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CHLOROFORM-INDUCED GLUTATHIONE DEPLETION AND TOXICITY IN FRESHLY ISOLATED HEPATOCYTES

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Abstract—Chloroform hepatotoxicity was studied in hepatocytes isolated from phenobarbital-treated rats. A single dose of 6.2 μmoles/ml chloroform decreased the intracellular level of GSH to about 15 per cent of control values within 60 min. Less than 5 per cent of the dose administered was recovered as water-soluble or protein-bound metabolites. The rate of metabolite formation after 60 min was low due to rapid evaporation of the chloroform. Hepatocytes exposed to a single dose of chloroform did not replenish their GSH when maintained in a medium supplemented with amino acid precursors for GSH synthesis. Their GSH level remained below 15 per cent of control values and during the second hour of incubation malondialdehyde accumulated. The accumulation of malondialdehyde was associated with cellular lysis, whereas inhibition of lipid peroxidation prevented cell destruction. It is suggested that there are two phases of importance for chloroform toxicity: a first phase characterized by chloroform metabolism, GSH depletion and protein alkylation; and a second phase characterized by GSH deficiency, lipid peroxidation and cell destruction.

Chloroform hepatotoxicity is potentiated by phenobarbital pretreatment [1] and it has recently been shown that phosgene is produced during microsomal oxidation of this widely used halomethane [2–4]. Phosgene thus formed can be expected to bind irreversibly to cellular glutathione (GSH) or tissue macromolecules [4]. The availability of GSH in the liver during chloroform metabolism seems to be of importance in preventing necrosis *in vivo* [1]. GSH may protect the liver cell in two ways: it can prevent the alkylation of vitally important cellular structures by activated chloroform metabolites [5]; and it may act as an antioxidant, as suggested by Brown *et al.* [1].

The LD_{50} for chloroform (for mice) is 6 mmoles/kg, while water solubility is more than 70 mmoles per liter [6]. This relationship makes chloroform suitable for toxicity studies with isolated hepatocytes. We have previously used this model system for studies on GSH turnover under the influence of amino acids and GSH-depleting agents [7–9]. We report here that chloroform rapidly depletes GSH in hepatocytes isolated from phenobarbital-treated rats and suggest a sequential toxic mechanism which may involve both protein alkylation and lipid peroxidation.

METHODS

Male Sprague–Dawley rats (180–250 g) were used throughout these experiments. Phenobarbital was present in the drinking water (1 mg/ml) for one week before the animals were killed. Hepatocytes were prepared by collagenase perfusion using basically the procedure described by Högberg *et al.* [7]. Incubations were performed in rotating round-bottom flasks (50 ml) with continuous gassing (O_2, 95% and CO_2, 5%). Each flask contained 16×10^6–30×10^6 cells suspended in an amino acid free Krebs–Henseleit buffer, pH 7.4, at a final volume of 20 ml [7].

Chloroform was added directly to the medium with a microsyringe. Metabolism was studied using [^{14}C]$CHCl_3$ (New England Nuclear, 5.4 mCi/mmole; 3.1 or 6.2 mM final concentration), which was added at zero time. Chloroform was extracted from 1 ml of the cell suspension with 2×2 ml ethylacetate (p.a.). In all, 95–96 per cent of the unmetabolized drug was extracted by this procedure. Binding to proteins was measured by precipitating the protein from 1 ml cell suspension with 10% trichloroacetic acid, filtration through a Whatman GF/C filter paper, washing the filtrant with methanol, and determining radioactivity on the filter in a Beckman LS 150 liquid scintillator. The rate of [^{14}C]CO_2 incorporation into proteins via arginine can be expected to be very low in our preparations, because arginine was absent from all solutions used (c.f. Ref. 10). Radiolabel associated with proteins is thus considered to be a good estimate of covalent binding of reactive metabolites to proteins.

