

Urinary Metabolites of Halothane in Man

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The urinary metabolites of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) were investigated in five individuals given trace doses (25 μ Ci), and in three individuals given large doses (1 mCi) of radioactively labeled 14 C-halothane. The latter were donor subjects for heart transplant operations. Separation of the nonvolatile urinary metabolites of halothane was accomplished by chemical extraction, electrophoresis, ion-exchange and high-pressure liquid chromatography, and gas chromatography. Identification of the individual metabolites was by nuclear magnetic resonance and mass spectrometry. Three major metabolites were identified: trifluoroacetic acid, N-trifluoroacetyl-2-aminoethanol, and N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine. Smaller unidentified radioactive peaks were also found. The presence of both ethanolamide and cysteine conjugates of halothane is of concern. These urinary products imply the presence of reactive intermediates. The conjugation of such intermediates to proteins and phospholipids may give rise to the high-molecular-weight covalently bound metabolites demonstrated to be present in the liver following halothane anesthesia. Elucidation of the structures of the urinary metabolites provides information important to an understanding of halothane metabolism and its potential hepatotoxicity. (Key words: Anesthetics, volatile, halothane; Biotransformation, halothane.)

DESPITE A SERIES of carefully performed animal experiments, Duncan and Raventos were unable to demonstrate metabolites of halothane in expired air, blood, or urine.¹ However, subsequent investigators, using more sensitive radioisotopic techniques, succeeded in establishing nonvolatile metabolite(s) of halothane in the urine of several animal species. Thus, Van Dyke and Chenoweth² found small residues of 36 Cl-labeled

metabolite in the urine following intraperitoneal injection of 36 Cl-halothane in the rat. Using an alternative approach, Stier³ measured an increase in urinary bromide excretion in the same species. In additional studies, Stier⁴ identified trifluoroacetic acid in the rabbit as a urinary end-product of halothane metabolism. Subsequent clinical studies by Stier *et al.*,^{5,6} Rehder *et al.*,⁷ and Cascorbi *et al.*⁸ demonstrated the presence of nonvolatile halothane metabolites in human urine. On the basis of measurements of urinary fluorine excretion in man, the amount of metabolite eliminated over a 13–21-day period was estimated to be 12 to 24.8 per cent of the halothane absorbed.^{7,8} By means of infrared spectra and paper chromatography methods, Stier⁴ suggested that trifluoroacetic acid was the only aliphatic metabolite of halothane in man.

Although halothane hepatotoxicity is a rare event, there is little doubt that it does occur. Unfortunately, despite considerable effort, the etiology of such "halothane hepatitis" remains presently unresolved. It is likely, however, that the toxicity of halothane is associated with the metabolism of the anesthetic. A direct relationship between metabolism and long-term toxicity has been established for a number of the inhalation anesthetics.^{9–14} Reduction in anesthetic metabolism produced by enzyme inhibitors such as SKF-525A¹⁵ or disulfuram¹⁶ prevents the toxicity of both chloroform and fluorene. On the other hand, increased toxicities of these anesthetics follow the administration of enzyme-inducing compounds such as 3-methylcholanthrene or phenobarbital.^{13–15}

Although it has been difficult to establish a dose-response relationship for halothane hepatotoxicity, it has been suggested that increased levels of toxicity might result from repeated anesthetic administrations^{17,18} or from chronic low-dose exposures.¹⁹ On the other hand, adverse reactions following the first administration of halothane have also been reported.^{20,21} The precise explanation of these

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Received from the Department of Anesthesia, Stanford University School of Medicine, Stanford, California 95305. Accepted for publication April 16, 1975. Supported by N.I.H. Grant GM-12527.

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unpredictable phenomena remains to be clarified. Since a dose-response relationship to halothane toxicity has not been defined, one must assume significant individual variability in response. The possibilities include the hypersensitivity or immune type of response, an abnormally high rate of halothane metabolism, aberrant routes of metabolism, or an inability to excrete the toxic metabolites formed. In each explanation the assumption of anesthetic metabolism is implicit.

Previous studies of halothane metabolism have been carried out in this laboratory with both mice and monkeys. These studies confirm the presence of nonvolatile halothane metabolites in the urine, and indicate that the molecular weights of a significant proportion exceed 1,000.²² There are also important species differences among these animals. In view of these differences and the presence of high-molecular-weight compounds in the urine (rarely found in man), the necessity for study of halothane metabolism in man was apparent. The present investigation was therefore designed to identify and quantify the urinary metabolites of halothane in man as a first step towards defining the routes of halothane metabolism and establishing a possible relationship between anesthetic metabolism and hepatotoxicity.

