EVIDENCE FOR PHOSGENE FORMATION DURING LIVER MICROSOMAL OXIDATION OF CHLOROFORM

Daniel MANSUY*, Philippe BEAUNE**, Thierry CRESTEIL**, Marc LANGE*, and Jean-Paul LEROUX**

* Laboratoire de Chimie de l'Ecole Normale Supérieure, Laboratoire associé du CNRS n°32, 24. rue Lhomond F-75231 PARIS CEDEX 05.

** Laboratoire de Biochimie : INSERM U 75, CHU Necker-Enfants Malades 156. rue de Vaugirard F-75730 PARIS CEDEX 15.

Received September 8, 1977

SUMMARY

When aerobically incubated with liver microsomes and NADPH, chloroform produces a stable adduct with cysteine as a nucleophilic trapping agent. The adduct was identified by thin layer chromatography, gas-liquid chromatography and combined gas chromatography-mass spectrometry as the reaction product of cysteine with phosgene.

INTRODUCTION

Chloroform ($CHCl_3$) hepatotoxicity has been associated with the covalent binding of reactive metabolites to tissue macromolecules. There is ample evidence to suggest that such metabolites are formed during microsomal cytochrome P 450-dependent oxidation of chloroform (1, 2, 3). The nature of the reactive metabolites is not yet known ; however they should be electrophilic as indicated by the decrease of hepatic glutathione levels observed in $CHCl_3$-treated rats (4). Moreover, recent studies in our laboratory indicated that $[^{14}C]$-$CHCl_3$, incubated aerobically with microsomes and NADPH, forms stable adducts with low molecular weight nucleophiles, e.g. cysteine, added to the incubation medium (5).

Among the possible reactive intermediates formed upon $CHCl_3$ oxidation, phosgene, which has been previously postulated as a chloroform metabolite (6, 3), appeared to us as a very probable reactive intermediate which could mediate chloroform hepatotoxicity for the following reasons : it is a well known product of chemical oxidation of $CHCl_3$; it may derive from microsomal hydroxylation of $CHCl_3$ to CCl_3OH followed by a fast loss of HCl ; it is a highly reactive electrophile.

ISSN 0006-291X

In the results presented here, we have used cysteine as a nucleophilic trapping agent in order to detect the formation of this postulated metabolite during microsomal oxidation of chloroform.

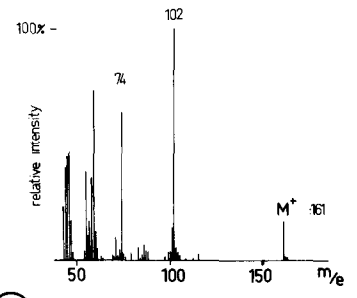
MATERIALS AND METHODS

Male adult rats (Sprague Dawley Strain) weighing between 200 and 250 g were used throughout this study. Liver microsomes were prepared from phenobarbital-treated rats (80 mg/kg i.p. daily for 3 days) as previously described (7). Microsomal suspensions (25 nmoles of cytochrome P 450 per ml of 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and 150 mM KCl) were incubated for 15 min with 0.4 mM NADPH or a NADPH-generating system, 2 mM chloroform (freshly purified on alumina column) with or without 2 mM cysteine (Sigma) in stopped glass tubes with air as gas phase. For thin layer chromatography $[^{14}C]$-$CHCl_3$ from New England Nuclear (100 μCi/mmol) was used. Incubation was stopped by addition of 2 vol of absolute ethanol. After centrifugation, the supernatant was evaporated to dryness and washed twice by pure $CHCl_3$. The residue was dissolved in HCl-acidified water (pH 2-3)and extracted three times by ether. Thin layer chromatography (TLC) was performed on TLC plastic sheets cellulose F_{254} (Merck) with butanol-acetic acid-water 4/1/5 (v/v/v) as solvent. Each 13 cm plate was divided into 13 adjacent parts which were scraped off and counted for radioactivity in a Packard spectrometer after addition of 5 ml of toluene-PPO-POPOP. Ethereal extracts of incubation mixtures were methylated by addition of an excess of ethereal diazomethane at ambiant temperature (8). Gas chromatography analyses (GLC) were performed in a Varian 3 700 gas chromatograph with a hydrogen flame ionization detector. Two columns were used : a stainless steel column (1,5 m x 3 mm I.D.) packed with 3 % OV 1 on chromosorb gas chrom Q (column temperature 130°C, injector and detector temperature 170°C, gas carrier N_2 at a flow rate of 30 ml/min) or a glass column (2 m x 3 mm I.D.) packed with 10 % OV 17 on chromosorb gas chrom Q (column temperature 190°C, injector and detector temperature 200°C and N_2 flow of 15 ml/min). For combined gas chromatography-mass spectrometry, a Girdel chromatograph 30 (equipped with an OV 17 column, operating conditions as above except that He replaced N_2 as a carrier gas) was coupled with a Riber 1000 mass spectrometer (electron ionization, electron energy 100 eV, trap current 200 μA) and a PDP 8 computer.

