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Hepatic microsomal lipid damage induced by carbon tetrachloride



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# HEPATIC MICROSOMAL LIPID DAMAGE INDUCED BY CARBON TETRACHLORIDE

by

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DISSERTATION

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# ABSTRACT

*In vitro* model systems have been used extensively in experiments relating carbon tetrachloride administration to injury of the endoplasmic reticulum. It was of interest to elucidate the nature of the membrane lesion occurring *in vitro* and compare it to the *in vivo* lesion. The *in vitro* system could provide a model which closely reflects the membrane changes that are occurring *in vivo*. An attempt was made to understand the nature of the increased sensitivity of the phenobarbital pretreated liver following carbon tetrachloride administration. Attention was directed to the changes in membrane composition following phenobarbital stimulation and the resulting destruction of the membrane after carbon tetrachloride challenge. The *in vivo* oral administration of carbon tetrachloride-induced lipid damage was compared to the response produced by a model system employing purified hepatic microsomes to which carbon tetrachloride and the necessary electron donor, NADPH, were added.

In order to provde insight into the specificity of the carbon tetrachloride-induced membrane destruction, experiments were conducted in the presence of general lipid oxidants. NADPH, the electron donor, was employed by itself and FeADP, an electron carrier, was employed in conjunction with NADPH.

Phenobarbital pretreatment alone increased phosphatidylethan-

olamine content of the endoplasmic reticulum, while the relative quantities of arachidonic and docosahexenoic fatty acids (C20:4 and C22:6, respectively) decreased in phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine but not in phosphatidylinositol. Linoleic acid was found to increase in all phospholipids.

Carbon tetrachloride intoxication in vivo decreased phosphatidylethanolamine content, while diene conjugation was highest in phosphatidylserine. Loss of C20:4 and C22:6 occurred in all phospholipids, especially in phosphatidylserine. Carbon tetrachloride challenge of phenobarbital-pretreated animals accentuated changes quantitatively without qualitative modification. NADPH induced peroxidation caused a loss of phosphatidylethanolamine while diene conjugation levels were similar in all phospholipids. Carbon tetrachloride added to this in vitro system accentuated phosphatidylethanolamine loss and produced a hierarchy of phospholipid peroxidation PS PI PC and PE. Microsomes from phenobarbital pretreated animals were generally less affected. Both in vivo and in vitro carbon tetrachloride addition resulted in significant losses in stearic acid (C18:0), C20:4 and C22:6 in phosphatidylserine which was not mimicked by NADPH or FeADP systems.

These results suggest that carbon tetrachloride peroxidation is locus specific, that a fatty acid topology exists in the endoplasmic reticulum and that phosphatidylserine may be important in

mixed function oxidase activity. Phosphatidylserine destruction is enhanced with phenobarbital-induced endoplasmic reticulum. This membrane is more susceptible to endogenous peroxidation in spite of a lower content of long chain unsaturated fatty acids.

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# INTRODUCTION

Halogenated hydrocarbons are important anesthetics, pesticides, herbicides, and industrial solvents such as degreasers, many of which cause liver injury when ingested or inhaled in other than trace amounts.

Halomethanes, the class of compounds to which carbon tetrachloride and chloroform belong, were employed as anesthetics until they were linked with hepatotoxic side effects. These early observations prompted research on experimental animals which has since provided a large body of evidence on the probable mechanisms in which these compounds exert their toxic effects in the liver.

Administration of chloroform and carbon tetrachloride to rats resulted in centrilobular necrosis (1) and fatty degeneration (2) of the liver. The earliest morphological and biochemical changes that have been detected in the liver cell fractions have been in the endoplasmic reticulum (3). Within thirty minutes after carbon tetrachloride administration protein synthesis is decreased by 50 percent (4). The decrease was not associated with a decrease in amino acid uptake or protein release from the synthetic machinery (3,5) but with a reduced capacity of the ribosome particle (4). The changes in protein synthesis occur so early and are so diffuse at low doses of carbon tetrachloride indicate that the disorder in the protein synthetic apparatus is one of the very early events in the pathogenesis of carbon tetrachloride injury to the liver.

Enzymes localized in the rat liver endoplasmic reticulum such as glucose-6-phosphatase (6) and aminopyrene demethylase (7) are markedly depressed in activity within the first few hours after the *in vivo* administration of carbon tetrachloride (8).

Phospholipids are known to be required for the normal function of glucose-6-phosphatase (9) and the enzymes involved in the drug metabolizing system localized in the endoplasmic reticulum (10). When rat liver microsomes undergo peroxidative decomposition *in vitro* glucose-6-phosphatase activity is rapidly lost (11) as well as the aminopyrene demethylase activity (12). The association of lipid peroxidation and the decline in activity in these enzymes *in vitro* may be associated with decreases in these enzymes *in vivo* as a result of lipid peroxidation.

Oral administration of $^{14}C$-carbon tetrachloride results in rapid incorporation of the labeled metabolites into the lipids and proteins of the endoplasmic reticulum since five minutes after the administration of the toxin the liver microsomal lipids have half-maximal incorporation of the label (13). The rapidity of the microsomal lipid binding correlated with the very rapid stimulation of lipid peroxidation as measured by diene conjugation; lipids are peroxidized to half-maximal five minutes after administering the compound (14). The authors concluded that the data provide evidence that carbon tetrachloride administration initiates lipid

peroxidation which may be an important feature of the hepatotoxic action of carbon tetrachloride.

The influence of lipid peroxidation on the activity of glucose-6-phosphatase and the drug metabolizing system was demonstrated when EDTA was shown to completely prevent lipid peroxidation and the loss in enzyme activity (15). Since neither the in vitro aerobic metabolism of carbon tetrachloride to carbon dioxide (16) nor the in vitro anaerobic conversion of carbon tetrachloride to chloroform (17) is inhibited by EDTA addition, this suggests that carbon tetrachloride destruction of the membrane-bound enzymes involves lipid peroxidation.

Evidence that carbon tetrachloride by itself is not responsible for changes in the liver is threefold: there is a lack of synchrony in tissue levels and destruction. When carbon tetrachloride is administered via gastric intubation it is present first in the stomach, the small intestine and the hepatic portal system before reaching the liver. However, none of these organs display the degree of destruction found in the liver (18). Although the concentration of carbon tetrachloride is relatively high in such tissues as the brain and adipose, the lack of drug metabolizing enzymes present in these tissues is correlated with a lack of reactivity to the toxin.

A positive correlation was found to exist between the extent of tissue damage caused by carbon tetrachloride and the levels of the drug metabolizing enzymes present. When drug metabolizing

activity is induced by phenobarbital, rats become hypersensitive to carbon tetrachloride (19). Smuckler et al. (20) reported that there was a rapid loss of cytochrome P-450 after carbon tetrachloride administration which paralleled a decreased activity of microsomal oxidative demethylation of drugs. Starvation and administration of a protein deficient diet results in a decrease in the drug metabolizing activity of the liver and correlates with resistance to carbon tetrachloride toxicity (21). The addition of SKF-525A, a competitive inhibitor of drugs with the cytochrome P-450 molecule results in a decrease in carbon tetrachloride toxicity (8). It was discovered that rats will survive when challenged with an ordinarily lethal dose of carbon tetrachloride within 24 hours after administration of a small intragastric dose of carbon tetrachloride (12). It is thought that the protective effect of the small dose is the result of a marked depression in activity of the liver drug metabolizing enzyme system with a resulting decrease in the activation of carbon tetrachloride to a reactive intermediate.

Further support for the metabolism of carbon tetrachloride to a more reactive intermediate is provided by the observation that carbon tetrachloride, when added to isolated organelles, produces little or no responsiveness of these organelles to the parent compound. When carbon tetrachloride is added to polysome preparations the functional and structural changes observed with

carbon tetrachloride addition to microsomal or in vivo preparations are not observed (4). In support of the lipid peroxidation theory in which carbon tetrachloride administration results in peroxidized lipids as a vector of cell injury large amounts of carbon tetrachloride do not depress the activities of glucose-6-phosphatase or aminopyrene demethylase provided lipid peroxidation is not allowed to occur (22).

The liver contains several metabolic pathways in which halomethane metabolism has been observed. Heppel and Porterfield (23) demonstrated that rat liver extracts, slices or homogenates are capable of dehalogenating a number of compounds. This system was found to be more active in an atmosphere of nitrogen than oxygen and kidney slices were more active than liver slices.

A non-enzymatic heat insensitive system was identified in which the conversion of carbon tetrachloride into chloroform was found to occur in the presence of glutathione, cysteine or ascorbic acid (24).

The enzymatic dehalogenation of carbon tetrachloride by rat liver homogenates which is believed to be the most important pathway, was found to require both the microsomal and soluble fractions of the homogenate for activity (25). Subsequently it was discovered that the process is dependent on the presence of nicotinamide adenine dinucleotide in the reduced form (NADPH) and oxygen (16). Reconstitution of the drug metabolizing system has revealed that

in addition to NADPH the metabolism of carbon tetrachloride requires a flavoprotein, cytochrome P-450 NADPH reductase and the hemeprotein, cytochrome P-450 (26). The cytochrome P-450 NADPH reductase catalyzes the reduction of the cytochrome P-450 molecule with reducing equivalents obtained from NADPH. This complex, in the presence of oxygen, catalyzes the oxidation of a great variety of endogenous substrates such as steroid hormones, cholesterol, fatty acids and other lipid-soluble compounds (27) as well as many lipid-soluble exogenous compounds (xenobiotics) of diverse structure.

