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Reaction of Glutathione with a Free Radical Metabolite of Carbon Tetrachloride

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SUMMARY

Carbon tetrachloride and bromotrichloromethane are both metabolized by cytochrome P-450 in the presence of phenyl-*N*-*t*-butyl nitrone (PBN) to the PBN/ trichloromethyl (PBN/·CCl_3) and the PBN/carbon dioxide anion (PBN/·CO_2^-) radical adducts in the liver. The formation of the latter but not the former species in perfused liver was reduced markedly by prior depletion of hepatic glutathione with either diethyl maleate or buthionine sulfoximine treatments. In microsomal incubations, the PBN/·CO_2^- radical adduct was detected only upon the addition of cytosol. In microsomal incubations containing PBN, CCl_4, and GSH, but no added cytosol, a novel radical adduct with distinctive coupling constants was detected. This radical adduct's ESR spectrum exhibited ^{13}C isotope effects when it was formed in an incubation containing $^{13}CCl_4$ or $Br^{13}CCl_3$. The presence of GSH in the radical adduct is postulated based on the radical adduct's hydrophilicity and slow rate of rotation in solution. The detection of this new radical adduct, PBN/[GSH-·CCl_3], establishes the reaction of GSH with a CCl_4-derived free radical as a significant event in the metabolism of $CBrCl_3$ and CCl_4. The cytosolic conversion of PBN/[GSH-·CCl_3] into PBN/·CO_2^- has been demonstrated and characterizes the PBN/·CO_2^- radical adduct as the product of metabolism of PBN/[GSH-·CCl_3], a primary radical adduct. Thus, it is concluded that GSH rather than oxygen is obligatory for the formation of PBN/·CO_2^- from ·CCl_3 in intact cells.

The hepatotoxicity of carbon tetrachloride (CCl_4) and bromotrichloromethane ($CBrCl_3$) is generally accepted to result from metabolic activation of the halocarbons by cytochrome P-450-dependent monooxygenases to free radical metabolites (1–4). The formation of the trichloromethyl free radical (·CCl_3), a reductive dehalogenation product of both halocarbons, has been stabilized with nitrone spin traps and detected with ESR (5). The PBN/·CCl_3 radical adduct was identified in microsomal (6, 7) and hepatocyte incubations (8, 9) and extracts of rat liver (6, 9), based on ^{13}C isotope effects. A second radical adduct derived from CCl_4 metabolism, the carbon dioxide anion radical adduct of PBN (PBN/·CO_2^-), was found in the effluent perfusate of the isolated perfused liver following infusion of halocarbon (10). This new radical adduct had ESR hyperfine coupling constants and ^{13}C isotope effects identical to those of PBN/·CO_2^- formed by a Fenton system containing [^{13}C]formate. LaCagnin *et al.* (11) demonstrated that PBN/·CO_2^- moved from the cell into the perfusate via anion transport channels, based on sensitivity to DIDS. Further, they reported that the rate of production of PBN/·CO_2^- by the perfused liver correlated very well with hepatocellular damage, as indexed by the release of lactate dehydrogenase. Furthermore, Janzen *et al.* (12) detected PBN/·CO_2^- in microsomal preparations, and Knecht and Mason (13) found both PBN/·CCl_3 and PBN/·CO_2^- in bile of rats exposed to CCl_4 and PBN *in vivo*.

One possible pathway for the formation of the PBN/·CO_2^- radical adduct is via the trichloromethyl peroxyl radical formed from the reaction of the trichloromethyl radical with oxygen (14); however, recent work has cast doubt on this possibility. For example, PBN/·CO_2^- has been detected in microsomal incubations under vacuum (12), and LaCagnin *et al.* (11) and Knecht and Mason (13) detected more PBN/·CO_2^- in the effluent perfusate of rat livers perfused with nitrogen-saturated buffer and in the bile of rats deprived of oxygen, respectively, than in corresponding normoxic controls. With a role for molecular oxygen and peroxyl radicals questionable, the metabolic pathway(s) by which the PBN/·CO_2^- radical adduct is formed remains unclear.

Zeigler[2] suggested that nucleophilic attack by the anion of glutathione (GS^-) on the electron-deficient carbon in the trichloromethyl radical could play a role in the formation of PBN/·CO_2^-. This possibility seemed particularly interesting in view

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ABBREVIATIONS: PBN, phenyl-*N*-*t*-butyl nitrone; BSO, buthionine sulfoximine; DEM, diethyl maleate; sulfonated PBN, sodium 2-sulfonate phenyl-*t*-butylnitrone; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid.

of the high concentration of GSH present in the liver. Thus, the objective of this work was to test the hypothesis that glutathione is involved in the formation of the PBN/$\cdot CO_2^-$ radical adduct.

Materials and Methods

PBN, sulfonated PBN, BSO, NADPH, DTNB, EDTA Tris, reduced and oxidized glutathione, L-cysteine, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium chloride, DEM, CCl_4, and $CBrCl_3$ (analytical grade) were from Fisher Scientific (Pittsburgh, PA). [^{13}C]Carbon tetrachloride and [^{13}C]bromotrichloromethane were the products of MSD Isotopes (St. Louis, MO).

Female Sprague-Dawley rats (Zivic-Miller; 250–300 g) were treated with sodium phenobarbital (1 mg/ml) in drinking water for at least 7 days to induce cytochrome P-450, before perfusion experiments or microsome preparation. For depletion of glutathione, either DEM or BSO was administered by intraperitoneal injection. DEM (0.7 g/kg) was given to fed rats 1 hr before liver perfusion. BSO or saline vehicle was administered to fasted rats in eight doses as follows: 4 mmol/kg every 3 hr for four doses (i.e., at noon, 3 p.m., 6 p.m., and 9 p.m.), 8 mmol/kg at midnight, and then 4 mmol/kg every 2 hr for three doses starting at 7 a.m. Livers were perfused within 2 hr of the final dose.

GSH levels in isolated perfused liver tissue were determined using a method modified from that of Moron *et al.* (15). Liver tissue (100 mg) was homogenized in 7% perchloric acid (1 ml) and centrifuged. A 200-μl aliquot of the supernatant was added to Ellman's reagent (2 ml), which consisted of 0.3 mM DTNB, 15 mM EDTA, and 0.04% bovine serum albumin in sodium phosphate buffer, pH 7.2. Samples were vortexed and allowed to stand for 15 min before absorbance was measured at 412 nm. Blank samples contained 200 μl of perchloric acid in 2 ml of Ellman's reagent. Reduced glutathione concentrations in liver were calculated using an extinction coefficient of 13.9, as reported by Ellman (16).

Livers were perfused with Krebs-Henseleit bicarbonate buffer, pH 7.4, 37°, saturated with O_2/CO_2 (95:5) or N_2/CO_2(95:5) in a nonrecirculating system, as described previously (17). The perfusate was pumped into the liver via a cannula placed in the portal vein and left 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 or sulfonated-PBN (5 mM) was dissolved in the perfusate, whereas CCl_4 or $CBrCl_3$ (final concentration in perfusate, 1 mM) was bound to albumin (final concentration in perfusate, 0.2%) by stirring slowly for 16 hr. Perfused liver samples were homogenized in perfusion buffer (5 ml/g), extracted with $CHCl_3/CH_3OH$ (2:1) solution (5 ml/g), and centrifuged for 10 min at 2500 rpm. The organic layer was removed, dried with anhydrous sodium sulfate, bubbled with nitrogen for 3 min, and placed in a quartz sample tube for ESR analysis.

