The Formation of a Novel Free Radical Metabolite from CCl₄ in the Perfused Rat Liver and in Vivo*

Henry D. Connor†, Ronald G. Thurman§, Marlene D. Galiziğ, and Ronald P. Mason†

From the †Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 and the §Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Electron spin resonance spectroscopy has been used to monitor free radicals formed during CCl₄ metabolism by perfused livers from phenobarbital-treated rats. Livers were perfused simultaneously with the spin trap phenyl N-t-butyl nitrotrone and with either [¹²C]CCl₄ or [¹³C]CCl₄. Perfusate samples and CHCl₃:CH₂OH extracts of perfusate and liver samples were analyzed for phenyl N-t-butyl nitrotrone radical adducts of reactive free radicals. In the organic extracts, hyperfine coupling constants and ¹³C isotope effects observed in the ESR spectra indicated the presence of the radical adduct of the trichloromethyl radical. Surprisingly, an additional free radical signal about two orders of magnitude more intense than that of the phenyl N-t-butyl nitrotrone/CCl₃ radical adduct was observed in the aqueous liver perfusate. This adduct was also detected by ESR in rat urine 2 h after intragastric addition of spin trap and CCl₄. This radical adduct had hyperfine coupling constants and ¹³C isotope effects identical with the radical adduct of the carbon dioxide anion radical (CO₂). Analysis of the pH dependence of the coupling constants yielded a pKₐ for C₃ of 2.8 for the CO₂ radical adduct formed either in the perfused liver or chemically. Carbon tetrachloride is converted into CCl₃ by cytochrome P-450 through a reductive dehalogenation. The trichloromethyl free radical reacts with oxygen to form the trichloromethyl peroxy radical, CCl₂OO⁻, which may be converted into COCI and then trapped. This radical adduct would hydrolyze to the carboxylic acid form, which is detected spectroscopically. Alternatively, the carbon dioxide anion free radical could form through complete dechlorination and then react with the spin trap to give the CO₂ radical adduct directly.

CCl₄ is dehalogenated reductively to the trichloromethyl free radical by cytochrome P-450 (1). The PBN/CCl₃ radical adduct was identified in microsomal incubations containing NADPH, CCl₃, and PBN based on the similarity of its six-line ESR spectrum to that of the free radical formed by UV photolysis of a CCl₃ solution containing PBN (2). Pöpper et al. (3) studied the microsomal metabolism of CCl₃ using [¹³C]carbon tetrachloride. A 12-line PBN/¹³CCl₃ ESR spectrum appeared after a lag period (3), during which time a [¹³C]CCl₄ is dehalogenated reductively to the trichloromethyl free radical by cytochrome P-450 (1). The PBN/CCl₃ radical adduct was identified in microsomal incubations containing NADPH, CCl₃, and PBN based on the similarity of its six-line ESR spectrum to that of the free radical formed by UV photolysis of a CCl₃ solution containing PBN (2). Pöpper et al. (3) studied the microsomal metabolism of CCl₃ using [¹³C]carbon tetrachloride. A 12-line PBN/¹³CCl₃ ESR spectrum appeared after a lag period (3), during which time a [¹³C]CCl₄ was detected. The PBN/¹³CCl₃ radical adduct has also been formed in vivo and detected in liver extracts of rats given [¹³C]CCl₄ and PBN orally (3, 6). The failure to detect the PBN/CCl₃OO⁻ radical adduct under aerobic conditions, despite the high rate constant for the reaction of CCI₃ with O₂, has been attributed to the high reactivity of CCl₃ with the instability of the PBN-peroxyl adduct (5).

We have examined rat urine and effluent perfusate from rat liver for PBN adducts formed during CCl₄ metabolism. In addition to detecting the trichloromethyl radical in the liver and in extracts of effluent perfusate, a novel free radical metabolite of carbon tetrachloride was discovered in the effluent perfusate. This free radical intermediate is a product of the reaction of CCI₃ with oxygen.

MATERIALS AND METHODS

PBN, sodium formate, ferrous sulfate, bovine serum albumin, catalase, and ascorbate oxidase were purchased from Sigma and were used without modification. Hydrogen peroxide (30%) (American Chemical Society certified) and carbon tetrachloride (analytical reagent grade) were from Fisher. [¹³C]Sodium formate and [¹³C]carbon tetrachloride were from MSD Isotopes.

