0026-895X/88/030351-07$02.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 33:351-357

MOL PHARM

The Carbon Dioxide Anion Radical Adduct in the Perfused Rat Liver: Relationship to Halocarbon-Induced Toxicity

LYNN B. LaCAGNIN, HENRY D. CONNOR,[1] RONALD P. MASON, and RONALD G. THURMAN

Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27514 (L.B.L., R.G.T.) and Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 (L.B.L., H.D.C., R.P.M.)

Received August 24, 1987; Accepted December 10, 1987

SUMMARY

CCl_4 has been shown previously to be metabolized to the trichloromethyl radical ($\cdot CCl_3$) and to a novel oxygen-containing carbon dioxide anion radical ($\cdot CO_2^-$) in the perfused rat liver and *in vivo*. Since the role of free radicals in CCl_4-induced hepatotoxicity is unclear, these studies were designed to determine if a relationship between $\cdot CO_2^-$ formation and halocarbon-induced hepatotoxicity exists. CCl_4 or bromotrichloromethane ($CBrCl_3$) was infused into livers from control or phenobarbital-treated rats perfused with either nitrogen- or oxygen-saturated Krebs-Henseleit bicarbonate buffer. Samples of effluent perfusate and chloroform/methanol extracts of liver were analyzed by ESR spectroscopy for free radical adducts following infusion of halocarbon and the spin trap, phenyl-*t*-butylnitrone (PBN). Hyperfine coupling constants and ^{13}C-isotope effects observed in the ESR spectra of organic extracts of liver demonstrated the presence of the PBN radical adduct of $\cdot CCl_3$ from both halocarbons. Radical adducts in aqueous extracts of liver and effluent perfusate had hyperfine coupling constants and ^{13}C-isotope effects identical to those of PBN/$\cdot CO_2^-$ generated chemically from formate. The PBN/$\cdot CO_2^-$ radical adduct was also observed in urine following the intragastric administration of $CBrCl_3$ and PBN. Detection of PBN/$\cdot CO_2^-$ adducts in the effluent perfusate was decreased 3- to 4-fold by DIDS (0.2 mM), an inhibitor of the plasma membrane anion transport system. The rate of formation of PBN/$\cdot CO_2^-$ was decreased 2- to 3-fold following inhibition of cytochrome P-450-dependent monooxygenases by metyrapone (0.5 mM) and was increased about 2-fold by induction of cytochrome P-450 by phenobarbital pretreatment. Toxicity of halocarbons in the perfused liver was assessed by measuring the release of lactate dehydrogenase (LDH) into the effluent perfusate in livers from phenobarbital-treated rats under conditions identical to those employed to detect radical adducts (i.e., during the infusion of CCl_4 or $CBrCl_3$ into livers perfused with either nitrogen- or oxygen-saturated perfusate). Under all conditions studied, PBN/$\cdot CO_2^-$ was detected in the effluent perfusate within 2–4 min. Metabolism of halocarbons to PBN/$\cdot CO_2^-$ was 6- to 8-fold faster during perfusion with nitrogen-saturated rather than with oxygen-saturated perfusate. Concomitantly, liver damage detected from LDH release occurred much sooner during halocarbon infusion in the presence of nitrogen-saturated rather than oxygen-saturated perfusate. A good correlation between the rate of formation of PBN/$\cdot CO_2^-$ and the time of onset of LDH release following halocarbon infusion was observed. Therefore, it is concluded that PBN/$\cdot CO_2^-$ is a useful marker for free radical intermediates which may be related causally to halocarbon-induced hepatotoxicity.

It has been well established that the metabolism of CCl_4 by cytochrome P-450-dependent monooxygenases is involved in its hepatotoxicity (1–4). The immediate consequences of the metabolic activation of CCl_4 are lipid peroxidation in microsomes (3–5) and covalent binding of ^{14}C and ^{36}Cl from labeled CCl_4 to microsomal lipids and proteins (6, 7). However, the relative contribution of lipid peroxidation and covalent binding as well as the subsequent events leading to centrilobular necrosis of the liver remain unclear.

Experiments employing ESR and the spin-trapping technique have demonstrated that the metabolism of CCl_4 produces carbon-centered free radicals. The trichloromethyl radical ($\cdot CCl_3$), a reductive dehalogenation product of CCl_4, has been detected as the PBN/$\cdot CCl_3$ radical adduct in a number of biological systems, including liver microsomes (8, 9), isolated hepatocytes (8), and the isolated perfused liver (10) as well as in livers of rats given CCl_4 *in vivo* (8, 9). Recently, the PBN radical adduct of a novel oxygen-containing radical metabolite of CCl_4, the carbon dioxide anion radical adduct (PBN/$\cdot CO_2^-$), was discovered in the effluent perfusate of the isolated perfused liver following infusion of CCl_4 (10). The PBN/$\cdot CO_2^-$ radical

This work was supported, in part, by National Institute of Environmental Health Sciences (NIEHS) Grant ES-02759 (R.G.T.). The costs of publication of this article were defrayed, in part, by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was done while L. B. L. held a National Research Council-NIEHS/National Institutes of Health Research Associateship.

[1] Present address: Department of Chemistry, Kentucky Wesleyan College, Owensboro, KY 42301.

ABBREVIATIONS: PBN, phenyl *N*-*t*-butylnitrone (with the IUPAC name *N*-*tert*-butyl-α-phenylnitrone); LDH, lactate dehydrogenase; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

adduct was also detected in the urine of rats which had been given CCl_4 intragastrically (10).

