Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2678–2682, April 1982
Medical Sciences

Reductive metabolism of carbon tetrachloride by human cytochromes *P*-450 reconstituted in phospholipid vesicles: Mass spectral identification of trichloromethyl radical bound to dioleoyl phosphatidylcholine

(liver necrosis/free radicals/lipid peroxidation/spin trapping/human proteins)

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Communicated by James P. Collman, January 18, 1982

ABSTRACT **It has been proposed that covalent binding of reactive metabolites to liver membrane constituents may be responsible for the hepatoxicity of carbon tetrachloride. This study demonstrates that trichloromethyl free radical is the major reductive metabolite of carbon tetrachloride by cytochrome *P*-450 and that this free radical is capable of binding to double bonds of fatty acyl chains of the phospholipids in the membrane surrounding cytochrome *P*-450. The structural identification of the reactive free radical metabolite and the product of its addition to phospholipids was accomplished by use of a reconstituted system of human cytochromes *P*-450, NADPH-cytochrome *P*-450 reductase, and cytochrome b_5 in phospholipid vesicles. The reconstituted vesicles contained a mixture of dioleoyl phosphatidylcholine and egg phosphatidylethanolamine that served as both structural components and targets for trichloromethyl free radical binding. After incubation of these vesicles under a N_2 atmosphere in the presence of NADPH with $^{14}CCl_4$, the phospholipids were extracted and then separated by high-pressure liquid chromatography. The dioleoyl phosphatidylcholine fraction was transesterified and the resulting single ^{14}C-labeled fatty acid methyl ester was purified by reverse-phase chromatography. Desorption chemical ionization mass spectrometry with ammonia as reagent gas as well as desorption electron-impact mass spectrometry permitted identification of the molecular structure as a mixture of 9- and 10-(trichloromethyl)stearate methyl esters.**

Carbon tetrachloride has been known for over 40 years to cause acute centrilobular hepatic necrosis in mammals (1). In 1961 a unique form of reductive metabolism of carbon tetrachloride was described that involved chloroform production via homolytic fission of a carbon–chlorine bond (2). The trichloromethyl free radical was postulated as an intermediate in this reaction and, on the basis of its potential reactivity, was suggested to be involved in carbon tetrachloride hepatotoxicity (2–5). Evidence for the formation of carbon-centered free radicals from metabolism of carbon tetrachloride has also come from electron paramagnetic resonance studies of spin-trap adducts (6, 7). However, difficulties in the interpretation of hyperfine splitting in EPR spectra have made a definitive structural identification difficult (8). Moreover, the mechanism by which these free radicals bind to constituents of biological membranes has not been previously demonstrated. In addition, the existence of a reactive dichlorocarbene has been proposed (5) on the basis of spectral studies of complex formation between halocarbon metabolites and cytochrome *P*-450. The observed formation of carbon monoxide from carbon tetrachloride is in accord with two successive one-electron reductions catalyzed by cytochrome *P*-450 (9).

Products of the metabolism of carbon tetrachloride are known to be capable of covalent binding to both lipids and proteins (3, 4, 10). However, the molecular structures of such reactive metabolites have not been determined and there has been no characterization of adducts formed or their mechanism of binding to tissue molecules. Past attempts to isolate adducts formed after metabolism of ^{14}C-labeled carbon tetrachloride have been unsuccessful due to the heterogeneity of radioactive products (11).

