Table 1. Methane chemical ionization mass spectra*

Treatment	Ion sought	Ion observed [m/z (relative abundance)]
+ $^{14}CCl_4$ *in vivo*	M + 1	507 (78%)
	M + 1	505 (100%)
	M + 1 − $^{14}CHCl_3$	385 (78%)
+ CCl_4 *in vivo*	M + 1	505 (100%)
	M + 1	503 (53%)
	M + 1 − $CHCl_3$	385 (95%)
+ γ-Irradiated CCl_4 plus cholesterol	M + 1	505 (100%)
	M + 1	503 (42%)
	M + 1 − $CHCl_3$	385 (67%)

* Finnigan 4000 quadruple mass spectrometer, operated in chemical ionization mode with methane at 0.5 torr and ionization source temperature 100°. The chosen diagnostic ions were monitored in the selective ion monitoring mode.

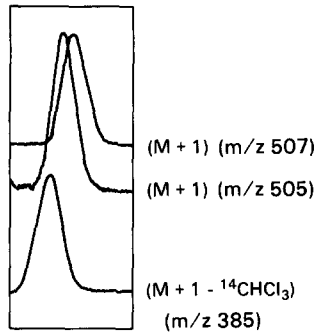

Fig. 1. Selective ion monitoring profile of ^{14}C-trichloromethylated cholesterol.

sistent with the three ions sought. A typical selective ion monitoring spectrum from an animal given $^{14}CCl_4$ is presented in Fig. 1. These results are indicative of the formation of trichloromethylated cholesterol *in vitro* and *in vivo*.

Further studies to determine the specific activity and to definitively characterize the structure and stereochemistry of the observed trichloromethyl cholesterol product are in progress. Chemical studies by others [7, 8] on the interaction of cholesterol with several types of free radicals indicate that the C-7 is the most vulnerable site for free radical interaction.

In summary, we have presented the first chemical evidence, based on methane ionization mass spectrometry, for *in vivo* binding of a $^{\cdot}CCl_3$ radical metabolite to cholesterol.

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REFERENCES

1. E. S. Reynolds and M. T. Moslen, *Biochem. biophys. Res. Commun.* **57**, 747 (1974).
2. E. Gordis, *J. clin. Invest.* **48**, 203 (1969).
3. A. Benedetti, A. F. Casini, M. Ferrali and M. Comporti, *Chem. Biol. Interact.* **17**, 151 (1977).
4. E. S. Reynolds and M. T. Moslen, in. *Free Radicals in Biology* (Ed. W. A. Pryor), Vol. IV, pp. 49–94. Academic Press, New York (1980).
5. F. S. Shenstone, in. *Biochemistry and Methodology of Lipids* (Eds. A. R. Johnson and J. B. Davenport), p. 185–7. John Wiley, New York (1971).
6. M. S. P. Munson and F. H. Field, *J. Am. chem. Soc.* **88**, 2621 (1966).
7. O. Hellinger, H. Heusinger and O. Hug, *Biophysik* **6**, 193 (1970).
8. L. L. Smith, *Cholesterol Autoxidation*, p. 180. Plenum Press, New York (1981).

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Inhibition of hepatic γ-glutamyl-cysteine synthetase by chloroform

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Centrilobular liver necrosis induced by chloroform is related to glutathione (GSH) depletion in hepatic parenchymal cells [1]. These effects can be studied in isolated hepatocytes from phenobarbital-pretreated rats, and in a previous report we showed that chloroform-metabolizing cells rapidly lost GSH and eventually lysed [2]. It was also

shown that addition of sulfur amino acid neither replenished GSH levels nor prevented lysis [2]. In cytosol from chloroform-exposed hepatocytes the rate of GSH synthesis from precursor amino acids was reduced, indicating an irreversible inhibition of GSH-synthesizing enzymes [3].

GSH is formed from glutamate, cystein and glycine. γ-Glutamyl-cysteine synthetase (E.C. 6.3.2.2) catalyses the dipeptide formation and glutathione synthetase (E.C. 6.3.2.3) catalyses the tripeptide formation. Both enzymes are cytosolic. The activity of γ-glutamyl-cysteine synthetase is rate limiting and the synthesis of GSH may be regulated by feed-back inhibition of this enzyme [4]. In this paper we present evidence that chloroform, under certain conditions, selectively inhibits γ-glutamyl-cysteine synthetase in rat hepatocytes. It is suggested that this inhibition is of importance for chloroform-induced necrosis.