Although the formation of free radical metabolites of CCl_4 has been demonstrated, their role in the mechanism of CCl_4-induced hepatotoxicity remains unclear. It was the objective of this study to determine if a quantitative relationship between the rate of free radical formation and liver damage exists. The metabolism of CCl_4 and $CBrCl_3$, which, like CCl_4, is metabolized to the trichloromethyl radical, was examined using the isolated perfused rat liver as a model. ESR spectroscopy was used to detect the PBN/$\cdot CO_2^-$ radical adduct in the effluent perfusate, and LDH release was measured as an index of irreversible cell death.

Materials and Methods

PBN, DIDS, metyrapone, ascorbate oxidase, catalase, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Fremy's salt (potassium nitrosodisulfonate, 95%) was obtained from Alfa Products (Danvers, MA). Hydrogen peroxide (10%; American Chemical Society certified), 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).

Fed, 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 prior to perfusion experiments. Livers from normal or phenobarbital-treated rats 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 (11). The perfusate was pumped into the liver at a rate of 4 ml/g/min via a cannula placed in the portal vein, and perfusate 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 (5 mM) or DIDS (0.2 mM) was dissolved in the perfusate, whereas CCl_4 or $CBrCl_3$ (final concentration of 1 mM) was bound to albumin (final concentration of 0.2%) by stirring for 16 h. Metyrapone was dissolved in perfusate and infused into the liver at a final concentration of 0.5 mM. LDH activity in effluent perfusate was determined by standard enzymatic procedures (12).

Liver samples were homogenized in perfusion buffer (5 ml/g), extracted with a $CHCl_3$/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, gassed with nitrogen for 3 min, and placed in a quartz sample tube for ESR analysis. The aqueous layer of the extract and aqueous perfusate samples were bubbled with oxygen for 10 min and then with nitrogen for 5 min prior to ESR analysis for the following reason. We found that the ESR spectrum from a given sample of perfusate increased in intensity for several hours. Presumably, this is due to oxidation by oxygen of the hydroxylamine formed by the partial reduction of the nitroxide moiety of the radical adduct. We found, however, that perfusate samples bubbled with oxygen for 10 min and then with nitrogen for 5 min yielded stable ESR signals identical to the spectra of untreated samples allowed to remain at room temperature for several hours. On the basis of these findings, we routinely treated the aqueous layer of liver extracts and the aqueous perfusate samples by bubbling with oxygen for 10 min and then with nitrogen for 5 min. Bubbling with nitrogen decreases oxygen-dependent ESR line broadening of the radical adduct.

For the analysis of PBN adducts in urine, fasted (24 hr) rats were given PBN (0.02 g/kg) and $CBrCl_3$ (0.6 g/kg) in corn oil intragastrically three times at 0.5-hr intervals. About 2 hr after the last dose, rat urine was collected in a Petri dish and was washed into a small (3-ml) glass vial with an equal volume of perfusion buffer. Ascorbate oxidase (4 μl containing 1 unit) and catalase (4.7 μl containing 1 unit) were added and the solution was bubbled with oxygen for 15 min followed by nitrogen for 5 min to decrease the ascorbate free radical ESR signal. The urine sample was then transferred to an ESR quartz flat cell for analysis.

The rate of formation of the PBN/$\cdot CO_2^-$ radical adduct was quantitated by comparing the amplitude of the maximized ESR spectral lines of PBN/$\cdot CO_2^-$ to that of 0.1 mM Fremy's salt in 10 mM K_2CO_3 (13). The concentration of Fremy's salt was determined spectrophotometrically at 248 nm using an extinction coefficient of 1690 cm^{-1} M^{-1} (14). The amplitude of the spectral lines of PBN/$\cdot CO_2^-$ or Fremy's salt was maximized by adjusting the microwave power and modulation amplitude of the ESR spectrometer.

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 centered in an ER-4103 TM microwave cavity for analysis.

Results

The effects of PBN and $CBrCl_3$ on oxygen uptake by the isolated, perfused liver are illustrated in Fig. 1. The basal rate of O_2 uptake was 120 μmol/g/hr. Infusion of albumin into the perfused liver increased oxygen uptake to approximately 127 μmol/g/hr, most likely due to the metabolism of contaminating fatty acids present in the albumin. PBN (5 mM) increased oxygen uptake initially to about 150 μmol/g/hr, which declined subsequently to a new steady state level of approximately 144 μmol/g/hr, possibly resulting from monooxygenation of the spin trap. Infusion of $CBrCl_3$ (1 mM) produced a small, transient increase followed by a progressive decrease in O_2 uptake to a value of approximately 40 μmol/g/hr after 60 min.

ESR analysis of aqueous perfusate, which was collected during infusion of $^{12}CBrCl_3$ and PBN, yielded a stable six-line spectrum (a^N = 15.88 G and a_β^H= 4.65 G) which was identified as the carbon dioxide anion radical adduct similar to that detected previously during CCl_4 infusion (10). During infusion of $^{13}CBrCl_3$, the corresponding ESR spectrum had 12 lines with hyperfine coupling constants of a^N = 15.90 G; a_β^H = 4.60 G; and a_β^{13C} = 11.86 G (Table 1). A six-line spectrum similar to that