To circumvent the problem of heterogeneity of microsomal phospholipids, we have reconstituted purified cytochrome *P*-450 from human liver, NADPH-cytochrome *P*-450 reductase, and cytochrome b_5 in phospholipid vesicles that contained dioleoyl phosphatidylcholine (Ole_2-PtdCho) as the sole phosphatidylcholine (2, 12–14). In this study, a mixture of human cytochromes *P*-450 was used in order to encompass all the different possible metabolic pathways that may result from the many isoenzymes of cytochrome *P*-450. Human cytochromes *P*-450 were used to increase the relevance to human hepatotoxicity in that major species differences in pathways of halocarbon metabolism have been reported (15). Cytochrome b_5 was included in the reconstituted system in the same molar ratio as it occurs in the endoplasmic reticulum because it has been shown to participate in electron transfer from NADPH-cytochrome *P*-450 reductase to cytochrome *P*-450 (16, 17). The Ole_2-PtdCho was shown to act as a structural component of the vesicle, to support catalytic activity of the reconstituted enzyme system, and to serve as an effective target for reactive metabolites that may bind covalently to the double bonds in fatty acyl chains. 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 mono-unsaturated 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 Ole_2-PtdCho as the only phosphatidylcholine in the reconstituted system because the single double bond in the oleoyl chains results in the most simple mixture of adducts after free radical addition (9- or 10- substitution). The reconstituted system was fully characterized as to protein and phospholipid content, metabolic activity toward a variety of substrates, and dependence on NADPH for activity (12, 18).

We have used the reconstituted vesicle system described above in an extensive study of the reductive metabolism of the

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Abbreviations: Ole_2-PtdCho, dioleoyl phosphatidylcholine; egg PtdEtn, egg phosphatidylethanolamine; ODS, octadecylsilica.

inhalation anesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane). We have shown that only in the presence of NADPH do ^{14}C-labeled metabolites bind to phospholipids in reconstituted vesicles in a manner similar to that in microsomes. We were able to demonstrate that 1-chloro-2,2,2-trifluoroethyl radical is the major reductive metabolite of halothane and that it binds to the double bond of the fatty acyl chain of Ole_2-PtdCho (14, 18). In a separate study we demonstrated that the halothane free radical adduct formed metabolically in the reconstituted system is identical to that formed by free radicals created by ultraviolet irradiation of halothane in the presence of methyl oleate (19).

MATERIALS AND METHODS

Human livers were obtained from heart donors in the Stanford Heart Transplantation program within 15 min after cessation of blood flow. Human cytochromes *P*-450 were purified 12-fold from liver microsomes by DEAE-cellulose and hydroxylapatite chromatography to a specific content of 4.8 nmol/mg of protein (20). This preparation is a mixture of the major forms of human cytochromes *P*-450 (13). NADPH-cytochrome *P*-450 reductase was purified from liver microsomes of phenobarbital-pretreated rabbits to an activity of 32 μmol/min per mg of protein (21). Cytochrome b_5 was purified from the same microsomes as the reductase to a purity of over 90% (22). Ole_2-PtdCho was obtained from Sigma, and egg phosphatidylethanolamine (egg PtdEtn) was prepared from fresh eggs under an argon atmosphere by the method of Singleton (23). The lipids were repurified by HPLC on Lichrosorb Si-100 prior to use. $^{14}CCl_4$ with greater than 99% purity was purchased from New England Nuclear.

Reconstitution of the three purified proteins into phospholipid vesicles was achieved by a modification of the slow cholate dialysis method (12, 13). The mixture of human cytochromes *P*-450 in 0.3 M potassium phosphate buffer, pH 7.5, containing 20% (vol/vol) glycerol was monomerized by the addition of 0.1% zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (24) followed by standing at 22°C for 2 hr. To a solution of 2.4 mg of human cytochrome *P*-450, solutions of 0.4 mg of cytochrome b_5 and 0.8 mg of cytochrome *P*-450 reductase were added to yield a molar ratio of 1:0.5:0.2. After addition of 24 mg of Ole_2-PtdCho and 12 mg of egg PtdEtn dissolved in 4% sodium cholate to the solution of the proteins, the final volume was 14 ml and the sodium cholate concentration was 2%. An equilibration time of 16 hr at 4°C was allowed for complete formation of mixed micelles (12). Dithiothreitol was present at 1 mM for further protection against peroxidation. Dialysis of the mixture was performed in a dialysis bag of 0.5 cm diameter suspended in a 1-cm inside diameter column with a continuous flow of 1 liter per day of N_2-saturated 20 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol and 0.5 mM dithiothreitol for 3 days followed by 1 day of the same buffer without dithiothreitol. Over 90% of cytochrome *P*-450 was recovered in the reconstituted vesicles and less than 6% cytochrome *P*-420 was observed.

