Application of Low-temperature Autoradiography to Studies of the Uptake and Metabolism of Volatile Anesthetics in the Mouse

III. Halothane

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The uptake and metabolism of halothane-2 ^{14}C in the mouse were studied with low-temperature whole-body autoradiography. Precise concentrations of the volatile anesthetic and nonvolatile metabolites were defined in various tissues through impulse counting of biopsy specimens. Immediately following inhalation of the halothane, the highest concentrations of radioactivity were present in the brown fat and liver. At ten minutes all tissues except brown fat and general body fat contained significant concentrations of nonvolatile metabolites. Within two hours, 9.22 per cent of the administered anesthetic could be recovered as ^{14}C-labeled metabolite. A marked accumulation of these materials was present in the liver. Two of the metabolites were separated, but not identified, following thin-layer radiochromatography of a liver extract. (Key words: Halothane; Metabolism; Low-temperature autoradiography; Liver.)

THE APPLICATION of low-temperature whole-body autoradiography to *in-vivo* studies of the uptake and metabolism of the volatile anesthetic agents (chloroform, diethyl ether) has been recently reported.[1, 2] Previous investigations involving the uptake of halothane utilized a variety of analytic techniques including gas–liquid chromatography,[3, 4, 5] infrared analysis,[6] and nuclear activation plus spectrofluorometry.[7] These investigations afforded much useful data, but technical difficulties prevented multiple analyses as well as a simultaneous scan of anesthetic concentration in body tissues. The metabolism of halothane has been studied by a number of workers, including Van Dyke *et al.*,[9] Stier and Alter,[10] and Rehder et al.[11] The former utilized ^{36}Cl- and ^{14}C-labeled halothane, while both Stier and Rehder measured nonvolatile halothane metabolites present in the urine as either the bromide or the fluoride ion.

The present investigation concerns the uptake, distribution, and metabolism of halothane-2 ^{14}C studied in the mouse by low-temperature whole-body autoradiography and related radioisotope techniques.

Procedure

Halothane-2 ^{14}C, specific activity 1.2 mC/mM, was obtained from commercial sources.‡ Purity of the material was established by radio gas chromatography. The labeled anesthetic was diluted twofold with nonradioactive halothane, and 20 μC of drug (equivalent to 2.2 per cent halothane vol/vol oxygen) were administered by inhalation for ten minutes to each of nine Swiss white mice, averaging 20 gm in weight. Following administration of the anesthetic, the animals were either immediately sacrificed or transferred to room air to recover from anesthesia. The latter animals subsequently were sacrificed at 15 and 120 minutes by immersion in liquid nitrogen. Details of the low-temperature autoradiographic procedure utilized, preparation of the biologic specimens, etc., have been reported.[1, 2] Since the vapor pressure of halothane was calculated to be less than 0.5 mm at −78 C.§ it was satisfactory to store all bio-

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‡ New England Nuclear Corporation, Boston, Massachusetts.

§ $\log p = 7.689 - 1555/T$

p = vapor pressure in mm Hg
T = absolute temperature C

(Abajian, J.: Physics and Chemistry, Chapter II, Halothane. Philadelphia, F. A. Davis Co., 1962, p. 8.) (Personal communication from J. C. Topham indicates the freezing point of halothane to be −118.3 C.)

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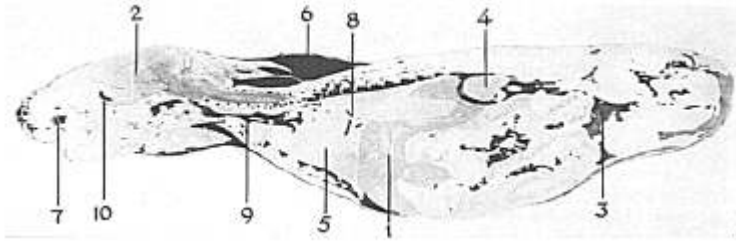

FIG. 1. Autoradiograph prepared from hemisection of mouse sacrificed immediately following inhalation of halothane-2 ^{14}C. 1, liver; 2, brain; 3, fat; 4, kidney; 5, heart; 6, brown fat; 7, nasal mucous membrane; 8, lung; 9, blood; 10, Harder's gland.

