Oxygen- and Carbon-centered Free Radical Formation during Carbon Tetrachloride Metabolism

OBSERVATION OF LIPID RADICALS IN VIVO AND IN VITRO

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Free radical reactions involved in the metabolism of carbon tetrachloride by rat liver have been considered to be a cause of at least part of the injury resulting from exposure to this halocarbon. In an earlier study employing electron spin resonance and spin-trapping techniques, we demonstrated that trichloromethyl (\(^{13}\)CCl\(_3\)) radicals are readily observed in rat liver microsomes metabolizing \(^{12}\)CCl\(_4\), and that the same radical could be shown to form \textit{in vivo} in the liver of intact rats given a single dose of \(^{13}\)CCl\(_4\). This report describes the production of lipid dienyl (L\(\bullet\)) and oxygen-centered lipid radicals (LO\(\bullet\) or LOO\(\bullet\) or both) \textit{in vitro} systems metabolizing \(^{13}\)CCl\(_4\), and also the formation of lipid dienyl radicals (L\(\bullet\)) in liver of intact animals exposed to CCl\(_4\). The radicals appear to be produced in a sequence of reactions governed among other things by the oxygen tension in the system. The lipid radicals (L\(\bullet\)) which form in intact liver of CCl\(_4\)-treated rats are apparently the result of an attack on lipids of the endoplasmic reticulum by \(^{13}\)CCl\(_4\) radicals formed by reductive cleavage to CCl\(_4\) and are the initial intermediates in the process of lipid peroxidation. These investigations demonstrate that while the events occurring in liver microsomes \textit{in vitro} appear to parallel those which take place in intact liver \textit{in vivo}, the conditions \textit{in vivo} make the spin-trapping studies of radicals in intact animals much more selective than when \textit{in vitro}. The results require the use of more than one type of spin-trapping agent to detect different radical species \textit{in vivo}.

Electron paramagnetic resonance experiments in this laboratory employing a spin-trapping agent were the initial demonstrations that the metabolism of carbon tetrachloride produces trichloromethyl radicals (CCl\(_3\)) in rat liver both \textit{in vitro} (1) and \textit{in vivo} (2). Formation of these radicals was shown to be observable in rat liver microsomes and in reconstituted mixed function oxidase systems using the spin-trap PBN.\(^1\) In the reconstituted system, the -CCl\(_3\) radical was observed only when a specific form of liver microsomal cytochrome P-450 (52,000 Da) was present (3). This particular cytochrome was quickly destroyed during the reaction. Loss of this 52,000-Da cytochrome P-450 appears to be the earliest demonstrable molecular change \textit{in vivo} in the liver of rats exposed to CCl\(_4\) (4). Our initial observations were evaluated by Kalyanaraman et al. (5), who suggested that the radical detected was a lipid dienyl radical rather than the CCl\(_3\) radical. The question was resolved in our laboratories by the use of \(^{13}\)CCl\(_4\) for both the \textit{in vitro} and \textit{in vivo} systems (6). The 12-line spectrum obtained with \(^{13}\)CCl\(_4\) as compared to the 6-line spectrum observed with \(^{12}\)CCl\(_4\) clearly demonstrated that the CCl\(_3\) radical was being formed. These results using \(^{13}\)C-labeled carbon tetrachloride have recently been confirmed by Albano et al. (7) using the same spin-trapping agent, PBN. A more detailed analysis of the spectra obtained from \(^{13}\)C-labeling studies indicated that Kalyanaraman et al. (5) were also correct in the sense that it now appears that more than one type of radical intermediate is produced during the metabolism of carbon tetrachloride by liver microsomes.

The information presented in this article supports the conclusion that a sequence of detectable radicals are produced \textit{in vitro} and apparently \textit{in vivo} also, and that this sequence is markedly influenced either by high or low levels of oxygen in the system. These results indicate that lipid dienyl radicals (L\(\bullet\)) are formed during the metabolism of CCl\(_4\) by rat liver microsomes metabolizing CCl\(_4\). Both lipid dienyl radicals (L\(\bullet\)) and lipid oxy (LO\(\bullet\)) or lipid peroxy (LOO\(\bullet\)) radicals (or both) appear to be the primary species trapped initially in the \textit{in vitro} systems incubated under low oxygen-containing atmospheres. In all systems, however, the very stable trichloromethyl radical adduct signal is ultimately the major feature of the ESR spectrum. In the intact animal studies, evidence is presented that, in addition to CCl\(_3\) radicals, there is also formation of carbon-centered lipid radicals which can be trapped \textit{in vivo} by (MO\(_3\))PBN. These investigations are being carried out as part of a program to determine how the sequence of early molecular events occurring in the liver relates to the peroxidation of lipids in the endoplasmic reticulum which occurs as a consequence of CCl\(_4\) exposure. This information

\(^1\) The abbreviations used are: PBN, phenyl \textit{t}-butyl nitrene; (MO\(_3\))PBN, 2,4,6-trimethoxyphenyl \textit{t}-butyl nitrene; HO(MO\(_3\))PBN, 2-hydroxy-4,6-dimethoxyphenyl \textit{t}-butyl nitrene; NADPH, nicotine adenine dinucleotide phosphate, reduced; L\(\bullet\), lipid radical; LO\(\bullet\) and LOO\(\bullet\), lipid oxy and lipid peroxy radicals, respectively; EPR, electron paramagnetic resonance.
should be useful in determining the possible role these processes may play in the hepatotoxicity of this halomethane (8).

