Biochemical Pharmacology, Vol. 21, pp. 2555–2561. Pergamon Press, 1972. Printed in Great Britain.

# COMPARISON OF EARLY INJURY TO LIVER ENDOPLASMIC RETICULUM BY HALOMETHANES, HEXACHLOROETHANE, BENZENE, TOLUENE, BROMOBENZENE, ETHIONINE, THIOACETAMIDE AND DIMETHYLNITROSAMINE*

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(*Received* 1 *February* 1972; *accepted* 5 *May* 1972)

**Abstract**—This study compares the effects *in vivo* of $CH_2Cl_2$, $CH_2Br_2$, $CHCl_3$, $CH_2I_2$, $CHBr_3$, $CCl_4$, $CHI_3$, $CBr_4$, $CI_4$, $C_2Cl_6$, $C_2HCl_5$, $C_6H_6$, $C_7H_8$, $C_6H_5Br$, ethionine, thioacetamide and dimethylnitrosamine on the functions and composition of liver endoplasmic reticulum 2 hr after poisoning. Within the halomethane series, the effects on lipid diene conjugate content, oxidative demethylase, $^{14}$C-glycine incorporation into protein and glucose 6-phosphatase of liver microsomes and cell sap RNA content, increase with decreasing effective negative charge on the halogen atoms ($\epsilon$), an indicator of increasing halomethane free radical reactivity (cf. B. P. Dailey, *J. chem. Phys.* **33**, 1641, 1960). Peak toxic effect is reached following $CCl_4$ and $CHI_3$. Glycine incorporation into protein is also decreased 2 hr after thioacetamide and dimethylnitrosamine. After dimethylnitrosamine, suppression of protein synthesis is concomitant with increases in lipid diene conjugate content and cell sap RNA. $C_6H_5Br$ and ethionine, both known hepatotoxins, do not effect microsomal composition and function at this time, nor do $C_6H_6$ or $C_7H_8$ both organic solvents with solubility properties similar to $CCl_4$. The findings support the hypothesis that free radical halomethane metabolites injure the endoplasmic reticulum by reacting with and chemically altering its constituents. While dimethylnitrosamine may act similarly, thioacetamide, bromobenzene and ethionine apparently do not.

THE HEPATOTOXICITY of $CCl_4$ has been attributed to the reaction of its free radical metabolites with lipid and protein constituents of the endoplasmic reticulum.[1–4] Since halomethanes constitute a homologous series whose reactivity in homolytic cleavage reactions increases with increasing halogenation,[5] a direct relationship should exist between their reactivity and the extent of the lesion.

While alterations after poisoning with carbon tetrachloride are the most thoroughly studied, some quantitative information is available about the effects of iodoform and chloroform. Within 1 hr after poisoning with $CCl_4$ or iodoform, lipid diene conjugate contents of the structural lipids of liver microsomes increase, and drug detoxification, protein synthesis and glucose 6-phosphatase activity are suppressed.[6–11] Concomitantly, halomethane-derived carbon and halogen are recoverable from liver lipids and proteins, particularly those of liver microsomes.[1,6] In addition to these changes primarily affecting the functions and composition of the endoplasmic reticulum, especially that of the centrolobular portion of the liver lobule, calcium transiently

* This work was supported by Grants GM-14323 and HE-06370 from the National Institutes of Health. The author is a Research Career Development Awardee (6-K-3-GM-7309) of the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service.

enters midzonal parenchyma during this period.[7] Although chloroform also produces centrolobular necrosis, neither glucose 6-phosphatase nor liver calcium is affected early in the course of poisoning with this agent.[7]

This study examines the effects of isomolar doses of a series of specific chloro, bromo, and iodomethanes on the composition and function of liver microsomes 2 hr after poisoning and correlates the alterations with the free radical reactivity of the series. In addition, the same effects are examined in a second group of compounds with properties other than free radical reactivity in common with $CCl_4$. Included are hexachloroethane, the ethane counterpart of $CCl_4$, benzene and toluene, lipid solvents with solubility properties similar to those of carbon tetrachloride, and four known hepatotoxins—bromobenzene, ethionine, thioacetamide and dimethylnitrosamine (DMNA).

