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IDENTIFICATION OF DICHLOROMETHYL CARBENE AS A METABOLITE OF CARBON TETRACHLORIDE

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Although indirect evidence has suggested that liver microsomal cytochrome P-450 can reductively dehalogenate several compounds to carbene metabolites, there has been no direct proof for the formation of these reactive species. We report in this paper that carbenes can be chemically trapped and identified as metabolites. For example, 1,1-dichloro-2,2,3,3-tetramethylcyclopropane was identified as a metabolite by gas chromatography mass spectrometry when carbon tetrachloride (CCl_4) was incubated anaerobically with rat liver microsomes, NADPH and 2,3-dimethyl-2-butene. The reaction required NADPH and was inhibited by carbon monoxide. These findings show that cytochrome P-450 in rat liver microsomes can reductively metabolize CCl_4 to dichloromethyl carbene ($:CCl_2$) which can be trapped with 2,3-dimethyl-2-butene to form 1,1-dichloro-2,2,3,3-tetramethylcyclopropane. A similar approach may be used for the identification of carbene metabolites of other compounds.

The results of several studies have indicated that the hepatotoxicity produced by carbon tetrachloride (CCl_4) is due to a reactive and toxic metabolite (1). For this reason, there has been considerable interest in determining the pathways of metabolism of CCl_4. The results of most studies indicate that the first step in the metabolism of CCl_4 is its one electron reductive dechlorination to trichloromethyl radical ($\bullet CCl_3$) by liver microsomal cytochrome P-450. If the oxygen concentration of the environment is high enough, the radical ($\bullet CCl_3$) reacts with O_2 to form trichloromethylperoxy radical ($CCl_3OO\bullet$), which appears to be an intermediate in the formation of the reactive species phosgene ($COCl_2$) (2-5) and electrophilic chlorine (6-10). At low oxygen tension, the radical ($\bullet CCl_3$) may abstract a hydrogen atom from a cellular component to form $CHCl_3$ (11-13) or react with unsaturated fatty acids of phospholipids (14). In addition it has been postulated that cytochrome P-450 may reduce the radical ($\bullet CCl_3$) by an additional electron to form trichloromethyl carbanion $(:CCl_3)^-$,

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which decomposes by α-elimination of chloride to form the reactive intermediate dichloromethyl carbene ($:CCl_2$) (12). In the past, the evidence for the formation of the carbene ($:CCl_2$) has been based solely on the formation of a unique spectral complex of cytochrome P-450 which absorbs at approximately 460 nm and the formation of carbon monoxide, which is a known hydrolysis product of the carbene.

To determine unequivocally whether the carbene ($:CCl_2$) is produced during the liver microsomal metabolism of CCl_4, we have conducted incubations in the presence of 2,3-dimethyl-2-butene (DMB), which is a highly efficient trap of $:CCl_2$ (15). We report that the carbene ($:CCl_2$) was trapped as 1,1-dichloro-2,2,3,3-tetramethylcyclopropane (DCTC, Fig.1) and that cytochrome P-450 appears to catalyze the formation of the carbene.

EXPERIMENTAL

Chemicals. NADPH was purchased from Sigma Biochemicals, St. Louis, MO. CCl_4 and DMB were purchased from Aldrich Chemical Co., Milwaukee, WI. DCTC was synthesized from DMB and $CHCl_3$ by the method of Doering and Henderson (15).

Trapping of $:CCl_2$ in microsomes. Liver microsomes were prepared from phenobarbital treated (80 mg/kg, in saline, i.p. for 4 days) male Sprague Dawley rats (80-120 g, Taconic Farms, Germantown, NY) as described elsewhere (4) and were resuspended in 20 mM HEPES buffer, pH 7.5. Unless otherwise indicated, incubation mixtures contained 6 nmoles of cytochrome P-450, 2 μmoles NADPH, 16 μmoles DMB, and 10 μmoles of CCl_4 in a total volume of 2 mls of 20 mM HEPES, pH 7.5. The incubations were conducted in sealed vials (rubber septum) at 37°C under an anaerobic atmosphere of N_2 or CO. The incubations were made anaerobic by evacuating ice cooled stoppered reaction mixtures (minus CCl_4 and DMB) with a mechanical pump. This was followed by the addition of N_2 or CO. The process was repeated ten times and the reactions were started by the addition of DMB and CCl_4 with a syringe through the rubber septa of

