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2-Hexanone Potentiation of [^{14}C]Chloroform Hepatotoxicity: Covalent Interaction of a Reactive Intermediate with Rat Liver Phospholipid[1]

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2-Hexanone Potentiation of [^{14}C]Chloroform Hepatotoxicity: Covalent Interaction of a Reactive Intermediate with Rat Liver Phospholipid. COWLEN, M. S., HEWITT, W. R., AND SCHROEDER, F. (1984). *Toxicol. Appl. Pharmacol.* 73, 478–491. Rats were treated with [^{14}C]chloroform ($^{14}CHCl_3$) in corn oil (CO) or corn oil alone 18 hr following pretreatment with 2-hexanone (2-HX) in corn oil or corn oil alone. Livers were removed, homogenized 1, 2, and 6 hr post-$^{14}CHCl_3$ administration, and glutathione (GSH) content, irreversible binding of $^{14}CHCl_3$-derived radiolabel, and phospholipid composition were determined. The combination of 2-HX + $CHCl_3$ reduced GSH content to 21% of control (CO + CO) 1 hr after $CHCl_3$ administration. No significant rebound of GSH was observed 24 hr post-$CHCl_3$ administration. In contrast, GSH was not altered by administration of $CHCl_3$ to CO-pretreated rats. Although $^{14}CHCl_3$-derived radiolabel was irreversibly bound to hepatic macromolecules of both CO- and 2-HX-pretreated rats, total irreversibly bound ^{14}C was significantly enhanced in 2-HX-pretreated rats at all time points. The latter observation was consistent with the decrease in GSH of 2-HX-pretreated rats. Total ^{14}C binding in 2-HX-pretreated rats reached a plateau 2 hr post-$^{14}CHCl_3$ administration and was distributed 52% in protein, 41% in lipid, and 7% in acid soluble fractions 6 hr post-$^{14}CHCl_3$ administration. 2-HX enhanced ^{14}C binding to protein and lipid at each time point. Radiolabel was not detected in neutral lipids of control or 2-hexanone-treated animals, but was enhanced 33-fold in phospholipids of 2-hexanone-treated animals. Phospholipid fatty acid methyl ester derivatives did not contain ^{14}C indicating the radiolabel was most likely associated with phospholipid polar head groups. Two dimensional thin layer chromatographic analysis of phospholipid from treated animals demonstrated that 87% of the total radiolabel was associated with a specific phospholipid (^{14}C-PL) which had a 1:1 molar ratio of phosphate to ^{14}C. The latter indicates that the ^{14}C-PL was a monophospholipid derivative of $^{14}CHCl_3$ reactive intermediate, generally thought to be phosgene. Concurrent decrease in phosphatidylethanolamine content from 23% of total phospholipid to 7%, accumulation of ^{14}C-PL to 2.6% of total phospholipid, and increase in lysophosphatidylethanolamine from 1 to 7% of total phospholipid during 2-hexanone + $^{14}CHCl_3$ treatment indicated that the amine moiety of phosphatidylethanolamine polar head groups was the probable target of phosgene–lipid interaction, and that a degradative pathway existed which removed the abnormal phospholipid from hepatic membranes. No phospholipid other than phosphatidylethanolamine was depleted. During model studies, 12% phosgene in toluene was reacted with liver phosphatidylethanolamine for 6 hr at 37°C. Two reaction products were formed, further substantiating that phosphatidylethanolamine (PE) may react with [^{14}C]phosgene in liver. Although the data presented here are insufficient to conclude that the depletion of PE is a major mechanism in $CHCl_3$ hepatotoxicity, documented studies in which altered phospholipid composition modified cellular function suggest the possibility that PE depletion may contribute to phosgene-induced liver injury.

The initial event in chloroform ($CHCl_3$) -induced hepatotoxicity appears to be the bioactivation of $CHCl_3$ to a reactive intermediate,

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presumably phosgene (Mansuy *et al.*, 1977; Pohl *et al.*, 1977, 1980; Pohl, 1979). Phosgene is then thought to attack nucleophilic sites on hepatic macromolecules and in doing so initiates a chain of events that results in hepatocellular necrosis (Pohl, 1979). Although covalent adduct formation with hepatic macromolecules has been postulated to be a key step in $CHCl_3$ liver injury, few investigations have focused on identifying the critical molecular targets of phosgene. This investigation was undertaken to characterize further the nature of the interaction between $CHCl_3$ (phosgene) and hepatic macromolecules *in vivo* in an attempt to identify critical targets for $CHCl_3$. To facilitate this objective, covalent adduct formation was examined in vehicle- and 2-hexanone-pretreated rats. 2-Hexanone (2-HX)[3] has been shown to potentiate $CHCl_3$ hepatotoxicity (Hewitt *et al.*, 1980; Branchflower and Pohl, 1981) in part by increasing the bioactivation of $CHCl_3$ to phosgene (Branchflower and Pohl, 1981). In theory, a higher degree of selective binding of $^{14}CHCl_3$-derived radiolabel to a macromolecule in 2-HX-pretreated rats would implicate that molecule as a critical target to $CHCl_3$ (phosgene) attack.

METHODS

Male, Fischer 344 rats (150 to 300 g) were purchased from Harlan Industries, Inc. (Indianapolis, Ind.) and maintained on Purina Lab Chow and water *ad libitum*. Animals were used after a suitable period of acclimation in the animal quarters.

Hepatic glutathione concentration. $CHCl_3$ (0.5 ml/kg, ip) in corn oil (CO, final volume of solution, 410 ml/kg) or corn oil alone (4.0 ml/kg, ip) were administered to rats 18 hr following pretreatment (po) with a single, 10 mmol/kg dosage of 2-hexanone (in corn oil) or corn oil alone (10 ml/kg). Rats were killed at times ranging from 1 to 25 hr following $CHCl_3$ administration. The nonprotein sulfhydryl concentration expressed as glutathione (GSH) was determined as described by McMurty *et al.* (1978), with authentic glutathione standards. The results were expressed as micromoles GSH/per gram wet weight of liver.

Irreversible binding of $^{14}CHCl_3$-derived radiolabel. The methods employed to determine the irreversible binding of $^{14}CHCl_3$ to liver constituents were minor modifications (Cianflone *et al.*, 1980) of those described by Maling *et al.* (1974). $^{14}CHCl_3$ (0.5 ml/kg, 100 μCi/kg) was administered (ip) to rats 18 hr following a single dosage of 2-hexanone (10 mmol/kg, po) or corn oil (10 ml/kg, po). The rats were killed 1, 2, or 6 hr after $^{14}CHCl_3$ administration. Three naive rats were also killed to determine normal hepatic lipid content.