Cell membrane permeability, measured as the latency of NADH oxidation, was routinely determined [7]. Glutathione was measured either as small molecular weight thiols according to Saville [11] or specifically with the fluorometric method of Hissin and Hilf [12]. Amino acid uptake was measured using 1-ml aliquots of the cell suspension as previously described by Högberg and Kristoferson [13]. Lipid peroxidation was monitored as the formation of malondialdehyde according to Högberg *et al.* [14].

Each experiment has been repeated at least three times with different batches of cells.

RESULTS

There was considerable evaporation of chloroform in our model system and relatively high concentrations had to be used to induce toxicity. The chloroform concentration fell rapidly during the first 20 min of incubation when measured as ethylacetate extractable radiolabel (Fig. 1A). There was also a

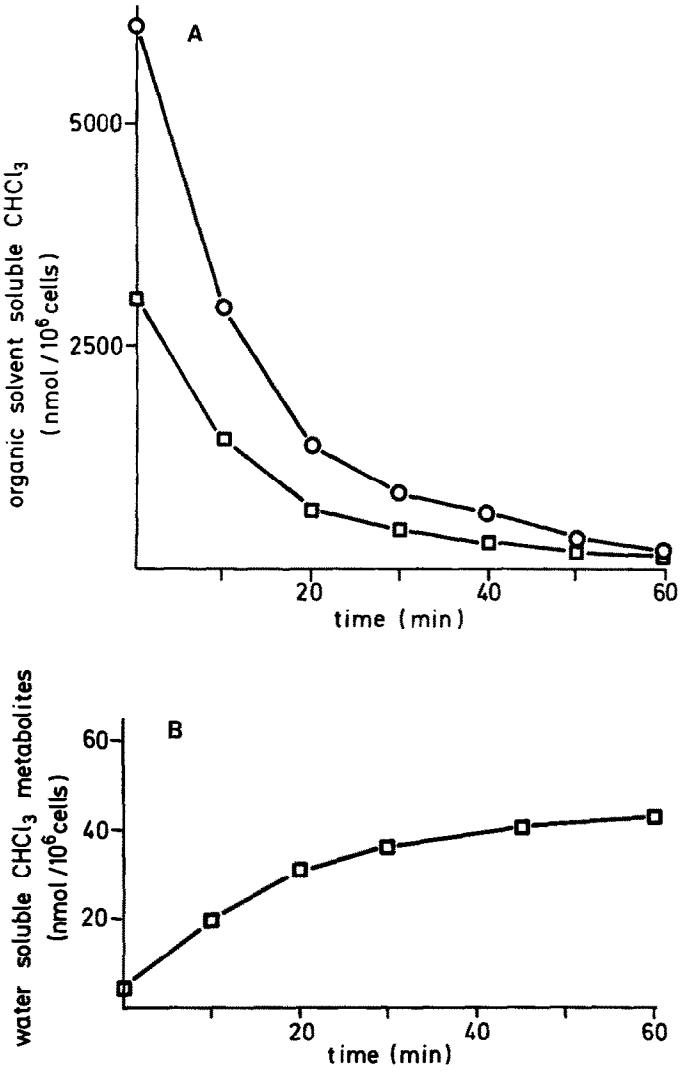

Fig. 1. Metabolism of chloroform in phenobarbital-induced isolated hepatocytes. Panel A shows the amount of radiolabel extractable with ethylacetate from the complete incubation medium. □—□, 3.1 mM $CHCl_3$; ○—○, 6.2 mM $CHCl_3$. Panel B shows the amount of non-volatile radiolabel remaining in the water phase after ethylacetate extraction. □—□, 6.2 mM $CHCl_3$.

roughly parallel drop in the total radioactivity in the medium. Only a small fraction of the initial dose was recovered as non-volatile radiolabel in the ethylacetate-extracted water phase after incubation with hepatocytes isolated from phenobarbital-treated rats (Fig. 1B).