Rat microsomes were prepared by differential centrifugation, according to published methods (18), from homogenate prepared in a buffer containing KCl (150 mM) and Tris•HCl (50 mM), pH 7.4. During microsomal preparation, postmitochondrial S-9 fraction and cytosol from the homogenate were collected and stored at −70°, along with the microsomal pellets and samples of homogenate. Before use, homogenate, postmitochondrial S-9 fraction, and cytosolic samples were thawed, swirled in a nitrogen stream, and kept in a nitrogen atmosphere at 0° before being added to incubations without further dilution. Microsomal pellets were resuspended in 3 ml of incubation medium (KCl/Tris•HCl buffer at pH 7.4)/pellet (25 mg of protein/ml), swirled in a nitrogen stream, and kept in a nitrogen atmosphere. In all experiments involving cell fractions, the incubation medium containing PBN and GSH (where indicated) was placed in a sealed septum bottle and bubbled with nitrogen for 10 min. Cell fractions were injected into the bottle while a stream of nitrogen was flowing. Subsequently, nitrogen flow was stopped and halocarbon and NADPH were injected. Samples were then aspirated immediately into a flat cell in the ESR cavity (19) and the ESR spectrum was recorded at appropriate time intervals. Final concentrations of ingredients of the incubation mixture were 150 mM KCl, 50mM Tris•HCl, 80 mM PBN, 1 mM NADPH, 1 mM halocarbon, and 10 mM GSH, in a total volume of 3 ml.

Organic extractions of microsomal experiments were performed on 6-ml microsomal incubations. Half of the incubation was analyzed by ESR to determine the amount of GSH-dependent radical adduct (PBN/[GSH-$^{\cdot}CCl_3$]) present. The other half was extracted with an equal volume of nitrogen-saturated chloroform. After centrifugation in a sealed centrifuge tube to prevent exposure to air, both the aqueous and organic phases were analyzed by ESR.

ESR spectra were obtained using an IBM-200 ESR spectrometer operating at 9.7 GHz with a 100 KHz modulation frequency. Aqueous samples were aspirated into a quartz flat cell positioned in an ER-4103 TM microwave cavity.

Results

Effect of prior depletion of GSH on PBN/$\cdot CO_2^-$ adduct formation in the perfused liver. As reported previously (10), the PBN/$\cdot CO_2^-$ and PBN/$^{\cdot}CCl_3$ radical adducts were detected in the effluent perfusate and organic solvent extracts of liver, respectively, following infusion of either CCl_4 or $CBrCl_3$ into the perfused liver. The possible involvement of GSH in the formation of these two radicals was investigated by depleting it in rat liver by treatment with DEM or BSO before perfusion experiments. Following treatment with DEM or BSO, GSH levels decreased by 95 and 87%, respectively, compared with levels in untreated rats (Table 1). Analysis of aqueous perfusate obtained following halocarbon infusion from livers of DEM- or BSO-treated rats indicated that production of PBN/$\cdot CO_2^-$ was decreased dramatically, to undetectable levels (Fig. 1, C and E), when compared with controls (Fig. 1A). In comparison, formation of PBN/$^{\cdot}CCl_3$ (Fig. 1B) was not altered by depletion of GSH (Fig. 1, D and F). These results indicate that glutathione is involved in the formation of PBN/$\cdot CO_2^-$ but not of PBN/$^{\cdot}CCl_3$ in perfused rat liver.

Intracellular formation of PBN/$\cdot CO_2^-$ in the perfused liver. The negative charge on the sulfate group of sulfonated PBN most likely restricts its diffusion through hepatocyte membranes, and the bulk of any radical trapping would thus be expected to occur extracellularly. With sulfonated PBN as the spin trap in perfusion experiments, only a residual $\cdot CO_2^-$ radical adduct signal was detectable in either perfusate or homogenate of perfused livers (data not shown). The sulfate group is not likely to interfere with the radical-trapping process, because a formate-containing Fenton system containing the sulfonated nitrone produced a sulfonated PBN/$\cdot CO_2^-$ radical

TABLE 1

Hepatic glutathione concentrations in perfused livers

Rats were treated with DEM or BSO as described in Materials and Methods. Livers were perfused as described in Materials and Methods up to the time of halocarbon infusion (10–15 min), removed from the perfusion system, and stored at −70°. After thawing, liver samples were analyzed for GSH concentrations as described in Materials and Methods. Values are mean ± standard error.

Nutritional state	Treatment	Hepatic glutathione concentration	
		mM	% of control
Fed	None	5.44 ± 0.40	100.0
Fed	DEM	0.31 ± 0.03	5.7
Fasted	None	3.69 ± 0.45	67.8
Fasted	BSO	0.90 ± 0.05	16.5

