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DEUTERIUM ISOTOPE EFFECT IN IN VIVO BIOACTIVATION OF CHLOROFORM TO PHOSGENE

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The results of several investigations suggest that a reactive metabolite of chloroform ($CHCl_3$) is responsible for its hepatotoxicity and possibly its carcinogenicity and renal toxicity [1-5]. Consistent with this view, phosgene ($COCl_2$) was identified as a metabolite when $CHCl_3$ was incubated with rat liver microsomes [6-8].

Recently it was found that deuterium-labeled chloroform ($CDCl_3$) was less hepatotoxic than $CHCl_3$ and was metabolized to $COCl_2$ by liver microsomes at approximately half the rate of $CHCl_3$ [9]. Since these observations indicated that the cleavage of the C-H bond of $CHCl_3$ was the rate-determining step both in the in vitro formation of $COCl_2$ and in the process leading to hepatotoxicity, it seems likely that $COCl_2$ was responsible, at least in part, for the hepatotoxicity produced by $CHCl_3$ [9].

The present investigation provides evidence that $COCl_2$ is a metabolite of $CHCl_3$ in vivo and that a deuterium isotope effect appears to occur in the formation of this metabolite.

In a typical experiment, male Sprague-Dawley rats (180-200 g, Hormone Assay) that were allowed free access to water and food (Purina Lab Rat Chow) were pretreated with phenobarbital (80 mg/kg, in saline, i.p.) 72, 48 and 24 hr before the beginning of the study. On the day of the experiment, the rats were injected first with L-cysteine (1 g/kg, in saline, i.p.) and then with $CHCl_3$ (4.98 m-moles/kg, in sesame oil, i.p.) 30 min later. After 1 hr, the rats were killed by decapitation and their livers were removed and homogenized in 1 vol. of 0.02 M Tris 1.15% KCl buffer, pH 7.7. The homogenate was extracted with hexane (15 ml, 3 times), ethyl acetate (15 ml, 3 times) and then acidified with concentrated hydrochloric acid (1 ml) to pH approximately 1. The acidic homogenate was then extracted with ethyl acetate (15 ml, 4 times). The ethyl acetate extracts of the acidified homogenate were combined, dried with $MgSO_4$, and then evaporated under vacuum to dryness. The resulting black residue was redissolved in methanol (100 µl), and methylated with diazomethane which was generated from Diazald (Aldrich) following the directions given on the reagent bottle. After 15 min the reaction mixture was evaporated to dryness under N_2. The sample was then redissolved in methanol (50 µl) and analyzed by gas chromatography electron ionization mass spectrometry, in a VG Micromass 16F spectrometer (accelerating voltage, 4 kV; electron energy 70 eV; ionizing current 100 µA; ion source temperature 200°), which was equipped with a Varian 1400 gas chromatograph containing a glass column (181 mm x 2 mm i.d.) packed with 3% SE -30 on Gas Chrom Q, 100/120 mesh. Helium was used as the carrier gas at a rate of flow of approximately 25 ml/min. One µl of sample was injected (injector temperature 200°) onto the column (125°), which after 1 min was programmed up to 200° at 20°/min.

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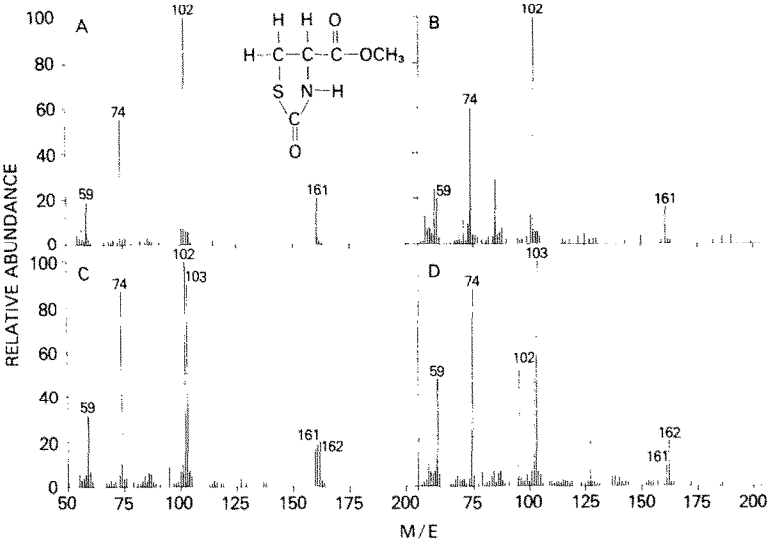

