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Genotoxic and Mutagenic Assays of Halothane Metabolites in Bacillus subtilis *and* Salmonella typhimurium

Krishna Sachdev, Ph.D., Ellis N. Cohen, M.D.,† Vincent F. Simmon, Ph.D.‡*

Reactions of *N*-acetylcysteine with the halothane metabolite, 2-chloro-1,1-difluoroethylene (CF_2CHCl), and two related probable metabolites, 2-bromo-1,1-difluoroethylene (CF_2CHBr) and 2-bromo-2-chloro-1,1difluoroethylene (CF_2CBrCl), afforded the saturated conjugates, $RSCF_2CH_2Cl$, $RSCF_2CH_2Br$, and $RSCF_2CHBrCl$, as well as the unsaturated analogs, RSCFCHCl and RSCFCHBr; R = $-CH_2CH(COOH)NHCOCH_3$. The mutagenic and genotoxic potential of these conjugates was evaluated in the *Salmonella*/microsome system described by Ames and a "*rec*" DNA repair system developed by Kada employing recombination proficient and deficient strains of *Bacillus subtilis*. When screened for mutagenicity with *Salmonella typhimurium* strains TA1535 and TA100, the saturated and the unsaturated conjugates were found to be nonmutagenic. However, in a preliminary test using strain TA100 in logarithmic growth, compounds $RSCF_2CHBrCl$ and RSCFCHCl were mutagenic. Furthermore, screening for DNA-damaging ability in the *B. subtilis* "*rec*" assay with strains H17 and M45 revealed that the urinary halothane metabolite, $RSCF_2CHBrCl$, and the unsaturated analogs, RSCFCHCl and RSCFCHBr, preferentially inhibited the growth of strain M45, which is deficient in its ability to repair DNA. In view of the reported correlation between known mutagens and their differential lethal action on *rec*$^-$ versus *rec*$^+$ bacteria, the present findings of the DNA-damaging effects of the nonvolatile halothane metabolites and related probable metabolites suggest a possible relationship between halothane metabolism and reported toxic effects associated with occupational anesthetic exposure. (Key words: Anesthetics, volatile: halothane, metabolites. Biotransformation: halothane; fluorometabolites. Metabolism: metabolites. Toxicity: carcinogenicity; mutagenicity.)

NUMEROUS STUDIES over the past few years have established that halothane ($CF_3CHBrCl$) undergoes biodegradation to volatile and nonvolatile metabolites, with the liver being the major site of activity.[1–5] Although halothane administered in clinical concentrations is neither toxic nor mutagenic,[6] the possibility exists that it undergoes bioconversion to more toxic species. Rare cases of hepatotoxicity, as well as spontaneous abortion, congenital abnormalities, and cancer in women during occupational anesthetic exposure, have been attributed to the production of reactive intermediates, which upon covalent binding to tissue nucleophiles may interfere with normal cellular functions, resulting in cell damage.[7,8] *In-vivo* and *in-vitro* studies with ^{14}C-halothane have demonstrated that metabolic radioactivity is transferred to the hepatic microsomes, cellular proteins, and phospholipids,[9,10] and that binding to tissue components is enhanced under anaerobic conditions.[11–12] The precise nature of the bound radioactive moiety, however, remains unknown.

In a recent study by Cohen *et al.*,[3] three major urinary metabolites of halothane in man were identified as trifluoroacetic acid, N-trifluoroacetyl-2-aminoethanol, and N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine. In a separate study by Sharp *et al.*,[4] two volatile halothane metabolites, 1,1,1-trifluoro-2-chloroethane (CF_3CH_2Cl) and 2-chloro-1,1-difluoroethylene (CF_2CHCl), and a metabolite-decomposition product, 2-bromo-2-chloro-1,1-difluoroethylene (CF_2CBrCl), were identified by gas chromatography–mass spectrometry in the exhaled breath of patients anesthetized with halothane in semiclosed and totally closed anesthesia circuits. Reductive biodegradation of halothane involving free radicals and carbenes was suggested to account for the formation of these metabolites. The mutagenic potential of the volatile halothane metabolites has recently been investigated[13–15] in the *Salmonella* rodent microsome system[16] and in its liquid suspension modification. These studies revealed that the saturated compound CF_3CH_2Cl is not mutagenic[14,15] while the olefins CF_2CHCl and CF_2CBrCl are weakly mutagenic in *Salmonella typhimurium* TA100.[13,15]

A variety of halogenated ethylenes, including vinyl chloride, vinylidene chloride, and trichloroethylene, have been extensively studied in relation to their metabolism and possible hepatotoxic, mutagenic, and carcinogenic effects.[17–22] One mechanism of the bioactivation of olefins involves epoxidation by the mixed-function oxidases of mammalian hepatic microsomes. The resulting oxiranes, if not detoxified by the microsomal epoxide hydrase or by reaction with soluble glutathione-epoxide transferase, are capable of alkylating various nucleophilic centers on tissue macromolecules either directly or via 2-haloaldehydes. For lipid-soluble halogenated hydrocarbons or their metabolic products, conjugation with glutathione results in formation of water-soluble mercapturic acids (N-

* Research Associate in Anesthesia.