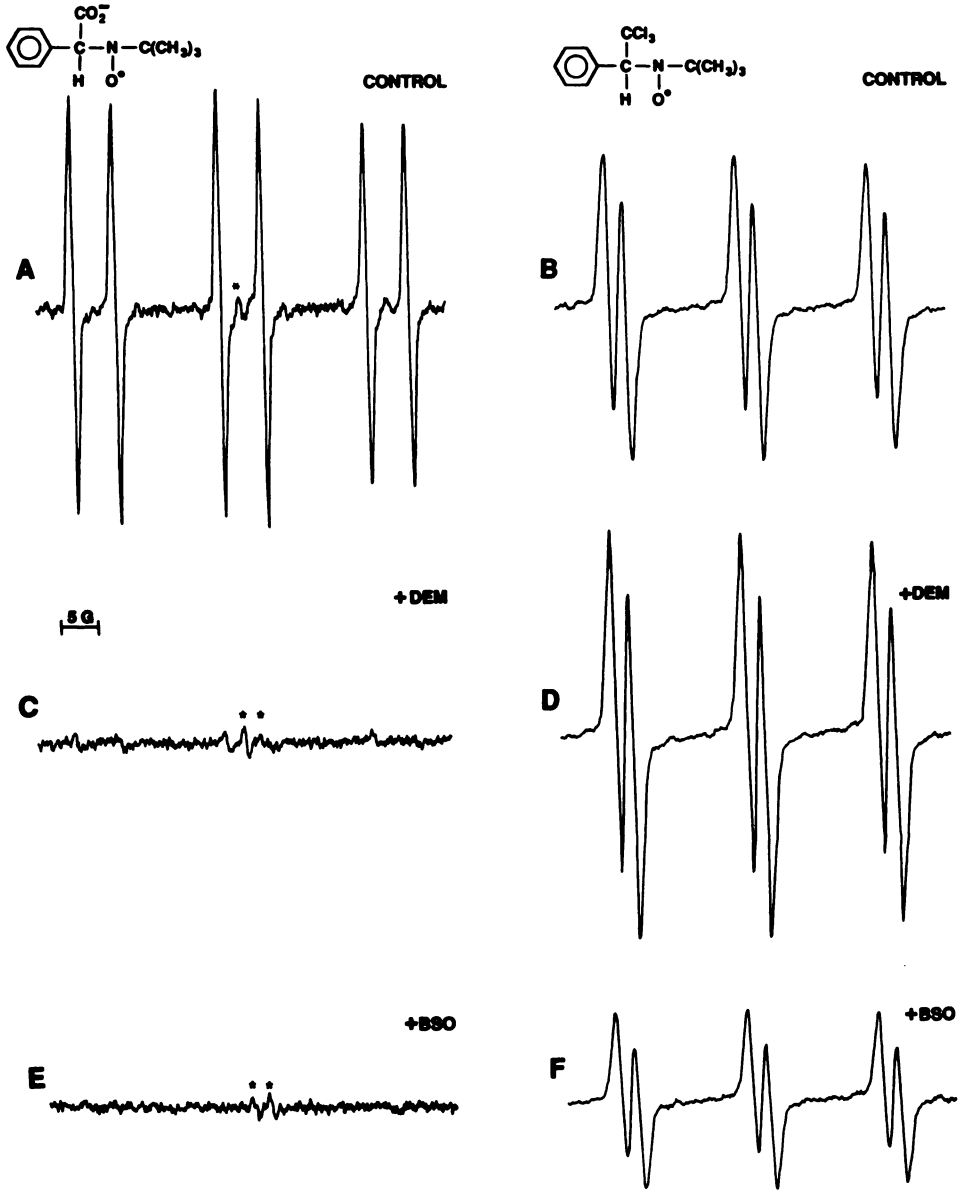

Fig. 1. Effects of GSH depletion on ESR spectra of samples from perfused liver. Perfusate and liver extracts from livers perfused with PBN (5 mM) and $CBrCl_3$ (1 mM) were as in Materials and Methods. Perfusate was saturated with 95:5% N_2/CO_2. A, Spectrum of effluent perfusate from a liver with normal GSH levels. Perfusate sample was collected 15 min after $CBrCl_3$ infusion was initiated. Spectrometer settings: modulation amplitude, 0.7 G; microwave power, 20 mW; gain, 5×10^5; scan time, 500 sec; time constant, 0.64 sec. *Ascorbyl semidione radical. B, Spectrum of organic extract of the liver used in A. Spectrometer settings were the same as in A, except that the receiver gain was 2×10^5. C, Spectrum of effluent perfusate from a liver after treatment of the rat with DEM as described in Materials and Methods. Perfusate sample was collected 15 min after $CBrCl_3$ infusion was initiated. Spectrometer settings as in A. D, Spectrum of organic extract of the liver used in C. Spectrometer settings as in B. E, Spectrum of effluent perfusate from a liver after treatment of the rat with BSO as described in Materials and Methods. Perfusate sample was collected 15 min after $CBrCl_3$ infusion was initiated. Spectrometer settings as in A. F, Spectrum of organic extract of the liver used in E. Spectrometer settings as in B.

adduct (data not shown), as previously described for PBN (10). Lack of significant sulfonated PBN/$\cdot CO_2^-$ formation, coupled with the fact that substantial PBN/$\cdot CO_2^-$ was formed in identical experiments, supports the postulate that $\cdot CO_2^-$ radical adduct formation occurs in the cell. This conclusion is supported by the observation that PBN/$\cdot CO_2^-$ in the effluent perfusate was diminished dramatically by DIDS, an inhibitor of organic anion transport through hepatocyte membranes (11).

Production of PBN/$\cdot CO_2^-$ in subcellular fractions of liver. In an attempt to elucidate the mechanism by which GSH is involved in the formation of PBN/$\cdot CO_2^-$, halocarbons were incubated with various subcellular fractions from liver including homogenate, the postmitochondrial S-9 fraction, the microsomal fraction, and cytosol. When rat liver homogenate was incubated with $^{13}CBrCl_3$, the PBN/$\cdot ^{13}CO_2^-$ radical adduct was produced. The ESR spectrum was characterized by the hyperfine splitting constants $a^N = 15.8$ G, $a_\beta^H = 4.6$ G, and $a_\beta^{^{13}C} = 11.7$ G (Fig. 2A, Table 2). Use of $^{13}CCl_4$ instead of $^{13}CBrCl_3$ in the same system gave the same ESR spectrum but with reduced amplitude (Fig. 2B). The formation of the PBN/$\cdot CO_2^-$ radical adduct was dependent on the presence of halocarbon (Fig. 2C) and liver homogenate (Fig. 2D). Substitution of the postmitochondrial S-9 fraction for homogenate gave the same results (Fig. 3); however, when halocarbon was incubated with the microsomal fraction in the presence of NADPH and PBN, the PBN/$\cdot CO_2^-$ radical adduct was not detected (Fig. 4D). This result is in contrast to a report by Janzen *et al.* (12) that microsomes can produce PBN/$\cdot CO_2^-$. One explanation for the difference between their result and those reported in this study may be the methods used in the preparation of the microsomal fraction. In the study by Janzen *et al.*, the microsomes were not washed, whereas those used in the present study were washed twice. When microsomes were prepared as described by Janzen *et al.*, incubation of halocarbon with the unwashed microsomal fraction in the presence of NADPH and PBN indeed produced a spectrum of the PBN/$\cdot CO_2^-$ radical adduct (data not shown). Thus, it is likely that unwashed microsomes are contaminated with cytosolic components required for the formation of the PBN/$\cdot CO_2^-$ radical adduct. As described below,