Methods and Results

Two opportunities for the study of urinary metabolites of halothane in man were made available. The urines of five individuals[†] given tracer doses (25 μ Ci) of ¹⁴C-labeled halothane^{8,23} were examined, together with the urines of three subjects each given a large dose (1 mCi) of radioactively labeled ¹⁴C-halothane.^{**} The latter studies were made possible through cooperation of the heart-

transplant team at Stanford University. Since the donor subjects for these heart transplant operations are legally dead, they provide appropriate subjects for human experimentation.^{††} Six to eight hours customarily elapse between the decision to transplant the heart from the donor and the time required for preparation of a suitable recipient. This period, in which the body is maintained in as normal a physiologic state as possible, allowed us the opportunity to administer non-anesthetic doses of radioactive halothane (small volumes of ¹⁴C-halothane of high specific activity) to three heart transplant donor subjects and to examine anesthetic metabolism until such time as the heart was removed. The quantitative aspects of these radioactive studies were ensured by endotracheal intubation and by collection of all condensable volatiles in silicone oil cooled with dry ice. The urine was collected through an indwelling urethral catheter. A centrally placed intravenous catheter permitted continuous blood sampling. At operation, after removal of the heart, biopsy samples were obtained from selected organs. Urine and blood specimens were collected and examined at hourly intervals.

Various radioactive components in the urine were initially separated on an anion-exchange (AG-1) column (fig. 1). Separation of the radioactive components on the AG-1 column was accomplished by elution with water followed by a pyridinium acetate stepped gradient. The first component (Peak A) was eluted with water, and the remaining fractions with increasing concentrations of pyridinium acetate. These separations indicated the presence of three major and several minor radioactive metabolites in the urine. The presence of each of the three major radioactive peaks was demonstrated in urines of all eight subjects. In urines of the three heart transplant donor subjects given large amounts of radioactivity, several minor metabolites were also found. These minor metabolites were qualitatively identical among the three urines.

By examining each hourly urine collection separately, the relative amounts and time

[†] Radioactive urine samples supplied through the courtesy of Drs. Helmut Cascorbi and David Blake.

^{**} The radioactive purity of the halothane (1-¹⁴C-2-bromo-2-chloro-1,1,1-trifluoroethane) used in these donor subjects was in excess of 98 per cent. Minor impurities included trifluorotrichloroethane, cis- and trans-dichlorohexafluorobutene, trifluoromonochloroethane, and trifluoromonobromomethane.²³ The specific activity of this material, obtained from the New England Nuclear Corporation, was 1.0 mCi/mM.

^{††} All human studies were approved by the Stanford Human Subjects Committee.

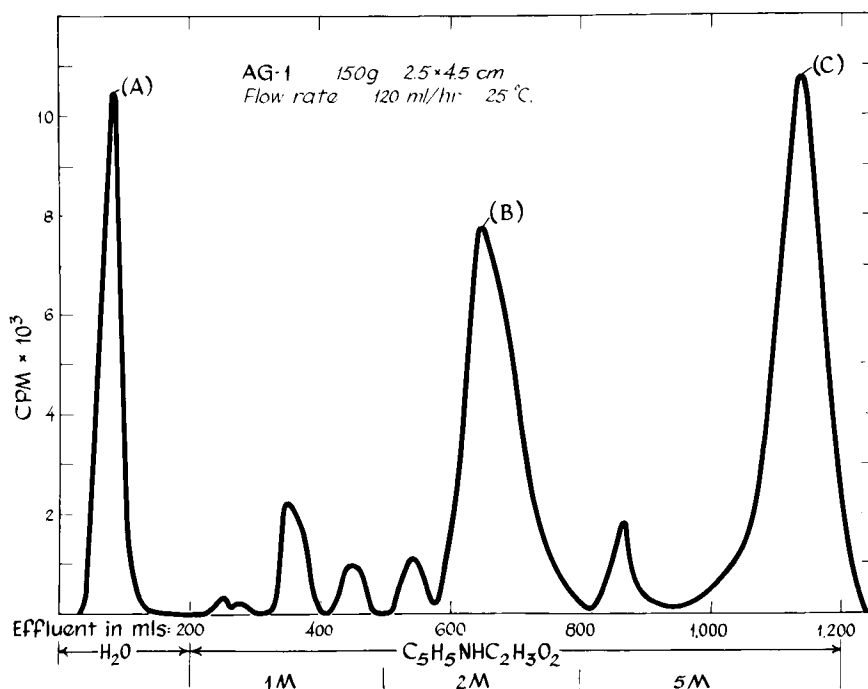


FIG. 1. Separation of the urinary radioactivity on an AG-1 ion exchange column demonstrates three major (A, B, C) and several minor metabolites. The neutral component is eluted with water and the remaining peaks with an increasing gradient of pyridinium acetate. Urine specimen shown was taken from third hour of collection.