When phenobarbital-induced hepatocytes were exposed to chloroform, [^{14}C]-label could be recovered as protein bound material (Fig. 2) and there was a rapid loss of cellular GSH (Fig. 3). An approximately linear binding rate for 40 min was observed when the initial chloroform concentration was 6.2 mM (Fig. 2). With the same chloroform concentration the intracellular GSH concentration fell from 35 to 5 nmoles/10^6 cells within an hour and almost complete depletion was eventually obtained (Fig. 3). There was no significant increase in the concentration of oxidized glutathione. With the lower concentration of chloroform (3.1 mM), a further loss of GSH was seen when more chloroform was added after 60 min. The extent of protein binding and GSH depletion was considerably lower in control hepatocytes (Figs. 2 and 3).

Cell membrane permeability started to increase in phenobarbital-induced cells during the second hour (Fig. 4). This delay in toxicity was characteristic for concentrations below 30 mM, whereas higher concentrations tended to induce an immediate lysis. Exposure of cells from untreated animals to chloroform did not affect their permeability.

Cysteine and methionine were added to the cell suspensions to determine whether these amino acids could protect the cells from lysis by facilitating resynthesis of GSH (cf. Ref. 9). However, no noticeable effect on GSH levels was observed when the amino acids were added after 30 min of chloroform metabolism (as shown in Figs. 5A and B) or after 60 min (not shown), at which time the metabolism of chloroform was much slower. This lack of effect of sulfur-containing amino acids on GSH levels could

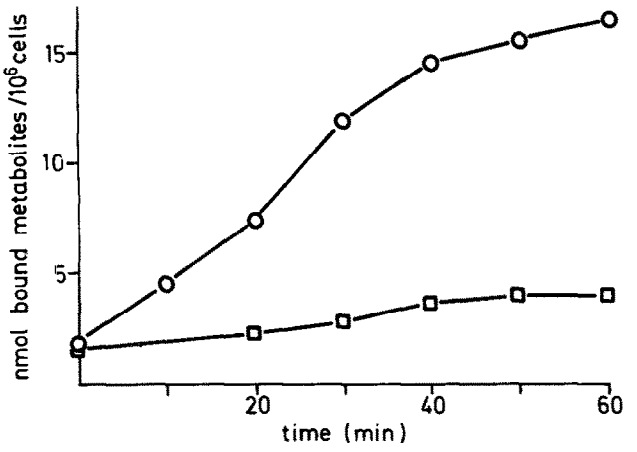

Fig. 2. [^{14}C]-chloroform binding to proteins in phenobarbital-induced and control isolated hepatocytes. $CHCl_3$ concentration was 6.2 mM initially. Binding to protein was measured as described in Methods. □—□, Control hepatocytes; ○—○, phenobarbital-induced hepatocytes.

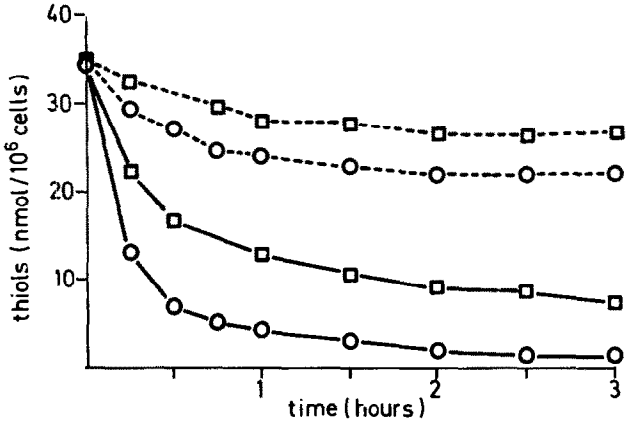

Fig. 3. Effect of chloroform metabolism on GSH levels in phenobarbital-induced and control hepatocytes. GSH was measured as acid-soluble thiols. Dotted lines, control cells; continuous lines, phenobarbital-induced cells. □, 3.1 mM $CHCl_3$; ○, 6.2 mM $CHCl_3$.

