MOLECULAR PHARMACOLOGY, 21:710–717

Reductive Metabolism of Halothane by Human and Rabbit Cytochrome P-450

Binding of 1-Chloro-2,2,2-Trifluoroethyl Radical to Phospholipids

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Received September 4, 1981; Accepted December 15, 1981

SUMMARY

The 2-chloro-1,1,1-trifluoroethyl radical is a postulated metabolite of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) by cytochrome P-450 under anaerobic reductive conditions. The formation and identification of a lipid-bound metabolite was made possible by the use of human cytochrome P-450$_{HA_2}$ or rabbit liver cytochrome P-450$_{LM_2}$ reconstituted with NADPH cytochrome P-450 reductase and cytochrome b_5 into phospholipid vesicles containing a mixture of dioleoylphosphatidylcholine and egg phosphatidylethanolamine as structural components of the vesicles as well as targets for free radical binding. The reconstituted vesicles were incubated under an argon atmosphere in the presence of NADPH with 1-[^{14}C]halothane for 1 hr. The phospholipids were extracted, and the dioleoylphosphatidylcholine fraction was isolated by high-pressure liquid chromatography and then subjected to transesterification. Separation of the resulting fatty acid methyl esters by reverse-phase high-pressure liquid chromatography resulted in a single radioactive fraction with a retention time less than that of methyl oleate. This fraction was further purified by a second reverse-phase high-pressure liquid chromatography and then subjected to capillary gas chromatography-mass spectrometry. Interpretation of the mass spectra showed that a free radical metabolite added to the double bond of oleic acid and produced a mixture of 9- and 10-(1-chloro-2,2,2-trifluoroethyl)-stearate methyl esters. A similar anaerobic incubation of halothane with liver microsomes from phenobarbital-pretreated rabbits revealed that halothane metabolites bind in similar amounts to the phosphatidylcholine and phosphatidylethanolamine fractions. Separation of the metabolites bound to microsomal phospholipids after transesterification resulted in a broad distribution of radioactivity such as would result from random binding of metabolites to any of the double bonds in the many different fatty acyl chains of phospholipids found in microsomes. Cytochrome P-450 reconstituted in a vesicle with a target phospholipid containing a single double bond proved to be the optimal system for isolation and structure elucidation of the free radical metabolite of halothane.

INTRODUCTION

Metabolism of xenobiotics in the liver usually proceeds by addition of activated oxygen to substrates by cytochrome P-450. However, under conditions of anaerobic incubation *in vitro*, cytochrome P-450 can act as a one- or possibly two-electron reductant to substrates. If halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is the substrate, reductive metabolism results in increased binding of metabolites to phospholipids of the endoplasmic reticulum, reduction of content of glutathione, increase in content of conjugated dienes in the fatty acid chains, loss of structural integrity of the endoplasmic reticulum, and liver necrosis. It has often been suggested that this metabolite could be a free radical. In 1961 Butler (1) proposed that reductive metabolism of another halocarbon, carbon tetrachloride, would result in a free radical metabolite. Binding of a free radical metabolite of halothane to a double bond of a fatty acid chain in a phospholipid would be consistent with the observation of increased conjugation of dienes in the fatty acid chains following reductive metabolism (2). Two volatile reduc-

This research was supported by Grant OH 00978 from the National Institute of Occupational Safety and Health and by an award from the Alexander von Humboldt Stiftung (to B. B.).

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0026-895X/82/030710-08$02.00/0

tive metabolites of halothane, 2-chloro-1,1,1-trifluoroethane and 2-chloro-1,1,difluoroethylene, have been detected in the expired air of rabbits (3) and human patients (4). We have suggested that production of a single free radical species, 1-chloro-2,2,2-trifluoroethyl radical, by reduced cytochrome P-450 could account for both of these volatile metabolites—hydrogen radical abstraction in the case of the haloethane and expulsion of a fluorine free radical in the case of the haloethylene (4). The molecular structure of the 1-chloro-2,2,2-trifluoroethyl radical as a major reductive metabolite of halothane is consistent with the following previous studies: 1:3 stoichiometry between ^{14}C-containing metabolites and fluoride ion in the livers of mice exposed to [^{14}C]halothane (5), an approximate 1:1 ratio of ^{36}Cl:^{14}C in the phospholipid extracts of rats exposed under hypoxic conditions to either [^{36}Cl]halothane or [^{14}C]halothane (2), and an approximate 1:1 ratio of ^{3}H:^{14}C in the $CHCl_3$:MeOH extract of anaerobic incubations of rat liver microsomes with either [^{3}H]halothane or [^{14}C]halothane (6).

In addition, there is substantial spectral evidence for the production of a 2,2,2-trifluoroethylcarbene during reductive metabolism of halothane by cytochrome P-450 (7, 8). Incubation of halothane under anaerobic conditions with reduced cytochrome P-450 results in a peak in absorption at 470 nm. The same absorption spectrum also can be produced by adding 2,2,2-trifluoroethylcarbene, produced by the precursor molecule 2,2,2-trifluorodiazoethane, to cytochrome P-450 (7).