Before incubation with $^{14}CCl_4$, the vesicle suspension was deoxygenated by a stream of N_2 for 15 min followed by 30 min of gentle stirring at 30°C after the addition of 5 mM D-glucose 6-phosphate, glucose oxidase (activity 115 μmol/min per mg) at 20 μg/ml, and thymol-free catalase (activity 125 μmol/min per mg) at 10 μg/ml. Then an NADPH-generating system was added through a septum to yield a final concentration of 0.5 mM NADP, 5 mM D-glucose 6-phosphate, and glucose-6-phosphate dehydrogenase at 1 international unit/ml. Finally, $^{14}CCl_4$ [diluted with CCl_4 to a specific activity of 0.72 μCi/μl, 70.1 μCi/mmol (1 Ci = 3.7×10^{10} becquerels)] was added to give 0.4 μl/ml, and the mixture was stirred for 1 hr at 30°C. Excess unreacted $^{14}CCl_4$ was removed by repeated extractions with ethyl acetate. The phospholipids were extracted with 80 ml of $CHCl_3$/CH_3OH, 2:1 (vol/vol). The solvents were evaporated, and the phospholipids were dissolved in hexane/isopropyl alcohol/water, 6:8:1 (vol/vol), and then applied to a 1 × 25 cm Lichrosorb Si-100 HPLC column. The PtdEtn fraction was eluted at a flow rate of 2.5 ml/min with hexane/isopropyl alcohol/water, 6:8:1 (vol/vol); at 14 min the solvent system was changed to hexane/isopropyl alcohol/water, 6:8:1.8 (vol/vol), to elute the Ole_2-PtdCho fraction. Elution of material from the column was monitored by refractive index and absorbance at 208 nm.

The phosphatidylcholine fraction from the Lichrosorb Si-100 column was vacuum dried and then subjected to transesterification with 2 ml of BCl_3 in methanol at 45°C for 2 hr. The resulting methyl esters were extracted into 5 ml of hexane that was backwashed with 4 ml of water. The hexane extract was subjected to HPLC on a 1 × 25 cm Lichrosorb reverse-phase C-18 column eluted with methanol/water, 96:4 (vol/vol), at a flow rate of 2.5 ml/min. The major radioactive fraction, which eluted from this column at 41 min (Fig. 1), was taken to dryness under argon and rechromatographed on a 0.46 × 25 cm reverse-phase Lichrosorb C-8 HPLC column eluted with methanol/water, 90:10 (vol/vol). The major radioactive fraction that eluted from the Lichrosorb C-8 HPLC column was further purified on a 0.46 × 25 cm reverse-phase Spherosorb octadecylsilica (ODS) (Altex, Berkeley, CA) column by elution with methanol/water, 96:4 (vol/vol), at a flow rate of 0.6 ml/min. The single radioactive peak was subjected to analysis by mass spectrometry. Direct inlet electron impact mass spectra were measured on a Varian CH-7 mass spectrometer at 20 eV. Desorption electron impact mass spectra were measured on a Ribermag R10-10B by desorption of the sample from a tungsten coil that was heated by increasing the current in the coil at 10 mA/sec from 50 mA to 500 mA (Fig. 2). The source temperature was maintained at 130°C; the filament current was 0.2 mA. The desorption chemical ionization mass spectra were measured on the Ribermag mass spectrometer, using 0.1 torr (13 Pa) of ammonia as the reagent gas with the same heating rate of the tungsten coil.

