

Binding of Halothane-free Radicals to Fatty Acids Following UV Irradiation

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A previous study indicated that exposure of mice to halothane vapor that had been irradiated by ultraviolet light resulted in rapid and severe toxicity. Two of the major decomposition products of irradiated halothane are identical to reductive halothane metabolites that were detected in the expired air of rabbits and human patients as well as in the head space over incubations of liver microsomes with halothane. In this study the authors established the existence of a 1-chloro-2,2,2-trifluoroethyl free radical produced by ultraviolet radiation. The halothane free radical was studied by two techniques. First, ^{14}C -halothane was irradiated with ultraviolet light in the presence of methyl oleate, an unsaturated fatty acid representative in its reactivity toward free radicals of those found in phospholipids that make up the membrane of the lungs and liver endoplasmic reticulum. Gas chromatography-mass spectrometry revealed free radical addition to the 9-10 double bond of methyl oleate resulting in the corresponding saturated substituted methyl stearate. Irradiation under an oxygen atmosphere resulted in two products: a CF_3CHClOO -methyl oleate and a CF_2CHCl methyl-stearate. In a second set of experiments, halothane vapor diluted in argon was passed over an ultraviolet light source and then into a solution of a free radical spin trap. The electron paramagnetic resonance spectrum of this spin trap solution revealed the existence of both a halothane free radical as well as the secondary production of hydroxyl free radicals due to interaction of the initial halothane free radical with traces of water or oxygen in the trapping solution. Both of these experiments suggest that ultraviolet light sources should be used with caution in the presence of halothane and that free radical addition is a plausible mechanism for the initiation of cellular damage in liver cells. (Key words: Anesthetics, volatile: halothane. Biotransformation: fluoro-metabolites. Liver: toxicity. Membrane: lipid peroxidation; phospholipids. Toxicity: hepatic; metabolites.)

A RECENT STUDY by Karis *et al.* demonstrated that exposure of mice to halothane vapor that had been irradiated with ultraviolet light resulted in pulmonary edema, elevated serum SGTP, decreased hepatic microsomal hemoproteins, and decreased hepatic glutathione content.¹ Two of the ultraviolet irradiation decomposition products of halothane identified by these authors were $\text{CF}_3\text{CH}_2\text{Cl}$ and CF_2CHCl . These products are identical to two volatile reductive metabolites of halothane detected in the expired air of rabbits by Mukai *et al.*,² in the expired air of human patients in our laboratory,³ and in anaerobic incubations of liver micro-

somes by Maiorino *et al.*⁴ We have previously suggested that the metabolic production of these volatile metabolites could be explained by a common free radical intermediate, a 1-chloro-2,2,2-trifluoroethyl radical, that could abstract a hydrogen radical from solvent to form the $\text{CF}_3\text{CH}_2\text{Cl}$ molecule or, alternatively, a fluorine atom could leave as a free radical to produce the unsaturated CF_2CHCl .³

Our investigation of the possible production of halothane free radicals by ultraviolet irradiation has two separate motivations. First, ultraviolet lights are used in operating rooms and in some analytical instrumentation. It is likely that diagnostic and therapeutic X-ray irradiation will affect halothane by the same mechanism as ultraviolet light.⁵ We feel that it is important to demonstrate why halothane should not be used in the presence of ionizing radiation. Second, the production of 1-chloro-2,2,2-trifluoroethyl radicals by ultraviolet light may provide an excellent model system for understanding the molecular mechanisms by which reductive metabolism of halothane may induce damage in the endoplasmic reticulum of liver cells. Binding of a 1-chloro-2,2,2-trifluoroethyl radical to phospholipids in the endoplasmic reticulum is consistent with the following experiments: Cohen *et al.* demonstrated a stoichiometry of three fluorines per ^{14}C bound in an extract of lipids from livers of mice exposed to ^{14}C -halothane⁶; Wood *et al.* reported ^{36}Cl and ^{14}C bound in similar magnitude to lipid extracts from rat liver⁷; and Gandolfi *et al.* demonstrated a nearly 1:1 stoichiometry between ^{14}C and tritium in lipid extracts from microsomal suspensions incubated with either ^{14}C or tritiated halothane.⁸ Of course, the results also could be explained by a fortuitous mixture of several radical species.

