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The Formation of a Novel Free Radical Metabolite from CCl_4 in the Perfused Rat Liver and *in Vivo**

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Electron spin resonance spectroscopy has been used to monitor free radicals formed during CCl_4 metabolism by perfused livers from phenobarbital-treated rats. Livers were perfused simultaneously with the spin trap phenyl *N-t*-butylnitrone and with either $^{12}CCl_4$ or $^{13}CCl_4$. Perfusate samples and $CHCl_3$:CH_3OH extracts of perfusate and liver samples were analyzed for phenyl *N-t*-butylnitrone radical adducts of reactive free radicals. In the organic extracts, hyperfine coupling constants and ^{13}C 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*-butylnitrone/$CCl_3^\bullet$ 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 CCl_4. This radical adduct had hyperfine coupling constants and ^{13}C isotope effects identical with the radical adduct of the carbon dioxide anion radical (CO_2^-). Analysis of the pH dependence of the coupling constants yielded a pK_a of 2.8 for the CO_2^- radical adduct formed either in the perfused liver or chemically. Carbon tetrachloride is converted into $CCl_3^\bullet$ by cytochrome P-450 through a reductive dehalogenation. The trichloromethyl free radical reacts with oxygen to form the trichloromethyl peroxyl radical, $CCl_3OO^\bullet$, which may be converted into $\dot{C}OCl$ 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 CO_2^- radical adduct directly.

CCl_4 is dehalogenated reductively to the trichloromethyl free radical by cytochrome P-450 (1). The PBN[1]/$CCl_3^\bullet$ radical adduct was identified in microsomal incubations containing NADPH, CCl_4, and PBN based on the similarity of its six-line ESR spectrum to that of the free radical formed by UV photolysis of a CCl_4 solution containing PBN (2). Poyer *et al.* (3) studied the microsomal metabolism of CCl_4 using [^{13}C] carbon tetrachloride. A 12-line PBN/$^{13}CCl_3^\bullet$ ESR spectrum appeared after a lag period (3), during which time a ^{13}C invariant ESR spectrum was detected and eventually assigned to a PBN radical adduct of a lipid alkoxy radical (4).

Tomasi *et al.* (5) obtained similar results with extracts of microsomes or isolated hepatocytes incubated for 10 min with $^{13}CCl_4$ and PBN, either aerobically or anaerobically. Again, a 12-line ESR spectrum indicative of the PBN/$^{13}CCl_3^\bullet$ radical adduct was detected. The PBN/$^{13}CCl_3^\bullet$ radical adduct has also been formed *in vivo* and detected in liver extracts of rats given $^{13}CCl_4$ and PBN orally (3, 6). The failure to detect the PBN/$CCl_3OO^\bullet$ radical adduct under aerobic conditions, despite the high rate constant for the reaction of $CCl_3^\bullet$ with O_2, has been attributed to the high reactivity of $CCl_3OO^\bullet$ and the instability of the PBN-peroxyl adduct (5).

We have examined rat urine and effluent perfusate from rat liver for PBN adducts formed during CCl_4 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 $CCl_3^\bullet$ 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. [^{13}C]Sodium formate and [^{13}C]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 at least 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 O_2:CO_2 (95:5) 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 O_2 electrode and was collected in polyethylene bottles for ESR analysis. PBN (10 mM) was dissolved in the perfusate and carbon tetrachloride was bound to albumin by stirring with a 22.5% aqueous albumin solution for 16 h.

Liver samples (7 g) were homogenized in perfusion buffer (30 ml) and extracted with 30 ml of a $CHCl_3$:CH_3OH (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 3-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 $CHCl_3$:CH_3OH (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 2.2 M CCl_4 in corn oil intragastrically three times at ½-h intervals.

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[1] The abbreviation used is: PBN, phenyl *N-t*-butylnitrone, with the IUPAC name *N-tert*-butyl-α-phenylnitrone.

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 formate, a hydroxyl radical scavenger, was used to independently synthesize the PBN/CO_2^- radical adduct. The PBN/OH· radical adduct was prepared by the addition of 50 μl of a 0.36 M 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/CO_2^- radical adduct was generated by the addition of [^{12}C]- or [^{13}C]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/$^{13}CO_2^-$ 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_{110} microwave cavity. Organic solutions were transferred to the ESR sample tube side arm which was then centered in an E-231 TE_{102} microwave cavity for analysis. Some preliminary experiments and the pK_a determination of the PBN/CO_2^- 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 CCl_4 (1 mM) produced a small increase followed by a progressive decrease in O_2 uptake for the next 30 min of perfusion (Fig. 1).

As in previous studies of CCl_4 metabolism (3, 6), ESR spectra were taken of organic extracts of the liver after perfusion with CCl_4 and PBN. Experiments utilizing $^{12}CCl_4$ produced a stable six-line ESR spectrum due to the PBN radical adduct of the trichloromethyl radical characterized by its hyperfine coupling constants of a^N = 14.45 G and a_β^H = 1.85 G (Fig. 2*A*). Confirmation of this spectral assignment was provided by the 12-line ESR spectrum obtained from the organic extract of a liver into which $^{13}CCl_4$ was infused. This spectrum exhibited an additional hyperfine coupling of 9.20 G attributable to the nuclear spin of ^{13}C (Fig. 2*B*).