The livers were excised immediately, rinsed with ice-cold saline, and blotted dry. A small sample (0.2 g) was removed immediately for determination of total $^{14}CHCl_3$-derived radioactivity (volatile + nonvolatile) (Maling *et al.*, 1974; Cianflone *et al.*, 1980). The balance of each liver was homogenized in sufficient Tris–KCl buffer (1.15% KCl in 0.02 M Tris–KCl, pH 7.4) to yield a 20% (w/v) homogenate. Aliquots of this homogenate were taken immediately for determination of the irreversible binding (nonvolatile radiolabel) of $^{14}CHCl_3$-derived radiolabel to the protein, lipid, and acid-soluble fractions (Maling *et al.*, 1974; Cianflone *et al.*, 1980). The results were expressed as nanomoles $^{14}CHCl_3$ bound per gram wet weight of liver. The remainder of the homogenate was stored at −70°C until prepared for analysis of ^{14}C binding to specific lipid fractions.

Lipid determinations. All glassware was washed in sulfuric acid–dichromate prior to use, and organic solvents were glass distilled. A 0.1-ml aliquot of liver homogenate was diluted 30× in phosphate buffered saline, and protein concentration was determined by the method of Lowry *et al.* (1951). Total lipid was extracted with 0.5 ml of liver homogenate and 3.5 ml of phosphate buffered saline, pH 7.2, as described by Bligh and Dyer (1959). Neutral and polar lipids were separated by silicic acid column chromatography with Unisil (100 to 200 mesh) washed with methanol and activated overnight at 100°C. Prior to use, columns were washed with 5 ml chloroform, 4 ml methanol, and again with 5 ml chloroform. Lipids were applied to columns in 1.0 ml chloroform. Neutral lipids were eluted with 3.0 ml chloroform followed by polar lipids which were eluted with 4.0 ml methanol as described by Schroeder *et al.* (1976). Quantitation of total lipid, neutral lipid, and polar lipid fractions was accomplished by the method of Marzo *et al.* (1971). Covalently attached ^{14}C in lipids from $^{14}CHCl_3$-derived radiolabel was analyzed with a Beckman LS 100 C liquid scintillation system as follows: A 20-μl aliquot was removed from 2.0 ml lipid in chloroform:methanol, 1:1, placed in a scintillation vial, dried under N_2, and resuspended in 5.0 ml scintillation mixture (1 liter toluene, 0.1 g dimethyl POPOP, 4.0 g

[3] Abbreviations used: ^{14}C-PL, ^{14}C-labeled phospholipid; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; CL, cardiolipin; 2-HX, 2-hexanone; CO, corn oil; GSH, glutathione; POPOP, 4-methyl-5-phenyloxazoylbenzene; PPO, 2-5-diphenyloxazole.

PPO, and 330 ml Triton X-100). Each sample was counted for 20 min. A 1/10 aliquot of each phospholipid fraction was used to prepare fatty acid methyl ester derivatives to measure radiolabel associated with the fatty acid moieties (Schroeder, 1982).

Phospholipids from the silicic acid column were resolved by two dimensional thin layer chromatography on 250-μm silica gel G precoated plates activated in acetone (Schroeder *et al.*, 1976). Individual phospholipid spots were visualized with I_2 vapor. Spots were scraped and eluted with chloroform:methanol:1.5% NH_4OH (6:5:1), and total phosphate was determined by the method of Ames (1966). Each fraction was also analyzed for ^{14}C as described above. Phospholipids on the plate were identified by cochromatographing with the following standards: phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, phosphatidylserine, lysophosphatidylethanolamine, cardiolipin, phosphatidylglycerol, and lysophosphatidylcholine. All samples were stored in ultrapure benzene flushed with N_2 and kept at −70°C.

Phosgene–phospholipid experiments. Phospholipids were resolved by two dimensional thin layer chromatography as described above. A 200-μg sample of phosphatidylethanolamine was split into two aliquots and 4.0 ml of toluene was added to one, while 4.0 ml of 12% phosgene in toluene was added to the other. Samples were flushed with N_2, tightly capped, and incubated at 37°C for 6 hr with a modular Temp-Blok heater (Labline Inst., Melrose Park, Ill.). Phosgene and toluene were evaporated under N_2 to terminate the reaction, and samples were resolved by two dimensional thin layer chromatography as described above. Total phosphate was determined by the method of Ames (1966). Phosphatidylserine, dioleoylphosphatidylcholine, and dipalmitoylphosphatidylcholine standards (Supelco, Inc., Bellefonte, Pa.) were also reacted with 12% phosgene in toluene or toluene alone, and the products were resolved by thin layer chromatography as described above.

Statistical analyses. Data on hepatic GSH concentration and irreversible binding of ^{14}C were submitted to statistical analysis by a completely randomized design analysis of variance. Treatment means were tested by the Student–Newman–Keuls or Tukey's *w* procedures (Sokal and Rohlf). Data on all other experiments were submitted to statistical analysis with the Student *t* test. The 0.05 level of probability was used as the criterion of significance.

Materials. [^{14}C]Chloroform (sp act: 5.4 mCi/mmol) was obtained from New England Nuclear (Boston, Mass.). Dimethyl-POPOP and -PPO were purchased from Packard Instrument Co. (LaGrange, Ill.). Triton X-100 is a product of Research Products Int. (Elk Grove, Ill.). Ultrapure benzene was purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.). Boron trifluoride in methanol was obtained from Supelco, Inc. (Bellefonte, Pa.). Silicic acid (Unisil, 100–200 mesh) was purchased from Clarkson Chemical Company, Inc. (Williamsport, Pa.).

The following standards were used to identify phospholipids by cochromatography on 200-μm-thick silica gel G plates (Analtech, Newark, NJ) by two dimensional thin layer chromatography as described by Schroeder *et al.* (1976): phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, phosphatidylserine, lysophosphatidylethanolamine, and cardiolipin standards (Serdary Research Labs., London, Ontario, Canada); phosphatidylglycerol (Applied Science Labs., State College, Pa.); lysophosphatidylcholine (Supelco).

Twelve percent phosgene in toluene was obtained from MCB Reagents, a division of E. Merck (Darmstadt, Germany). All other chemicals were obtained at the highest commercial purity available and used as supplied.