RESULTS

After anaerobic incubation of the reconstituted vesicles with $^{14}CCl_4$ and NADPH the phospholipids were extracted and subjected to HPLC. As shown in Table 1, reactive metabolites de-

Table 1. Binding of ^{14}C-labeled metabolites to phospholipid fractions from reconstituted vesicles incubated with $^{14}CCl_4$

Phospholipid fraction	Total ^{14}C-metabolite bound per fraction		nmol ^{14}C-labeled per nmol cyt. *P*-450	% of total phospholipid labeled
	dpm	μg		
Egg PtdEtn	20,000	130	3.6	1.4
Ole_2-PtdCho	19,900	128	3.6 (3.5)	0.7 (0.7)

Reconstituted vesicles were incubated under argon for 1 hr at 30°C with NADPH and $^{14}CCl_4$. The phospholipids were extracted and applied to a Lichrosorb Si-100 HPLC column to separate egg PtdEtn from Ole_2-PtdCho. The quantity of metabolite-bound phospholipids, the nmol of ^{14}C-containing metabolite formed per nmol of cytochrome *P*-450, and the percentage of metabolite-bound phospholipids in the vesicles were calculated from the specific activity of $^{14}CCl_4$. The numbers in parentheses are results of a repetition of the experiment with separately reconstituted vesicles made from the same stock of proteins and phospholipids.

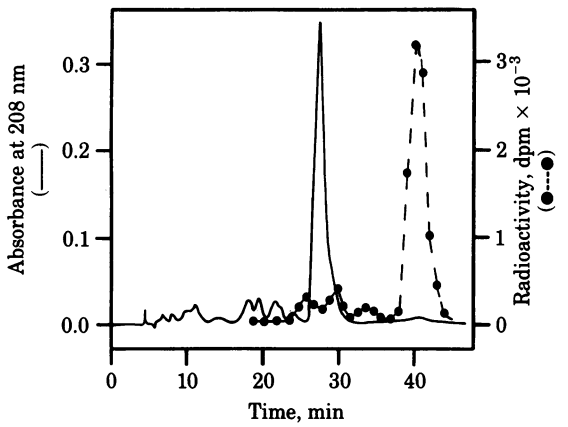

FIG. 1. HPLC profile of fatty acid methyl esters from Ole_2-PtdCho fraction. An aliquot of the Ole_2-PtdCho fraction obtained by preparative HPLC (Table 1) was subjected to transesterification. The resulting fatty acid methyl esters were separated on a 1 × 25 cm reverse-phase C-18 column with methanol/water, 96:4 (vol/vol), at 2.5 ml/min. The radioactivity in each fraction is shown; the absorbance was monitored continuously at 208 nm. Only one major radioactive peak is seen, at 41 min, well separated from the unchanged methyl oleate peak at 28 min.

rived from the $^{14}CCl_4$ bound in similar quantities to both phospholipid fractions. A repetition of the experiment with separately reconstituted vesicles from the same stock of proteins and phospholipids gave the nearly identical values in parentheses. This suggests that the reactive intermediate from metabolism of $^{14}CCl_4$ reacts to a similar extent with the mono-unsaturated acyl chains of Ole_2-PtdCho as with the polyunsaturated acyl chains of egg PtdEtn. From the specific activity of $^{14}CCl_4$, the total quantity of bound radioactive material is calculated to be 260 μg, which is equivalent to 7.2 nmol of metabolite bound per nmol of cytochrome *P*-450 in 60 min. This corresponds to labeling of approximately 2% of the total phospholipids present in the reaction mixture. No binding of metabolites to the methyl oleate fraction was observed when the incubation was carried out in the absence of NADPH (18).

The Ole_2-PtdCho fraction was transesterified and subjected to chromatography on a Lichrosorb C-18 reverse-phase column.