MATERIALS AND METHODS

$\beta$-Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and oxidized form (NADP), glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Co., St. Louis, MO; phenobarbital sodium was purchased from Merck Co., Inc., Rahway, NJ; PBN was obtained from Eastman Organic Chemicals, Rochester, NY; $^{3}C_{13}$ (99 atom % $^{13}C$) was from Stohler Isotopes Chemicals and Merck Co., Inc., Rahway, NJ; AtmosBag (the flexible inflatable polyethylene chamber with built-in glove port) was used to work in a totally isolated and controlled environment) was purchased from Aldrich Chemical Co., Milwaukee, WI; nitrogen gas and oxygen were obtained from Liquid Air, Inc., San Francisco, CA; argon gas was from Big Three Industries Inc., Houston, TX; crystalline (MO)PBN was synthesized in our laboratory by the procedure described by Sommermeyer and Seiffert (9). The structure and purity of (MO)PBN were confirmed by mass spectrometry and nuclear magnetic resonance in accordance with the criteria outlined by Sommermeyer and Seiffert (9).

Male Sprague-Dawley rats (300–400 g) that had been maintained on a commercial rat ration were injected with phenobarbital sodium intraperitoneally (30 mg/kg body weight) for 4 days. Treatment of rats with phenobarbital is not required to observe the effects, but does enhance the intensity of ESR spectra observed. The rats were killed and the livers were removed. Liver microsomes were prepared by homogenizing the livers in 0.15 M potassium phosphate buffer, pH 7.4, to a total protein concentration of 1.0 ml of buffer. The microsomes were subjected to centrifugation at 10,000 × g for 15 min. The supernatant fraction was then centrifuged at 105,000 × g for 1.0 h. The microsomal pellets obtained were then frozen and kept for later use. The thawed pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.4, so that the microsomes from 1 g of liver were suspended in 1.0 ml of buffer. The protein concentration of microsomal suspensions was determined by the method of Lowry et al. (10). The ESR spectrometer settings were as follows: microwave power, 25 milliwatts; modulation amplitude, 1 G; time constant, 10 s; scan range, 100 G; and scan time, 16 or 30 min. The spectra were taken at room temperature, 25 °C, unless otherwise indicated.

The incubation systems not carried out in an air atmosphere were assembled in a sealed AtmosBag which was flushed with either argon gas, nitrogen gas, or oxygen. If argon gas or nitrogen gas was used, all the components of the reaction mixture were gassed slowly with argon gas or nitrogen gas for at least 5 min. The reaction mixture consisted of liver microsomes (3.3 mg of protein), 0.2 M PBN, 0.1 M $^{13}C_{13}$, and either 0.3 mM NADPH or an NADPH-generating system which was composed of 5 mM glucose 6-phosphate, 0.3 mM NADP, and 0.5 Kornberg unit of glucose-6-phosphate dehydrogenase/ml of reaction system. All the components of the system were contained in the buffer system that was subjected to controlled collapse of the thin part of the tube. This technique was used to sediment the microsomes toward the tip end of the pipette so that the particulates were within the magnetic field of the spectrometer. Microsomes which have been frozen prior to use sediment easily at the g force indicated.

In experiments in which PBN was added at various times after $^{13}C_{13}$, metabolism was initiated, the experimental conditions were the same as described above. At the end of the initial incubation period at 25 °C, 13 mg of PBN was added to the reaction mixture (to achieve 0.1 m final concentration), and then the system was incubated for another 15 min at 25 °C. The ESR spectrum of the reaction system was then determined by placing the mixture in a Pasteur pipette as described above.

In the in vivo experiments, the rats were fasted for 24 h before being administered a mixture of $^{13}C_{13}$, and either PBN or (MO)PBN. A mixture of $^{13}C_{13}$ (80 μl/100 g of body weight) and 1.0 ml of a 0.05 M solution of (MO)PBN in glass-distilled water was homogenized in 0.3 ml of stripped corn oil. This emulsion was administered to the rats by stomach tube. After 15 min, the rats were killed, the livers were removed, and the liver lipids were immediately extracted by the method of Folch et al. (11). The concentrated lipid extracts were placed in the tip of Pasteur pipettes for scanning in an EPR spectrometer (Varian E-90). The separation of the spin adducts of $^{13}C_{13}$ from the lipid radicals was accomplished as follows. The lipid extracts were shaken with 30 ml of acetonitrile. The mixture was centrifuged resulting in a layered separation of the lipid and the acetonitrile. The acetonitrile layer was recovered and concentrated. EPR spectroscopy of both the acetonitrile extract and the remaining lipid was performed.

The parameters used for the computer simulated EPR spectra are given in Table I.

RESULTS AND DISCUSSION

In Vitro Production of Radicals by Rat Liver Microsomes

In vitro experiments were conducted under air and either oxygen-poor, or oxygen-rich conditions in which the gas phase of the chamber where the systems were assembled and incubated was thoroughly flushed either with O$_2$ or with nitrogen or argon gas which contained about 25 and 1 ppm O$_2$, respectively. Situating the reaction system at the bottom of a Pasteur pipette in which the thawed, resuspended liver microsomes sediment toward the bottom of the sealed tip provides for an extended diffusion gradient with respect to oxygen in the gas phase, not dissimilar to the gradient between the blood stream and the endoplasmic reticulum in intact liver tissue in terms of oxygen access. Depending on the gas phase employed, a distinctively different progression of ESR signals was observed during the course of the reaction. The signals were all dependent, however, on an NADPH-dependent rat liver microsomal enzyme activity.

The in vitro studies described in the present report were conducted under conditions similar to those employed in our earlier reports (6) except for the variation in some cases of the time and temperature of incubation and the atmospheric composition of the systems.