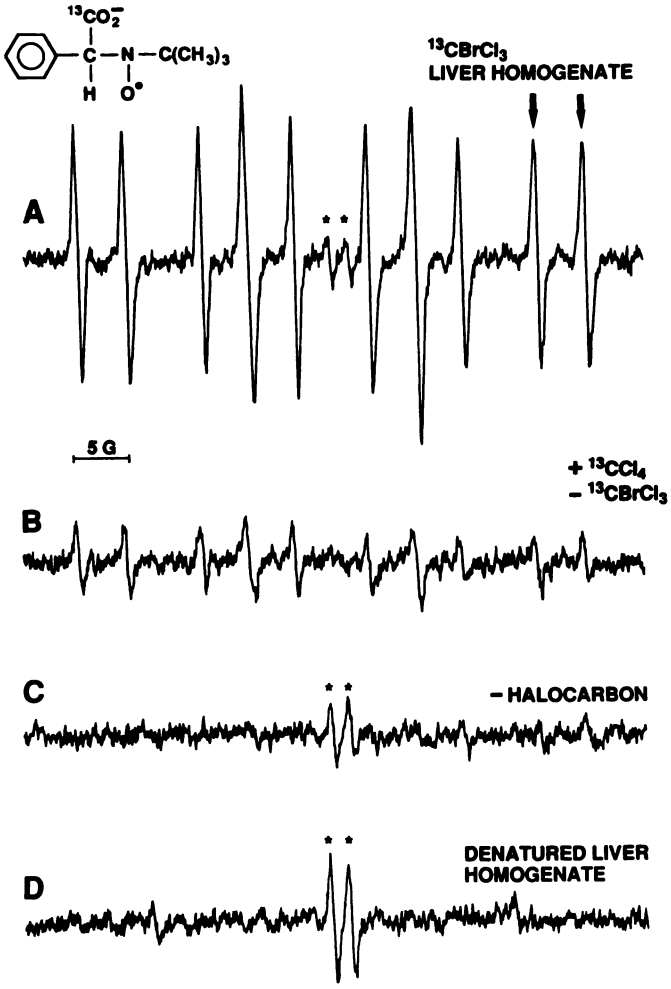

Fig. 2. Metabolism of $^{13}CCl_4$ or $^{13}CBrCl_3$ to PBN/$\cdot CO_2^-$ by rat liver homogenate. ESR spectra of complete nitrogen-saturated incubations containing halocarbon (1 mM), PBN (80 mM), and rat liver homogenate (7 mg of protein/ml) 500 sec after initiation of incubation. Spectrometer settings: modulation amplitude, 0.7 G; microwave power, 20 mW; gain, 8×10^5; scan time, 500 sec; time constant, 0.64 sec. *Ascorbyl semidione radical. A, ESR spectrum of PBN/$\cdot CO_2^-$ produced by a complete incubation system containing $^{13}CBrCl_3$. B, ESR spectrum of PBN/$\cdot CO_2^-$ produced by a complete incubation system containing $^{13}CCl_4$. C, ESR spectrum of an incubation system that did not contain halocarbon. D, ESR spectrum of an incubation system containing $^{13}CBrCl_3$ and denatured liver homogenate.

addition of cytosol to a microsomal incubation containing halocarbon is required for production of PBN/$\cdot CO_2^-$. The results of these experiments indicate that both microsomal and cytosolic components are required for the formation of the PBN/$\cdot CO_2^-$ radical adduct.

GSH-dependent radical adduct in microsomes. It is well documented that the microsomal component necessary for the metabolism of CCl_4 or $CBrCl_3$ is the cytochrome P-450-dependent monooxygenase enzyme system. The cytosolic component(s) required for PBN/$\cdot CO_2^-$ formation is not known. However, as suggested by Zeigler[2] and by the results from studies with the isolated perfused liver, GSH may be involved. To test this hypothesis, $^{13}CBrCl_3$ was incubated with microsomes in the presence of NADPH, PBN, and GSH. ESR analysis of the incubation indicated that a new radical adduct was produced (Fig. 4A). Coupling constants were $a^N = 15.36$ G, $a_\beta^H = 2.84$ G, and $a_\beta^{^{13}C} = 11.44$ G and were distinctly different from values for PBN/$^\cdot CCl_3$ and PBN/$\cdot CO_2^-$ (see Table 2). A similar spectrum

TABLE 2

Hyperfine coupling constants of carbon tetrachloride-derived radical adducts

Source	Structure	Hyperfine splittings			Reference
		a_β^H	a^N	$a_\beta^{^{13}C}$	
Microsomal system with $^{13}CCl_4$	PBN/$\cdot{}^{13}CCl_3$	1.5	13.9	9.5	Ref. 7
Organic extract of $^{13}CCl_4$ liver perfusion	PBN/$\cdot{}^{13}CCl_3$	1.8	14.45	9.20	Ref. 10
Effluent perfusate of $^{13}CCl_4$ liver perfusion	PBN/$\cdot{}^{13}CO_2^-$	4.6	15.8	11.7	Ref. 10
Microsomal system with $^{13}CCl_4$, GSH, and cytosol	PBN/$\cdot{}^{13}CO_2^-$	4.6	15.8	11.7	Fig. 2
Microsomal system with $^{13}CCl_4$ and GSH	PBN/[GSH-$^\cdot CCl_3$]	2.84	15.36	11.44	Fig. 4

with lower amplitude was produced when $^{13}CCl_4$ was used instead of $^{13}CBrCl_3$ (Fig. 4B). The formation of this new radical adduct was dependent on the presence of halocarbon (Fig. 4C) and GSH (Fig. 4D). Furthermore, the ESR spectrum obtained from $^{13}CBrCl_3$ in the absence of NADPH (Fig. 4E) was substantially less intense than that of the complete system, demonstrating the expected dependence on NADPH. As expected, the radical adduct was not detected in an incubation containing heat-denatured microsomes (Fig. 4F).

Formation of the new radical adduct was dependent on GSH concentration (Fig. 5). As GSH concentrations were increased to 1 mM, a dose-dependent increase in ESR intensity was observed; however, formation of the new radical adduct was independent of GSH concentrations between 1 and 20 mM (Fig. 5). Substitution of GSSG for GSH in the incubation medium produced a low intensity ESR spectrum attributable to trace amounts of GSH present in the GSSG. The new radical adduct was not formed in incubations where GSH was replaced by cysteine or *N*-acetylcysteine (10 mM) (data not shown).

Efforts to characterize the polarity of the new radical adduct were complicated by its sensitivity to oxygen. The ESR spectrum of the new radical adduct was lost rapidly unless all extraction procedures were conducted in a nitrogen atmosphere. However, analysis of the organic layer of an anaerobic chloroform extract of a microsomal incubation that contained the new radical adduct yielded the ESR spectrum of PBN/$^\cdot CCl_3$. The new radical adduct was detected only in the aqueous phase of the extraction, leading to the conclusion that it is charged or highly polar.

Kinetic evidence that the new GSH-dependent radical (PBN/[GSH-$^\cdot CCl_3$]) is a precursor of the PBN/$\cdot CO_2^-$ radical adduct. Time courses for radical adduct formation are shown in Fig. 6. The ESR signal of the PBN/[GSH-$^\cdot CCl_3$] radical adduct reached maximum intensity 500 sec after addition of NADPH to the microsomal system (Fig. 6A, *trace a*) and remained nearly constant during the next 500 sec. Fig. 6A, *trace b*, shows that the PBN/$\cdot CO_2^-$ radical does not form at any time in the absence of cytosol. Addition of rat liver cytosol to a microsomal incubation containing the PBN/[GSH-$^\cdot CCl_3$] radical adduct resulted in a rapid ($t_{0.5}$ = about 10 sec) decline in signal intensity of the GSH-dependent radical adduct to almost zero (Fig. 6B, *trace c*). Concomitantly, the intensity of the PBN/$\cdot CO_2^-$ radical adduct signal increased with a similar

