

Mechanisms of Acute Hepatic Toxicity:

Chloroform, Halothane, and Glutathione

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The effects of hepatic microsomal enzyme induction with phenobarbital and depletion of hepatic glutathione (GSH) with diethyl maleate on the acute hepatotoxic responses to chloroform and halothane anesthesia were studied in rats. Phenobarbital pretreatment markedly increased the hepatotoxic response to chloroform anesthesia, but had little effect on halothane hepatotoxicity. Hepatic GSH levels were decreased 70–80 per cent by 2 hours of chloroform anesthesia in induced rats, but were unchanged in non-induced rats and in animals anesthetized with halothane. Marked destruction of microsomal electron transfer components was observed in the chloroform-anesthetized, induced animals only. Induction caused a large increase in *in-vitro* covalent binding of $^{14}\text{CCHCl}_3$ metabolites to microsomal protein, which could be prevented by GSH. Diethyl maleate pretreatment lowers GSH content approximately 80 per cent. Chloroform anesthesia produced hepatic necrosis and destruction of microsomal enzymes in the absence of induction, but halothane did not. Hepatotoxicity of chloroform appears to be related to two factors: 1) rate of biotransformation; 2) availability of the hepatic antioxidant, GSH. Halothane hepatotoxicity does not proceed by the same sequence of events as does that of chloroform. (Key words: Anesthetics, volatile, halothane; Anesthetics, volatile, chloroform; Biotransformation, enzyme induction; Toxicity, hepatic; Liver, hepatotoxicity.)

THE HEPATOTOXICITY of alkyl halides such as carbon tetrachloride and chloroform has been attributed to the interaction of highly reactive intermediates (perhaps free radicals)

with tissue macromolecules. This interaction results in an alteration of cellular integrity which leads to tissue necrosis.¹⁻³ It is generally assumed that these active intermediates are produced by action of the hepatic microsomal enzymes.⁴⁻⁶ For example, Scholler⁷ demonstrated that increasing the rate of chloroform metabolism by hepatic microsomal enzyme induction greatly enhanced the hepatic necrosis produced by chloroform, whereas inhibiting metabolism of the anesthetic or supplying antioxidants reduced the severity of the necrosis. Normally, the liver possesses antioxidants or free radical scavengers that can protect important structural or biochemical lipids and proteins from interaction with the active intermediates.^{8,9} However, if the liver is exposed to an abnormally high concentration of active intermediates (as with enzyme induction), or if the levels of endogenous protective agents are reduced, tissue destruction may occur.

Recent findings have indicated that glutathione (GSH), a sulfhydryl tripeptide found in high concentrations in the liver, may play a key role in protecting the liver from the toxic effects of bromobenzene,¹⁰ acetaminophen¹¹ and carbon tetrachloride.¹² The actual mechanism by which GSH reduces tissue damage is not known, but it is suggested that the covalent binding of the active intermediates of these compounds to tissue macromolecules is reduced.^{11,13,14} Also, it appears that the microsomal lipid peroxidation induced by CCl_4 is prevented by GSH.¹⁵

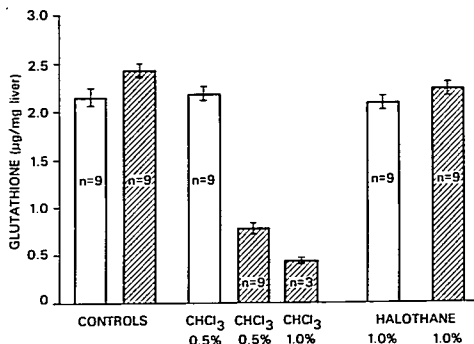
The present studies were performed to determine whether GSH affords protection against the acute hepatotoxicity following chloroform anesthesia. Halothane, an inhalation anesthetic implicated in sporadic clinical cases of hepatic necrosis,¹⁶⁻¹⁸ and recently reported to produce hepatotoxicity in phenobarbital-pretreated rats,¹⁹ was also investigated.

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FIG. 1. Liver reduced glutathione content (means \pm SE) following 2 hours of anesthesia. Hatched bars are results in rats pretreated with phenobarbital. Depletion of glutathione was significant, $P < 0.001$ level, in the induced animals anesthetized with chloroform.



