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EFFECT OF DIBROMOTETRAFLUOROETHANE INHALATION ON HEPATIC DRUG METABOLISM IN MICE

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Abstract—Inhalation of dibromotetrafluoroethane, 0·63 to 1·0%, for 5 hr daily, for 3 or 4 days, reduced hexobarbital sleeping time and zoxazolamine paralysis time 2-fold in mice. Increased metabolism of hexobarbital and zoxazolamine by the hepatic 9000 *g* microsomal supernatant fraction prepared from exposed mice correlated well with the effects determined *in vivo*. Addition of 2,4-dichloro-6-phenyl-phenoxyethyldiethylamine (SKF 525-A) to the hepatic 9000 *g* microsomal supernatant fraction prevented the dibromotetrafluoroethane-induced increase in hepatic drug metabolism.

HALOGENATED alkanes as a class are noted for their hepatotoxicity. Outstanding are the chlorinated compounds carbon tetrachloride, chloroform, 1,2-dichloroethane and 1,1,2,2-tetrachloroethane.[1–3] Other short-chain halogenated hydrocarbons such as methylchloroform (1,1,1-trichloroethane), an industrial solvent and degreaser, stimulate hepatic drug metabolism. Short term methylchloroform inhalation has been shown to decrease the duration of action of hexobarbital, zoxazolamine and meprobamate with increased metabolism *in vitro* by hepatic microsomes of hexobarbital, zoxazolamine and aminopyrine in rats.[4] Lal and Shah[5] reported that exposure to methylchloroform vapors reduced hexobarbital narcosis and increased the hepatic microsomal oxidation of hexobarbital in mice.

Generally, the fluorinated alkanes would not be considered significant hepatotoxins, owing to firm C—F bonds which do not dissociate easily.[6]

The following fluoroalkanes have come under investigation in this laboratory: bromotrifluoromethane (b.p. −57°), a fire extinguishing agent and aerosol propellant; 1,1,2-trichloro-1,2,2-trifluoroethane (b.p. +47·6°), a solvent and refrigerant; and 1,2-dibromo-1,1,2,2-tetrafluoroethane* (b.p. +47·3°), a proposed fire extinguishing agent.

Personnel handling these compounds would be most likely to receive the quantitatively most important exposures through inhalation because of their low boiling points. Preliminary studies of the effects of dibromotetrafluoroethane exposure on the duration of hexobarbital sleep and zoxazolamine paralysis suggested the possibility that the compound induced hepatic microsomal enzymes.[7] The present study was conducted to test further this hypothesis.

* Dibromotetrafluoroethane (Freon-114B2), E. I. DuPont De Nemours & Company, Inc., Freon Products Division, Deepwater, N.J.; also known as Halon 2402.

MATERIALS AND METHODS

Animals. Male CF-1 Albino mice, 18–22 g, obtained from Carworth Laboratories Midwest (Portage, Mich.), were bedded on Ab-Sorb-Dri. The animals were maintained on Purina laboratory chow and water *ad lib.* All sleep time studies and chamber exposures were performed during the same time periods each morning to eliminate daily rhythmic variation.[8]

Inhalation exposure. The animals were exposed in a Plexiglas chamber to a mixture of dibromotetrafluoroethane in air at ambient temperature. The composition of the exposure atmosphere was maintained by controlled delivery of liquid dibromotetrafluoroethane into a vaporizer column utilizing a dual syringe feeder.* The oxygen and carbon dioxide levels of the atmosphere in the exposure chamber were determined periodically using a Scholander micrometer gas analyzer.[9] A gas chromatograph† equipped with thermal conductivity detector (thermistor bead type) was used to monitor the concentration of dibromotetrafluoroethane in the exposure chamber. Five-hr daily exposures were conducted for 3 and 4 consecutive days to permit sufficient time to produce an effect on the enzyme system.