Radical Production in Vitro under an Air Atmosphere—

When rat liver microsomes, NADPH, PBN, and $^{13}C_{13}$, are incubated under an air atmosphere for 30 min to 1 h, a 12-line spectrum due to the spin-trapping of the $^{13}C$-centered trichloromethyl radical adduct of PBN is always observed (Fig. 1). The 12-line spectrum makes this $^{13}C$-centered radical ($g = 1.8$ for $^{13}C$) easily identifiable ($g_c = 13.9$, $g_a = 1.5$, $a_c^{14}C = 9.5$, $a_a^{13}C = 0.23$ G) since the $^{13}C$-centered radical adduct of PBN has only a 6-line spectrum ($g_a = 14.1$, $g_a^{13}C = 18.5$). However, if this system is observed at earlier time intervals, a different sort of spectrum is obtained. There is a rapid appearance (with 5 min of a signal in the form of a 6-line spectrum shown in Fig. 2 (first trace). This spectrum is reproducible. Under the conditions used for these experiments, the spectrum intensity is relatively weak and the lines are broad. The latter feature must be due in part to the presence of oxygen in the system. However, it should be noted that the high field doublet is broadened more than the other doublets, indicating that the adducts generating the signal are bulky molecules. These initial radical adducts must be derived from membrane lipid radicals because the total signal is present in the microsomal pellet isolated from the system, and can be recovered from the thoroughly washed chloroform phase of a PBN extract. Total extraction of the adduct into

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2 E. G. Janzen and C. M. DuBose, manuscript to be submitted elsewhere which will contain more information on this type of spin-trapping chemistry.
Oxygen- and Carbon-centered Lipid Free Radicals in Liver

Parameters for computer-simulated ESR Spectra

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<th>Fig.</th>
<th>(a_N)</th>
<th>(a_H^\beta)</th>
<th>(a_{C^{13}}^\beta)</th>
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<th>(%L^a)</th>
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\(a_N\) \% Lorentzian; \(G\) \% Gaussian. 
\(a_{\text{other}}\) Relative areas of component spectra.
\(\Delta^g\) \(g\) value shift in gauss relative to other spectra, e.g. 0.5 means center is shifted 0.5 G upfield.

![Fig. 1. EPR spectrum of the \(^{13}\)CCl\(_3\) radical.](image)

![Fig. 2. Evidence for lipid oxy radical formation during \(^{13}\)CCl\(_3\) metabolism by rat liver microsomes.](image)

Alkylperoxy adducts typically have smaller nitrogen- and \(H\)-hydrogen-coupling constants \((a_N = 13.8, a_H^\beta = 2.2 G)\) and can only be detected below room temperature \((-80^\circ \text{C})\). Therefore, this 6-line spectrum can be assigned to a lipid oxy radical (LO-).

Within 30 min after appearance of the LO- radical adduct, the \(^{13}\)CCl\(_3\) signal becomes detectable and grows rapidly during the next hour to become the dominant feature of the spectrum which is stable for several days. The initial 6-line spectrum continues to diminish.

The advantage of using \(^{13}\)C-labeled CCl\(_3\) (other than for the definitive identification of the signal produced by the PBN adduct of \(^{13}\)CCl\(_3\)) is that the effect of the isotope is to shift the CCl\(_3\) spectral peaks away from the position where other \(^{13}\)C-centered and oxygen-centered radical adduct signals would appear. In order to obtain more information about the nature of these early signals, the oxygen content of the systems was decreased to a low level by flushing with either nitrogen or argon gas, or was increased by flushing the systems with \(O_2\).

Radical Production In Vitro under Conditions of Low Oxygen Tension—When the microsomal metabolism of \(^{13}\)CCl\(_3\) is carried out in the presence of PBN at room temperature for 15
min under a nitrogen atmosphere containing approximately 25 ppm O₂, there was simultaneous observation of at least three radical species (Fig. 3A). In addition to the 12-line ¹³-CCl₃ signal, there is a set of doublets with a larger 1H hyperfine splitting constant (aₓ = 14.4, aᵧ = 3.25 G) characteristic of a carbon-centered radical (marked by triangles). In addition, shoulders on the low field and center doublets, plus the unusual cancellation of peaks within the high field doublet, clearly indicate that an oxygen-centered radical has also been trapped (marked by daggers). A computer simulation of these three components is also shown (Fig. 3B). The carbon in the carbon-centered radical is not ¹⁵C, and, therefore, not derived from ¹³CCl₃. The diminished signal at the high field end of the spectrum indicates a bulky radical, and, since all of the spin adducts in this system are extractable with the membrane lipids, it is highly likely that these are spin adducts of lipid radicals (L⁻) and lipid oxy radicals (LO⁻).

The sequence of radical production in a system in which air is flushed from both the aqueous and gas phase above the system using argon is shown in Fig. 4. The effect of incubation temperature is also shown. The O₂ concentration of the system is reduced to approximately 1.0 ppm O₂. At 25 and 37 °C, a 6-line signal characteristic of the L⁻ adduct (aₓ = 14.1 G, aᵧ = 3.3 G) appears very rapidly (within 1 min), and is much more intense at 37 and 25 °C than when the same reaction is carried out at 0 °C (ice bath). At all temperatures, trapping of ¹³-CCl₃ is observed subsequently, but occurs much earlier at 0 than it does at 25 and 37 °C. The spectrum observed at 1 min is probably due to overlapping L⁻ and LO⁻ adduct signals since the intensity of the second pair of doublets is considerably greater than that of the first, especially at 37 °C (Fig. 4); that is, as in Fig. 3, the center doublet indicates summation of more than one radical species, and this is reflected as peak broadening in the other doublets of this spectrum.

Fig. 5 shows that, after 40 min, at all three temperatures, the ¹³-CCl₃ signal has now become the dominant feature of the EPR spectrum, while the initially observed 6-line signal has diminished, especially in the systems incubated at 25 and 37 °C where this signal was previously strong. All of these signals are extractable from the system by Folch extraction with chloroform-methanol.