† Professor of Anesthesia.

‡ Manager, Microbial Genetics Program, SRI International. Present address: Genex Corporation, 6110 Executive Blvd., Rockville, Maryland 20850.

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Address reprint requests to Dr. Sachdev: IBM, East Fishkill Facility, D/96K, Zip 41D, Route 52, Hopewell Junction, New York 12533.

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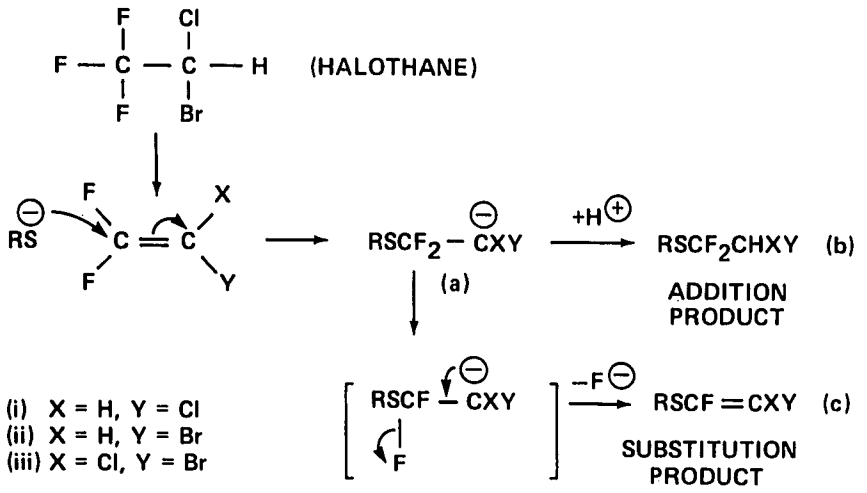

FIG. 1. Reactions of fluoroolefins with sulfhydryl nucleophils: Interaction of sulfhydryl anion, RS^- (generated by the abstraction of proton from RSH by a base) with the difluorocarbon end of a 1,1-difluoroolefin, resulting in a carbanion intermediate (*a*). The carbanion may pick up a proton from available proton-donating species to form saturated product (*b*) corresponding to the addition of RSH across the double bond. Alternatively, in the absence of a readily available proton, displacement of fluoride ion (F^-) may take place, with the formation of fluorovinylthioethers (*c*).

acetylcysteine derivatives), which provides a detoxification mechanism through renal excretion. For example, in the metabolic studies with vinyl chloride,[22] N-acetyl-S-(2-hydroxyethyl)-cysteine ($CH_3CONHCH(COOH)CH_2SCH_2CH_2OH$) was identified as one of the major urinary metabolites. Halothane metabolism studies in man have demonstrated[3] the presence of N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine as one of the urinary metabolites. Its formation may be visualized by initial enzymatic dehydrofluorination of halothane to 1,1-difluoro-2-bromo-2-chloroethylene followed by the reaction of the latter with glutathione or cysteine sulfhydryl anion. Such a reaction may be quite facile due to the presence of a highly electron-withdrawing CF_2 group in 1,1-difluoroethylenes CF_2CHCl, CF_2CHBr, and CF_2CBrCl. Reactions of difluoroalkenes with electron-rich species such as alkoxide (RO^-), thiolate anion (RS^-) and amines (RNH_2) are well known.[23,24] The urinary metabolite of halothane in man, $CH_3CONHCH(COOH)CH_2SCF_2CHBrCl$, and similar potential conjugates from CF_2CHCl and CF_2CHBr are structurally related to N-acetyl S-(2-chloroethyl)-cysteine. The latter is a monofunctional sulfur mustard which has been shown to be mutagenic in *Drosophila*[25] and in *S. typhimurium* TA100.[26]

The present investigation was directed toward the possibility that the halothane metabolite, CF_2CHCl, and the potential metabolites, CF_2CHBr and CF_2CBrCl, may undergo reaction with sulfhydryl nucleophiles by two different pathways, including addition across the double bond or displacement of fluorine, as shown in figure 1. The olefinic products resulting from the latter pathway are expected to undergo activation by the hepatic enzymes to epoxides, which may react with DNA and thus are likely to be carcinogenic. Furthermore, both the addition and substitution products may participate directly in the alkylation of nucleophilic centers of various macromolecules. With these considerations, compounds **1–6** (fig. 2) were synthesized and subjected to mutagenic screening employing *Salmonella*/microsome assay[16] and the *Bacillus subtilis* "*rec*" DNA repair assay[27] for detecting mutagens and carcinogens. The S-(2-chloroethyl)-cysteine, a known mutagen,[26] was also synthesized and included in the mutagenicity tests as a positive reference compound.