The cytochrome P-450 electron transport system, or monooxygenase system of the liver is of considerable pharmacological and toxicological interest. The duration and intensity of drug action are largely determined by the rate at which drugs are metabolized in the body. In the case of xenobiotics, their metabolism by the monooxygenase system can lead to enzyme induction of the system (28) and/or the formation of reactive metabolites which can be carcinogens, mutagens or toxic in other ways (29).

Although the detailed mechanisms by which carbon tetrachloride is metabolized is unknown, in a highly non-polar environment such as exists in the endoplasmic reticulum in the region of the drug metabolizing sequence, a homolytic cleavage has been suggested (24):

$$CCl_4 + R\cdot \longrightarrow CCl_3^{\cdot} + RCl$$

The mechanism is dependent on an interaction of carbon tetrachloride with an endogenous free radical. At least two free radicals are present in the drug metabolizing system, the NADPH cytochrome P-450 reductase which oscillates between the fully reduced and the semiquinone radical and the cytochrome P-450 molecule which contains oxygen radicals (30).

According to the lipid peroxidation hypothesis in which peroxidized lipids are a vector in the production of cell injury, carbon tetrachloride is activated to the highly reactive and short-lived trichloromethyl radical by the monooxygenase system of the liver. Subsequent to the cleavage of the carbon-chlorine bond there is a free-radical attack on the methylene group hydrogen of the polyenoic fatty acids of membrane lipids (8) producing a chain reaction of peroxidative changes in the adjacent unsaturated fatty acids. Recently the hypothesis gained support by the trapping of the trichloromethyl radical (31). The free radical attack of the unsaturated fatty acids culminates in the decomposition of polyenoic fatty acids with the production of malonaldehyde (MDA), a breakdown product of fatty acid peroxides as well as numerous other products which have not been identified but are believed to cause cell injury at sites distant from the locus where it was produced (32). In addition to the loss in glucose-6-phosphatase and drug metabolizing ability peroxidation of lipids was associated with a loss of the cytochrome P-450 protein (33)

and the loss of the hemeprotein occurred when microsomes were incubated aerobically in the presence of NADPH without the addition of carbon tetrachloride (33). Lipid peroxidation also results in the destruction of the fatty acid constituents of the membrane phospholipids (34). The maintenance of the functional and structural integrity of membranes is being recognized as an important factor in providing a necessary spatial organization and chemical environment that may influence the reactivity of electron transport proteins. The compartmentalization of the endoplasmic reticulum within the hepatocyte may be important in maintaining the homeostatic balance within the cell and physical disruption of this highly ordered organelle by peroxidative changes may provide insight into the molecular mechanisms of cell injury.

Carbon tetrachloride, both a pro-oxidant and a producer of free radicals, provides a good chemical model for studying time sequences and mechanisms which precede liver cell injury and death. Although the results are applicable to carbon tetrachloride and closely related halomethanes, the influence of the compound on the tissue has broad importance in understanding the developmental sequence of necrosis. As a histologically well-defined sequence, research on cell injury and necrosis has led to a coordination of many disciplines in providing biochemical information which relate to the histological changes which occur. Additionally, carbon

tetrachloride has stimulated appreciation of the role of free radical mechanisms in cell injury. The production of extraneous free radicals in vivo will have a multiplicity of effects on natural components which can be predicted to have deleterious effects on normal metabolism. Trichloromethyl radicals produced in the membranes of the endoplasmic reticulum can be expected to react with thiol groups, nucleic acid bases and unsaturated bonds that occur in fatty acids associated with membranes.

The relationship of carbon tetrachloride-induced peroxidative destruction of the phospholipids of the endoplasmic reticulum and the numerous cell lesions produced as a consequence of peroxidation (32) have been difficult to distinguish primarily because the endoplasmic reticulum is a complex composite of protein and lipid interactions. The recent report that some product of microsomal lipid peroxidation, other than malonaldehyde, can travel and effect damage at sites distant from the locus of peroxidation (32) may provide insight into the observation that very small quantities of carbon tetrachloride have hepatotoxic effects elicited by the whole animal (35). The contribution of lipid peroxidation to cell injury and death, whether directly or by the reaction of toxic product diffusion, remains to be elucidated. The liver demonstrates an increased susceptibility to carbon tetrachloride administration when the drug metabolizing system has been stimulated by pretreating the animal with phenobarbital (19). Phenobarbital also results in a 2-fold increase in the endoplasmic reticulum (28), the site of the monooxy-

genase system. In an attempt to understand the nature of the increased sensitivity of the phenobarbital pretreated liver to carbon tetrachloride administration attention was directed to the changes in membrane composition following phenobarbital stimulation and the resulting destruction of the membrane following carbon tetrachloride administration. The response of in vivo oral administration of carbon tetrachloride was compared to a model system employing purified preparations of microsomes to which carbon tetrachloride and the necessary electron donor, NADPH, were added. Since the model system has been used extensively in experiments relating carbon tetrachloride administration to injury of the endoplasmic reticulum (12,21,35) it was of interest to identify the nature of the membrane lesion occurring in vitro and compare it to the in vivo lesion. If membrane response to carbon tetrachloride administration is similar in both in vivo and in vitro, the in vitro system would provide a good model which closely reflects the membrane changes that are occurring in vivo.

In order to provide insight into the specificity of the carbon tetrachloride-induced membrane destruction experiments were conducted in the presence of general lipid pro-oxidants. NADPH, the electron donor, was employed by itself and FeADP, an electron carrier, was employed in conjunction with NADPH.

## EXPERIMENTAL APPROACH

Male, pathogen-free Sprague-Dawley rats, (Charles River Breeding Laboratories, Wilmington, Delaware), weighing 180-200 grams were separately housed in cages with steel-screened bottoms and maintained on Purine Rodent Chow and water ad libitum for 4 days prior to experimentation. Food was removed 14-16 hours prior to sacrificing although free access to water was permitted.

### Carbon tetrachloride-induced lipid peroxidation *in vivo*

Phenobarbital (Elkins-Simm, Cherry Hill, New Jersey) was injected intraperitoneally as a solution in saline (50 mg/kg body weight) at a concentration of 5 mg/ 1.0 ml solution/ 100 gm body weight for 4 days prior to sacrificing. Control animals were given equal volumes of saline containing 0.38 percent absolute alcohol and 2.61 percent propylene glycol (the carrier present in the phenobarbital injections). Carbon tetrachloride (Mallinkrodt, St. Louis, Missouri; analytical reagent grade) was administered as a solution in mineral oil, 1:1 ratio, by gastric intubation at a dosage of 2.5 ml/kg body weight. Control animals were given equal volumes of mineral oil alone. Animals were sacrificed one hour post carbon tetrachloride administration.

Preparation of microsomes

Under ether anesthesia the livers were perfused in situ with 0.9 percent saline via the thoracic aorta. The blanched livers were removed, weighed in tared beakers, and homogenized in 3.4 volumes of 0.1 M potassium phosphate buffer, pH 7.4. The supernatant obtained from a 10,000 x g spin was sedimented at 105,000 x g for 1 hour in a Beckman L-50 ultracentrifuge with a titanium 60 fixed angle rotor. Pellets were resuspended in 15 volumes of 50 mm Tris-HCl buffer, pH 7.4 containing 175 mm KCl and resedimented at 105,000 x g for 1 hour. The final pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4. Protein was measured as described by Lowry et al. (36) using bovine serum albumin as standards.

Carbon tetrachloride-induced lipid peroxidation with in vitro microsomal incubations

Microsomes were prepared from 14-16 hour fasted experimental (phenobarbital pretreated) or control (saline pretreated) animals as described in the previous paragraph. The final microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.4 and following protein determinations were diluted to 2.0 mg protein per ml and incubated with the following additions: potassium phosphate buffer (controls); nicotinamide adenine dinucleotide phosphate (reduced form) 570 um final concentration (Sigma, St. Louis, Missouri); carbon tetrachloride and NADPH at 1.4 percent and 570 um final concentrations, respectively; and FeADP and NADPH additions with final concentrations of 286 um ADP, 857 um ferric ion and 570

um NADPH. Final protein concentrations after additions were made were 1.02 mg/ml in a total volume of 45 mls per incubation. The reaction was started by the addition of the NADPH and the flasks were placed in a 37°C water bath and incubated for 20 minutes with constant shaking. Following incubation aliquots were removed to ascertain whether peroxidation had occurred by the thiobarbituric acid assay. Microsomal aliquots (0.8 ml) were mixed with 0.4 ml of a trichloroacetic acid-HCl solution (50 percent TCA in 5.0 M HCl). The protein was precipitated on ice for 10 minutes and sedimented with a 10 minute centrifugation (800 x g) at 4°C. The supernatant (0.5 ml) was then assayed for malonaldehyde production, a by-product of lipid peroxidation, by the addition of 1.0 ml of a 1.0 percent solution of thiobarbituric acid (Eastman, Rochester, New York) and incubated at 100°C for 10 minutes for chromagen development. After cooling to room temperature samples were read at 535 nm.