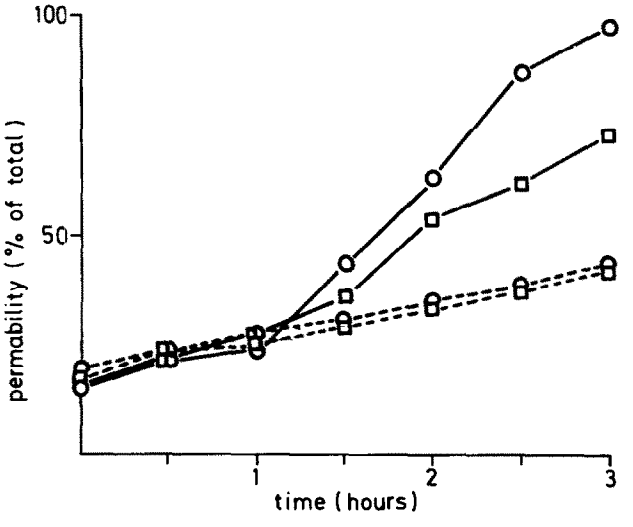

Fig. 4. Effect of chloroform metabolism on cell membrane permeability in phenobarbital-induced and control hepatocytes. The degree of permeability was measured as the latency of NADH oxidation in control cells (dotted lines) and phenobarbital-induced cells (continuous lines). □, 3.1 mM $CHCl_3$; ○, 6.2 mM $CHCl_3$.

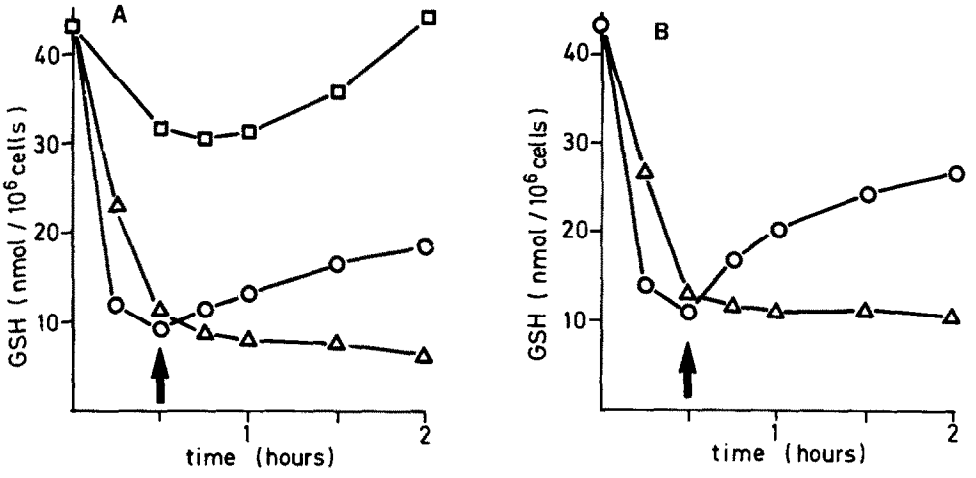

Fig. 5. Effect of methionine and cysteine on GSH levels in chloroform-exposed isolated hepatocytes. Panel A shows the effect of methionine addition (0.5 mM). Panel B shows the effect of cysteine addition (0.5 mM). Amino acids were added after 30 min. □—□, no addition; △—△, 6.2 mM $CHCl_3$; ○—○, 50 μM iodoacetamide.

also be demonstrated in phenobarbital-induced cells exposed to 3.2 mM or 1 mM chloroform (not shown). When the lower concentration (1 mM) was used, the experiments were performed in filled and sealed flasks without gas flow using 1-hr incubations.