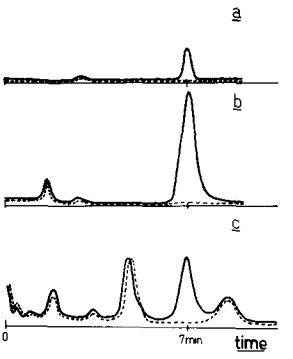
RESULTS

When a mixture of $CHCl_3$, NADPH and liver microsomes from phenobarbital-treated rats was aerobically incubated with cysteine, a stable adduct was formed, and shown to be identical with the product of chemical reaction of phosgene with cysteine, 4-carboxy-thiazolidine-2-one, 1. In fact, we prepared an authentic sample of the carboxylic acid, 1, mp : 170°C (dec), by reaction of phosgene with cysteine in 7 M potassium hydroxide. Its methyl ester was obtained by treatment of an ethereal solution of 1 with an excess of diazomethane. The structure and purity of these two compounds were verified by I.R. and N.M.R. spectroscopy, mass spectrometry and elemental analysis.

As shown by TLC analysis, the ethereal extracts of the incubation



(1)



(2)

Fig 1 : Mass spectrum of the major product present in the methylated ethereal extracts of microsomes incubated with cysteine, chloroform and NADPH.

The mass spectrum corresponds to the GLC peak which has the same retention time as the methylated authentic acid, 1. Experimental conditions are described under material and methods. The mass spectrum of the methylated authentic acid, 1, was strictly identical.

Fig 2 : Computer-reconstructed mass chromatograms of the products of the methylated ethereal extract of a cysteine-chloroform microsomal incubation mixture, exhibiting a mass spectrum peak at m/e = 161 (a), 102 (b) or 74 (c).

Conditions as in Fig 1. Solid lines : incubation in the presence of NADPH ; dashed lines : incubation without NADPH.

mixtures of microsomes with $[^{14}C]$-$CHCl_3$ and NADPH (after ethanolic deproteinization) contained one major product with a R_f 0.70 $\pm$ 0.01 (for conditions see materials and methods) identical with that of authentic 4-carboxy-thiazolidine-2-one. After elimination of excess $[^{14}C]$-$CHCl_3$, 50-60 % of the total ether-extracted radioactivity was recovered in this spot where no radioactivity was detectable when NADPH had been omitted in the incubation mixture. As measured by total radioactivity after blank subtraction, about 0.2 nmoles of phosgene were formed per mg protein in 1 min and trapped by cysteine (5).

Ethereal extracts were also analyzed by gas chromatography after diazomethane methylation of the cysteine-derived carboxylic acid. The chromatograms obtained on two columns (OV 1 and OV 17) exhibited a peak showing retention times identical with those of the methylated authentic acid, 1. This peak was not observed when cysteine or NADPH had been omitted in the incubation mixture.

