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Formation of electrophilic chlorine from carbon tetrachloride—involvement of cytochrome P-450

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Despite many years of intensive research, the molecular processes which initiate carbon tetrachloride hepatotoxicity are not completely characterized. Although it is generally accepted that a reactive metabolite of carbon tetrachloride is responsible for its hepatotoxic effects [1–4], there are several possible reactive species which may be responsible for carbon tetrachloride toxicity. The chemically reactive compounds trichloromethyl radical [5, 6], dichloromethyl carbene [7, 8], and phosgene [9–11] are all products of carbon tetrachloride metabolism. We have shown recently than an electrophilic form of chlorine is a new product of aerobic carbon tetrachloride metabolism by rat liver microsomes [12, 13]. In the present report, we have shown that the formation of this unique metabolite is an enzymatic process catalyzed by cytochrome P-450.

Male Sprague–Dawley rats (180–250 g) were obtained from Taconic Farms, Germantown, NY. The animals were allowed free access to water and food (Purina Lab Rat Chow). Groups of three animals were treated daily with phenobarbital (80 mg/kg in saline) for 4 days prior to the experiment. Twenty-four hours after the last phenobarbital injection, liver microsomes were prepared as described elsewhere [11] and resuspended in 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (pH 7.5). Protein content was determined by the method of Lowry *et al.* [14] with bovine serum albumin as a standard. The content of microsomal cytochrome P-450 was assayed by the method of Omura and Sato [15].

Unless otherwise indicated, incubation mixtures contained 4 mg of microsomal protein from phenobarbital-pretreated rats, 1.0 mM NADPH, 20 mM HEPES buffer (pH 7.5), 5 mM carbon tetrachloride, and 1 mM 2,6-dimethylphenol (DMP) in a total volume of 2 ml. The mixtures were incubated in sealed vials (20 ml) at 37° under an atmosphere of air. Electrophilic chlorine was quantitated by trapping the electrophilic chlorine with DMP to form 4-chloro-2,6-dimethylphenol (4-ClDMP). The amount of 4-ClDMP formed was measured by gas chromatography/electron ionization mass spectrometry as described previously [12, 13].

NADPH–cytochrome P-450 reductase was purified by the method of Yasukochi and Masters [16] to a specific activity of 12,000 units/mg protein. The reductase activity was assayed as described by Masters *et al.* [17]. The major form of cytochrome P-450 found in phenobarbital-pretreated male Sprague–Dawley rats was purified by the method of West *et al.* [18] to a specific content of 5.0 nmoles/mg protein. Although the values of specific activity or content of these purified proteins were lower than the reported maxima, sodium dodecyl sulfate (SDS) gel electrophoresis of the preparations by the method of Laemmli [19], as modified by Guengerich [20], revealed a single, apparently homogenous band. Loss of the protein prosthetic group during purification is the most likely cause of the low specific content [21, 22].

Cytochrome P-450 (2.0 nmoles) was reconstituted with dilaurylphosphatidyl choline (40 μg, suspended by sonication), NADPH–cytochrome P-450 reductase (10,000 units), DMP (1 mM) and carbon tetrachloride (5 mM) in 20 mM HEPES buffer (pH 7.5) in a final volume of 2 ml. The reaction was initiated by addition of NADPH (1 mM). After 60 min, the samples were analyzed for 4-ClDMP as described above.

Although the formation of electrophilic chlorine has been shown previously to require both oxygen and NADPH [12], the enzymatic nature of this process has not been fully studied. The rate of electrophilic chlorine formation, as measured by the trapping of electrophilic chlorine with DMP to form 4-ClDMP, was constant up to 30 min (data not shown). The correlation coefficient obtained after linear regression of six data points within this time period was 0.96. After incubation for 30 min under these conditions, only 20% of the cytochrome P-450 is lost. The rate of electrophilic chlorine formation was proportional to the protein concentration between 0.25 and 2.0 mg of microsomal protein/ml. The linear rates of electrophilic chlorine formation with respect to time and protein concentration demonstrate that enzyme cofactors were not rate limiting.

When the concentration of carbon tetrachloride in the incubation mixtures was varied from 0.25 to 10.0 mM (six data points), the rate of electrophilic chlorine formation reached a maximum asymptotically. The V_{max} and K_m, calculated by unweighted nonlinear least-squares regression [23], were 320 pmoles per 4 mg microsomal protein per 30 min and 0.98 mM respectively. Analysis of the data by means of a Lineweaver–Burk plot yielded similar values (V_{max} = 307 pmoles per 4 mg protein per 30 min, K_m = 0.88 mM, R^2 = 0.99). A maximum rate of 4-ClDMP formation was observed (Fig. 1) at a concentration of DMP of 0.5 mM although 1 mM yielded similar results. Higher concentrations of DMP (2.5 to 10 mM) decreased the rate of 4-ClDMP formation.

The role of cytochrome P-450 in the bioactivation of carbon tetrachloride to electrophilic chlorine was examined with inhibitors of cytochrome P-450 (Table 1) and with purified components of the mixed function oxidase system

Table 1. Effect of inhibitors of cytochrome P-450 on the formation of electrophilic chlorine from carbon tetrachloride

Condition	(pmoles 4-ClDMP/30 min)	% Inhibition
Standard incubation*	362 ± 30†	0
+ SKF-525A (0.5 mM)	292 ± 17	19‡
+ Piperonyl butoxide (1.0 mM)	141 ± 19	61‡
+ Nitrogen/Oxygen (8:2)	346 ± 31	0
+ Carbon monoxide/Oxygen (8:2)	154 ± 29	55§

* Standard incubation mixtures contained 4 mg of microsomal protein from phenobarbital-pretreated rats, 1.0 mM NADPH, 20 mM HEPES buffer (pH 7.5), 5 mM carbon tetrachloride, and 1 mM 2,6-dimethylphenol (DMP) in a total volume of 2 ml.