Table 2. Binding of ^{14}C-labeled metabolites of $^{14}CCl_4$ to methyl esters of fatty acids derived from Ole_2-PtdCho

Chromatographic fraction	Total ^{14}C-metabolite bound per fraction		% of total
	nmol	μg	
Original Ole_2-PtdCho	163	128	100
Transesterified Ole_2-PtdCho	112	46	69
Lichrosorb C-18 peak	82	34	50
Lichrosorb C-8 peak	57	24	35
Spherosorb ODS peak	37	15	23

Reconstituted vesicles were incubated with NADPH and $^{14}CCl_4$ under argon at 30°C for 1 hr. After extraction of phospholipids, the Ole_2-PtdCho fraction was isolated by HPLC, transesterified with BCl_3/MeOH, and chromatographed on a Lichrosorb C-18 column. The isolated radioactive material was rechromatographed on a Lichrosorb C-8 and then a Spherosorb ODS column. From the specific activity of $^{14}CCl_4$ the binding of radioactive metabolite to the separated material could be quantitated, permitting estimation of recovery at each chromatographic step.

Fig. 1 shows the elution of a single peak of radioactive material at 41 min distinct from methyl oleate and other UV-absorbing components. This radioactive peak was rechromatographed, first on a Lichrosorb C-8 column and then on a Spherosorb ODS reverse-phase column. The percentage recoveries of radioactive material at each chromatographic step together with calculated quantities of bound metabolites are shown in Table 2. The recovery of radioactive material at each chromatographic step was approximately 70%.

The radioactive methyl ester fraction purified by HPLC was subjected to mass spectrometric analyses by three techniques. Direct inlet electron-impact ionization on a Varian CH-7 mass spectrometer yielded a spectrum with only low-mass fragment ions. Fig. 2 shows the desorption electron-impact mass spectrum on a Ribermag R10-10B mass spectrometer. This spectrum is almost identical to that obtained on the direct probe analysis on the Varian CH-7. The large peak at *m/e* 297 corresponds to loss of a trichloromethyl substituent, a possibility anticipated from our studies on phospholipid binding of reductive metabolites of another halocarbon, the inhalation anesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) (14, 18, 19).

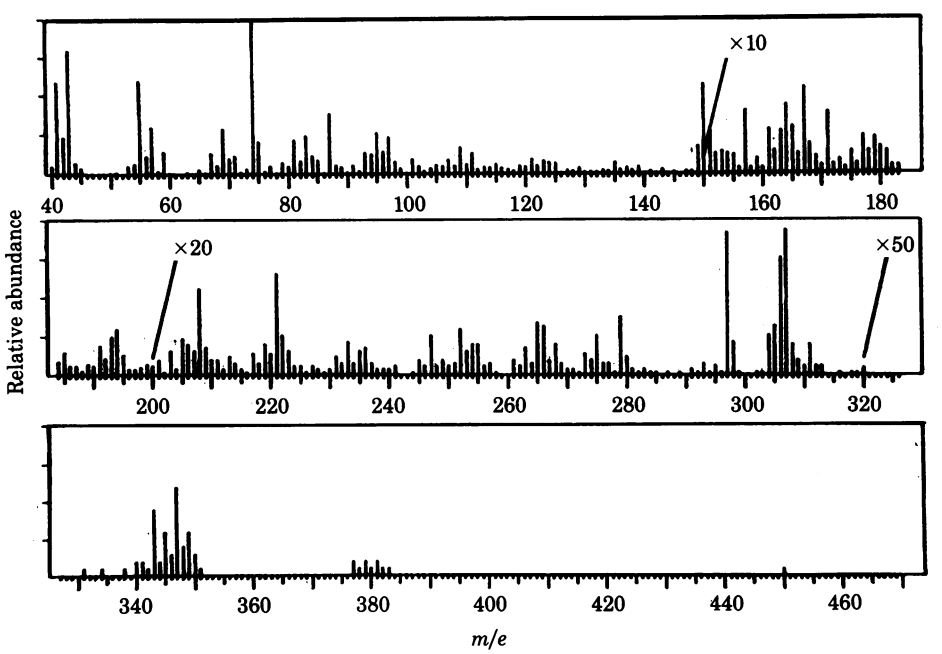

FIG. 2. The Ole_2-PtdCho fraction isolated in Fig. 1 was transesterified and the resulting ^{14}C-containing fatty acid methyl esters were purified on three successive reverse-phase HPLC columns. The single radioactive fraction obtained from the third HPLC column was subjected to desorption electron-impact mass spectrometry on a Ribermag R10-10B mass spectrometer. The mass spectrum is plotted as percent relative abundance of the peak at mass-to-charge ratio *m/e* 74. The slanted lines indicate the positions at which the relative abundance was multiplied by the indicated amount to make the peak more easily seen. No molecular ion peak at *m/e* 414 is observed.