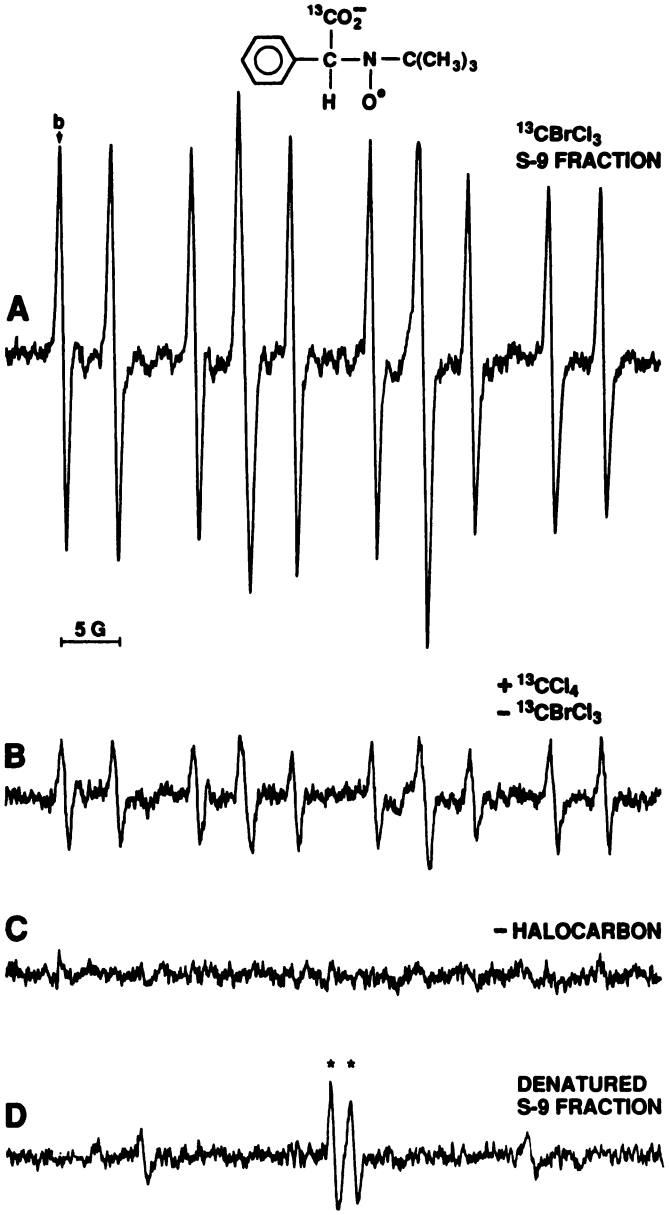

Fig. 3. Metabolism of $^{13}CCl_4$ or $^{13}CBrCl_3$ to PBN/$\cdot CO_2^-$ by rat liver postmitochondrial fraction. ESR spectra of complete nitrogen-saturated incubations containing halocarbon (1 mM), PBN (80 mM), and postmitochondrial S-9 liver fraction (5 mg of protein/ml) 500 sec after initiation of incubation. Spectrometer settings: modulation amplitude, 0.7 G; microwave power, 20 mW; gain, 8×10^6; scan time, 500 sec; time constant, 0.64 sec. *Ascorbyl semidione radical. A, ESR spectrum of PBN/$\cdot CO_2^-$ produced by a complete incubation system containing $^{13}CBrCl_3$. See Discussion for explanation of *arrow* at low field line. B, ESR spectrum of PBN/$\cdot CO_2^-$ produced by a complete incubation system containing $^{13}CCl_4$. C, ESR spectrum of an incubation system that did not contain halocarbon. D, ESR spectrum of an incubation system containing $^{13}CBrCl_3$ and denatured postmitochondrial S-9 liver fraction.

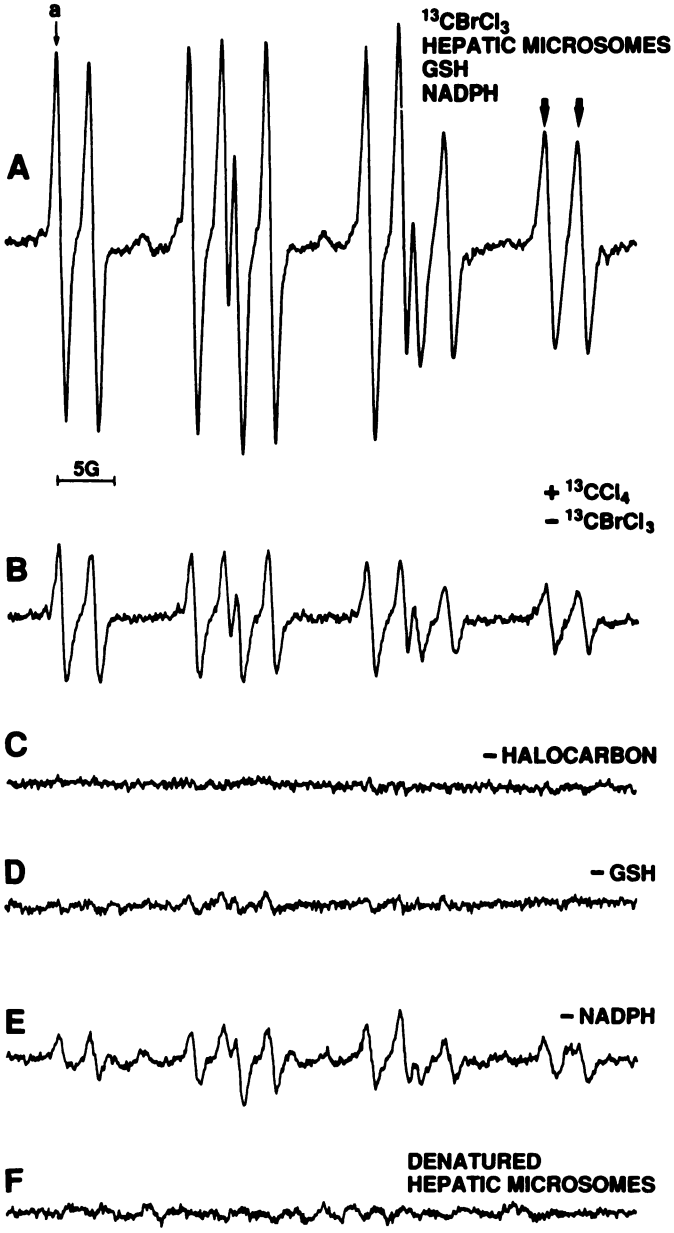

Fig. 4. Metabolism of CCl_4 by rat liver microsomes. ESR spectra of hepatic microsomal (2.5 mg of protein/ml) nitrogen-saturated complete incubations containing GSH (10 mM), halocarbon (1 mM), NADPH (1 mM), and PBN (80 mM) 500 sec after initiation of incubation. Spectrometer settings: modulation amplitude, 0.7 G; microwave power, 20 mW; gain, 4×10^5; scan time, 500 sec; time constant, 0.64 sec. A, ESR spectrum of PBN radical adduct produced by a complete incubation system containing $^{13}CBrCl_3$. See Discussion for explanation of *arrows*. B, ESR spectrum of PBN radical adduct produced by a complete incubation system containing $^{13}CCl_4$. C, ESR spectrum of an incubation system that did not contain halocarbon. D, ESR spectrum of an incubation system that contained $^{13}CBrCl_3$ but did not contain GSH. E, ESR spectrum of an incubation system that contained $^{13}CBrCl_3$ but did not contain NADPH. F, ESR spectrum of an incubation system that contained $^{13}CBrCl_3$ and denatured hepatic microsomes.

time-course after the addition of cytosol (Fig. 6B, *trace d*). The ESR signal intensity of the PBN/$\cdot CO_2^-$ radical adduct 100 sec after the addition of cytosol was approximately the same as the intensity of the PBN/[GSH-$^{\cdot}CCl_3$] radical adduct before the addition of cytosol.