The remainder of the microsomal suspensions were diluted with iced buffer and centrifuged at 105,000 x g for 1 hour. The pellets were scraped into glass tubes for lipid extraction.

Lipid extraction and purification

Pellets from the in vivo and in vitro microsomes were extracted in 20 volumes of a chloroform-methanol solution (37) dispersed with a Polytron homogenizer (Brinkman, Westbury, New York) and filtered through Whatman #1 Phase Separators, (Whatman, Clifton, New Jersey). The filtrate was washed with 0.9 percent aqueous saline, 10 percent

by volume. The lower, organic phase was filtered through a Fluoropore filter (Millipore Corp., Bedford, Massachusetts) with a 1.0 um diameter pore in a Swinnex, 13 mm holder. Phospholipid phosphorous was determined by the method of Bartlett (38). Lipids were stored in 0.01 percent BHT at -20°C for periods which did not exceed 10 days.

Lipid peroxidation was quantitated by determining diene conjugation absorption spectra at 232-234 nm in a Cary 118 double beam spectrophotometer using hexane as the reference solution and as the lipid solvent. The amount of diene conjugates per mg phospholipids, that is, the specific activity, was calculated by dividing the optical density at 232-234 nm by the mg of phospholipid present in the sample.

Phospholipids were purified by high performance liquid chromatography (HPLC) using a Hewlett-Packard 1084A chromatograph equipped with a Si60 stainless steel column (Hewlett-Packard, Palo Alto, CA). Phospholipids were eluted by a slight modification of the method of Geurts Van Kessel et al. (39) employing a linear gradient ranging from 100 percent solvent A (hexane:propanol:water, in the ratio of 6:8:0.75) at 0 minutes to 100 percent solvent B (hexane:propanol:water, in the ratio of 6:8:1.5) at 5 minutes. The flow rate was 1.5 mls/minute run at room temperaturs. The effluent was monitored at 206 nm and fractions containing the various phospholipids were collected and identified by comparing retention times to phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and

phosphatidylcholine (Supelco, Bellefonte, PA) as well as comparing $R_f$ values on thin layer chromatography.

Fatty acid analysis

Methylation was accomplished by placing 1.0 ml of the phospholipid solution into a 10 ml vial with a teflon lined screw cap. To each aliquot 1.0 ml of benzene was added, swirled and followed by 1.0 ml of methanolic base reagent (Supelco). The vials were sealed and heated in an 80°C water bath for 15 minutes. When the solutions cooled to room temperature, 3.0 mls each of distilled, deionized water and diethylether were added and vortexed. The top layer was washed in 2.0 mls of water, dried over anhydrous sodium sulfate, and evaporated under a nitrogen stream in a Meyer N-Evap analytical evaporator (Organomation, Northborough, MA). The aliquots were analyzed in a Hewlett-Packard 5830A gas chromatograph equipped with a glass column, 1/8 inch I.D., with 10 percent SP-2330 on a 100/120 Chromosorb WAW support (Supelco).

Column temperature at the beginning of the run was 170°C, the flow rate was 20 ml/minute with helium as the carrier gas. Detection was by flame ionization. After 16 minutes column temperature was increased to 200°C at a rate of 30°C/minute for the remainder of the run. Peaks were identified by palmitic, stearic, oleic, arachidonic, docosahexenoic and eicosatrienoic fatty acid standards (Supelco).

Identification of phospholipids by thin layer chromatography

Silica G plates (Merck, Darmstadt) were activated at 100°C for 1 hour. The solvent system (40) contained chloroform:methanol: water:acetic acid in the ratio of 25:15:4:2. Additional thin layer chromatography using a slight modification of a method previously reported (39) employed hexane:2-propanol:water in the ratio of 6:8: 1.0. Plates were stained for primary amines (ninhydrin), phosphorous and lipids (rhodamine B).

# RESULTS

## Effects of phenobarbital administration on the liver

Phenobarbital administered intraperitoneally once daily for four consecutive days in the dosage of 50 mg/kg body weight resulted in a 20 percent gain in total liver weight with a 60 percent increase in the amount of microsomal protein with a concomitant increase of 100 percent in microsomal phospholipids (Table 1).

## Effects of phenobarbital administration on the phospholipid composition of the microsomes

The phospholipid composition of the microsomes was found to change with phenobarbital administration. The percentage of phosphotidylethanolamine in the microsomal phospholipids increased by approximately 4 percent with phenobarbital administration. This is demonstrated with microsomes extracted directly from treated animals (Table 2) as well as from phenobarbital-pretreated animals in which the endoplasmic reticulum was purified into microsomes that were incubated *in vitro* (Table 10).

Phenobarbital administration also results in a slight decrease in the proportions of phosphatidylinositol, phosphatidylserine and phosphatidylcholine in the liver microsomal phosphlolipids (Table 2). It appears that the increase in the phosphatidylethanolamine fraction results in a decreased proportion in the percentages of phosphatidylinositol and phosphatidylserine (Table 8).

Table 1 The effect of phenobarbital administration on rat liver protein and phospholipid content

| | Control | Phenobarbital |
|---|---|---|
| Liver size (percentage of total body weight) | 3.2 ± 0.2 | 3.75 ± 0.3 |
| Microsomal protein (mg/g) | 16.2 ± 1.2 | 21.80 ± 1.9 |
| Microsomal protein/liver (250 g rat) | 129.6 ± 10.8 | 207.0 ± 12.3 |
| Phospholipid (mg PL/mg microsomal protein) | 0.5 ± 0.02 | 0.6 ± 0.04 |
| Microsomal phospholipid (total mg PL/liver) | 62.2 ± 2.7 | 120.1 ± 5.3 |

Male Sprague-Dawley rats were either given intraperitoneal injections of phenobarbital (50mg/kg body weight) or saline daily for 4 days prior to experimentation. Animals were starved 14-16 hours prior to sacrificing. Livers were perfused with physiological saline via the thoracic aorta while the animals were under ether anesthesia. Blanched livers were weighed in tared beakers containing iced phosphate buffer, pH 7.4, minced and homogenized with a teflon pestled motor driven homogenizer. After removing cell debris (10,000 x g for 10 minutes) the supernatant was centrifuged at 105,000 x g for one hours. The pellet was washed with 15 volumes of 50 mm Tris-HCl buffer, pH 7.4 containing 175 mm KCl and resedimented at 105,000 x g for one hour. The final pellet was taken up in phosphate buffer. Aliquots were removed for protein determination. Lipids were extracted with chloroform: methanol and phospholipid phosphorous determined (see Experimental Approach section for details).

Table 2 Phospholipid composition of control and phenobarbital induced microsomes In Vivo[a]

| | phosphatidylethanolamine | phosphatidylinositol | phosphatidylserine | phosphatidylcholine |
|---|---|---|---|---|
| Control | 23.7 ± 0.20[b] | 12.1 ± 0.16[b] | 3.2 ± 0.08[b] | 61.6 ± 1.3[b] |
| Phenobarbital | 28.1 ± 0.23* | 11.8 ± 0.21 | 2.0 ± 0.18 | 57.6 ± 1.0 |

(a) Animals were treated with either phenobarbital or saline and microsomes prepared as described in Table 1. The lipids were extracted in chloroform:methanol (2:1) and the solvent system was replaced with hexane. The total lipid extract was injected into the HPLC and eluted with an increasingly polar solvent system. The fractions containing the various phospholipids were collected, checked for purity on TLC and the phosphorus determined (see Experimental Approach section for details).

(b) Each value represents the percentage of the total phospholipid eluate.

* $P<0.01$ when compared to control PE

Table 3 Fatty acid changes in total microsomal phospholipids following phenobarbital administration(a)

| Fatty Acids | | Controls | Phenobarbital |
|---|---|---|---|
| Palmitic | (16:0) | 15.8 ± 0.05(b) | 15.6 ± 0.06(b) |
| Stearic | (18:0) | 25.4 ± 0.04 | 25.8 ± 0.07 |
| Oleic | (18:1) | 5.7 ± 0.03 | 5.6 ± 0.04 |
| Linoleic | (18:2) | 9.3 ± 0.08 | 11.8 ± 0.09* |
| Arachidonic | (20:4) | 26.7 ± 0.10 | 24.5 ± 0.15* |
| Docosahexenoic | (22:6) | 9.0 ± 0.15 | 7.2 ± 0.08* |

(a) Animals were injected with either phenobarbital or saline. Microsomes were prepared and the lipids extracted (see Table 1). The total phospholipid extract was transesterified and the methylated fatty acids were analyzed by GLC.

(b) Figures represent the percentage of each fatty acid in the total microsomal lipid extract.

* $P < 0.01$ when compared to control values

The phospholipid composition of the microsomes was influenced also by *in vitro* incubations. There was a slight but definite loss of phosphatidylethanolamine from the uninduced control and the phenobarbital-induced microsomes (Table 10), the loss being somewhat greater for the phenobarbital-pretreated animals.