Studies in vivo. The duration of hexobarbital sleeping time and zoxazolamine paralysis time was determined on groups of 30 mice exposed 5 hr daily for 3 consecutive days. Hexobarbital (120 mg/kg) sleeping time or zoxazolamine‡ (100 mg/kg) paralysis time was defined as that interval between the loss and return of the righting reflex. Efforts were made throughout the study to hold constant extraneous factors that might alter mouse drug responsiveness.[10] Animals were treated with phenobarbital sodium (80 mg/kg) or with 3-methylcholanthrene (80 mg/kg) to test *in vivo*, the alterations of drug metabolism and to test *in vitro*, the metabolizing system of the hepatic 9000 *g* supernatant.[11,12]

Studies in vitro. Eighteen to 24 hr after exposure or treatment (the next a.m.), the mice were killed by cervical dislocation, the gall bladder was excised and the liver was removed and placed immediately in cold KCl (1·15%). All tissue and homogenate handling was carried out at 0°–4°. Homogenates were centrifuged at 9000 *g* for 20 min in a Sorvall RC-2 refrigerated centrifuge. The supernatant fraction was aspirated and used for the determination of enzyme activity.

The compound SKF 525-A§ (5×10^{-5} M/flask) was added 30 min prior to incubation to pooled liver preparations in a concentration expected to result in a 50 per cent inhibition of the oxidation of hexobarbital.[13]

Enzyme assay. Microsomal suspension was incubated at 37° in phosphate buffer pH 7·35 containing KCl, glucose 6-phosphate, $MgSO_4$, nicotinamide, NADP and the appropriate substrate. Hexobarbital metabolism was determined by the method of Cooper and Brodie[14] following substrate disappearance; zoxazolamine metabolism was determined by the method of Juchau *et al.*[15] Substrate metabolized was the difference between zero time and 30-min incubation. Conditions of incubation, cofactors

* Dual syringe feeder: syringe type pump of unlimited volume with external reservoir, type 63, designed by the Dow Chemical Company. Manufactured by Modern Metal Craft, Midland, Mich.

† Perkin–Elmer Vapor Fractometer, model 154, Perkin–Elmer Corp., Norwalk, Conn. The column, 1/4 in. O.D. S.S. containing Pora Pak Q, was maintained at a temperature of 140°, the detector at 140° and thermistor bead voltage at 8·2 V d.c. Helium with a flow of 100 ml/min was the carrier gas. The sample loop was 5·0 ml volume.

‡ Zoxazolamine was kindly supplied by McNeil Laboratories, Inc.

§ SKF 525-A (2,4-dichloro-6-phenyl-phenoxyethyldiethylamine) was kindly supplied by the Smith, Kline & French Laboratories.

used and concentrations were optimal and similar to those described by Gram and Fouts.[16] Linear relationships were demonstrated for hexobarbital oxidase activity up to and beyond 30-min incubation of hepatic 9000*g* supernatant for control and dibromotetrafluoroethane-treated animals. In addition, linear response was demonstrated for hexobarbital and zoxazolamine activity relative to the protein concentrations incubated. Aliquots of each hepatic 9000 *g* supernatant sample were assayed for protein content according to the method of Lowry *et al.*[17] Data are expressed as nanomoles of substrate metabolized per milligram of 9000 *g* supernatant protein per incubation period.

Light and electron microscopy. Livers of 1 % dibromotetrafluoroethane-exposed mice and control mice were examined by light and electron microscopy according to the technique described by Weinstein *et al.*[18]

Statistical analysis. Excluded from the sleep and paralysis time data are those occasional immobilization times that lie beyond the mean plus and minus two standard deviations, a range that would be expected to include approximately 95 per cent of all points of normally distributed data. The significance of differences between group means was determined by use of the Student's *t*-test. Hexobarbital oxidase and zoxazolamine hydroxylase specific activities were each treated as a mixed model, 3-level nested analysis of variance.[19] Significant differences among means in the analysis of variance were determined using Duncan's multiple range test.[20]

RESULTS AND DISCUSSION

Hexobarbital sleeping time and duration of zoxazolamine paralysis. Inhalation of dibromotetrafluoroethane in mice reduced the hexobarbital sleeping time and the duration of zoxazolamine paralysis by amounts up to 50 per cent, 18 hr after the termination of exposures. Figures 1 and 2 illustrate the results of exposure of mice to

