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THE METABOLISM OF VOLATILE ANESTHETICS—II.
IN VITRO METABOLISM OF METHOXYFLURANE AND HALOTHANE IN RAT LIVER SLICES AND CELL FRACTIONS

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Abstract—The *in vitro* metabolism of halothane and methoxyflurane has been investigated. With either chlorine-36 or carbon-14-labeled material, it has been found that the chlorine is enzymatically removed from both anesthetics by rat liver slices and, more specifically, by microsomes. In this same system, methyl-^{14}C-methoxyflurane is converted to $^{14}CO_2$, indicating a cleavage of the ether linkage. However, 1-^{14}C-halothane is not oxidized to CO_2.

EVIDENCE has previously been presented from this laboratory that certain volatile anesthetics—chloroform, diethyl ether, halothane, and methoxyflurane—are metabolized *in vivo*.[1] The end point of the metabolism of these anesthetics was shown to be carbon dioxide or halide ions and urinary metabolites of unknown composition.

Among the anesthetics studied, halothane* and methoxyflurane† are certainly the most rewarding because of their widespread use in general anesthesia and their interesting structures. As indicated by their formulae, they contain halogens which, if metabolism to CO_2 is to occur, must be removed either enzymatically or nonenzymatically. In the case of methoxyflurane, there is additionally an ether linkage, which must be broken in order to complete the biodegradation.

Ether linkages have been found to be broken enzymatically by enzymes present in microsomes.[2] Halogens are known to be removed enzymatically, since the work of Heppel and Porterfield[3] in 1948 showed evidence for the existence of a halidase which removed bromine and chlorine from certain halomethanes and haloethanes. They were able to purify partially the enzyme that was found in the supernatant fraction of the cell. Bray *et al.*[4] and Butler[5] have shown that chlorinated hydrocarbons can be nonenzymatically dehalogenated to a certain extent in the presence of sulfhydryl compounds.

Recently, Paul and Rubinstein[6] have reported on the metabolism of carbon tetrachioride and chloroform; they gave evidence for the conversion of these two materials to carbon dioxide. Presumably, the dehalogenation proceeded either by enzymatic or nonenzymatic means or both, but the oxidation of the carbon occurred by enzymatic means.

It is the purpose of the present communication to report on certain aspects of the dechlorination of halothane and methoxyflurane by means of ^{36}Cl-labeled materials.

* 1,1,1-Trifluoro-2-bromochloroethane.
† 2,2-Dichloro-1,1-difluoroethyl methyl ether.

This will be compared with the conversion of these two compounds to CO_2 by utilizing ^{14}C label.

MATERIALS AND METHODS

Preparation of labeled anesthetics

The addition of the ^{36}Cl to the anesthetic precursors (supplied by us) was performed by New England Nuclear Corp. The final purification of the ^{36}Cl-labeled halothane and methoxyflurane and the preparation of the ^{14}C-methoxyflurane was carried out by Dr. Eric Larsen of the Halogens Research Laboratory, The Dow Chemical Co. The purification was performed by vapor-phase chromatography; there were no detectable impurities. The final specific activities were as follows: ^{36}Cl-methoxyflurane, $1{\cdot}29 \times 10^7$ dpm/0·1 ml; ^{36}Cl-halothane, $2{\cdot}13 \times 10^7$ dpm/0·1 ml; ^{14}C-methoxyflurane, $1{\cdot}66 \times 10^7$ dpm/0·1 ml.

Preparation of tissue samples

The investigations were carried out in livers from adult male rats of a Wistar strain. Tissue slices weighing 300 mg each were sectioned in a Stadie-Riggs tissue slicer and were placed in the main compartment of a Warburg flask with 3 ml of buffer. The rat liver homogenates were prepared in a Potter-Elvehjem hand homogenizer with phosphate buffer (0·2 M) at pH 7·4; the buffer contained 50 μmoles of nicotinamide. Three ml of the homogenate was used per incubation by placing this amount in the main compartment of the Warburg flask. This homogenate was also used to prepare the mixture of microsomes and cell supernate according to the method of Axelrod.[7] The homogenate was centrifuged at 9,000 *g* for 15 min to remove mitochondria, nuclei, and cell debris. The supernate from the centrifugation, consisting of microsomes and cell supernate, was used either directly for the incubation or for the preparation of microsomes and the cell supernatant fraction. This was accomplished by recentrifuging the 9,000 *g* supernate at 105,000 *g* for 1·5 hr. The microsomes so obtained were resuspended in phosphate buffer (pH 7·4) to a volume equal to the volume of the original homogenate. The cell supernatant fraction was dialyzed against cold water and handled according to the method of Booth *et al.*[8]