Materials and methods

Male Sprague–Dawley rats (180–250 g) were used throughout. Phenobarbital was given in the drinking water (1 mg/ml) for one week prior to use. [^{14}C]-$CHCl_3$ and [^{14}C]-L-glutamate were purchased from New England Nuclear (New England, ND), [^{3}H]-L-glutamate and [^{35}S]-cysteine from Amersham (Arlington Heights, IL).

Hepatocytes were prepared by collagenase perfusion using basically the same procedure as described in [5]. Incubations were performed in rotating, round bottom flasks with continuous gassing (95% O_2 and 5% CO_2). Each flask contained 30–60 × 10^6 cells in 20 ml amino acid free Krebs–Henseleit buffer, pH 7.4. Chloroform was added directly to the medium with a microsyringe. 105,000 *g* supernatant from incubated hepatocytes was prepared by sonication (30 sec) [6] and centrifugation (1 hr) in 25 mM Tris–125 mM KCl buffer (pH 7.4).

The *in vivo* experiments were performed with phenobarbital (as above) and diethylmaleate (1 ml/kg given one hr prior to chloroform administration) pretreated animals. Chloroform (1036 mg/kg) was given orally by stomach tube. The rats were decapitated and the liver was removed at the times indicated in Fig. 1. Supernatant fraction was prepared from liver homogenates (20% w/v) in 25 mM Tris–125 mM KCl buffer (pH 7.4), by centrifugation at 105,000 *g* for 1 hr.

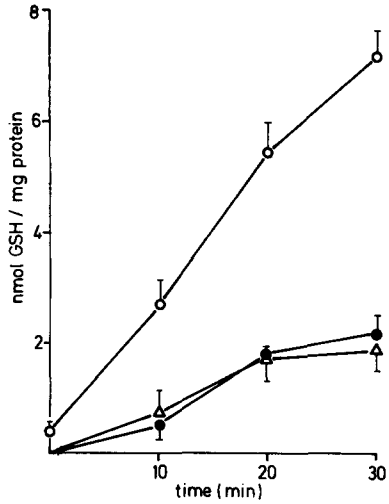

Fig. 1. GSH synthesis in hepatic 105,000 *g* supernatant from rats given chloroform *in vivo*. All rats were pretreated with phenobarbital and had been given 1 ml DEM/kg (i.p.). One hour after the DEM-treatment some of the rats were given 1036 mg $CHCl_3$/kg. These rats were decapitated 4 hr (○——○) or 6 hr (△——△) after $CHCl_3$-treatment. Control rats (○——○) were decapitated 7 hr after DEM-treatment. The livers were homogenized and supernatants were incubated to assay GSH synthesis. GSH was quantitated in aliquots of the incubation mixture with the fluorimetric method. Each point gives the mean ± S.D. of results obtained with four rats.

The rate of GSH synthesis was assayed in a 100 mM Tris–HCl buffer (pH 8.2), containing supernatant from either lysed cells or homogenized liver (1 mg protein/ml), KCl (50 mM), $MgCl_2$ (20 mM), EDTA (2 mM), ATP (10 mM), L-glutamate (10 mM), L-cysteine (10 mM) and L-glycine (10 mM). In some experiments glycine was omitted. Incubation temperature was 37°. Samples were taken periodically and analyzed either fluorometrically as described by Hissin and Hilf [7] or by high performance liquid chromatography (HPLC) essentially as described by Reed *et al.* [8]. Briefly, the analysis was carried out on a Waters Associates ALC/202 HPLC equipped with a Waters μ-Bondapak-NH_2 analytical column. An ammonium acetate–acetic acid–methanol gradient was used for elution. Solvent A was methanol:water (4:1, v/v) and solvent B prepared as follows; 154 g of ammonium acetate, 122 ml water and 378 ml acetic acid were mixed and 200 ml of the solution was added to 800 ml of solvent A.

The conditions for the analysis were 10 min isocratic elution with 12% solvent B in A followed by an exponential gradient (No. 4) to 95% of solvent B. The flow rate was 2 ml per min and the effluent was monitored at 365 nm.