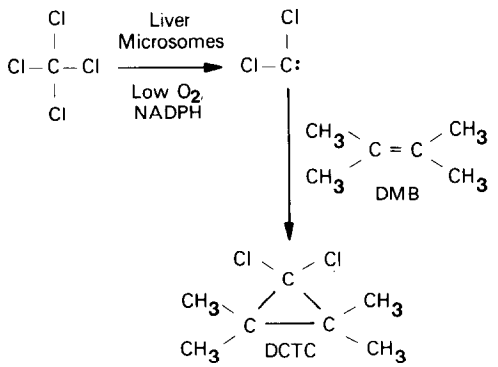

Fig. 1. Trapping of dichloromethyl carbene with DMB during the metabolism of CCl_4 by rat liver microsomes.

the reaction vials. After 60 min, the incubation mixtures were extracted with hexane (0.5 ml) and a sample of the extract (1 µl) was analyzed by gas chromatography electron ionization mass spectrometry (GC/EIMS).

GC/EIMS analyses. The GC/EIMS analyses were performed with a V.G. Micromass Model 16 F spectrometer (accelerating voltage, 4 kV; electron energy 70 eV; ionizing current 100 µAmp; ion-source temperature 220°C) that was interfaced with a Hewlett Packard 5710A gas chromatograph equipped with a J and W fused silica DB-1 capillary column (0.32 mm i.d. x 20 M). Helium was used as the carrier gas at a flow rate of approximately 1 ml/min. The samples were injected onto the column through a splitless injector at an injector temperature of 200°C and a column temperature of 60°C. Quantitation was performed in the selective ion mode by monitoring ion current at m/z 131. The amount of DCTC formed in the incubation mixtures was determined by comparison of the peak height at m/z 131 to a standard curve that was prepared from standards of DCTC. Sixty percent of DCTC (602 pmoles) added to a standard reaction was recovered after 1 hr of incubation; this value was used to correct the amounts of DCTC obtained metabolically from CCl_4. The limit of detection of the assay was approximately 150 pmoles/6 nmoles P-450/60 min.

RESULTS AND DISCUSSION

When CCl_4 was incubated with rat liver microsomes, NADPH and DMB under an atmosphere of nitrogen, a metabolite was formed that had the same GC retention time and EIMS as authentic DCTC (Fig.2). The fragment ion multiplets at m/z 151 and 153, and m/z 131 and 133 corresponded to losses of a methyl group and

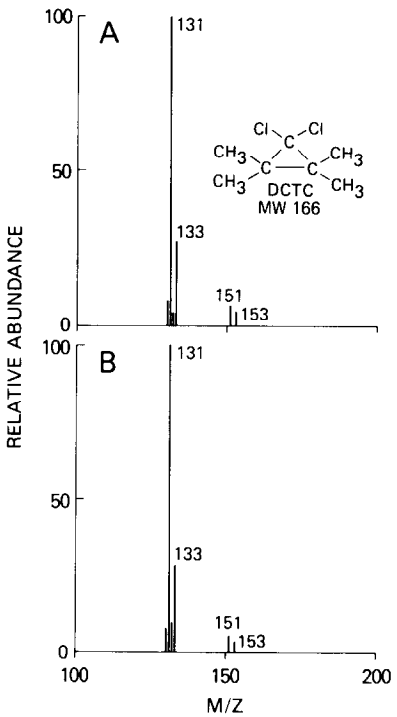

Fig. 2. GC/EIMS of (A) authentic DCTC, retention time 3.0 min and (B) metabolite from the incubation of CCl_4 and DMB with rat liver microsomes and NADPH, retention time 3.0 min.

a chlorine atom respectively from the unstable molecular ion. Approximately 1.48 ± 0.08 nmoles (mean ± S.E., n = 3) of DCTC/6 nmoles of microsomal cytochrome P-450 was detected after 60 min of incubation. Cytochrome P-450 appeared to catalyze the reaction because DCTC was not detected as a metabolite when NADPH was omitted from the incubation mixtures or when the atmosphere of nitrogen was replaced with that of carbon monoxide (16).