## EXPERIMENTAL

Healthy young male rats (Charles River Laboratories, Wilmington, Mass.) weighing between 150 and 300 g each were maintained on a diet of Purina chow and water *ad lib*. Animals fasted for 16 hr were fed a single dose of $CH_2Cl_2$, $CH_2Br_2$, $CHCl_3$, $CH_2I_2$, $CCl_4$, $CHI_3$, $CBr_4$, $CI_4$, $C_2Cl_6$, $C_6H_6$, $C_7H_8$ and $C_6H_5Br$, 2600 $\mu$moles/100 g animal (equivalent to 0·25 ml $CCl_4$), in an equal volume of mineral oil by polyethylene stomach tube. Because $CHBr_3$ and $C_2HCl_5$ produce profound anesthesia and death at this dose level prior to 2 hr, a lower dose of 830 $\mu$moles/100 g animal was given. Ethionine (260 $\mu$moles/100 g), thioacetamide (2600 $\mu$moles/100 g) and dimethylnitrosamine (2600 $\mu$moles/100 g) were dissolved in a minimal volume of saline prior to feeding. Control animals received 0·5 ml mineral oil or saline. Thirty min prior to sacrifice 25 $\mu$c $^{14}$C-glycine (uniformly labeled, 115 mc/m-mole, New England Nuclear Corp.) was given intraperitoneally. All animals in the experimental series were either awake, or if anesthetized by the agent, responsive to pain at the time of sacrifice (see Table 2).

Animals were processed in groups of twelve six on the first day and six on the succeeding day. Each group contained at least one mineral oil fed control and one 2-hr $CCl_4$ fed experimental as references which were randomly distributed in the rank order of sacrifice. All chemical and functional parameters of liver microsomes were determined in duplicate on aliquots of microsomes from each experimental animal.

Immediately after decapitation and exsanguination, the liver was removed, homogenized in 5 vol. of 0·25 M sucrose and centrifuged at 12,500 *g* for 20 min to remove residue and mitochondria. The 12,500 *g* supernatant was recentrifuged at 105,000 *g* for 30 min and the microsomal pellets were weighed and resuspended by homogenization in 2·0 ml 0·25 M sucrose. Aliquots of 105,000 *g* supernatant were set aside for measurement of RNA. All preparations were carried out at 0–4°.

RNA content of microsomes and cell sap, conjugated diene content of microsomal lipids and glucose 6-phosphatase activity of microsomes were determined as previously described.[6] Oxidative demethylation of antipyrine was assayed according to the method of Orrenius[12] and nicotinamide adenine dinucleotide phosphate (NADPH)–neotetrazolium (NT) reductase determined according to the method of Dallner.[13] Microsomal protein, isolated by a modified Schmidt–Thannhauser procedure, was dried to

constant weight (104°), dissolved in a small amount of 0·3 M KOH and counted at infinite thinness (less than 200 $\mu g/cm^2$) in a thin-window gas flow counter.[6]

Coefficients of variation for the determination of protein and RNA were 0·03; and for oxidative demethylase, glucose 6-phosphatase, $^{14}C$-glycine incorporation, NADPH–NT reductase and lipid conjugated diene, 0·05.

## RESULTS AND DISCUSSION

The effects of $CH_2Cl_2$, $CH_2Br_2$, $CHCl_3$, $CH_2I_2$, $CHBr_3$, $CBr_4$, and $CI_4$, ranked in order of decreasing effective negative charge on the halogen atom, i.e. increasing free radical reactivity (Table 1), on the endoplasmic reticulum at 2 hr are compared with those of carbon tetrachloride and iodoform at 1 and 2 hr in Table 2 and Fig. 1. One hr after carbon tetrachloride, diene conjugate content of microsomal lipids more than doubles; cell sap RNA, an indicator of the extent of degranulation of the endoplasmic reticulum, increases; oxidative demethylase activity decreases to half; and glycine incorporation into protein *in vivo* drops to one-third control values (Fig. 1). NADPH–NT reductase, protein content and glucose 6-phosphatase activities are unchanged at this time. After iodoform, changes at 1 hr are qualitatively similar but of lesser