ESR analysis of the aqueous layer in the extract of a liver exposed to $^{12}CCl_4$ yielded a stable siz-line spectrum (a^N = 15.8 G and a_β^H = 4.6 G) (Fig. 2*C*) which was not similar to PBN/$CCl_3^·$. Following infusion of $^{13}CCl_4$, the corresponding ESR spectrum yielded a 12-line spectrum where 2 lines are nearly superimposed (Fig. 2*D*) with an additional hyperfine coupling from ^{13}C ($a_\beta^{C\text{-}13}$ = 11.7 G). There was no evidence of this new radical in the organic phase.

PBN Radical Adducts in Effluent Perfusate—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).

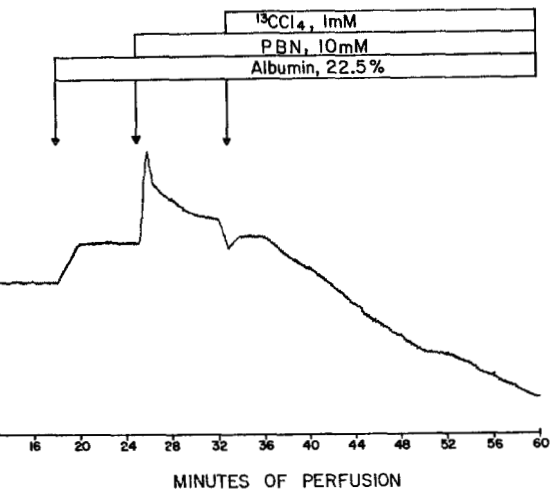
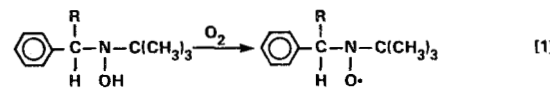


FIG. 1. **Effect of PBN and $^{13}CCl_4$ on O_2 uptake by the isolated, perfused liver.** Liver from a fed, phenobarbital-treated rat was perfused with Krebs-Henseleit bicarbonate buffer for times indicated. [O_2] was monitored continuously with a Clark-type O_2 electrode and values were converted into rates employing the influent-effluent concentration difference, the flow rate, and the liver wet weight. Additions are depicted by *horizontal bars* and *arrows*. Typical experiment.



[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/$CCl_3^·$ 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. 4*A* and 5*A*). The ESR spectrum from the effluent perfusate of a liver exposed to $^{13}CCl_4$ clearly indicated the presence of the PBN/$^{13}CCl_3^·$ radical adduct. Extracts of control samples collected during perfusion with PBN prior to CCl_4 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 weak 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 $^{13}CCl_4$ was obtained (Fig. 5*B*), the ^{13}C coupling was deleted and relative amplitudes were adjusted slightly to yield a composite spectrum (Fig. 4*B*) which closely matched the ESR spectrum of the extract from the effluent