course of each metabolite could be determined (fig. 2). Of the three large radioactive peaks, the fraction eluted with 2-M pyridinium acetate (Peak B) appeared in high concentration in the early samples, and by six hours had essentially disappeared. Conversely, the radioactive peak eluted with 5-M pyridinium acetate (Peak C) did not appear in the urine in measurable amount until the second hour of collection. By the sixth hour, however, its relative fraction had increased such that it was the major urinary metabolite. The neutral radioactive peak, eluted with water (Peak A), appeared early and maintained an essentially constant level throughout the study.

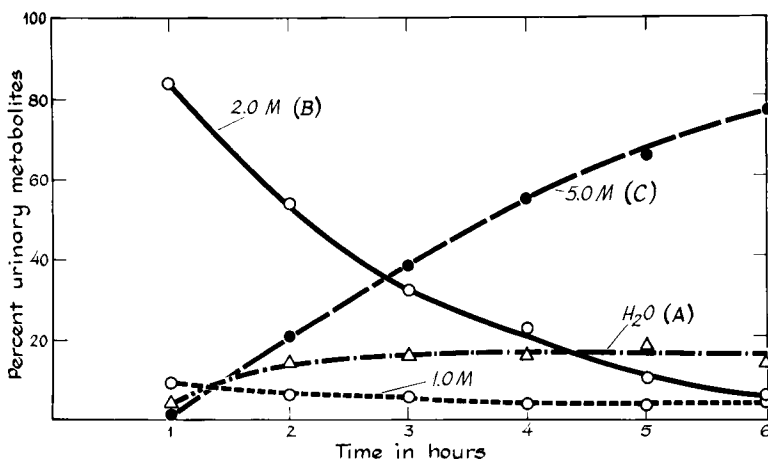
Isolation of the three major halothane metabolites found in urine was accomplished by a combination of techniques, including chemical extraction, ion-exchange chromatography, thin-layer chromatography, high-pressure liquid chromatography, and electrophoresis. Final separation and identification of the metabolites was by radio-gas chroma-

tography, nuclear magnetic resonance, and mass spectrometry.††

The most acidic fraction (Peak C) was lyophilized to dryness and converted to a methyl ester with diazomethane. The methyl derivative was then gas-chromatographed on Carbowax 400 on Porosil. The metabolite of interest was identified from among those non-radioactive compounds normally present in the urine by detecting the radioactivity associated with the ^{14}C -halothane portion. This identification was obtained by comparison of peaks between the flame ionization and radioactivity detectors on a radio-gas chromatograph (fig. 3). Subsequent combined gas chromatography-mass spectrometry indicated the derivative to be methyl trifluoroacetate, establishing the original urinary metabolite as trifluoroacetic acid (fig. 4).

†† A detailed description of the multiple separation and analytic steps involved, and additional structural confirmatory procedures, are to be presented in a separate publication.

FIG. 2. Percentage concentration of each metabolite obtained at hourly urine collections. Note the initial absence of the 5.0-M peak, C, which gradually increased by 6 hours to become the major radioactive metabolite. Conversely, the 2.0-M peak, B, rapidly decreased during the same interval. See text for details.

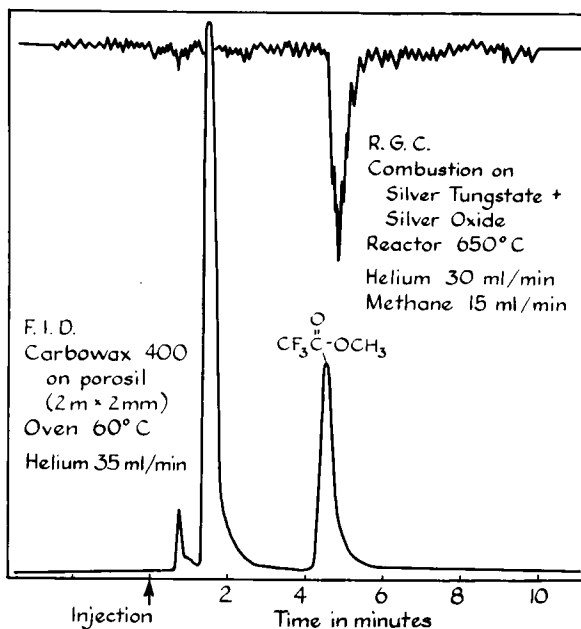


The neutral fraction (Peak A), eluted from the anion exchange column with water, was concentrated and purified by thin-layer chromatography on Chromar 1000. Elution with methanol-chloroform 1:25 indicated a radioactive band at R_f 0.4. This material was derivatized with acetic anhydride and examined by radio-gas chromatography using a two-meter OV-225 column (fig. 5). In turn, combined gas chromatography-mass spectrometry identified the derivative as N-(2-acetoxyethyl)-trifluoroacetamide (fig. 6). Au-