Radical adduct concentrations determined from ESR spectra were used to investigate the stoichiometric relationship of formation of the PBN/$\cdot CO_2^-$ radical adduct from the PBN/[GSH-$^{\cdot}CCl_3$] radical adduct. Relative values of radical adduct concentrations were obtained using Eq. 1 (20).

$$C \propto A(LW)^2 \qquad (1)$$

In this equation, C represents the radical concentration in arbitrary units, A represents the amplitude of a nonoverlapped

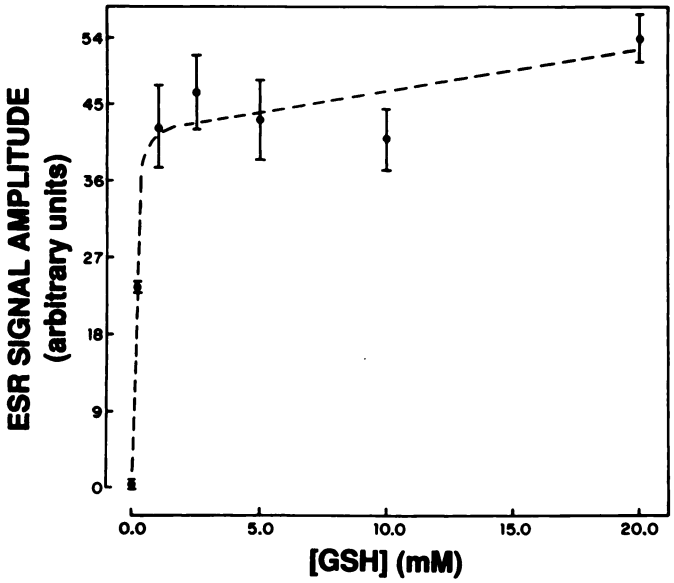

Fig. 5. ESR signal amplitude of GSH-dependent adduct as a function of glutathione concentration. Microsomal (2.5 mg of protein/ml) nitrogen-saturated incubations contained $^{13}CBrCl_3$ (1 mM), NADPH (1 mM), PBN (80 mM), and GSH as indicated on the *abscissa*. ESR signal amplitude measured 500 sec after initiation of incubation.

ESR line, and LW represents the line width of the ESR line. Because the low-field ESR lines of both radical adducts occur due to the same nuclear interactions with an unpaired electron (^{14}N, ^{1}H, ^{13}C), a concentration ratio can be determined (Eq. 2)

$$\frac{C_I}{C_{II}} = \frac{A_I(LW_I)^2}{A_{II}(LW_{II})^2} \qquad (2)$$

In this equation, I refers to PBN/[GSH–$^{\bullet}CCl_3$] and II refers to PBN/$\cdot CO_2^-$. A concentration ratio of 1.0 would indicate a perfect stoichiometric relationship between the two radical adducts. This relationship was evaluated with two experimental approaches. In the first method, the low-field ESR line of PBN/[GSH-$^{\bullet}CCl_3$] was evaluated at its time of maximum intensity, 500 sec after the incubation was initiated. Cytosol was then added to the incubation and the low-field ESR line for PBN/$\cdot CO_2^-$ was evaluated 100 sec later. After adjustment for dilution effects, C_I/C_{II} was found to be 0.9 ± 0.1. In the second design, either cytosol or buffer was added, immediately before initiation of the reaction with NADPH, to a GSH-containing microsomal incubation, and the ESR amplitude and linewidth were measured 500 sec later. Systems to which cytosol were added produced PBN/$\cdot CO_2^-$, whereas systems to which buffer was added produced only the PBN/[GSH-$^{\bullet}CCl_3$] radical adduct. Using the above equation, a relative concentration of 1.1 ± 0.2 was produced. Thus, within experimental error, there is a 1:1 stoichiometric relationship between the two radical adducts.

The ESR spectrum of the PBN/$\cdot CO_2^-$ radical adduct produced by incubation of $^{13}CBrCl_3$ with both microsomes and cytosol is shown in Fig. 7A. A similar spectrum of weaker intensity was produced when $^{13}CCl_4$ was used instead of $^{13}CBrCl_3$ (Fig. 7B). Production of the PBN/$\cdot CO_2^-$ radical adduct is completely halocarbon-dependent (Fig. 7C). Incubation in the absence of GSH (Fig. 7D) or NADPH (Fig. 7E) produced significant concentrations of radical adduct, due to the presence of GSH and NADPH in cytosol. ESR analysis of an incubation to which heat-denatured cytosol was added gave an ESR spectrum of PBN/[GSH-$^{\bullet}CCl_3$] (Fig. 7F). No spectrum was obtained when heat-denatured microsomes were incubated (Fig. 7G). To determine whether the cytosolic factor responsible for transformation of PBN/[GSH-$^{\bullet}CCl_3$] into PBN/$\cdot CO_2^-$ was a protein, cytosol was dialyzed. Dialysis using 10,000 and 12,000–14,000 molecular weight semipermeable membranes failed to remove the factor, as demonstrated by conversion of the PBN/[GSH-$^{\bullet}CCl_3$] radical adduct into the PBN/$\cdot CO_2^-$ when dialysate of cytosol was added to a GSH containing microsomal incubation.

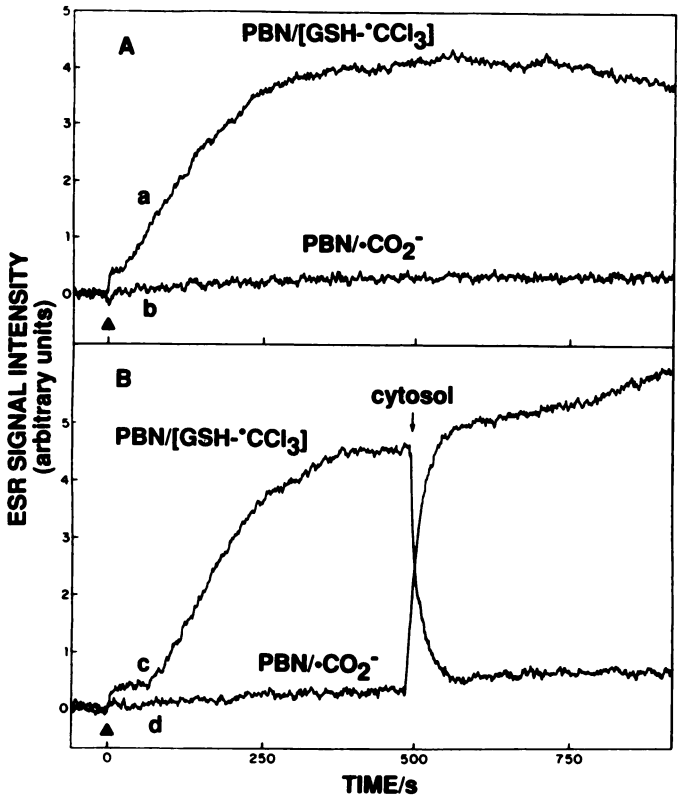

Fig. 6. Time course for formation of the PBN/$\cdot CO_2^-$ and GSH-dependent (PBN/[GSH-$^{\bullet}CCl_3$]) radical adducts. A, ESR signal intensity as a function of time for the microsomal incubation described in Fig. 4 with the magnetic field set on the low field line of the PBN/[GSH-$^{\bullet}CCl_3$] radical adduct (*a* in Fig. 4A) (*a*) and the magnetic field set on the low field line of the PBN/$\cdot CO_2^-$ radical adduct (*b* in Fig. 3A) (*b*). ▲, Initiation of the microsomal incubation with NADPH. B, ESR signal intensity as a function of time for the microsomal incubation/cytosol addition system described in Fig. 7 with the magnetic field set on the low field line of the PBN/[GSH-$^{\bullet}CCl_3$] radical adduct (*a* in Fig. 4A) (*c*) and the magnetic field set on the low field line of the PBN/$\cdot CO_2^-$ radical adduct (*b* in Fig. 3A) (*d*). ▲ Initiation of the microsomal incubation with NADPH; ↓, addition of cytosol.