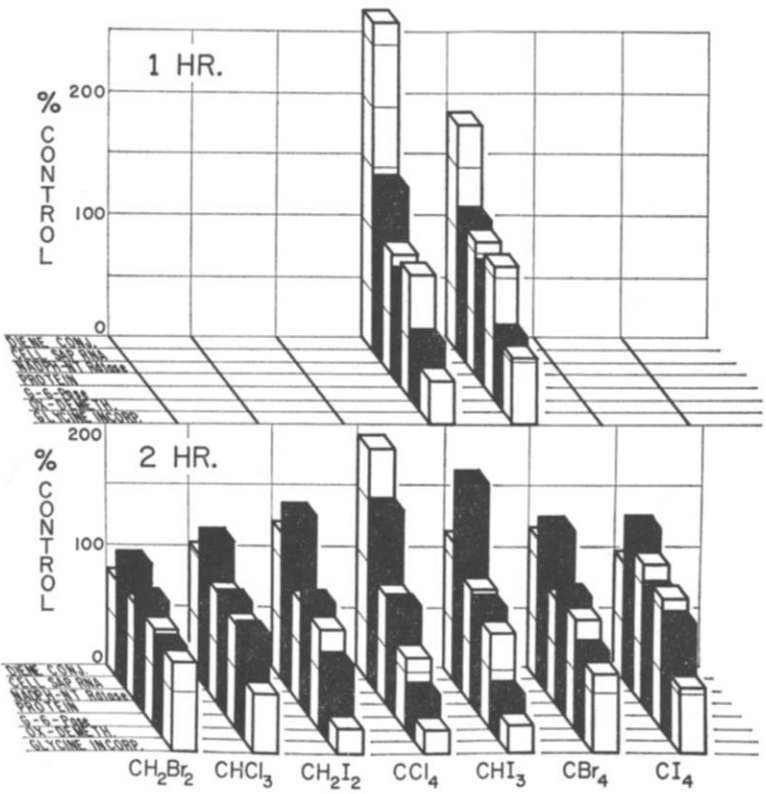


FIG. 1. Effect of halomethanes on microsomal composition and function 1 and 2 hr after poisoning with 2600 $\mu$moles halomethane/100 g rat. Halomethanes are arranged from left to right in order of decreasing effective negative charge on the halogen atoms ($\epsilon$). Experimental values, expressed as per cent control, are obtained from the data of Table 2.

magnitude, and the increase in cell sap RNA is not yet significant. Between 1 and 2 hr after both carbon tetrachloride and iodoform, lipid diene conjugate contents decrease (that of iodoform approaching control values), while changes in cell sap RNA, glycine incorporation and oxidative demethylation continue to increase. Glucose 6-phosphatase activity, normal at 1 hr, is suppressed at 2 hr particularly after carbon tetrachloride. The microsomal lesion appears fully developed at 2 hr after $CCl_4$ and $CHI_3$ and progresses but little during the next 2-hr interval.[6,8]

TABLE 1. WATER SOLUBILITY AND EFFECTIVE NEGATIVE CHARGE ON HALOGEN ATOMS ($\epsilon$) OF COMPOUNDS FED

| Compound | Water solubility at 30°* ($M \times 10^3$) | $\epsilon$† |
|---|---|---|
| Halomethanes | | |
| $CH_2Cl_2$ | 9·5 | −0·70 |
| $CH_2Br_2$ | 66 | −0·59 |
| $CHCl_3$ | 63 | −0·52 |
| $CH_2I_2$ | 53 | −0·47 |
| $CHBr_3$ | 13 | −0·42 |
| $CCl_4$ | 5·5 | −0·39 |
| $CHI_3$ | 0·25 | −0·32 |
| $CBr_4$ | 0·73 | −0·31 |
| $CI_4$ | < 0·01 | −0·24 |
| Chloroethanes | | |
| $C_2HCl_5$ | 2·5 | |
| $C_2Cl_6$ | 0·3 | |
| Lipid solvents | | |
| $C_6H_6$ | 22 | |
| $C_7H_8$ | 5·8 | |
| Hepatotoxins | | |
| $C_6H_5Br$ | 2·9 | |
| Ethionine | VS‡ | |
| Thioacetamide | VS | |
| Dimethylnitrosamine | VS | |

* Values obtained from Refs. 14 and 15.
† Values obtained from Ref. 5.
‡ VS = very soluble.