Female Sprague-Dawley rats (Zivic-Miller, 250–350 g) were treated with sodium phenobarbital (1 mg/ml) in drinking water for 5 days to induce cytochrome P-450 prior to perfusion experiments. Livers were perfused with Krebs-Henseleit bicarbonate buffer (pH 7.6, 37 °C) saturated with O₂:CO₂ in a non-recirculating system as described previously (7). The buffer was pumped into the liver via a cannula placed in the portal vein and out of the liver via a cannula in the inferior vena cava. The effluent perfusate flowed past a Teflon-shielded, Clark-type O₂ electrode and was collected in polyethylene bottles for ESR analysis. PBN (10 mM) was dissolved in the perfusate and carbon tetra-chloride was bound to albumin by stirring with a 22.5% aqueous albumin solution for 16 h.

Livers samples (7 g) were homogenized in perfusion buffer (30 ml) and extracted with 30 ml of CHCl₃:CH₂OH (2:1) solution. The mixture was centrifuged for 10 min at 2500 rpm. The organic layer was removed, dried with anhydrous sodium sulfate, and placed in a Pyrex sample container with a 6-mm outside diameter side arm for ESR analysis. Samples were degassed using standard vacuum techniques and stored in liquid nitrogen until ESR analysis. The aqueous layer of the extraction was bubbled with oxygen for 10 min and then with nitrogen for 5 min prior to ESR analysis. Aqueous perfusate samples were treated in a similar manner. Effluent perfusate was extracted using the same procedure except that 400 ml of perfusate was extracted with 15 ml of CHCl₃:CH₂OH (2:1).

For the analysis of PBN adducts in urine, rats were fasted for 24 h. Then they were given 0.5 ml of 0.05 M PBN solution and 0.3 ml of 2.3 M CCl₄ in corn oil intragastrically three times at 1.5-h intervals.

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¹The abbreviation used is: PBN, phenyl N-t-butyl nitrotrone, with the IUPAC name N-tet-butyl-o-phenylnitrotrone.

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About 2 h after the last dose, rat urine was collected in a Petri dish and was washed into a small ampoule with an equal volume of the perfusion buffer. The urine sample was transferred to an ESR flat cell, and 4 µl containing 1 unit of ascorbate oxidase and 4.7 µl containing 1 unit of catalase were added. The solution was then bubbled with oxygen for 10 min, followed by nitrogen for 5 min.

A hydroxyl radical-generating Fenton system containing ferrous sulfate, a hydroxyl radical scavenger, was used to independently synthesize the PBN/OH· radical adduct. The PBN/OH· radical adduct was prepared by the addition of 90 µl of a 0.36 mM ferrous sulfate solution to 5 ml of a pH 7.6 solution of perfusion buffer containing 3.3 mM hydrogen peroxide and 15 mM PBN. The PBN/OH· radical adduct was generated by the addition of [15C]- or [13C]sodium formate (100 mM) to perfusion buffer prior to the addition of ferrous sulfate.

In the study of the pH dependence of the hyperfine couplings of the PBN/13CO2 radical adduct, 200 µl of the solution described above were added to 3 ml of perfusion buffer which was pH-adjusted by the addition of 1 N HCl or NaOH. After mixing thoroughly, 2 ml of the sample was aspirated into the ESR cavity and its spectrum was recorded within 2 min. The final pH of the remaining portion of the solution was measured after the ESR spectrum was recorded. In the corresponding analysis of the radical from the liver perfusate, the portion of perfusate with the highest free radical concentration was identified first. The ESR spectrum and pH value of a mixture of 200 µl of this solution and 3 ml of pH-adjusted perfusate were then obtained.

The ESR spectra were obtained using a Varian E-109 spectrometer operating at 9.5 GHz with a 100-kHz modulation frequency. Aqueous samples were aspirated into a quartz flat cell centered in an E-238 TM.10c microwave cavity. Organic solutions were transferred to the ESR sample tube side arm which was then centered in an E-331 TD30c microwave cavity for analysis. Some preliminary experiments and the pH determination of the PBN/CO2 radical adduct were conducted using an IBM ER-200 ESR spectrometer operating at 9.7 GHz with a 100-kHz modulation frequency and equipped with an ER-4103 TM microwave cavity. The simulations of ESR spectra were performed on a Hewlett-Packard HP 9835B computer equipped with a Varian data acquisition system.