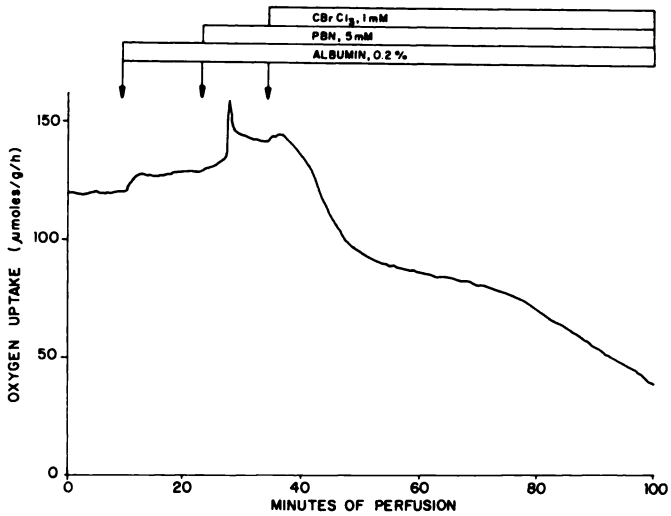

Fig. 1. Effect of PBN and $CBrCl_3$ on O_2 uptake by the isolated perfused liver. Liver from a fed, phenobarbital-treated rat was perfused with Krebs-Henseleit bicarbonate buffer for the times indicated. Oxygen concentration was monitored continuously with a Clark-type O_2 electrode and values were converted into rates employing the influent-effluent concentration difference, the flow rate, and the liver wet weight. Additions are depicted by *horizontal bars* and *arrows*. A typical experiment is shown.

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

TABLE 1

Hyperfine coupling constants of radical adducts derived from bromotrichloromethane or carbon tetrachloride in rat liver

Source	Structure	Hyperfine coupling constants (gauss)			
		a_β^H	a^N	a_β^{13C}	Source
Effluent perfusate of $^{13}CBrCl_3$ liver perfusion	PBN/$^{13}\cdot CO_2^-$	4.60	15.90	11.86	This work
Effluent perfusate of $^{13}CCl_4$ liver perfusion	PBN/$^{13}\cdot CO_2^-$	4.60	15.80	11.70	Ref. 10
Rat urine after $CBrCl_3$ administration	PBN/$^{13}\cdot CO_2^-$	4.40	15.80		Fig. 2A
Organic extract of $^{13}CBrCl_3$ liver perfusion	PBN/$^{13}\cdot CCl_3$	1.85	14.38	9.15	This work
Organic extract of $^{13}CCl_4$ liver perfusion	PBN/$^{13}\cdot CCl_3$	1.85	14.45	9.20	Ref. 10

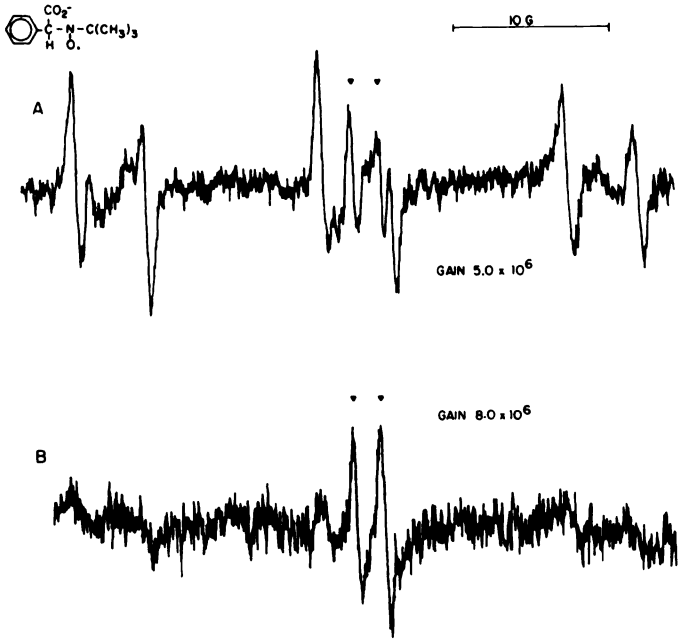

Fig. 2. ESR spectrum of rat urine. A. Spectrum of urine collected from rat 2 hr after treatment with PBN (0.02 g/kg) and $CBrCl_3$ (0.6 g/kg) in corn oil. Spectrometer settings were: scan range, 50 G; modulation amplitude, 1.0 G; microwave power, 20.9 mW; scan time, 1.4 hr; time constant, 5 sec; gain, 5.0×10^6. B. Spectrum of urine collected from rat 2 hr after treatment with PBN (0.02 g/kg) alone in corn oil. Spectrometer settings were the same as in A except gain, 8.0×10^6. Ascorbate free radical spectrum (▼) was decreased by addition of ascorbate oxidase and bubbling with oxygen (details are under Materials and Methods).

produced from CCl_4 and identified as the PBN adduct of the trichloromethyl radical was observed upon ESR analysis of organic extracts of the liver after perfusion with $CBrCl_3$ and PBN. Confirmation of this spectral assignment was provided by the 12-line ESR spectrum ($a^N = 14.38$ G; $a_\beta^H = 1.85$ G; $a_\beta^{13C} = 9.15$ G) obtained from the organic extract of a liver into which $^{13}CBrCl_3$ was infused (Table 1). No ESR spectra were detected in the perfusate or liver extracts when PBN was perfused in the absence of halocarbon. ESR spectra with hyperfine coupling constants characteristic of the PBN/$\cdot CO_2^-$ radical adduct were observed in rat urine collected 2 hr after intragastric administration of PBN and $CBrCl_3$ (Fig. 2A, Table 1). The PBN/$\cdot CO_2^-$ radical adduct was not detected in urine of rats treated with PBN and corn oil alone (Fig. 2B).