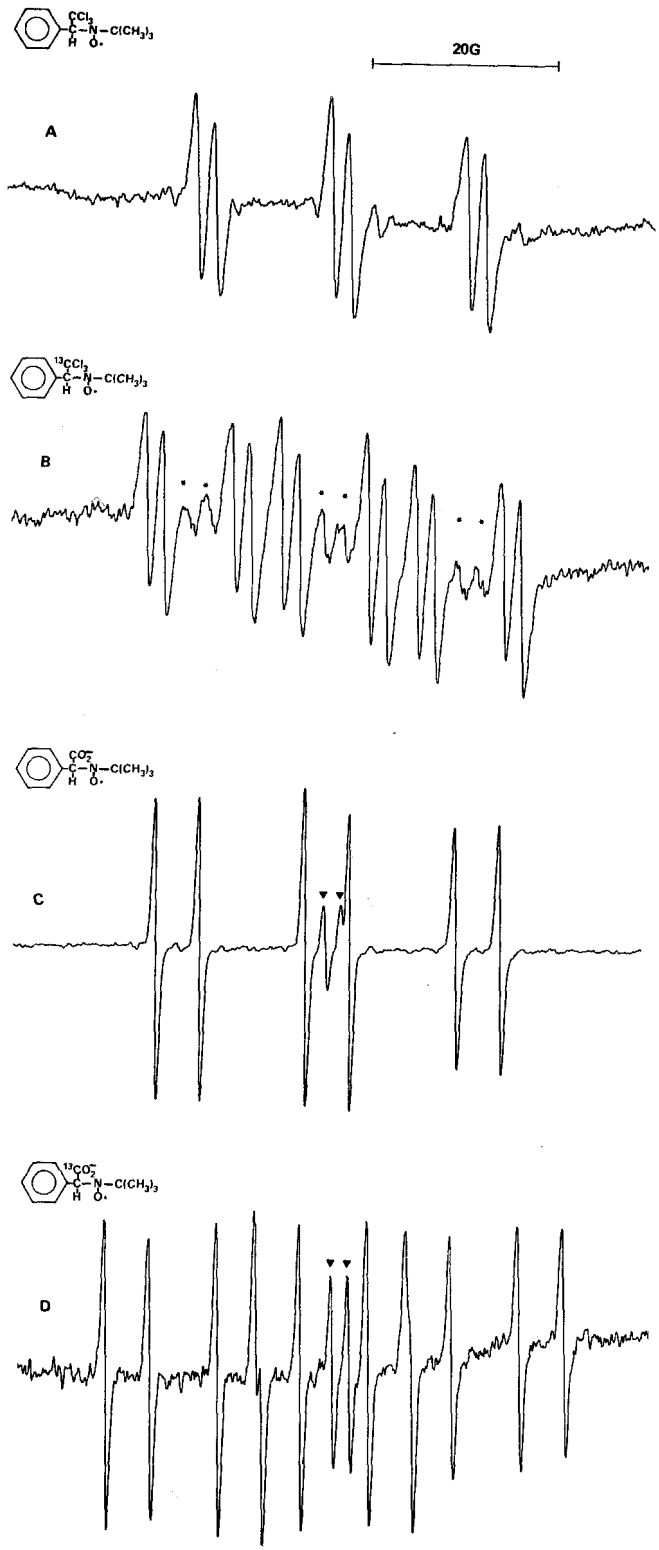


FIG. 2. **ESR spectra of liver extracts.** *A*, spectrum of organic extract of liver after perfusion with PBN and CCl_4. Spectrometer settings were: scan range, 80 G; modulation amplitude, 1.6 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 8 s. *B*, spectrum of organic extract of liver after perfusion with PBN and $^{13}CCl_4$. Spectrometer settings were the same as in *A* (● = predominately PBN/$^{12}CCl_3$ spectrum). *C*, spectrum of aqueous extract of liver after perfusion with PBN and CCl_4. Spectrometer settings were the same as in *A* except modulation amplitude was 0.53 G (▼ = ascorbate semidione radical spectrum). *D*, spectrum of aqueous extract of liver after perfusion with PBN and $^{13}CCl_4$. Spectrometer settings were the same as in *C* (▼ = ascorbate semidione radical spectrum).

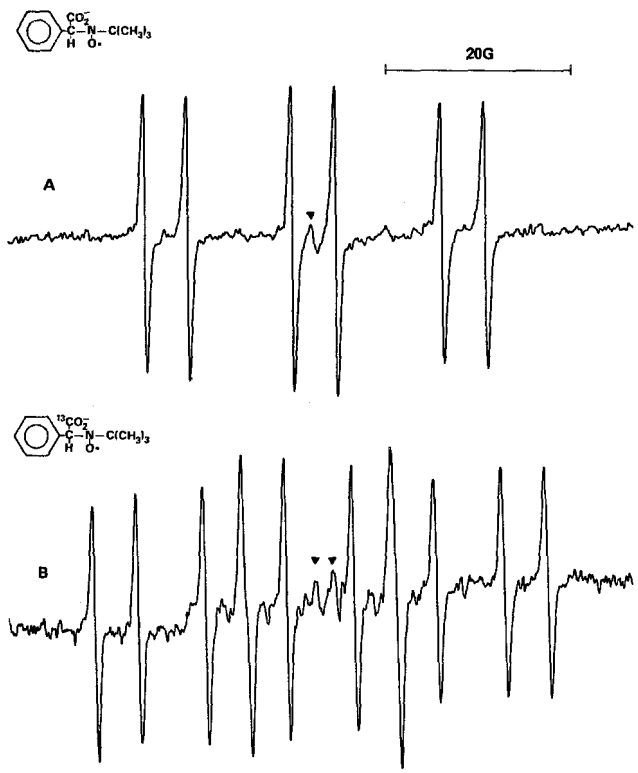


FIG. 3. **ESR spectra of effluent perfusate.** *A*, spectrum of effluent perfusate from a liver perfused with PBN (10 mM) and CCl_4 (1 mM). Spectrometer settings were: scan range, 80 G; modulation amplitude, 0.53 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 8 s (▼ = ascorbate semidione radical spectrum). *B*, spectrum of effluent perfusate from a liver perfused with PBN and $^{13}CCl_4$. Spectrometer settings were the same as in *A* (▼ = ascorbate semidione radical spectrum).

perfusate of a liver perfused with $^{12}CCl_4$ (Fig. 4*A*): In the future, studies of CCl_4 metabolism involving ESR analysis of perfusate extracts will require the use of $^{13}CCl_4$ to minimize difficulties associated with these impurity species.

In Vitro Preparation of PBN/CO_2^- Radical Adduct—A Fenton system containing formate was used to generate the PBN/CO_2^- 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).

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + OH^- + \cdot OH \quad [2]$$

$$H-\underset{\underset{O}{\|}}{C}-O^- + \cdot OH \longrightarrow \cdot\underset{\underset{O}{\|}}{C}-O^- + H_2O \quad [3]$$

$$\cdot\underset{\underset{O}{\|}}{C}-O^- + \text{PBN} \longrightarrow \text{PBN}/CO_2^- \quad [4]$$

Reasonably concentrated solutions (1 mM) of the PBN/CO_2^- radical adduct could be formed which were stable for several hours at 0 °C. The ESR hyperfine coupling constants for these PBN/CO_2^- radical adducts (a^N = 15.8 G, a_β^H = 4.6 G, $a_\beta^{C\text{-}13}$ = 11.7 G) were identical with those obtained from aqueous perfusate.

