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FACTORS INFLUENCING THE FORMATION OF THE CARBON DIOXIDE RADICAL ANION ($\cdot CO_2^-$) SPIN ADDUCT OF PBN IN THE RAT LIVER METABOLISM OF HALOCARBONS

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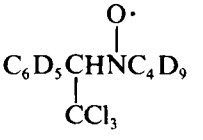
Spin trapping techniques have been used to detect free radicals generated from the *in vitro* metabolism by rat liver microsomes of carbon tetrachloride (CCl_4) and bromotrichloromethane ($BrCCl_3$) under conditions of varying oxygen tension and pH. Dispersions of rat liver microsomes incubated with $^{12}CCl_4$, $^{13}CCl_4$ or $Br^{12}CCl_3$, α-phenyl-*tert*-butyl nitrone (PBN) and NADPH/NADH in a phosphate buffer varying in pH from 6.6 to 8.0 under varying oxygen tensions produced various amounts of four different PBN adducts: PBN-CCl_3, PBN-L, PBN-OL and PBN-CO_2^- where L is a carbon-centered lipid type radical and LO is an oxygen-centered lipid type radical. The relative amount of PBN-CO_2^- increases with the absence of oxygen. With the use of ^{31}P-NMR *in vivo* spectroscopy it was possible to detect a pH change from 7.4 to 6.8 in the livers of rats treated with CCl_4 or $BrCCl_3$. These results suggest that halocarbon metabolism in biological systems may depend on both oxygen tension as well as pH.

KEY WORDS: ESR, spin trapping, trichloromethyl radicals, $\cdot CCl_3$, carbon dioxide radical anion, $\cdot CO_2^-$, carbon tetrachloride, bromotrichloromethane, α-phenyl N-*tert*-butyl nitrone, rat liver microsomes, ^{31}P-NMR *in vivo* spectroscopy, pH determinations.

INTRODUCTION

The application of spin trapping in developing an understanding of the mechanism of CCl_4 toxicity in liver has been mentioned in many reviews.[1–4] Four different spin adducts have been detected to date by ESR in the rat liver metabolism of carbon tetrachloride using PBN as a spin trap. The trichloromethyl radical adduct was the first to be correctly identified in *in vitro* experiments.[5] Later the same radical was found in *in vivo* trials with rats.[6] Others have confirmed these results.[7] The use of $^{13}CCl_4$ [8] and deuterated PBN [9] has greatly improved the spin trapping technique to the extent that three different spin adducts (I, II and III, see below) can be simultaneously recorded in an organic extract of the rat liver microsomal (RLM) preparations.[9] Recently the carbon dioxide radical anion ($\cdot CO_2^-$) spin adduct of PBN (IV) was also detected in an aqueous liver perfusate and in rat urine when both CCl_4 and PBN were administered.[10]



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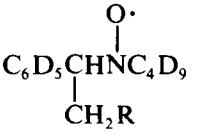

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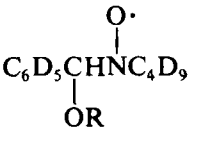

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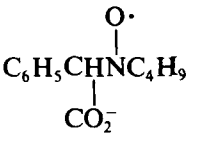

IV

In further studies on these systems we have found that the detection of the $\cdot CCl_3$ and $\cdot CO_2^-$ spin adducts *in vitro* depends on the pH of the phosphate buffer as well as the oxygen tension in RLM preparations. Results of these experiments are presented.

MATERIALS AND METHODS

Preparation of rat liver microsomes

Wistar rats (one month old, male) were killed by CO_2 asphyxiation and their livers removed. The livers were perfused via the hepatic portal vein with isotonic saline (0.85% NaCl) supplemented with EDTA (0.1 mM). The perfused livers were homogenized in sucrose/EDTA buffer (0.25 M/0.1 mM, pH 7.4). The microsomal fraction from the perfused livers was collected as a 105,000 × g pellet by further centrifugation of a 10,000 × g supernatant from the liver homogenate. The final concentration of the microsomal protein was diluted to 30 mg/ml, and determined as by Lowry *et al.*[11] Microsomal preparations were reconstituted in a 0.10 M phosphate buffer (KH_2PO_4 and $Na_2HPO_4 \cdot 7H_2O$) at an appropriate pH (pH 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.6, 7.8, 8.0) and kept frozen at −70°C until ready for use.