RESULTS

PBN Radical Adducts in Liver Extracts—Introduction of oxygenated perfusate containing PBN (10 mM) into a perfused liver resulted in an increase in oxygen uptake of about 15%, possibly due to the partial mixed-function oxidation of the spin trap (Fig. 1). The subsequent infusion of CCl4 (1 mM) produced a small increase followed by a progressive decrease in O2 uptake for the next 30 min of perfusion (Fig. 1).

As in previous studies of CCl4 metabolism (3, 6), ESR spectra were taken of organic extracts of the liver after perfusion with CCl4 and PBN. Experiments utilizing 14CCl4 produced a stable six-line ESR spectrum due to the PBN radical adduct of the trichloromethyl radical characterized by its hyperfine coupling constants of aH = 14.45 G and aC = 1.85 G (Fig. 2A). Confirmation of this spectral assignment was provided by the 12-line ESR spectrum obtained from the organic extract of a liver into which 13CCl4 was infused. This spectrum exhibited an additional hyperfine coupling of 9.20 G attributable to the nuclear spin of 13C (Fig. 2B).

ESR analysis of the aqueous layer in the extract of a liver exposed to 14CCl4 yielded a stable six-line spectrum (aH = 15.8 G and aC = 4.6 G) (Fig. 2C) which was not similar to PBN/CCl4. Following infusion of 13CCl4, the corresponding ESR spectrum yielded a 12-line spectrum where 2 lines are nearly superimposed (Fig. 2D) with an additional hyperfine coupling from 13C (aC' = 11.7 G). There was no evidence of this new radical in the organic phase.

PBN Radical Adducts in Effluent Perfusion—Direct ESR analysis of aqueous perfusate also yielded the spectra attributable to the new radical adduct (Fig. 3, A and B). The ESR spectrum from perfusate increased in intensity for several hours. Samples bubbled with oxygen for 10 min and then with nitrogen for 5 min exhibited a stable ESR signal that was identical with the spectrum of untreated samples allowed to sit at room temperature for several hours. Oxygen could either oxidize nitroxide-reducing species such as ascorbate or the sulfhydryl groups of proteins or oxidize the hydroxylamine form of the radical adduct directly to the nitroxide form (Equation 1).

\[ \text{[1]} \]

Subsequent bubbling with nitrogen decreased the concentration of dissolved oxygen to levels below which ESR line-broadening was not significant.

The amount of PBN/CCl4 radical adduct in the perfusate was below the sensitivity limits of the ESR spectrometer; therefore, it was concentrated by extraction. The organic layers from extractions with organic:aqueous ratios of 1:40 produced ESR spectra that were composites of spectra from three free radical species (Figs. 4A and 5A). The ESR spectrum from the effluent perfusate of a liver exposed to 14CCl4 clearly indicated the presence of the PBN/13CCl4 radical adduct. Extracts of control samples collected during perfusion with PBN prior to CCl4 exposure produced a distorted six-line spectrum most likely due to di-t-butyl nitroxide and an unassigned free radical adduct of PBN. This latter weaker spectrum, also produced by the addition of PBN (10 mM) to perfusate followed by extraction, is probably due to an impurity and is not a radical adduct. These impurity spectra changed slightly in time, possibly due to exposure to fluorescent light and the high potential for radical adduct formation due to the high PBN concentration (approximately 0.4 M if all PBN was extracted into the organic phase). Computer simulation was necessary to resolve the three contributing spectra in the composite spectrum from the organic extract. After a satisfactory simulation of the spectrum from the experiment involving 13CCl4, was obtained (Fig. 6B), the 13C coupling was deleted and relative amplitudes were adjusted slightly to yield a composite spectrum (Fig. 4B) which closely matched the ESR spectrum of the extract from the effluent
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Fig. 2. ESR spectra of liver extracts. A, spectrum of organic extract of liver after perfusion with PBN and CCl₄. Spectrometer settings were: scan range, 80 G; modulation amplitude, 1.6 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 8 s. B, spectrum of organic extract of liver after perfusion with PBN and ¹³CCl₄. Spectrometer settings were the same as in A (V = ascorbate semidione radical spectrum). C, spectrum of aqueous extract of liver after perfusion with PBN and CCl₄. Spectrometer settings were the same as in A except modulation amplitude was 0.53 G (V = ascorbate semidione radical spectrum). D, spectrum of aqueous extract of liver after perfusion with PBN and ¹³CCl₄. Spectrometer settings were the same as in C (V = ascorbate semidione radical spectrum).