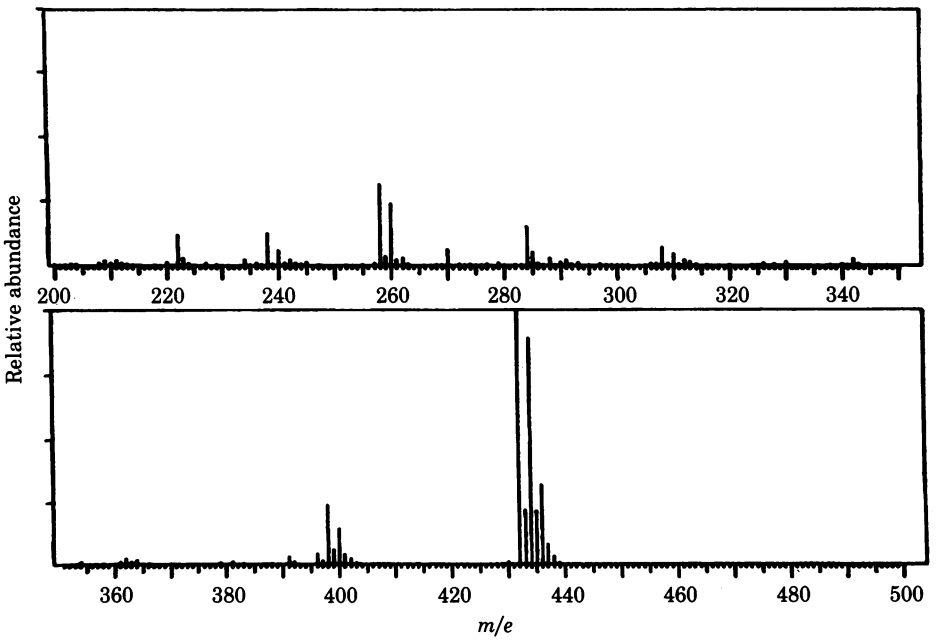

FIG. 3. The Ole_2-PtdCho fraction isolated in Fig. 1 was transesterified and the resulting ^{14}C-containing fatty acid methyl esters were purified on three successive reverse-phase HPLC columns. The single radioactive fraction obtained from the third HPLC column was subjected to desorption chemical ionization mass spectrometry using ammonia as a reagent gas in a Ribermag R10-10B mass spectrometer. The mass spectrum is plotted as percent relative abundance of the largest peak. A pseudomolecular ion was observed at *m/e* 432 ($M + NH_4^+$), which is the expected addition product of a molecule of molecular weight 414 under conditions of chemical ionization with ammonia as reagent gas. The isotope pattern at *m/e* 432, 434, and 436 is consistent with a molecule containing three chlorine atoms.

It is often difficult to observe a molecular ion (M) in the electron-impact mass spectra of polychlorinated organic molecules (25). However, the use of desorption chemical ionization mass spectrometry with ammonia as reagent gas in the Ribermag R10-10B gave a pseudomolecular ion peak at *m/e* 432 ($M + NH_4^+$) (Fig. 3). This pseudomolecular ion is expected for a molecule of molecular weight 414 under conditions of chemical ionization mass spectrometry with ammonia as reagent gas. The ratios of relative abundance of ions at *m/e* 432, 434, and 436 are those expected from a molecule containing three chlorine atoms as a consequence of the natural abundance of ^{35}Cl to ^{37}Cl being 3:1 (26). The spectrum in Fig. 3 is consistent with a mixture of 9- and 10-(trichloromethyl)stearate methyl esters. This product mixture would be expected from addition of a trichloromethyl radical to either atom of the double bond of oleic acid followed by abstraction of a hydrogen radical from a neighboring molecule.