ESR analysis of PBN spin adducts

The incubation experiments were either carried out in air at 1 atmosphere for 15 min. at 25°C and subsequently degassed under N_2 for 15 min. or subjected to vacuum by the method of freeze-pump-thaw (3 cycles) at liquid nitrogen temperatures. The reaction mixtures consisted of rat liver microsomes (30 mg/ml of protein in 0.10 M phosphate buffer at an appropriate pH), 0.10M PBN, 0.20 M CCl_4 or $^{13}CCl_4$ (99 atom % ^{13}C) and 0.3 mM each of NADPH and NADH. ESR spectra of the whole microsomal reaction mixture was obtained with the use of a flat cell (degassed sample) or a sealed capillary cell (*in vacuo* sample), placed in a ST-ESR cavity. Rat liver lipid extracts were obtained from the whole microsomal reaction mixture with benzene. The benzene solution was transferred to a round cell and degassed under N_2 for 15 min. ESR spectra were determined with the use of a ST-ESR cavity.

ESR spectra were recorded with the use of a Bruker EPR ER-200D X-band spectrometer (center field = 3480 Gauss; sweep width = 100 kHz; microwave power = −10 db (20.4 mW); gain = 5.0×10^5; modulation amplitude = 0.5 G). Spectral accumulation and averaging was done using a bruker ER-140 (Aspect 2000) data system. Spectral assignments were confirmed with the use of a computer simulation program.[12]

The PBN-CCl_3 g-values in WRLM (whole rat liver microsomes) and benzene were determined by using a solution of Fremy's salt as a reference (g-value = 2.00550 ± 0.00005; a_N = 13.091 ± 0.004) (13, 14). All other g-values were

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determined by using the g-values for PBN-CCl_3 as an internal standard in combination with computer simulation techniques.

Formation of PBN-CO_2^- spin adduct

Sodium formate (0.5 M), sodium persulfate (0.01 M), and PBN (0.05 M), were used to synthesize the PBN-CO_2^- radical adduct.

$$Na_2S_2O_8 \longrightarrow 2\cdot SO_4Na \text{ (thermal at room temperature)}$$

$$\cdot SO_4^- + HCO_2Na \longrightarrow NaHSO_4 + \cdot CO_2^-$$

pH measurements using ^{31}P-NMR in vivo spectroscopy

Wistar rats were administered a dose of CCl_4 or $BrCCl_3$ (160 μg/kg of body weight, with 5% Emulphor in 0.85% saline) via i.p. injection and subsequently anaesthetized with sodium pentobarbital (i.p.; 1 ml/2.27 kg of body weight). A 1 cm-diameter surface coil was positioned over the liver area of the rat. NMR measurements were made with the use of a modified Bruker CXP 200 spectrometer equipped with a 40 cm horizontal-bore superconducting magnet operating at 2.35 T, corresponding to a Larmor frequency for phosphorus of 40.5 MHz. The spatial localization in the liver was defined from phantom studies by adjusting the excitation pulse length. Depth pulses were also used to narrow the zone of excitation as well as eliminate high flux regions.[15]

Spectra were obtained over a 12 h period by Fourier transformation of accumulated free induction decays (FIDs) obtained with a repetition interval of 0.8 s. The pH values were derived from Pi (pH sensitive) and α-ATP (reference) peaks by means of a calibration chart obtained at 37°C from solutions of 10 mM KH_2PO_4, 20 mM NaCl, 12.5 mM ATP and 12.5 mM PCr.

RESULTS

The hyperfine splitting constants[9,10] and g-values of the four known PBN spin adducts in either WRLM or in a benzene extract of the former, are summarized in Table I. The ESR spectra shown in Figures 1 and 2 illustrate the formation of the PBN-CO_2^- spin adduct as a function of pH in RLM preparations exposed to either CCl_4 (Figure 1) or $BrCCl_3$ (Figure 2) and air. Table II gives the measured hyperfine splitting constants as a function of pH.