We attempted to establish the existence of halothane free radicals and to determine their structure by two techniques. First, halothane was irradiated with ultraviolet light in the presence of methyl oleate. Methyl oleate is a 9-10 unsaturated fatty acid methyl ester that comprises 16 per cent of the unsaturated fatty acyl chain in the phospholipids that make up the membranes of lung and liver cells. Its single double bond has been shown to react with halothane free radicals in a similar manner to polyunsaturated fatty acyl chains.⁹ If a halothane free radical were produced, it would be expected to add preferentially to the double bond of methyl oleate to produce a substituted saturated methyl stearate, *e.g.*, with a 1-chloro-2,2,2-trifluoroethyl substituent added to either the

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9 or 10 position of the fatty acid chain. Second, we attempted to trap halothane free radicals by exposing halothane vapor diluted in argon to an ultraviolet light source and then bubbling it into a solution of a free radical spin trap. This spin trap, phenyl-t-butyl nitron (PBN) is able to react rapidly with free radicals and retain the unpaired electron for a time sufficient to observe a free radical spectrum with an electron paramagnetic resonance spectrometer (EPR). If the initial radical spin trap adduct is stable, hyperfine splittings in the EPR spectrum often allow interpretation of the structure of the free radical.¹⁰

Materials and Methods

For the study of binding of halothane free radicals to unsaturated fatty acids, 10 mg of chromatographically pure methyl oleate were mixed with 10 μ l of ¹⁴C halothane (1-¹⁴C-2-bromo-2-chloro-1,1,1-trifluoroethane obtained from New England Nuclear and re-purified by preparative gas chromatography in our laboratory) and injected into each of two 4 mm ID quartz tubes. The samples were sealed under an atmosphere of either argon or oxygen and then irradiated for 15 min at a 2-cm distance from a General Electric G4T4-1 low pressure ultraviolet lamp. Following irradiation, the samples were subjected to high vacuum for three hours to remove residual ¹⁴C-halothane and each was then applied to a 1 cm \times 25 cm reverse phase C-18 high pressure liquid chromatography (HPLC) column and eluted at 20° C with a 96:4 methanol:water mixture. The major ¹⁴C-containing peaks preceding the elution of methyl oleate were subjected individually to either direct inlet mass spectrometry or to combined capillary gas chromatography-mass spectrometry (GC-MS) on a 10 m \times 0.25 mm OV-17 fused silica capillary column coupled to a Varian CH-7 mass spectrometer. The capillary column was operated at a linear helium gas velocity of 30 cm/s, and was programmed from 50° C to 190° C at 10° C/min.

A separate set of spin trapping experiments was performed to demonstrate that halothane free radicals were primary products of UV irradiation. Thymol was removed from halothane by filtration through a short aluminum oxide column. The halothane was then placed in a 25-ml two-neck flask through which argon was bubbled for one hour at 0° C to remove oxygen. The halothane was then warmed to 20° C to produce a vapor of halothane in argon that was passed through a 4 mm ID quartz tube positioned 2 cm away from a General Electric G4T4-1 ultraviolet lamp. Argon was used as a carrier gas because it is known not to be ionized by ultraviolet radiation. The irradiated vapor was conducted through a quartz capillary tube into a solution of 0.1 M phenyl-tert-butyl nitron (PBN) in redistilled deoxygenated ethyl