Comparative experiments with phenobarbital-induced cells showed that both amino acids stimulated GSH accumulation when the GSH level was decreased by iodoacetamide instead of chloroform (Figs. 5A and B). In control hepatocytes, exposed to both iodoacetamide and chloroform, there was also an accumulation of GSH when methionine or cysteine was added after 30 min of incubation (Fig. 6). Furthermore, it was noted that neither methionine nor cysteine had any effect on chloroform-induced cellular lysis in phenobarbital-treated cells, whereas both amino acids effectively prevented iodoacetamide-induced lysis in control as well as in phenobarbital-induced hepatocytes (not shown).

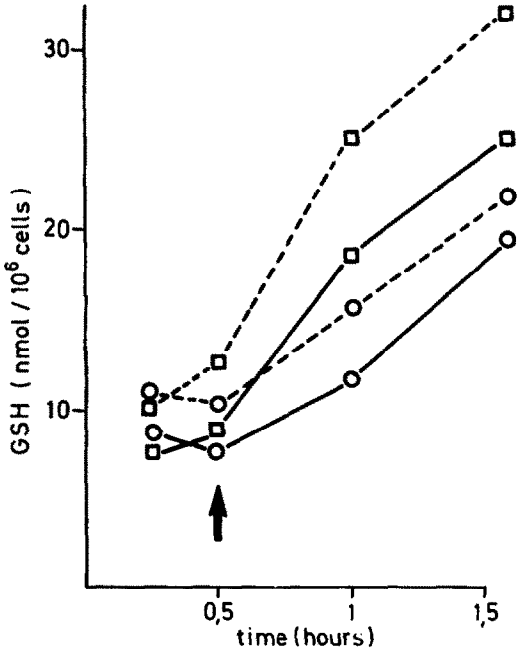

Fig. 6. Effect of chloroform metabolism on methionine and cysteine supported GSH accumulation in isolated control hepatocytes. Iodoacetamide (40 μM) with (continuous lines) or without (dotted lines) $CHCl_3$ was added at zero time. $CHCl_3$ concentration was 6.2 mM. Amino acids were added after 30 min. □, cysteine (0.5 mM); ○, methionine (0.5 mM).

The possibility that the transport of cysteine and methionine across the plasma membrane was inhibited by chloroform metabolism was then studied. Table 1 shows that the uptake of cysteine at the end of the first hour in non-exposed cells is almost doubled, a phenomenon similar to that described by Kletzien *et al.* [15]. In chloroform-exposed cells the rate of cysteine uptake remained essentially unchanged for 1 hr, as did the rate of methionine uptake.

GSH deficient hepatocytes accumulate malondialdehyde [9]. Figure 7A shows the amount of malondialdehyde formed in chloroform-exposed cells. The accumulation of malondialdehyde started when the GSH concentration was low but before there was a significant increase in membrane permeability (Fig. 7B). The response was related to the dose of chloroform and to the degree of cell permeability. As previously reported [9], paracetamol was a potent inhibitor of malondialdehyde accumulation. Figure 8 shows that the inhibition is dose-dependent and that 5 mM paracetamol prevented malondialdehyde accumulation as well as delayed chloroform-induced cellular lysis (not shown). This was the case when paracetamol was added at zero time (as in Fig. 8) or after 30 min. GSH depletion induced by chloroform, however, was not affected by paracetamol addition after 30 min incubation.