Methods

Mature male Sprague-Dawley rats (180–250 g) of the same breeding strain were employed. All animals were conditioned and insured freedom from contact with environmental inducing agents for one week before experiments began. Animals were fed *ad lib.* until the time the experiments were performed. The rats were divided into two major groups: control and phenobarbital-induced. Pretreatment with the microsomal enzyme inducing agent was accomplished for 10 days by adding 1 mg phenobarbital/ml drinking water. Enzyme induction was confirmed by noting increases in hepatic microsomal contents of cytochrome P-450, NADPH cytochrome c reductase, cytochrome b₅, and enhanced *in vitro* biotransformation of aniline and hexobarbital. Some animals of both control and phenobarbital-induced groups were given diethyl maleate (0.6 ml/kg, ip, in corn oil) 15 minutes before anesthesia. Diethyl maleate rapidly reduces levels of glutathione without producing acute hepatic toxicity, the maximal effect occurring 30 minutes after oral administration.^{8,9}

Animals were anesthetized for 2 hours in a 19-1 plexiglass chamber. A conventional anesthesia vaporizer outside the chamber supplied chloroform or halothane volatilized in air at a flow rate of 6 l/min. Samples from within the chamber were periodically analyzed for anesthetic concentration by gas chromatog-

raphy. The anesthetic was either chloroform (0.5 or 1.0 per cent) or halothane (1.0 per cent). At the termination of anesthesia, some animals were immediately sacrificed for examination; the rest were sacrificed 18 hours later.

The following analyses were performed on the livers of these animals. Reduced glutathione (GSH) was measured by the method of Grunert and Phillips²⁰ and expressed as µg/mg liver; microsomal electron transfer components were assayed by previously described techniques²¹ (cytochrome P-450 expressed as µmol/mg microsomal protein; NADPH cytochrome c reductase as µmol/min/mg microsomal protein; cytochrome b₅ as µmol/mg microsomal protein); liver triglycerides were determined by the method of Butler *et al.*²² and expressed as mg/g liver; liver microsomal diene conjugates (a measure of lipoperoxidation) were assayed by a previously described ultraviolet spectrophotometric technique²³; histologic sections of livers were prepared by buffered-formalin preservation, paraffin imbedding, and staining with hematoxylin and eosin.

The covalent binding of chloroform and halothane metabolites was determined by modification of the *in-vitro* procedure described by Corsini *et al.*¹⁴ Hepatic microsomal pellets were preincubated at 37 C for 3 minutes. Each reaction vial contained 1 mg microsomal protein, 0.2 mM NADP, 2 mM glucose-6-phosphate, and 1 I.E.U. glucose-6-phos-

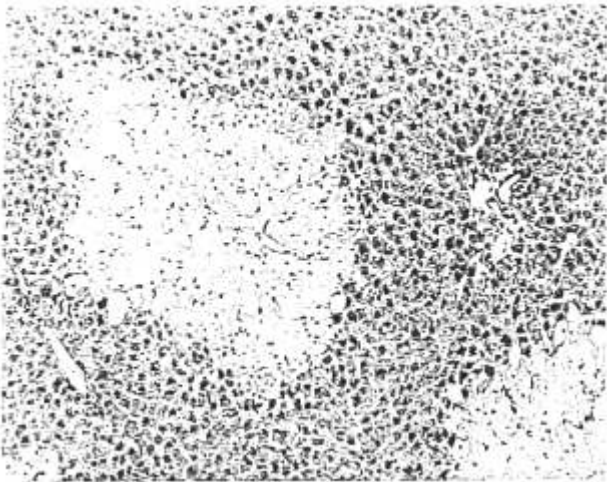


FIG. 2. Typical centrilobular necrosis observed in phenobarbital-pretreated rats anesthetized with chloroform (18 hours after chloroform). $\times 25$.

phate dehydrogenase in a total volume of 1 ml 0.02 M Tris, 1.15 per cent KCl buffer. The reaction was initiated by addition of 10 μ l of either $^{14}\text{C}\text{HCl}_3$ or $1\text{-}^{14}\text{C}$ -halothane (obtained from New England Nuclear Corp.) in dimethyl-formamide (10^{-3} M concentration, specific activity 1 $\mu\text{Ci}/\mu\text{mol}$). The vials were stoppered and incubated at 37 C. Reactions were terminated at 2, 5, 10, 20, and 40 min by addition of 1 ml 10 per cent trichloroacetic acid. The precipitate was removed by centrifugation and washed six times with 5 ml hot (50 C) methanol: ether (3:1) to remove noncovalently bound radioactivity. The washed precipitate was dissolved in 1.0 ml 1 N NaOH and samples were taken for determination of radioactivity by liquid scintillation counting and protein content by the method of Lowry.²¹ Blank values were determined. Results were expressed as picomols ^{14}C bound per mg protein. Control chloroform binding at zero time was 240 picomols/mg microsomal protein; that of halothane, 260 picomols. These values were subtracted from mean of all other results.