thentic N-(2-acetoxyethyl)-trifluoroacetamide was prepared synthetically. This compound was characterized by mass spectrometry, nuclear magnetic resonance, infrared absorption, and elemental analysis. As a final step, base hydrolysis of the synthesized compound yielded a product with a gas chromatographic retention time and mass spectrum identical to those of the original urinary metabolite, which was then established as N-trifluoroacetyl-2-aminoethanol.

The moderately acidic radioactive fraction

FIG. 3. Gas chromatographic separation of the Peak C on Carbowax 400 on Porosil using combined flame ionization and radioactivity detectors. The upper tracing indicates the presence of a single radioactive peak.



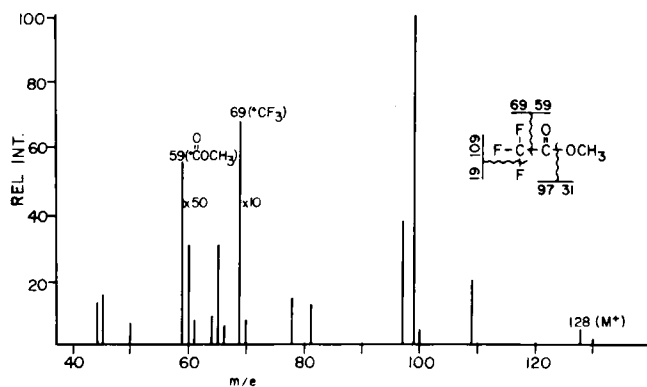


FIG. 4. Mass spectrum of Peak C obtained following gas chromatographic separation. The molecular weight (m/e) of each fragment is plotted against its abundance (relative intensity) expressed as percentage of the most abundant fragment. The mass spectrum may be interpreted by observing the peak at m/e 69, which is characteristic of a trifluoromethyl group, a major peak at m/e 59, (relative intensity scale reduced $\times 50$), which is characteristic of a methyl ester, and a peak at m/e 128, which is the correct molecular weight. Identity of the compound is thus determined as the methyl derivative of trifluoroacetic acid.

(Peak B) was eluted from the anion-exchange column with 2 M pyridinium acetate. It was accompanied by several minor radioactive compounds and by the presence of many non-radioactive urinary products. Repeated attempts to form a volatile derivative for injection into the gas chromatograph were unsuccessful due to its instability at elevated temperature. Ultimately, satisfactory purification for mass spectral analysis was achieved by high-pressure liquid chromatography. Reverse-phase liquid chromatography was carried out on a Poragel PT column, and the radioactive eluent converted to a methyl

ester by reaction with diazomethane. This derivative was rechromatographed on a second reverse-phase column, Corasil C-18. The purified derivative was finally subjected to high-pressure liquid chromatography on a silica gel (LiChrosorb-60) column.

At this point, we had sufficient information about the compound to suspect that it was a mercapturic acid derivative of halothane. Radioactive N-acetyl-L-cysteine was reacted with halothane under argon at 45 C in the presence of methanol and NaOH, and the synthetic product produced was purified as described above. Although the synthetic com-