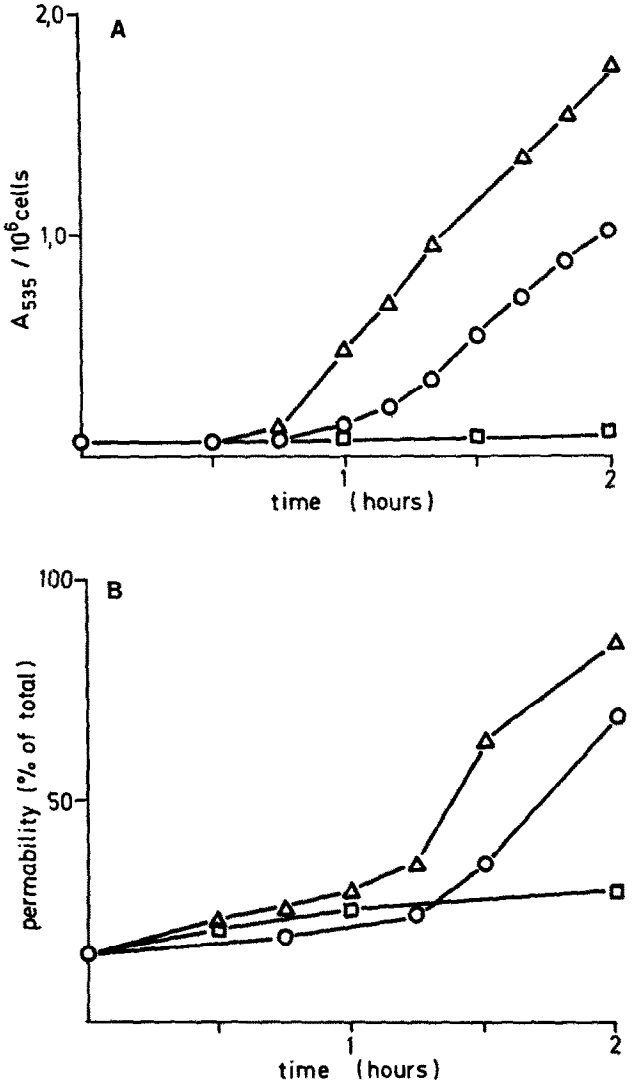

Fig. 7. Effect of chloroform metabolism on malondialdehyde levels (panel A) and cellular permeability (panel B) in phenobarbital-induced hepatocytes. □—□, no addition; ○—○, 3.1 mM $CHCl_3$; △—△, 6.2 mM $CHCl_3$.

Table 1. Uptake of cysteine and methionine in phenobarbital-induced isolated hepatocytes

	Cysteine uptake (nmoles/10^6 cells/5 min)		Methionine uptake (nmoles/10^6 cells/5 min)	
Time (min)	Without $CHCl_3$	$CHCl_3$ (6.2 mM)	Without $CHCl_3$	$CHCl_3$ (6.2 mM)
0	2.24	2.00	1.06	0.93
5	—	—	—	1.01
10	—	1.87	—	1.05
15	2.56	2.02	1.28	0.91
20	—	2.26	—	1.12
30	3.31	2.13	1.30	0.96
45	3.95	2.14	1.12	0.89
60	4.12	2.09	0.81	0.94

Hepatocytes were incubated as described in Methods, with or without chloroform. Aliquots (1 ml) of the cell suspension were transferred to test tubes at times indicated, either radiolabeled cysteine or methionine was added, and the incubation was continued in an oscillating water bath (37°) for 5 min. The final amino acid concentration was 0.2 mM.

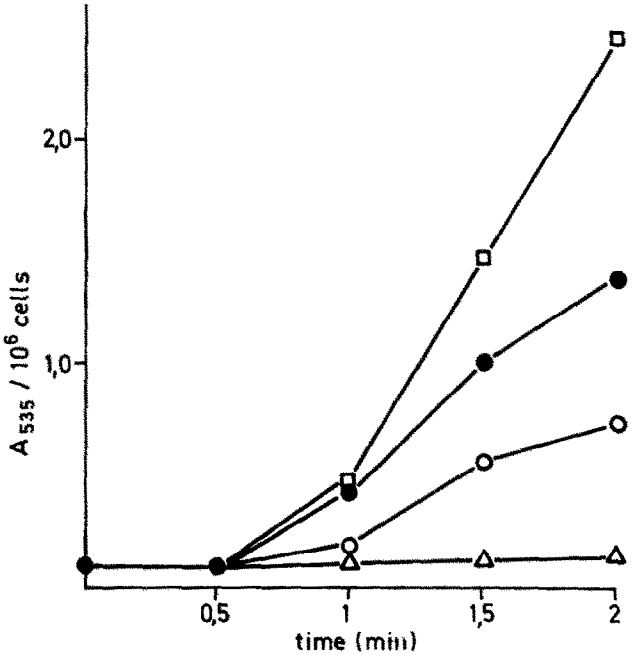

Fig. 8. Effect of paracetamol on lipid peroxidation in phenobarbital-induced and $CHCl_3$-exposed isolated hepatocytes. $CHCl_3$ concentration was 6.2 mM. □—□, no addition; ●—●, 1 mM paracetamol; ○—○, 2.5 mM paracetamol; △—△, 5 mM paracetamol.