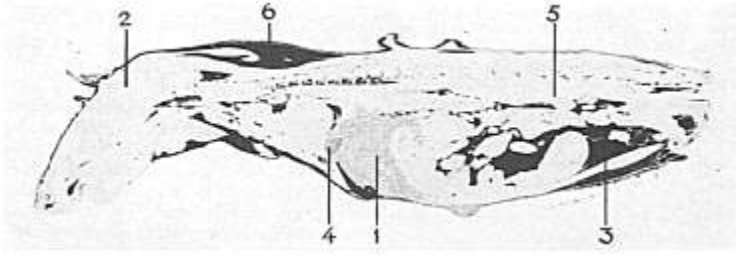

FIG. 2. Autoradiograph prepared from hemisection of mouse sacrificed 15 minutes following inhalation of halothane-2 ^{14}C. 1, liver; 2, brain; 3, fat; 4, gallbladder; 5, muscle; 6, brown fat.

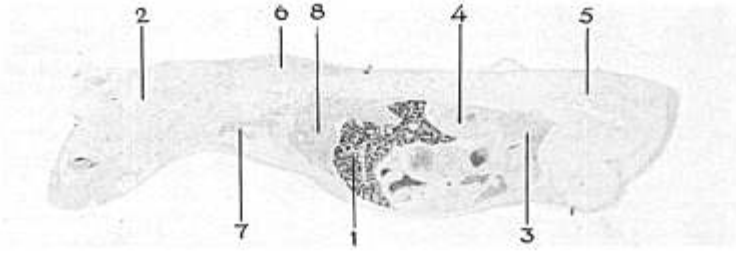

FIG. 3. Autoradiograph prepared from hemisection of mouse sacrificed 120 minutes following inhalation of halothane-2 ^{14}C. 1, liver; 2, brain; 3, fat; 4, kidney; 5, muscle; 6, brown fat; 7, blood; 8, lung.

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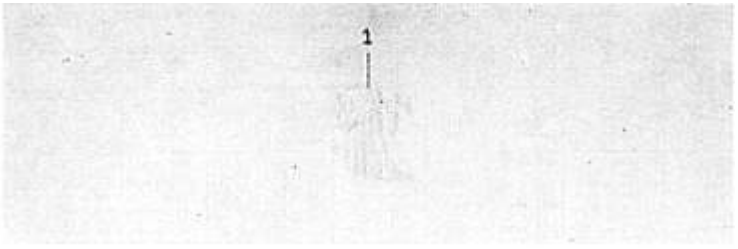

FIG. 4. Autoradiograph of a 40-μ section of mouse sacrificed immediately following inhalation of halothane-2 ^{14}C. Section dried for 48 hours at −15 C, then heated to 80 C for four hours. 1, liver.

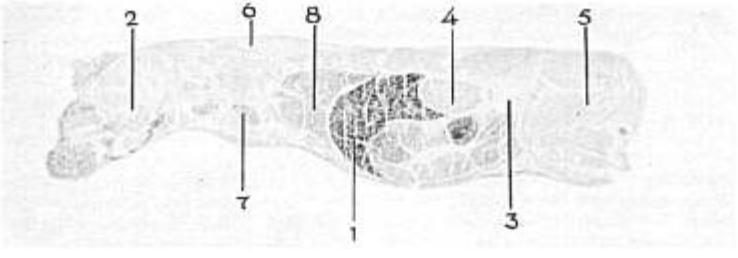

FIG. 5. Autoradiograph of a 40-μ section of mouse sacrificed 120 minutes following inhalation of halothane-2 ^{14}C. Section dried for 48 hours at −15 C, then heated to 80 C for four hours. 1, liver; 2, brain; 3, fat; 4, kidney; 5, muscle; 6, brown fat; 7, blood; 8, lung.

logic specimens in a light-tight insulated box over a deep base of solid carbon dioxide during the six-day period required for photographic exposure.

Following preparation of whole-body autoradiographs from the frozen hemisections, thin sections (40 μ) were cut from half of the blocks and used for anatomic reference and for autoradiographic study of the nonvolatile metabolites. Presence of the latter was verified by drying the thin sections for 48 hours at −15 C, and subsequently heating to 80 C for four hours to remove all volatile radioactivity. The alternate frozen hemisections were used for tissue biopsies and their radioactivity content quantified by impulse-counting techniques. Four biopsies were taken from each tissue specimen. Two biopsies were quickly sealed in a vial containing 1.0 ml N.C.S. solubilizer [c] and 0.5 ml heptane. The other specimens were taken to dryness and then solubilized in 1.0 ml N.C.S. solution. Ten ml of toluene cocktail [cc] were added to each vial prior to scintillation counting.