CCl_4 or $CBrCl_3$ was infused into livers perfused with either nitrogen- or oxygen-saturated perfusate and the time course of PBN/$\cdot CO_2^-$ formation and release of LDH was measured. Under all perfusion conditions studied, PBN/$\cdot CO_2^-$ was detected in the effluent perfusate within 2–4 min (Figs. 3A and 4A). During the infusion of CCl_4 or $CBrCl_3$ in the presence of oxygen-saturated perfusate, the rate of formation of PBN/$\cdot CO_2^-$ was relatively constant (10–15 nmol/g/hr) for 60 min. The production of PBN/$\cdot CO_2^-$ was 6- to 8-fold greater during perfusion with nitrogen-saturated rather than oxygen-saturated perfusate. LDH was released initially into the effluent perfusate within 15–30 min of onset of halocarbon infusion (Figs. 3B and 4B). The rate of release reached a maximum

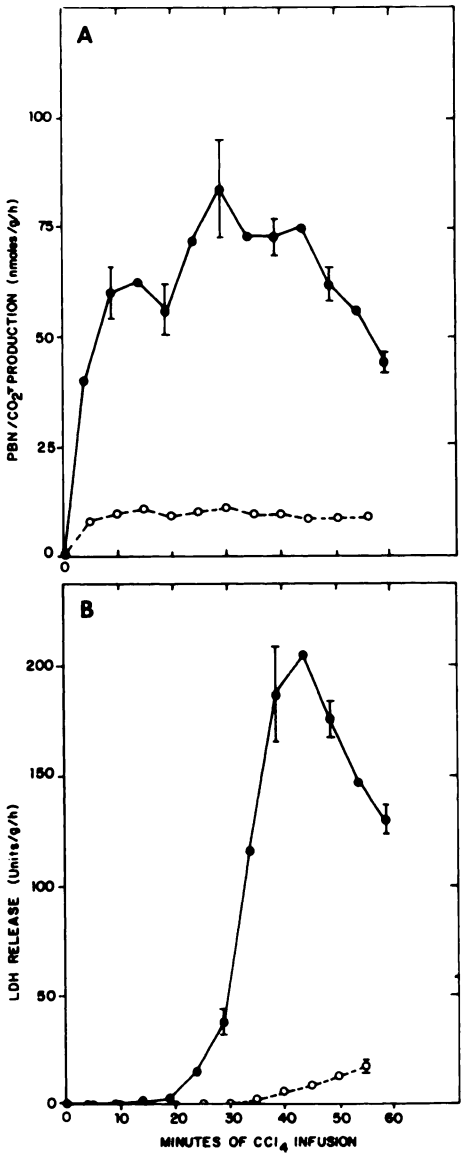

Fig. 3. The effect of nitrogen on the metabolism and toxicity of CCl_4 in the perfused rat liver. CCl_4 (1 mM) was infused into livers from phenobarbital-treated rats perfused with oxygen-saturated (○) or nitrogen-saturated perfusate (●) as described under Materials and Methods. The rate of formation of PBN/$\cdot CO_2^-$ (A) or the rate of LDH release (B) was plotted as a function of time of CCl_4 infusion. Values are expressed as the mean (±standard error) of three to six livers.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

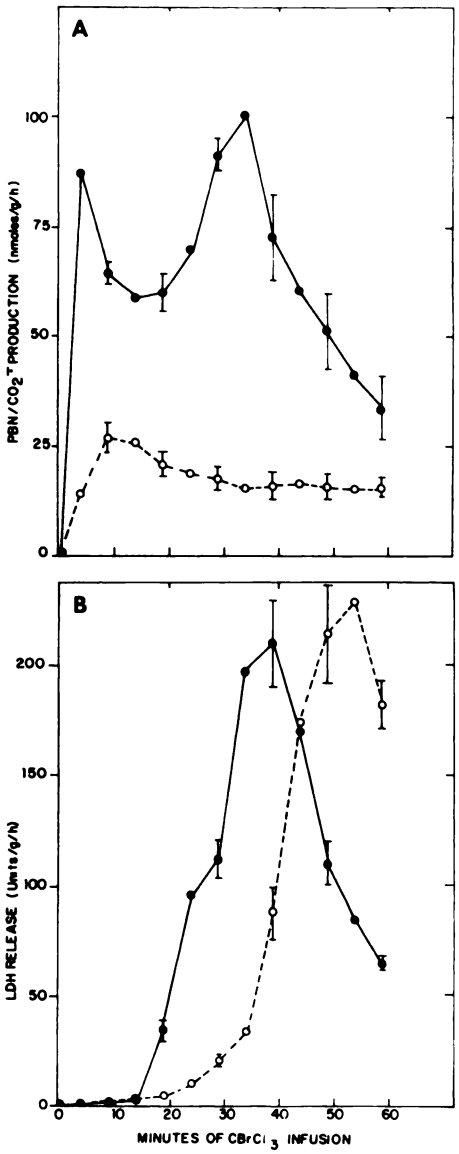

Fig. 4. The effect of nitrogen on the metabolism and toxicity of $CBrCl_3$ in the perfused rat liver. $CBrCl_3$ (1 mM) was infused into livers from phenobarbital-treated rats perfused with oxygen-saturated (○) or nitrogen-saturated perfusate (●) as described under Materials and Methods. The rate of formation of PBN/$\cdot CO_2^-$ (A) or the rate of LDH release (B) was plotted as a function of time of $CBrCl_3$ infusion. Values are expressed as the mean (±standard error) of four livers.

value of approximately 240 units/g/hr in 40–50 min under all conditions studied with the exception of CCl_4 infusion in the presence of oxygen-saturated perfusate, where it only reached a maximum value of approximately 25 units/g/hr (Fig. 3B). Liver damage reflected by LDH release occurred more rapidly during infusion of either halocarbon in the presence of nitrogen-saturated rather than oxygen-saturated perfusate. In the absence of halocarbon, LDH release was not affected by nitrogen-saturated perfusate. A good correlation between the rate of formation of PBN/$\cdot CO_2^-$ and the time of onset of LDH release in the effluent perfusate was observed (Fig. 5). No LDH was released into the effluent perfusate during perfusion in the absence of halocarbon. PBN did not protect against halocarbon-induced LDH release, presumably because it traps only a small fraction of halocarbon-derived radicals.