Results

As previously reported, *in vitro* exposure to chloroform markedly inhibits GSH synthesis in hepatocytes from phenobarbital-pretreated rats [2]. In a series of experiments, the mean rate of GSH synthesis by the cytosol from lysed hepatocytes was 52% of control. These hepatocyte incubations were performed in a medium without amino acids. To obtain more *in vivo*-like conditions, experiments were also performed with cysteine or methionine added to the medium. However, these amino acids did not prevent the inhibition of GSH synthesis, even at concentrations above 1 mM. Figure 1 shows that doses causing necrosis given *in vivo* to rats, inhibited the rate of GSH synthesis by the 105,000 g supernatant from rat liver homogenate. The inhibition became prominent after 4–6 hr. At 3 hr the inhibition was less clear The hepatic GSH concentration was 12 and 8% of the control concentration at 4 and 6 hr, respectively. The expected centrilobular necrosis could be seen after 6 hr exposure.

To further characterize the inhibition of GSH synthesis, the effects of chloroform on the isolated enzymes were studied. Partly purified preparations of γ-glutamyl-cysteine synthetase and glutathione synthetase were incubated with chloroform and with and without microsomes plus NADPH-generating system. However, so far we have not been able to relate significant changes in GSH-synthesizing activity to these treatments.

In another approach, glycine was excluded from the assay systems in which the rate of GSH synthesis was measured. As glycine is one of the substrates for glutathione synthetase, its exclusion would present an opportunity to assay γ-glutamyl-cysteine synthetase activity. An HPLC-technique was used to quantitate γ-glutamyl-cysteine as well as GSH. Figure 2 shows five chromatograms, three from incubations with supernatants from control cells (panel A, B and C) and two with supernatants from chloroform-exposed cells (panel D and E). At zero time, only trace amounts of GSH (Rt: 29 min) and GSSG (Rt: 31 min) were found (panel A). After 30 min incubation in a medium containing all three amino acid precursors there was a prominent increase in the GSH peak (panel B). This, as well as three smaller peaks contained radiolabel from glutamate. When glycine was excluded, the peak eluting just after GSH increased (Rt: 30 min) (panel C). When supernatants from chloroform-treated cells were used all the radiolabelled peaks became lower. Panel D shows the

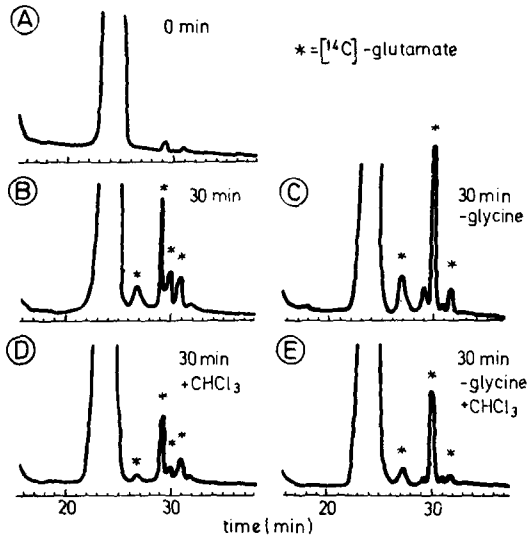

Fig. 2. GSH synthesis in 105,000 *g* supernatant from control and chloroform-exposed, isolated hepatocytes. Hepatocytes were incubated with or without chloroform (6.2 mM). 105,000 *g* supernatant were then prepared from lysed cells and incubated to assay GSH synthesis. Glycine was excluded in some of these incubates, and [^{14}C]-labelled glutamate was used throughout. Aliquots were prepared for HPLC-analysis. The figure shows traces obtained with supernatants from cells incubated under control conditions (panels A, B and C) and from chloroform-exposed cells (panels D and E). Panels A, B and D show the results obtained in the presence of glycine, and panels C and E in the absence of glycine. The supernatants were incubated for 30 min except in panel A, where the incubation time was 0 min.

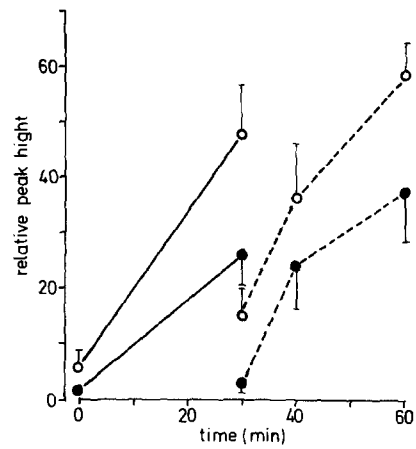

Fig. 3. γ-Glutamyl-cysteine and GSH synthesis in 105,00 *g* supernatant from control and chloroform-exposed cells. Hepatocytes were incubated for 60 min, with or without chloroform (6.2 mM). 105,000 *g* supernatants were then prepared from lysed cells and incubated to assay γ-glutamyl-cysteine and GSH synthesis. Glycine was added after 30 min. Aliquots were prepared for HPLC-analysis at times indicated. The figure gives the mean peak heights ± S.D. from three experiments with separate batches of cells. Continuous lines: γ-glutamyl-cysteine disulfide; hatched lines: GSH + GSSG. Open circles: supernatants from cells incubated under control conditions; closed circles: supernatants from chloroform-exposed cells.

effect of chloroform when glycine was included in the assay system and panel E the effect when glycine was omitted.