† Mean ± standard deviation of four determinations.

‡ Percent inhibition calculated relative to standard incubation.

§ Percent inhibition calculated relative to incubation mixture with generated atmosphere of nitrogen/oxygen (8:2).

Table 2. Formation of electrophilic chlorine from carbon tetrachloride with purified components of the mixed-function oxidase system

Condition	(pmoles 4-ClDMP/2 nmoles P-450/60 min)
Complete system	144 ± 14*
−NADPH–cytochrome P-450 reductase	ND†
−cytochrome P-450	ND
−cytochrome P-450, -lipid	ND
Liver microsomes	213‡

* Mean ± standard deviation of four determinations.

† Not detectable; limit of detection was 12.5 pmoles.

‡ Based on V_{max} determination with 1.5 nmoles P-450/mg protein with microsomes from animals treated with phenobarbital.

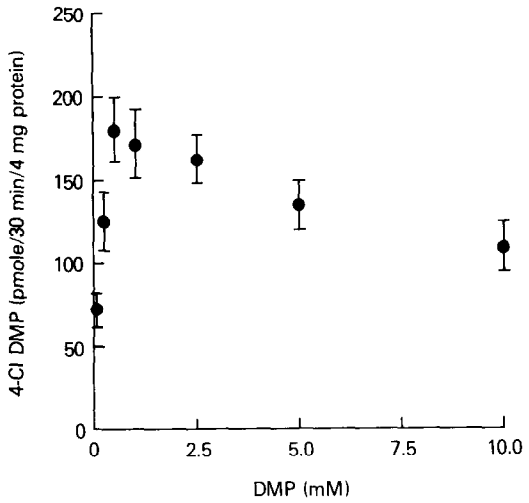

Fig. 1. Effect of DMP concentration on the rate of electrophilic chlorine formation from carbon tetrachlorine. DMP concentration was varied from 0.10 to 10 mM in standard incubation mixtures.

(Table 2). The reduction of electrophilic chlorine formation by an atmosphere of carbon monoxide:oxygen (8:2, 55% decrease) and piperonyl butoxide (1 mM, 61% decrease) suggests a cytochrome P-450-dependent pathway. SKF-525A, however, inhibited the reaction by less than 21%. This is consistent with previous studies of carbon tetrachloride metabolism which showed that SKF-525A does not markedly inhibit the covalent binding of carbon tetrachloride [24] or phosgene formation [10].

The involvement of cytochrome P-450 in the formation of electrophilic chlorine was unambiguously established in studies with purified components of the mixed-function oxidase system. With a reconstituted system consisting of lipid, NADPH–cytochrome P-450 reductase and cytochrome P-450, electrophilic chlorine was produced at a rate similar to that observed in rat liver microsomes (Table 2). Exclusion of cytochrome P-450, cytochrome P-450 and lipid, or NADPH–cytochrome P-450 reductase from the reconstituted system completely abolished electrophilic chlorine formation (Table 2). The present data preclude NADPH–cytochrome P-450 reductase as the site of carbon tetrachloride activation to electrophilic chlorine.

Although additional studies are needed to determine the mechanism of electrophilic chlorine formation and the relevance of this process to carbon tetrachloride-induced hepatotoxicity, this study firmly establishes that the formation of electrophilic chlorine from carbon tetrachloride is catalyzed by cytochrome P-450.

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Structure–genotoxicity relationship for aliphatic epoxides

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In a recent commentary on the role of reactive metabolites in the carcinogenicity of halogenated ethylenes Bolt *et al.* [1] have discussed the relationship between the stability of the corresponding epoxides and activity (both carcinogenic and mutagenic) in the light of Henschler's structural theory [2]. They have also presented data for the oncogenic effects of halogenated ethylenes in comparison with covalent macromolecular binding, mutagenicity in bacterial tests and carcinogenicity in animal bioassays. The interpretation of this data and of genotoxic response more generally outlined by these authors, particularly with regard to the *optimum* (as opposed to minimum) stability of the epoxide ring for genotoxic response, parallels closely that put forward independently by the present authors shortly afterwards [3]. Here, we wish to develop this theme still further.

The essential feature of our approach to the mutagenicity

Table 1. Structure–activity relationships for haloethylenes

Compound	Two-centre energy of the corresponding epoxide (eV)	Mutagenicity*	Oncogenicity†
$CCl_2{=}CCl_2$	−14.89	100	0
$CCl_2{=}CHCl$	−14.1	232	0
$CCl_2{=}CH_2$	−13.16	229	0.15
$CHCl{=}CHCl$	−14.38	100	ND
$CHCl{=}CH_2$	−13.4	663	18.2
$CH_2{=}CHF$	−13.24	ND	14.3
$CH_2{=}CF_2$	−12.89	ND	1.81

* Per cent spontaneous mutation rate for the arginine operon of *E. coli* K_{12} [2].
† Per cent foci theoretically produced by 1 mole metabolites per kg body wt [1].
ND = no experimental data available.