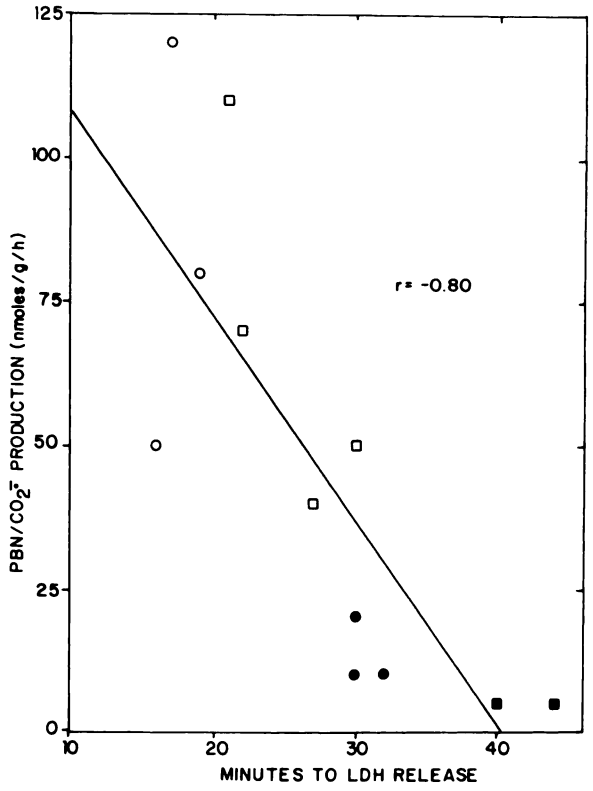

Fig. 5. Correlation between the rate of formation of PBN/$\cdot CO_2^-$ and the time of onset of LDH release. CCl_4 (□, ■) or $CBrCl_3$ (○, ●; 1 mM) was infused into livers from phenobarbital-treated rats perfused with oxygen-saturated (■, ●) or nitrogen-saturated (□, ○) perfusate. The rate of formation of PBN/$\cdot CO_2^-$ was plotted versus the time to onset of LDH release. Each *symbol* represents data from one liver.

When cytochrome P-450 content was increased by phenobarbital pretreatment, PBN/$\cdot CO_2^-$ production increased 2-fold when compared to untreated controls following infusion of CCl_4 into livers perfused with nitrogen-saturated perfusate (Fig. 6A). In addition, LDH release occurred 10–15 min sooner in perfused livers from phenobarbital-treated rats than in those from untreated rats (Fig. 6B). Metyrapone (0.5 mM), an inhibitor of cytochrome P-450 monooxygenases, decreased the formation of PBN/$\cdot CO_2^-$ 2- to 3-fold (Fig. 6A).

The concentration of PBN/$\cdot CO_2^-$ in the effluent perfusate was decreased 3- to 4-fold in the presence of DIDS (0.2 mM), an inhibitor of anion transport (Fig. 7). DIDS did not decrease the PBN/$\cdot CCl_3$ or PBN/$\cdot CO_2^-$ radical adducts in the organic or aqueous layers of liver extracts, respectively (data not shown); therefore, it is concluded that DIDS did not inhibit the formation of PBN/$\cdot CCl_3$ or PBN/$\cdot CO_2^-$ in the perfused liver.

Discussion

These studies demonstrate that CCl_4 and $CBrCl_3$ are metabolized to carbon-centered free radicals in a similar manner in the perfused rat liver. The lipid-soluble trichloromethyl radical adduct (PBN/$\cdot CCl_3$) was detected in organic extracts of livers infused with either CCl_4 or $CBrCl_3$ (Table 1). Furthermore, the carbon dioxide anion radical adduct of PBN (PBN/$\cdot CO_2^-$) was detected in the aqueous layer of liver extracts and in the effluent perfusate following CCl_4 or $CBrCl_3$ infusion (Table 1). Halocarbon metabolism to $\cdot CO_2^-$ was 6- to 8-fold greater during perfusion under hypoxic conditions (i.e., nitrogen-saturated