In a series of experiments reported here, we found that anaerobic incubations of halothane with liver microsomes produced reactive metabolites that bound at random to double bonds in microsomal phospholipids. However, we were unable to isolate a single metabolite-substituted fatty acid in sufficient quantity and purity for structural elucidation by mass spectrometry. In order to circumvent the problem due to the heterogeneity of microsomal phospholipids, we developed a system in which highly purified cytochrome P-450, NADPH cytochrome P-450 reductase, and cytochrome b_5 were reconstituted in phospholipid vesicles (9–11). The reconstituted system allowed great flexibility in the choice of classes of phospholipids or particular cytochromes P-450 and exhibited high metabolic activity.

The two most likely reductive metabolites of halothane described above would be predicted to add to a double bond of an unsaturated phospholipid in the following ways. The 1-chloro-2,2,2-trifluoroethyl radical should add to one carbon atom of a double bond and produce a free radical on the adjoining carbon position. This free radical would then abstract a hydrogen radical from another molecule to result in the corresponding substituted and saturated fatty acyl chain. The 2,2,2-trifluoroethylcarbene should add across a double bond to form a trifluoromethyl-substituted cyclopropane ring on the fatty acyl chain (12). Therefore the most simple component to add to a reconstituted system as a target for radical or carbene addition would be a straight-chain hydrocarbon or a fatty acid with a single double bond. However, simple monounsaturated small molecules such as hexene or methyl oleate are themselves good substrates for cytochrome P-450 and are therefore unsuitable. For this reason we used DOPC[2] as the only phosphatidylcholine in the reconstituted system, since the phospholipid would act both as a potential target for the radical or carbene metabolite and as a structural component of the phospholipid vesicle.

Our previous work had shown that the metabolic activity and stability of the reconstituted system was greater when egg PE was present (9). Although the multiple double bonds found in the fatty acid chains of the PE could themselves trap the reactive metabolites of halothane just as do the phospholipids in microsomes, it is possible to separate the DOPC from the PE in a single chromatographic step following the incubation. The DOPC could then be transesterified to yield a mixture of methyl oleate and 9- or 10-metabolite-substituted methyl stearates. In a previous study we demonstrated that a major product of ultraviolet radiation of halothane is 1-chloro-2,2,2-trifluoroethyl radical. When this radical was formed in the presence of methyl oleate it added to the 9,10-double bond of methyl oleate to form a mixture of the 9- and 10-(1-chloro-2,2,2-trifluoroethyl)-stearate methyl esters (13). These substituted methyl stearates could be easily separated from the parent methyl oleate target molecule. Therefore we were confident that we could separate the transesterified fatty acid methyl esters derived from DOPC by the same technique of reverse-phase HPLC.

Most studies of metabolism and long-term toxicity of halocarbons have been performed in small animals. However, this knowledge is applicable to human toxicity only to the extent that all cytochrome P-450 forms produce the same reductive metabolites. For this reason, in this study we compared the most well-studied cytochrome P-450, phenobarbital-induced rabbit cytochrome P-450$_{LM_2}$ (14), with the major component of noninduced human liver cytochrome P-450 that was isolated in our laboratory, cytochrome P-450$_{HA_2}$ (10). Cytochrome b_5 has been shown to be important in the transfer of electrons from NADPH cytochrome P-450 reductase to cytochrome P-450 (15). For this reason, we included cytochrome b_5 in the reconstituted systems in order to better duplicate the conditions that exist in the endoplasmic reticulum of liver cells. Furthermore, we have studied the human and rabbit reconstituted cytochrome P-450 systems in parallel with an intact microsomal suspension of comparable cytochrome P-450 content in order to measure whether the rate of metabolite binding to phospholipids previously observed in microsomes (2) is duplicated in the reconstituted systems.

MATERIALS AND METHODS

Cytochrome P-450$_{LM_2}$ was purified from livers of phenobarbital-pretreated rabbits by DEAE-cellulose and hydroxylapatite chromatography (14) to a purity of 17 nmoles/mg of protein. The cytochrome was identified by SDS-PAGE (16). Human cytochrome P-450$_{HA_2}$ was prepared from human liver by DEAE-cellulose, hydroxyl-

[2] The abbreviations and common names used are: DOPC, dioleoylphosphatidylcholine; PE, phosphatidylethanolamine; HPLC, high-pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

apatite, and CM-Sephadex chromatography essentially as previously described (10) to a purity of 12 nmoles/mg of protein. It consisted of over 70% of one component of molecular weight 52,500 and two minor components as judged by SDS-PAGE. NADPH cytochrome P-450 reductase was purified from livers of phenobarbital-pretreated rabbits by affinity chromatography on 2′,5′-ADP-Sepharose (17) to yield a specific activity toward cytochrome *c* of 32–40 μmoles/min/mg of protein. Cytochrome b_5 was purified from livers of phenobarbital-pretreated rabbits as previously described (18) to a purity of over 90% as judged by SDS-PAGE. All protein preparations were concentrated and dialyzed against 0.3 M potassium phosphate buffer (pH 7.5) with 20% glycerol and 0.5% sodium cholate and then stored in liquid nitrogen until used.