The pH dependence of the ESR hyperfine couplings for the PBN/CO_2^- radical adduct was measured (Fig. 7). Identifica-

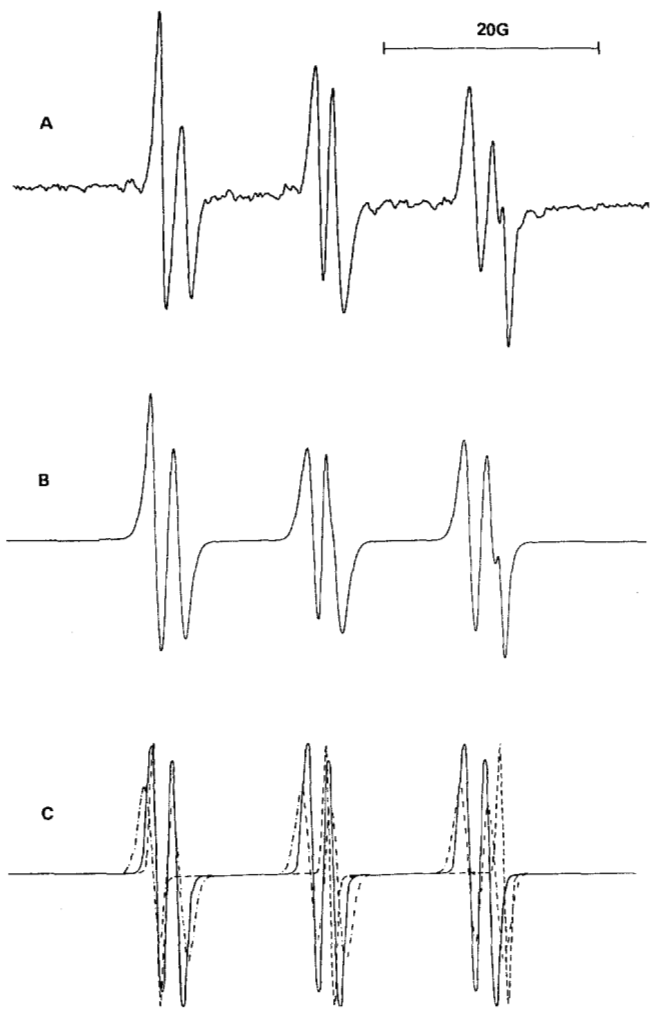


FIG. 4. **ESR spectrum of organic extract of effluent perfusate.** *A*, spectrum of organic extract of effluent perfusate from a liver perfused with PBN and CCl_4. Spectrometer settings were: scan range, 80 G; modulation amplitude, 1.0 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 8 s. *B*, composite of the simulated spectra for PBN/$CCl_3^{\cdot}$ radical adduct, di-*t*-butyl nitroxide, and unassigned PBN radical adduct. *C*, simulation for the PBN/$CCl_3^{\cdot}$- (——), di-*t*-butyl nitroxide (– – –), and unassigned PBN radical adduct (—·—·—). Parameters used were: PBN/$CCl_3^{\cdot}$, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_{1/2}$ = 0.6 G, a^N = 14.45 G, and a_β^H = 1.85 G; di-*t*-butyl nitroxide, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_{1/2}$ = 0.4 G and a^N = 16.10 G; unassigned PBN radical adduct, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_{1/2}$ = 0.9 G, a^N = 14.65 G, and a_β^H = 2.50 G. The spectrum of the di-*t*-butyl nitroxide is shifted *versus* the other two spectra by +0.31 G.

tion of the pH at the midpoint of the pH-sensitive region of the hyperfine couplings gave a pK_a value of 2.85 for this radical adduct produced from either the Fenton system or effluent perfusate (Fig. 7).

Formation of the PBN/$CO_2^{\cdot-}$ Radical Adduct in Vivo—The absence of any mention of the PBN/$CO_2^{\cdot-}$ radical adduct in previous spin-trapping studies of CCl_4 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 [^{13}C]carbon tetrachloride (Fig. 8). Initial ESR spectra of urine samples exhibited strong ascorbate semidione free radical peaks which partially obscured the PBN/$CO_2^{\cdot-}$ radical adduct spectrum. Treatment