RESULTS

Glutathione Content

The hepatic content of GSH was not altered by administration of the $CHCl_3$ challenge dosage (0.5 ml/kg) to CO-pretreated rats (Table 1). Although pretreatment of rats with 2-HX alone produced a slight depression (30%) of GSH stores, this effect was transient. In contrast, the combination of 2-HX + $CHCl_3$ produced a rapid and prolonged fall in hepatic GSH content. Thus, GSH content was reduced to approximately 21% of control (CO + CO) values 1 hr following $CHCl_3$ administration to 2-HX-pretreated rats. A rebound in GSH stores was not observed in the 2-HX + $CHCl_3$-treated rats; GSH content was 43% of control 24 hr post-$CHCl_3$ administration (Table 1).

Incorporation of $^{14}CHCl_3$ into Hepatic Macromolecules

$^{14}CHCl_3$-derived radiolabel was irreversibly bound to hepatic macromolecules of both CO- and 2-HX-pretreated rats (Table 2). The total amount of irreversibly bound ^{14}C increased with time in CO-pretreated rats; maximum binding was observed 6 hr post-$^{14}CHCl_3$ administration. At equal time points, bound ^{14}C was distributed in approximately equivalent amounts in the protein and lipid fractions with

TABLE 1

EFFECT OF 2-HEXANONE PRETREATMENT ON THE $CHCl_3$-INDUCED DEPLETION OF HEPATIC GLUTATHIONE CONTENT IN MALE, F344 RATS[a]

Time (hr) Pretreatment: challenge:	Hepatic GSH content (μmol/g liver)			
	Corn oil Corn oil	Corn oil $CHCl_3$ (0.5 ml/kg)	2-Hexanone (10 mmol/kg) Corn oil	2-Hexanone (10 mmol/kg) $CHCl_3$ (0.5 ml/kg)
1	5.53 ± 0.19 (4)	4.65 ± 0.38 (4)	3.86 ± 0.30 (4)[b]	1.14 ± 0.22[c,d]
2	4.78 ± 0.42 (4)	4.70 ± 0.34 (4)	3.42 ± 0.23 (4)	1.46 ± 0.37[c,d]
4	4.86 ± 0.23 (4)	4.66 ± 0.34 (4)	4.93 ± 0.30 (4)	1.34 ± 0.22[c,d]
6	4.94 ± 0.61 (4)	4.65 ± 0.30 (4)	4.66 ± 0.33 (4)	2.02 ± 0.31[c,d]
24	6.04 ± 0.37 (4)	8.15 ± 0.96 (4)	7.45 ± 0.27 (4)	2.62 ± 0.26[c,d]

[a] $CHCl_3$ (0.5 ml/kg, ip) was administered 18 hr following a single oral dose of 2-hexanone (10 mmol/kg). The animals were killed at times ranging from 1 to 24 hr following $CHCl_3$ administration. Values represent the mean ± SE determined in (*n*) rats.

[b] Significantly different than the corn oil + corn oil group, $p < 0.05$.

[c] Significantly different than the 2-hexanone + corn oil group, $p < 0.05$.

[d] Significantly different than the corn oil + $CHCl_3$ group, $p < 0.05$.

smaller amounts present in the acid soluble fraction from CO-pretreated rats (Table 2). Total irreversibly bound ^{14}C reached a plateau 2 hr following $^{14}CHCl_3$ administration to 2-HX-pretreated rats. In keeping with the marked depletion of hepatic GSH, binding of ^{14}C to hepatic macromolecule was significantly increased in 2-HX-pretreated rats at all times examined (Table 2). The increase in total ^{14}C bound was reflected by significant increases in ^{14}C binding to protein and lipid at each time point; with the exception of the 1-hr interval, bound ^{14}C in the acid soluble fraction was not different in CO- and 2-HX-pretreated rats (Table 2). ^{14}C was distributed 52% in protein, 42% in lipid, and 7% in acid soluble fractions 6 hr post-$^{14}CHCl_3$ administration in 2-HX pretreated rats (Table 2).

Total lipid was separated into neutral lipid and phospholipid fractions by silicic acid column chromatography, and ^{14}C incorporation into the two fractions was subsequently determined (Fig. 1). Incorporation of the radiolabel into the neutral lipid fraction was not detected with either treatment regimen. However, ^{14}C was incorporated into the phospholipid fraction of both treatment groups with incorporation in 2-HX-pretreated rats (2200 dpm/mg phospholipid) being 33-fold greater than controls (67 dpm/mg phospholipid). This incorporation of ^{14}C was maximal 2 hr post-treatment. Fatty acid methyl esters derivatized from phospholipids of 2-HX- and CO-pretreated animals did not contain ^{14}C at any time (Fig. 2). Therefore, the ^{14}C radiolabel was most likely attached to the phospholipid polar head group moiety.

Incorporation of $^{14}CHCl_3$ into Specific Phospholipids

The ^{14}C was not randomly distributed among the various phospholipid classes. Instead it was incorporated into a specific phospholipid of animals pretreated with 2-HX (Fig. 3B), but in CO-pretreated rats such specific incorporation was not detected (Fig. 3A). The new ^{14}C phospholipid partially cochromatographed with cardiolipin (CL) on two dimensional thin layer chromatographic plates (Fig. 3B). For radioisotope determinations, these phospholipids were therefore scraped together and eluted as one spot. The ^{14}C-PL + CL phospholipid contained 2.86, 12.00, and 7.00 dpm/nmol phospholipid and 85.03, 87.51,

TABLE 2

EFFECTS OF 2-HEXANONE PRETREATMENT ON THE IRREVERSIBLE BINDING OF $^{14}CHCl_3$-DERIVED RADIOLABEL TO LIVER CONSTITUENTS IN MALE, F344 RATS[a]

Time:	1 hr		2 hr		6 hr	
pretreatment:	Corn oil	2-Hexanone	Corn oil	2-Hexanone	Corn oil	2-Hexanone
Total radiolabel (nmol/g liver)	1700 ± 334 (6)	4294 ± 822 (6)[b]	2278 ± 325 (6)	6696 ± 1037 (6)[b]	1283 ± 135 (5)	4110 ± 429 (5)[b]
Irreversibly bound radiolabel						
protein (nmol/g liver)	84 ± 10 (6)	933 ± 196 (6)[b]	162 ± 14 (6)	2220 ± 528 (6)[b]	206 ± 6 (5)	1881 ± 160 (4)[b]
Lipid (nmol/g liver)	72 ± 13 (6)	609 ± 141 (6)[b]	123 ± 20 (6)	1132 ± 216 (6)[b]	264 ± 46 (5)	1464 ± 89 (4)[b]
Acid soluble (nmol/g liver)	66 ± 14 (6)	377 ± 89 (6)[b]	78 ± 21 (3)	152 ± 38 (3)	149 ± 26 (5)	243 ± 29 (4)
Total (nmol/g liver)	222 ± 36 (6)	1980 ± 415 (6)[b]	374 ± 87 (3)	3434 ± 1472 (3)[b]	619 ± 39 (5)	3588 ± 239 (4)[b]

[a] $^{14}CHCl_3$ (0.5 ml/kg, 100 μCi/kg, ip) was administered 18 hr following a single oral dose of 2-hexanone (10 mmol/kg) or corn oil. The rats were killed 1, 2, or 6 hr following $^{14}CHCl_3$ administration. Values are expressed as the mean ± SE determined in (*n*) rats.