Among the short-term microbial assay procedures available for the detection of chemical carcinogens and mutagens, the *in-vitro* mutagenicity assay with *S. typhimurium* has been shown to be effective in detecting a large number of carcinogens.[16] Introduction of a microsomal activation system into the assay enables the detection of mutagens requiring metabolic activation. Approximately 65–95 per cent of the known carcinogens examined in this system are mutagenic. The test uses a special set of histidine-requiring mutants of *S. typhimurium* and, in the presence of a suspected mutagen, the number of bacteria reverting back to ability to grow without added histidine are measured. An alternate approach to detect DNA-damaging capability of certain chemicals involves the use of DNA repair-proficient and repair-deficient bacterial strains. Two repair-assay procedures, namely the "*pol*" assay with *Escherichia coli* developed by Slater, Anderson and Rosenkranz,[28] and the "*rec*" assay with *B. subtilis* developed by Kada,[27] have recently become available for the evaluation of environmental chemical mutagens. In these tests, the ability of chemicals to alter DNA is characterized by their increased lethal action toward repair-deficient compared with repair-proficient strains. Positive test data are taken as an indication of high probability that the test compound

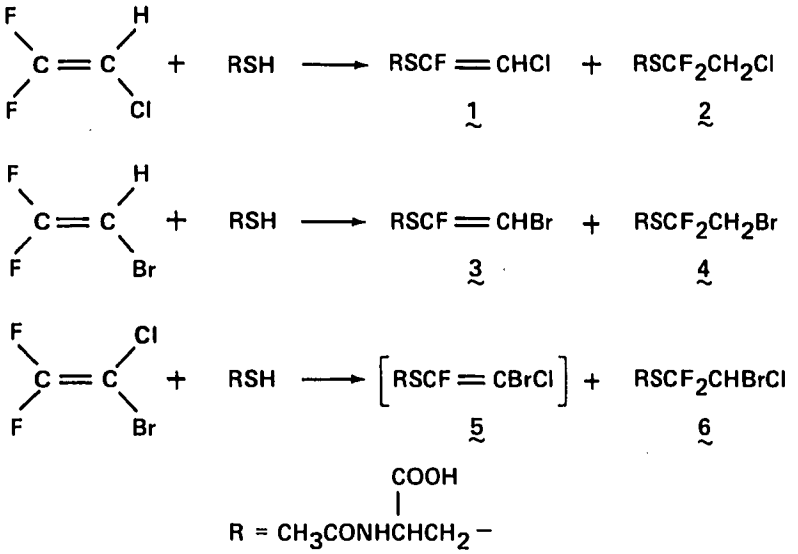

FIG. 2. Mercapturates synthesized from the known (CF_2CHCl) and probable (CF_2CHBr and CF_2CBrCl) halothane metabolites: Reaction of N-acetylcysteine and 1,1-difluoroolefins in the presence of a base results in products corresponding to addition across the double bond and substitution of vinylic fluoride. Saturated conjugates **2**, **4**, and **6** and unsaturated conjugated **1** and **3** (also the corresponding methyl ester) have been isolated and characterized. Compound **5**, a possible product of the reaction of 2-bromo-2-chloro-1,1-difluoroethylene with N-acetylcysteine, has not yet been obtained in pure form.

is likely to cause mutagenic and possibly carcinogenic effects.

Methods

The difluoroalkenes employed as starting materials for preparation of compounds **1–6** (fig. 2) were obtained from PCR Chemicals, Inc., Gainesville, Florida. The commercial samples of CF_2CHCl and CF_2CHBr were better than 99 per cent pure by gas chromatographic analysis. A pure sample of CF_2CBrCl was obtained by preparative gas chromatography of a mixture containing 63 per cent of the desired olefin, using a 3-m × 4.6-mm column packed with 15 per cent Carbowax® 20 M on 80–100-mesh Chromosorb® W-AW-DMCS at 60 C. The saturated conjugates **2**, **4** and **6** (fig. 2) were prepared by the reactions of CF_2CHCl, CF_2CHBr, and CF_2CBrCl, respectively, with N-acetyl-L-cysteine (Aldrich Chemical Co.) in aqueous methanol with the added presence of one equivalent of sodium hydroxide. For the olefins CF_2CHCl and CF_2CHBr, a closed system consisting of a glass pressure vessel equipped with a pressure gauge was employed. The unsaturated conjugates **1** and **3** (fig. 2) were formed as major products when aprotic solvent as dimethylformamide was used in conjunction with triethylamine or sodium hydride. Pure samples of **1**, **2**, **4**, and **6**, and their respective methyl esters, were obtained by crystallization. Elemental analysis and spectroscopic data in each case were consistent with the assigned structures. Compound **3** and its methyl ester was obtained in 85 per cent purity, the saturated analog **4** being the remaining 15 per cent. Compound **5** has not yet been obtained in pure form. Details of reaction procedure, product isolation, purification, and characterization are described in a separate communication (manuscript in preparation).