Reconstitution of the purified proteins into vesicles was achieved by a modification of the slow cholate dialysis method previously described (9, 10). A stock solution of phospholipids was prepared by adding 2.5 ml of a solution of 20% sodium cholate in water to 24 mg of DOPC and 12 mg of egg PE. The lipids were dissolved by sonication in a bath for 1 min, 14 mg of dithiothreitol were added, and the mixture was kept at 20° under an argon atmosphere for 1 hr. A portion (1 ml) of this phospholipid stock solution was added to 11.5 ml of a solution of 1.5 mg (28 nmoles) of human cytochrome P-450_{HA_2}, 1.1 mg (14 nmoles) of rabbit NADPH cytochrome P-450 reductase, and 0.24 mg (14 nmoles) of cytochrome b_5 in 0.3 M potassium phosphate buffer (pH 7.5) containing 20% glycerol. The mixture was allowed to equilibrate overnight at 4° under argon to assure complete formation of mixed micelles. Vesicles were prepared by dialysis against 10 changes of 700 ml of 20 mM potassium phosphate buffer (pH 7.5) with 0.5 mM dithiothreitol, 0.5 mM EDTA, and 20% glycerol for 4 days at 4° under a nitrogen atmosphere. The last two changes of buffer did not contain dithiothreitol or EDTA. Over 90% of the three enzymes was recovered, and no cytochrome P-420 was detectable. Three additional reconstituted vesicle suspensions prepared in a manner similar to that described above contained one of the following mixtures: rabbit cytochrome P-450_{LM_2}, NADPH cytochrome P-450 reductase, and cytochrome b_5; NADPH cytochrome P-450 reductase as the only protein; or no proteins, but both phospholipids.

Before incubation with halothane, each of four 5-ml vesicle suspensions and a 2 ml (10 mg of protein) microsomal suspension was deoxygenated by a stream of nitrogen and the addition of 5 mM β-D-glucose, glucose oxidase (50 μg/ml) (activity 115 μmoles/min/mg), and thymol-free catalase (10 μg/ml) (activity 125 μmoles/min/mg) for 1 hr at 30°. A solution of an NADPH-generating system was then added through a septum to result in a final concentration of 0.5 mM NADP, 5 mM D-glucose 6-phosphate, and 10 IU of glucose 6-phosphate dehydrogenase (Calbiochem, San Diego, Calif.). Formation of NADPH was tested by the increase in absorption at 340 nm before use. Finally, 1-[^{14}C]halothane was diluted with thymol-free halothane to yield a specific activity of 1.3 μCi/μl and then 2 μl were added to each of the 5-ml incubation mixtures with careful exclusion of oxygen. The incubations were stirred for 1 hr at 30° and then stopped by freezing in dry ice. Suspensions of the same reconstituted cytochrome P-450_{LM_2} and of rabbit microsomes were incubated under identical conditions except for addition of an oxygen atmosphere.

Aliquots (2 ml) of each of the incubation mixtures were treated in an identical manner: the mixture was extracted twice with 3 ml of ethyl acetate; the aqueous phase was then extracted with 10 ml of $CHCl_3$:MeOH (2:1, v/v); and the $CHCl_3$ phase was removed, washed with 2 ml of water, and taken to dryness under argon. The resulting phospholipid extract was applied to a Lichrosorb Si-100 HPLC column (1 × 25 cm). The PE fraction was eluted with hexane:isopropanol:water (6:8:1) at a flow rate of 2.5 ml/min. At 14 min, the solvent system was changed to hexane:isopropanol:water (6:8:1.8) to elute the phosphatidylcholine fraction. Elution of material from the column was monitored by refractive index and absorption at 208 nm.

The phosphatidylcholine fractions from the Si-100 column were vacuum-dried and then subjected to transesterification with 2 ml of BCl_3:MeOH at 45° for 2 hr. The resulting methyl esters were extracted into 5 ml of hexane which was back-washed with 4 ml of water. The hexane extract was dried and subjected to HPLC on a Lichrosorb reverse-phase C-18 column (1 × 25 cm) eluted with MeOH:water (96:4, v/v) at a flow rate of 2.5 ml/min. The major radioactive fraction that eluted from this column at 23 min was taken to dryness under argon and rechromatographed on a Lichrosorb reverse-phase C-8 HPLC column (0.46 × 25 cm) eluted with MeOH:water (90:10, v/v). The major radioactive fraction that eluted from the C-8 column was taken to dryness and applied to a fused silica OV-17 wall-coated capillary gas chromatographic column programmed from 50 to 190° at 10°/min with a linear helium velocity of 30 cm/sec. The column was directly coupled to a Varian CH-7 mass spectrometer on which mass spectra were measured at 20 eV.

Chloroform, ethyl acetate, *n*-hexane, 2-propanol, and methanol were HPLC grade from Baker Chemical Company (Philadelphia, Pa.). L-α-DOPC was obtained from Sigma Chemical Company (St. Louis, Mo.). The phosphatidylserine was obtained from Supelco, Inc. (Bellafonte, Pa.). Methyl arachidonate, methyl linoleate, and methyl oleate were obtained from Serdary Research Laboratories (London, Ont., Canada).

L-α-DOPC (Sigma Chemical Company) and egg PE [prepared by the method of Singleton *et al.* (19)] were each repurified by preparative HPLC on a Lichrosorb Si-100 column (25 × 1 cm) using mixtures of hexane:isopropanol:water.