Because the formation of spin adducts in systems where PBN is added at time 0 provides no indication of the duration of production of a particular form of radical over the total period of the incubation, the addition of the spin-trapping agent was made at different time intervals during the reaction. Essentially, only radicals formed from the time of addition onward would be subject to trapping. Microsomal systems metabolizing ¹³CCl₃ under nitrogen were incubated for 30 min at 25 °C before the addition of PBN, and then allowed to incubate for another 15 min. As long as 30 min after assembling the CCl₃-metabolizing system, lipid radicals and some oxygen-centered radicals as well are still being generated in sufficient amounts to provide a substantial signal which is observed along with that of the ¹³-CCl₃ adduct (Fig. 6). Addition of PBN 120 min after starting the reaction indicates that production of L⁻ radicals has diminished to levels undetectable by this technique. The formation of ¹³-CCl₃ radicals, however, is still continuing. These results provide further indications that the generation times for LO⁻ radicals under conditions of very low oxygen tension is brief. L-radical formation continues for a longer time. For example, oxygen-centered radicals were a major feature of the spectrum if PBN is added initially but not if PBN is added at 30 min. Also L⁻ can still be trapped after the reaction had proceeded for up to 1 h, but usually not if PBN was added after that time. The ·CCl₃ adduct is still observed if PBN is added 2 h after initiating the reaction.

Radical Production in Vitro under an Oxygen Atmosphere—When this same microsomal system is incubated with O₂, the carbon-centered lipid radical is not observed, but an adduct appears within 15 min which has the characteristics of the oxygen-centered radical (aₓ = 13.88, aᵧ = 2.17 G) (Fig. 7A). This signal is most likely due primarily to the LO⁻ adduct of PBN since it is also membrane-bound and totally lipid soluble. This signal predominates in the system at least for several hours. By 24 h, the ¹³-CCl₃ radical has appeared and the intensity of the lipid oxy radical is diminishing (Fig. 7B).

Thereafter, the much more stable ¹³-CCl₃ radical adduct is the only signal detected. This suggests that the domain in which the ¹³-CCl₃ radicals are formed can become locally depleted of both oxygen and lipids. Even though there is an abundance of oxygen in these systems, the geometry of the incubation vessels, with the reaction system sedimented at the tip end of sealed Pasteur pipettes, could provide for loci sufficiently low in O₂ to permit some adduct formation be-

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Fig. 3. Formation of L⁻, LO⁻, and ¹³-CCl₃ under low O₂ tension. A, EPR spectrum of a microsomal system metabolizing ¹³CCl₃ under a nitrogen atmosphere. The nitrogen gas used in these experiments contained about 25 ppm of oxygen. The composition and conditions of the reaction system are as described in Fig. 1 except for the incubation time (15 min) and the gas phase. The latter was equilibrated with the various components of the system before assembly in a closed chamber as described under "Materials and Methods." The spectrum shows clear evidence for the trapping of three radicals in this system (¹³-CCl₃, L⁻, and LO⁻) during the 15-min time period. The doublets for the L⁻ signal are indicated by † and for the LO⁻ signal by 4. The rest of the signal constitutes the 12-line ¹³-CCl₃ signal. The LO⁻ (aₓ = 13.8, aᵧ = 2.0 G) and L⁻ (aₓ = 14.4, aᵧ = 3.25 G) partially overlap, resulting in the broadened peaks with shoulders as indicated, and the summation effect in the center pair of doublets. The diminished intensity of the high field L⁻ and LO⁻ radical signals indicates relatively bulky radicals have been trapped. B, computer simulation of ¹³-CCl₃ (aₓ = 13.9, aᵧ = 1.5), L⁻ (aₓ = 14.5, aᵧ = 3.25), and LO⁻ (aₓ = 13.5, aᵧ = 2.0) (see Table 1).
oxygen-centered lipid radical adducts have largely decayed, allowing reaction scheme:

\[ \text{CCI}_3 + \text{O}_2 \rightarrow \text{LOO} \cdot \]

Under argon atmosphere) than it does under air or oxygen. When \( \text{CCI}_3 \) occurs earlier under very low oxygen concentrations (i.e., 1 ppm of \( \text{O}_2 \)) but under an argon atmosphere, it is not possible to observe \( \text{L} \cdot \) and \( \text{LO} \cdot \) doublets predominate. This may reflect the decreased fluidity of membrane lipids at 9 °C which could hinder the availability of the lipids to react with the \( \text{CCI}_3 \) radicals being formed. The low temperature together with the very low oxygen tension in the system, would enable PBN to compete for the \( \text{CCI}_3 \) radicals more effectively at 0 than at 25 °C or 37 °C (C). In the latter two systems, L· radicals are dominant at 1.0 min.

The results can be accommodated by considering the following reaction scheme:

\[
\begin{align*}
\text{CCI}_3 + \text{H}_2 \text{O} & \rightarrow \text{CCl}_2 \cdot + \text{H}_2 \text{O} \cdot \\
\text{CCl}_2 \cdot + \text{H}_2 \text{O} & \rightarrow \text{CCl}_3 + \text{H}_2 \text{O} \\
\text{CCl}_3 + \text{PBN} & \rightarrow \text{CCl}_3 \cdot + \text{PBN} \\
\text{L} \cdot + \text{CCl}_3 \cdot & \rightarrow \text{LO} \cdot + \text{L} + \text{PBN} \\
\text{LO} \cdot + \text{PBN} & \rightarrow \text{LO} \cdot - \text{PBN} \\
\end{align*}
\]

**SCHEME 1**

**FIG. 4.** EPR spectrum of a microsomal system metabolizing \( ^{13}\text{CCI}_3 \) under an argon atmosphere. The argon gas employed contained about 1 ppm of \( \text{O}_2 \). The reaction system was assembled as in Fig. 3 except for incubation temperatures as indicated. The incubations were carried out for 1.0 min at either 0, 25, or 37 °C. A, the 0 °C spectrum shows that the 12-line \( ^{13}\text{CCI}_3 \) radical is observed more readily at this early time than at 25 (B) or 37 °C (C) where the overlapping \( \text{L} \cdot \) and \( \text{LO} \cdot \) doublets predominate. This may reflect the decreased fluidity of membrane lipids at 9 °C which could hinder the availability of the lipids to react with the \( ^{13}\text{CCI}_3 \) radicals being formed. The low temperature together with the very low oxygen tension in the system, would enable PBN to compete for the \( ^{13}\text{CCI}_3 \) radicals more effectively at 0 than at 25 (B) or 37 °C (C). In the latter two systems, L· radicals are dominant at 1.0 min.