Mutagenicity Tests

Salmonella/microsome Assay (Ames Test): The standard mutagenicity assay using *S. typhimurium* strains TA1535 and TA100, which detect base-pair substitution mutagens, and TA1538 and TA98, which detect frameshift mutagens, were carried out according to the procedures described by Ames and co-workers.[16] For experiments to determine the effect of metabolic activation, S-9 mix, prepared from the livers of randomly bred adult male Sprague-Dawley rats pretreated with the polychlorinated biphenyl Aroclor 1254, were incorporated in the top agar with bacteria and the test chemical. In a variation[29] of the standard Ames test, a mixture of 0.05 ml of the bacterial culture, 1 ml of S-9 mix, and 0.2 ml solution of the test compound was incubated at room temperature for 20 min, then equal portions of the mixture in duplicate were added onto 2 ml molten top agar at 45 C and plated. The S-9 mix was prepared immediately prior to use by diluting freshly thawed liver supernatant 1 to 10 with 100 mM sodium phosphate, *p*H 7.4, containing 5 mM glucose-6-phosphate, 4 mM NADP, 8 mM $MgCl_2$, and 33 mM KCl. Each experiment included solvent controls as well as a known direct-acting mutagen and a mutagen that required metabolic activation. Results of these assays are presented in table 1.

Bacillus subtilis: The *"rec"* assay procedure for detecting genotoxic agents based on their ability to interact with DNA was introduced by Kada *et al.*[27] This test employs Marburg strains of *B. subtilis rec*$^+$ (H17) and *rec*$^-$ (M45). Single-colony isolates of each strain were grown overnight in a nutrient broth and adjusted to the same OD_{550} by appropriate dilution. A 0.1-ml aliquot of a test strain was incorporated in a thin overlay of 2 ml top agar on yeast-complete plates, with

TABLE 1. Results of *in-vitro* Assays with *Salmonella typhimurium*

Compound* (μg/Plate)	his+ Revertants/Plate†							
	TA1535‡		TA100‡		TA1535§		TA100‡	
	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)
RSCF=CHCl								
0	27	21	108	116	26	22	103	114
0.6	26	16	125	138	—	—	—	—
1.5	30	8	117	132	—	—	—	—
3.0	25	0	122	86	—	—	—	—
5.0	—	—	—	—	17	7	138	164
6.0	23	0	116	97	—	—	—	—
10.0	—	—	—	—	25	0	131	118
30.0	5	0	45	0	17	0	122	0
$RSCF_2CH_2Cl$¶								
0	27	21	108	116	26	22	103	114
60	27	30	118	157	24	27	107	114
100	—	—	—	—	22	18	119	153
150	22	25	109	164	—	—	—	—
300	30	18	119	170	19	17	122	136
500	—	—	—	—	18	14	130	145
750	26	6	126	69	17	11	109	100
1500	21	0	127	26	17	7	127	33
3000	15	0	154	12	16	0	112	14
COOH | $H_2NCHCH_2SCH_2CH_2Cl$								
0	30	22	127	128	26	16	115	94
60	—	—	—	—	337	305	585	563
150	—	—	—	—	770	616	1252	730
300	—	—	—	—	1289	1087	1394	1284
750	—	—	—	—	1126	1028	1534	1314
1500	—	—	—	—	908	927	900	1214
3000	—	—	—	—	121	850	144	1545
Sodium azide	495	—	604	—	450	—	481	—
2-Anthramine	30	173	99	463	25	135	115	938

* R = $CH_3CONHCH(COOH)CH_2$-. The compounds were added from solution in 0.1 M phosphate buffer (*p*H 7.4) except in the case of S-(chloroethyl)-cysteine hydrochloride, which was dissolved in water immediately before use.

† The numbers corresponding to (−) and (+) are the revertant colonies without and with the incorporation of metabolic activation system, respectively.

‡ Data are from experiments in which the bacteria were incubated for 20 minutes at room temperature in liquid culture medium with the test compound and the S-9 mix or an equivalent volume of buffer, prior to adding onto molten top agar at 45 C and plating for assay of mutagenesis.

§ These data were obtained by adding bacteria, the test compound solution and the S-9 mix, in this order, to 2 ml top agar at 45 C. The contents are quickly mixed and plated on minimal glucose medium.