The reduced NADP-generating system used in certain incubations was composed of 0·2 μM NADP, 5×10^{-3} M glucose-6-phosphate, 1 to 5 units of glucose-6-phosphodehydrogenase, 25 μmoles $MgCl_2$. The amount of anesthetic used was varied; it was placed in the side arm of the flask either directly, in which case 5–10 μliter was used, or it was first dissolved in propylene glycol (25 μliter anesthetic in 2 ml propylene glycol) and an aliquot (0·2 ml) of the propylene glycol–anesthetic solution added to the side arm. In this latter manner, the rate of diffusion of the anesthetic into the main compartment was reduced, as was the total anesthetic concentration in the buffer. The incubations were carried out at 37° for 2 hr. In all cases reported either Tris buffer at pH 7·4 or phosphate buffer (0·2 M, pH 7·4) was used. It was determined that there was no difference in the results if either of these buffers or Ringer-phosphate buffer[9] was used.

Assay of tissue sample activity

If the CO_2 was to be collected and analyzed for $^{14}CO_2$, then NaOH solution was placed in the center well. The NaOH was analyzed for $^{14}CO_2$ according to a method

described previously.[1] If dechlorination was to be studied, then ^{36}Cl-labeled anesthetic was placed in the side arm, incubated, and the incubation stopped by adding concentrated HNO_3. The tissue slice and buffer were then analyzed for inorganic chloride. This was accomplished by removing the precipitated protein and washing the protein precipitate once with 1 ml of 0·9% saline and several times with 1 ml aliquots of water. The saline in the wash acted as a carrier for the $^{36}Cl^-$ formed in the incubation. These washings were combined with the original supernate. The chloride was precipitated with silver nitrate added in excess. The silver chloride precipitate was washed several times with water prior to dissolving in concentrated ammonium hydroxide. An aliquot of this was added to a scintillation vial containing 3·5% Cab-o-sil in toluene plus Liquiflor (PPO (2,5-diphenyloxazole)) and POPOP [*p-bis*-2 (5-phenyloxazolyl) benzene] scintillation fluid. This solution maintained the AgCl well dispersed for counting in the scintillation counter.

RESULTS

The results of the studies in Table 1 on the metabolism of ^{14}C-methoxyflurane to CO_2 *in vitro* demonstrate that the metabolism is dependent upon the presence of

TABLE 1. $^{14}CO_2$ FORMATION FROM ^{14}C-METHOXYFLURANE *IN VITRO*

Tissue	Atmos.	Additions	$^{14}CO_2$ (dpm/300 mg wet wt)
Liver slices	O_2	none	718 ± 40
Liver slices	N_2	none	0
Liver slices (boiled)	O_2	none	0
None (buffer only)	O_2	none	0
Liver slices	O_2	Citrate (10^{-2} M)	833 ± 11
Liver slices	O_2	Acetate (10^{-2} M)	744 ± 12
Liver slices	O_2	Glucose (10^{-2} M)	941 ± 58
Liver slices	O_2	Malonate (10^{-2} M)	450 ± 53
Liver slices	O_2	NaCN (10^{-2} M)	78 ± 12
Liver homogenate	O_2	none	450 ± 10
Liver slices	O_2	Dimedon (10^{-3} M)	110 ± 5

oxygen. It has not been determined whether the oxygen demand is for the rupture of the ether linkage or for the ultimate oxidation of the carbon. As would be expected, the production of CO_2 does not proceed in those flasks that contain the heated slices. The addition of citrate, acetate, and glucose to viable slices results in only a slight enhancement of the conversion to CO_2. Malonate, NaCN, and dimedon* addition result in an inhibition of the conversion. The conversion of the ^{14}C label to CO_2 is probably a multistep reaction and therefore we can not be certain whether the effect of the addition of malonate or NaCN is on the ether cleavage or on the resulting 1-carbon oxidation. Dimedon addition resulted in a diminution of counts appearing in the CO_2, although this inhibition can be readily predicted; its significance is considered in the discussion. The low conversion of methoxyflurane to CO_2 by the whole homogenate is due in part to the inhibition by the nuclei and mitochondria and in part to the more rapid and greater uptake of anesthetic by the

* 5,5-Dimethyl-1,3-cyclohexanedione.

homogenate than by the liver slice, in which case the anesthetic may act as an inhibitor at the concentrations reached in the homogenate.

The conversion of 1-^{14}C-halothane to CO_2 *in vivo* occurs to only a minor extent.[7] Therefore, the *in vitro* measurements would also be expected to show little or no conversion of 1-^{14}C-halothane to CO_2. As predicted, there was no production of CO_2 from ^{14}C-halothane *in vitro*.