Fig. 4 shows the intensities of the pseudomolecular ion ($M + NH_4^+$) at *m/e* 432 as well as the total ion current as a function of time during heating of the desorption coil. The similarity between the profiles of the mass chromatogram at *m/e* 432 and the total ion current suggests that the sample is homogeneous.

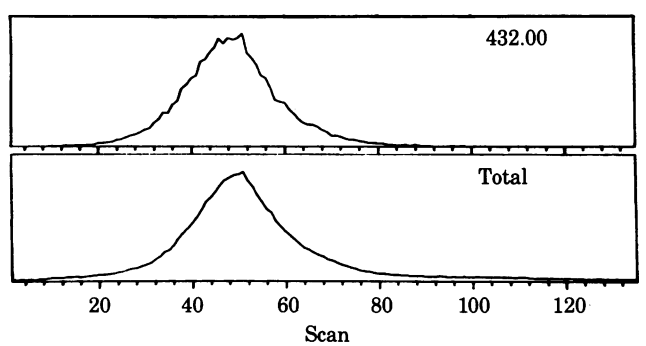

FIG. 4. The highly purified ^{14}C-metabolite bound to fatty acid was applied to a tungsten desorption coil for measurement of a chemical ionization mass spectrum as described for Fig. 3. As the heating current through the coil was increased from 50 to 500 mA at 10 mA/sec, a series of 125 mass spectra were recorded. In the upper mass chromatogram the abundance of the pseudomolecular ions at *m/e* 432 is displayed and compared to the abundance of the total ion current in the lower trace. The close similarity of the abundance of the pseudomolecular ions to the total ion current suggests that the sample was relatively homogeneous.

DISCUSSION

The present identification of the molecular structure of the adduct formed via metabolite binding to the fatty acyl chain of Ole_2-PtdCho confirms the assignment of the reactive intermediate as the trichloromethyl radical as proposed by many investigators (6, 7, 27, 28). A mechanism for production of the observed mixture of 9- and 10-(trichloromethyl)stearate methyl esters is proposed in Fig. 5. Binding of carbon tetrachloride to cytochrome *P*-450 as a substrate is followed by one-electron reduction to form the trichloromethyl radical (B). In the absence of oxygen this radical leaves the substrate binding site of the cytochrome and diffuses into the phospholipid bilayer. If the radical abstracts a hydrogen radical from a neighboring phospholipid, it will form chloroform (2) and a free radical on the phospholipid which may then be subject to peroxidation or diene conjugation reactions. Alternatively, the $\cdot CCl_3$ radical may add to one carbon of a double bond in a fatty acyl chain to produce a free radical on the adjacent carbon atom (C). The resultant fatty acyl free radical may undergo diene conjugation if it is polyunsaturated or lipoperoxidation if it interacts with oxygen, or it may form a saturated acyl chain by abstraction of a hydrogen atom from a neighboring molecule (28) to form a substituted, saturated acyl chain (D). In our experiments, the 9- and 10-(trichloromethyl)stearate methyl esters (E) were formed by transesterification of the substituted phospholipid (D).

Although this study demonstrates that the trichloromethyl free radical is a major product of the reductive metabolism of carbon tetrachloride, it does not rule out formation of a cytochrome *P*-450–dichlorocarbene complex (5, 9) and its subsequent dissociation to yield small amounts of reactive dichlorocarbene intermediates. From considerations of the rates of carbon monoxide versus chloroform formation during reductive metabolism of carbon tetrachloride (9), the quantities of any dichlorocarbene formed and possibly bound to phospholipids in our vesicle studies would be predicted to be less than 5% of the bound free radicals.