Certain progressions in the degree of injury to the endoplasmic reticulum with increasing halomethane-free radical reactivity are evident 2 hr after poisoning (Table 2 and Fig. 1). The least reactive halomethanes, $CH_2Cl_2$, and $CH_2Br_2$, with relatively high $\epsilon$ are without significant effect. Maximum effect is reached after carbon tetrachloride and iodoform, and decreases after $CBr_4$ and $CI_4$. Indeed, the effect of $CI_4$, limited to increased cell sap RNA and decreased glycine incorporation, resembles that of $CHCl_3$. Decreased toxic effect on the endoplasmic reticulum after $CBr_4$ and $CI_4$, considered to be the most readily homolytically cleavable compounds in this series, may be due to their marked water insolubility limiting the dose of halocarbon reaching the endoplasmic reticulum of the liver (Table 2). $CHBr_3$ given in approximately

TABLE 2. CHEMICAL AND FUNCTIONAL PROPERTIES OF LIVER MICROSOMES AT 1 AND 2 HR AFTER POISONING*

| Agent† | Dose (μmoles/ 100 g) | Time (hr) | Protein content (mg/g microsomes wet wt.) | Oxidative demethylase (μmoles product /g microsomes wet wt.) | Glucose 6-phosphatase (μmoles product /g microsomes wet wt.) | NADPH–NT reductase (μmoles product /g microsomes wet wt.) | $^{14}$C-glycine incorporation (cpm/g protein) | Cell sap RNA (μmoles/g liver wet wt.) | Lipid conjugated diene content (μmoles/g microsomes wet wt.) |
|---|---|---|---|---|---|---|---|---|---|
| Control (25) | | | 72·3 ± 1·2 | 0·319 ± 0·017 | 21·4 ± 1·0 | 1·65 ± 0·07 | 458 ± 38 | 2·96 ± 0·14 | 0·233 ± 0·014 |
| Halomethanes‡ | | | | | | | | | |
| $CH_2Cl_2$ (4) | 2600 | 2 | 68·8 ± 1·2 | 0·265 ± 0·023 | 22·1 ± 1·9 | 1·73 ± 0·14 | 393 ± 67 | 2·61 ± 0·35 | 0·210 ± 0·050 |
| $CH_2Br_2$ (4) | 2600 | 2 | 67·1 ± 3·3 | 0·247 ± 0·033 | 17·1 ± 1·5 | 1·32 ± 0·16 | 339 ± 149 | 3·08 ± 0·10 | 0·185 ± 0·034 |
| $CHCl_3$ (6) | 2600§ | 2 | 68·0 ± 1·9 | 0·282 ± 0·016 | 18·4 ± 1·5 | 1·49 ± 0·12 | 218 ± 82‖ | 3·63 ± 0·26‖ | 0·233 ± 0·024 |
| $CH_2I_2$ (6) | 2600§ | 2 | 67·5 ± 3·5 | 0·209 ± 0·022¶ | 15·5 ± 1·0‖ | 1·37 ± 0·07¶ | 94 ± 29¶ | 4·25 ± 0·32¶ | 0·280 ± 0·015 |
| $CHBr_3$ (5) | 830§ | 2 | 67·0 ± 4·4 | 0·247 ± 0·015 | 18·7 ± 2·2 | 1·74 ± 0·11 | 407 ± 55 | 3·03 ± 0·32 | 0·354 ± 0·029¶ |