¶ Analogous data indicative of lack of mutagenic response were obtained for the related conjugates, $RSCF_2CH_2Br$ and $RSCF_2CHBrCl$.

the hepatic microsomal activation system included in a parallel experiment. After the overlay had solidified (25 C for 1–2 hours), 6-mm sterile filter paper discs were placed onto the center of the surface. Each disc was inoculated with 10 μl of a solution of the test compound in DMSO or in 0.1 M phosphate buffer (*p*H 7.4). After incubation at 37 C for 16–18 hours, the plates were scored for zones of growth inhibition around the filter discs. Toxicities and DNA interactions of the various compounds tested were assessed by comparison of the diameters of the zones of growth inhibition between the DNA-repair-deficient and DNA-repair-proficient strains. The degree of growth inhibition is dependent on the toxicity and the diffusion properties of the test compound. Methyl methanesulfonate (MMS), which is known to interact with DNA, was used as a positive control, while the non-DNA-altering bactericidal agent kanamycin was employed as negative control. Relevant data are summarized in table 2.

Escherichia coli (polA⁻/polA⁺). The *E. coli* DNA polymerase-deficient assay procedure for detecting environ-

mental mutagens developed by Slater *et al.*[28] was employed for testing compounds **1–4** and **6**. These assay procedures have been described.[30]

Results

Pathways for addition across the double bond and displacement of fluorine in the reaction of 1,1-difluoroethylenes with sulfhydryl nucleophils are outlined in figure 1. Saturated conjugates **2**, **4**, and **6** (fig. 2), corresponding to the addition of N-acetylcysteine to CF_2CHCl, CF_2CHBr, and CF_2CBrCl, respectively, were formed when the reactions were carried out in aqueous methanol. Unsaturated conjugates **1** and **3** (fig. 2) were the major products in the reactions of CF_2CHCl and CF_2CHBr, respectively, with N-acetylcysteine under aprotic conditions.

When screened for mutagenicity in the standard Ames test with *S. typhimurium* TA1535 and TA100, or the alternative procedure involving incubating the mixture of bacteria, S-9 mix and the test compound for 20 min prior to adding onto top agar, none of the compounds described was found to be mutagenic. However, as characterized by their lethal actions, the unsaturated conjugates **1** and **3** were considerably more toxic to the test organism than the saturated conjugates **2**, **4**, and **6**, and this toxicity increased significantly with the added presence of a metabolic activation system. Furthermore, the lethal actions of these olefinic substrates seemed more pronounced with strain TA1535 than with strain TA100. In contrast to the lack of mutagenic response with compounds **1–4** and **6**, the reference compound, S-(2-chloroethyl)-cysteine, induced a dose-related increase in the number of histidine-independent revertants. Representative data are summarized in table 1. In a preliminary experiment employing TA100 during logarithmic growth, the unsaturated conjugate **1** and the saturated urinary halothane metabolite **6** gave a weak mutagenic response that appeared to be dose-related.

Further testing in the *Bacillus subtilis "rec"* assay procedure[27] revealed that compounds **1**, **3**, and **6** preferentially inhibited growth of recombination repair-deficient strain M45. Figure 3 is representative of the differential toxicity zones observed relative to a positive and a negative control. Compounds **2** and **4**, under the same conditions, gave essentially equal zones of toxicity with rec^+ and rec^- strains. When a metabolic activation system was incorporated in the top agar, clearer zones were obtained, but the diameter of growth inhibition zones was essentially the same as that observed with no S-9 mix present. For the unsaturated conjugates a dose-related selective toxicity was observed at concentrations of 0.2 to 2 mg per plate, while with the saturated conjugate **6**, due to its lesser toxicity, a similar dose–response relationship was evident only at concentrations of 1–10 mg per plate. Kanamycin and methylmethane sulfonate served as negative and positive controls, respectively, in all tests. The results of repair assays are presented in table 2.

TABLE 2. Preferential Inhibition of Recombination-deficient Mutant of *Bacillus subtilis*

Compound	mg/Disc*	Growth Inhibition Zone Diameter in mm†	
		H17, rec^+	M45, rec^-
Kanamycin‡	0.1	18	18
$CH_3SO_2—O—CH_3$(MMS)	1.0	18	48
COOH | $CH_3CONHCHCH_2SCH=CHCl$ **1**	0.2	8 (8)	18 (16)
	0.7	12 (11)	24 (23)
	2.0	13 (13)	26 (25)
$COOCH_3$ | $CH_3CONHCHCH_2SCF=CHBr$ **3** (methyl ester)	0.3	8 (8)	20 (18)
	2.2	16 (14)	28 (28)
	2.5	16 (15)	29 (30)
COOH Cl | | $CH_3CONHCHCH_2SCF_2CHBr$ **6**	1.1	8	16
	1.7	12 (12)	20 (20)
	7.0	11	22
	10.0	10	19
Dimethylsulfoxide	10 μl	0	0

* In each case 10 μl of solution in DMSO were deposited on the disc.

† The numbers in parentheses indicate the toxicity zones with the added presence of metabolic activation.

‡ A 10-μl volume of a freshly prepared 1 per cent solution of kanamycin sulfate in water was employed as a "negative" control.

Preliminary tests for DNA-modifying activity of these compounds in the *"pol"* assay with the *E. coli* DNA-polymerase system[28] did not indicate any differential toxicity.