**FIG. 5.** EPR spectrum of a microsomal system metabolizing \( ^{13}\text{CCI}_3 \) under an argon atmosphere. The reaction system and experimental conditions are the same as in Fig. 4 except that the incubation time in these experiments was 40 min. Incubations were done at 0 (A), 25 (B), and 37 °C (C). After 40 min at 0 °C, the spectrum displays the 12-line \( ^{13}\text{CCI}_3 \) signal but at a lesser intensity than the system incubated at 25 °C. The intensity of the \( \text{L} \cdot \) and \( \text{LO} \cdot \) radical signal is decreased by this time at both 25 and 37 °C but is essentially unchanged at 0 °C, indicating that the spin adducts of \( \text{L} \cdot \) and \( \text{LO} \cdot \) are relatively unstable at 25 °C or above. Whereas the \( ^{13}\text{CCI}_3 \) signals were barely detectable at 1.0 min in the 25 and 37 °C systems (Fig. 4, B and C), this radical adduct is a major feature of the spectra of these systems at 40 min (B and C).

Given that the reaction is initiated at some rate depending on the local concentration of \( \text{CCI}_3 \) and the appropriate cytochrome P-450 (assumed to be in the reduced form), the \( \text{CCI}_3 \) radicals can either react with the lipid molecules in the immediate vicinity of the reaction site or with PBN. The rate constant for spin-trapping \( \text{CCI}_3 \) radicals by PBN is much smaller (13) (estimated to be less than \( 10^9 \text{ M}^{-1} \text{s}^{-1} \)) in contrast, the addition of radicals like \( ^{13}\text{CCI}_3 \) to olefins in hydrocarbon solvents is faster (14) (e.g., \( 3 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) for \( \text{CF}_3 \cdot + \text{CH}_2 = \text{CH}_2 \) in cyclohexane or heptane). Since hydrogen atom abstraction is probably slower (this value is unknown but could be about \( 10^5 \text{ M}^{-1} \text{s}^{-1} \)), the major route for the initial reactions of \( ^{13}\text{CCI}_3 \) radicals should be addition to the unsaturated sites of lipid molecules. Moreover, the organization of the lipid molecules in relation to the enzyme system producing the \( ^{13}\text{CCI}_3 \) radicals may be such that the reaction of the radicals with the lipids in the immediate vicinity may be facilitated. However, as the accessible lipid molecules are depleted, the reaction of \( ^{13}\text{CCI}_3 \) radicals with PBN begins to predominate (60–90 min in the experiment at 25 °C).

Simultaneously, of course, \( \text{L} \cdot \) can be trapped or react with oxygen. The latter reaction will always be much faster in the presence of even a small amount of oxygen since the rate constant of the reaction of carbon-centered radicals with oxygen is essentially the rate of diffusion of oxygen to the radical (\( 10^8-10^9 \text{ M}^{-1} \text{s}^{-1} \)). In comparison, the rate constant for spin-trapping carbon-centered radicals by PBN is much smaller (15, 16) (2 to 5 \( 10^7 \text{ M}^{-1} \text{s}^{-1} \) for 1-hexenyl in benzene at 40 °C: 6.8 \( 10^5 \text{ M}^{-1} \text{s}^{-1} \) for 6-hepten-2-yl in benzene at 40 °C). Thus, \( \text{L} \cdot \) can only be detected when oxygen is absent or completely consumed in the vicinity of the spin trap.

In regard to the possible trapping of the lipid peroxy radical,
FIG. 6. EPR spectrum observed in a microsomal system metabolizing $^{13}$CCl$_4$ when PBN is added 30 min after the reaction is initiated. The composition and conditions of the reaction are the same as in Fig. 3 except that PBN was added to the system after an initial 30-min incubation followed by an additional 15-min incubation. The spectrum shows that, even after 30 min, production of some LO$^\cdot$ is still occurring but L$^\cdot$ and the 12-line $^{13}$CCl$_3$ radical adducts are the dominant features of the signal at this time. The presence of the PBN adduct of LO$^\cdot$ is indicated by the doublet consisting of the shoulder on the third peak from the left together with the fourth peak (same feature as in Fig. 2).

It has been shown that most peroxy spin adducts of PBN decompose at room temperature (12). Thus, it is quite possible that LO$^\cdot$ radicals are trapped but do not survive under the conditions used for the experiments. However, LO$^\cdot$ radical adducts are quite clearly obtained under conditions of lipid peroxidation, whether these radicals are formed as a "natural" result of the system under investigation or whether the LO$^\cdot$ adducts of PBN decay to give LO$^\cdot$ adducts is not known at this time. Further investigations with spin traps where the peroxy spin adducts are more persistent are planned.

Additional reactions possible within this system require comment in spite of the fact that no results obtained provide explicit experimental information about them: namely, the reaction of $^{13}$CCl$_3$ with oxygen, and subsequent reactions:

\[
\begin{align*}
\text{CCl}_3 + O_2 &\rightarrow \text{Cl}_3\text{COO}\cdot \\
\text{Cl}_3\text{COO}\cdot + \text{lipid} &\rightarrow \text{L} \\
\text{Cl}_3\text{COO}\cdot + \text{PBN} &\rightarrow \text{Cl}_3\text{COO-PBN} \\
\text{Cl}_3\text{COO}\cdot &\rightarrow \rightarrow \rightarrow \text{Cl}_3\text{CO} \\
\text{Cl}_3\text{CO} + \text{PBN} &\rightarrow \text{Cl}_3\text{CO-PBN} \\
O &\rightarrow \text{Cl} + \text{Cl} + \text{Cl} \\
\end{align*}
\]