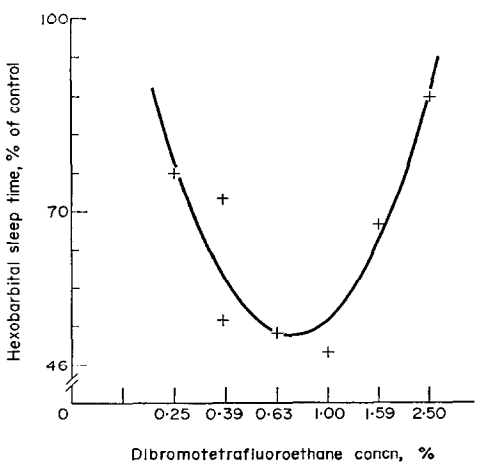

FIG. 1. Effect of dibromotetrafluoroethane exposure on the duration of hexobarbital sleeping in mice. Animals were injected, i.p., 18 hr post exposure. Sleep time is represented along the abscissa as percentages of control, and dibromotetrafluoroethane concentration is represented along the ordinate on a logarithmic scale. The parabola plotted through the points represents a curve determined by the method of least squares and had a coefficient of correlation of 0·91. Each point represents 13–16 mice.

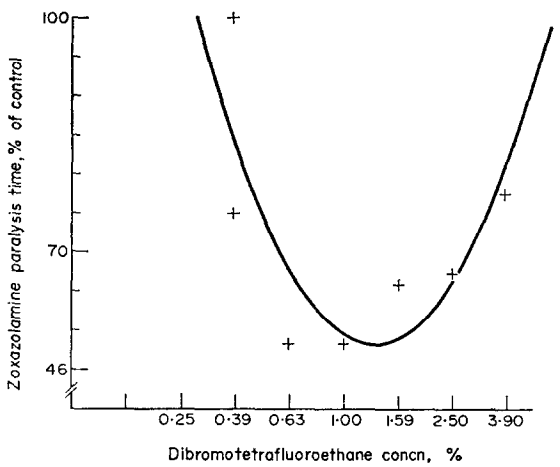

FIG. 2. Effect of dibromotetrafluoroethane exposure on the duration of zoxazolamine paralysis. Paralysis time is represented along the abscissa as percentage of control, and dibromotetrafluoroethane concentration is represented along the ordinate on a logarithmic scale. The parabola plotted through the points represents a curve determined by the method of least squares and had a coefficient of correlation of 0·80. Each point represents 13–14 mice.

concentrations ranging from 0·25 to 3·9 per cent. The duration of hexobarbital sleeping time and zoxazolamine paralysis time, expressed as a percentage of control, shows similar time courses and appears to be concentration dependent. The parabolic relationship suggests a phasic effect, with increased metabolic rate (induction) up to 1·0 per cent and possible hepatotoxic effect beyond that concentration.[21] Maximum reduction in sleeping and paralysis time followed exposure to concentrations of about 1 per cent. The pharmacologic effects of exposures were convulsions at levels of 2–3 per cent narcosis at 4–5 per cent and death at 5–6 per cent. Similar signs observed in rats, exposed to somewhat higher concentrations, were reported by Rainaldi.[22]

Study in vitro. Studies were conducted on the enzyme activity of hepatic 9000 *g* supernatant fraction of control animals and animals exposed to those concentrations that had elicited maximum changes *in vivo* (0·63 and 1·00 per cent).

Data presented in Table 1 indicate that, at the 0·63 and 1·0 per cent concentrations, the livers from the dibromotetrafluoroethane-exposed animals metabolized hexobarbital and zoxazolamine at rates significantly higher than those of the control animals. The observation was made that a single 5-hr exposure and a 2-day (5 hr/day) exposure had no apparent effect on hexobarbital sleeping time or zoxazolamine paralysis time at a concentration of 1·0% dibromotetrafluoroethane. Therefore, it was felt unnecessary to test further 1- and 2-day exposures. Increasing the exposure to 3 days (5 hr/day) produced a significant increase in microsomal activity over the control. Extension of the exposure period from 3 to 4 days (5 hr/day) further increased the hexobarbital oxidase activity of exposed mice over the control animals.

Effect of phenobarbital and 3-methylcholanthrene on the enzyme systems. Mice treated with phenobarbital or 3-methylcholanthrene to ascertain their effects on the enzyme systems metabolized hexobarbital and zoxazolamine at rates significantly greater than controls, as seen in Table 2. Also observed was a decreased hexobarbital sleeping time and zoxazolamine paralysis time.