The ESR signal due to the trichloromethyl adduct of PBN is detected over the entire pH range studied (pH 6.6–8.0). Conversely, the PBN-CO_2^- spin adduct is only detectable in the pH range of 6.7 to 7.6. A weak signal due to PBN-CO_2^- is initially observed at pH 6.76 along with a stronger signal due to the trichloromethyl spin adduct. The major spectral pattern detected for microsomal suspensions incubated with either CCl_4 or $BrCCl_3$ in the pH range of 6.89 to 7.05 is from the PBN-CO_2^- adduct with only a weak contribution from the PBN-CCl_3 adduct. At pH's ranging from 7.05 to 7.61 both PBN spin adducts can be detected simultaneously in WRLM incubated with CCl_4, although at pH's above 7.05 the PBN-CO_2^- adduct dies rapidly ($t_{1/2} \cong 30$ min.)

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TABLE I
Hyperfine splitting constants[a] and g-values of PBN spin adducts obtained from whole rat liver microsomes[b] or a benzene extract of a WRLM preparation

	I[c]		II[d]	III[e]	IV[f]
	(benzene)	(WRLM)	(benzene)	(benzene)	(WRLM)
a_N	13.88	14.1	14.49	13.70	15.8
a_β^H	1.61	1.8	3.35	1.88	4.6
$a_\beta^{C^{13}}$	9.67	9.6	–	–	11.7
a_γ^H	–	–	0.53	–	–
g-value	2.00628	2.00616	2.00612	2.00631	2.00570

[a]All hyperfine splitting constants in gauss
[b]Whole rat liver microsomes = WRLM
[c]I = PBN-CCl_3
[d]II = PBN-CH_2-R ("carbon-centered" lipid type adduct)
[e]III = PBN-OL ("oxygen-centered" lipid type adduct)
[f]IV = PBN-CO_2^-

leaving behind PBN-CCl_3 as the major species. At pH's greater than 7.61 the PBN-CO_2^- spectra is essentially too weak for good resolution.

During lipid extractions of the WRLM suspensions at pH's where both the PBN-CCl_3 and the PBN-CO_2^- adducts are present it was observed that only the PBN-CCl_3 adduct is extracted into benzene or hexane. The PBN-CO_2^- adduct is never detected in the organic solvent phase (Figure 3). Since $^{13}CCl_4$ was not used for all the solutions studied, the presence of other carbon and/or oxygen-centred radical adducts[16] was not monitored as a function of pH.

Experiments were performed in the total absence of oxygen by using a specially designed ESR cell with two chambers separated by a "break seal". The microsomal suspension in a phosphate buffer of appropriate pH was evacuated (via 3 cycles of the freeze-pump-thaw technique) separately from a mixture of CCl_4, PBN and NADPH/NADH. Mixing of the contents of the two chambers was done just prior to ESR analysis. In the complete absence of oxygen at pH 7.0 only the PBN-CO_2^- adduct is observed (Figure 4), with no detection of the PBN-CCl_3 adduct. This observation is in sharp contrast to the spectra found in samples incubated under aerobic conditions open to the atmosphere. It should be noted that simple degassing with nitrogen bubbling is not sufficient to eliminate the effect of oxygen on spin adduct formation.

^{31}P-NMR *in vivo* spectroscopy was used to study the effect of CCl_4 or $BrCCl_3$ intoxication as a function of liver intracellular pH. Over a 12 hr. period of exposure to either halocarbon the cytosolic pH (measured from the change in chemical shift of the "inorganic phosphate" peak (Pi)) shifted from a pH of 7.4 (control conditions) to a more acidic value of pH 6.8. The pH changes observed *in vivo* (pH 7.4–6.8) due to halocarbon induced cytosolic acidosis correspond very well with the pH range dependence for the detection of the $\cdot CO_2^-$ radical anion in the microsomal system (*in vitro*).

DISCUSSION

Although the details of the trichloromethyl radical production by WRLM are still speculative it is generally believed that reductive cleavage takes place to produce the chloride ion and trichloromethyl radicals.