Discussion

Recent studies[13–15] have shown that 1,1-difluoro-2-chloroethylene (CF_2CHCl), a halothane metabolite, and 1,1-difluoro-2-bromo-2-chloroethylene (CF_2CBrCl), a probable biotransformation product of halothane, are mutagenic in the *Salmonella* mutagenicity assay, and that a metabolic activation system is not required for the observed mutagenesis. This suggests a direct alkylating potential of these compounds consistent with the generally facile alkylation reactions[23] of 1,1-difluoro-2-haloolefins with various nucleophilic species, including RO^-, NH_2, S^-, and F^-.

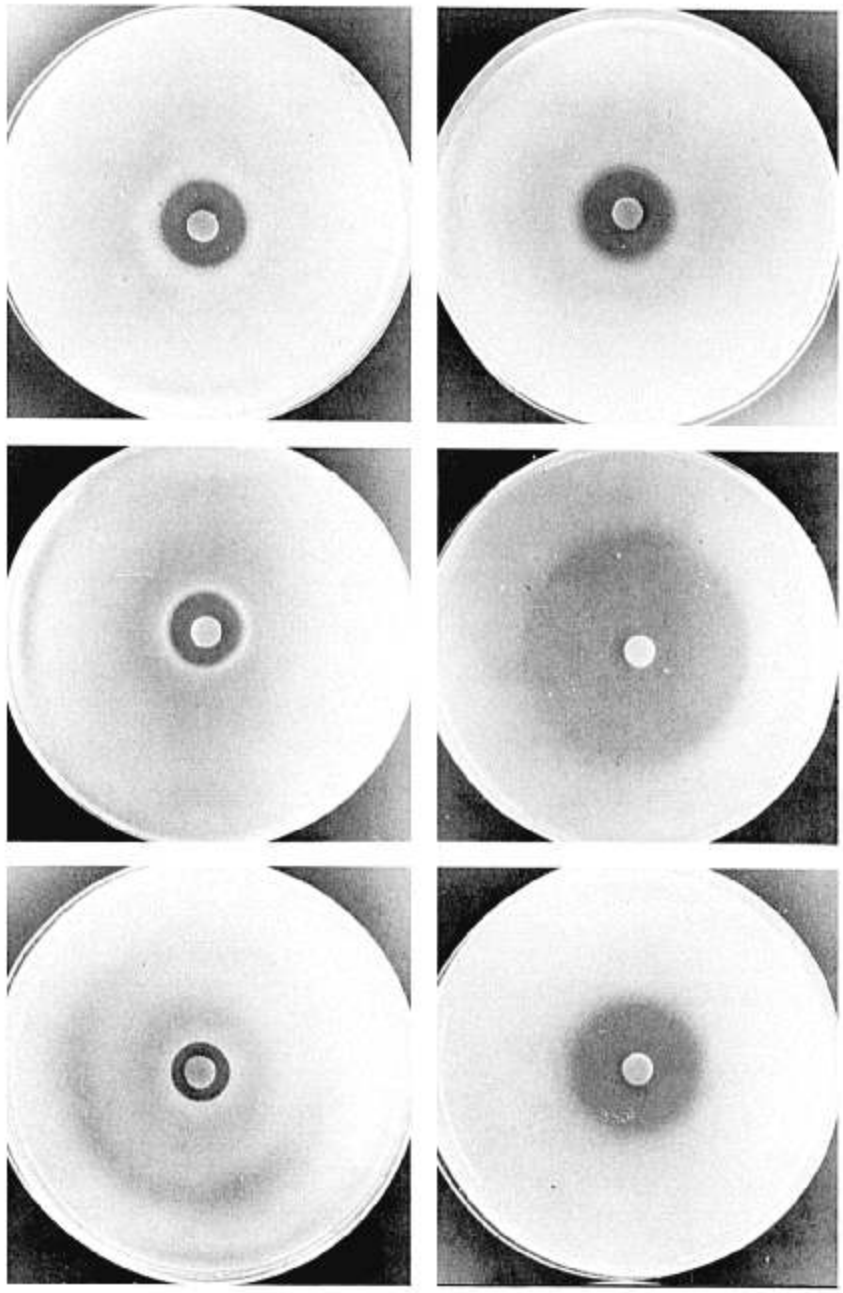

FIG. 3. Effects on the growth of *Bacillus subtilis* H17-*rec*$^+$ (*left*) and M45-*rec*$^-$ (*right*): *A*, kanamycin used as a "negative" control (0.1 mg/plate) causes cytotoxicity by a mechanism other than DNA interaction and has equal zones of growth inhibition; *B*, methylmethanesulfonate (CH_3SO_2—O—CH_3, MMS) employed as a "positive" control (1 mg/plate in DMSO) is a known carcinogen and shows preferential inhibition of *rec*$^-$ strain; *C*, N-acetyl-S-(2-chloro-1-fluoroethenyl)-L-cysteine (CH_3-CONHCH(COOH)CH_2SCF=CHCl), a potential halothane metabolite, at 2 mg/plate from DMSO solution, shows a differential effect similar to that of MMS.