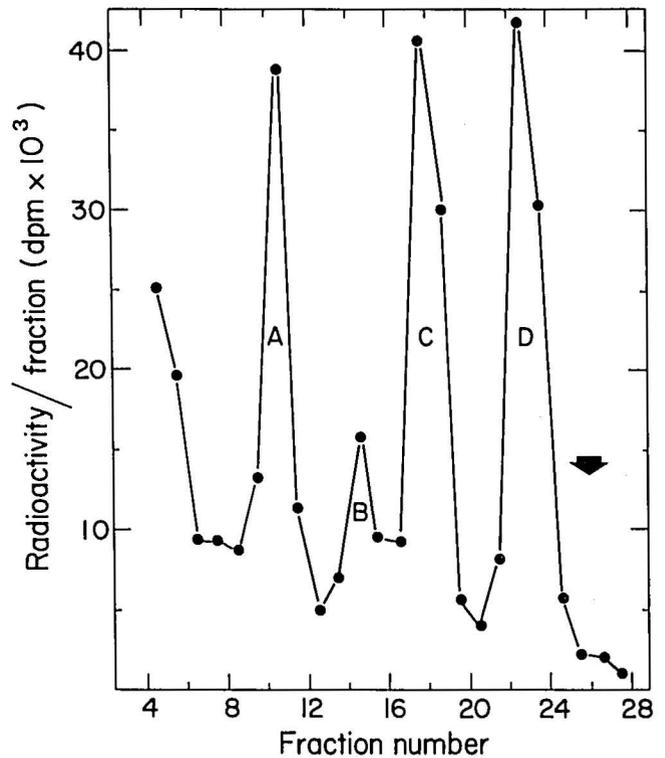


FIG. 1. Elution with 96:4 methanol:water of a mixture of methyl oleate and ¹⁴C-halothane that was irradiated with ultraviolet light and then applied to a C-18 reverse phase HPLC column produced the chromatogram above. Fractions 4-6 contain unchanged ¹⁴C-halothane, fraction 26 contains unchanged methyl oleate and is marked with a large black arrow.

acetate. After 15 min, the PBN solution was concentrated fivefold and then 50 μ l were placed in a quartz EPR sample tube under an argon atmosphere. EPR spectra of the spin trap solution then were measured at 22° C with a Varian E-104A spectrometer.

Results

Separation of the UV-irradiated methyl oleate derivatives by HPLC with a C-18 reverse phase column produced chromatograms that were different depending on whether an argon or oxygen atmosphere was used during irradiation. In the case of irradiation of ¹⁴C-halothane and methyl oleate under an oxygen atmosphere, four radioactive peaks were observed preceding the elution of the parent methyl oleate (fig. 1).

Application of the major component (peak D, fig. 1) to GC-MS produced a mass spectrum (fig. 2) with a molecular ion of m/e 414. This molecular weight is consistent with addition of a CF₃CH₂Cl radical to the double bond of methyl oleate followed by abstraction of a hydrogen radical from a neighboring donor (fig. 3, I). The doublet at m/e 414 and 416 with a 3:1 intensity ratio