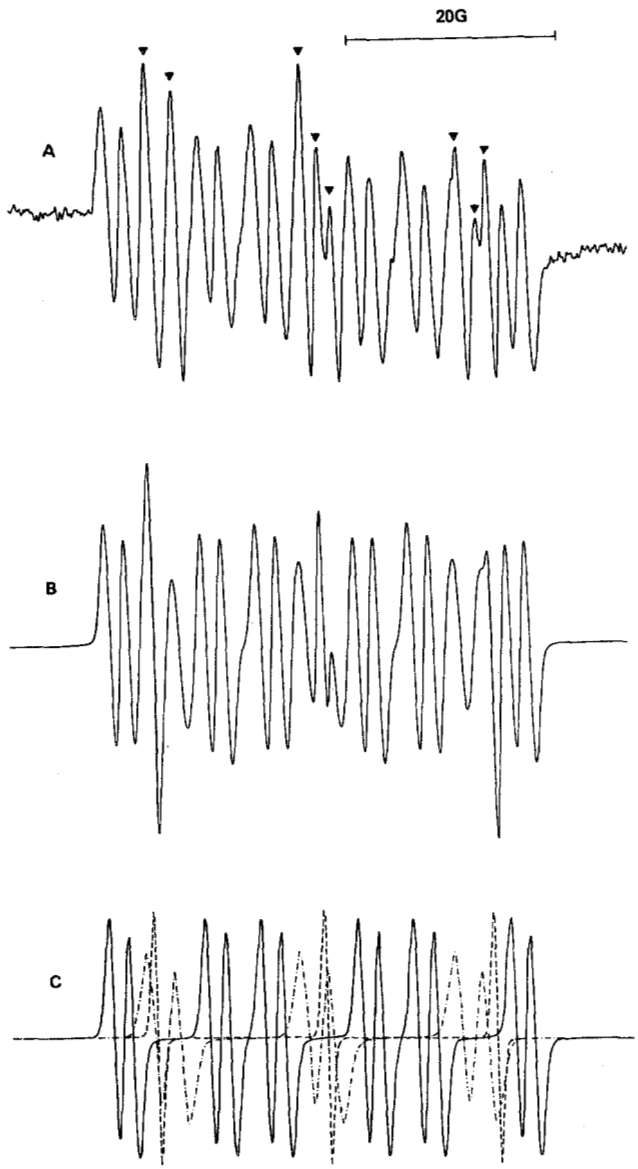


FIG. 5. **ESR spectrum of organic extract of effluent perfusate with $^{13}CCl_4$.** *A*, spectrum of organic extract of effluent perfusate from a liver perfused with PBN and $^{13}CCl_4$. Spectrometer settings were: scan range, 80 G; modulation amplitude, 1.0 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 8 s (▼ = di-*t*-butyl nitroxide or unassigned PBN radical adduct spectra). *B*, composite of the simulated spectra for PBN/$^{13}CCl_3^{\cdot}$ radical adduct, di-*t*-butyl nitroxide, and unassigned PBN radical adduct. *C*, simulation for the PBN/$^{13}CCl_3^{\cdot}$- (——), di-*t*-butyl nitroxide (– – –), and unassigned PBN radical adduct (—·—·—). Parameters used were: PBN/$^{13}CCl_3^{\cdot}$, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_{1/2}$ = 0.6 G, a^N = 14.45 G, a_β^H = 1.85 G, and $a_\beta^{C\text{-}13}$ = 9.2 G; di-*t*-butyl nitroxide, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_{1/2}$ = 0.4 G and a^N = 16.10 G; unassigned PBN radical adduct, 30% Lorentzian line shape and 70% Gaussian line shape with $\Delta H_{1/2}$ = 0.9 G, a^N = 14.65 G, and a_β^H = 2.50 G. The spectrum of the di-*t*-butyl nitroxide is shifted *versus* the other two spectra by +0.31 G.

of the sample with oxygen, ascorbate oxidase, and catalase to oxidize ascorbate to dehydroascorbate and to convert H_2O_2 into H_2O reduced the ascorbate free radical ESR peaks significantly (Fig. 8). The PBN/$CO_2^{\cdot-}$ 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

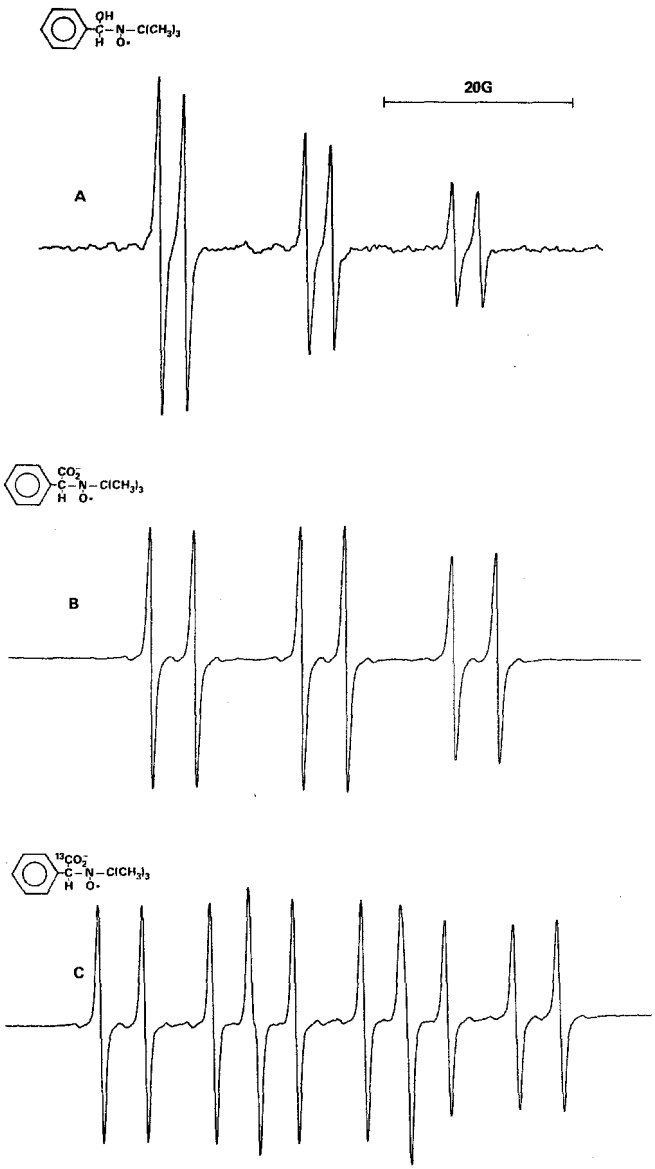


FIG. 6. **ESR spectra of radical adducts produced by the Fenton system.** *A*, spectrum of PBN/OH· radical adduct prepared as described under "Materials and Methods." Spectrometer settings were: scan range, 80 G; modulation amplitude, 0.33 G; microwave power, 20 milliwatts; scan time, 1 min; time constant, 0.064 s. *B*, spectrum of PBN/CO_2^- radical adduct prepared as described under "Materials and Methods." Spectrometer settings were: scan range, 80 G; modulation amplitude, 0.53 G; microwave power, 20 milliwatts; scan time, 16 min; time constant, 0.25 s. *C*, spectrum of PBN/$^{13}CO_2^-$ radical adduct prepared as described under "Materials and Methods." Spectrometer settings were the same as in *B*.