Results

After two hours hepatic GSH content in phenobarbital-pretreated animals was decreased 70 per cent by 0.5 per cent chloroform anesthesia, and 83 per cent by 1 per cent chloroform. The difference between GSH contents with these two concentrations of chloroform was significant, $P < 0.001$. There was no change in GSH concentrations in non-induced chloroform-anesthetized animals or in animals of either halothane-anesthetized group. These results are summarized in figure 1. Mortality rates at 18 hours were approximately 20 per cent for phenobarbital-pretreated rats anesthetized with 0.5 per cent chloroform and 80 per cent with 1.0 per cent chloroform. There was no death in the non-induced chloroform series or in any of the halothane series. Figure 2 shows a section of liver from an induced rat 18 hours after anesthesia with 0.5 per cent chloroform. Marked centrilobular necrosis is evident. Although this histologic pattern was seen in all induced rats anesthetized with chloroform,

non-induced animals did not show necrosis after 24 hours. Neither phenobarbital-pretreated nor control rats showed necrosis following halothane.

Phenobarbital induction *per se* does not increase microsomal diene conjugates. The mean of the non-anesthetized control and phenobarbital-pretreated groups was Δ OD 0.204 ± 0.018 . Using Δ OD = 0.204 as control, figure 3 illustrates the increases in diene conjugates in induced animals produced by chloroform (0.5 per cent) and halothane (1 per cent).

Eighteen hours after anesthesia, microsomal electron transfer components were assayed. Only induced chloroform-anesthetized animals displayed destruction of these enzymes. In neither group was halothane detrimental to these components. Chloroform in phenobarbital-pretreated animals caused marked destruction of cytochrome P-450 and NADPH cytochrome c reductase. These animals also had increased microsomal cytochrome b_5 . This effect is probably due to leakage of this enzyme from destroyed mitochondria. Cytochrome b_5 levels were not depressed as were those observed by Barker *et al.* following carbon tetrachloride toxicity.²⁴ The proper controls for this study were phenobarbital-induced non-anesthetized animals, since no change was observed in any of the non-induced groups. Table I summarizes these data.

Diethyl maleate administration produces a

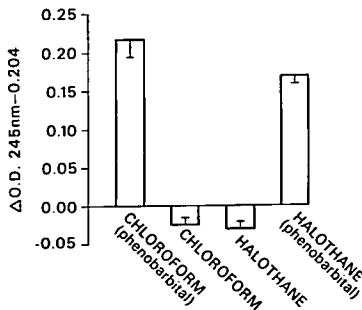


FIG. 3. Microsomal diene conjugate concentration following anesthesia (means \pm SE), calculated as Δ OD = 0.204. Only induced animals anesthetized with chloroform and halothane showed increases in this product of lipoperoxidation.

rapid reduction in GSH content. Two hours after an ip dose, the mean GSH level in rats was $0.474 \pm 0.03 \mu\text{g}/\text{mg}$ liver ($n = 6$). This is a decrease of 78 per cent from control value of $2.15 \pm 0.01 \mu\text{g}/\text{mg}$ liver. Hepatic microsomal enzymes, diene conjugates, and hepatic histology are unaffected by diethyl maleate-produced GSH depletion in these groups: control, halothane-anesthetized, and phenobarbital-pretreated halothane-anesthetized. In contrast, chloroform anesthesia (0.5 per cent) in the presence of GSH depletion increased diene conjugates in non-

TABLE I. Microsomal Enzyme Transfer Components (Means \pm SE) 18 Hours after Anesthesia with 0.5 Per Cent Chloroform in Induced and Non-induced Rats

	Induced		Non-induced	
	Control	Chloroform	Control	Chloroform
Cytochrome P-450 $\mu\text{mol}/\text{mg}$ ($n = 9$)	1.26 ± 0.03	$0.281 \pm 0.025^*$	0.487 ± 0.016	0.453 ± 0.08
NADPH Cytochrome Reductase μmol cyt c red/min/mg ($n = 9$)	120.6 ± 5.7	$40.5 \pm 5.9^*$	87.1 ± 2.7	84.4 ± 4.8
Cytochrome b_5 $\mu\text{mol}/\text{mg}$ ($n = 9$)	0.469 ± 0.013	$0.965 \pm 0.02^*$	0.302 ± 0.01	0.345 ± 0.036

§ Significantly different from control, $P < 0.001$ l.