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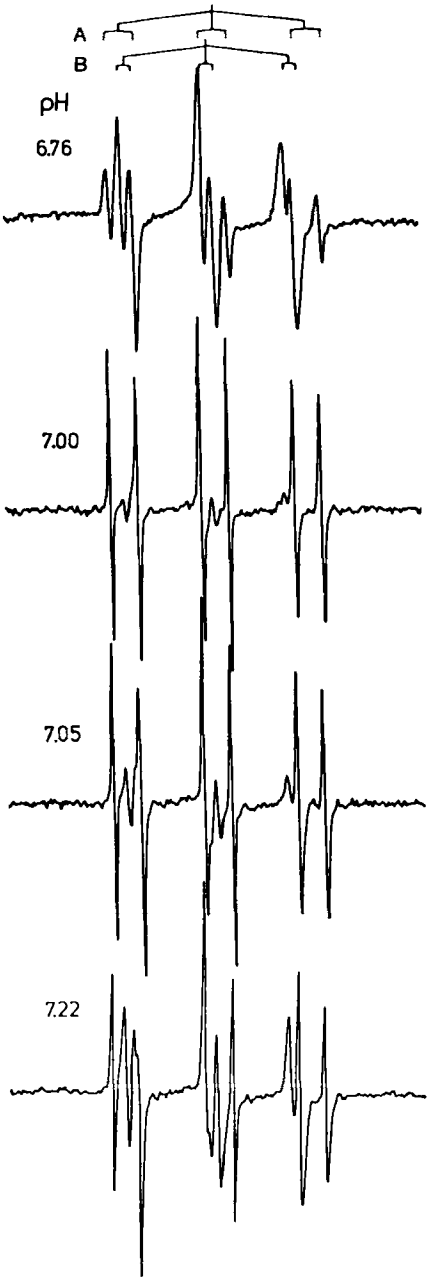

FIGURE 1 The detection of PBN-CO_2^- (A) and PBN-CCl_3 (B) spin adducts in rat liver microsomes (incubated with CCl_4, PBN and NADPH/NADH) as a function of pH.

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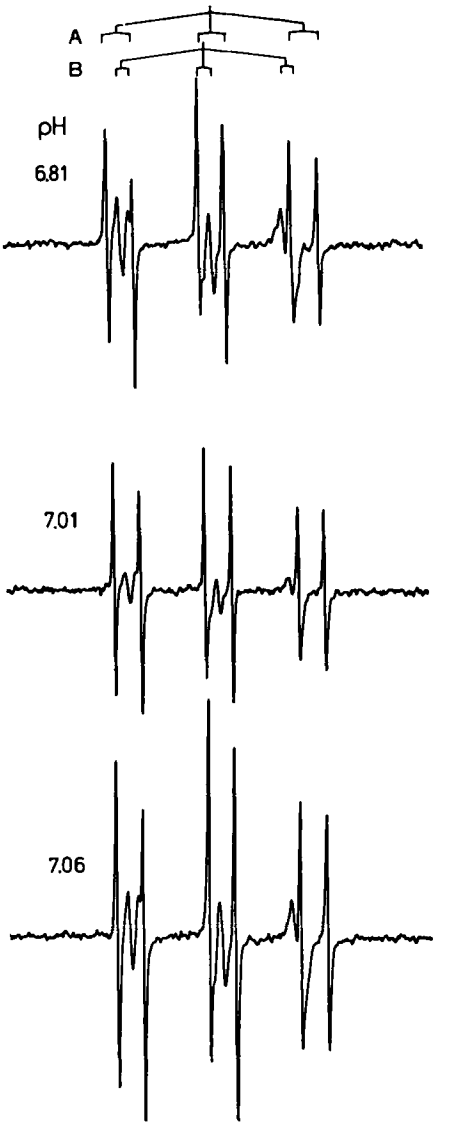

FIGURE 2 The detection of PBN-CO_2^- (A) and PBN-CCl_3 (B) spin adducts in rat liver microsomes (incubated with $BrCCl_3$, PBN and NADPH/NADH) as a function of pH.