In other aspects of the study, a liver homogenate was prepared from those animals sacrificed at 120 minutes. This was taken to dryness, extracted in equal volumes of methanol and ethyl acetate, and the radioactive com-

[c] Amersham/Searle, Des Plaines, Illinois.
[cc] 2,5-diphenyloxazole; 5 gm/l toluene.

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TABLE 1. Concentrations of Radioactivity (Halothane Plus Metabolites) in Various Tissues of the Mouse*

Tissue	0 Minutes			15 Minutes			120 Minutes		
	Total† Radioactivity	Nonvolatile Radioactivity	Per Cent Metabolite	Total Radioactivity	Nonvolatile Radioactivity	Per Cent Metabolite	Total Radioactivity	Nonvolatile Radioactivity	Per Cent Metabolite
Blood	796 ± 134	137 ± 18	17.2	388 ± 35	231 ± 12	59.5	836 ± 40	686 ± 31	82.1
Muscle	565 ± 91	46 ± 11	8.1	148 ± 24	92 ± 21	62.2	273 ± 17	251 ± 27	91.1
Lung	666 ± 102	186 ± 44	27.9	487 ± 53	272 ± 35	55.9	722 ± 38	414 ± 46	57.3
Fat	592 ± 54	19 ± 1.8	3.2	540 ± 54	50 ± 15	9.3	283 ± 14.9	136 ± 56	48.1
Kidney	1,064 ± 204	274 ± 47	24.8	411 ± 33	280 ± 28	68.1	561 ± 60	475 ± 76	84.7
Brain	1,136 ± 177	92 ± 6.0	8.1	388 ± 68	147 ± 12	37.9	433 ± 24	355 ± 16	82.0
Liver	1,178 ± 149	300 ± 30	25.5	767 ± 115	631 ± 80	82.3	1,044 ± 46	869 ± 29	83.2
Brown fat	13,232 ± 5,314	48 ± 5.6	0.4	4,639 ± 1,647	70 ± 10	1.5	218 ± 12	192 ± 15	77.4

* Animals sacrificed at 0, 15, and 120 minutes following a ten-minute inhalation of halothane-2 ^{14}C. Data represent duplicate determinations in each of three animals at each time sequence (± SE).
† Count/min/mg.

ponents separated by thin-layer radiochromatography.

In additional studies, 8.6 μC of halothane-2 ^{14}C were injected into the tail veins of four mice. These animals were sacrificed two hours after injection of the anesthetic, and the livers removed. The carcasses were homogenized in an omnimixer.†† Biopsies of the liver and aliquots of the homogenized tissue were dried and treated like the other biopsy material.

An attempt was also made to correlate the fluorine content with the ^{14}C label of the nonvolatile liver metabolites by injecting a measured amount of ^{14}C-labeled halothane into two mice. These animals were then sacrificed five and 24 hours after administration of the halothane, and the livers assayed for ^{14}C count *vs.* elemental fluorine content.‡‡

Results

Autoradiograph figures 1 to 3 illustrate the uptake and elimination of halothane-2 ^{14}C in the mouse. The animal represented in figure 1 was sacrificed immediately after a ten-minute inhalation of halothane. Although radioactivity was widely distributed through the body, the highest concentrations were present in the brown fat and liver. By 15 minutes (fig. 2) there had been a decrease in total radioactivity, but proportionate increases were present in body fat and in the liver. At 120 minutes (fig. 3), the highest relative concentrations of radioactivity were present in the liver and intestine, with lesser amounts found in the blood, lung, kidney, brain, and body fat.

Autoradiograph figure 4 represents a thin (40-μ) section taken from an animal sacrificed immediately following a ten-minute inhalation of halothane. These sections, taken to dryness and heated to 80 C, indicate the accumulation of nonvolatile metabolite(s) in the liver within ten minutes of induction of anesthesia. Figure 5 represents a thin section taken from an animal sacrificed 120 minutes after termination of the halothane anesthesia. At this time the highest concentration of nonvolatile radioactivity was found in the liver but, in addition, significant amounts of nonvolatile metabolite were present throughout the body with the marked exception of brown fat and general body fat. In other respects a close similarity between autoradiographic figures 3 and 5 can be seen.

TABLE 2. Concentrations of Radioactivity in Livers of Mice Sacrificed 0, 15, and 120 Minutes Following Inhalation of Halothane-2 ^{14}C

Radioactivity*	0 Minutes	15 Minutes	120 Minutes
Total	1,178 ± 149	767 ± 115	1,044 ± 46
Volatile	878 ± 124	136 ± 33	175 ± 13
Nonvolatile	300 ± 30	631 ± 80	869 ± 29

* Count/min/mg liver tissue.