RESULTS AND DISCUSSION

When liver microsomes from phenobarbital-pretreated rabbits were incubated with 1-[^{14}C]halothane under aerobic and/or anaerobic conditions, similar amounts of radioactivity were recovered in the $CHCl_3$:MeOH extracts of the microsomal phospholipids (Table 1). However, when these phospholipid extracts were subjected to HPLC on a Lichrosorb Si-100 column, over 90% of the radioactivity from the aerobic incubation appeared in the solvent peak which eluted before the major phospholipid

TABLE 1

Radioactivity in phospholipid fractions from rabbit hepatic microsomal preparations incubated with [^{14}C]halothane

Phenobarbital-treated rabbit hepatic microsomal preparations were incubated with [^{14}C]halothane under aerobic or anaerobic conditions as described under Materials and Methods. The dried $CHCl_3$:MeOH extracts (A) were subjected to HPLC on a Si-100 column to separate major phospholipids, including PE (B) and phosphatidylcholine (PC) (C). The phosphatidylcholine fraction was transesterified and the resultant methyl esters were separated by reverse-phase HPLC on a C-18 column (D). Radioactivity represents total disintegrations per minute in each fraction derived from a microsomal incubation containing 10 mg of microsomal protein.

Incubation condition	A: total phospholipids	B: PE fraction	C: PC fraction	D: Total methyl esters from C
		dpm		
Aerobic	41,300	<1,000	<100	<20
Anaerobic	36,800	15,700	17,200	8,900

fractions, whereas the extract from the anaerobic incubation had most of its radioactivity associated with the PE and phosphatidylcholine fractions (Table 1; Fig. 1). The lack of binding to phospholipids under oxidative conditions is consistent with the finding of Wood *et al.* (2) in that, when their $CHCl_3$:MeOH extract of aerobic incubations of microsomes with halothane was corrected for the high background seen in control microsomes, little or no binding of ^{14}C attributable to metabolism was observed. It is seen that the nonvolatile radioactivity in the $CHCl_3$:MeOH extract is similar in all of the incubations. In the case of the incubations performed under oxygen the radioactive components in this extract elute from a Lichrosorb Si-100 column with hexane:isopropanol:water (6:8:1) in a broad peak distinct from that of [^{14}C]halothane but far ahead of the phospholipid fractions. The assumption that nonvolatile radioactivity in the $CHCl_3$:MeOH extract was equivalent to binding to phospholipids has possibly confounded the results of some previous studies on reductive metabolism of halothane.

In an initial series of experiments, identification of the halothane metabolite bound to fatty acid chains of phospholipids in microsomes was attempted by subjecting the phosphatidylcholine fraction purified by preparative HCLC as shown in Fig. 1 to transesterification with BCl_3:MeOH. Unfortunately, following HPLC on a reverse-phase Lichrosorb C-18 column, the broad distribution of radioactive metabolites bound to fatty acid methyl esters shown in Fig. 2 was observed. In Fig. 2, the retention times of methyl arachidonate, methyl linoleate, and methyl oleate are shown for reference. In a previous study we demonstrated that, when the 1-chloro-2,2,2-trifluoroethyl radical is generated by ultraviolet photolysis of halothane, it adds to either position 9 or position 10 of the double bond in methyl oleate (13). We suggest that a metabolically produced radical will add to double bonds in a similar fashion, and the broad distribution of radioactivity in Fig. 2 is consistent with random addition of a halothane metabolite to any of the double bonds in the many different unsaturated fatty acids found in microsomes. In the radioactivity profile in Fig. 2, each peak represents microgram amounts of a specific metabolite-bound methyl ester superimposed on a background of

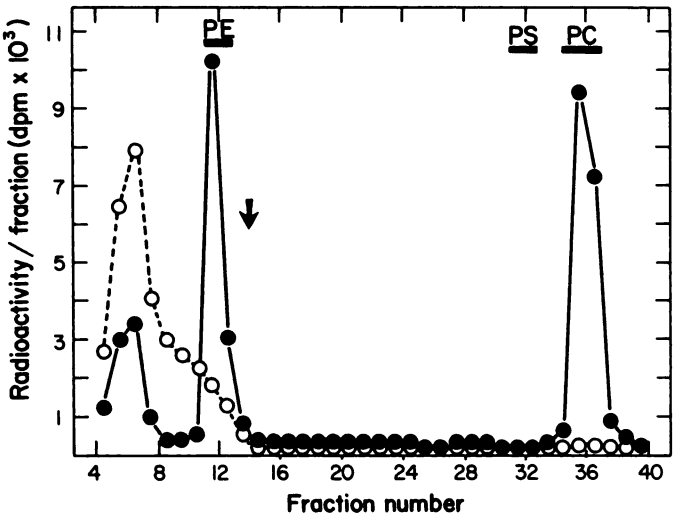

FIG. 1. *HPLC of the $CHCl_3$:MeOH extract of liver microsomes from phenobarbital-pretreated rabbits after anaerobic and aerobic incubation with 1-[^{14}C]halothane for 1 hr*

HPLC on a Si-100 silica gel column (25 × 1 cm) eluted from 0 to 14 min with hexane:isopropanol:water (6:8:1) and then the eluting mixture was changed at 14 min (*arrow*) to hexane:isopropanol:water (6:8:1.8) at 2.5 ml/min. Under these chromatographic conditions the retention times of PE, phosphatidylserine (*PS*), and phosphatidylcholine (*PC*) are shown by *heavy black lines* along the top. The radioactivity in each 2.5-ml fraction is shown in the case of microsomes incubated under reductive conditions (●) and under oxygen (○).