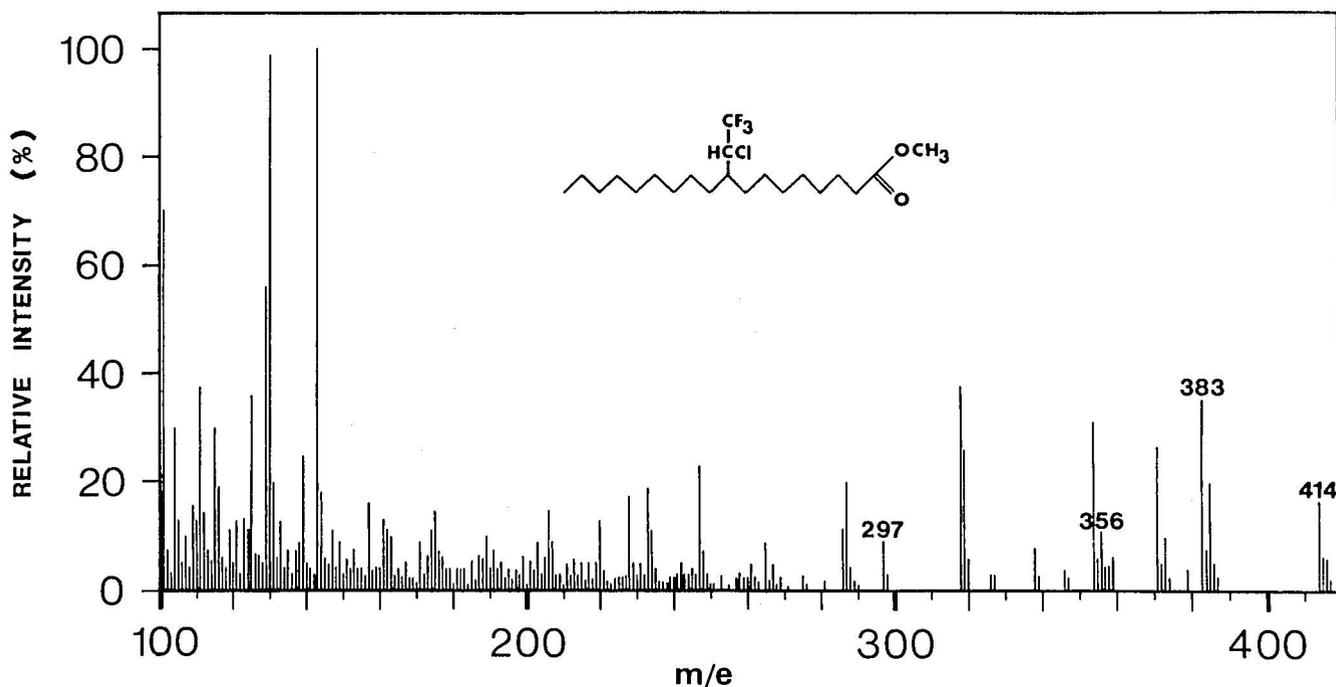


FIG. 2. The mass spectrum of a mixture of 9- and 10-(1-chloro-2,2,2-trifluoro)-methyl stearate produced by UV irradiation of a mixture of halothane and methyl oleate. The quantity of each fragment is measured as the per cent relative abundance of the largest peak in the spectrum. The molecular weight of each fragment is expressed as the ratio of mass to charge (m/e). The doublets at 414 and 416, 383 and 385, as well as 356 and 358 are characteristic of a methyl ester of a fatty acid containing a single chlorine atom.

is also consistent with a structure containing one chlorine atom because the natural abundance ratio of ³⁵Cl and ³⁷Cl is 3:1. The fragments of m/e 383 ($M^+ - CH_3O$) and m/e 356 ($M^+ - CH_2COHCH_3$) are indicative of a methyl ester of a fatty acid.

The methyl oleate adduct in peak A (fig. 1) was further purified by HPLC on a C-8 reverse phase column with a 60:40 MeOH:H₂O eluting mixture. The major component after this purification was not sufficiently stable for GC-MS so it was introduced directly into the ion

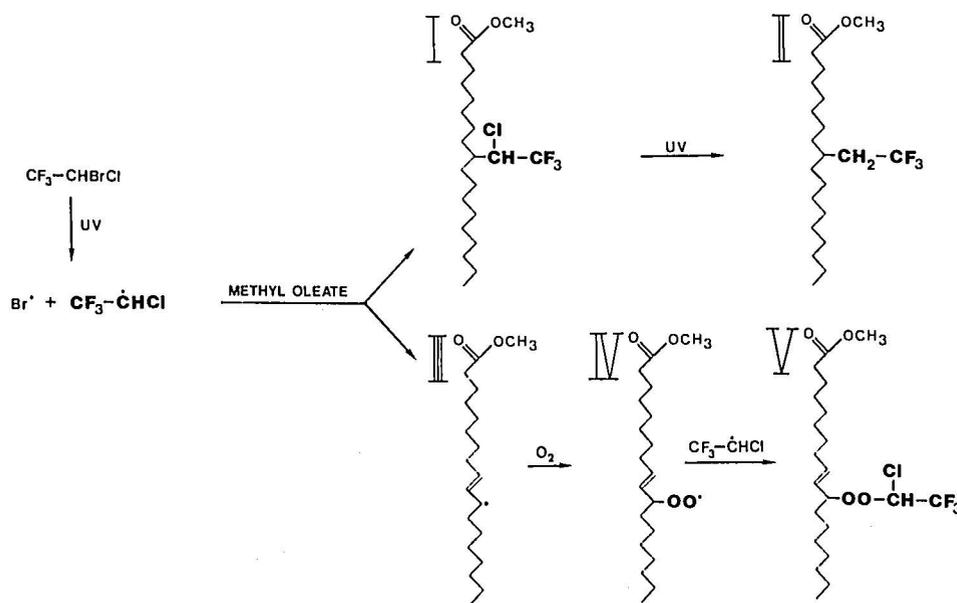


FIG. 3. Halothane exposed to ultraviolet radiation loses a bromine radical to form a 1-chloro-2,2,2-trifluoroethyl radical. This radical may react with methyl oleate by two separate pathways. A halothane free radical added to the double bond produces the saturated substituted methyl stearate (I), the adduct isolated as Peak D in figure 1. Continued ultraviolet irradiation of structure (I) yields structure (II), Peak C in figure 1. Alternatively, the halothane free radical may abstract a hydrogen radical from methyl oleate to yield structure (III). This structure may react with molecular oxygen to form the peroxy radical (IV). This could decompose to form conjugated dienes, aldehydes, peroxidized lipid fragments, or, as shown, react with another 1-chloro-2,2,2-trifluoroethyl radical to produce the unsaturated peroxy adduct (V), Peak A in figure 1.