[b] Significantly different than the respective, corn oil pretreated group, $P < 0.05$.

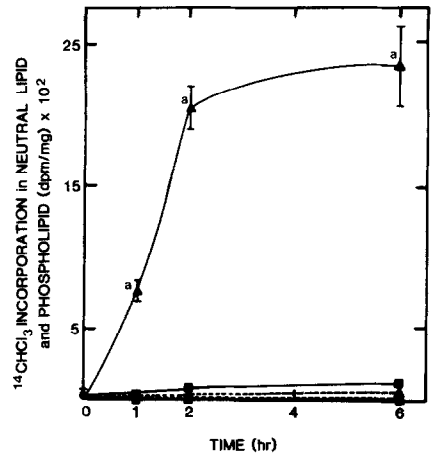


FIG. 1. Incorporation of ^{14}C into the neutral lipid and phospholipid fraction of rat liver following 0.5 ml/kg $^{14}CHCl_3$, ip, 18 hr after the administration of 10 mmol/kg 2-hexanone in corn oil (▲——▲, polar; ▲- - -▲, neutral) or corn oil control (■——■, polar; ■- - -■, neutral), po, as a function of time after $^{14}CHCl_3$ administration. 0-hr time point shown (●). [a]Significantly different from corn oil group of phospholipid fractions and both groups of neutral lipid fractions at respective time, $p < 0.05$.

and 73.22% of the ^{14}C spotted on the plate at 1, 2, and 6 hr post-$^{14}CHCl_3$ exposure, respectively (Table 3). The percentage of total phosphate represented by the ^{14}C-PL spot alone was determined by subtracting the amount of cardiolipin in control samples from the ^{14}C-

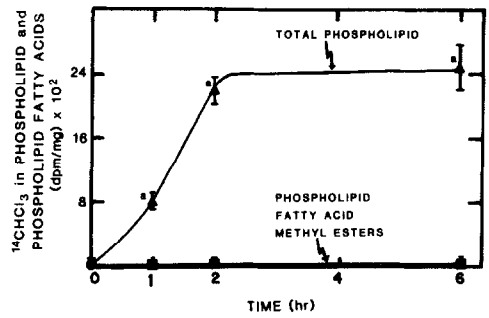


FIG. 2. Incorporation of ^{14}C into the polar lipid fraction (▲) and into fatty acid methyl esters of the polar lipid fraction (■) of rat liver following 0.5 ml/kg $^{14}CHCl_3$, ip, 18 hr after the administration of 10 mmol/kg 2-hexanone in corn oil as a function of time after $^{14}CHCl_3$ administration. [a]Significantly different from fatty acid methyl ester at respective time, $p < 0.05$.

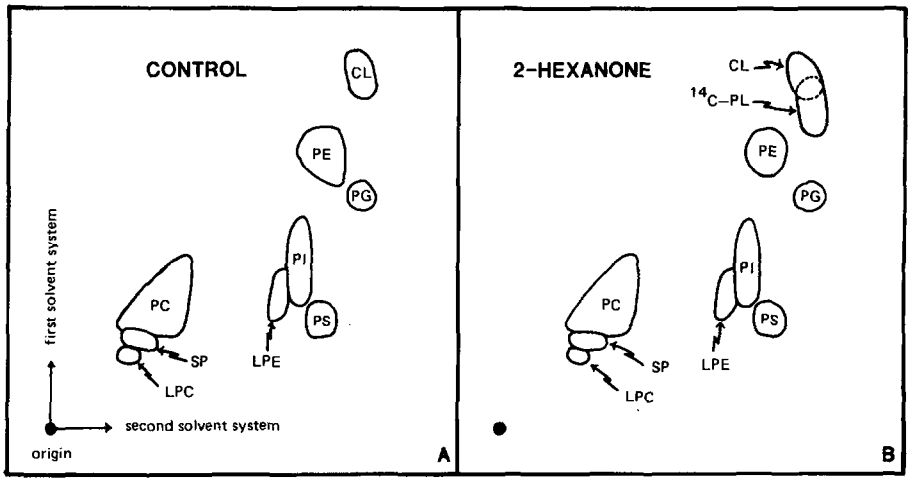


FIG. 3. Two dimensional thin layer chromatographic analysis of phospholipids from rats pretreated with (A) corn oil control or (B) 10 mmol/kg 2-hexanone in corn oil administered po 18 hr prior to the administration of 0.5 ml/kg $^{14}CHCl_3$, ip. First solvent system = chloroform:methanol:water, 65:25:4. Second solvent system = butanol:acetic acid:water, 6:2:1.75. LPC = lysophosphatidylcholine; SP = sphingomyelin, PC = phosphatidylcholine, LPE = lysophosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine, PG = phosphatidylglycerol, PE = phosphatidylethanolamine, CL = cardiolipin, ^{14}C-PL = ^{14}C-labeled phospholipid.

PL + CL spot. The percentage composition of total phospholipid represented by ^{14}C-PL remained near 0 during the 1st hr of post-$^{14}CHCl_3$ exposure time, then increased to approximately 2.6% during the 2nd hr, and remained just above 2% of the total phospholipid for the next 4 hr (Fig. 4B). When corrected for the overlap of the ^{14}C + PL and cardiolipin spots, the specific activity of the ^{14}C-PL was found to be 31.42 dpm/nmol PO_4. The specific activity of ^{14}C in $^{14}CHCl_3$ was 35.7 dpm/nmol; thus, the molar ratio of phosphate to ^{14}C was 1.14, corresponding to reaction of one molecule of $^{14}CHCl_3$ reactive intermediate with one molecule of phospholipid. Cross-linked products (one molecule of $^{14}CHCl_3$ reactive intermediate per two molecules of phospholipid) were not detected.