Table 1 provides an analysis of the total radioactivity present in selected tissues at the time of sacrifice. The concentration of nonvolatile radioactivity and the percentage of

†† Ivan Sorvall Inc., Norwalk, Connecticut.
‡‡ Fluorine assay performed by Mr. Ernst Meier, Department of Organic Chemistry, Stanford University, Stanford, California.

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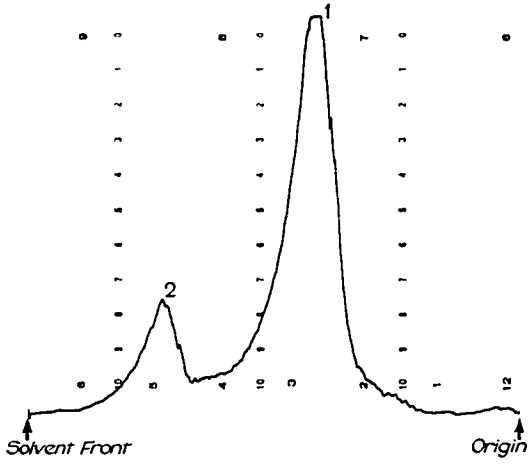

FIG. 6. Radiochromatogram from nonvolatile metabolites extracted from liver. Mouse sacrificed 120 minutes after inhalation of halothane-2 ^{14}C. Solvent system utilized: chloroform = 65; methyl alcohol = 35; water = 8. Peak 1 has R_f 0.41; Peak 2 has R_f of 0.73.

metabolite is shown for each tissue. Immediately after termination of the anesthesia, the highest concentrations of total radioactivity were present in the brown fat and the liver. With the former tissue, the radioactivity largely represented volatile materials (presumably unchanged halothane). The liver (and the kidney and lung) also contained high concentrations of nonvolatile radioactivity. Within 15 minutes, the relative concentrations of nonvolatile radioactivity had increased in all tissues, with the greatest change occurring in the kidney and liver. By 120 minutes, the total radioactivity content had increased in many tissues, reflecting an accumulation of nonvolatile metabolite(s). Brown fat and general body fat showed marked decreases in concentration of radioactivity.

Immediately after termination of the anesthesia, the highest concentrations of nonvolatile metabolite(s) were found in the liver (table 2). Nonvolatile metabolites constituted 25.5 per cent of the total radioactivity present in this organ. By 120 minutes, these nonvolatile metabolites accounted for 83.2 per cent of the total radioactivity.

A homogenate of the livers of mice sacrificed 120 minutes after anesthesia was extracted into equal volumes of methanol and ethyl acetate, and the radioactive materials isolated by thin-layer radiochromatography. Figure 6 demonstrates the isolation of two nonvolatile radioactive components. Aliquots of these peaks were separated from the silica gel and re-extracted into methanol and ethyl acetate. The samples were taken to dryness, redissolved in chloroform, and adjusted to *p*H 7.1 with phosphate buffer. An aliquot was then treated with 6,000 units bacterial β-glucuronidase, type II, in a water bath at 37 C for 24 hours. Control studies without β-glucuronidase were made. Re-extraction of the radioactivity was followed by thin-layer chromatography. No quantitative changes nor alteration in R_f resulted, suggesting the absence of glucuronide formation in the extract examined.

Four additional animals were sacrificed 120 minutes after the intravenous injection of 4 μC ^{14}C-halothane. The livers were removed and the carcasses homogenized. Representative aliquots taken to dryness and counted in the scintillation chamber indicated that by two hours 9.22 ± 1.0 per cent of the injected radioactivity was still present in the animal as nonvolatile metabolite. On a weight basis the

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concentration of radioactivity remaining in the liver was calculated to be two to three times that present in the homogenized body tissue.

A measured amount of cold halothane with tracer concentrations of $^{14}CF_3CHBrCl$ was administered to two animals. These were sacrificed five and 24 hours after administration of the anesthetic, the livers removed and assayed for ^{14}C. Assuming the ^{14}C-label to remain associated with three fluorine atoms as $^{14}CF_3$, one can calculate the possible fluorine content of the liver. This value, in turn, was compared with an elemental analysis of fluorine actually found in the liver:

	Calculated F/gm liver	Measured elemental F/gm liver
5-hour liver	117.5 μg	113.0 μg
24-hour liver	74.6 μg	60.0 μg

The close agreement between the calculated and observed data suggests a direct association of fluorine with the ^{14}C in the nonvolatile metabolite(s).