Phosphatidylcholine was also recovered in decreased amounts from the control microsomes incubated *in vitro* compared to recoveries of phospholipids from *in vivo* experiments. The loss represented approximately 5 percent of the phosphatidylcholine fraction (Table 10 vs. Table 2). No similar loss of phosphatidylcholine was observed for microsomes prepared from phenobarbital-pretreated animals.

The loss of phosphatidylethanolamine resulted in an increased proportion of phosphatidylinositol and phosphatidylserine in both control and phenobarbital-induced membranes. The additional loss of phosphatidylcholine from *in vitro* control microsomes resulted in an even greater proportion of phosphatidylinositol and phosphatidylserine in these microsomes compared to the phenobarbital-pretreated membranes (Table 10).

Effects of phenobarbital administration on the fatty acid composition of the microsomal phospholipids

The changes in total fatty acid composition of the microsomal membrane with phenobarbital administration demonstrates a 30 percent increase in linoleic acid, accompanied by a 14 percent decrease in arachidonic acid (20:4) and a 20 percent decrease in docosahexenoic acid (22:6). The saturated:unsaturated ratio of fatty acid constit-

Table 4 Fatty acid changes in purified microsomal phospholipids after phenobarbital administration[a]

| | Fatty Acids | | | | | |
|---|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 | 20:4 | 22:6 |
| PE | | | | | | |
| Control | 12.2±1.2[b] | 27.7±2.4 | 4.5±0.3 | 7.4±0.6 | 28.6±1.1 | 11.1±1.8 |
| Phenobarbital | 16.1±1.2* | 27.1±1.5 | 5.5±0.4 | 10.5±0.8* | 23.8±1.4* | 9.5±0.7 |
| PI | | | | | | |
| Control | 4.3±0.2 | 42.1±0.9 | 1.9±0.6 | 1.6±0.1 | 35.1±0.8 | 4.8±0.1 |
| Phenobarbital | 4.2±0.5 | 42.2±2.9 | 2.5±0.2 | 3.8±0.4 | 34.5±3.3 | 4.0±0.7 |
| PS | | | | | | |
| Control | 6.8±0.4 | 44.0±2.4 | 4.2±0.5 | 3.2±0.6 | 21.5±0.8 | 11.2±2.4 |
| Phenobarbital | 9.8±0.5 | 45.4±2.8 | 3.8±0.2 | 5.3±0.3 | 18.1±0.8* | 8.2±0.7 |
| PC | | | | | | |
| Control | 18.5±1.2 | 26.8±0.5 | 6.2±2.5 | 12.0±0.5 | 27.8±1.0 | 6.6±0.5 |
| Phenobarbital | 16.9±0.8 | 27.2±0.5 | 6.1±0.4 | 14.0±0.9 | 25.4±0.6 | 5.5±0.5 |

(a) Lipids were extracted and purified from the microsomes of control and phenobarbital pretreated animals as previously described (see Table 2). The purified phospholipid fractions were methylated and analyzed with GLC.

(b) Figures represent the percentage of each fatty acid present in the purified phospholipid fractions.

* $P < 0.05$ when compared to control values

Table 5 The saturated:unsaturated fatty acid ratios in individual phospholipids after phenobarbital administration[a]

| Phospholipid | Control | Phenobarbital |
|---|---|---|
| Phosphatidylethanolamine | 0.84[b] | 0.83[b] |
| Phosphatidylinositol | 1.07 | 1.09 |
| Phosphatidylserine | 0.95 | 1.21 |
| Phosphatidylcholine | 0.85 | 0.82 |

(a) Purified phospholipids from phenobarbital pretreated animals and controls were extracted from the microsomes, methylated and analyzed by GLC as previously described (see Table 4).

(b) Ratios were obtained by dividing the percent of saturated by the percent of unsaturated fatty acids for each phospholipid.

Table 6 Comparison of phospholipid components of microsomes prepared from rats following phenobarbital administration and carbon tetrachloride challenge - In Vivo

| | PE | PI | PS | PC |
|---|---|---|---|---|
| Control | 23.7 ± 0.2[b] | 12.1 ± 0.16[b] | 3.2 ± 0.08[b] | 61.6 ± 1.3[b] |
| Control/$CCl_4$ | 21.6 ± 0.26 | 14.1 ± 0.22 | 4.3 ± 0.12 | 61.1 ± 1.8 |
| Phenobarbital | 28.1 ± 0.23 | 11.8 ± 0.21 | 2.0 ± 0.18 | 57.6 ± 1.0 |
| Phenobarbital/$CCl_4$ | 27.7 ± 0.31 | 13.8 ± 0.28 | 2.5 ± 0.23 | 56.1 ± 1.8 |

(a) Rats were either given phenobarbital or saline injections. Fasted animals were given either carbon tetrachloride in a 1:1 solution of mineral oil by means of gastric intubation (2.5 ml $CCl_4$/kg body weight) or mineral oil alone. After one hour the livers were processed and the lipids were extracted from the purified microsomes. Phospholipids were purified by HPLC and quantitated by phosphorous determinations. (See Experimental Approach for details).

(b) Figures represent the percent contribution of each phospholipid fraction in the total lipid eluate.

uents of purified phospholipids in phenobarbital-induced endoplasmic reticulum reflects an increase in saturation in the phosphatidylserine fraction, while the other phospholipids maintain the same ratio observed in the uninduced control microsomes (Table 5).

The fatty acid changes are reflected to a greater extent in the purified phospholipids. There is a general increase in linoleic acid in all phospholipid fractions with phenobarbital administration, however, the extent of increase varies with each phospholipid. The increase in linoleic acid ranges from a 70 percent increase in phosphatidylinositol to a 20 percent increase in phosphatidylcholine, with phosphatidylethanolamine and phosphatidylserine reflecting a 35 and 40 percent increase, respectively (Table 4).

With the exception of phosphatidylinositol there is a general decrease in the polyunsaturated fatty acids, arachidonic and docosahexenoic. The decrease varies depending on the particular phospholipid. The decrease in these polyunsaturated fatty acids is greatest in phosphatidylethanolamine and phosphatidylserine, while the decrease in phosphatidylcholine is slight (Table 4).

Effect of carbon tetrachloride challenge on the phospholipid composition of uninduced control membranes

The changes in the proportions of phospholipids with carbon tetrachloride challenge which are observed *in vitro* (Table 11), while more marked than the *in vivo* system (Table 6), follow the same pattern. There is an approximate 40 percent loss of phosphatidylethanolamine from microsomes incubated *in vitro* compared with a 10

Table 7 A comparison of the fatty acid composition of purified phospholipids of control and phenobarbital-induced microsomes after carbon tetrachloride-induced peroxidation - In Vivo[a]

| | Control | Control $CCl_4$ | % Decrease | PB | PB $CCl_4$ | % Decrease |
|---|---|---|---|---|---|---|
| PE | | | | | | |
| (20:4) | 0.29 ± 0.03[b] | 0.25 ± 0.02[b] | 15 | 0.24 ± 0.01[b] | 0.23 ± 0.01[b] | 5 |
| (22:6) | 0.12 ± 0.01 | 0.11 ± 0.01 | 10 | 0.11 ± 0.01 | 0.10 ± 0.01 | 10 |
| PI | | | | | | |
| (20:4) | 0.37 ± 0.01 | 0.32 ± 0.02* | 15 | 0.35 ± 0.02 | 0.31 ± 0.10 | 10 |
| (22:6) | 0.04 ± 0.01 | 0.02 ± 0.001 | 30 | 0.03 ± 0.001 | 0.03 ± 0.01 | 13 |
| PS | | | | | | |
| (18:0) | 0.36 ± 0.01 | 0.34 ± 0.03 | 10 | 0.38 ± 0.01 | 0.26 ± 0.01 | 30 |
| (20:4) | 0.26 ± 0.001 | 0.20 ± 0.03* | 25 | 0.21 ± 0.01 | 0.09 ± 0.02** | 60 |
| (22:6) | 0.12 ± 0.01 | 0.08 ± 0.01 | 35 | 0.12 ± 0.02 | 0.04 ± 0.01** | 70 |
| PC | | | | | | |
| (20:4) | 0.27 ± 0.02 | 0.26 ± 0.02 | - | 0.25 ± 0.01 | 0.24 ± 0.01 | 5 |
| (22:6) | 0.07 ± 0.10 | 0.05 ± 0.01 | 40 | 0.05 ± 0.01 | 0.08 ± 0.01 | 30 |

(a) Animals were either pretreated with phenobarbital or saline and ingested either carbon tetrachloride - mineral oil or mineral oil alone. One hour post-ingestion the rats were sacrificed, the microsomes purified and the lipids extracted. Each purified phospholipid was methylated and subsequent fatty acid analysis performed.

(b) Figures represent the fraction of each fatty acid found in each purified phospholipid.

* $P < 0.05$ when compared to control values

** $P < 0.01$

Table 8 Comparison of phospholipids from control and phenobarbital-induced endoplasmic reticulum - In Vivo[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| Control | 231 ± 20[b] | 121 ± 15[b] | 36 ± 9[b] | 616 ± 17[b] |
| Control-$CCl_4$ | 216 ± 25 | 141 ± 20 | 43 ± 10 | 670 ± 20 |
| Phenobarbital | 288 ± 20 | 118 ± 20 | 28 ± 5 | 676 ± 26 |
| Phenobarbital-$CCl_4$ | 277 ± 24 | 138 ± 26 | 32 ± 10 | 561 ± 25 |

(a) Rats were pretreated and the microsomes purified and extracted as previously outline (see Table 7).