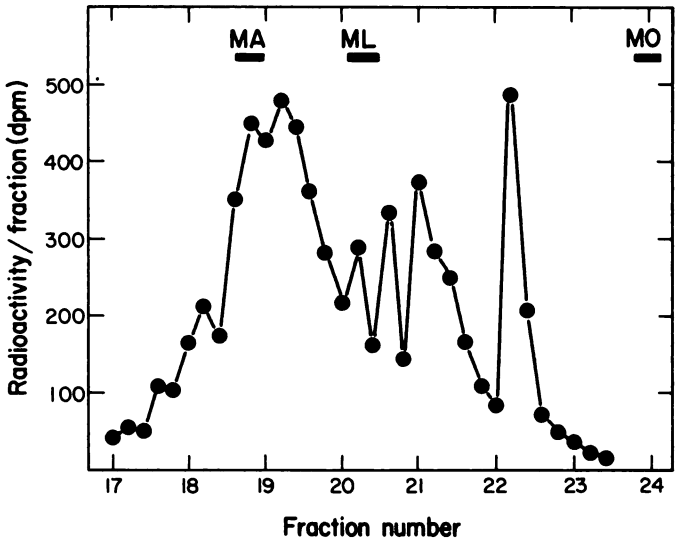

FIG. 2. *HPLC of fatty acid methyl esters from microsomal phospholipids*

The phosphatidylcholine fraction obtained from the chromatography in Fig. 1 after extraction of microsomes incubated under anaerobic conditions with 1-[^{14}C]halothane was transesterified with BCl_3:MeOH at 45° for 2 hr. The resulting mixture of fatty acid methyl esters was applied to a C-18 reverse-phase HPLC column (25 × 1 cm) eluted with MeOH:water (96:4) at 2.5 ml/min. The radioactivity in each 2.5-ml fraction is indicated. Under these chromatographic conditions the retention times of methyl arachidonate (*MA*), methyl linoleate (*ML*), and methyl oleate (*MO*) are indicated along the *top* by *heavy black lines*. It is seen that the [^{14}C]halothane metabolite binds to many fatty acyl chains.

approximately 5 mg of fatty acid methyl esters that are not labeled. Any attempted separation of a single labeled fatty acid from the much larger quantity of unlabeled methyl esters would be most difficult given the 1-μg capacity of a capillary gas chromatography column.

To circumvent the isolation problem encountered in microsomes, we used a reconstituted phospholipid vesicle system in which the single double bond of oleic acid presented the only target for free radical addition in the phosphatidycholine fraction. Anaerobic incubation of 1-^{14}C]halothane with a suspension of either human cytochrome P-450$_{HA_2}$ or rabbit cytochrome P-450$_{LM_2}$ reconstituted in phospholipid vesicles with NADPH cytochrome P-450 reductase and cytochrome b_5 (9–11) resulted in similar binding of radioactive metabolites in terms of disintegrations per minute per nanomole of phospholipid in the egg PE and DOPC fractions (Table 2; Fig. 3). There appears to be little direct correlation between phospholipid labeling under reductive conditions and the *N*-demethylation activity of each system in the presence of air or oxygen. Rates of formation of CH_2O from benzphetamine were 2.5 and 36 nmoles/nmole of cytochrome P-450 per minute for the human and rabbit cytochrome P-450-reconstituted systems, respectively. The latter rate of *N*-demethylation corresponds well with data reported for other rabbit cytochrome P-450-reconstituted systems.

When the DOPC fraction which was purified by preparative HPLC (Fig. 3) was subjected to transesterification with BCL$_3$:MeOH and then applied on the same reverse-phase HPLC column as in Fig. 2, the single radioactive peak shown in Fig. 4 was obtained. The radioactive peak eluted at 22 min whereas the retention time of methyl oleate was 24.5 min. The radioactive fraction was contaminated with some methyl oleate, and therefore it was re-chromatogaphed on a Lichrosorb C-8 HPLC column yielding the separations seen in Fig. 5.

The radioactive fraction obtained by preparative chromatography as shown in Fig. 5 was subjected to capillary