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

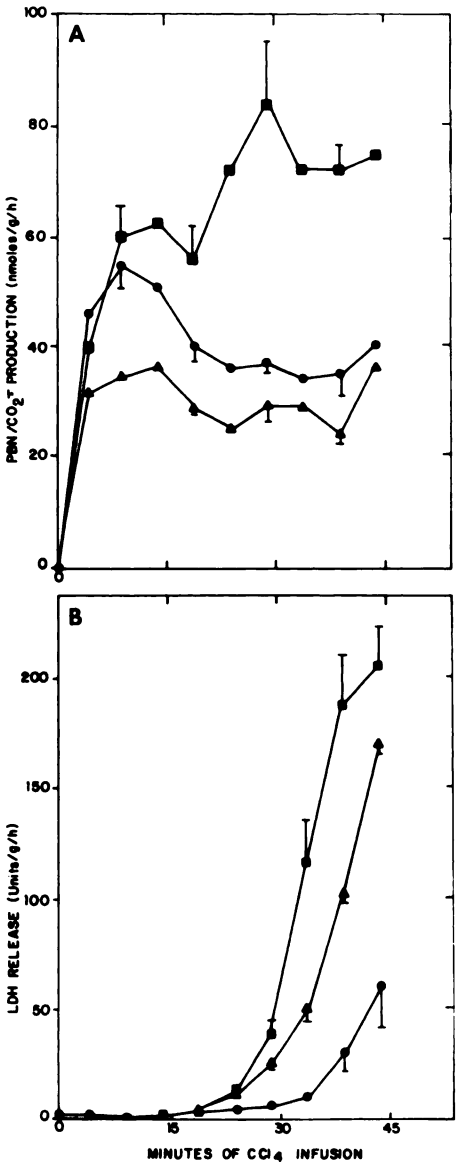

Fig. 6. The effect of metyrapone and phenobarbital treatment on the metabolism and toxicity of CCl_4 in the perfused liver. CCl_4 (1 mM) was infused into livers from phenobarbital-treated (■) or untreated (●) rats or into livers from phenobarbital-treated rats in the presence of 0.5 mM metyrapone (▲) perfused with nitrogen-saturated Krebs-Henseleit buffer as described under Materials and Methods. The rate of formation of PBN/$\cdot CO_2^-$ (A) or the rate of LDH release (B) was plotted as a function of time of CCl_4 infusion. Values are expressed as the mean (±standard error) of four to six livers.

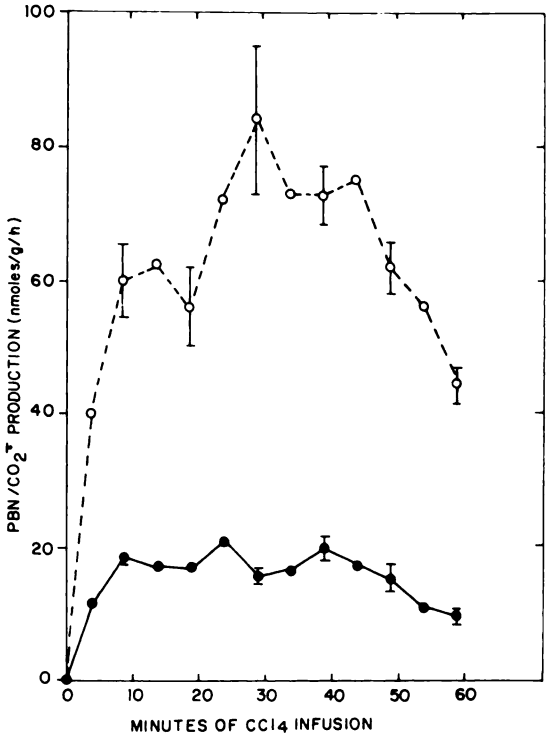

Fig. 7. The effect of DIDS on PBN/$\cdot CO_2^-$ concentration in the effluent perfusate. CCl_4 (1 mM) was infused into livers from phenobarbital-treated rats perfused with nitrogen-saturated Krebs-Henseleit buffer in the presence (●) or absence (○) of DIDS (0.2 mM) as described under Materials and Methods. Values are expressed as the mean (±standard error) of four to six livers.

perfusate) than under normal oxygen tension (Figs. 3A and 4A). It has been clearly established that CCl_4 is metabolically activated under anaerobic conditions to give a much higher yield of covalently bound product (presumably $\cdot CCl_3$) than is found under aerobic conditions (15). Even studies on CCl_4-induced lipid peroxidation show enhanced metabolic activation by hypoxia (16, 17). Since the rate of formation of PBN/$\cdot CO_2^-$ was faster at low oxygen tension, it is concluded that the carbon dioxide anion radical is derived from the trichloromethyl radical and, therefore, may serve as a marker for $\cdot CCl_3$ production. The PBN/$\cdot CO_2^-$ radical adduct was also found in the urine after pretreatment with $CBrCl_3$ (Fig. 2), confirming earlier studies with CCl_4 (10).

It was somewhat surprising that the metabolism of $CBrCl_3$ was only slightly greater than that of CCl_4 in the perfused rat liver (Figs. 3A and 4A), since $CBrCl_3$ is metabolized to $\cdot CCl_3$ faster than CCl_4 in isolated microsomes. For example, Slater and Sawyer (18) reported that the bond dissociation energy for the hemolytic cleavage of the C-Br bond of $CBrCl_3$ is considerably less than for cleavage of the C-Cl bond of CCl_4, implying a greater tendency for free radical formation. Similarly, Mico *et al.* (19) reported that approximately 35 times more electrophilic chlorine was formed in rat liver microsomes incubated with $CBrCl_3$ than with CCl_4. One explanation for differences between the results of this study and those of others may involve the experimental model employed. Subcellular components, such as microsomal suspensions, were used in previously reported investigations, whereas the isolated perfused liver, which is a whole cell, nearly physiological model, was employed in the work reported here. In studies utilizing subcellular components, NADPH, a necessary cofactor in the metabolism of $CBrCl_3$ or CCl_4, was supplied in excess. This is not the case in the perfused liver where NADPH supply is regulated and may be compromised by hypoxia and/or halocarbon addition (20, 21). Therefore, NADPH supply in the cell may limit $\cdot CCl_3$ formation from both halocarbons and may be responsible for the observation that PBN/$\cdot CO_2^-$ was formed at similar rates with $CBrCL_3$ and CCl_4. (Figs. 3A and 4A).

Since PBN/$\cdot CO_2^-$ is a charged species, it is most likely transported across biological membranes via an anion transport carrier system. To evaluate this possibility, DIDS, an inhibitor of sulfate-hydroxide anion exchange, sulfate-bicarbonate exchange, and bicarbonate-chloride exchange in hepatocytes (22–

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

24) and sulfate exchange in the perfused rat liver (25), as well as bicarbonate-chloride exchange in erythrocytes (26, 27) and Ehrlich ascites tumor cells (28), was perfused during infusion of halocarbon. Rates of efflux of PBN/$\cdot CO_2^-$ from the liver were decreased 3- to 4-fold by DIDS (Fig. 7), supporting the hypothesis that the anion radical adduct leaves the cell via a carrier-mediated transport process. In addition, PBN/$\cdot CO_2^-$ may be released from the cell following halocarbon-induced cell lysis.