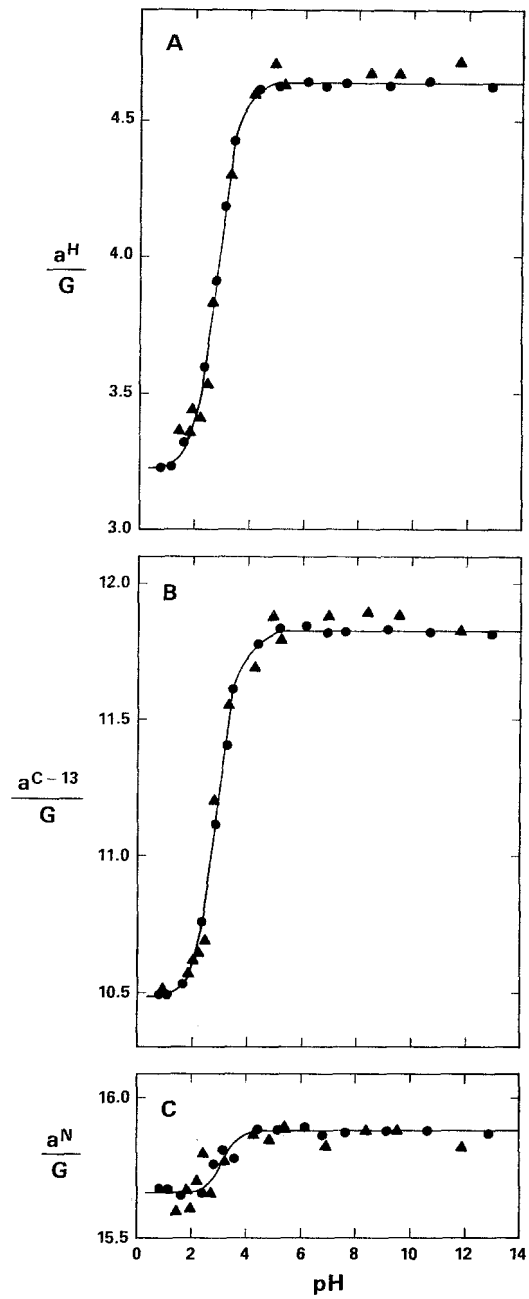


FIG. 7. **pH variation of ESR hyperfine coupling constants of PBN/$^{13}CO_2^-$.** *A*, pH variation of ESR hyperfine coupling for β-hydrogen of PBN/$^{13}CO_2^-$ radical adduct prepared as described under "Materials and Methods." *B*, pH variation of ESR hyperfine coupling for β-carbon-13 of PBN/$^{13}CO_2^-$ radical adduct. *C*, pH variation of ESR hyperfine coupling for nitrogen of PBN/CO_2^- radical adduct (▲ = liver perfusion, ● = Fenton system).

(Fig. 8, Table I) to those obtained from the effluent perfusate and the Fenton system justifies the assignment of the free radical in the urine as the PBN/CO_2^- radical adduct.

DISCUSSION

Production of $CCl_3^·$ by Perfused Liver—It is well established that $CCl_3^·$ is produced from the metabolism of CCl_4 by cytochrome P-450 (Equation 5) (1).

$$CCl_4 \xrightarrow[\text{P-450}]{+e^-} \cdot CCl_3 + Cl^- \xrightarrow{O_2} Cl_3COO\cdot \qquad [5]$$

Physical evidence for the formation of the $CCl_3^·$ 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/$CCl_3^·$ radical adduct and the radical adduct observed in organic extracts of livers perfused with CCl_4 (Table I). A substantial fraction of this species remained in the liver. To obtain measurable quantities of the PBN/$CCl_3^·$ radical adduct from the aqueous perfusate, it was necessary to use concentrating extractions. It is concluded that PBN/$CCl_3^·$ is formed from CCl_4 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 CCl_4—Using pulse radiolysis, the trichloromethyl radical has been shown to react with O_2 to form the $Cl_3COO·$ peroxyl

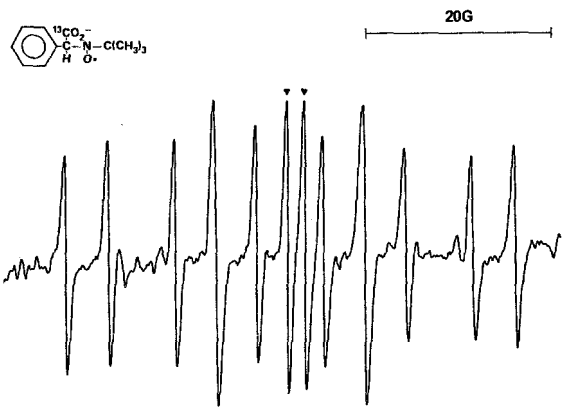


FIG. 8. **ESR spectrum of rat urine.** Spectrum of urine collected from rat 2 h after treatment with PBN and $^{13}CCl_4$. Ascorbate free radical spectrum (▼) was decreased by addition of ascorbate oxidase and bubbling with oxygen (details are under "Materials and Methods"). Spectrometer settings were: scan range, 80 G; modulation amplitude, 0.53 G; microwave power, 20 milliwatts; scan time, 2 h; time constant, 16 s.