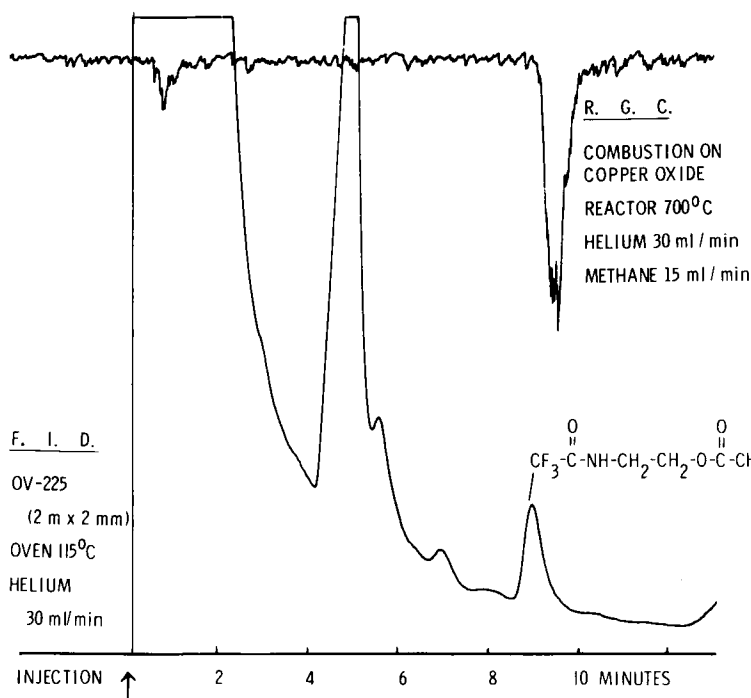


FIG. 5. Gas chromatographic separation of Peak A on an OV-225 detector. Although multiple peaks are present on the lower (flame ionization detector) tracing, the upper radioactive tracing yields only a single peak.

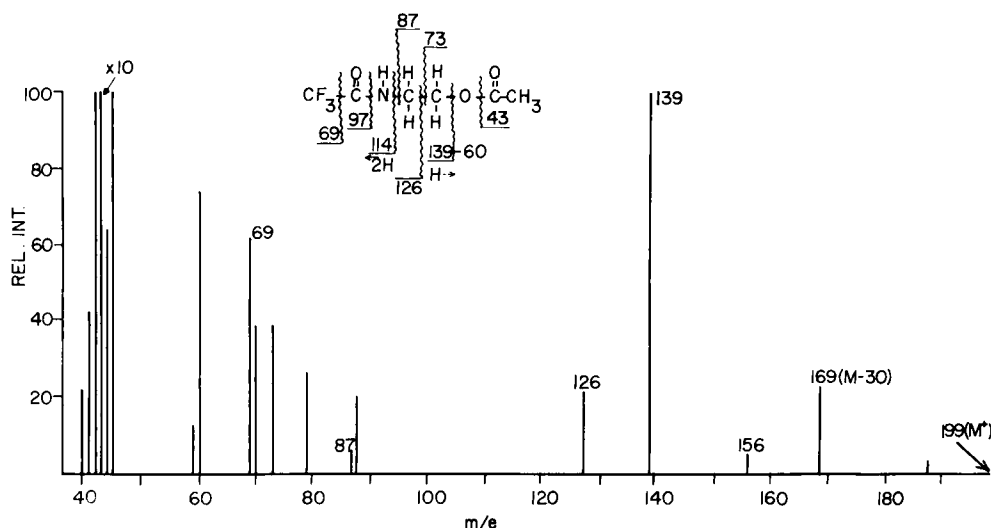


FIG. 6. Mass spectrum of Peak A obtained following gas chromatographic separation. The fragmentation pattern establishes the identity of the radioactive peak determined as the acetylated derivative of N-trifluoroacetyl-2-aminoethanol.

compound was expected to result from a displacement of the bromine of halothane by the cysteine anion, as previously demonstrated,^{24,25} the major product isolated was 1-bromo-1-chloro-2,2-difluoroethyl cysteine. After acetylation of the amino group, this synthetic compound demonstrated equal electrophoretic mobility and cochromatography with the biologic material on Corasil C-18 using water-methanol 6:4 as a solvent system, and on LiChrosorb-60 using both ethyl acetate-hexane 1:1, and isopropanol-hexane 1:1 as solvent systems. Thus, the two compounds appeared identical.

Due to the very small amount of biologic material available, conditions for a direct-inlet mass spectral analysis were initially established using the synthetic material. A similar mass spectrum was subsequently obtained for the biologic material, identifying the urinary metabolite as an N-acetyl-cysteine conjugate (fig. 7). Data from high-resolution mass spectrometry^{§§} provided confirmatory evidence for the structure of the proposed halothane mercapturic acid. The results of these high-resolution spectra indicated the sulfhydryl group of N-acetyl-cysteine did not react directly with halothane, but rather with

its dehydrofluorination product, 2-bromo-2-chloro-1,1-difluoroethylene (fig. 8).