DISCUSSION

The experiments presented here clearly indicate a relationship between chloroform metabolism and toxicity in the isolated cell system. The delayed lysis of phenobarbital-induced cells and the lack of similar effects in control cells show that chloroform *per se* is not toxic at the concentrations used. The loss of GSH and the covalent binding of radiolabel to proteins further support the hypothesis [2–5] that a reactive intermediate is formed as a function of monooxygenase activity.

Due to limitations in the model system used the period of maximal chloroform metabolism was short. The K_m value for chloroform oxidation in microsomes (1.4 mM; cf. Ref. 4) and the evaporation curve indicate that this period was less than 20 min when 6.2 mM was used. A similar conclusion can be drawn from Fig. 1B. The rate of covalent binding to proteins, on the other hand, continued essentially unchanged for almost 40 min, but this apparent linearity may be due to a greater proportion of the metabolites being bound to proteins at the end than at the beginning of the incubation. In any event, these results show that only a relatively short period of chloroform metabolism is required to induce persistent changes which may be of vital importance for the hepatocytes. Thus, chloroform-exposed hepatocytes rapidly lost the ability to replenish their GSH level and did not regain it when chloroform metabolism declined. This finding distinguishes chloroform metabolism from that of several other xenobiotics. A general observation in previous studies from this laboratory was that GSH levels could be replenished in depleted cells as long as sulfur amino acids were transported across the plasma membrane [9, 16].

Lipid peroxidation was detectable at a later stage of incubation, when the chloroform concentration was low and its metabolism apparently had declined to a low rate. Lipid peroxidation can thus hardly explain the lost ability to replenish cellular GSH. Indicative is also that sulfur amino acids prevented lipid peroxidation and cellular lysis in the experiments with iodoacetamide (Fig. 5). The importance of lipid peroxidation seems to be its coupling with cellular lysis. Lipid peroxidation preceded cellular lysis and lysis was prevented when lipid peroxidation was inhibited. Paracetamol was chosen to inhibit the reaction for two reasons: it is a non-volatile substrate for cytochrome P-450, the enzyme which has been suggested to be essential for the propagation of lipid peroxidation in rat liver microsomes [17]. Furthermore, paracetamol metabolites bind covalently to macromolecules [18–21] and when added in our model system the formation of reactive metabolites can be expected to have continued.

The delayed accumulation of malondialdehyde indicates that GSH depletion was a necessary prerequisite for lipid peroxidation to occur (cf. Ref. 9), but the critical control for this conclusion, i.e. prevention of lipid peroxidation by GSH resynthesis, is unfortunately lacking. Of further interest is that the delayed accumulation of malondialdehyde permits a separation of effects into two main phases, both of possible importance for chloroform toxicity. During the first phase chloroform metabolism brings about persistent changes in GSH turnover and then apparently declines. During the second phase lipid peroxidation destroys the cells. The probable link between these two phases is a state of GSH deficiency.

It has recently been reported that chloroform is a very weak inducer of *in vivo* lipid peroxidation as compared to carbon tetrachloride; conjugated dienes could not be detected after 30 min exposure [22]. It appears from the results presented here that both the mechanism and the time scale for chloroform induced lipid peroxidation are different from that induced by carbon tetrachloride. Our results indicate that the longer exposure time used by Brown *et al.* [1] to demonstrate conjugated dienes was critical.

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