(b) Figures represent micrograms of phospholipid per milligram of the total microsomal phospholipid.

Table 9 Specific activity of peroxidation in phospholipid fractions with phenobarbital-induced endoplasmic reticulum challenged with carbon tetrachloride - In Vivo[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| Control | 0.44 ± 0.02[b] | 0.44 ± 0.14[b] | 1.18 ± 0.30[b] | 0.21 ± 0.06[b] |
| Control $CCl_4$ | 0.53 ± 0.16 | 0.74 ± 0.28 | 4.33 ± 1.84* | 0.55 ± 0.13 |
| Phenobarbital | 0.73 ± 0.22 | 0.90 ± 0.28 | 3.04 ± 0.93 | 0.33 ± 0.10 |
| PB $CCl_4$ | 0.67 ± 0.11 | 1.25 ± 0.21 | 8.28 ± 1.37* | 0.89 ± 0.15 |

(a) Rats were pretreated with phenobarbital and were given carbon tetrachloride via gastric intubation (controls were given mineral oil). Livers were subsequently processed as described in Table 6. Purified phospholipids were scanned at 232-234 nm to obtain diene absorption quantitation.

(b) Specific activities were obtained by dividing the absorbance at 232-234 nm by the milligrams of phospholipid per sample.

* $P < 0.01$

Table 10 The effect of phenobarbital administration on phospholipid composition of microsomes - In Vitro[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| Control | 21.6 ± 0.68[b] | 15.5 ± 0.59[b] | 4.4 ± 0.45[b] | 56.5 ± 1.6[b] |
| Phenobarbital | 25.4 ± 0.80* | 12.2 ± 0.71 | 4.0 ± 0.60 | 57.4 ± 1.3 |

(a) Rats were either injected with saline (controls) or phenobarbital (50 mg/kg) I.P. for 4 days. Fasted (14-16 hrs.) animals were anesthetized and the livers perfused in situ with saline. Blanched livers were weighed in tared beakers with phosphate buffer, homogenized in a teflon-pestled glass homogenizer. The microsomes were purified with differential centrifugation (see Experimental Approach section for details). The purified microsomes were suspended in phosphate buffer at a concentration of 2 mg protein/ml. The microsomal suspensions were incubated with buffer additions to bring the final concentration to 1 mg protein /ml. Flasks were swirled in a water bath at 37°C for 20 min. Aliquots were removed for TBA assay. The reaction was stopped by the addition of cold buffer. Microsomes were pelleted at 105,000 x g for 1 hour. The pellets were scraped into glass vials and the lipids extracted in chloroform:methanol (2:1). After filtering the lipids were separated by HPLC and quantitated by phosphorous determinations.

(b) Figures represent the phospholipid phosphorous per phospholipid fraction in the total HPLC eluate, expressed as a percentage of the total.

* $P < 0.05$ when compared to control values

percent loss of phosphatidylethanolamine from microsomal lipids when carbon tetrachloride is administered in vivo. The effect of carbon tetrachloride administration on the phospholipid proportions in the microsomes is to decrease the phosphatidylethanolamine content both in vivo and in vitro and this loss is reflected in the greater representation of the phosphatidylinositol and phosphatidylcholine fractions (Tables 6 and 11). While the phosphatidylserine fraction increases in proportion to the loss of phosphatidylethanolamine with carbon tetrachloride challenge in vivo (Table 6), some phosphatidylserine may be lost with the in vitro experiments since the phosphatidylserine fraction does not reflect the changes in phosphatidylethanolamine concentration as does the phosphatidylinositol fraction (Table 11).

Susceptibility of phospholipids to peroxidative changes with carbon tetrachloride administration - Uninduced control membranes

Phosphatidylserine had the highest specific acitivity for peroxidation followed by phosphatidylinositol, with phosphatidylethanolamine and phosphatidylcholine being least affected. This pattern of peroxidation was observed both in vivo (Table 9) as well as in vitro (Table 17).

When carbon tetrachloride was administered in vivo, there was an approximate 4-fold increase in peroxidation over endogenous levels in the phosphatidylserine fraction, while the other phospholipids demonstrated a decreased response; a 2-fold (PC) or less, 1.7-fold (PI), and a 1.25-fold increase (PE) (Table 9).

Table 11 The effect of carbon tetrachloride, NADPH, FeADP/NADPH and buffer on the phospholipid composition of microsomes from control animals - In Vitro[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| Buffer | 21.6 ± 0.68 | 15.5 ± 0.59 | 4.40 ± 0.45 | 56.5 ± 1.62 |
| $CCl_4$/NADPH | 13.2 ± 1.52* | 20.0 ± 1.92 | 3.91 ± 0.67 | 62.8 ± 6.04 |
| NADPH | 15.0 ± 1.32* | 19.7 ± 1.40 | 3.80 ± 0.86 | 60.2 ± 4.51 |
| FeADP/NADPH | 15.5 ± 1.73* | 19.9 ± 1.36 | 2.88 ± 0.71 | 61.7 ± 4.87 |

(a) Animals were injected with saline for 4 days. All subsequent steps for microsome purification were carried out as previously described (see Table 10). Microsomal suspensions (2 mg protein/ml) were incubated with buffer, carbon tetrachloride, NADPH or FeADP/NADPH so that the final protein concentration was 1 mg/ml. The reaction was started with the addition of NADPH. All concentrations were as previously described (see Experimental Approach section). After 20 minutes incubation at 37°C in a shaking water bath the microsomes were pelleted and the lipids extracted and purified by HPLC.

(b) Figures represent the phospholipid phosphorous per phospholipid fraction as a percentage of the total HPLC eluate.

* $P < 0.01$ when compared to control values

Purified microsomes, challenged with carbon tetrachloride resulted in a 6-fold increase in peroxidation over endogenous levels in phosphatidylserine in vitro, however, the increases in the peroxidation of the other phospholipids had increased to disproportionately higher levels; phosphatidylcholine demonstrated an 8-fold increase and phosphatidylinositol and phosphatidylethanolamine had a 5 and 6-fold increase, respectively.

The peroxidative activity is higher in vitro and somewhat generalized by the greater increases in peroxidation occurring in phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine compared to the increase observed in phosphatidylserine. When the in vivo data is compared to the in vitro results it appears that the response of the endoplasmic reticulum to carbon tetrachloride challenge is more specific to the phosphatidylserine in vivo. While total peroxidative damage to the phosphatidylserine fraction in vitro was only 2-fold higher (Table 17) than in vivo (Table 9), the other phospholipids experienced a 3-fold or higher increase in peroxidation.

Changes in the phospholipid composition with carbon tetrachloride challenge in phenobarbital-induced membranes

There are some changes in the proportions of phospholipids with carbon tetrachloride challenge which are observed in vivo (Table 2) which differ with the in vitro pattern (Table 9). While only 5 percent loss of phosphatidylethanolamine was observed when carbon tetrachloride was administered in vivo (Table 2), a 45 percent loss of this phospholipid occurred when carbon tetrachloride challenge was

carried out in vitro (Table 9). It appears that phosphatidylethanolamine from the phenobarbital-induced endoplasmic reticulum is lost to the same degree or less when compared with control membranes when carbon tetrachloride challenge occurs in vivo. However, once the phenobarbital-induced endoplasmic reticulum is purified into microsomes and then challenged with carbon tetrachloride in vitro, phosphatidylethanolamine loss exceeds the loss occurring in microsomes prepared from control rats (Tables 11 and 12). The loss of phosphatidylethanolamine resulted in an increased proportion of phosphatidylinositol and phosphatidylserine, however, since the phenobarbital-induced microsomes did not lose phosphatidylcholine during microsomal incubation (Table 10) the phosphatidylinositol and phosphatidylserine increase was not as great as the control microsomes demonstrated.

Susceptibility of phospholipids to peroxidative changes with carbon tetrachloride administration - Phenobarbital-induced membrane

The phenobarbital-induced membranes have a higher endogenous level of peroxidation than uninduced control membranes, both in vivo (Table 9) as well as in vitro (Table 18).

As in the control membranes in vivo, carbon tetrachloride challenge resulted in phosphatidylserine having the highest specific activity for peroxidation, followed by phosphatidylinositol, phosphatidylcholine with phosphatidylethanolamine being unaffected (Table 9). In vivo, carbon tetrachloride administration results in an approximate 3-fold increase in peroxidation over endogenous levels, both in the phosphatidylserine and phosphatidylcholine fractions with only a 1.3-

Table 12 The effect of carbon tetrachloride, NADPH, FeADP/NADPH and buffer on the phospholipid composition of microsomes from phenobarbital-pretreated animals - In Vitro[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| PB-Buffer | 26.4 ± 0.87 | 12.2 ± 0.71 | 4.02 ± 0.60 | 57.4 ± 1.31 |
| PB-$CCl_4$/NADPH | 14.6 ± 2.08** | 21.1 ± 1.36 | 3.20 ± 1.04 | 61.1 ± 5.03 |
| PB-NADPH | 20.4 ± 1.65* | 18.7 ± 1.21 | 3.96 ± 0.98 | 57.0 ± 4.53 |
| PB-FeADP/NADPH | 19.0 ± 2.20* | 16.8 ± 1.82 | 4.75 ± 1.22 | 59.5 ± 5.36 |

(a) Rats were injected with phenobarbital (50 mg/kg) for 4 days. Microsomes were prepared, incubated and the lipids extracted and purified as previously described (see Table 11).