| $CCl_4$ (3) | 2600 | 1 | 64·4 ± 3·2‖ | 0·206 ± 0·020‖ | 20·9 ± 1·2 | 1·79 ± 0·19 | 161 ± 59‖ | 4·74 ± 0·52‖ | 0·541 ± 0·085¶ |
| $CCl_4$ (22) | 2600 | 2 | 65·6 ± 1·3¶ | 0·121 ± 0·008¶ | 12·9 ± 0·7¶ | 1·47 ± 0·08 | 96 ± 17¶ | 4·40 ± 0·16¶ | 0·440 ± 0·025¶ |
| $CHI_3$ (5) | 2600 | 1 | 67·9 ± 2·8 | 0·218 ± 0·016¶ | 21·9 ± 2·0 | 1·90 ± 0·15 | 233 ± 25‖ | 3·88 ± 0·55 | 0·360 ± 0·035¶ |
| $CHI_3$ (7) | 2600 | 2 | 65·7 ± 2·6‖ | 0·125 ± 0·019¶ | 17·0 ± 1·5 | 1·56 ± 0·24 | 107 ± 31¶ | 5·05 ± 0·28¶ | 0·259 ± 0·016 |
| $CBr_4$ (5) | 2600 | 2 | 71·7 ± 2·6 | 0·234 ± 0·035‖ | 19·2 ± 2·2 | 1·42 ± 0·13 | 299 ± 45 | 3·93 ± 0·63‖ | 0·268 ± 0·043 |
| $CI_4$ (5) | 2600 | 2 | 73·0 ± 3·4 | 0·309 ± 0·011 | 22·8 ± 0·7 | 1·90 ± 0·27 | 240 ± 76‖ | 4·00 ± 0·28‖ | 0·221 ± 0·030 |
| Chloroethanes | | | | | | | | | |
| $C_2HCl_3$ (5) | 830§ | 2 | 66·7 ± 3·3 | 0·272 ± 0·021 | 21·9 ± 1·8 | 1·75 ± 0·56 | 322 ± 50 | 3·19 ± 0·34 | 0·255 ± 0·035 |
| $C_2Cl_6$ (4) | 2600 | 2 | 73·5 ± 3·8 | 0·263 ± 0·020 | 24·2 ± 2·1 | 1·55 ± 0·10 | 581 ± 53 | 3·53 ± 0·56 | 0·257 ± 0·015 |
| Lipid solvents | | | | | | | | | |
| $C_6H_6$ (4) | 2600 | 2 | 72·7 ± 2·9 | 0·282 ± 0·016 | 21·2 ± 2·5 | 1·70 ± 0·05 | 447 ± 26 | 2·44 ± 0·44 | 0·260 ± 0·057 |
| $C_7H_8$ (3) | 2600 | 2 | 68·1 ± 3·0 | 0·290 | 29·2 ± 5·0 | 1·65 ± 0·11 | 483 ± 209 | 2·49 ± 0·21 | 0·215 ± 0·027 |
| Hepatotoxins | | | | | | | | | |
| $C_6H_5Br$ (3) | 2600 | 2 | 73·3 ± 7·9 | 0·283 ± 0·004 | 22·9 ± 2·4 | 1·39 ± 0·19 | 438 ± 131 | 2·69 ± 0·13 | 0·274 ± 0·039 |
| Ethionine (3) | 260 | 2 | 70·7 ± 1·9 | 0·312 ± 0·061 | 20·2 ± 2·7 | 1·85 ± 0·27 | 422 ± 60 | 3·45 ± 0·22 | 0·245 ± 0·030 |
| Thioacetamide (4) | 2600 | 2 | 70·0 ± 3·5 | 0·340 ± 0·059 | 20·9 ± 2·1 | 1·70 ± 0·21 | 129 ± 11¶ | 2·77 ± 0·40 | 0·241 ± 0·027 |
| Dimethyl-nitrosamine (5) | 2600 | 2 | 71·3 ± 2·5 | 0·299 ± 0·027 | 22·7 ± 2·4 | 1·40 ± 0·16 | 187 ± 23‖ | 4·17 ± 0·23¶ | 0·380 ± 0·035¶ |

* Compositional and enzymatic activities expressed as mean ± standard error.

† Number in parentheses is number of animals.

‡ Halomethanes are ranked in order of decreasing effective negative change on the halogen atoms ($\epsilon$).

§ Lethargic, or lightly anesthetized at time of sacrifice.

‖ $0.05 > P > 0.005$.

¶ $0.005 > P$.

one-third the dose of the other halomethanes also produces a significant increase in lipid diene conjugate content (Table 2).