**Scheme 2**

Although very little is known about the reaction chemistry of $^{13}$CCl$_3$ and oxygen, it can be assumed that the rate would be very fast to produce the peroxy radical. The trichloromethyl peroxy radical would behave similarly to a lipid peroxy radical, although a greater reactivity might be expected because of the electron withdrawing nature of the trichloromethyl group. The rate constant for the addition of Cl$_3$COO$^\cdot$ to PBN has been estimated (13) \(5.6 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) in CCl$_4$. However, this adduct does not appear to be stable at room temperature. The trapping of trichloromethoxy radical seems very unlikely since $\beta$-cleavage to give chlorine atoms appears to be a rapid process.$^3$

Thus, the spectral data are consistent with the concept that, initially, the reaction of the $\cdot$CCl$_3$ radical with O$_2$ and with the microsomal lipid proceeds at a significantly greater rate than it does with PBN. The addition of CCl$_4$ metabolites to unsaturated sites of the lipid has previously been detected by using $^{13}$CCl$_4$ (17). The resulting L$^\cdot$ radicals would also rapidly react with O$_2$ to form LO$^\cdot$. As the limited oxygen supply is depleted, L$^\cdot$ would be the principal radical that is spin-trapped by PBN, producing the type of mixed signal seen in Figs. 3 and 5. As the number of available unreacted lipid and O$_2$ molecules at the site of radical generation decreases, reaction of $^{13}$CCl$_4$ with PBN to form the very stable $^{13}$CCl$_3$-PBN spin adduct could occur at an increasing rate. The diminished signal for the lipid radicals at 0 compared to 25 or

$^3$ C. M. DuBose, unpublished observations.

FIG. 7. EPR spectrum of a microsomal system metabolizing CCl$_4$ under an oxygen atmosphere. The composition and conditions of the incubation system are the same as in Fig. 1 except for the oxygen gas phase instead of air. A, the EPR spectrum observed after 15 min of incubation. Only the LO$^\cdot$ doublets are seen at this time whereas under an air atmosphere, L$^\cdot$ and some $^{13}$CCl$_3$ radical adducts are also observed at this time. B, the spectrum of the same system observed 24 h after assembly. Enzyme-catalyzed radical production has stopped and the less stable lipid radical adducts have degraded substantially, leaving the much more stable $^{13}$CCl$_3$ adducts as the primary feature of the spectrum.
at the bottom of the sealed tip end of a Pasteur pipette (see text).

Treatment of the rats and the procedure for rapid recovery of the lipid spectrum extract are described under "Materials and Methods." The EPR actually due to a radical adducts of this type form adducts of (M0)3PBN which demethylate spontaneously to yield the 2-hydroxy derivative (see text).

From the liver of an animal given a mixture of "CCl4 and (MO)5PBN in corn oil 15 min earlier by stomach tube. The treatment of the rats and preparation of the liver lipids as well as the extraction of the lipid with acetonitrile are described under "Materials and Methods." The EPR signal was found in the lipid extract of the liver as in the case of 13Ccl4, the spectrum shown in Fig. 8 was observed. When (MO)5PBN was administered to animals in vivo along with 13Ccl4, the spectrum shown in Fig. 8 was observed. The signal was found in the lipid extract of the liver as in the case of 13Ccl4 radical trapped by PBN. No signal is observed if fresh liver tissue, (MO)5PBN, and 13Ccl4 are subjected to a Folch extraction together, demonstrating that the signal observed in the liver extract of animals treated with (MO)5PBN and CCl4 in vivo is not an artifact of the extraction procedure. In order to ascertain if the small additional peaks observed in Fig. 8 were possibly due to the 13Ccl4 adduct, the liver lipid containing the spin adducts was extracted with acetoneitrile and concentrated to a small volume. Fig. 9A shows a weak but distinct 13Ccl3 adduct in the acetonitrile extract. Fig. 9B indicates that the adduct with the narrow doublet shown in Fig. 8 still remains in the lipid material. The fact that triplets of doublets with relatively small β-H splittings were observed was puzzling, since authentic adducts of (MO)5PBN have β-H coupling constants in the range of 3.7–12.00 G. Further investigation of the spin-trapping chemistry of (MO)5PBN disclosed that certain carbon-centered adducts of (MO)5PBN demethylate in solutions exposed to air to give HO(MO)2PBN, for example, the n-butyl adduct:  

\[
\begin{array}{cccc}
\text{Radical} & \text{Adducts of (MO)5PBN} & \text{Adducts of HO(MO)2PBN} \\
& a_N & a_H^N & a_N & a_H^N \\
\text{CH}_3(CH_2)_2 & 14.79 & 10.41 & 16.09 & 2.30 \\
(CH_2)_2CH_2 & 14.23 & 7.62 & 16.09 & 1.95 \\
(CH_2)_3CO & 14.18 & 9.27 & 13.83 & 2.53 \\
\end{array}
\]

37 °C (Fig. 5) appears to be a reflection of the mobility of the lipid molecules in the microsomal membrane which, in turn, could influence their availability for reaction with 13Ccl3 within the domain of the radical formation. Decreased fluidity resulting in restricted availability of the lipids at that site at low temperatures would favor an earlier reaction of -CCl3 with PBN, which is the effect that was observed.

Recently, Albano et al. (7) reported the spin-trapping of lipid radicals in peroxidizing fatty acids and in rat liver microsomes metabolizing CCl4 using 2-methyl-2-nitrosopropene as the trapping agent. However, in view of the report by Mason et al. (18) that 2-methyl-2-nitrosopropene can react with double bonds of unsaturated fatty acids by a pseudo-Diels-Alder mechanism based on reactions of similar nitroso spin traps (19), the use of 2-methyl-2-nitrosopropene to detect radicals in biological systems should be done with that information in mind (20, 21).