In addition to direct alkylation, these olefins may manifest toxicity via metabolism to an epoxide and subsequent rearrangement to α-haloaldehydes and α-haloacid halides, similar to the mechanism proposed for the mutagenicity of vinyl chloride, related halogenated alkenes and other indirect-acting alkylating carcinogens. Several possible modes of biotransformation of 1,1-difluoroethylenes are outlined in figure 4.

Conjugation of reactive intermediates with glutathione is generally considered to be a mechanism of detoxification. However, it is conceivable that the reactive halothane metabolites, CF_2CHCl and CF_2CBrCl, and structurally similar metabolite, CF_2CHBr, may react with sulfhydryl and amine nucleophiles to form genotoxic products. The monofunctional sulfur or nitrogen mustards having the —NCH_2CH_2X and/or —SCH_2-CH_2X (X = Cl,Br) moiety are highly reactive alkylating agents and potent carcinogens. A rationale for the unusual reactivity of β-halosulfides and β-haloamines involves neighboring group participation by sulfur or nitrogen in the displacement of halide in the β-position[31] (figure 5). The resulting three-membered intermediate may then effectively alkylate available nucleophiles. However, corresponding mustards carrying a difluorocarbon adjacent to sulfur, as in compounds **2**, **4**, and **6** (fig. 2), are less likely to undergo similar

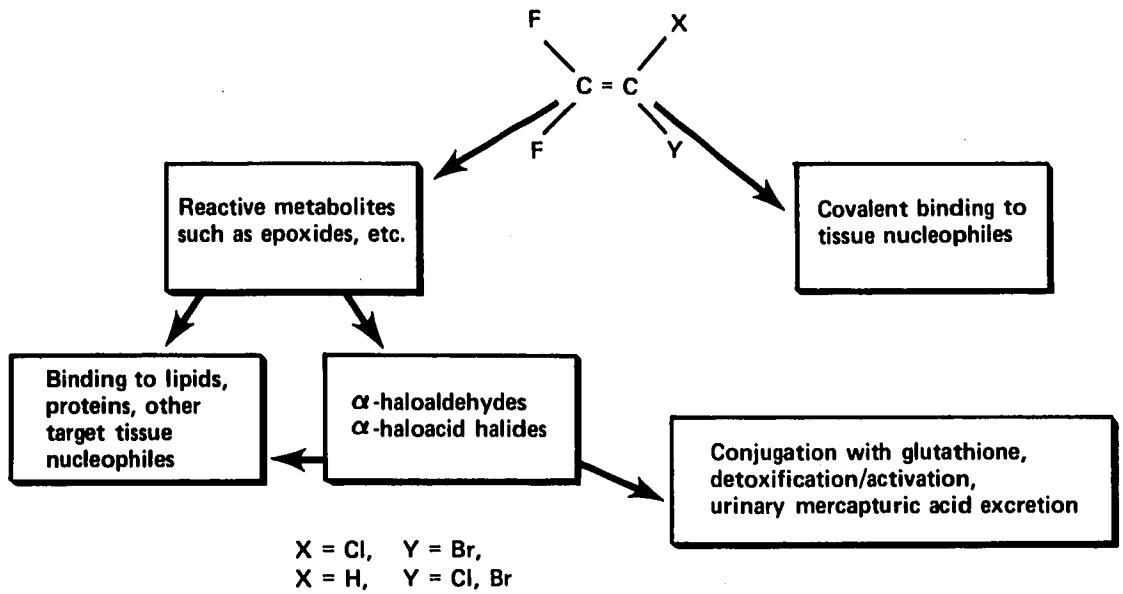

FIG. 4. Possible modes of biotransformation of 1,1-difluoroethylenes.

nucleophilic reactions due to possible electron withdrawal by the CF_2 group adjacent to sulfur. On the other hand, in view of the lability of fluorine at an Sp^2 carbon, the fluorovinylthioethers **1**, **3**, and **5** are expected to participate in displacement reactions or undergo further metabolic conversion to epoxides with covalent bonding to tissue macromolecules. Such interactions would have a bearing on the possible correlation between halothane metabolism and reported hepatic toxicity, genetic abnormalities, increased risks of cancer, and teratogenic effects.