(b) Figures represent the phospholipid phosphorous per phospholipid fraction expressed as the percentage of the total HPLC eluate.

* $P < 0.05$

** $P < 0.01$

Table 13 The influence of phenobarbital administration on the purified phospholipids recovered from microsomes after in vitro incubation [a]

| | | | | |
|---|---|---|---|---|
| Control | 210 ± 22[b] | 161 ± 15[b] | 65.3 ± 18[b] | 567 ± 20[b] |
| Phenobarbital | 260 ± 15 | 160 ± 21 | 62.2 ± 17 | 582 ± 15 |

(a) Rats were treated and lipids were extracted from microsomes incubated as described (see Table 10).

(b) Figures represent the micrograms of purified phospholipids per milligram of total phospholipid HPLC eluate.

Table 14 The influence of carbon tetrachloride, NADPH, FeADP/NADPH and buffer on the quantities of phospholipids from control microsomes after in vitro incubation[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| Buffer | 210 ± 22[b] | 161 ± 15[b] | 65 ± 18[b] | 567 ± 20[b] |
| $CCl_4$/NADPH | 151 ± 36 | 170 ± 28 | 53 ± 12 | 619 ± 17 |
| NADPH | 182 ± 19 | 180 ± 19 | 48 ± 15 | 601 ± 15 |
| FeADP/NADPH | 162 ± 21 | 165 ± 34 | 49 ± 14 | 620 ± 24 |

(a) Rats were treated and microsomes prepared, incubated and extracted as described (see Table 11).

(b) Figures represent micrograms of phospholipid per milligram of total HPLC phospholipid eluate.

Table 15 The influence of carbon tetrachloride, NADPH, FeADP/NADPH on the quantities of phospholipids recovered from phenobarbital-induced microsomes after in vitro incubation[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| PB-Buffer | 244 ± 15[b] | 150 ± 21[b] | 58 ± 17[b] | 547 ± 15[b] |
| PB-$CCl_4$/NADPH | 160 ± 28 | 173 ± 28 | 49 ± 10 | 617 ± 19 |
| PB-NADPH | 212 ± 22 | 157 ± 34 | 55 ± 14 | 586 ± 21 |
| PB-FeADP/NADPH | 191 ± 32 | 170 ± 22 | 53 ± 9 | 590 ± 12 |

(a) Rats were treated with phenobarbital for 4 days prior to experimentation. The livers were removed, weighed, homogenized, and the microsomes purified by differential centrifugation. Following incubation, the microsomes were pelleted and the lipids extracted (see Experimental Approach section for details), and purified.

(b) Figures represent micrograms of phospholipid per milligram total HPLC phospholipid eluate + one standard deviation.

fold increase in phosphatidylinositol while no peroxidation was apparent in the phosphatidylethanolamine fraction (Table 9). The high levels of endogenous peroxidation present in the in vivo membrane may have influenced the differences of susceptibility of phospholipids to carbon tetrachloride metabolism which resulted in lower increases in peroxidation, although the relatively lower endogenous peroxidation levels present in the phosphatidylethanolamine fraction did not result in a higher degree of specific activity of peroxidation when carbon tetrachloride was administered (Table 9).

In terms of the specificity of the phenobarbital-induced endoplasmic reticulum response to carbon tetrachloride challenge in vivo, phosphatidylcholine appears to be equally susceptible to peroxidation as phosphatidylserine, since both had a 3-fold increase in peroxidation over endogenous levels. Phosphatidylcholine in both the uninduced and phenobarbital-induced endoplasmic reticulum responds to carbon tetrachloride administration to an equal extent demonstrating a 2.7-fold increase in peroxidation over endogenous levels. However, phosphatidylserine demonstrates a high level of endogenous peroxidation in the phenobarbital-induced membrane and its response to carbon tetrachloride challenge may be compromised by these pre-existing high levels of peroxidized lipids. Consequently, the in vivo response to carbon tetrachloride administration in the induced membrane is less than the uninduced controls (Table 9) as well as less specific in peroxidative response with carbon tetrachloride metabolism.

When microsomes are prepared from phenobarbital-pretreated animals and are exposed to carbon tetrachloride in vitro, phosphati-

dylserine is peroxidized to the greatest extent, while phosphatidylinositol and phosphatidylcholine are equally affected with phosphatidylethanolamine having the lowest specific activity of peroxidation (Table 18). Phosphatidylserine demonstrated a 4-fold increase in peroxidation over endogenous levels, phosphatidylcholine a 6-fold increase while a 3-fold increase in the phosphatidylinositol and phosphatidylethanolamine fractions was observed (Table 18). Thus the phenobarbital-induced microsomes challenged with carbon tetrachloride exhibit a lower activity of lipid peroxidation than the *in vitro* controls (Table 17), however, the pattern of peroxidation to the *in vitro* controls is similar; phosphatidylserine had the highest amount of peroxidation present, phosphatidylinositol and phosphatidylcholine are followed by phosphatidylethanolamine (Table 18).

The endogenous levels of peroxidation present in the phenobarbital-induced membranes was not as high as the levels present in the *in vivo* system (Table 9) and may have exerted little influence on the pattern of peroxidation observed in this group. There was a decrease in peroxidative activity present in the phenobarbital-induced microsomes (40 percent) which is greater than the higher initial levels of peroxidation present compared to control microsomes. The endogenous peroxidation levels in the induced microsome group, in particular phosphatidylserine, is only 15 percent higher than the phosphatidylserine fraction of the control microsomes yet the controls experienced a 6.5-fold increase in peroxidation with carbon tetrachloride challenge while the phosphatidylserine from the phenobarbital-induced microsomes had only a 4-fold increase (Tables 17

Table 16 Specific activity of endogenous peroxidation of phenobarbital-induced microsomal phospholipids compared with untreated control microsomes after in vitro incubation[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| Control | 0.32 ± 0.10 | 0.43 ± 0.15 | 1.25 ± 0.35 | 0.22 ± 0.10 |
| Phenobarbital | 0.43 ± 0.22 | 0.56 ± 0.20 | 1.49 ± 0.25 | 0.31 ± 0.23 |

(a) Rats were either injected with saline (controls) or phenobarbital (50 mg/kg) for 4 days prior to experimentation. Microsomes were purified and incubated in buffer for 20 minutes at 37°C, pelleted and the lipids extracted and purified (see Table 10). Each fraction was scanned at 232-234 nm for diene absorption.

(b) Figures represent the specific activity of peroxidation per sample which was determined by expressing the absorbance at 232 per mg phospholipid.

Table 17 The influence of carbon tetrachloride, NADPH and FeADP/NADPH on the specific activity of phospholipid peroxidation in control microsomes incubated in vitro[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| Buffer | 0.32 ± 0.10[b] | 0.43 ± 0.15[b] | 1.25 ± 0.35[b] | 0.22 ± 0.10[b] |
| $CCl_4$/NADPH | 1.87 ± 0.19** | 2.08 ± 0.14** | 8.02 ± 3.63*** | 1.72 ± 0.65** |
| NADPH | 1.33 ± 0.29** | 1.64 ± 0.46** | 5.50 ± 2.12** | 1.22 ± 0.37** |
| FeADP/NADPH | 1.25 ± 0.22** | 1.76 ± 0.14** | 6.06 ± 2.57** | 1.12 ± 0.38** |

(a) Rats were injected with saline for 4 days prior to experimentation. The livers were processed as previously described (see Table 11) and suspended in buffer (2 mg protein/ml). The appropriate additions were made and incubated for 20 min. at 37°C. Microsomes were pelleted, the lipids were extracted and purified as described (see Table 11). Each fraction was scanned at 232-234 nm for diene absorption.

(b) Figures represent the specific activity of peroxidation per sample which was determined by expressing the absorbance at 232 per milligram phospholipid.

** $P < 0.01$ when compared to control values.

*** $P < 0.001$

Table 18 The influence of carbon tetrachloride, NADPH, and FeADP on the specific activity of phospholipid peroxidation in phenobarbital-induced microsomes incubated in vitro[a]

| | PE | PI | PS | PC |
|---|---|---|---|---|
| PB-Buffer | 0.43 ± 0.22[b] | 0.56 ± 0.20[b] | 1.50 ± 0.25[b] | 0.31 ± 0.23[b] |
| PB-$CCl_4$/NADPH | 1.48 ± 0.28** | 1.76 ± 0.50** | 5.90 ± 1.40** | 1.74 ± 1.03** |
| PB-NADPH | 0.87 ± 0.13* | 1.15 ± 0.14* | 4.39 ± 1.41* | 0.71 ± 0.33* |
| PB-FeADP/NADPH | 0.81 ± 0.50 | 1.04 ± 0.40 | 1.94 ± 1.16 | 0.43 ± 0.20 |

(a) Rats were pretreated with phenobarbital (50 mg/kg) for 4 days prior to experimentation. The microsomes were purified, incubated, extracted and purified as for Table 17. Each fraction was scanned at 232-234 nm for diene absorption.