To further characterize the material eluting in the peak at 30 min as a glutamate-cysteine derivative, [^{3}H]-glutamate and [^{35}S]-cysteine were used in experiments similar to that presented in panel C (Fig. 2). Major amounts of both labels, and with expected specific activities, eluted with the peak at 30 min only. Smaller amounts eluted with the peak at 27 min.

In another series of experiments, glycine was added to the incubate after 30 min, whereupon incubation was continued for another 30 min. Figure 3 shows changes in the height of the peak eluting at 30 min, as well as changes in the sum of the heights of the GSH and GSSG peaks. In the absence of glycine, during the first 30 min of incubation, there was a more rapid accumulation of glutamate-cysteine derivative with control supernatants than with chloroform-exposed ones. After the addition of glycine the height of this peak progressively decreased (not shown). Of further interest is that in the presence of glycine, during a 30–40 min incubation, there was no difference in the accumulation rate of glutathione, under the conditions used in this experiment (Fig. 3).

Several samples analyzed with the HPLC-technique were also analyzed with the fluorometric technique used in Fig. 1. About equally high readings were obtained both in the presence and absence of glycine, indicating a lack of specificity of the fluorescence assay for GSH. The lower readings obtained with this technique after chloroform exposure (Fig. 1) thus further support the results obtained with the HPLC-technique. No accumulation of γ-glutamyl-cysteine occurred to compensate for the loss of GSH.

Discussion

The present results strongly suggest that chloroform metabolism leads to an inhibition of γ-glutamyl-cysteine synthetase: there was a less rapid accumulation of the glutamate-cysteine derivative when chloroform-exposed supernatants were used in the glycine-lacking system, and in the presence of glycine there was no indication that γ-glutamyl-cysteine or its disulfide accumulated. The labelling experiments leave little doubt that the 30 min peak contained γ-glutamyl-cysteine, formed in the absence of glycine. According to Reed *et al.* [8] γ-glutamyl-cysteine has a much shorter retention time than 30 min, while the retention time of the corresponding disulfide is more compatible with our data. It is thus possible that γ-glutamyl-cysteine is rapidly oxidized in the assay system and that the peak contained the disulfide.

Another indication that only γ-glutamyl-cysteine synthetase was inhibited by chloroform metabolism is that GSH synthesis increased to control rates after a preincubation period in the absence of glycine (Fig. 3). The data presented in Fig. 3 indicate that the activity of glutathione synthetase was unaffected by the chloroform exposure, which inhibited γ-glutamyl-cysteine synthetase. It is assumed that γ-glutamyl-cysteine disulfide, under these conditions, equilibrated with the thiol, catalyzed by cytosolic thiol transferases, or spontaneously [9], and thus served as a substrate for glutathione synthetase.

The mechanism behind this inhibition is not clear. γ-Glutamyl-cysteine synthetase is sensitive to thiol reagents [4], and a plausible mechanism would be an interaction with the chloroform metabolite, phosgene [10]. The observation that chloroform metabolism in isolated subcellular fractions has so far failed to inhibit this enzyme may be taken as an evidence against such a theory. However, it is also possible that an intact hepatocellular topography

was important. The distance from the site of metabolism to the site of action has been claimed to be critical for the genetic activity of chloroform [11], and it is possible that such a factor might have influenced our results as well.

An inhibition of γ-glutamyl-cysteine synthetase fully explains the previously described inhibition of GSH synthesis [2, 3]. The possible *in vivo* significance of these findings was indicated by the loss of activity prior to morphological cell damage. Leakage of enzymes from the liver might have influenced the results, but the activity of lactate dehydrogenase in peripheral plasma is not markedly increased 4 hr after chloroform treatment [12]. Furthermore, the observation that GSH synthesis was equally affected at 4 and 6 hr indicates that leakage was of minor importance.

We conclude that chloroform metabolism may lead to γ-glutamyl-cysteine synthetase inhibition, while GSH synthetase activity is unaffected. This inhibition might be of importance for chloroform-induced liver necrosis.