**In Vivo Production of Radicals in Rat Liver**

Several years ago, the feasibility of spin-trapping radicals in livers of intact animals was reported from our laboratory after exposure of rats to 13Ccl4 (2) and to halothane (22). In the case of 13CCl3, we have only been able to detect the 13CCl3 radical in the liver with PBN even though, in the in vitro investigations reported above, several other radical species were detected with this spin trap. (MO)5PBN is a spin trap which forms radical adducts with quite different hyperfine splitting parameters than PBN (9). Specifically, the β-H-splitting constants are larger and, thus, it was thought that the L- and LO- adducts could be more easily distinguished in the spin adduct spectra.4

When (MO)5PBN was administered to animals in vivo along with 13CCl3, the spectrum shown in Fig. 8 was observed. The signal was found in the lipid extract of the liver as in the case of 13CCl3 radical trapped by PBN. No signal is observed if fresh liver tissue, (MO)5PBN, and 13CCl3 are subjected to a Folch extraction together, demonstrating that the signal observed in the liver extract of animals treated with (MO)5PBN and CCl3 in vivo is not an artifact of the extraction procedure. In order to ascertain if the small additional peaks observed in Fig. 8 were possibly due to the 13CCl3 adduct, the liver lipid containing the spin adducts was extracted with acetoneitrile and concentrated to a small volume. Fig. 9A shows a weak but distinct 13CCl3 adduct in the acetonitrile extract. Fig. 9B indicates that the adduct with the narrow doublet shown in Fig. 8 still remains in the lipid material.

4 E. G. Janzen and C. M. DuBose, unpublished observations.

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**Fig. 8.** EPR spectrum of a washed lipid extract obtained from the liver of an animal given a mixture of 13CCl3 and (MO)5PBN in corn oil 15 min earlier by stomach tube. The treatment of the rats and the procedure for rapid recovery of the lipid extract are described under "Materials and Methods." The EPR spectrum of the lipid extract was obtained after placing the sample at the bottom of the sealed tip end of a Pasteur pipette (see text). The coupling constants are $a_N = 15.45$, $a_H^N = 2.07$. The signal is actually due to a L- adduct of HO(MO)2PBN since carbon-centered radical adducts of this type form adducts of (MO)5PBN which demethylate spontaneously to yield the 2-hydroxy derivative (see text).

**Fig. 9.** EPR spectra observed when a lipid extract of liver from a rat given 13CCl3 and (MO)5PBN orally in corn oil is further extracted with acetonitrile. The treatment of the rats and preparation of the liver lipids as well as the extraction of the lipid with acetonitrile are described under "Materials and Methods." A, acetonitrile extract of the lipid shows a weak EPR signal characteristic of 13CCl3 which was not observable in the more dilute original lipid extract. B, EPR signal of lipid residue remaining after acetonitrile extraction.
The mechanism of this reaction will be discussed elsewhere.\(^4\) The spin adducts of \(\text{HO(OM)}\) PBN give relatively small \(\beta\)-H hyperfine coupling constants. A comparison of some carbon-centered and oxygen-centered radical adducts of \(\text{MO})\) PBN and \(\text{HO(OM)}\) PBN is shown in Table II.

The spectrum in Fig. 8 consists of a nitrogen triplet of 15.45 G and a \(\beta\)-H doublet of 2.07 G. Because of the small size of the \(\beta\)-H doublet, it is concluded that an adduct of \(\text{HO(OM)}\) PBN has been detected. Since the value for the nitrogen splitting is relatively high, the spectrum is assigned to a carbon-centered adduct on the basis of the similarity of the spectrum in Fig. 8 to those obtained from the \(n\)-butyl and isopropyl adducts (\(a_\beta = 16.09\) and \(a_\beta = 2.30-1.95\) G).

Thus, we assign the spectrum obtained from the \textit{in vivo} experiments to a lipid adduct (\(L\)) of \(\text{HO(OM)}\) PBN. The line broadening of the high field doublet is additional evidence that the molecule trapped has a relatively large molecular weight. It is assumed that \(\text{MO})\) PBN actually traps the lipid radical but that a rapid chemical reaction converts it to the lipid adduct of \(\text{HO(OM)}\) PBN. It appears that lipid adducts of \(\text{HO(OM)}\) PBN are more persistent than lipid adducts of either \(\text{PBN}\) or \(\text{MO})\) PBN.

The spectrum in Fig. 8A is clearly due to a \(\text{\textsuperscript{13}}\text{CCl}_3\) adduct. Since the hyperfine coupling constants are similar to those of the \(\text{\textsuperscript{13}}\text{CCl}_3\) adduct of \(\text{PBN}\), the spectrum is assigned to the \(\text{\textsuperscript{13}}\text{CCl}_3\) adduct of \(\text{HO(OM)}\) PBN (\(a_\beta = 14.31, a_\beta = 2.35, a_\beta = 9.02\) G). Again it is assumed that \(\text{MO})\) PBN is the actual \textit{in vivo} trap for \(\cdot\text{CCl}_3\) radicals and the conversion to the trichloromethyl adduct of \(\text{HO(OM)}\) PBN happens spontaneously.

These results indicate that, depending on the spin-trapping agent employed in investigations on \(\text{\textsuperscript{13}}\text{CCl}_3\) metabolism \textit{in vivo}, different radicals may be observed, and this suggests that a combination of spin-trapping agents may be useful in such experiments. The reason for not observing carbon-centered \(L\) adducts \textit{in vivo} with \(\text{PBN}\), whereas \(L\) adducts of this spin trap are readily observed in microsomes \textit{in vitro} during \(\text{\textsuperscript{13}}\text{CCl}_3\) metabolism, appears to be due to decay of the \(L\) adducts during lipid extraction and concentration. The carbon-centered \(L\) adducts of \(\text{MO})\) PBN are much more stable to such manipulation.