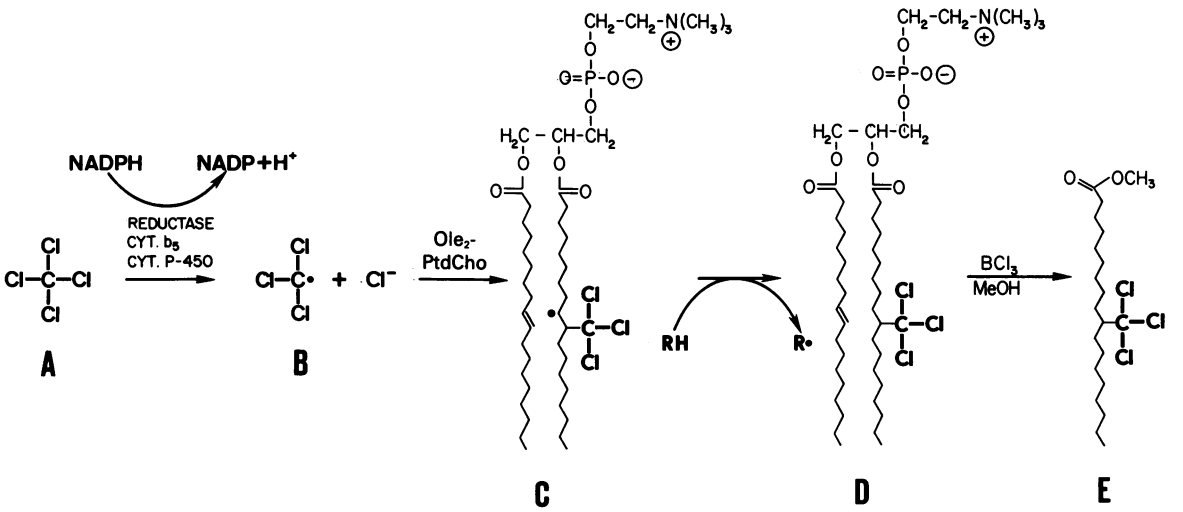

FIG. 5. Possible mechanism for formation of 9- or 10-substituted trichloromethylstearate methyl esters from CCl_4 and Ole_2-PtdCho. Under anaerobic conditions, in the presence of NADPH and cytochrome *P*-450, CCl_4 is subject to one-electron reduction to the trichloromethyl radical (**B**). Addition of this radical to one carbon of a double bond in a fatty acyl chain of the phospholipid can produce a free radical on the adjacent carbon atom (**C**). Abstraction of a hydrogen atom from a neighboring molecule results in formation of a substituted saturated acyl chain (**D**). Transesterification produces a mixture of 9- and 10-(trichloromethyl)stearate methyl esters (**E**).

Reaction of trichloromethyl free radicals with phospholipid molecules provides a possible mechanism for the observed covalent binding of carbon tetrachloride metabolites to hepatic microsomal lipids and proteins (4, 9–11). Under the present experimental conditions, approximately 2% of the total phospholipids in the vesicles were labeled after incubation with $^{14}CCl_4$. In the case of microsomes, where the protein-to-phospholipid ratio is 10 times that of the reconstituted vesicles, the same rate of production and binding of $\cdot CCl_3$ radicals would result in a correspondingly higher percentage of the phospholipids being attacked.

We thank Ms. Annemarie Wegmann and Dr. Carl Djerassi for making the Ribermag desorption chemical ionization mass spectrometer available to us through National Institutes of Health Grant GM-28352-21 to their mass spectrometry laboratory and to Ms. Annemarie Wegmann for measuring the mass spectra. This study would have been impossible with a conventional electron impact mass spectrometer. We also thank Ms. Marie Bendix for expert technical assistance, Ms. Audrey Stevens for editorial assistance, and the Stanford Cardiac Anesthesia and Cardiac Transplantation teams for their help in obtaining the human liver specimens. This study was supported by Grant OH 00978 from the National Institute of Occupational Safety and Health and a stipend to B.B. from the Alexander von Humbolt-Stiftung.

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