Since such a reaction would involve the elimination of fluoride ion, we investigated the formation of the bromochlorodifluorocysteine conjugate in a synthetic system. In aqueous methanolic sodium hydroxide containing halothane and cysteine, fluoride ion was produced as rapidly as the sulfhydryl groups of cysteine disappeared. Moreover, the reaction of halothane with sodium hydroxide in aqueous methanol produced fluoride ion at the same rate as when the sulfhydryl group of cysteine was present. This suggests that, at least in this synthetic system, formation of bromochlorodifluoroethylene precedes the subsequent rapid reaction with the cysteine sulfhydryl groups. Assignment of the carbon atom of the bromochlorodifluoroethane to which sulfur is bound was inferred from model reactions of N-butyl mercaptan with halothane in aqueous alkaline methanol. Subsequent gas chromatography-mass spectrometry of the resultant butyl thioether established its structure as 2-bromo-2-chloro-1,1-difluoroethyl butyl thioether. The identity of the metabolite is thus N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-cysteine.

Discussion

The use of the human heart transplant donor as the subject for these studies is

§§ High-resolution mass spectrometry was provided by Drs. F. Walls and A. Burlingame, NIH Research Resource, Space Sciences Laboratory, University of California, Berkeley.

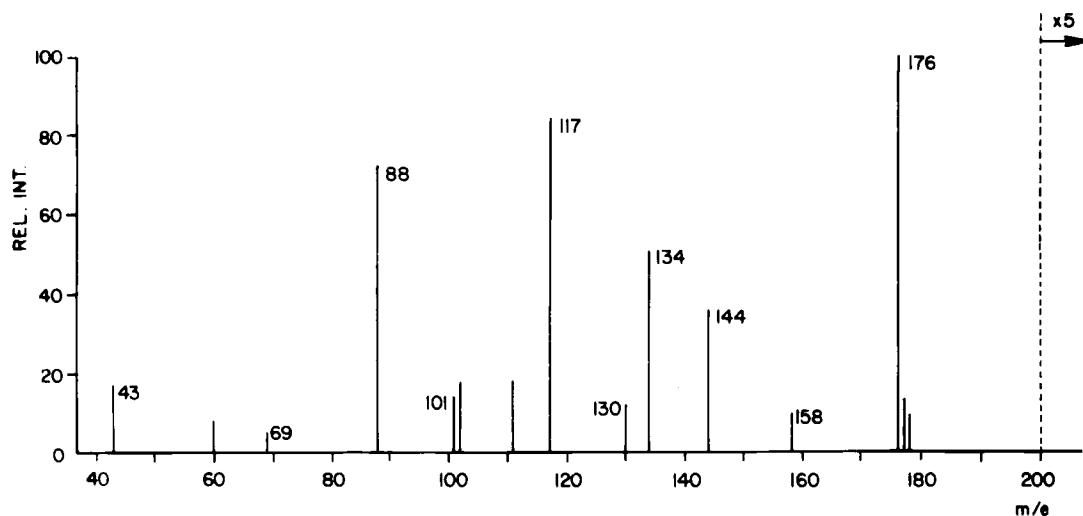


FIG. 7. Mass spectrum of Peak B obtained following purification, methylation, and direct inlet introduction. This spectrum was interpreted as follows. The molecular weight of the compound is m/e 353. The peaks immediately above 353 are indicative of the presence of bromine and chlorine. An empirical formula of $C_8H_{11}O_3N_1S_1Br_1Cl_1F_2$ was confirmed by high-resolution mass spectrometry. The fragment at m/e 176 is characteristic of the methyl ester of N-acetylcysteine. The other fragmentations predicted in the small diagram of the molecule in the upper right-hand corner and observed in the mass spectrum substantiate