Although several halogenated compounds have been converted to carbenes by model porphyrin systems (12,17), to the best of our knowledge, this report represents the first unequivocal identification of a carbene metabolite of a xenobiotic. The results also support the idea that cytochrome P-450 may reduce carbon centered radicals by one electron to form transient carbanions (12,13,18-22). The procedure outlined for the trapping of $:CCl_2$ should be useful for the study of the metabolism of other compounds that are suspected of being metabolized to carbenes (17-19,21).

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REFERENCES

1. Recknagel, R.O. (1983) Life Sci. 33, 401-408.
2. Shah, H., Hartman, S.P. and Weinhouse, S. (1979) Cancer. Res. 39, 3942-3947.
3. Kubic, V.L. and Anders, M.W. (1980) Life Sci. 26, 2151-2155.
4. Pohl, L.R., Branchflower, R.V., Highet, R.J., Martin, J.L., Nunn, D.S., Monks, T.J., George, J.W., and Hinson, J.A. (1981) Drug Metab. Disp. 9, 334-339.
5. Pohl, L.R., Morris, P.L., Schulick, R.D. and George, J.W. (1983) The Toxicologist 3, 115.
6. Mico, B.A., Branchflower, R.V., Pohl, L.R., Pudzianowski, A.T., and Loew, G.H. (1982) Life Sci. 30, 131-137.
7. Mico, B.A., Branchflower, R.V. and Pohl, L.R. (1983) Biochem. Pharmacol. 32, 2357-2359.
8. Pudzianowski, A.T., Loew, G.H., Mico, B.A., Branchflower, R.V. and Pohl, L.R. (1983) J. Amer. Chem. Soc. 105, 3434-3438.
9. Pohl, L.R. and Mico, B.A. Trends Pharmacol. Sci., in press.
10. Mico, B.A. and Pohl, L.R. Arch. Biochem. Biophys., in press.
11. Wolf, C.R., Harrelson, W.G., Nastainczyk, W., Philpot, R.M., Kalyanaraman, B. and Mason, R.P (1980) Mol. Pharmacol. 18, 553-558.
12. Ahr, H.J., King, L.J., Nastainczyk, W., and Ullrich, V. (1980) Biochem. Pharmacol. 29, 2855-2861.
13. Kubic, V.L. and Anders, M.W. (1981) Chem. Biol. Interactions 34, 201-207.
14. Trudell, J.R., Bosterling, B. and Trevor, A.J. (1982) Proc. Natl. Acad. Sci. 79, 2678-2682.
15. Doering, W.E. and Henderson, W.A. (1958) J. Amer. Chem. Soc. 80, 5274-5277.

16. Gillette, J.R., Davis, D.C., and Sasame, H.A. (1972) Ann. Rev. Pharmacol. 12, 57-84.
17. Mansuy, D. (1980) Pure and Appl. Chem. 52, 681-690.
18. Wolf, C.R., Mansuy, D., Nastainczyk, W., Deutschmann, G., and Ullrich, V. (1977) Mol. Pharmacol. 13, 698-705.
19. Ahr, H.J., King, L.J., Nastainczyk, W., and Ullrich, V. (1982) Biochem. Pharmacol. 31, 383-390.
20. Nastainczyk, W., Ahr, H.J. and Ullrich, V. (1982) Biochem. Pharmacol. 31, 391-396.
21. Morris, P.L., Burke, T.R., and Pohl, L.R. (1983) Drug Metab. Disp. 11, 126-130.
22. Mansuy, D. and Fontecave, M. (1983) Biochem. Pharmacol. 32, 1871-1879.