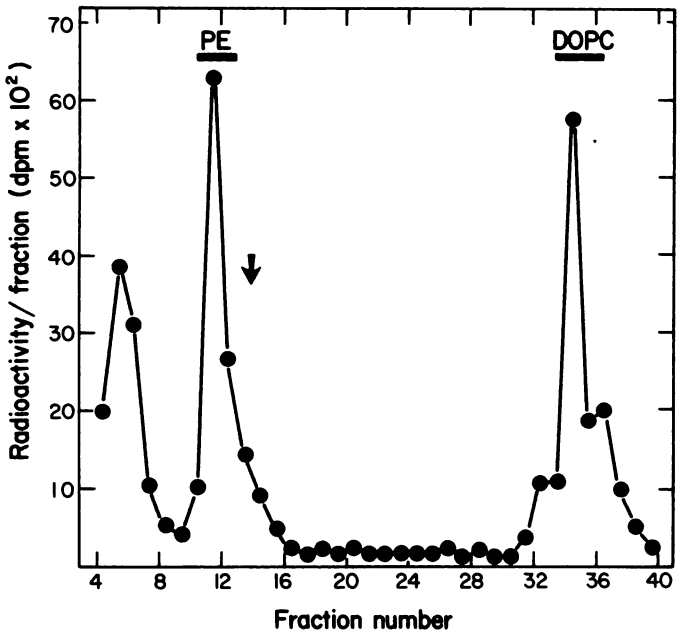

FIG. 3. *HPLC of phospholipids from vesicle of reconstituted cytochrome P-450*

Cytochrome P-450$_{LM_2}$ from phenobarbital-pretreated rabbits was reconstituted into phospholipid vesicles containing a mixture of egg PE and DOPC and then incubated under an argon atmosphere with NADPH and 1-[^{14}C]halothane for 1 hr. The phospholipids were extracted and applied to a Si-100 silica gel HPLC column (25 × 1 cm) and eluted with hexane:isopropanol:water (6:8:1) from 0 to 14 min and then with hexane:isopropanol:water (6:8:1.8) beginning at 14 min (*arrow*). Under these elution conditions, the retention times of standard PE and DOPC are indicated along the *top* by *heavy black lines*. The radioactivity of each 2.5-ml fraction is indicated.

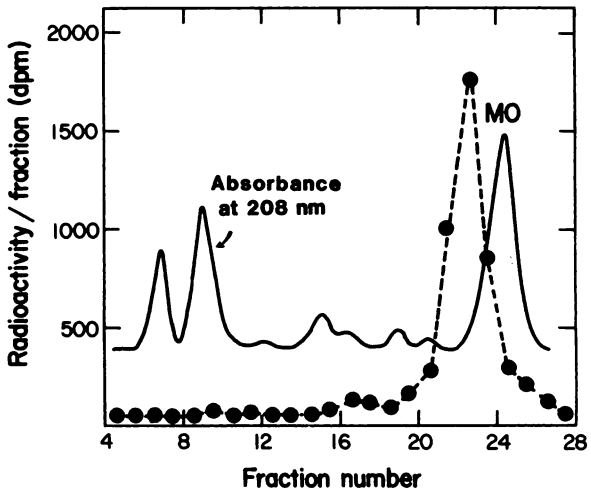

FIG. 4. *HPLC of fatty acid methyl esters from phosphatidylcholine fraction of reconstituted vesicles*

The DOPC fraction obtained by preparative HPLC as shown in Fig. 3 was subjected to transesterification with BCl$_3$:MeOH at 45° for 2 hr. The mixture of fatty acid methyl esters was applied to a reverse-phase C-18 column (25 × 1 cm) and eluted with methanol:water (96:4) at 2.5 ml/min. The radioactivity in each 1.25-ml fraction is shown (●). The absorbance of the eluate was continuously monitored at 208 nm (- - -). The peak in Fractions 6–11 represent small amounts of oxidation and decomposition products with high extinction coefficients. Under these conditions, a single radioactive fraction eluted at 23 min and methyl oleate (*MO*) eluted at 25 min.

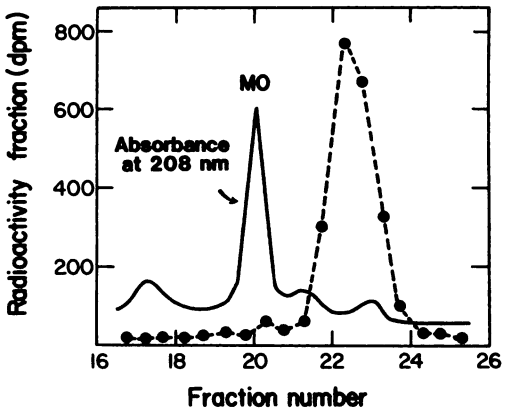

FIG. 5. *HPLC of ^{14}C-containing fatty acid methyl ester*

The radioactive fractions centered at 23 min by preparative HPLC in Fig. 4 were combined and rechromatographed on an analytical reverse-phase C-8 column (25 × 0.45 cm) eluted with methanol:water (80:20) at 0.6 ml/min. The radioactivity in each fraction was determined (●). The absorbance of the eluate was monitored at 208 nm (- - -). Under these conditions methyl oleate (*MO*) eluted at 20 min and a single radioactive fraction at 22.5 min.

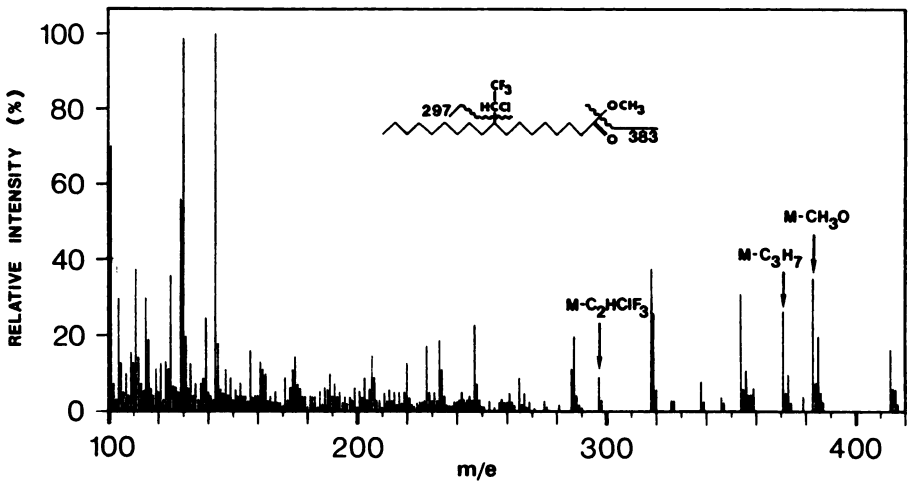

FIG. 6. *Mass spectrum of 9- and 10-(1-chloro-2,2,2-trifluoroethyl)stearate methyl ester*