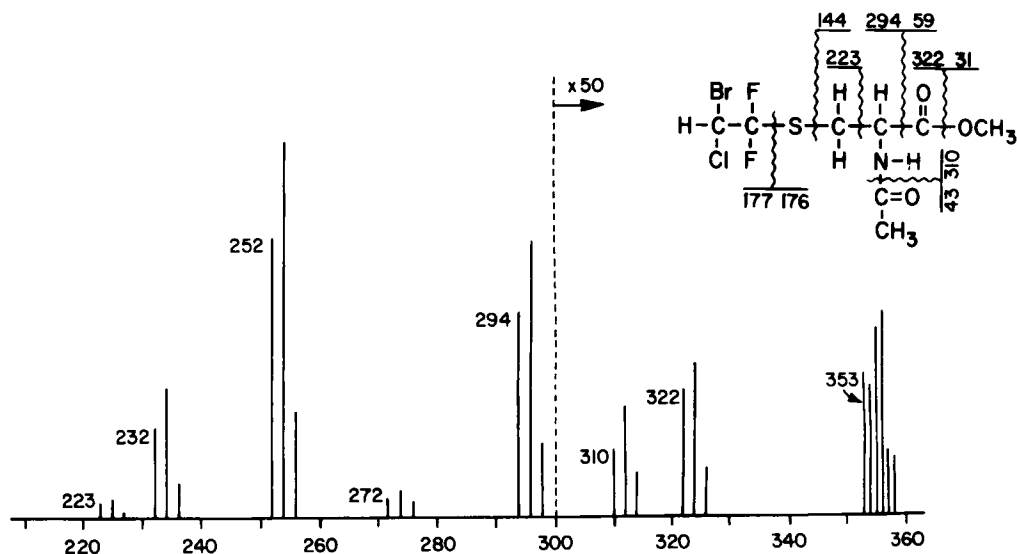
unique. The opportunity to administer large amounts of radioactivity and to obtain subsequent autopsy tissue samples for the measurement of drug metabolites offers an unusual advantage. On the other hand, caution must be used in the interpretation of these results as they might be applied to "normal" man. Although the donor subjects appeared to be in a state of reasonable physiologic balance as judged by circulation, temperature control, urinary output, etc., this state resulted from continuous mechanical ventilation and the administration of large amounts of supporting fluids, blood, vasopressor drugs, etc. We assume that hepatic metabolism under these circumstances proceeded in a normal fashion, although one could not state with certainty that the rate of drug metabolism or even the metabolic pathways themselves were normal. Nonetheless, the data are from a human source and thus of considerable interest.

The identification of trifluoroacetic acid as the major urinary metabolite of halothane was as predicted by earlier workers, although the present study provides the first mass spectrometric confirmation. Formation of the trifluoroacetyl radical may well represent a

key step in halothane metabolism. This is a highly reactive substance, and inference for its covalent bonding is available through study of radioactively labeled fragments; irreversibly bound halothane metabolites have been demonstrated in the liver both chemically^{22,26-29} and autoradiographically.³⁰

Although it has been shown in the experimental animal that trifluoroacetic acid given parenterally has limited toxicity,³¹ this observation bears little relationship to intracellular production of the trifluoroacetyl radical as a result of halothane metabolism. Trifluoroacetic acid itself is completely ionized at physiologic pH and thus unlikely to penetrate cellular barriers from the outside.

Further evidence for the covalent binding of halothane fragments is suggested by the presence of N-trifluoroacetyl-ethanolamide as a urinary metabolite. This is a very unusual conjugate, with the most likely source of the ethanolamide being phosphatidylethanolamine, a normal lipid constituent of cell membranes. This urinary metabolite suggests possible conjugation of the trifluoroacetyl radical with phosphatidylethanolamine within the cell membrane. Subsequent enzymatic cleavage of the molecule would then lead to



the cysteine fragment. The precise structure of the bromochlorodifluoroethane portion of the molecule was more difficult to interpret, although the presence of bromine and chlorine was inferred from the characteristic isotope pattern seen in the peak groups beginning with m/e 252, 272, 294, 310, 322, and 353. Identity of the radioactive peak was thus defined as methyl N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine.

the appearance of the N-trifluoroacetyl-ethanolamide fragment in the urine. Accumulation of this N-trifluoroacetyl-phosphatidylethanolamide metabolite within the cells of certain individuals might result from increased production of the trifluoroacetyl radical following increased halothane metabolism, or alternatively, occur as a result of reduced amounts of enzyme available for cleavage of the trifluoroacetyl-ethanolamide-conjugated molecule. A high concentration of N-trifluoroacetyl-phosphatidylethanolamide retained within the cell might result in interference with normal function.

The appearance in human urine of a cysteine conjugate of halothane is of considerable importance. Mercapturic acids have been isolated from the urine of several animal species, as well as man, after administration of a variety of halogenated hydrocarbon or aromatic compounds.^{24,32,33} Formation of a halothane mercapturic acid was expected to proceed by displacement of bromine from halothane by glutathione, thus resulting in 1-chloro-2,2,2-trifluoroethane mercapturate, similar to the bromine displacement from other haloalkanes.²⁴ The human urinary metabolite identified here contains

one chlorine, one bromine, but only two fluorine atoms per molecule. It is best rationalized as occurring when glutathione reacts with the dehydrofluorination product of halothane, 1-bromo-1-chloro-2,2-difluoroethylene.