TABLE 3

INCORPORATION OF ^{14}C INTO THE ^{14}C-LABELED PHOSPHOLIPID OF PURIFIED PHOSPHOLIPIDS OF $^{14}CHCl_3$-TREATED RAT LIVER[a]

Pretreatment	$^{14}CHCl_3$ postexposure time (hr)	dpm/nmol PL[b]	% ^{14}C[c]
2-Hexanone	1	2.86 ± 0.61	85.03 ± 5.89
2-Hexanone	2	12.00 ± 0.84	87.51 ± 3.97
2-Hexanone	6	7.00 ± 0.37	73.22 ± 8.42

[a] $^{14}CHCl_3$ administered ip, 0.5 ml/kg 18 hr after pretreatment with 10 mmol/kg 2-hexanone or corn oil administered po.

[b] Refers to dpm/nmol PO_4 in ^{14}C-PL + CL, in Fig. 3. PL, phospholipid; CL, cardiolipin.

[c] Refers to % ^{14}C in ^{14}C-PL + CL, Fig. 3 as compared to the remainder of phospholipid on plate.

Phospholipid Composition

Since a $^{14}CHCl_3$ intermediate reacted with a specific phospholipid, it seems probable that a corresponding loss of that phospholipid would occur. The percentage composition of the major phospholipids from the livers of control and 2-hexanone-pretreated animals appears in Fig. 4. While the percentage content of phosphatidylethanolamine did not change significantly with respect to naive rats (expressed as 0-hr point) at any time after $^{14}CHCl_3$ administration to CO-pretreated rats, there

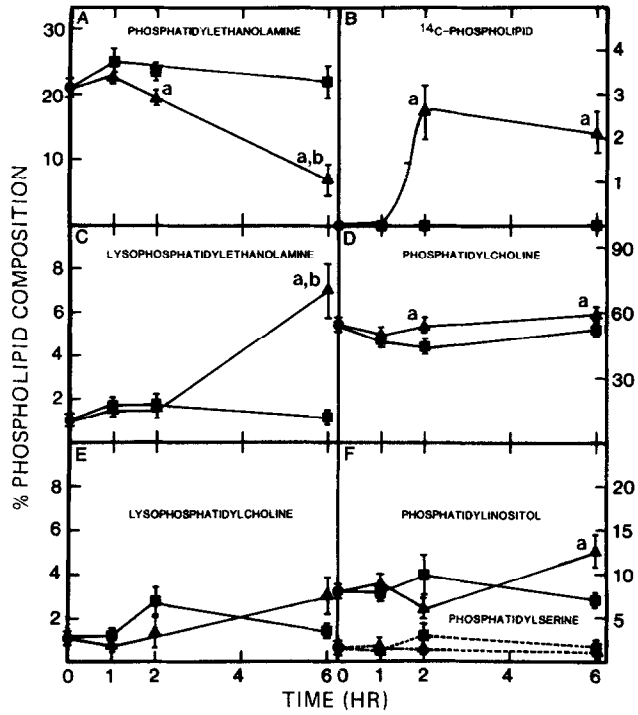


FIG. 4. Percentage phospholipid composition of (A) phosphatidylethanolamine, (B) ^{14}C-labeled phospholipid, (C) lysophosphatidylethanolamine, (D) phosphatidylcholine, (E) lysophosphatidylcholine, and (F) phosphatidylinositol (——) and phosphatidylserine (-----) of livers from rats treated ip with 0.5 ml/kg $^{14}CHCl_3$ 18 hr after the administration of 10 mmol/kg 2-hexanone in corn oil (▲) or corn oil control (■), as a function of time after $^{14}CHCl_3$ administration. [a]Significantly different from the corn oil group at the respective time, $p < 0.05$. [b]Significantly different from naive rats (0-hr time point, ●), $p < 0.05$.

was a significant decrease by 2 hr in the 2-HX-pretreated animals. In naive rats, the content of phosphatidylethanolamine was 21%. In the 2-hexanone-treated group, this value decreased to 19% after 2 hr; 6 hr after $^{14}CHCl_3$ treatment, the percentage composition of phosphatidylethanolamine decreased to 7% (Fig. 4A). Concurrently, the ^{14}C-labeled phospholipid reached a maximal content of 2.6% at 2 hr, but did not increase further (Fig. 4B). While lysophosphatidylethanolamine (LPE) was 1% in naive rats and did not change significantly at any time post-$^{14}CHCl_3$ administration in CO-pretreated rats, LPE was 7% of total phospholipids 6 hr post-$^{14}CHCl_3$ administration in 2-HX-pretreated rats (Fig. 4C). The percentage composition of phosphatidylcholine of 2-HX-pretreated animals increased from 54 to 59%, while CO-pretreated

TABLE 4

PHOSPHATIDYLCHOLINE / PHOSPHATIDYLETHANOLAMINE MOLAR RATIO OF LIVER FROM RATS EXPOSED TO $^{14}CHCl_3$[a]

Pretreatment	$^{14}CHCl_3$ postexposure time (hr)	PC/PE[b]
Naive rats		2.51 ± 0.39
Corn oil	1	1.98 ± 0.18
2-Hexanone	1	2.15 ± 0.16
Corn oil	2	1.92 ± 0.12
2-Hexanone	2	2.68 ± 0.27
Corn oil	6	2.57 ± 0.31
2-Hexanone	6	12.1 ± 3.63[c]

[a] $^{14}CHCl_3$ administered ip, 0.5 ml/kg 18 hr after pretreatment with 10 mmol/kg 2-hexanone or corn oil administered po.

[b] PC = phosphatidylcholine; PE = phosphatidylethanolamine.

[c] Significantly different than respective corn oil group and naive rats, $p < 0.05$.

animals showed no change (Fig. 4D). More importantly, the molar ratio of phosphatidylcholine to phosphatidylethanolamine increased from 2.5 in naive rats to 12.1 after 6 hr of $^{14}CHCl_3$ treatment in 2-HX-treated animals, while the ratio was not significantly altered in CO-pretreated rats (Table 4). Lysophosphatidylcholine was 1% in naive rats and did not change significantly with either treatment (Fig. 4E). The phosphatidylinositol content was greater than controls in 2-HX-pretreated animals after 6 hr of $^{14}CHCl_3$ exposure (Fig. 4F), while no significant changes in phosphatidylserine (Fig. 4F) or other phospholipids (not shown) occurred with respect to CO-pretreated controls. All of these data are consistent with the reaction of phosphatidylethanolamine amino group with a reactive intermediate derived from $^{14}CHCl_3$ in the liver.