source of the mass spectrometer in a gold cup on a solid probe. The mass spectrum revealed a molecular ion of m/e 444. This molecular weight is consistent with replacement of a hydrogen on a methyl oleate with a CF_3CHClO radical. If this radical species were added directly to methyl oleate, it would be expected to add to the double bond rather than replace a hydrogen atom, resulting in a molecular ion of m/e 446. We suggest that the observed ion is formed in the stepwise sequence shown in the lower pathway in figure 3: ultraviolet irradiation produces many 1-chloro-2,2,2-trifluoroethyl radicals, one of these radicals abstracts an alpha hydrogen from methyl oleate (III), the methyl oleate radical reacts with molecular oxygen (IV), this relatively stable dioxygen radical adds to another 1-chloro-2,2,2-trifluoroethyl radical to produce the m/e 444 adduct (V).

The mass spectrum of the adduct in peak B (fig. 1) revealed a molecular ion of m/e 294, which is two atomic mass units less than that of methyl oleate. The product of binding of a halothane moiety to methyl oleate must have a greater molecular weight than the parent compound. Therefore, the halothane-radical adduct isolated by HPLC must be thermally unstable under conditions of GC-MS or direct solid probe introduction mass spectrometry and must have decomposed to the m/e 294 fragment that represents the loss of two hydrogen atoms from methyl oleate. Possible structures that would decompose to such a fragment include the product of addition of a 1-chloro-2,2,2-trifluoroethyl radical to a methylene position alpha to the double bond of methyl oleate.

The molecular ion of the adduct in peak C (fig. 1) is m/e 380. This molecular weight formally corresponds to the addition of a 2,2,2-trifluoroethyl radical to the double bond of methyl oleate followed by capture of a hydrogen radical from a neighboring hydrocarbon. However, when the purified component of peak D (m/e 414) described above was mixed with 10 μ l unlabeled halothane, placed in a quartz tube, then irradiated under the conditions of the original experiment, a product identical to peak C (m/e 380) was isolated. Therefore, peak C is formed by photodechlorination of the $CF_3 CClH$ radical adduct and is not a true primary reaction product (Figure 3, II).

In the case of irradiation of ^{14}C -halothane and methyl oleate under an argon atmosphere, the HPLC chromatograms and the major products are identical to those produced under an atmosphere of oxygen except that the dioxygen-containing adduct, peak A in figure 1, is absent.

The EPR spectrum of the spin trap-adducts of the halothane free radicals is a composite of the spectra of at least two separate spin-trapped radicals (fig. 4A). We assigned the spectrum of the major component with a 2.9 Gauss splitting of the outer hyperfine extrema (arrows) to that resulting from direct binding of a halothane