Table 2 reveals the extent of dechlorination of ^{36}Cl-methoxyflurane *in vitro* by liver slices. These data indicate that there is an oxygen requirement and that the

TABLE 2. *IN-VITRO* REMOVAL OF ^{36}CL FROM ^{36}CL-METHOXYFLURANE

Tissue	Atmos.	Additions	^{36}Chloride (cpm/300 mg wet wt.)
Liver slices	O_2		1,015 ± 79
Liver slices (heated)	O_2		0
Buffer only	O_2		0
Liver slices	N_2		160 ± 7
Liver slices*	O_2		2,745 ± 166
Buffer	O_2	Cysteine	0
Buffer	O_2	Glutathione	0
Liver slices	O_2	NaF (3×10^{-3} M)	1,240 ± 18
Liver slices	O_2	Malonate (3×10^{-3} M)	1,081 ± 11
Liver slices	O_2	NaCN (3×10^{-4} M)	240 ± 10

* ^{36}-Cl-Methoxyflurane (25 μliter) mixed with propylene glycol (2 ml) prior to addition of 0·1-ml aliquot of mixture to the Warburg side arm.

TABLE 3. *IN-VITRO* REMOVAL OF ^{36}CL FROM ^{36}CL-HALOTHANE

Tissue	Atmos.	^{36}Chloride (cpm/300 mg wet wt.)
Liver slices	O_2	461 ± 36
Liver slices (boiled)	O_2	0
Liver slices	N_2	24 ± 5
Buffer only	O_2	0
Buffer + cysteine	O_2	0
Buffer + glutathione	O_2	0
Liver slices*	O_2	444 ± 99

* Halothane (25 μliter) mixed with 2 ml propylene glycol prior to addition of 0·2 ml of this mixture to the side arm of the Warburg.

ability to dechlorinate is heat labile. The effect of limiting the rate of diffusion of anesthetic from the side arm to the incubating medium is also shown. This is reflected in an increased total dechlorination. In addition, Table 2 shows the effect of certain stimulators and inhibitors on the dechlorination of methoxyflurane. The only significant effect is that of cyanide, which inhibitis the dechlorination.

Table 3 contains the data collected in the study of the dechlorination of ^{36}Cl-halothane: the dechlorination proceeds under the same conditions as those for the dechlorination of methoxyflurane; the data indicate a requirement for oxygen; the presence of sulfhydryl-containing compounds does not contribute to the dechlorination as it does with chloroform.[4–6]

We attempted to limit the rate of diffusion of halothane from the side arm to the incubating medium to increase the dechlorination by slices. As seen in Table 3, the dechlorination was not significantly altered by this.

Table 4 indicates the dechlorination of halothane and methoxyflurane by slices of some selected organ tissues. The data indicate that the liver is the most efficient organ for the dechlorination.

TABLE 4. *IN VITRO* REMOVAL OF ^{36}CL FROM ^{36}CL-HALOTHANE AND ^{36}CL-METHOXYFLURANE BY VARIOUS ORGANS OF A RAT

Tissue	Atmos.	^{36}Chloride (cpm/300 mg wet wt.)	
		^{36}Cl-Halothane	^{36}Cl-Methoxyflurane
Liver slices	O_2	460	960
Thymus	O_2	100	100
Kidney	O_2	90	400
Brain	O_2	trace	trace
Buffer only	O_2	0	0

Table 5 contains information on the uptake of anesthetic by the buffer with time and compares this with the extent of dechlorination at the same time. This has been performed with and without limiting the rate of diffusion of the anesthetic from the side arm by means of the propylene glycol.

TABLE 5. UPTAKE OF ^{36}CL-ANESTHETICS BY INCUBATION MEDIUM AND THE DECHLORINATION WITH TIME

Tissue	Time of incubation (min)	^{36}Cl-Methoxyflurane		^{36}Cl-Halothane	
		Total cpm in aqueous $\times 10^{-3}$*	^{36}Chloride liberated (cpm)	Total cpm in aqueous $\times 10^{-3}$*	^{36}Chloride liberated (cpm)
Liver slices	20†	40·3	0·67	8·22	0·21
	60†	48·5	1·06	8·49	0·39
	120†	51·2	2·79	9·06	0·46
Liver slices	30‡	165·6	0·343	9·23	0·31
	60‡	204·1	0·462	8·64	0·50

* Represents the total diffusion of anesthetic from the side arm of the Warburg to the main compartment.

† Anesthetic mixed with propylene glycol prior to placing in side arm (see Methods).

‡ Anesthetic added directly to the side arm.