Fig. 3. ESR spectra of effluent perfusate. A, spectrum of effluent perfusate from a liver perfused with PBN (10 mM) and CCl₄ (1 mM). Spectrometer settings were: scan range, 80 G; modulation amplitude, 0.53 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 8 s (V = ascorbate semidione radical spectrum). B, spectrum of effluent perfusate from a liver perfused with PBN and ¹³CCl₄. Spectrometer settings were the same as in A (V = ascorbate semidione radical spectrum). In the future, studies of CCl₄ metabolism involving ESR analysis of perfusate extracts will require the use of ¹³CCl₄ to minimize difficulties associated with these impurity species.

In Vitro Preparation of PBN/CO₂ Radical Adduct—A Fenton system containing formate was used to generate the PBN/CO₂ radical adduct independently (Fig. 6). The hydroxyl radical produced from the reaction of ferrous ion with hydrogen peroxide (Equation 2) abstracted the hydrogen atom from the formate ion (Equation 3) producing the carbon dioxide anion free radical which was trapped by PBN (Equation 4).

\[
\begin{align*}
\text{Fe}^{++} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{+++} + \text{OH}^- + \cdot \text{OH} \\
\text{H} - \text{C} - \text{O}^- + \cdot \text{OH} & \rightarrow \text{C} - \text{O}^- + \text{H}_2\text{O} \\
\end{align*}
\]

\[
\begin{align*}
\text{C} - \text{O}^- + \text{C} - \text{C} - \text{N} - \text{CICH}_3 & \rightarrow \text{CO}_2^- \\
\text{H} - \text{O}^- & \rightarrow \text{C} - \text{N} - \text{CICH}_3 \\
\end{align*}
\]

Reasonably concentrated solutions (1 mM) of the PBN/CO₂ radical adduct could be formed which were stable for several hours at 0 °C. The ESR hyperfine coupling constants for these PBN/CO₂ radical adducts (a₁ ≈ 15.8 G, a² ≈ 4.6 G, a³ ≈ 11.7 G) were identical with those obtained from aqueous perfusate.

The pH dependence of the ESR hyperfine couplings for the PBN/CO₂ radical adduct was measured (Fig. 7). Identifica-
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FIG. 4. ESR spectrum of organic extract of effluent perfusate. A, spectrum of organic extract of effluent perfusate from a liver perfused with PBN and CC₁₄. Spectrometer settings were: scan range, 80 G; modulation amplitude, 1.0 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 8 s. B, composite of the simulated spectra for PBN/CC₁₄ radical adduct, di-t-butyl nitroxide, and unassigned PBN radical adduct. C, simulation for the PBN/CC₁₄ radical adduct, di-t-butyl nitroxide, and unassigned PBN radical adduct. Parameters used were: PBN/CC₁₄ radical adduct, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_m = 0.6$ G, $a^G = 14.65$ G, and $a^L = 1.85$ G; di-t-butyl nitroxide, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_m = 0.4$ G and $a^G = 16.10$ G; unassigned PBN radical adduct, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_m = 0.9$ G, $a^G = 14.65$ G, and $a^L = 2.50$ G. The spectrum of the di-t-butyl nitroxide is shifted versus the other two spectra by +0.31 G.