$$CCl_4 + [e^-] \longrightarrow \cdot CCl_3 + Cl^-$$

The nature of the reducing agent is not known but a reduced form of a cytochrome P_{450} with molecular weight of 52,000 daltons is believed to be involved.[17] Superoxide anion radical can also reduce CCl_4.[4,18-2]

A more difficult question is the formation of PBN-CO_2^-. The obvious assumption, due to the use of $^{13}CCl_4$, is that the carbon dioxide radical anion is produced by the microsomal system from CCl_4 or $BrCCl_3$ and trapped by PBN:[16]

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TABLE II

Hyperfine splitting constants[a] of PBN spin adducts obtained from whole rat liver microsomes[b] exposed to CCl_4 or $BrCCl_3$ dispersed in solutions of different pH

CCl_4 exposure							$BrCCl_3$ exposure				
	I[c] WRLM		I[c] C_6H_6 extract		IV WRLM			I[c] WRLM		IV WRLM	
pH ±0.05	a_N	a_β^H	a_N	a_β^H	a_N	a_β^H	pH ±0.05	a_N	a_β^H	a_N	a_β^H
6.64	13.97	1.73	13.94	1.71	–	–	6.65	13.79	1.81	15.80	4.59
6.76	13.94	1.69	13.89	1.66	15.82	4.50	6.75	13.77	1.73	15.77	4.57
6.89	very weak		13.89	1.70	15.82	4.54	6.81	13.77	1.73	15.77	4.59
7.00	very weak		13.87	1.73	15.85	4.57	7.01	13.82	1.83	15.85	4.57
7.05	13.87	1.88	13.89	1.73	15.85	4.54	7.06	13.80	1.76	15.80	4.59
7.22	13.89	1.71	13.97	1.73	15.90	4.5					
7.38	13.84	1.83	13.97	1.70	15.89	4.5					
7.50	13.89	1.73	13.87	1.76	16.02	4.5					
7.61	14.01	1.76	13.92	1.73	very weak						

[a]All hyperfine splitting constants in gauss
[b]Whole rat liver microsomes = WRLM
[c]May contain small amounts of II and III

$$CCl_4 \longrightarrow \longrightarrow \cdot CO_2^-$$

The one electron reduction of phosgene might produce the chloroformyl radical which would probably hydrolyze to the carbon dioxide radical or be trapped by PBN:

$$Cl\text{–}\overset{\overset{O}{\|}}{C}\text{–}Cl + [e^-] \longrightarrow \cdot\overset{\overset{O}{\|}}{C}\text{–}Cl + Cl^-$$

$$\cdot\overset{\overset{O}{\|}}{C}\text{–}Cl + H_2O \longrightarrow \cdot\overset{\overset{O}{\|}}{C}\text{–}O^- + 2H^+ + Cl^-$$

The chloroformyl radical adduct of PBN would also be expected to hydrolyze to PBN-CO_2^-.

$$\cdot\overset{\overset{O}{\|}}{C}\text{–}Cl \xrightarrow{PBN} C_6H_5CH(O\cdot)NC_4H_9(C{=}O)Cl \xrightarrow{H_2O} \text{IV}$$

V

The formation of phosgene can readily be rationalized if the trichloromethyl radical is produced in the presence of oxygen:[22]