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REFERENCES

1. B. R. Brown, Jr., I. G. Sipes and A. M. Sagalyn, *Anesthesiology* **41**, 554 (1974).
2. T. Ekström and J. Högberg, *Biochem. Pharmac.* **29**, 3059 (1980).
3. J. Högberg, T. Ekström, I. Anundi and A. Kristoferson, *Toxicology* **17**, 113 (1980).
4. A. Meister and S. S. Tate, *A. Rev. Biochem.* **45**, 559 (1976).
5. J. Högberg and A. Kristoferson, *Eur. J. Biochem.* **74**, 77 (1977).
6. P. Gällerfors and B. D. Nelson, *Analyt. Biochem.* **93**, 200 (1979).
7. P. J. Hissin and R. Hilf, *Analyt. Biochem.* **74**, 214 (1976).
8. D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodie, W.W. Ellis and D. W. Potter, *Analyt. Biochem.* **106**, 55 (1980).
9. K. Ormstad, D. P. Jones and S. Orrenius, *J. Biol. Chem.* **255**, 175 (1980).
10. L. R. Pohl, J. L. Martin and J. W. George, *Biochem. Pharmac.* **29**, 3271 (1980).
11. D. F. Callen, C. R. Wolf and R. M. Philpot, *Mutat. Res.* **77**, 55 (1980).
12. J. L. Stevens and M. W. Anders, *Chem. Biol. Interact.* **37**, 207 (1981).

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Differential toxicity of carrier-bound methotrexate against tumor/bone marrow cells *in vivo*

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Chemotherapeutic agents attached to carrier molecules have been shown recently to be effective in overcoming drug resistance *in vivo* [1, 2] and in enhancing toxicity to specific tumor cells. Drugs linked either to antibodies against specific tumor cell-associated antigens [3, 4] or to polypeptide hormones with affinities for tumor cell receptors [5] were shown to be more toxic to these specific cells than the parent compound.

In a previous paper [6], we have shown that methotrexate (MTX)*, when covalently linked to a non-specific molecule, poly-*l*-lysine mol. wt 3,000 or 40,000–60,000 (MTX-PLL 3K or 40–60K), was more toxic *in vitro* to cells derived from human solid tumors than to human lymphocytes or bone marrow cells. Under the same conditions, free MTX or MTX linked to human serum albumin (MTX-HSA) showed no differential toxicity between the two cell types. The MTX-PLL derivatives were more readily taken up by tumor cells than by lymphocytes/bone marrow cells, and inhibition of both cell growth and [^{3}H]deoxyuridine (dUrd) incorporation into DNA by the MTX-PLL derivatives in tumor cells, but not lymphocytes, could be reversed by inhibitors of lysosomal proteases. This indicated that breakdown of the MTX-PLL derivatives by lysosomal proteases occurred more readily in the tumor cells, and that enhanced metabolism may be responsible for their increased toxicity to tumor cells.

In the studies reported below, we have determined whether the differential toxicity of MTX-PLL toward tumor cells *in vitro* could also be observed *in vivo*. We have studied the disposition of MTX-HSA and the MTX-PLL derivatives into tissues when injected *in vivo*, their therapeutic effects on human tumor cells transplanted in nude athymic mice, and their relative toxicities to tumors versus bone marrow cells *in vivo*.

Materials and methods

The following were obtained from commercial sources: MTX, sodium salt, Lederle Laboratories, Pearl River, NY; human serum albumin and poly-*l*-lysine, Sigma Chemical Co., St. Louis, MO; [3′,5′,7-^{3}H]MTX (20 Ci/mmole), Amersham Radiochemicals, Chicago, IL; and [6-^{3}H]dUrd (24.2 Ci/mmole), New England Nuclear Corp., Boston, MA. MTX-HSA and MTX-PLL derivatives (75–105 mg MTX/g) and the ^{3}H-derivatives (4–14 mCi/mmole) were synthesized as previously described [6].† The T24A astrocytoma and T17 ovarian carcinoma tumors carried in nude mice and non-tumor bearing mice were obtained from the

* Abbreviations: MTX, methotrexate; MTX-HSA, MTX-human serum albumin; MTX-PLL 3K and 40–60K, MTX-poly-*l*-lysine mol. wt 3,000 and 40,000–60,000; [^{3}H]dUrd, [^{3}H]deoxyuridine; and PBS, phosphate-buffered saline.

† All concentrations reported for MTX-HSA and MTX-PLL refer to concentrations of bound MTX.