TABLE I

Hyperfine coupling constants of carbon tetrachloride-derived radical adducts

Source	Structure	pH	Hyperfine splittings (Gauss) a_β^H	a^N	$a_\beta^{C\text{-}13}$	Source
Organic extract $^{13}CCl_4$ liver perfusion	PBN/$^{13}CCl_3^{\cdot}$		1.85	14.45	9.20	Fig. 2
Microsomal system metabolizing $^{13}CCl_4$	PBN/$^{13}CCl_3^{\cdot}$		1.5	13.9	9.5	Ref. 4
Rat hepatocytes metabolizing $^{13}CCl_4$	PBN/$^{13}CCl_3^{\cdot}$		1.75	14.0	9.7	Ref. 6
Effluent perfusate $^{13}CCl_4$ liver perfusion	PBN/$^{13}CO_2^{\cdot-}$		4.6	15.8	11.7	Fig. 3
Fenton system containing H^{13}COONa	PBN/$^{13}CO_2^{\cdot-}$	6.9	4.6	15.8	11.7	Fig. 6
Photochemical system	PBN/$CO_2^{\cdot-}$		4.6	15.9		Ref. 12
Urine	PBN/$^{13}CO_2^{\cdot-}$	6.0	4.5	16.0	11.8	Fig. 8

free radical (8, Equation 5). Since CCl_4 is metabolized to phosgene (9) and CO_2 (10) in biological systems, it is reasonable to assume that the $Cl_3COO^{\cdot}$ radical is formed; however, direct evidence for this peroxyl radical from ESR spectroscopy is lacking. When CCl_4 was added to the perfused liver, O_2 consumption increased 15–20 μmol/g/h (11). An increase in O_2 uptake of approximately 10 μmol/g/h was observed in the presence of PBN (Fig. 1). Thus, it is possible that oxygen-containing free radicals are produced in perfused livers exposed to CCl_4. Indeed, a unique ESR signal with coupling constants distinctly different from the $CCl_3^{\cdot}$ radical adduct was obtained in effluent perfusate from livers perfused with CCl_4. Moreover, the species in the aqueous perfusate was between one and two orders of magnitude more intense than the $CCl_3^{\cdot}$ radical adduct. Proof that this free radical arises from CCl_4 metabolism comes from the observation of an additional hyperfine coupling in the ESR spectra when a liver was exposed to $^{13}CCl_4$ (Fig. 3).

The ESR hyperfine coupling constants for the radical adduct (Table I) in effluent perfusate correspond closely to values reported for the PBN/$CO_2^{\cdot-}$ radical adduct generated photochemically (12) (Table I). Furthermore, the radical detected in the effluent perfusate had hyperfine couplings identical with those of the PBN/$CO_2^{\cdot-}$ radical adduct produced *in vitro* from a Fenton system containing formate (Fig. 6). It is concluded, therefore, that this new species is the PBN/$CO_2^{\cdot-}$ radical adduct. This conclusion was supported by studies of the effect of pH on the ESR hyperfine coupling constants. A pK_a of 2.85 was obtained when the PBN/$CO_2^{\cdot-}$ radical adduct was generated either by the perfused liver or the Fenton system (Fig. 7).

Most previous ESR studies utilized organic extracts of tissue or hydrophobic membrane preparations to study radicals formed during CCl_4 metabolism. Since the pK_a of the PBN/$CO_2^{\cdot-}$ radical is less than 3, it is nearly completely ionized at physiological pH. Therefore, since this species is charged, it does not appear in organic extracts (Figs. 4 and 5), but is observed in the effluent perfusate (Fig. 3). This ionic character may explain, in part, why the PBN/$CO_2^{\cdot-}$ radical adduct has been overlooked in past studies.

Pathways Responsible for the Formation of the PBN/$CO_2^{\cdot-}$ Radical Adduct in Perfused Liver—It is possible that the PBN/$CO_2^{\cdot-}$ radical adduct is formed from chloroform, phosgene, formate, or carbon dioxide. It is well established that small quantities of phosgene are produced from the metabolism of CCl_4 (9, Equations 5 and 6).

$$Cl_3COO^{\cdot} \xrightarrow{H^{\cdot}} Cl_3COOH \xrightarrow[-H_2O]{+2e^-+2H^+} Cl_3COH \xrightarrow{-HCl} Cl_2CO \xrightarrow[-2HCl]{+H_2O} CO_2 \quad [6]$$