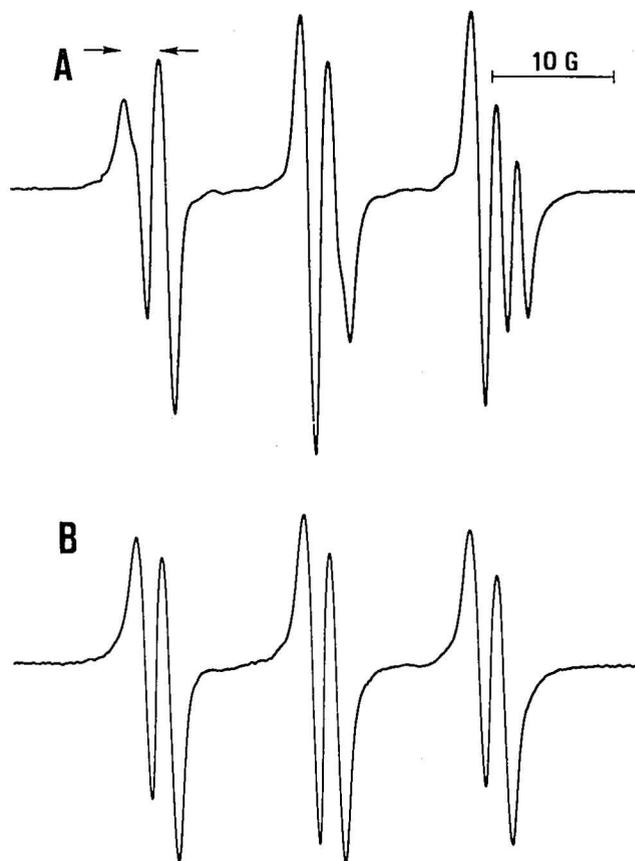


FIG. 4. The EPR spectrum of the spin trap phenyl-tert-butyl nitron (PBN) that has been exposed to halothane vapor irradiated with ultraviolet light under argon (A). The spectrum is a composite of both 1-chloro-2,2,2-trifluoroethyl radicals and hydroxyl radicals bound to PBN. The EPR spectrum of PBN exposed to halothane vapor irradiated with ultraviolet light in the presence of air (B). The spectrum is identical to that of a synthetically prepared PBN-hydroxyl radical adduct.

free radical to the PBN spin trap. The smaller component has outer hyperfine extrema with a splitting of 2.1 Gauss identical to that of the hydroxyl radical adduct of PBN shown in spectrum 4B. Spectrum 4B was obtained by adding oxygen to the spin trap solution during the halothane radical trapping experiment; it is identical to the spectrum of a synthetically-prepared PBN-hydroxyl adduct.¹¹ The hydroxyl radical adduct may occur by either of two possible mechanisms. The initially produced 1-chloro-2,2,2-trifluoroethyl radical may react with traces of oxygen in the spin trapping solution to produce the peroxy-1-chloro-2,2,2-trifluoroethyl radical. This radical may react with the PBN spin trap as shown below to form the unstable peroxide spin trap adduct which then decays to yield the hydroxyl radical spin trap adduct and the CF_3CHClO radical. A second possible mechanism for the observed hydroxyl radical adduct is that the primary 1-chloro-2,2,2-trifluoroethyl radical

reacts with a trace of water in the trapping solution to produce $\text{CF}_3\text{CH}_2\text{Cl}$, and a hydroxyl radical which is subsequently trapped by PBN.

Discussion

These results indicate that ultraviolet light irradiation from a standard germicidal lamp produces free radicals from halothane that have a sufficient lifetime to be trapped by a spin trap or to add to the double bond of fatty acid chains found in cellular membranes. Binding of the 1-chloro-2,2,2-trifluoroethyl radical to methyl oleate in figure 3 is consistent with many of the previous experimental findings of halothane metabolite binding to endoplasmic reticulum phospholipids. The intermediate free radical formed after the addition of the 1-chloro-2,2,2-trifluoroethyl radical to the double bond (figure 3) could initiate lipoperoxidation by reacting with oxygen to form the peroxy species which subsequently would decay to form malondialdehyde.¹² In the case of a similar radical addition to one of the double bonds of a poly-unsaturated fatty acid, such as arachidonic acid, the existence of the intermediate free radical addition product would allow hydrogen radical transfers that result in formation of conjugated dienes observed following microsomal incubations with halothane.^{7,12}

The spin trap data are also consistent with formation of halothane free radicals by ultraviolet irradiation. The spectrum obtained in the presence of higher amounts of oxygen in the trapping solution (fig. 4B) demonstrates that halothane radicals are able to produce either reactive alkylperoxy radicals or hydroxyl radicals, both of which are known to be cytotoxic.¹² However, our studies of trapping efficiency as a function of flow rates and path lengths for conductance of radicals between the ultraviolet light exposure and the solution of PBN spin trapping solution indicate that these radicals have a lifetime less than a second. In view of their short lifetime, it is very unlikely that operating room personnel would be directly exposed to free radical products of halothane; the free radicals would decay to the decomposition products CF_2CHCl , $\text{CF}_3\text{CH}_2\text{Cl}$, and CF_3CClO described by Karis *et al.*¹ The first of these decomposition products has been shown to be mutagenic in two separate bacterial assay systems,¹³ and clearly the mixture of these decomposition products is toxic when inhaled by mice.¹

These results show that the addition of a 1-chloro-2,2,2-trifluoroethyl radical to the double bond of a fatty

acid chain is a reasonable hypothesis for the initiation of halothane metabolite binding to phospholipids and subsequent damage to the endoplasmic reticulum. In fact, the binding of the UV-produced free radical from halothane to methyl oleate is identical to that of the chlorotrifluoroethyl free radical formed metabolically from halothane in reconstituted vesicle systems containing human hepatic microsomal enzymes.⁹ Moreover, they suggest that ultraviolet light or other ionizing radiation should be used with caution in the presence of halothane.

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