Discussion

Role of glutathione in radical adduct formation from halocarbons in the perfused liver. It is well known that treatment of rats with DEM and BSO depletes GSH (21, 22). The PBN/$\cdot CO_2^-$ radical adduct was not detected in the effluent perfusate from livers in which GSH had been depleted by either DEM or BSO treatment (Fig. 1, C and E) under conditions otherwise identical to those of control livers, which released PBN/$\cdot CO_2^-$ into the perfusate at high rates (Fig. 1A). In contrast, PBN/$^{\bullet}CCl_3$ was detected in treated livers in amounts similar to that of control livers, demonstrating that formation of $^{\bullet}CCl_3$ by cytochrome P-450 metabolism of halocarbons was

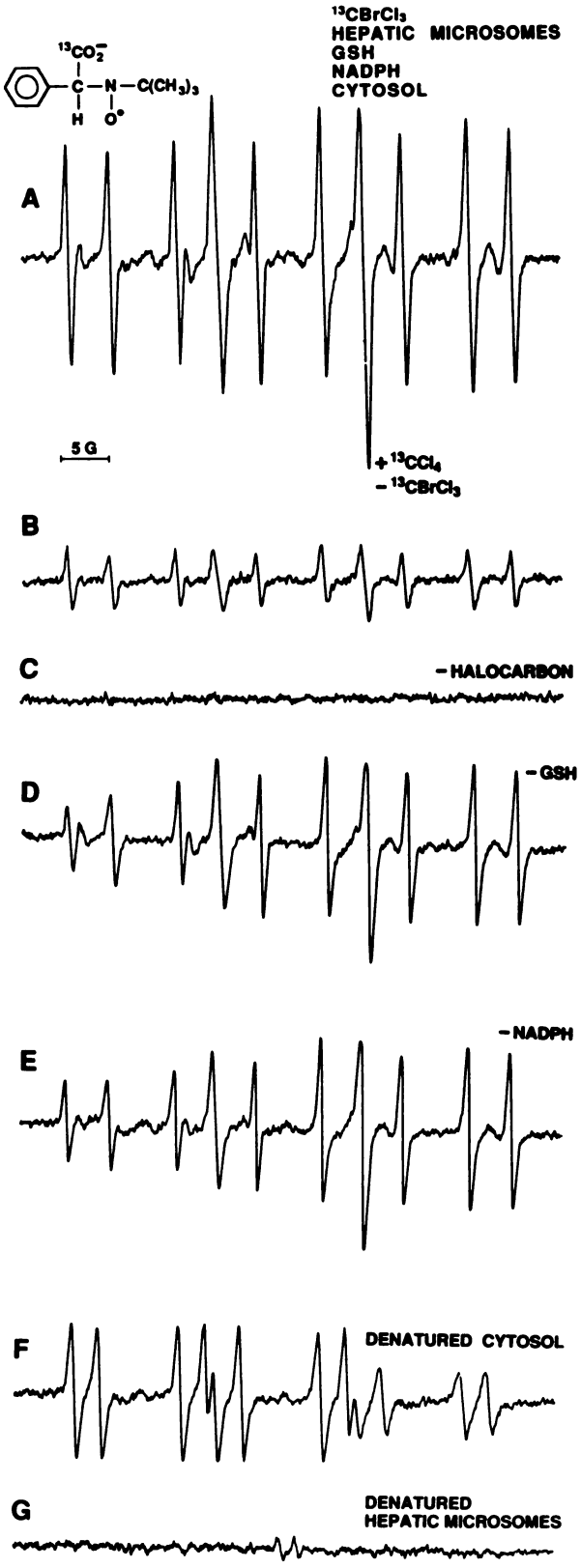

Fig. 7. Metabolism of CCl_4 by rat liver microsomes and cytosol. ESR spectra of microsomal systems described in Fig. 4, to which nitrogen-saturated cytosol (3 mg of protein/ml) was added 500 sec after the microsomal incubation was initiated with NADPH. Spectrometer settings: modulation amplitude, 0.7 G; microwave power, 20 mW; gain, 4×10^5; scan time, 500 sec; time constants, 0.64 sec. A, ESR spectrum of PBN/$\cdot CO_2^-$ produced by a complete incubation system containing $^{13}CBrCl_3$. B, ESR spectrum of PBN/$\cdot CO_2^-$ produced by a complete incubation system containing $^{13}CCl_4$. C, ESR spectrum of an incubation system that did not contain halocarbon. D, ESR spectrum of an incubation system containing $^{13}CBrCl_3$ that did not have GSH in the initial microsomal incubation. E, ESR spectrum of an incubation system containing $^{13}CBrCl_3$ that did not have NADPH in the initial microsomal incubation. F, ESR spectrum of an incubation system containing $^{13}CBrCl_3$ and heat-denatured cytosol. G, ESR spectrum of an incubation system containing $^{13}CBrCl_3$ and heat-denatured hepatic microsomes.

not affected by depletion of GSH. Because GSH was depleted in two different manners [i.e., by DEM by direct reaction with the thiol (21) and by BSO by inhibition of an enzyme involved in GSH formation (22)], the possibility that either agent was acting nonspecifically is unlikely. These results suggest strongly that GSH is involved in the formation of PBN/$\cdot CO_2^-$ from $^{\cdot}CCl_3$.

Intracellular localization of radical adduct formation from halocarbons. The high reactivity of the $^{\cdot}CCl_3$ radical is presumed to prevent it from diffusing far from its origin (23), hence ensuring that any trapping of radical products of $CBrCl_3$ or CCl_4 metabolism occurs within the cell. This hypothesis is supported by several experiments. First, LaCagnin *et al.* (11) found that DIDS, an anion transport inhibitor, decreased the efflux of PBN/$\cdot CO_2^-$ into the effluent of perfused liver but did not change the amount of PBN/$\cdot CO_2^-$ or PBN/$^{\cdot}CCl_3$ in the liver. They concluded that PBN/$\cdot CO_2^-$ formed within the cell is transported into the perfusate via an anion transporter mechanism. Second, the failure to detect radical adducts from perfusate or organic extracts of livers perfused with $CBrCl_3$ and charged sulfonated PBN suggested that radical formation and/or trapping did not occur outside the cell. Third, the cytosol-mediated production of PBN/$\cdot CO_2^-$ from a glutathione-dependent precursor showed that soluble intracellular components were necessary for PBN/$\cdot CO_2^-$ production. Thus, it is concluded that formation of PBN/$\cdot CO_2^-$ occurs in the cell.