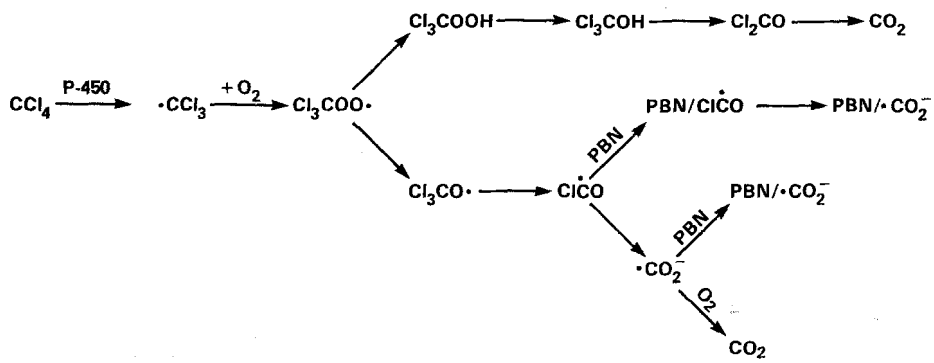
Thus, the reaction of phosgene or a phosgene-derived free radical with PBN to form the PBN/$CO_2^{\cdot-}$ radical adduct was considered. To evaluate this possibility, an experiment was undertaken using chloroform, which is metabolized to phosgene over 8 times faster than CCl_4 (9). However, no PBN/$CO_2^{\cdot-}$ radical adduct was observed in the effluent perfusate of livers exposed to $CHCl_3$; therefore, the involvement of phosgene is unlikely (data not shown). Similarly, infusion of formate failed to produce the PBN/$CO_2^{\cdot-}$ radical adduct, indicating that either this radical is not formed from formate produced from CCl_4 metabolism or formate is not absorbed. In experiments with ^{13}C-labeled CCl_4, the PBN/$CO_2^{\cdot-}$ radical adduct was not diluted by any ^{12}C carbon source, such as carbon dioxide. Taken together, these data indicate that the PBN/$CO_2^{\cdot-}$ radical does not arise from chloroform, phosgene, formate, or carbon dioxide. The possible dechlorination of PBN/$CCl_3^{\cdot}$ to form PBN/$CO_2^{\cdot-}$ is unlikely because PBN/$CO_2^{\cdot-}$ appears immediately in the perfusate and its concentration does not increase with time, whereas PBN/$CCl_3^{\cdot}$ accumulates in the liver and would provide an increasing source of PBN/$CO_2^{\cdot-}$.

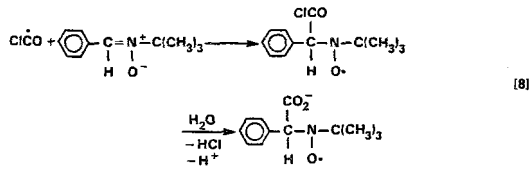
The reaction sequence most likely responsible for PBN/$CO_2^{\cdot-}$ radical adduct formation involves the trichloromethyl peroxyl radical ($CCl_3OO^{\cdot}$) (Equation 5). The trichloromethyl peroxyl radical is converted to the trichloromethoxy radical ($CCl_3O^{\cdot}$) by a two-electron reduction followed by protonation and elimination of a water molecule (Equation 7). The trichloromethoxy radical then reacts with hydroxide ion to produce the chlorocarbonyl radical $\dot{C}OCl$, chloride ion, and a molecule of hypochlorous acid (Equation 7).

$$Cl_3COO^{\cdot} \xrightarrow[-H_2O]{+2e^-+2H^+} Cl_3CO^{\cdot} \xrightarrow{OH^-} \dot{C}lCO + HOCl + Cl^- \quad [7]$$

Previous observations of a radical adduct of the chlorocarbonyl radical from the photolysis of CCl_4 provide additional support that this species reacts with PBN (13, Equation 8).

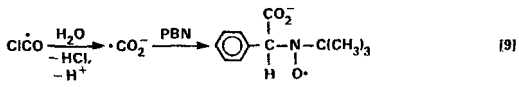
SCHEME 1





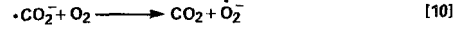
[8]

Upon contact with water, the PBN chlorocarbonyl radical adduct would hydrolyze to give the PBN/CO_2^- radical adduct (Equation 8). It is also possible that the CO_2^- free radical is generated directly from hydrolysis of the chlorocarbonyl radical (Equation 9).



[9]

The carbon dioxide anion radical then reacts with PBN to form the PBN/CO_2^- radical adduct (Equation 9). The CO_2^- radical is known to reduce oxygen to superoxide with a nearly diffusion-limited rate, 2.4×10^9 $M^{-1}s^{-1}$ (14). This reaction forms carbon dioxide, the final product of CCl_4 metabolism (10) and superoxide.



[10]

The PBN/O_2^- radical adduct (a^N = 14.8 G and a_β^H = 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_2^- is formed by the trapping of CO_2^-, then PBN must compete with O_2 with its 2.4×10^9 $M^{-1}s^{-1}$ 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 $CCl_3^·$ is trapped by PBN in spite of the 3.3×10^9 $M^{-1}s^{-1}$ rate constant for its reaction with oxygen (16).

Scheme 1 summarizes Equations 6–10. Although PBN/CO_2^- is clearly $CCl_3^·$-derived and presumably $CCl_3OO^·$-derived, other aspects of the proposed mechanisms(s) are speculative. The mechanism(s) by which $CCl_3^·$ loses its three chlorine atoms to form PBN/CO_2^- can only be suggested at this time (16).

Fate of PBN/CO_2^- 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_2^- 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_2^- was observed (Fig. 8). It was identical with the spectrum of the PBN/CO_2^- radical adduct generated *in vitro* or detected in the effluent perfusate. Thus, the PBN/CO_2^- 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_2^- radical adduct in the perfused liver presumably arises from $CCl_3OO^·$ peroxyl radical formation. The direct evidence for $CCl_3OO^·$ 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 $Cl_3COO^·$, a non-free radical pathway is also possible. In view of these limitations and until the $CCl_3OO^·$ radical can be detected in biological systems, the characterization of factors which influence PBN/CO_2^- formation may give insight into $CCl_3OO^·$ formation in liver.

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