Considering the relatively lower reactivity of β-halogen in systems such as $RSCF_2CH_2X$ (X = Cl,Br), it is not surprising that the saturated conjugates **2**, **4**, and **6** (fig. 2) lack the ability to induce mutations in *S. typhimurium* strains TA1535 and TA100 in the standard assay (table 1). In comparison, the usual β-halosulfides, $RSCH_2CH_2X$, are potent mutagenic alkylating agents which undergo rapid hydrolysis even in slightly alkaline aqueous environments. However, the apparent lack of mutagenic activity for the unsaturated conjugates **1** and **3** in the standard *Salmonella* assay is not readily explicable. Membrane permeability may not be a factor, as these conjugates were quite toxic to strains TA1535 and TA100. Toxicity increased considerably with the added presence of S-9 mix, suggesting that compounds **1** and **3** are metabolically transformed to more toxic species such as epoxides and their rearrangement products. Furthermore, the lethal action of these compounds seemed to be more pronounced with *S. typhimurium* strain TA1535 than with strain TA100. A similar difference in toxicities of these compounds toward TA1538 and TA98 was indicated in another study. Strains TA100 and TA98 are derived from TA1535 and TA1538, respectively, by the introduction of the resistance transfer factor, plasmid pKM101. The present preliminary observation of a difference in toxicities among strains of each set may indicate involvement of plasmids in providing a protective mechanism against the toxic effects of certain chemicals similar to the ultraviolet-protecting property of plasmid R46.[32]

When tested in the *Bacillus subtilis* "*rec*" assay with strains H17 and M45, compounds **1**, **3**, and **6** gave differential zones of growth inhibition (table 2, fig. 3), indicative of DNA-damaging effects. When the compounds **2** and **4** were tested at the same dose levels as compound **6**, essentially equal zones of toxicity were observed with both *rec*$^+$ and *rec*$^-$ strains. The diameters of the growth inhibition zones observed for strains H17 and M45 remained unchanged when the hepatic enzyme activation system was included in these tests, suggesting a direct alkylation mechanism for the toxic effects of the compounds under study.

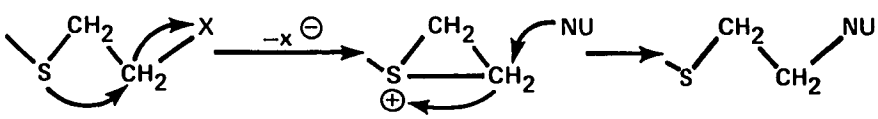

FIG. 5. Scheme for reaction of monofunctional sulfur or nitrogen mustards with displacement of halide in the β position.

The greater toxicity of the unsaturated conjugates versus their saturated analogs toward *S. typhimurium* and *B. subtilis* strains is consistent with the generally enhanced reactivity of the olefinic substrates, with the possibility of their metabolism to reactive intermediates. The difference in the results of the two bioassay systems employed for compounds **1**, **3**, and **6**, which are apparently nonmutagenic in the *Salmonella* plate assay but give reproducible dose-related differential inhibition of growth in the *Bacillus subtilis "rec"* assay, is not readily apparent. It may be mentioned that in spite of acceptance of the Ames test as the most reliable procedure for detection of potential chemical mutagens, its limitations are well known, especially when the test compound is strongly bactericidal but has only weak mutagenic activity. There are instances when compounds show negative or limited responses in the standard *Salmonella* assay but, nevertheless, have DNA-modifying activity in repair bioassay systems. For example, among a series of halogenated alkanes studied,[18] 1,1,2,2-tetrabromoethane showed the highest DNA-modifying activity in the *Escherichia coli* (*polA*$^-$/*polA*$^+$) assay, and was apparently nonmutagenic when tested in the standard *Salmonella* assay. With toxic but weak potential mutagens, the exponentially growing culture modification of the Ames test, which determines the number of induced mutants per number of survivors, has frequently been successfully employed for the detection of mutagenesis. The discrepancy between results of the two bioassays in the present studies might also be explained if significant differences in the cellular membrane permeability to these compounds existed between the *S. typhimurium* and *B. subtilis* strains.

Data from various studies indicate that all inhalational anesthetics are metabolized in the body, and that halothane-related hepatic toxicity may be mediated through its biotransformation to alkylating metabolites. The present study has demonstrated that N-acetylcysteine conjugates of the volatile olefinic products of halothane metabolism are genotoxic in the *Bacillus subtilis "rec"* assay system. In the standard Salmonella mutagenicity assay, these conjugates fail to induce revertants, but preliminary experiments using strain TA100 in logarithmic growth show mutagenic responses due to the saturated as well as the unsaturated conjugates. The latter are also found to be toxic to the *Salmonella* strains. Such observations are indicative of DNA damage in the bacteria and allude to the possibility of chemical interaction of mercapturates in the genetic material and attendant alterations in the structure of cellular nucleic acids and other critical cell constituents. While the concentration of halothane associated with long-term occupational exposure in the operating room is of the order of 10–30 ppm and only a small fraction of the inspired anesthetic is metabolized in the body, accumulation of these metabolites to toxic levels in the absence of rapid detoxification by endogenous defense systems may result in cellular damage. It is hoped that the results of the experiments described above, although obtained in susceptible bacterial strains, will contribute to better understanding of the mechanisms of potential toxicity of halothane and related inhalational anesthetics.

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