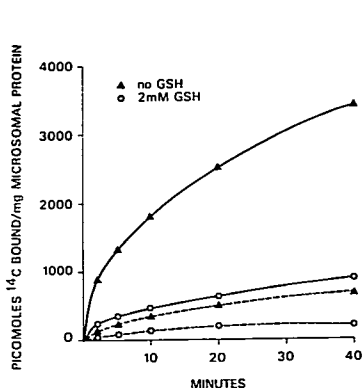


FIG. 4. *In-vitro* covalent binding of ^{14}C Cl $_2$ metabolites to hepatic microsomal protein. Solid lines represent induced microsomal protein; dashed lines, non-induced. Each point determined by the mean of results of two experiments.

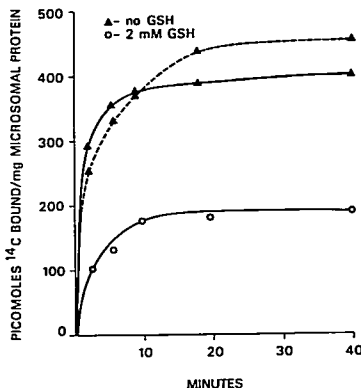


FIG. 5. Binding of $1\text{-}^{14}\text{C}$ -haloethane metabolites to hepatic microsomal protein. Solid line represents induced microsomes; dashed lines, non-induced. Each point determined by the mean of results of two experiments.

induced animals ($\Delta \text{OD} - 0.204 = 0.127 \pm 0.02$, $n = 6$), and produced destruction of cytochrome P-450 from 1.26 ± 0.03 to 0.373 ± 0.02 $\mu\text{mol}/\text{mg}$, $n = 6$. Content of this oxidase under these circumstances is decreased, $P < 0.001$. Typical centrilobular necrosis is produced by chloroform anesthesia in diethyl-maleate GSH-depleted rats.

Starvation increases the sensitivity of the liver to toxic actions of chloroform.²⁶ Nine control rats were starved for 48 hours and microsomal electron components measured and compared with those of fed rats. Enzyme contents in starved rats all increased significantly $P < 0.001$. The increases were: cyto-

chrome P-450, 86 per cent; NADPH cytochrome c reductase, 39 per cent; cytochrome b₅, 70 per cent.

In-vitro covalent binding of chloroform metabolites to induced and control microsomal protein is illustrated in figure 4. Microsomal protein from livers of phenobarbital-pretreated animals bound chloroform metabolites to a much greater extent than did microsomal protein from non-induced livers. Addition of GSH (2 mM) markedly inhibited binding of these metabolites by induced microsomes and reduced binding in non-induced microsomes. Binding of ^{14}C -haloethane metabolites was not great, and was

TABLE 2. Liver Triglyceride Content (Means \pm SE) 18 Hours after Anesthesia with Chloroform or Haloethane

	Control	Chloroform	Chloroform + Phenobarbital	Chloroform + Diethyl Maleate	Haloethane	Haloethane + Phenobarbital + Diethyl Maleate
Liver triglyceride Content (mg/g)	6.3 ± 0.8 (n = 9)	6.1 ± 0.71 (n = 9)	$13.15 \pm 0.76^*$ (n = 9)	$14.75 \pm 1.7^*$ (n = 4)	5.43 ± 0.5 (n = 9)	5.4 ± 1.6 (n = 6)

* Elevated triglycerides produced by chloroform anesthesia in phenobarbital-induced and in GSH-depleted rats ($P < 0.001$).

not different in either group (fig. 5). (Note that the y axes of figures 4 and 5 are of different orders of magnitude). ^{14}C -halothane metabolite binding in control and induced microsomes was quantitatively similar to $^{14}\text{CHCl}_3$ binding in non-induced microsomal protein. Addition of GSH did reduce halothane binding. Omitting the NADPH generating system did not inhibit binding in the ^{14}C -halothane experiments. Radioactive halothane has been found to have impurities by the technique of radiochromatography.²⁷ However, the disparity between chloroform binding and halothane binding was so great that even 80 per cent impurities could not account for the difference.

Another index of hepatic toxicity in these various circumstances was the hepatic triglyceride content. Table 2 summarizes these values and demonstrates that chloroform was toxic in induced animals and in control animals depleted of GSH.

Discussion

This study demonstrates that the metabolism of chloroform is a crucial factor in its hepatic toxicity. Phenobarbital pretreatment markedly increased the hepatotoxic response to chloroform. Burger and Herdson²⁴ found that phenobarbital caused proliferation of the endoplasmic reticulum predominantly in the centrilobular portions of the liver. Hence, chloroform would be expected to produce necrosis primarily in the centrilobular portions of the liver.