Fig. 1. Gas chromatography electron ionization mass spectra (GCMS). A. GCMS of authentic methyl 2-oxothiazolidine-4-carboxylate which was synthesized from 2-oxothiazolidine-4-carboxylic acid [10] and diazomethane; retention time 3.5 min. B. GCMS of metabolite isolated from rat liver 1 hr after the administration of $CHCl_3$; retention time 3.5 min. C. GCMS of metabolite isolated from rat liver 1 hr after the administration of a 1:1 mixture of $CHCl_3$ and $[^{13}C]CHCl_3$; retention time 3.5 min. D. GCMS of metabolite isolated from rat liver 1 hr after the administration of a 1:1 mixture of $CDCl_3$ and $[^{13}C]CHCl_3$; retention time 3.5 min.

As previously reported in the in vitro studies [6-9], $COCl_2$ was trapped with cysteine as 2-oxothiazolidine-4-carboxylic acid. Figure 1A represents the mass spectrum of the methylated derivative of this compound. The ion at m/e 161 represents the molecular ion, while the ion at m/e 102 corresponds to the loss of the carbomethoxy side chain from the molecular ion. The ion at m/e 74 was likely derived from the ion at m/e 102 by a loss of CO, whereas the fragment ion at m/e 59 corresponds to the carbomethoxy side chain. A nearly identical mass spectrum was obtained for a fraction having the same retention time as the synthetic standard (3.5 min) which was isolated from the liver of a phenobarbital-pretreated rat 1 hr after the administration of $CHCl_3$ (Fig. 1B).

To confirm that the ions in Fig. 1B were derived from $CHCl_3$, the in vivo experiment was repeated with a 1:1 mixture of $CHCl_3$ and ^{13}C-labeled $CHCl_3$ (Merck). A fraction eluting from the gas chromatograph at 3.5 min again contained ions of m/e 161, 102, 74 and 59 (Fig. 1C). However, it also contained ions at m/e 162 and 103, which were approximately the same intensity as the ions at m/e 161 and 102 respectively. These additional ions represented the molecular ion methyl 2-oxothiazolidine-4-carboxylate and its decarbomethoxylated fragment ion that were derived from $[^{13}C]CHCl_3$. The absence of intense ions at m/e 75 and 60 established that the 2-oxo carbon was not present in fragment ions m/e 74 and 59.

In order to determine whether a deuterium isotope effect occurred in the in vivo formation of $COCl_2$, the in vivo trapping experiment was repeated with a 1:1 mixture of $CDCl_3$ and $[^{13}C]CHCl_3$. Ion doublets at m/e 162, 161 and 103, 102 were again observed (Fig. 1D), but in this case, the ions at m/e 162 and 103 were more intense than the corresponding ions at m/e 161 and 102. These differences reflect a deuterium isotope effect in the formation of $COCl_2$ in vivo, since the ions at m/e 162 and 103 were derived from $[^{13}C]CHCl_3$, whereas the ions at m/e 161 and 102 were derived from $CDCl_3$. A similar finding was observed when the experiment was repeated independently in two additional rats. The calculated isotope effects from these experiments were $CHCl_3/CDCl_3$ of 2.10 ± 0.06 (mean ± standard error) which agrees closely to the finding of 2.11 obtained in vitro with rat liver microsomes [9].

Since $CDCl_3$ is also approximately 2 to 3 times less hepatotoxic than $CHCl_3$ [9],* the results of this study further support the idea that $COCl_2$ is a hepatotoxic metabolite of $CHCl_3$.

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