$$\cdot CCl_3 + O_2 \longrightarrow Cl_3COO\cdot$$

$$Cl_3COO\cdot + \cdot OOR \longrightarrow Cl_3CO\cdot + O_2 + \cdot OR$$

$$Cl_3CO\cdot \longrightarrow Cl_2CO + Cl\cdot$$

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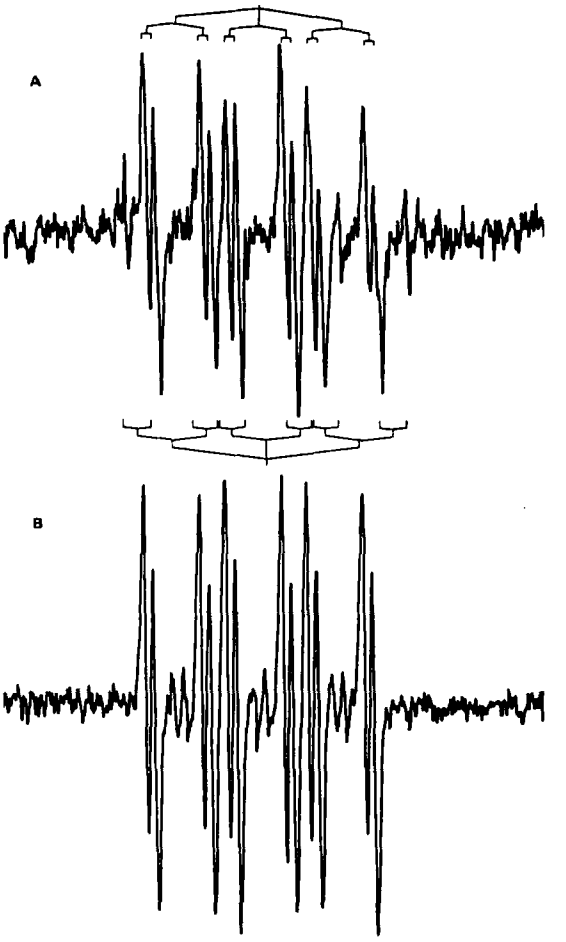

FIGURE 3 A. ESR spectra of PBN spin adducts in rat liver microsomal preparation incubated with $^{13}CCl_4$, PBN and NADPH/NADH (pH 7.22), subsequently degassed under nitrogen (15 min.). (i) PBN-$^{13}CCl_3$ spin adduct; a_β^H = 1.69, a_β^{13C} = 9.62 and a_N = 14.01 Gauss (G). (ii) PBN-$^{13}CO_2$ spin adduct a_β^H = 4.88, a_β^{13C} = 11.74 and a_N = 15.86 G. B. ESR spectra of a rat liver microsomal lipid extract (toluene) degassed under nitrogen (15 min.) (hyperfine splitting constants in (ii) and (iii) from computer simulation of spectrum). (i) PBN-$^{13}CCl_3$ spin adduct; a_β^H = 1.71, a_β^{13C} = 9.67 and a_N = 13.97 G. (ii) PBN-OR (alkoxyl) spin adduct; a_β^H = 1.88 and a_N = 13.55 G. (iii) PBN-R (alkyl) spin adduct; a_β^H = 3.35 and a_N = 14.40 G.

Analgous reactions as shown above are accepted for *tert*-butylperoxyl radicals[4,23] and the production of chlorine atoms has been verified by spin trapping with PBN when trichloromethylperoxyl radicals are produced in chloroform.[4,14]

Because PBN-CO_2^- is formed as the major ESR detectable product in the total absence of oxygen (samples were evacuated by a vacuum freeze-pump-thaw technique) we have considered an alternative mechanism not depending on the presence of oxygen which involves trapping of the dichlorocarbene which is believed to be produced under anaerobic conditions:[24]

$$\cdot CCl_3 + [e^-] \xrightarrow{P_{450}} [P_{450} - CCl_3]^- \longrightarrow :CCl_2 + Cl^-$$