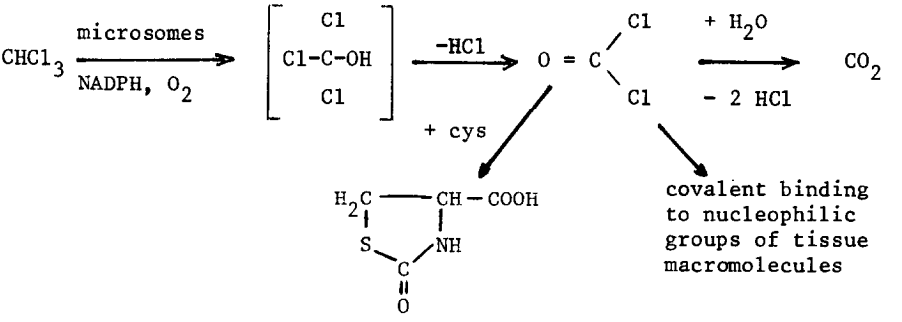
The mass spectrum of the product corresponding to this chromatographic peak, monitored by combined gas chromatography-mass spectrometry, was identical with the mass spectrum of the methyl ester of the authentic acid 1, and exhibited three characteristic ions at m/e 161 (M^+), 102 and 74 (Fig 1). The computer-reconstructed chromatograms of the ether-extractable product exhibiting an ion at m/e 161, 102 or 74 showed a major peak, with the same retention time as that of the methylated authentic acid 1. However the presence of the methylated acid 1 could not be detected, even by this very sensitive technique, in the ethereal extract of incubation mixture without NADPH (Fig 2).

DISCUSSION

Results show that phosgene is formed during NADPH and oxygen dependent microsomal oxidation of $CHCl_3$. It probably derives from a hydroxylation of $CHCl_3$ followed by a rapid loss of HCl (Scheme 1). In this respect, it is noteworthy that we could detect the formation of phosgene during oxidation of $CHCl_3$ by peracids like p-nitroperbenzoic or trifluoroperacetic acids (D. MANSUY and M. LANGE, unpublished results) which are considered as models of cytochrome P 450-dependent monooxygenases (9).

In the liver, phosgene, a very reactive electrophilic compound, will react with all possible nucleophiles (scheme 1) : it can be hydrolyzed to CO_2, the major metabolite of $CHCl_3$ in vivo (10, 11, 12), or react with some nucleophilic groups of the tissue macromolecules with formation of irreversible covalent bonds. This could explain, at least in part, the hepatotoxicity of chloroform.

Scheme 1



1

4-carboxy-thiazolidine-2-one.

ACKNOWLEDGEMENTS

The authors wish to thank RIBER-MAG for fruitful help in the gas chromatography/mass spectrometry analysis.

This work was supported by a grant from D.G.R.S.T. BRD n° 75-7-1614.

REFERENCES

1. Ilett, K.F., Reid, W.D., Sipes, I.G. and Krishna, G. (1973) Exp. Mol. Pathol. 19, 215-229.
2. Uehleke, H. and Werner, T. (1975) Arch. Toxicol. 34, 289-308.
3. Reynolds, E.S. and Yee, A.G. (1967) Lab. Invest. 16, 591-603.
4. Docks, E.L. and Krishna, G. (1976) Exp. Mol. Pathol. 24, 13-22.
5. Cresteil, Th., Beaune, Ph., Leroux, J.P. and Mansuy, D. submitted for publication.
6. Sipes, I.G., Krishna, G. and Gillette, J.R. (1977) Life Sci. 20, 1541-1548.
7. Degkwitz, E., Ullrich, V., Staudinger, H. and Rummel, W. (1969) Hoppe Seyler's Z. Physiol. Chem. 350, 547-553.
8. Vogel, A.I. (1956) Textbook of practical Organic Chemistry, p. 567.
9. Ullrich, V.(1972) Angew Chem. Int. Ed. 11, 701-712.
10. Paul, B. and Rubinstein, D. (1963) J. Pharmacol. 141, 141-148.
11. Van Dyke, R.A., Chenoweth, M.B. and Van Poznak, A. (1964) Biochem. Pharmacol. 13, 1239-1247.
12. Lavigne, J.G. and Marchand, C. (1974) Toxicol. Appl. Pharmacol. 29, 312-326.