Table 6 lists the results of the incubation of the homogenate and subcellular fractions. As indicated, there is a very low dechlorination by the whole homogenate. The incubation carried out with the 9,000-*g* supernate showed a greater ability to dechlorinate than did the whole homogenate, and this was further increased by the addition of a reduced NADP-generating system. The addition of the reduced glutathione to the 9,000-*g* supernate as well as the dialyzed 9,000-*g* supernate resulted in no increase in the dechlorination by either system. The addition of the reduced

glutathione to the dialyzed cell supernatant fraction also resulted in no dechlorination. The microsomes did, however, show an ability to dechlorinate.

Although not shown here, the purity of these two anesthetics plays an important role in the results obtained. Samples of both anesthetics of impure character (98–99% pure) were found to be dechlorinated by heated protein as well as by cysteine and glutathione. On the other hand, when the purity of both was increased to 99·9%, the nonenzymatic dechlorination decreased to zero. In all reported experiments we used a heated protein blank to check the purity of the materials.

TABLE 6. DECHLORINATION OF ^{36}Cl-METHOXYFLURANE AND ^{36}Cl-HALOTHANE BY RAT LIVER CELL FRACTION*

Cell fraction	Additions	^{36}Chloride (cpm/300 mg wet wt.)	
		from ^{36}Cl-methoxyflurane	from ^{36}Cl-halothane
Whole liver homogenate	NADPH	0	0
supernate, 9,000 *g*	none	1,346	285
Supernate, 9,000 *g*	NADPH	2,461	586
Microsomes	NADPH	783	306
Supernate, 105,000 *g*	NADPH	45	94
Supernate, 105,000 *g*	NADPH and glutathione	66	90

* NADPH added as a NADPH-generating system (see Methods). All incubations contained nicotinamide. Incubation carried out for 2 hr at 37° in an atmosphere of oxygen.

DISCUSSION

Heppel and Porterfield[3] have previously shown that a number of chlorinated compounds can be dechlorinated in mammalian systems and that the enzymes responsible for this are present in the supernatant fraction of the cell. Booth *et al.*[8] have also reported the presence in the cell supernatant fraction of an enzyme that will catalyze the replacement of chlorine atoms by the sulfhydryl of glutathione, which results in the bonding of 3,4-dichloronitrobenzene to glutathione and the release of inorganic chloride. This appears to be the case with the system reported by Heppel and Porterfield, who found that the dechlorinating activity was also in the cell supernatant fraction and also required glutathione for optimal activity. Reports by other investigators as well indicate that other chlorinated compounds are dechlorinated in the presence of sulfhydryl-containing compounds.[4,5]

The present communication has failed to show that glutathione is essential for the dechlorination of either ^{36}Cl-halothane or ^{36}Cl-methoxyflurane in a highly purified form. As stated previously in this paper, the purity of these materials determined the major pathway of dechlorination, that is, whether it appeared to be enzymatic or nonenzymatic. Relatively impure material showed a dechlorination by sulfhydryl-containing compounds, whereas pure material did not. It is conceivable therefore that the ease of dechlorination is a function of the nearest carbon substitution as well as other substitution on the chlorine-containing carbon. In other words, not all carbon–chlorine bonds exhibit the same biological stability. The strongest of these bonds would be refractory to everything except an enzymatic attack, and the weakest

would be subject to easy replacement by sulfhydryl groups. Between these two extremes lies a mixture of the two. Thus it is conceivable that one could find, as did Booth *et al.*[8] and Heppel and Porterfield,[3] that a compound is enzymatically dechlorinated and at the same time this dehalogenation is enhanced by sulfydryl-containing compounds. This report does show, however, that the dechlorination occurs in the microsomes, requires reduced NADP and oxygen, and is sensitive to the substrate concentration.

The fact that the conversion of ^{14}C-methoxyflurane to $^{14}CO_2$ is not so sensitive to substrate concentration as is the dechlorination suggests that there are two pathways for the metabolism of methoxyflurane. One involves the splitting of the ether linkage with the methyl portion possibly being metabolized via formaldehyde, which occurs, presumably, in the microsomes.[2] Furthermore, this possibility is enhanced by the fact that dimedon decreases the appearance of $^{14}CO_2$, presumably by combining with the formaldehyde as it appears. An investigation of this possibility is currently under way. The other pathway involves the dechlorination of the molecules to yield an intact dechlorinated ether. In the case of methoxyflurane, therefore, it is not necessary for the dechlorination to precede the ether linkage split, nor is it necessary for the ether to be destroyed prior to dechlorination. In the case of halothane, the dechlorination also proceeds independently of the carbon oxidation, since such an oxidation, at least of the 1-carbon, does not occur.[10]

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