The single radioactive fraction purified by preparative HPLC as shown in Fig. 5 was concentrated and applied to a fused silica capillary gas chromatographic column (10 m) with a linear helium velocity of 30 cm/sec programmed from 50 to 190° at 10°/min. The column was directly coupled to a mass spectrometer in which the mass spectrum was measured at 20 eV. The intensity of each fragment ion is measured as percentage of relative abundance of the largest ion at m/e 170. The doublet at m/e 414 and 416 is consistent with a molecular ion containing one chlorine atom, the fragments at m/e 383 and 385 are consistent with a fatty acid methyl ester, and the fragment ion at m/e 297 is that which would be expected from loss of the $CHClCF_3$ group.

gas chromatography coupled to a mass spectrometer to yield the mass spectrum shown in Fig. 6. A molecular ion at m/e 414 is consistent with addition of a CF_3CHCl radical to the double bond of oleic acid followed by abstraction of a hydrogen radical from a neighboring donor. The molecular ion is a doublet at m/e 414 and 416 with a 3:1 intensity ratio that is expected for a molecule containing one chlorine atom, in that the natural abundance ratio of ^{35}Cl and ^{37}Cl is 3:1. The fragment ion at m/e 383 corresponds to the loss of the CH_3O group characteristic of methyl esters of fatty acids, and the fragment at m/e 297 corresponds to the loss of the $CHClCF_3$ group from the molecular ion.

The molecular structure obtained from the mass spectrum in Fig. 6 can be explained by the reaction scheme shown in Fig. 7. When halothane is reduced with one electron by cytochrome P-450 it can lose a bromide ion and form the 1-chloro-2,2,2-trifluoroethyl radical (*A*). This free radical could then add to either end of the double bond in the two oleic acid chains in DOPC (*B*). This mixture of four isomers can be transesterified with BCl_3:MeOH to yield methyl oleate in the case of the fatty acid chains that did not react with the free radical, or a mixture of 9- and 10-(1-chloro-2,2,2-trifluoroethyl)-stearate methyl ester. As is shown in Figs. 4 and 5, this substituted methyl stearate is easily separated from methyl oleate.

Table 2 provides a summary of reconstituted systems of human and rabbit cytochromes P-450 and two control reconstitutions. The radioactivity found in the purified phosphatidylcholine and PE fractions gives a measure of [^{14}C]halothane metabolite binding to phospholipids. In both the reconstituted human and rabbit cytochrome systems under nitrogen atmosphere, the values of disintegrations per minute per nanomole of phospholipid are roughly equivalent. This equivalence in binding is important in that it allows us to eliminate one plausible pathway for free radical attack on a fatty acid chain in which a free radical is created at the methylene position *alpha* to a double bond and this fatty acid radical then initiates binding of the halothane molecule. We have shown that both phosphatidylcholine and PE have equal access to cytochrome P-450 in this reconstituted system

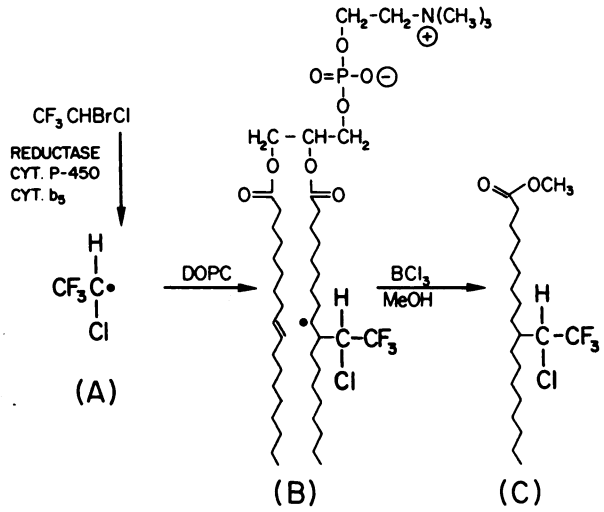

FIG. 7. *Metabolic production and isolation of 1-chloro-2,2,2-trifluoroethyl radical*

Halothane is metabolized under anaerobic conditions to form the 1-chloro-2,2,2-trifluoroethyl radical by cytochrome P-450 reconstituted in phospholipid vesicles with NADPH cytochrome P-450 reductase and cytochrome b_5 (*A*). This radical will add to a double bond of an oleic acid at either the α- or β-position of DOPC to yield a mixture of four possible isomers of the labeled phosphatidylcholine (*B*). Transesterification of the DOPC by BCl_3 in methanol yields a mixture of saturated methyl stearates with a 1-chloro-2,2,2-trifluoroethyl group at either position 9 or position 10 (*C*). The fragments in the mass spectrum shown in Fig. 6 are consistent with the structure of this substituted methyl stearate.