The biotransformation of chloroform theoretically can occur by homolytic fission and produce highly reactive free-radical intermediates.²⁸ The resultant organic free radical must acquire an electron to gain stability. Apparently, metabolites of chloroform may acquire this electron either from polyenoic unsaturated fatty acid esters of microsomal membranes or from -SH groups such as those of reduced glutathione (fig. 6). Acquisition of an electron from the fatty acids gives rise to an autocatalytic destruction of cell components, as emphasized by the lipid peroxidation theory of Recknagel. It appears that GSH is capable of neutralizing the free radicals without obvious harmful effect. The covalent binding studies indicate GSH is the

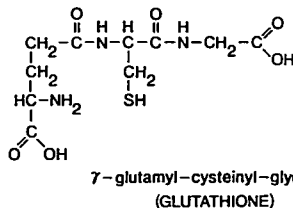


FIG. 6. Structural formula of glutathione.

preferred electron donor, unless its content is decreased to the extent that it cannot effectively reduce the activity of the chloroform metabolites. In induced animals, GSH is rapidly depleted by enhanced chloroform biotransformation such that it cannot prevent tissue destruction by inhibiting the covalent binding or lipid peroxidation.

Chloroform hepatotoxicity would seem to occur in the following sequence: 1) enhanced metabolism (either drug-induced or by starvation) results in increased production of free-radical intermediates (evidenced by increased diene conjugates); 2) GSH concentration is reduced to a critical level as it is consumed quenching the free radicals and perhaps conjugating with chloroform intermediates; 3) with GSH content reduced, continued metabolism of chloroform produces intermediates that covalently bind the microsomal (and mitochondrial?) protein; 4) an autocatalytic lipoperoxidation reaction occurs, destroying phospholipid-rich intracellular membranes and other structures; 5) this in turn leads to cellular necrosis and triglyceride accumulation. A decrease in GSH in the absence of induced metabolism of chloroform (*i.e.*, diethyl maleate administration) will produce a similar effect, since GSH is key in protection against free-radical effects. Control animals anesthetized with chloroform do not have this pronounced hepatotoxicity because the low generation of free radicals permits the liver to maintain an adequate supply of GSH. These findings are similar to those of Ilett *et al.*,³⁰ who reported massive chloroform-induced centrilobular necrosis in mice pretreated with phenobarbital but only slight centrilobular glycogen loss

in non-induced mice. Similarly, Sasame *et al.*²¹ found no P-450 destruction in non-induced rats receiving chloroform (1.25 ml/kg, orally). It is generally assumed that chloroform is hepatotoxic, but the data presented in this paper suggest that it is the condition of the liver that determines the toxicity. Factors such as drug pretreatment,⁷ stress, and starvation²⁶ determine the susceptibility of the liver, and these factors must be considered when comparing one published report with another.

Halothane, in induced or GSH-depleted animals, or even in induced and GSH-depleted animals, is not hepatotoxic to rats. It produces no decrease in GSH levels, even in induced animals. Speculation exists that the electron abstraction power of halothane intermediates is far less than that of chloroform. There is little covalent binding of halothane, and there is no difference between the extents of binding in microsomes in control and phenobarbital-pretreated animals. In the course of these halothane experiments it was observed that there was no change in ¹⁴C-halothane metabolite binding even when the NADPH generating system was omitted. This would indicate that halothane metabolite formation does not depend on a functional cytochrome P-450 system. GSH does inhibit ¹⁴C-halothane binding to some extent, although the present data do not define the mechanism of this observation. The enhanced diene conjugate formation observed in induced animals following halothane cannot be completely explained by these experiments, since it is not followed by hepatic necrosis. There are three possible explanations for this discrepancy. First, diene conjugate formation as a quantitative measure does not always parallel tissue destruction.²² Second, lipoperoxidation produced by halothane in induced animals may be quickly quenched by GSH and other intrinsic antioxidants before it propagates to produce cellular necrosis. Third, the increased ultraviolet absorption at 243 nm may be the result of a methanol:chloroform soluble ethanolamine-halothane metabolite complex, as described by Cohen *et al.*²³

Although clinical corollaries of such an animal study are fraught with dangers, it

would appear there may be some risk from chloroform anesthesia in individuals exposed to microsomal enzyme-inducing drugs. Also, these data, on the surface, appear to confirm the clinical retrospective studies of Greene,²⁴ who concluded that halothane anesthesia in induced patients is not hazardous.

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