FIG. 5. ESR spectrum of organic extract of effluent perfusate with $^{13}$CC₁₄. A, spectrum of organic extract of effluent perfusate from a liver perfused with PBN and $^{13}$CC₁₄. Spectrometer settings were: scan range, 80 G; modulation amplitude, 1.0 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 8 s. B, composite of the simulated spectra for PBN/$^{13}$CC₁₄ radical adduct, di-t-butyl nitroxide or unassigned PBN radical adduct spectra. C, simulation for the PBN/$^{13}$CC₁₄ radical adduct, di-t-butyl nitroxide, and unassigned PBN radical adduct. Parameters used were: PBN/$^{13}$CC₁₄ radical adduct, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_m = 0.6$ G, $a^G = 14.65$ G, and $a^L = 1.85$ G; di-t-butyl nitroxide, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_m = 0.4$ G and $a^G = 16.10$ G; unassigned PBN radical adduct, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_m = 0.9$ G, $a^G = 14.65$ G, and $a^L = 2.50$ G. The spectrum of the di-t-butyl nitroxide is shifted versus the other two spectra by +0.31 G.

Formation of the PBN/CO₂ Radical Adduct in Vivo—The absence of any mention of the PBN/CO₂ radical adduct in previous spin-trapping studies of CC₁₄ metabolism in vivo led to the conclusion that this species does not remain in the liver, the focus of previous work, but rather moves into the bloodstream and eventually is excreted in the urine. Indeed, the radical adduct was observed in rat urine collected 2 h after the rat had been treated with PBN and [¹³C]carbon tetrachloride (Fig. 8). Initial ESR spectra of urine samples exhibited strong ascorbate semidione free radical peaks which partially obscured the PBN/CO₂ radical adduct spectrum. Treatment of the sample with oxygen, ascorbate oxidase, and catalase to oxidize ascorbate to dehydroascorbate and to convert H₂O₂ into H₂O reduced the ascorbate free radical ESR peaks significantly (Fig. 8). The PBN/CO₂ radical adduct concentration was not increased by this procedure, indicating that the ascorbate semidione free radical does not significantly reduce this radical adduct in urine. The similarity of the ESR hyperfine couplings for the spectrum obtained from the rat urine...
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**DISCUSSION**

*Production of CCl₃ by Perfused Liver—*It is well established that CCl₃ is produced from the metabolism of CCl₄ by cytochrome P-450 (Equation 5) (1).

$$\text{CCl}_4 + e^{-} \rightarrow \text{CCl}_3 + \text{Cl}^- + \text{O}_2 \rightarrow \text{Cl}_3\text{COO}^-.$$

Physical evidence for the formation of the CCl₃ free radical in biological systems comes from studies of radical adduct formation (2–6). There is fair agreement between previously reported ESR hyperfine coupling constants for the PBN/CCl₃ radical adduct and the radical adduct observed in organic extracts of livers perfused with CCl₄ (Table 1). A substantial fraction of this species remained in the liver. To obtain measurable quantities of the PBN/CCl₃ radical adduct from the aqueous perfusate, it was necessary to use concentrating extractions. It is concluded that PBN/CCl₃ is formed from CCl₄ in the perfused liver as would be expected and is distributed based on its hydrophobicity.

**Evidence for a PBN Adduct of a Novel Radical Metabolite of CCl₄—**Using pulse radiolysis, the trichloromethyl radical has been shown to react with O₂ to form the Cl₃COO⁻ peroxyl...
was obtained in effluent perfusate from livers perfused with 
posed to CC1,. Indeed, a unique ESR signal with coupling 
the CC1, radical adduct. Proof that  this free radical arises 
was exposed to 13CCL (Fig.

Free radicals are produced in perfused livers ex-
consumption increased 15-20 pmol/g/h (11). An increase in 
direct evidence for this peroxyl radical from ESR spectroscopy 
is lacking. When CC1, was added to  the perfused liver, 
able to assume that  the  c1,cOo' radical is formed; however,

The ESR hyperfine coupling constants for the radical ad-

| Source                  | Structure      | pH | Hyperfine splittings (Gauss) | Source       |
|-------------------------|----------------|----|------------------------------|--------------|
| Organic extract         | PBN/13CC14     |    | a_N 18.5 14.45 9.20         | Fig. 2       |
| 13CCL liver perfusion   | PBN/13CC14     |    |                             |              |
| Microsomal system       | PBN/13CC14     |    |                             |              |
| metabolizing            | PBN/13CC14     |    |                             |              |
| Rat hepatocytes         | PBN/13CC14     |    |                             |              |
| metabolizing            | PBN/13CC14     |    |                             |              |
| Effluent perfusate      | PBN/13CO2      |    | a_N 4.6 15.8 11.7           | Fig. 3       |
| 13CCL liver perfusion   | PBN/13CO2      |    |                             |              |
| Fenton system           | PBN/13CO2      | 6.9| a_N 4.6 15.8 11.7           | Fig. 6       |
| containing H13COONa     | PBN/13CO2      |    |                             |              |
| Photochemical system    | PBN/13CO2      | 6.0| a_N 4.5 16.0 11.8           | Fig. 8       |
| Urine                   | PBN/13CO2      |    |                             |              |