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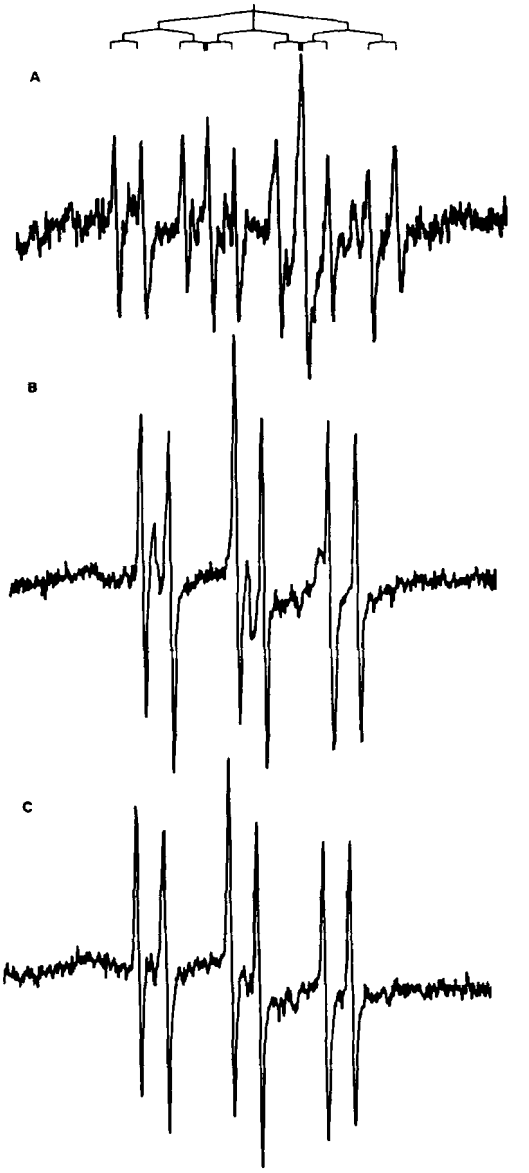

FIGURE 4 The detection of the PBN-CO_2^- spin adduct from rat liver microsomes (incubated with $^{13}CCl_4$ or $^{12}CCl_4$, PBN and NADPH/NADH at pH 7.00) under aerobic or anaerobic conditions. A. PBN-$^{13}CO_2^-$ – spin adduct obtained in an air atmosphere, followed by nitrogen flushing (15 min.) prior to ESR analysis; $a_\beta^H = 4.64$, $a_\beta^{13C} = 11.74$ and $a_N = 15.94$ G. B. PBN-$^{12}CO_2^-$ spin adduct obtained under conditions similar to A; $a_\beta^H = 4.54$ and $a_N = 15.97$ G. PBN-$^{12}CO_2^-$ spin adduct obtained *in vacuo* (3 cycles of freeze-pump-thaw); $a_\beta^H = 4.66$ and $a_N = 15.89$ G.

The reaction product of dichlorocarbene with PBN is unknown but one might expect the formation of the following oxazetidine four membered ring heterocyclic compound:

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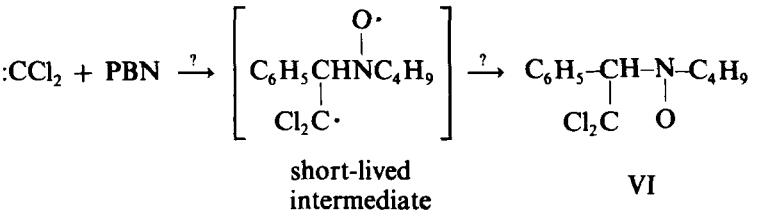
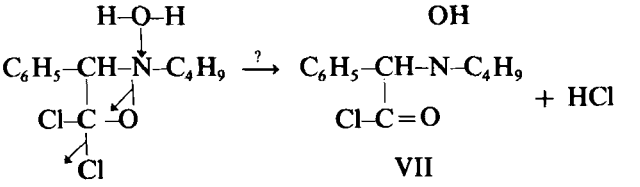


short-lived intermediate

VI

Since VI is an ESR silent molecule it would have gone undetected until now. Inspection of the literature indicates that reference to such heterocyclic compounds (oxazetidines) are rare and very little chemistry of these compounds is known.[25] Hydrolysis with ring opening should be enhanced by the presence of the electron withdrawing dichloromethylene group. Formation of the hydroxylamine of the chloroformyl adduct of PBN would be expected:



VII

Subsequent oxidation of the hydroxylamine VII to the nitroxide V would need to be accomplished in the biochemical system even in the absence of air. This suggested route provides IV without the express intermediacy of $\cdot CO_2^-$! Experiments are planned in this laboratory to test this hypothesis.

CONCLUSIONS

We have shown that different spin adducts are detected from whole rat liver microsomal preparations depending on pH. Since the distribution of cytochrome P_{450}'s may not be homogenous in the liver[26] and different regions of the liver exposed to halocarbons may have different pH's, the possibility should be considered that different radicals are produced from CCl_4 in different parts of the liver. Further work on these systems is underway.

Acknowledgements

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