It has been suggested that reactive alkenes formed by dehydrohalogenation of parent compounds are intermediates in mercapturic acid formation.²⁵ Similarly, subsequent to glutathione reaction with the difluoroethylene derivative, degradation in the mercapturic acid pathway followed by acetylation in the liver would lead to the cysteine conjugate observed. Such a highly reactive difluoroethylene derivative might be formed by proton extraction from halothane by cytochrome P-450.^{34,35} The resulting anion might proceed under oxidative conditions to trifluoroacetic acid or under reductive conditions to bromochlorodifluoroethylene.³⁶ The latter is an extremely reactive molecule and would be expected to be a very potent alkylating agent.

It is possible that only a small amount of the difluoroethylene intermediate may react with glutathione, while the remainder alkylates other cellular constituents. It has been suggested that glutathione plays a protective

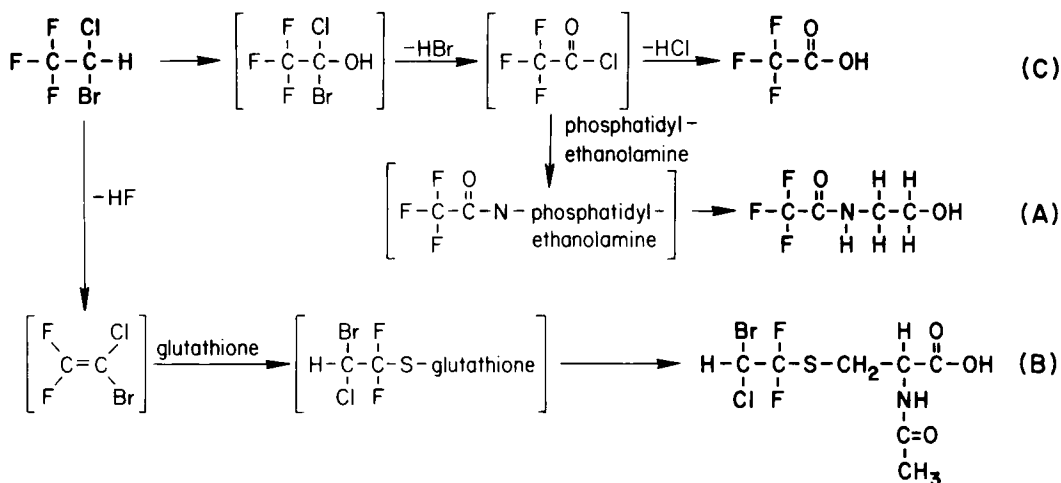


FIG. 8. This scheme proposes metabolic routes for the production of the three major urinary metabolites. The first route begins with the hydroxylation of halothane, followed by spontaneous loss of HBr to form trifluoroacetyl chloride. This molecule may then either hydrolyze to yield trifluoroacetic acid, or react with cellular constituents such as phosphatidylethanolamine.³⁸ Subsequent enzymatic cleavage of the conjugated phospholipid would yield N-trifluoroacetyl-2-aminoethanol. Enzymatic dehydrofluorination of halothane to yield 2-bromo-2-chloro-1,1-difluoroethylene is a likely route to the observed cysteine conjugate.²⁵

role in the body through preferential conjugation to active intermediates formed during the metabolism of certain foreign compounds. Thus, conjugation of glutathione to bromobenzene,³⁷ acetaminophen,³⁸ or carbon tetrachloride³⁹ serves as a detoxification mechanism. Recent *in-vitro* studies treating microsomes prepared from enzyme-induced rats with ¹⁴C-labeled chloroform and halothane indicate the ability of glutathione to inhibit markedly the binding of chloroform metabolites, and to a lesser degree the binding of halothane metabolites.⁴⁰ *In-vivo* studies in phenobarbital-treated animals indicate that chloroform anesthesia significantly decreases glutathione levels in the liver, although this could not be demonstrated for halothane.

The binding of halothane metabolites to cell protein and phospholipids has been demonstrated.²⁷⁻²⁹ If the bromochlorotrifluoroethane anion we propose did not eliminate fluoride ion to produce bromochlorodifluoroethylene, it would then be capable of becoming a free radical or a carbene, which also could react with protein and phospholipid components of liver cell organelles.

Identification of the major urinary metabolites of halothane in man provides an important first step towards our understanding of the metabolism of this anesthetic (fig. 8). The covalent binding of the trifluoroacetyl group to ethanolamine (likely to be phos-

phatidylethanolamine) and the conjugation of halothane with glutathione indicate the production of potentially destructive reactive intermediates. Knowledge of the precise chemical structure of the urinary metabolites provides data from which one can infer the characteristics of the metabolites covalently bound to the liver. Such information may prove important in defining mechanisms for long-term organ toxicity.

Ms. Nancy Cline provided expert technical assistance. The authors thank Drs. C. Henrick and J. Brauman for helpful discussions.

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