Phosgene–Phospholipid Interactions in Vitro

To determine the probable identity of the ^{14}C-PL, several model studies were performed with phosgene, the most likely reactive intermediate produced from $^{14}CHCl_3$ in the liver (Pohl, 1979). The incubation of rat liver phosphatidylethanolamine with toluene for 6 hr

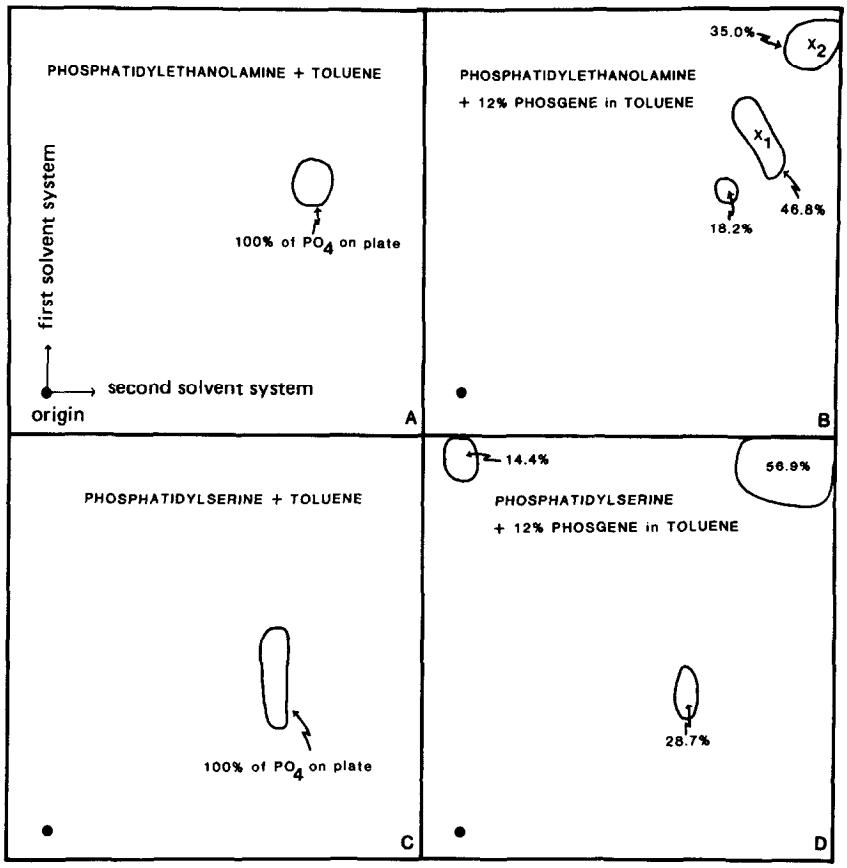
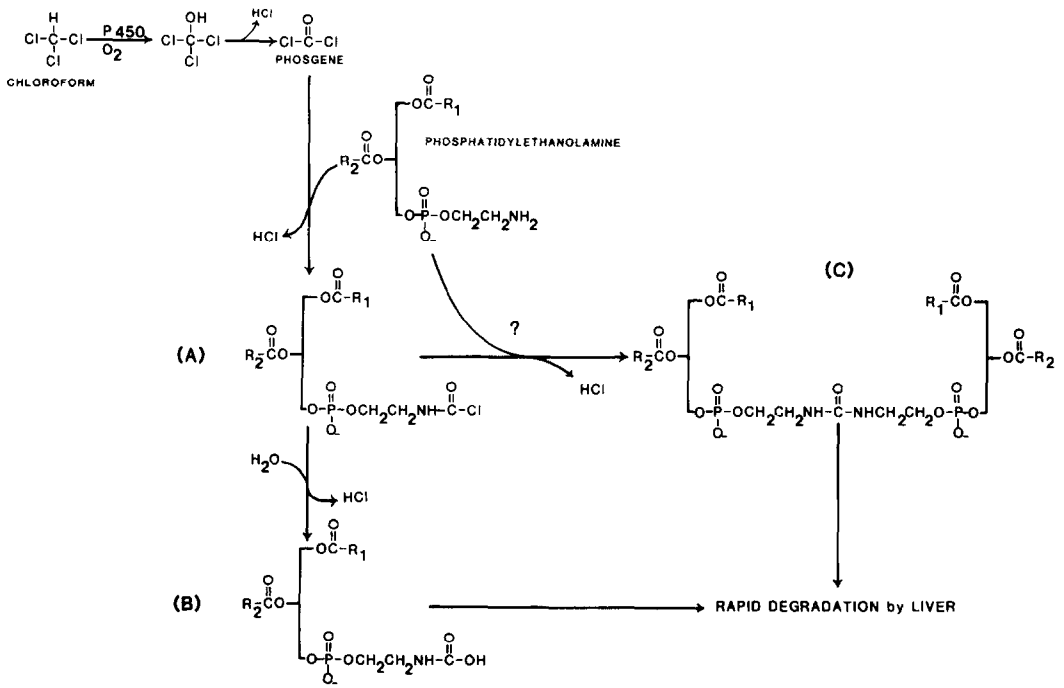


FIG. 5. Two dimensional thin layer chromatographic analysis of phospholipids incubated with toluene or 12% phosgene in toluene at 37°C for 6 hr. Numbers refer to percentage of total phosphate on plate. First solvent system = chloroform:methanol:water, 65:25:4. Second solvent system = butanol:acetic acid:water, 6:2:1.75.

at 37°C and subsequent two dimensional thin layer chromatographic analysis showed that 100% of the PO_4 on the plate was associated with phosphatidylethanolamine (Fig. 5A). On the other hand, incubation of 12% phosgene in toluene and phosphatidylethanolamine under identical conditions indicated that only 18.2% of the PO_4 on the plate remained unaltered as phosphatidylethanolamine, while 46.8% was within a spot designated X_1, and 35.0% within a spot designated X_2 (Fig. 5B). Although Spot X_1 had nearly identical R_f values on the chromatography plates as ^{14}C-PL (Fig. 3B), ^{14}C-PL and X_1 did not cochromatograph. However, the interaction of phosgene with a nucleophile in an organic environment, such as toluene, would not be expected to yield products identical to those produced from the interaction of phosgene with a nucleophile in an aqueous environment (*in vivo*). This theory is supported by a pathway for phosgene–nucleophile interaction proposed by Cresteil *et al.* (1979), in which a nucleophile–carbonylchloride adduct is converted nonenzymatically to a nucleophile–carboxyl adduct in the presence of H_2O. This step would be expected *in vivo* (Scheme 1B), but not in a toluene–phosgene–PE reaction mixture, which should produce the compound in Scheme 1A. In addition, no spot in Fig. 3 corresponded with X_2 of Fig. 5B.