The objective of these investigations was to determine whether a correlation between rates of PBN/$\cdot CO_2^-$ formation and liver damage exists. Various factors affecting PBN/$\cdot CO_2^-$ production were examined in conjunction with the measurement of LDH release into the effluent perfusate as an index of irreversible cell injury following infusion of CCl_4 or $CBrCl_3$ in the isolated perfused liver. It was found that PBN/$\cdot CO_2^-$ production was highly correlated with the time required for LDH release to occur (Fig. 5). For example, rates of PBN/$\cdot CO_2^-$ formation were enhanced significantly and the time of onset of LDH release into the effluent perfusate was decreased when either halocarbon was metabolized in the presence of nitrogen-saturated rather than oxygen-saturated perfusate (Figs. 3 and 4). A decrease in halocarbon metabolism to free radicals under aerobic perfusion conditions due to oxygen inhibition of halocarbon reduction (29, 30) may account for the decrease and/or delay in hepatoxicity observed in the isolated perfused liver. It follows that these factors would play an even greater role in the protective effect of hyperbaric oxygen against carbon tetrachloride poisoning reported by Truss and Killenberg (31). Taken together, these observations support the hypothesis that free radical metabolites of CCl_4 or $CBrCl_3$ are directly involved in halocarbon-induced hepatic injury. Additional support for this hypothesis was obtained by altering the level of cytochrome P-450 monooxygenases responsible for halocarbon metabolism by pretreatment with phenobarbital. Rates of PBN/$\cdot CO_2^-$ production increased 2-fold and the time of onset of LDH release was approximately 10–15 min faster in perfused livers from phenobarbital-treated rats than in those from untreated controls (Fig. 6).

In conclusion, we have demonstrated that halocarbon metabolism to PBN/$\cdot CO_2^-$ is highly correlated with hepatocellular damage reflected by the time of onset of LDH release. The role that the carbon dioxide anion radical plays in the sequence of events leading to cell death is not known. It may only serve as a marker for other, more reactive radical metabolites of CCl_4 and $CBrCl_3$, which are causally involved in halocarbon-induced hepatotoxicity. Early investigators attributed CCl_4-induced injury to $\cdot CCl_3$ (32). Later, it was recognized that $\cdot CCl_3$ is converted rapidly to a much more reactive radical, $CCl_3OO\cdot$, when oxygen is present (33, 34). Both radical species can bind covalently to lipids and proteins and initiate lipid peroxidation (34, 35). The results reported in this communication demonstrate that oxygen tension in the cell is a determining factor in both the rate of formation of free radical species as well as the extent of toxicity observed. In the future, studies using the isolated perfused liver, a whole cell, nearly physiological model, with electron spin resonance may be useful in studying mechanisms of free radical-induced toxicity.