(b) Figures represent the specific activity of peroxidation per sample which was determined by expressing the absorbance at 232-234 nm per mg phospholipid.

* $P < 0.05$ when compared to control values

** $P < 0.01$

and 18).

When the specificity (as determined by the increase in peroxidation over endogenous levels) of the *in vitro* phenobarbital-induced system is compared to the *in vitro* controls, they are similar. Phosphatidylcholine has the highest increase in peroxidation, followed in decreasing order of peroxidation by phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol. Thus there is a more generalized peroxidative response to carbon tetrachloride challenge in both control and induced membranes *in vitro*.

In the phenobarbital-induced microsomes both the *in vivo* and *in vitro* system, when challenged with carbon tetrachloride resulted in the peroxidative destruction of the phospholipids with phosphatidylserine the most severely affected. *In vivo* phosphatidylinositol had the next highest level of peroxidation and was significantly more peroxidized than phosphatidylcholine (Table 9). However, when peroxidation occurs *in vitro*, the increased level of peroxidation in the phosphatidylcholine fraction results in phosphatidylinositol and phosphatidylcholine having the same degree of peroxidation (Table 18). The phosphatidylethanolamine recovered from *in vivo* carbon tetrachloride challenge had approximately the same specific activity as the phosphatidylethanolamine obtained from the controls which were exposed to mineral oil, indicating that some phosphatidylethanolamine present in the membranes is stable to carbon tetrachloride-induced peroxidation.

*In vitro*, the phosphatidylethanolamine fraction recovered from

microsomes challenged with carbon tetrachloride is peroxidized, although the specific activity in this fraction is the lowest for all the phospholipid fractions (Table 18).

In vivo carbon tetrachloride administration resulted in an approximate 3-fold increase in phosphatidylserine and phosphatidylcholine specific activities over control endogenous levels, while phosphatidylinositol had only a 30 percent increase and phosphatidylethanolamine demonstrated no increase at all (Table 9). The difference in the in vivo response in this group as compared with the uninduced control membranes could reflect that high endogenous levels of peroxidation present in the phenobarbital-induced endoplasmic reticulum could result in a lower percentage increase in peroxidation with carbon tetrachloride challenge. When a carbon tetrachloride challenge is presented to phenobarbital-induced microsomes in vitro phosphatidylcholine and phosphatidylethanolamine become more susceptible to peroxidation in vitro than do phosphatidylinositol and phosphatidylserine. The result is that phosphatidylcholine and phosphatidylserine have about the same increase in peroxidation in vitro, while phosphatidylethanolamine approached phosphatidylinositol in susceptibility.

When the total peroxidation of each phospholipid fraction which occurred in vivo is compared to peroxidation in vitro with carbon tetrachloride administration, phosphatidylserine is peroxidized to only 70 percent of the in vivo level (Table 9). Phosphatidylinositol in vitro has a 40 percent increase in peroxidation over the in vivo amounts while phosphatidylcholine and phosphatidylethanolamine dem-

onstrate a 2-fold increase in peroxidation with the *in vitro* challenge. The data suggest that carbon tetrachloride challenge *in vivo* results in the peroxidation of phosphatidylserine and phosphatidylinositol. *In vitro*, phosphatidylserine and phosphatidylinositol demonstrate a slight increase in peroxidation when challenged with carbon tetrachloride as opposed to phosphatidylcholine and phosphatidylethanolamine which peroxidize strongly *in vitro* yet have little or no increase *in vivo*.

The general peroxidizing agents NADPH and FeADP with NADPH were compared to the peroxidation resulting from carbon tetrachloride challenge *in vitro*, both in extent and specificity. Neither the electron donor (NADPH) nor the electron carrier (FeADP/NADPH) peroxidized the microsomal lipids to the same extent and in the same manner as carbon tetrachloride (Table 11). Phosphatidylethanolamine recovered from both the NADPH and FeADP/NADPH systems represented 70 percent of buffer-incubated controls, compared to a 60 percent recovery of phosphatidylethanolamine from carbon tetrachloride challenge. Unlike the phosphatidylethanolamine loss from carbon tetrachloride-challenged microsomes in which the phenobarbital-induced membranes lost 5 percent more phosphatidylethanolamine than control microsomes, when control or phenobarbital-induced microsomes are exposed to NADPH or FeADP/NADPH phosphatidylethanolamine is lost to the same extent (Table 12).

Specific activity of peroxidation in control and phenobarbital-induced microsomes with NADPH and FeADP/NADPH as the pro-oxidants

Phosphatidylserine was the most highly peroxidized phospholipid in both control (Table 17) and phenobarbital-induced (Table 18) membranes. However, the extent of peroxidation differed. The NADPH -stimulated peroxidation of phosphatidylserine from the induced membranes was only 80 percent of the control levels but fell to only 30 percent of control levels when the FeADP/NADPH system was employed (Table 18).

Phosphatidylinositol had the next highest response to NADPH and FeADP/NADPH, although the phenobarbital-induced membranes had only 75 percent and 55 percent, respectively, of the specific activity of control microsomes.

The phosphatidylethanolamine fraction recovered in both the control and phenobarbital-induced microsomes had a slightly higher specific activity than the phosphatidylcholine fraction, however, the peroxidative response of the phosphatidylethanolamine in the phenobarbital treated membranes was 30 percent less (Table 18) when compared to the specific activity of phosphatidylethanolamine from control, uninduced microsomes (Table 17).

Phosphatidylcholine had the lowest level of peroxidation. From the induced microsome this phospholipid had only 60 percent of the peroxidation of control microsomes when NADPH alone was employed, but when FeADP/NADPH was administered instead, the response of phosphatidylcholine in this membrane fell to 40 percent of the uninduced control level and, in fact, was not significantly greater than endogenous levels of peroxidation when microsomes are incubated in buffer alone

(Table 18).

The overall peroxidation of phenobarbital-induced microsomes with NADPH and FeADP/NADPH was significantly less than the peroxidation levels induced in control microsomes. Also, peroxidation in the individual phospholipid fractions recovered from both control and phenobarbital-induced membranes (Tables 17 and 18, respectively) demonstrated lower specific activities than that which occurred with carbon tetrachloride challenge.

The specificity of the peroxidative response, determined by the increase in peroxidation over endogenous levels, was approximately 4-fold higher for all phospholipids in the control, uninduced microsomes (Table 17). The higher endogenous levels of peroxidation present in the phenobarbital-induced membranes and the decrease in activity of peroxidation resulted in only a 2-fold increase in the specific activity of these phospholipids over endogneous levels, with the exception of phosphatidylcholine which was resistant to peroxidation in the presence of FeADP/NADPH. The fact that each phospholipid was peroxidized to an equal extent over endogenous levels indicates a lack of specificity of these agents to any one particular phospholipid, unlike the carbon-tetrachloride-induced peroxidation in which a hierarchy of phospholipid peroxidation was evident.

The changes in the fatty acid composition of the microsomal phospholipids with peroxidation

Phosphatidylserine was the most severly affected microsomal phos-

pholipid with *in vivo* losses of stearic (18:0), arachidonic (20:4), and docosahexenoic (22:6) being 2-fold greater in the phenobarbital stimulated membrane than in controls. The other phospholipids had peroxidative losses only in arachidonic and docosahexenoic acids. The *in vivo* pattern of fatty acid loss was consistent with both controls and induced membranes; with the exception of phosphatidylserine the control membranes had a slightly greater fatty acid loss than the phenobarbital-induced group (Table 7).

*In vitro* the degree of fatty acid loss in the phosphatidylserine fraction is less with the induced microsomes when compared with controls (Tables 20 and 21). The loss of fatty acids in also high in phosphatidylinositol and phosphatidylethanolamine with phosphatidylcholine being the least affected on a percentage decrease of the total amount.

Comparing the response of control and phenobarbital-induced microsomes to NADPH and FeADP/NADPH *in vitro*, the controls demonstrate a greater loss of fatty acids in response to these oxidants than the experimentals. Phosphatidylethanolamine and phosphatidylcholine of the induced microsomes are particularly resistant to the peroxidizing activity of NADPH and FeADP.

In comparing the fatty acid compositional changes with control and phenobarbital-induced membranes *in vivo* and *in vitro* systems, the data indicate that the controls are somewhat more effective in producing peroxidative destruction with carbon tetrachloride metabolism, and NADPH and FeADP/NADPH additions as well. The tendency of the

controls to have higher peroxidative response is enhanced when the *in vitro* system is employed. Phosphatidylserine was the most affected phospholipid, since the relatively few molecules present had the highest degree of fatty acid destruction and loss when compared with other phospholipids. The increase in phosphatidylcholine peroxidation was high but the destructive effects of the peroxidative agents used was distributed over many molecules. The *in vitro* system exposed the lipids to saturating amounts of carbon tetrachloride and to buffer effects which resulted in a more widespread response to peroxidative effects than that produced by the *in vivo* system. However, the pattern of peroxidation when carbon tetrachloride was employed *in vivo* or *in vitro* was similar in both systems and resulted in a hierarchy of phospholipid susceptibility (Table 22). Oxidants, NADPH and FeADP/NADPH, were found to be non-selective in their pro-oxidant attack of microsomal lipids *in vitro*.