Thus, the reaction of phosgene or a phosgene-derived free radical with PBN to form the PBN/CO2 radical adduct was considered. To evaluate this possibility, an experiment was undertaken using chloroform, which is metabolized to phos- 
gene over 8 times faster than CCl4 (9). However, no PBN/ 
CO2 radical adduct was observed in the effluent perfusate of 
lepers exposed to CHCl3; therefore, the involvement of phos-
gene is unlikely (data not shown). Similarly, infusion of 
formate failed to produce the PBN/CO2 radical adduct, in-
dicating that either this radical is not formed from formate 
produced from CCl4 metabolism or formate is not absorbed. 
In experiments with 13C-labeled CCl4, the PBN/CO2 radical adduct was not diluted by any 13C carbon source, such as carbon dioxide. Taken together, these data indicate that the PBN/CO2 radical does not arise from chloroform, phosgene, formate, or carbon dioxide. The possible dechlorination of PBN/CCl3 to form PBN/CO2 is unlikely because PBN/ 
CO2 appears immediately in the perfusate and its concentra-
tion does not increase with time, whereas PBN/CCl3 accumu-
lates in the liver and would provide an increasing source of PBN/CO2.

The reaction sequence most likely responsible for PBN/ 
CO2 radical adduct formation involves the trichloromethyl 
peroxyl radical (CCl3OO') (Equation 5). The trichloromethyl 
peroxyl radical is converted to the trichloromethoxy radical 
(CCl3O') by a two-electron reduction followed by protonation 
and elimination of a water molecule (Equation 7). The 
trichloromethoxy radical then reacts with hydroxide ion to pro-
duce the chlorocarbonyl radical COCl, chloride ion, and a 
molecule of hypochlorous acid (Equation 7).

Previous observations of a radical adduct of the chlorocar-
bonyl radical from the photolysis of CCl4 provide additional 
support that this species reacts with PBN (13, Equation 8).
Upon contact with water, the PBN chlorocarbonyl radical adduct would hydrolyze to give the PBN/CO$_3^-$ radical adduct (Equation 8). It is also possible that the CO$_3^-$ free radical is generated directly from hydrolysis of the chlorocarbonyl radical (Equation 9).

The carbon dioxide anion radical then reacts with PBN to form the PBN/CO$_3^-$ radical adduct (Equation 9). The CO$_3^-$ radical is known to reduce oxygen to superoxide with a nearly diffusion-limited rate, $2.4 \times 10^{9}$ M$^{-1}$s$^{-1}$ (14). This reaction forms carbon dioxide, the final product of CCl$_4$ metabolism (10) and superoxide.

The PBN/O$_3^-$ radical adduct ($a^{n}_N = 14.8$ G and $a^{l}_l = 2.75$ G) was not observed under any conditions. Apparently the abundant hepatic superoxide dismutase, which can totally suppress the formation of this radical adduct (15), disproportionated superoxide before it could be trapped in detectable concentrations. If PBN/CO$_3^-$ is formed by the trapping of CO$_3^-$, then PBN must compete with O$_2$ with its 2.4 $\times 10^9$ M$^{-1}$s$^{-1}$ rate constant (14). Apparently, this is possible only because, at the site of reaction, the concentration of PBN is much greater than that of O$_2$. It is noteworthy that CCl$_4$ is trapped by PBN in spite of the $3.3 \times 10^9$ M$^{-1}$s$^{-1}$ rate constant for its reaction with oxygen (16).

Scheme 1 summarizes Equations 6–10. Although PBN/CO$_3^-$ is clearly CCl$_4$-derived and presumably CClOO$^-$-derived, other aspects of the proposed mechanisms(s) are speculative. The mechanism(s) by which CCl$_4$ loses its three chlorine atoms to form PBN/CO$_3^-$ can only be suggested at this time (16).