\textit{In vitro} experiments with \([\text{MO})\) PBN\] with rat liver microsomes have been performed and gave similar results to those performed \textit{in vivo}. Spectra consisting of two components with very broad lines were obtained after 30 min (Fig. 10A) (see e.g. Fig. 10A after 30 min). Analysis of these spectra indicates that an adduct (or adducts) of \(\text{HO(OM)}\) PBN are detected in addition to \(t\)-butyl hydroxylamine, (\(\text{CH}_3\))\) LCNHO. The latter radical is a common oxidation product of \(t\)-butyl hydroxylamine which comes from the hydrolysis of the nitron (\(a_\alpha = 14.5, a_\beta = 13.8\) G) which occurs to some extent under these \textit{in vitro} conditions:

\[
\begin{align*}
\text{O}^- & \quad \text{R-CH=N-CMe}_3 \quad \text{H}_2\text{O} \quad \text{R-CHO} + \text{MeCNHOH} \\
+ & \end{align*}
\]

The spin adducts of \(\text{HO(OM)}\) PBN obtained from the \textit{in vitro} studies are more difficult to assign at this time since the nitrogen hyperfine coupling constants obtained from the still broad lines after 24 h in Fig. 10B (\(a_\alpha = 14.94, a_\beta = 2.15\) G) fall between those obtained from oxygen- and carbon-centered spin adducts (\(a_\alpha = 13.8\) and 16.09 G, respectively). Perhaps both \(L\) - and \(L\) - adducts of \(\text{HO(OM)}\) PBN are present in the mixture.

With the exception that the \(L\) - radical has yet to be observed \textit{in vivo}, the findings thus far indicate that the events occurring \textit{in vitro} during \(\text{CCl}_3\) metabolism by the liver also are occurring \textit{in vivo}. It now appears possible to trap radicals formed during lipid peroxidation in tissues of intact animals.

There is substantial evidence indicating that lipid peroxidation must occur in the endoplasmic reticulum during the metabolism of \(\text{CCl}_3\) in mammalian liver tissue \textit{in vivo} (23). Detection of conjugated dienes in microsomal lipids (24), loss
of polyunsaturated fatty acids from the endoplasmic reticulum (25), and exhalation of pentane and ethane in CCl₄-treated rats (26) constitute part of this evidence. Similar findings are observed in vitro with liver microsomal preparations (27). Prior treatment of experimental animals with free radical scavenging compounds provides a degree of protection against the toxicity of CCl₄ (28). These compounds also inhibit lipid peroxidation in the endoplasmic reticulum (and in microsomes in vitro) during the metabolism of CCl₄ (27), but do not appear to inhibit the binding of CCl₄ to lipids or macromolecules (27). Hence, the possibility exists that the toxicity of CCl₄ to hepatocytes may be exerted to a significant degree as a consequence of the injury to the endoplasmic reticulum resulting from the peroxidative breakdown of lipids in that membrane.

The sequence of early radical events occurring during CCl₄ metabolism in vivo as envisioned in this report is consistent with the following: CCl₄ enters the hepatocyte, diffuses to the endoplasmic reticulum and is reductively cleaved to CCl₃ radicals by an NADPH-dependent cytochrome P-450 system requiring the 52,000-Da form of this group of heme proteins (3). Presumably, CCl₄ radicals are produced at a locus vicinal to unsaturated phospholipids and addition to the double bonds can occur. Also, CCl₃ radicals react with O₂ to form Cl₃COO⁻. Peroxidative degradation of the lipids is also initiated by reaction of the CCl₃ and Cl₂COO⁻ radicals with these lipids as indicated previously. The lipid radicals then react with oxygen to form peroxy radicals which initiate further peroxidation of other lipids via the oxygen radical:

\[ \text{LOO} + \text{H} \rightarrow \text{LOOH} + \text{L} \]  
\[ 2\text{LOO} \rightarrow \text{LO}_2 + \text{O}_2 \]  
\[ \text{LOOH} + \gamma \text{ (reduction)} \rightarrow \text{LO} + \text{OH}^- \]

Any of the L⁻, LO⁻, and LOOH radicals can, in principle, be trapped by PBN, but the LOO-PBN adduct is very unstable and may degrade to LO-PBN.

The data indicate that CCl₃ must react with the microsomal lipid extensively before beginning to react with PBN since, as described above, the CCl₃ radical adduct is not observed during the initial stages of the reaction while lipid radicals are observed. The latter adduct formation could only be expected in domains where oxygen is essentially depleted. Both PBN and (MO)₃PBN are sufficiently lipid-soluble to provide for trapping of the lipid radicals so that the availability of the spin-trapping agent to the CCl₃-forming site seems likely. Hence, oxygen and lipid (and probably other organic membrane components as well) must be competing with PBN for CCl₃ radicals during the early phase of the reaction. The reaction at this time appears to be occurring in highly localized sites in the endoplasmic reticulum since initially there is a very rapid, specific polymerization of the 52,000-Da cytochrome P-450 which appears to be the primary heme protein associated with the production of the Cl₂C₃ radical in liver tissue (29). Since free radical scavengers provide a significant degree of protection against the lipid peroxidation and the membrane damage caused by CCl₄ to animals (27), it is reasonable to conclude that the free radical events under observation are causally related to the injury process. It has been claimed that lipid peroxidation in the hepatic endoplasmic reticulum was not a consistent feature of CCl₄-induced liver damage because mice, which are very susceptible to CCl₄ poisoning, did not show evidence that lipid peroxidation occurred after CCl₄ administration (30). However, it has now been demonstrated that the mouse responds to CCl₄ treatment with an early peroxidation of lipids which is more marked than that occurring in the rat (31). Unpublished observations in our laboratory have also revealed that the chicken, which is very resistant to CCl₄ toxicity (32), produces only an extremely weak CCl₄ EPR signal in the liver following CCl₄ treatment. CCl₄ treatment in the chicken also causes little or no lipid peroxidation in vivo (33). Hence, there is a correlation between CCl₄ production and lipid peroxidation in the liver with the hepatotoxicity of CCl₄ that suggests a causal relationship, but further investigation will be required to demonstrate an obligatory link between production of the radicals which have been observed in vivo and the subsequent liver damage.

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