New, glutathione-dependent, PBN radical adduct (PBN/[GSH-$^{\cdot}CCl_3$]) in halocarbon metabolism. When $CBrCl_3$ or CCl_4 was incubated with microsomes in the presence of GSH, a new radical adduct was discovered. This radical adduct was totally dependent on the presence of GSH in the incubation (Fig. 5) and exhibited a ^{13}C hyperfine splitting when formed with $^{13}CBrCl_3$ or $^{13}CCl_4$ (Fig. 4), indicating that it was halocarbon-derived. Recently, Reiter and Burk (24) reported that a GSH-dependent CCl_4 metabolite was formed in greatest concentration at low oxygen tension. They determined through ^{14}C and ^{35}S labeling studies that this metabolite contained one GSH unit for each CCl_4 metabolized. They also indicated that structural determinations were hindered due to the instability of the GSH adduct. These observations are analogous to the following characteristics of the PBN/[GSH-$^{\cdot}CCl_3$] radical adduct described in this study. The new PBN radical adduct, derived from halocarbon, requires GSH for its formation. The retention of the adduct in the aqueous phase of organic extractions of microsomal incubations is characteristic of charged radical adducts (10); the charge on this radical adduct is most likely due to ionization of glutathione at physiological pH. Line width increases in the ESR spectrum due to slower free radical rotation in solution support the presence of GSH in the new radical adduct. Nitroxide free radicals that rotate more slowly in aqueous solution due to large substituents such as GSH produce ESR spectra in which the high-field lines have broader line widths and hence lower amplitudes (25). Consistent with

this possibility, the high-field lines of the GSH-dependent radical adduct (Fig. 4A, at *arrows*) are shorter and wider than the corresponding lines of the PBN/$\cdot CO_2^-$ radical adduct (Fig. 2A, at *arrows*). Immobilization, such as occurs in microsomal lipid bilayers, could also slow rotation; however, the hydrophilicity of the radical adduct makes this possibility unlikely. Finally, the PBN/[GSH-$^\cdot CCl_3$] radical adduct was not stable in the presence of oxygen. As in the studies of Reiter and Burk (24), this instability will probably prevent complete structural determination of the radical adduct. Taken together, these observations justify representation of this new radical adduct as PBN/[GSH-$^\cdot CCl_3$], for which the brackets indicate our uncertainty of the radical adduct's precise chemical structure.

Conversion of PBN/[GSH-$^\cdot CCl_3$] into PBN/$\cdot CO_2^-$. Experiments utilizing the two components of the postmitochondrial S-9 fraction, i.e., microsomes and cytosol, demonstrate that the PBN/[GSH-$^\cdot CCl_3$] radical adduct (Fig. 3) was converted rapidly by a cytosolic component into the PBN/$\cdot CO_2^-$ radical adduct (Figs. 6 and 7). The addition of cytosol to a microsomal incubation caused the PBN/[GSH-$^\cdot CCl_3$] radical adduct to disappear while the PBN/$\cdot CO_2^-$ radical adduct appeared simultaneously and stoichiometrically (Fig. 6B, *traces c* and *d*). The ratio of the concentrations of these two radical adducts was near unity. These data strongly support the postulate that the PBN/[GSH-$^\cdot CCl_3$] radical adduct is converted quantitatively into the PBN/$\cdot CO_2^-$ radical adduct. The heat-sensitive cytosolic constituent that causes this transformation appears to be a protein of molecular weight greater than 12,000, based on dialysis results. Therefore, we conclude that a previously unidentified GSH-containing radical adduct, formed during the metabolism of CCl_4, is converted by a cytosolic factor to PBN/$\cdot CO_2^-$.

Formation of the PBN/$\cdot CO_2^-$ radical adduct during $CBrCl_3$ and CCl_4 metabolism in the whole cell. It was suggested previously that the formation of the PBN/$\cdot CO_2^-$ radical adduct from $^\cdot CCl_3$ involved molecular oxygen (10). In this proposed pathway, the trichloromethylperoxyl radical, formed from the reaction of $^\cdot CCl_3$ with O_2, was a secondary product of the reductive metabolism of CCl_4. Decomposition of the trichloromethylperoxyl radical to $\cdot C(=O)-Cl$ could be followed by hydrolysis of $\cdot C(=O)-Cl$ to $\cdot CO_2^-$; PBN would then react with $\cdot CO_2^-$ to give PBN/$\cdot CO_2^-$. However, the only evidence for this proposal was that PBN/$\cdot CO_2^-$ contained oxygen, and the $^\cdot CCl_3$ radical is highly reactive with oxygen (14).

Recent studies, however, have cast doubt on the role of O_2 in PBN/$\cdot CO_2^-$ formation. First, Janzen *et al.* (12) described the formation of PBN/$\cdot CO_2^-$ in a vacuum-degassed microsomal incubation. Second, both LaCagnin *et al.* (11) and Knecht and Mason (13) reported much more PBN/$\cdot CO_2^-$ formation in hypoxic compared with normoxic rat liver systems. Taken together, these results suggest strongly that O_2 is not involved in the mechanism of PBN/$\cdot CO_2^-$ radical adduct formation. Further, these data imply that PBN/$\cdot CO_2^-$ is not formed by the reaction of PBN with $\cdot CO_2^-$ or $\cdot C(=O)-Cl$.

The most likely explanation for the formation of PBN/$\cdot CO_2^-$ without involvement of molecular O_2 is a hydrolytic reaction that converts C—Cl bonds to C—O bonds. In this scheme, both oxygens in PBN/$\cdot CO_2^-$ would come from water. Two possible mechanisms are (a) direct conversion of PBN/$^\cdot CCl_3$ into PBN/$\cdot CO_2^-$ and (b) formation of PBN/[GSH-$^\cdot CCl_3$] from a free radical GSH conjugate of $^\cdot CCl_3$ or another CCl_4-derived radical, with subsequent conversion of this radical adduct into PBN/$\cdot CO_2^-$. The first process would involve a sequence of spontaneous hydrolysis and elimination reactions to convert PBN/$^\cdot CCl_3$ into PBN/$\cdot CO_2^-$. ESR analysis of the organic extracts of rat livers with significantly decreased GSH concentrations gave strong spectra of PBN/$^\cdot CCl_3$ (Fig. 1, D and F), whereas PBN/$\cdot CO_2^-$ was not detected in perfusate from these livers. Thus, PBN/$^\cdot CCl_3$ was formed in these livers but was not transformed into PBN/$\cdot CO_2^-$. This makes the former proposal unlikely. On the other hand, a critical role for GSH in the transformation of $^\cdot CCl_3$ into PBN/$\cdot CO_2^-$ has been established in this study. The data support a process in which $^\cdot CCl_3$ or a $^\cdot CCl_3$-derived radical reacts with GSH to form a GSH conjugate of a free radical. A GSH conjugate of $^\cdot CCl_3$ or a $^\cdot CCl_3$-derived radical has been trapped by PBN and subsequently detected by ESR. Furthermore, this PBN/[GSH-$^\cdot CCl_3$] radical adduct is transformed stoichiometrically by a cytosolic component into PBN/$\cdot CO_2^-$. Thus, it is concluded that both oxygens in PBN/$\cdot CO_2^-$ arise from water via an intermediate containing glutathione.

In the perfused liver, PBN/$\cdot CO_2^-$ is formed from halocarbon and is a marker for carbon tetrachloride free radical metabolite formation. Further, it has been demonstrated that formation of PBN/$\cdot CO_2^-$ correlates with cell death (11). This study demonstrates that hepatic GSH concentrations play an important role in this overall process.

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