TABLE 1. EFFECT OF DIBROMOTETRAFLUOROETHANE INHALATION ON METABOLISM *in vitro* OF THE SUBSTRATES HEXOBARBITAL AND ZOXAZOLAMINE BY THE 9000 *g* SUPERNATANT FRACTION OF MOUSE LIVERS*

Inhalation exposure (%)	Duration (days)	Enzyme activity (mean ± S. E. M.)					
		Hexobarbital oxidase			Zoxazolamine hydroxylase		
		Control†	Treated†	Sig. (P)	Control†	Treated†	Sig. (P)
0·63	3	15·89 ± 3·72 (8)	22·59 ± 3·92 (8)	<0·05	12·53 ± 0·63 (8)	16·30 ± 0·47 (8)	<0·01
1·0	3	18·58 ± 1·14 (8)	24·10 ± 2·17 (8)	<0·05	13·23 ± 0·74 (8)	17·34 ± 1·11 (8)	<0·05
1·0	4	14·64 ± 0·94 (16)	23·04 ± 1·43 (16)	<0·01			

* Microsomal 9000 *g* supernatant fraction prepared from livers of mice exposed to dibromotetrafluoroethane 5 hr daily. Livers were removed 18 hr after termination of exposure and pooled, five livers per group; the number of group observations are in parentheses.

† Values quoted are the mean ± S. E. M. expressed as nanomoles of hexobarbital or zoxazolamine metabolized per milligram of 9000 *g* supernatant protein per 30 min.

TABLE 2. EFFECT OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE TREATMENT ON THE HEPATIC 9000 *g* SUPERNATANT METABOLISM OF HEXOBARBITAL AND ZOXAZOLAMINE IN MICE*

	Enzyme activity (mean ± S. E. M.)					
	Hexobarbital oxidase			Zoxazolamine hydroxylase		
Treatment	Control†	Treated†	Sig. (P)	Control†	Treated†	Sig. (P)
Phenobarbital sodium (80 mg/kg, i.p., daily/4 days)	8·12 ± 1·01 (8)	19·53 ± 1·69 (8)	<0·01	11·29 ± 0·74 (8)	20·26 ± 0·76 (8)	<0·01
3-Methylcholanthrene (80 mg/kg, i.p., daily/3 days)				11·59 ± 1·05 (8)	14·69 ± 0·45 (8)	<0·05

* Microsomal 9000 *g* supernatant fraction prepared 24 hr post treatment, five livers per group; the number of group observations are in parentheses.

† Values quoted are the mean ± S. E. M. expressed as nanomoles of hexobarbital or zoxazolamine metabolized per milligram of 9000 *g* supernatant protein per 30 min.

Effect of SKF 525-A on the metabolism of livers from dibromotetrafluoroethane-treated mice. The concentration of SKF 525-A required for 50 per cent inhibition of the metabolism of hexobarbital *in vitro* by mouse liver microsomes has been reported to be about 5×10^{-5} M.[13] Table 3 illustrates the effect of this concentration on hexobarbital metabolism in microsomes prepared from untreated and dibromotetrafluoroethane-treated mice. SKF 525-A reduced hexobarbital oxidase mean activities in unexposed groups from 14·04 to 7·92 nmoles/30 min/mg of supernatant protein and in 1% dibromotetrafluoroethane-exposed groups from 20·44 to 10·25. The mean values representing the liver hexobarbital oxidase activities from the four treatment groups differed significantly from each other. Estimation of the variance components (Table 4) indicated that 1·00 per cent of the total variance was attributable to measurement error, 8·80 per cent to differences among pools of livers, and 90·20 per cent to treatment (dibromotetrafluoroethane and SKF 525-A) effects.

The observation that SKF 525-A inhibited hexobarbital oxidase activity in livers from both control and H-2402-exposed mice supported the view that the induction effect of H-2402 was the consequence of a quantitative increase in oxidative enzyme rather than the induction of a new or different enzyme system.[23]

Light and electron microscopy. Light and electron micrographs of livers from control and dibromotetrafluoroethane-exposed mice were indistinguishable by visual examination.