References

1. Butler, T. C. Reduction of carbon tetrachloride *in vivo* and reduction of carbon tetrachloride and chloroform *in vitro* by tissues and tissue constituents. *J. Pharmacol. Exp. Ther.* **134**:311–319 (1961).
2. Recknagel, R. O., and E. A. Glende, Jr. Carbon tetrachloride hepatotoxicity: an example of lethal cleavage. *CRC Crit. Rev. Toxicol.* **2**:263–297 (1973).
3. Slater, T. F. *Free Radical Mechanisms in Tissue Injury*. Pion Limited, London, 85–170 (1972).
4. McCay, P. B., and J. L. Poyer. Enzyme-generated free radicals as initiators of lipid peroxidation, in *The Enzymes of Biological Membranes* (A. Martonisi, ed.). Plenum Press, New York, 239–256 (1976).
5. Rao, K. S., and R. O. Recknagel. Early onset of lipoperoxidation in rat liver after carbon tetrachloride administration. *Exp. Mol. Pathol.* **9**:271–278 (1968).
6. Reynolds, E. S. Liver parenchymal cell injury. IV. Pattern of incorporation of carbon and chlorine from carbon tetrachloride into chemical constituents of liver *in vivo*. *J. Pharmacol. Exp. Ther.* **155**:117–126 (1967).
7. Gordis, E. Lipid metabolites of carbon tetrachloride. *J. Clin. Invest.* **48**:203–209 (1969).
8. Tomasi, A., E. Albano, K. A. K. Lott, and T. F. Slater. Spin trapping of free radical products of CCl_4 activation using pulse radiolysis and high energy radiation procedures. *FEBS Lett.* **122**:303–306 (1980).
9. Poyer, J. L., P. B. McCay, E. K. Lai, E. G. Janzen, and E. R. Davis. Confirmation of assignment of the trichloromethyl radical spin adduct detected by spin trapping during ^{13}C-carbon tetrachloride metabolism *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.* **94**:1154–1160 (1980).
10. Connor, H. D., R. G. Thurman, M. D. Galizi, and R. P. Mason. The formation of a novel free radical metabolite from CCl_4 in the perfused rat liver and *in vivo*. *J. Biol. Chem.* **261**:4542–4548 (1986).
11. Scholz, R., W. Hansen, and R. G. Thurman. Interaction of mixed function oxidation with biosynthetic processes. I. Inhibition of gluconeogenesis by amino pyrine in perfused rat liver *Eur. J. Biochem.* **38**:64–72 (1973).
12. Bergmeyer, H. U. *Metabolism der Enzymatischen Analyse*, Ed. 2. Verlag Chemie, Weinheim (1979).
13. Mason, R. P., and J. L. Holzmann. The mechanism of microsomal and mitochondrial nitroreductase. Electron spin resonance evidence for nitroaromatic free radical intermediates. *Biochemistry* **14**:1626–1632 (1975).
14. Murib, J. H., and D. M. Ritter. Decomposition of nitrosyl disulfonate ion. I. Products and mechanism of color fading in acid solution. *J. Am. Chem. Soc.* **74**:3394–3398 (1952).
15. Uehleke, H., K. H. Hellmer, and S. Tabarelli-Poplawski. Binding of ^{14}C-carbon tetrachloride to microsomal proteins *in vitro* and formation of $CHCl_3$ by reduced liver microsomes. *Xenobiotica* **3**:1–11 (1973).
16. De Groot, H., and T. Noll. The crucial role of low steady state oxygen partial pressures in haloalkane free-radical-mediated lipid peroxidation. *Biochem. Pharmacol.* **35**:15–19 (1986).
17. Noll, T., and H. De Groot. The critical steady-state hypoxic conditions in carbon tetrachloride-induced lipid peroxidation in rat liver microsomes. *Biochim. Biophys. Acta* **795**:356–362 (1984).
18. Slater, T. F., and B. C. Sawyer. The stimulatory effects of carbon tetrachloride and other halogeno-alkanes on peroxidative reactions in rat liver fractions *in vitro*: general features of the systems used. *Biochem. J.* **123**:805–814 (1971).
19. Mico, B. A., R. V. Branchflower, L. R. Pohl, A. T. Pudzianowski, and G. H. Loew. Oxidation of carbon tetrachloride, bromotrichloromethane, and carbon tetrabromide by rat liver microsomes to electrophilic halogens. *Life Sci.* **30**:131–137 (1982).
20. Thurman, R. G., and F. C. Kauffman. Factors regulating drug metabolism in intact hepatocytes. *Pharmacol. Rev.* **31**:229–251 (1979).
21. Belinsky, S. A., L. A. Reinke, R. Scholz, F. C. Kauffman, and R. G. Thurman. Rates of pentose cycle flux in perfused rat liver. Evaluation of the role of reducing equivalents from the pentose cycle for mixed-function oxidation. *Mol. Pharmacol.* **28**:371–376 (1985).
22. Hugentobler, G., and P. J. Meier. Multispecific anion exchange in basolateral (sinusoidal) rat liver plasma membrane vesicles. *Am. J. Physiol.* **251**:G656–G664 (1986).
23. Hagenbuch, B., G. Stange, and H. Murer. Transport of sulphate in rat jejunal and rat proximal tubular basolateral membrane vesicles. *Pflügers Arch.* **405**:202–208 (1985).
24. von Dippe, P., and D. Levy. Analysis of the transport system for inorganic anions in normal and transformed hepatocytes. *J. Biol. Chem.* **257**:4381–4385 (1982).
25. Bracht, A., A. Kelmer-Bracht, A. J. Schwab, and R. Scholz. Transport of inorganic anions in perfused rat liver. *Eur. J. Biochem.* **114**:471–479 (1981).
26. Cabantchik, Z. I., and A. Rothstein. The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *J. Membr. Biol.* **10**:311–330 (1972).
27. Cabantchik, Z. I., and A. Rothstein. Membrane proteins related to anion permeability of human red cells. *J. Membr. Biol.* **15**:207–226 (1974).
28. Hoffman, E. K. Anion exchange in the anion-cation CO transport systems in mammalian cells, in *The Binding and Transport of Anions in Living Tissues* (R. D. Keynes and J. C. Ellory, eds.). Royal Society, London, 153–170 (1982).
29. Nastainczyk, W., H. J. Ahr, and V. Ullrich. The reductive metabolism of halogenated alkanes by liver microsomal cytochrome P-450. *Biochem. Pharmacol.* **31**:391–396 (1982).
30. Burk, R. F., K. Patel, and J. M. Lane. Reduced glutathione protection against

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

rat liver microsomal injury by carbon tetrachloride. *Biochem. J.* **215**:441–445 (1983).
31. Truss, C. D., and P. G. Killenberg. Treatment of carbon tetrachloride poisoning with hyperbaric oxygen. *Gastroenterology* **82**:767–769 (1982).
32. Recknagel, R. O., E. A. Glende, Jr., and A. M. Hruszkewycz. Chemical mechanisms in carbon tetrachloride toxicity, in *Free Radicals in Biology* (W. A. Pryor, ed.), Vol. III. Academic Press, New York, 97–132 (1977).
33. Packer, J. E., T. F. Slater, and R. L. Willson. Reactions of the carbon tetrachloride related peroxy free radical ($CCl_3O_2^{\cdot}$) with amino acids: pulse radiolysis evidence. *Life Sci.* **23**:2617–2620 (1978).
34. Forni, L. G., J. E. Packer, T. F. Slater, and R. L. Willson. Reaction of the trichloromethyl and halothane-derived peroxy radicals with unsaturated fatty acids: a pulse radiolysis study. *Chem. Biol. Interact.* **45**:171–177 (1983).
35. Mico, B. A., and L. R. Pohl. Reductive oxygenation of carbon tetrachloride:trichloromethylperoxyl radical as a possible intermediate in the conversion of carbon tetrachloride to electrophilic chlorine. *Arch. Biochem. Biophys.* **225**:596–609 (1983).

Send reprint requests to: Dr. Ronald G. Thurman, Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27514.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012