Incubation of phosphatidylserine with phosgene decreased phosphatidylserine from 100 to 28.7% of PO_4 on plate with respect to phosphatidylserine + toluene control, and facilitated the formation of two new spots containing 14.4 and 56.9% of the PO_4 (Figs. 5C and D). However, neither of these spots appeared as radioactive phospholipid in the rat liver phospholipid (Fig. 3). Phosgene also reacted with dioleoylphosphatidylcholine decreasing its content from 100 to 60.1% and resulted in formation of five new compounds in the process (Figs. 6A and B). These products may be the result of phosgene reacting with the unsaturated fatty acid moiety and possibly



SCHEME 1. Proposed pathway for the interaction of chloroform with rat liver phosphatidylethanolamine.

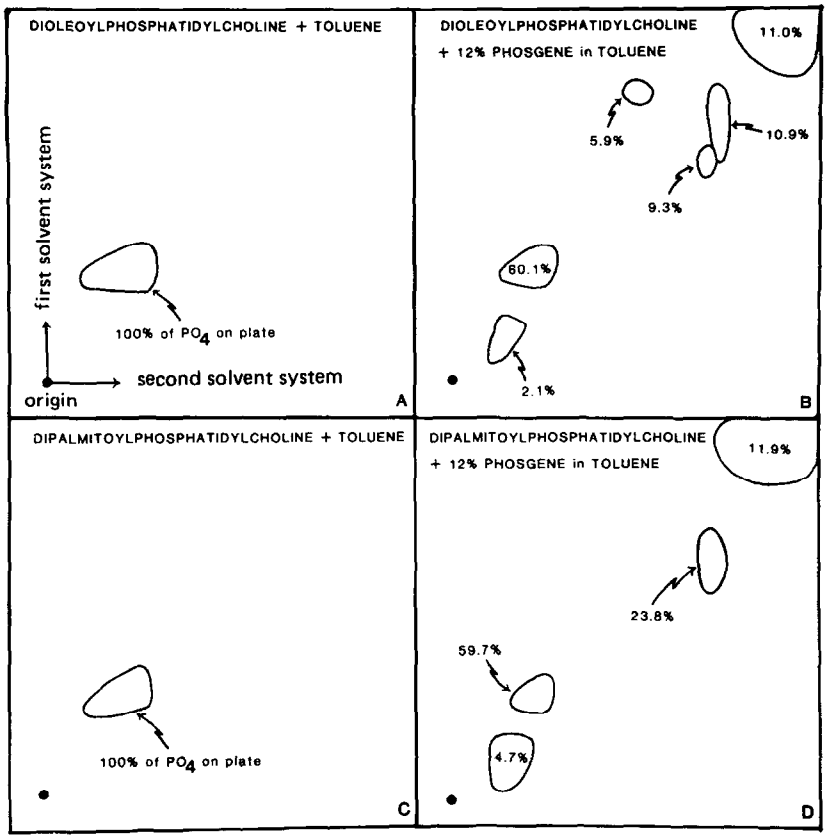


FIG. 6. Two dimensional thin layer chromatographic analysis of phospholiids incubated with toluene or 12% phosgene in toluene at 37°C for 6 hr. Numbers refer to percentage of total phosphate on plate. First solvent system = chloroform:methanol:water, 65:25:4. Second solvent system = butanol:acetic acid:water, 6:2:1.75.

subsequent crosslinking with other phospholipid molecules. However, as shown with the saturated dipalmitoylphosphatidylcholine, this was not necessarily the case. Dipalmitoylphosphatidylcholine exhibited a similar decrease to 59.7% in the presence of phosgene (as was seen with dioleoylphosphatidylcholine), but with the appearance of only three of the five spots seen with dioleoylphosphatidylcholine (Figs. 6C and D). In summary, phosgene is certainly capable of reacting with almost all of the phospholipid species in organic solvents when the phosgene is present at high concentrations (12%). This system likely does not reveal what actually occurs in liver, but is consistent with the reaction of phosphatidylethanolamine with [^{14}C]phosgene as a reactive metabolite of $^{14}CHCl_3$.

DISCUSSION

The three most salient observations of this study were: (1) 2-HX markedly increased $CHCl_3$ bioactivation to a reactive intermediate (phosgene) capable of depleting GSH and forming covalent adducts with hepatic macromolecules; (2) [^{14}C]phosgene generated from $^{14}CHCl_3$ appeared to selectively attack the amino head group of phosphatidylethanolamine (PE); and (3) total hepatic PE content was reduced in rats receiving 2-HX + $CHCl_3$

presumably as a consequence of the formation of a phosgene-PE adduct.

The enhanced degree of depletion of GSH (Table 1) produced by $CHCl_3$ in 2-HX-pretreated rats along with the 2-HX-induced stimulation of ^{14}C binding to hepatic macromolecules (Table 2) confirmed and extended the observation of Branchflower and Pohl (1981). These investigators demonstrated that 2-HX increased hepatic mixed function oxidase and the *in vivo* and *in vitro* formation of diglutathionyldithiocarbonate (GSCOSG). GSCOSG formation was accompanied by a degression of hepatic GSH content and subsequently an increased severity of $CHCl_3$ liver injury (Branchflower and Pohl, 1981). The failure of hepatic GSH content to rebound in rats receiving 2-HX plus $CHCl_3$ was also consistent with previous studies (Ekstrom and Hogberg, 1980; Ekstrom *et al.*, 1982) indicating that hepatic glutamyl-cysteine synthetase activity was markedly reduced in $CHCl_3$-poisoned rats. This enzyme may be a specific critical target for phosgene particularly in view of the lack of inhibition of glutathione synthetase, a related enzyme in the GSH synthetic pathway (Ekstrom *et al.*, 1982). Taken as a whole these observations support the hypothesis that 2-HX potentiates $CHCl_3$ hepatoxicity by increasing $CHCl_3$ biotransformation to a toxic, electrophilic intermediate (i.e., phosgene).