The induction effect reported here is of interest in view of other reports on structurally related compounds, most notably halothane. Halothane has been suggested to induce hepatic microsomal enzyme activity[24] and also reported not to induce such activity.[25] Generally, however, few data have been published concerning the effects of this class of halogenated compounds on the mixed-function oxidases of the hepatic endoplasmic reticulum.

TABLE 3. EFFECT OF 2,4-DICHLORO-6-PHENYL-PHENOXYETHYLDIETHYLAMINE (SKF 525-A) ON THE METABOLISM OF *in vitro* THE SUBSTRATE HEXOBARBITAL BY THE 9000 *g* SUPERNATANT FRACTION OF MOUSE LIVERS AFTER DIBROMOTETRAFLUOROETHANE EXPOSURE*

	Treatment							
	Untreated controls		SKF 525-A†		1% H-2402		1% H-2402 + SKF 525-A†	
	Liver pools		Liver pools		Liver pools		Liver pools	
	1	2	1	2	1	2	1	2
	13·77‡	14·13	6·32	8·95	21·05	20·69	12·13	8·45
	14·40	13·83	6·32	10·07	19·23	20·80	11·96	8·45
Group mean ± S.D.	14·04 ± 0·28		7·92 ± 1·90		20·44 ± 0·82		10·25 ± 2·08	

* Microsomal fraction prepared from livers of mice exposed to 1% dibromotetrafluoroethane (H-2402) 5 hr daily/4-day duration. Livers were removed 18 hr after termination of exposure and pooled, five livers per group.

† SKF 525-A, 5×10^{-5} M/flask.

‡ Variates in body of table are expressed as nanomoles of hexobarbital metabolized per milligram of 9000 *g* supernatant protein per 30 min.

TABLE 4. ANALYSIS OF VARIANCE TABLE FOR MOUSE LIVER HEXOBARBITAL OXIDASE ACTIVITIES*

Source of variation	df	Sum of squares	Mean squares	F	Significance
Among treatment groups	3	359·194	119·731	20·835	$P < 0.01$†
Among pools of livers	4	23·493	5·873	18·507	$P < 0.01$‡
Within pools (error between duplicate determinations)	8	2·538	0·317		

* The results of Duncan's multiple range test indicated that all pairs of treatment-group means differed significantly from each other.
† $F(0.01, df = 3,4) = 16.7$.
‡ $F(0.01, df = 4,8) = 7.01$.

The observation was made in the present study that inhalation of dibromotetrafluoroethane at concentrations ranging from 0·25 to 1·00 per cent reduced the duration of hexobarbital sleeping time and zoxazolamine paralysis time. Similarly, hexobarbital oxidase and zoxazolamine hydroxylase activities were increased.

Our inability to demonstrate quantitative alterations in the smooth endoplasmic reticulum (SER) of dibromotetrafluoroethane-exposed mice can be explained in two ways. First, the possibility exists that there is no proliferation of SER membranes with exposure to the compound. The induction effect on the microsomal enzyme systems as demonstrated in these experiments would seem to argue against this possibility. Alternatively, SER membrane surface area could have been mildly increased, yet the increase might not be apparent with the techniques used in this study. Subtle changes in SER membrane (e.g. 30 per cent) may be impossible to detect without direct measurement of membrane profiles using morphometric techniques.[26] These techniques were not used in this study. Although we have not demonstrated an increase in the SER of liver cells, it is entirely possible that our biochemical data are compatible with our morphologic observations.

Exposure of mice to dibromotetrafluoroethane decreased hexobarbital sleeping time and zoxazolamine paralysis time *in vivo* and increased hepatic hexobarbital oxidase and zoxazolamine hydroxylase activities *in vitro*. SKF 525-A *in vitro* inhibited the inductive effect of dibromotetrafluoroethane, which suggested that the increase in the oxidase activities represented a quantitative increase in the pre-existing system rather than the induction of a new system. In the absence of a morphometric analysis of the liver electron micrographs, it could not be stated that exposure to the fluorocarbon was not accompanied by a proliferation of hepatic endoplasmic reticulum.

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The experiments reported herein were conducted according to the *Guide for Laboratory Animal Facilities and Care* (1965), prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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