TABLE 2

Radioactivity in phospholipid fractions from vesicle reconstituted cytochrome P-450 systems incubated anaerobically with [^{14}C] halothane

Vesicle reconstituted systems were prepared and incubated with [^{14}C]halothane as described under Materials and Methods. The dried $CHCl_3$:MeOH extracts (A) were subjected to HPLC on a Si-100 column to separate PE (B) and DOPC (C). The DOPC fraction was transesterified, and the resultant methyl esters were separated by reverse-phase HPLC on a C-18 column. Column D represents the radioactive peak from the C-18 column that was distinct from that of methyl oleate. Radioactivity represents the total disintegrations per minute in each fraction derived from incubations that contained 3.2 mg of egg PE and 6.4 mg of DOPC.

System	A: $CHCl_3$:MeOH extract	B: PE fraction	C: DOPC fraction	D: methyl ester peak from transesterified DOPC (C)
			dpm	
Human cytochrome P-450$_{HA_2}$ + cytochrome P-450 reductase + cytochrome b_5 + DOPC and egg PE	26,200	3,680	2,960	1,030
Rabbit cytochrome P-450$_{LM_2}$ + cytochrome P-450 reductase + cytochrome b_5 + DOPC and egg PE	22,800	1,840	2,440	720
Cytochrome P-450 reductase + DOPC and egg PE	18,000	840	1,300	<20
DOPC and egg PE	4,600	920	1,375	<20

(20). However, the polyunsaturated fatty acids in egg PE have many methylene groups that are between two double bonds and therefore are much more activated toward loss of a hydrogen radical than any of the methylenes on oleic acid. If hydrogen radical abstraction was an important step in the binding of halothane metabolites to phospholipids, it would be expected that egg PE would have exhibited a much greater amount of binding of ^{14}C per nanomole of phospholipid.

It is seen that, in the anaerobic incubation of microsomes, similar values of disintegrations per minute per nanomole of phospholipid were observed in the phosphatidycholine and PE fractions whereas much less radioactivity appeared in the phosphatidylserine or phosphatidylinositol fractions. In a previous publication (20) we demonstrated that the negatively charged phosphatidic acid is preferentially localized near cytochrome P-450 in reconstituted vesicles. One might have expected that the negatively charged phosphatidylserine would occupy a similar position close to the positively charged cytochrome P-450 and be an especially effective target for free radical metabolites.

It is seen in Table 2 that when NADPH cytochrome P-450 reductase is reconstituted in DOPC and PE no binding of halothane metabolites to the transesterified fatty acyl chains of the phospholipid fractions is observed. This is somewhat surprising in that we have recently shown that reconstituted NADPH cytochrome P-450 reductase is capable of reducing molecular oxygen to superoxide radical anion (21). The reduction potential of the reductase is high enough that it also could directly reduce halothane. It would appear that reductase does not have a binding site that is capable of reducing the activation energy for electron transfer.

Conversion of the values of disintegrations per minute per nanomole of cytochrome P-450 into nanomoles of ^{14}C bound to phospholipid per nanomole of cytochrome P-450 reveals that approximately 1 nmole of ^{14}C is bound per nanomole of cytochrome P-450. This suggests that production of a halothane free radical may result in inactivation of a cytochrome P-450. This suggestion is consistent with the apparent stability of the halothane metabolite-cytochrome P-450 complex that produces the absorption peak at 470 nm under anaerobic conditions (8). Our reconstituted human cytochrome P-450$_{HA_2}$ system also produces an absorption peak near 470 nm after incubation under anaerobic conditions with NADPH. However, only a single radioactive fraction containing a free radical metabolite was observed following transesterification of DOPC and purification of methyl esters on two reverse-phase HPLC columns. The proposed 9,10-cyclopropane-substituted methyl stearate that would have resulted from addition of the 2,2,2-trifluoroethyl carbene was not observed. It is likely that this metabolite would be stable under the conditions of isolation. The absence of the product of addition of a carbene to a double bond in our study suggests that either the cytochrome P-450-carbene complex is so stable that it does not dissociate once formed, that the lifetime of the carbene is too short to diffuse to a double bond, or that the peak in absorption at 470 nm is not due to a carbene complex.

CONCLUSION

These results indicate that cytochrome P-450 is able to form a free radical from a halocarbon by one-electron reduction and to release the free radical into the matrix of surrounding phospholipids. NADPH cytochrome P-450 reductase does not carry out this reduction in the absence of cytochrome P-450. It is likely that this pathway of reductive metabolism will be a general one for halocarbons in incubations *in vitro* of reconstituted cytochrome P-450 under anaerobic conditions and in exposures *in vivo* under conditions that cause very low hepatic oxygen concentrations. The free radical formed following the first step of 1-chloro-2,2,2-trifluorethyl radical addition to a double bond is the most likely intermediate for the subsequent steps of conjugation of double bonds and lipoperoxidation that may be the initiating steps of hepatic necrosis.

ACKNOWLEDGMENTS

This study fulfills a goal of an investigation of halothane metabolism begun by Ellis N. Cohen, M.D., over 15 years ago. We are indebted to him for much support and helpful suggestions through the years. We would like to thank Dr. Chad Miller for discussions of free radical chemistry, Ms. Marie Bendix for expert technical assistance, and Ms. Buff Emslie for laboratory management. We would like to thank the

Stanford Cardiac Anesthesia and Cardiac Transplantation teams for their help in obtaining the human liver specimens.

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