The novel observation of this study was that the nucleophilic amino head group of PE appeared to be a primary target for the electrophile phosgene. The identification of the unknown ^{14}C-labeled phospholipid (^{14}C-PL, Fig. 3B) as a phosgene–PE adduct was derived from several pieces of data. The consistent appearance of a single radiolabeled spot (^{14}C-PL) following 2-dimensional TLC of hepatic phospholipids suggested that phosgene formed a covalent adduct with a single phospholipid class. The concurrent decrease in hepatic PE content (Fig. 4A) and increase in the unidentified ^{14}C-labeled phospholipid (Fig. 4B) indicated that PE was the major phospholipid class involved. The observation that phosgene and PE can interact to form modified phospholipid products *in vitro* is consistent with the formation of a phosgene–PE adduct *in vivo*. Finally, the lack of significant amounts of ^{14}C activity in fatty acid methyl esters prepared from the total phospholipid fraction (Fig. 2) led to the conclusion that the amino head group of PE was the target of phosgene. Based on a pathway originally suggested by Cresteil *et al.* (1979), we have presented a possible mechanism of interaction between chloroform and liver membrane phosphatidylethanolamine (Scheme 1).

Organohalogen–PE adducts and/or degradation products resulting from the interaction of an electrophilic metabolite with the nucleophilic amino head group of PE have been reported. Methylthioacetylaminoethanol is a urinary metabolite of 1,1-dichloroethylene in rats and has been proposed to originate from the attack of chloroacetic acid chloride on the amino head group of PE followed by degradation of this adduct (Reichert *et al.*, 1979). Similarly, *N*-trifluoroacetyl-α-aminoethanol, a halothane metabolite found in human urine, may be a degradation product of a hepatic PE–trifluoroacetylhalide interaction (Cohen *et al.*, 1975). In support of this hypothesis, Müller and Steir (1982) demonstrated that halothane treatment of rabbits modified the composition of hepatic microsomal lipids in that: (1) an *N*-trifluoroacetyl–PE adduct was formed (approximately 4% of total microsomal phospholipid); and (2) the PE content of microsomal membranes was depressed.

Although these observations are consistent with an attack on the amino head group of PE, it is unclear why the additional *in vitro* reaction products of phosgene and PE were not detected *in vivo*. The second product formed *in vitro* from phosgene and PE (X_2, Fig. 5B) may represent the diphosphatidylethanolamine–phosgene derivative (DPEP); DPEP would originate from two PE moieties crosslinked at the amino head groups by the carbonyl moiety of phosgene (Scheme 1C). Since

PE exists in clusters within membranes (Marinetti, 1977), the proximity of PE molecules could facilitate their crosslinking to form DPEP. However, the molar ratio (1.14) of PO_4 to ^{14}C observed in ^{14}C-PL (Fig. 3B) suggested that only the mono-PE–phosgene adduct was present in appreciable quantities in rat treated with 2-HX + $^{14}CHCl_3$ (Scheme 1B). While DPEP was not detectable *in vivo,* the possibility that this adduct may be formed, rapidly degraded, and excreted should not be ignored. In addition, crosslinking of PE with membrane bound proteins by phosgene-derived carbonyl bridge may be another means by which functional phosphatidylethanolamine is removed from membranes. Marinetti and Love (1976) have shown such crosslinking occurred between protein and dinitrodiphenylsulfone derivatives of ethanolamine.

The content of the phosgene–PE adduct reached a plateau 2 hr post-$^{14}CHCl_3$ administration (Fig. 4B) whereas total PE content continued to decrease (Fig. 4A). This apparent discrepancy may imply that the abnormal phospholipid formed activated a phospholipase thereby initiating a degradative process capable of producing a greater decrease in total hepatic PE content than indicated by the amount of ^{14}C-PE adduct detected. Consistent with this interpretation, lysophosphatidylethanolamine increased sevenfold 6 hr post-$^{14}CHCl_3$ administration (Fig. 4C), indicative of phospholipase A_2 activation. It has been demonstrated that a rat liver microsomal phospholipase A (Bjornstad, 1966a) and a rat liver mitochondrial phospholipase A (Bjornstad, 1966b) hydrolyzed phosphatidylethanolamine in preference to phosphatidylcholine, forming lysophosphatidylethanolamine. Lysophosphatidylcholine was not significantly enhanced in animals treated with 2-HX + $^{14}CHCl_3$ (Fig. 4E). Indeed the urine metabolites, methylthioacetylaminoethanol and *N*-trifluoracetyl-2-aminoethanol, suggest that a degradative pathway is activated when organohalogen–PE adducts are formed. However, such a pathway cannot account for the failure to observe phosgene adducts of other phospholipids *in vivo* since no decrease in hepatic content of other phospholipid classes was observed (Fig. 4). The occurrence of these PL–phosgene adducts *in vitro* may be due to the high phosgene concentration (12%) employed in the model test system.

Although suggestive, the data presented above are insufficient to assign the phosgene-induced depletion of PE content a major role in $CHCl_3$ hepatotoxicity. However, the inability to detect PE adduct formation or a reduction of PE content in rats treated with CO + 0.5 ml/kg $^{14}CHCl_3$ was consistent with previous studies (Brown and Hewitt, 1983) demonstrating that this $CHCl_3$ dosage produces minimal liver injury (SGPT activity = 265 ± 55 units/ml, n = 31) in CO-pretreated rats as compared to 2-HX-pretreated rats (SGPT activity = 7835 ± 509 units/ml, n = 6). Thus, it is tempting to speculate that PE is a critical target for phosgene and that the alteration in membrane fluidity known to occur with changes in membrane phosphatidylcholine/phosphatidylethanolamine ratios (Table 4; Blume and Ackerman, 1974), and the toxicity of increased lysophosphatidylethanolamine concentration (Fig. 4C, Lehninger, 1970) may contribute to the production of hepatocellular necrosis by $CHCl_3$. Data from the literature supportive of phospholipid alterations modulating cellular function include: (1) regulation of membrane bound enzyme activities such as adenylate cyclase (Engelhard *et al.,* 1976); (2) regulation of the selective permeability of membranes (McElhaney, 1975); (3) alteration of the asymmetric, transbilayer distribution of lipids in plasma membranes (Schroeder, 1980); (5) regulation of endocytosis (Schroeder, 1981); (6) modulation of concanavalin A agglutinability (Hampton *et al.,* 1980; Schroeder, 1983); and (7) inhibition of calcium pump activity (Chein *et al.,* 1978; Hidalgo *et al.,* 1982). Although the above examples do not pertain specifically to $CHCl_3$-induced hepatocellular necrosis, they strongly suggest that the phospholipid alter-

ations reported herein may contribute to the manifestation of chloroform hepatotoxicity.

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