Discussion

It is well recognized that a combination of respiratory, circulatory, and physicochemical factors influence the uptake and distribution of the inhalation anesthetics. Differences in uptake between individual anesthetic agents to a large extent depend upon the wide variation in their blood/gas and tissue/blood solubility coefficients. Such data have been well defined for halothane,[12] as well as for other anesthetics.

Although the information presented in figures 1–3 and in table 1 agrees in many respects with predicted [13] and previous [3–7] experimental results, there are certain differences in interpretation. Previous *in-vivo* studies have not considered the significant metabolism of halothane which occurs during its administration. This metabolism takes place continuously, and as is noted in table 1, the degree of metabolism may be considerable within ten minutes of induction of anesthesia. Nonvolatile metabolites in the blood constitute 17.2 per cent of the total radioactivity by that time. Fifteen minutes later, the relative concentration of nonvolatile metabolites has increased to 59.5 per cent, and by 120 minutes it equals 82.1 per cent of the total blood radioactivity. Proportionate increases in metabolite(s) are also present in certain other tissues.

There have been a number of reports concerned with the *in-vivo* metabolism of halothane. In an earlier study, Van Dyke *et al.*[8] demonstrated the production of $^{14}CO_2$ and unidentified ^{36}Cl urinary metabolites following the intraperitoneal injection of labeled anesthetic into the rat. Later studies by Stier *et al.*[10] provided evidence in man for the increased excretion of urinary bromide following halothane anesthesia. A more recent study by Rehder *et al.*[11] demonstrated the prolonged excretion of CF_3COOH in the urine of two patients following a 75-minute period of halothane anesthesia.

In the present studies, 9.22 per cent of the administered anesthetic had accumulated as nonvolatile labeled metabolite(s) within two hours. Metabolism has been shown to begin almost immediately and to reach the above levels rapidly.

Earlier studies in the mouse with chloroform [1] and diethyl ether [2] have established the accumulation of significant amounts of nonvolatile metabolites in these animals following their exposure to radioactively-labeled anesthetics. Preliminary identification of the major ether metabolites indicates them to be conjugated glucuronides and derivatives of long-chain fatty acids.[14]

It is of interest that the amount of nonvolatile metabolite(s) produced following halothane anesthesia is considerably greater than that from the other two labeled anesthetics studied:

Percentages of Injected Radioactivity Remaining in the Body as Nonvolatile Metabolites Two Hours after Anesthetic Administration

Diethyl ether	3.61 per cent
Chloroform	4.02 per cent
Halothane	9.22 per cent

Although identification of the nonvolatile metabolites of halothane remains to be accomplished, thin-layer radiochromatography has established the presence of at least two nonvolatile metabolites extractable from the liver. It has been further established that these materials are not glucuronides and do not represent either CF_3COOH (bp 72.4 C) or CF_3CH_2OH (bp 74.0 C), two suggested

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metabolites of halothane.[10] The evidence also indicates that the ratio of ^{14}C to F remains constant. It is, of course, possible that the materials are conjugates of either CF_3COOH or CF_3CH_2OH, although this remains to be proved.

It is of further interest that the high lipid solubility of the parent compound, halothane, has been lost in its nonvolatile metabolic products (compare autoradiographic figs. 3 and 5). This change to a more water-soluble compound represents an important step towards elimination of the molecule from the body.

The significance of the present study lies in the demonstration of a high rate of metabolism for halothane in the mouse, plus the formation and storage in the liver of more slowly eliminated nonvolatile by-products. Of critical importance is information regarding the specific nature of these materials—*i.e.*, their harmlessness or possible toxicity.

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14. In preparation.

Obstetrics and Pediatrics

MATERNAL HYPERVENTILATION Hyperventilation in obstetrical patients is common. To determine the effects of hyperventilation on the fetus, measurement of maternal and fetal capillary blood gases were made while conscious patients were in labor. In 86 patients, no relationship was found between maternal P_{CO_2} and fetal P_{O_2}. Six patients actively hyperventilated for as long as 18 minutes. Although maternal *p*H increased and P_{CO_2} decreased, no change in fetal P_{O_2} or base excess was found during hyperventilation or recovery. Spontaneous hyperventilation was found neither to harm nor to benefit the fetus. (*Lumley, J., and others: Hyperventilation in Obstetrics, Amer. J. Obstet. Gynec. 103: 847 (March) 1969.*)

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