**Rate of PBN/CO$_3^-$ in Vivo**—This is the first report of detection of the adduct of a free radical metabolite in a body fluid of a living whole animal. Because of the charged nature of the PBN/CO$_3^-$ radical adduct, we predicted that it should be filtered by the kidney and appear in the urine. Indeed, after treatment of a rat with PBN and $^{13}$CCl$_4$, an ESR spectrum identical with that characteristic of the PBN/CO$_3^-$ was observed (Fig. 8). It was identical with the spectrum of the PBN/CO$_3^-$ radical adduct generated in vitro or detected in the effluent perfusate. Thus, the PBN/CO$_3^-$ radical adduct is indeed formed in vivo. Although phosgene is presently thought to be the precursor to carbon dioxide formed in vivo from CCl$_4$ (Scheme 1), the near diffusion-limited rate of air oxidation of the carbon dioxide anion radical is consistent with at least some of the carbon dioxide formed in vivo being the result of this alternate route (Scheme 1).

The formation of the PBN/CO$_3^-$ radical adduct in the perfused liver presumably arises from CCl$_4$OO$^-$ peroxyl radical formation. The direct evidence for CCl$_4$OO$^-$ formation consists of in vitro kinetic (8, 16) and ESR (17) studies of irradiated CCl$_4$. Although the metabolism of CCl$_4$ to phosgene is thought to occur via CCl$_4$OO$^-$, a non-free radical pathway is also possible. In view of these limitations and until the characterization of factors which influence PBN/CO$_3^-$ formation may give insight into CCl$_4$OO$^-$ formation in liver.

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**REFERENCES**

1. Noguchi, T., Fong, K.-L., Lui, E. K., Alexander, S. S., King, M. M., Olson, L., Poyer, J. L., and McCoy, P. B. (1982) Biochem. Pharmacol. 31, 615–624.
2. Poyer, J. L., Floyd, R. A., McCoy, P. B., Janzen, E. G., and Davis, E. R. (1978) Biochim. Biophys. Acta 539, 402–409.
3. Poyer, J. L., McCoy, P. B., Lai, E. K., Janzen, E. G., and Davis, E. R. (1980) Biochem. Biophys. Res. Commun. 94, 1134–1160.
4. McCoy, P. B., Lai, E. K., Poyer, J. L., DuBose, C. M., and Janzen, E. G. (1984) J. Biol. Chem. 259, 2135–2143.
5. Tomasi, A., Albano, E., Lott, K. A. K., and Slater, T. F. (1980) FEBS Lett. 122, 303–306.
6. Albano, E., Lott, K. A. K., Slater, T. F., Stier, A., Symons, M. C. R., and Tomasi, A. (1982) Biochem. J. 204, 593–603.
7. Scholz, R., Hansen, W., and Thurman, R. G. (1973) Eur. J. Biochem. 38, 64–72.
8. Packer, J. E., Slater, T. F., and Willson, R. L. (1978) Life Sci. 23, 2517–2630.
9. Kubic, V. L., and Anders, M. W. (1980) Life Sci. 26, 2151–2155.
10. Paul, B. B., and Rubinstein, D. (1963) J. Pharmacol. Exp. Ther. 141, 141–148.
11. Thurman, R. G., Christenson, V., and Kauffman, F. C. (1984) Toxicologist 4, 43.
12. Aurian-Blajeni, B., Halmann, M., and Manassen, J. (1982) Photochem. Photobiol. 35, 157–162.
13. Hartgerink, J. W., Engberts, J. B. F. N., and de Boer, Th. (1971) Tetrahedron Lett. 2709–2712.
14. Adams, G. E., and Willson, R. L. (1969) Trans. Faraday Soc. 65, 2981–2987.
15. Kalyanaraman, B., Perez-Reyes, E., and Mason, R. P. (1980) Biochim. Biophys. Acta 630, 119–130.
16. Möött, J., Bahnemann, D., and Asmus, K.-D. (1983) Chem.-Biol. Interact. 47, 15–27.
17. Hesse, C., LeRay, N., and Roncin, J. (1971) Mol. Phys. 22, 137–145.