 (*65*) is believed to be the enzymically active form metabolizing halogenated hydrocarbons to free-radical products (*42,43*), both the purified "A" and "B" forms (*65*) were found to be capable of forming trichloromethyl free radicals in this system. That is, when dithionite was used as reducing agent, the microsomal system did not dependent on any enzymic activity, since heat-denatured microsomes could function as well as undenatured microsomes. Also, when microsomes were treated with 0.2% sodium dodecyl sulfate (SDS) to convert the endogenous cytochrome P-450 to cytochrome P-450 (observed spectrophotometrically), they were still capable of forming the $\cdot CCl_3$ radical from CCl_4 and dithionite. It is presumed that the sodium dithionite reduces the cytochrome P-450 to the ferrous form and that the ferrous form donates an electron to the halogenated hydrocarbon to form a free radical and a halide anion:

$$\text{Cyt. P-450}(Fe^{2+}) + \text{R-X} \rightarrow \text{Cyt. P-450}(Fe^{3+}) + \text{R}\cdot + X^- \quad (71)$$

This nonenzymic system may be of value in determining the capability of halogenated hydrocarbons to be metabolized to free radical products in the liver.

The metabolic conversion of CCl_4 to the trichloromethyl radical is thought to occur by an electron transfer (donation) from the ferrous form of cytochrome P-450 directly to the halocarbon to form the $\cdot CCl_3$ radical and a chloride ion. Therefore, it was of interest to determine what other electron-transfer (donation) reactions could produce $\cdot CCl_3$ radicals from CCl_4. It was found that aqueous solutions of thallous or ferrous ions, ferrous or cuprous ions chelated with 2,2-dipyridyl, Mn^{2+} protoporphyrin IX, and the one-electron reduced free-radical form of methyl viologen can all donate electrons to CCl_4 to form $\cdot CCl_3$ radicals, observed after spin-trapping with PBN. Several mechanisms for the metabolic formation of free radicals and other products from halogenated hydrocarbons have been proposed and are given in a review by McDonald (*66*).

Electron transfer from a hemoprotein, such as cytochrome P-450, to a halocarbon probably takes place through an "inner sphere" electron transfer process (*67,68*), by which the halocarbon becomes axially bonded to the Fe^{2+} of the hemoprotein and accepts an electron from the Fe^{2+} of the heme to break a halogen-carbon bond and form a carbon-centered free radical and a halide anion. The half-wave reduction potential of the halocarbon would have to be sufficiently positive for the halocarbon to accept an electron from the hemoprotein (*69–71*).

For the electron donation process to take place, the

axial ligands of the hemoprotein would have to be accessible to the halocarbon and capable of being displaced by the halocarbon, so that the inner sphere axial electron transfer could take place. In keeping with the table by Castro (*72*) these requirements are apparently not met by cytochrome c, and it does not donate electrons in the reduced Fe^{2+} state to CCl_4 to form $\cdot CCl_3$ radicals. However, as predicted by the same table, hemoglobin, myoglobin, carbon monoxymyoglobin, catalase and horse radish peroxidase, all in the Fe^{2+} state, will all donate electrons to CCl_4 to form $\cdot CCl_3$ radicals from CCl_4.

Since several hemoproteins will form free radical products from halogenated hydrocarbons, it is implied that organelles containing these hemoproteins would be susceptible to some injury when exposed to some halogenated hydrocarbons, although not as severe as that occurring in the liver of animals exposed to CCl_4.

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