Certain functional correlations appear among the changes within the halomethane group. The extent of centrolobular necrosis at 24 hr is roughly proportional to the magnitude of change in cell sap RNA and glycine incorporation at 2 hr—no necrosis occurring after $CH_2Cl_2$ and $CH_2Br_2$.[7] Decreased glycine incorporation into protein is inversely proportional to the increase in cell sap RNA ($r = -0{\cdot}808$, $P < 0{\cdot}01$). Of the functional alterations examined, protein synthesis is the most general and most sensitive indicator of injury, while glucose 6-phosphatase is the least sensitive but most selective, occurring after carbon tetrachloride and to a lesser extent after $CH_2I_2$ (Fig. 1 and Table 2). Diene conjugation cannot be used as a quantitative measure of injury at least beyond the first hr, since diene conjugate content decreases between 1 and 2 hr after poisoning. The differences in the extent of injury after each agent may reflect quantitative differences in halogenation, halomethylation, saturation and formation of branch chain fatty acids in the lipid components of the endoplasmic reticulum.[1,6,16–18] Less chloroform metabolite is recovered in microsomal lipid than $CCl_4$ metabolite while that of $CH_2Cl_2$ is barely incorporated into liver lipids at all.[17]

Among the non-halomethane compounds examined, $C_6H_6$ and $C_7H_8$, lipid solvents with solubility properties similar to those of $CCl_4$,[14,15] $C_2HCl_5$, $C_2Cl_6$, the ethane counterparts of $CHCl_3$ and $CCl_4$, bromobenzene and ethionine are without effect on microsomal function at 2 hr (Table 2). Lack of effect of $C_2HCl_5$ and $C_2Cl_6$, both of which are reductively dechlorinated by the liver in a reaction similar to that undergone by $CCl_4$,[2,19] may be due to limitation of dose reaching the liver because of their marked water insolubility (Table 1). On the other hand, the effects of dimethylnitrosamine are qualitatively similar to those of halomethanes (Table 2). Suppression of protein synthesis and increase in cell sap RNA 2 hr after DMNA are concomitant with increased lipid diene conjugate content! The presence of increased lipid diene conjugates indicates that structural lipids as well as RNA and proteins of microsomes may be chemically altered after poisoning with this biologically active methylating agent, and supports the hypothesis that DMNA methylates by a free radical mechanism.[20,21] However, in contrast to carbon tetrachloride, which preferentially labels lipids, DMNA metabolites are preferentially recovered primarily from microsomal RNA—lipid incorporating but little of the total counts bound[20]—a difference which may account for DMNA's lack of effect on oxidative demethylase and glucose 6-phosphatase activity.

Thioacetamide in doses of this magnitude (200 mg/100 g) virtually stops glycine incorporation into liver protein 2 hr after feeding without affecting any other microsomal parameters measured (Table 2). In smaller doses, thioacetamide's effect on protein synthesis becomes apparent at later times, an effect considered due to impairment either of the synthesis of messenger and transfer RNA, or their transfer from the nucleus.[22] The abrupt cessation of $^{14}$C-glycine incorporation into protein after this larger dose suggests that thioacetamide or its metabolites may also interfere with either $^{14}$C-glycine uptake by the liver cell, or more directly with protein synthesis through sequestration of required precursors, in a manner analogous to ethionine sequestration of adenosine.[23]

Neither bromobenzene, an intracellular sulfhydryl sequestering agent which affects intracellular cysteine and glutathione content and causes centrolobular necrosis at

24 hr,[24,25] nor ethionine which causes depletion of intracellular ATP[23] affects the parameters of function of the endoplasmic reticulum measured in this study at 2 hr after poisoning (Table 2). Although lack of effect in the case of ethionine could be due to sex difference,[23] similar negative results were obtained with female rats. After doses of ethionine of the magnitude used in this study (42 mg/100 g rat), decreased amino acid incorporation into microsomal protein lags the fall in liver ATP by 1 hr and is not apparent until the third hour of poisoning.[23] Similarly, bromobenzene is without effect on the protein synthetic functions of the endoplasmic reticulum until times beyond 12 hr after poisoning.[24–26]

Among the effects of the hepatotoxins examined in this study, two patterns of toxicity can be discerned. In the first (halomethanes and DMNA), failure of protein synthesis is associated with other compositional and functional alterations involving the endoplasmic reticulum early in the course of poisoning; in the second (thioacetamide, bromobenzene, ethionine), failure of protein synthesis is a relatively isolated event occurring relatively late in the course of poisoning. In the former, functional failure may occur as the result of direct chemical destruction of the endoplasmic reticulum by the hepatotoxin, while in the latter instance, failure of protein synthesis may be caused by a toxin-induced deficiency of required cofactors, or substrates for protein synthesis.

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