Table 19 Comparison of fatty acid composition of purified phospholipids from control and phenobarbital-induced microsomes incubated in vitro[a]

| | 18:0 | 20:4 | 22:6 |
|---|---|---|---|
| PE | | | |
| Control | | 0.29 ± 0.01[b] | 0.11 ± 0.02[b] |
| Phenobarbital | | 0.24 ± 0.01* | 0.09 ± 0.01* |
| Net decrease (% control) | | 17 | 18 |
| PI | | | |
| Control | | 0.35 ± 0.01 | 0.05 ± 0.01 |
| Phenobarbital | | 0.34 ± 0.03 | 0.04 ± 0.01 |
| Net decrease (% control) | | 3 | 20 |
| PS | | | |
| Control | 0.44 ± 0.02 | 0.21 ± 0.01 | 0.11 ± 0.02 |
| PB | 0.45 ± 0.03 | 0.18 ± 0.01* | 0.08 ± 0.01 |
| Net decrease (% control) | - | 14 | 27 |
| PC | | | |
| Control | | 0.28 ± 0.01 | 0.06 ± 0.01 |
| PB | | 0.25 ± 0.01* | 0.05 ± 0.01 |
| Net decrease (% control) | | 10 | 10 |

(a) Rats were injected with either saline (controls) or phenobarbital for 4 days prior to experimentation, fasted for 14-16 hours before sacrifice and the microsomes were processed as described (see Table 10). Purified phospholipid eluates from HPLC were transesterified, and the methylated fatty acids analyzed on GLC.

(b) Fatty acid components are expressed as the fraction of the total lipids per sample; net decreases are given as the percent decrease of control quantity.

* $P < 0.05$ when compared to control values.

Table 20 Comparison of fatty acid composition in phospholipid fractions from control microsomes after exposure to buffer, carbon tetrachloride, NADPH or FeADP/NADPH in vitro[a]

| Phospholipid | Fatty Acids and Associated Decrease | | | | | |
|---|---|---|---|---|---|---|
| | 18:0 | % dec. | 20:4 | % dec. | 22:6 | % dec. |
| PE - buffer | | | 0.29±0.01 | -- | 0.11±0.02 | -- |
| - CCl4/NADPH | | | 0.23±0.03 | 20 | 0.07±0.01 | 32 |
| - NADPH | | | 0.24±0.02 | 17 | 0.08±0.01 | 29 |
| - FeADP/NADPH | | | 0.24±0.02 | 17 | 0.08±0.01 | 29 |
| PI - buffer | | | 0.35±0.01 | -- | 0.05±0.01 | -- |
| - $CCl_4$/NADPH | | | 0.27±0.02* | 20 | 0.03±0.01 | 40 |
| - NADPH | | | 0.34±0.03 | 3 | 0.03±0.01 | 40 |
| - FeADP/NADPH | | | 0.32±0.04 | 10 | 0.03±0.01 | 40 |
| PS - buffer | 0.44+0.01 | -- | 0.21±0.01 | -- | 0.11±0.02 | -- |
| - $CCl_4$/NADPH | 0.34+0.03 | 23 | 0.10±0.02** | 50 | 0.03±0.02** | 75 |
| - NADPH | 0.44±0.02 | 0 | 0.16±0.02 | 22 | 0.06±0.01 | 45 |
| - FeADP/NADPH | 0.40±0.03 | 9 | 0.15±0.03 | 30 | 0.05±0.01 | 55 |
| PC - buffer | | | 0.28±0.01 | -- | 0.06±0.01 | -- |
| - $CCl_4$/NADPH | | | 0.23±0.02 | 15 | 0.04±0.01 | 34 |
| - NADPH | | | 0.25±0.01 | 9 | 0.05±0.01 | 17 |
| - FeADP/NADPH | | | 0.24±0.02 | 13 | 0.05±0.01 | 17 |

(a) Rats were injected with saline intraperitoneally for 4 days, fasted and the microsomal fraction was prepared as described in Table 10. Lipids were extracted and purified by HPLC. Fatty acid methyl esters were obtained by transesterification and analyzed by GLC.

(b) Fatty acid components are expressed as the fraction of the total fatty acids in the sample. Decreases are given as the percent decrease of the control quantity.

* $P < 0.05$ when compared to controls

** $P < 0.01$

Table 21 Comparison of fatty acid composition in phospholipid fractions from phenobarbital-induced microsomes after exposure to buffer, carbon tetrachloride, NADPH or FeADP/NADPH in vitro(a)

| Phospholipid | Fatty Acids and Associated Decrease | | | | | |
|---|---|---|---|---|---|---|
| | 18:0 | % dec. | 20:4 | % dec. | 22:6 | % dec. |
| PE - buffer | | | 0.24±0.01 | -- | 0.10±0.01 | -- |
| - $CCl_4$/NADPH | | | 0.21±0.02 | 14 | 0.07±0.01 | 22 |
| - NADPH | | | 0.23±0.02 | 2 | 0.08±0.02 | 6 |
| - FeADP/NADPH | | | 0.22±0.01 | 9 | 0.09±0.01 | 4 |
| PI - buffer | | | 0.34±0.03 | -- | 0.04±0.01 | -- |
| - CCl4/NADPH | | | 0.25±0.03 | 26 | 0.03±0.01 | 25 |
| - NADPH | | | 0.29±0.03 | 15 | 0.025±.01 | 35 |
| - FeADP/NADPH | | | 0.30±0.03 | 11 | 0.03±0.01 | 25 |
| PS - buffer | 0.45±0.03 | -- | 0.18±0.01 | -- | 0.08±0.01 | -- |
| - $CCl_4$/NADPH | 0.23±0.03 | 49 | 0.15±0.01* | 16 | 0.05±0.01** | 35 |
| - NADPH | 0.45±0.02 | -- | 0.15±0.01 | 16 | 0.08±0.01 | -- |
| - FeADP/NADPH | 0.38±0.03 | 16 | 0.14±0.01 | 20 | 0.07±0.01 | 12 |
| PC - buffer | | | 0.25±0.01 | -- | 0.055±.01 | -- |
| - $CCl_4$/NADPH | | | 0.22±0.01* | 12 | 0.04±0.01 | 7 |
| - NADPH | | | 0.24±0.01 | 4 | 0.04±0.01 | 7 |
| - FeADP/NADPH | | | 0.25±0.02 | -- | 0.057±.01 | -- |

(a) Rats were given daily injections of phenobarbital (50 mg/kg) for 4 days, fasted, and microsomes prepared with incubation additions as described (see Table 11). After incubating the microsomes were pelleted and the lipids extracted, purified and methylated. Fatty acid analysis was done by GLC.

(b) Fatty acid components are expressed as the fraction of the total fatty acids in the sample. Decreases are given as the percent decrease of the control quantity.

* $P < 0.05$ when compared to controls

** $P < 0.01$

Table 22 Fatty acid composition of microsomes challenged with carbon tetrachloride in vivo and *in vitro*[a]

| | IN VIVO | | | | IN VITRO | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | PB | % Decrease Ctrl. | % Decrease PB | Control | PB | % Decrease Ctrl. | % Decrease PB |
| PE | | | | | | | | |
| (20:4) | 0.29 ± 0.03[b] | 0.25 ± 0.01[b] | 15[c] | 5[c] | 0.29 ± 0.01[b] | 0.24 ± 0.01[b] | 20[c] | 14[c] |
| (22:6) | 0.12 ± 0.01 | 0.11 ± 0.01 | 10 | 10 | 0.11 ± 0.02 | 0.10 ± 0.01 | 33 | 26 |
| PI | | | | | | | | |
| (20:4) | 0.37 ± 0.01 | 0.35 ± 0.01 | 15 | 10 | 0.35 ± 0.01 | 0.34 ± 0.03 | 22 | 29 |
| (22:6) | 0.04 ± 0.002 | 0.03 ± 0.001 | 35 | 13 | 0.05 ± 0.001 | 0.04 ± 0.01 | 43 | 29 |
| PS | | | | | | | | |
| (18:0) | 0.36 ± 0.01 | 0.38 ± 0.01 | 10 | 30 | 0.44 ± 0.02 | 0.45 ± 0.03 | 23 | 51 |
| (20:4) | 0.26 ± 0.01 | 0.21 ± 0.01 | 25 | 60 | 0.21 ± 0.01 | 0.18 ± 0.01 | 53 | 20 |
| (22:6) | 0.11 ± 0.01 | 0.12 ± 0.02 | 35 | 70 | 0.11 ± 0.02 | 0.08 ± 0.003 | 78 | 44 |
| PC | | | | | | | | |
| (20:4) | 0.27 ± 0.02 | 0.25 ± 0.01 | - | 5 | 0.28 ± 0.01 | 0.25 ± 0.01 | 16 | 13 |
| (22:6) | 0.07 ± 0.01 | 0.05 ± 0.01 | 40 | 30 | 0.06 ± 0.01 | 0.06 ± 0.01 | 18 | 32 |

(a) Rats were either injected with saline (controls) or phenobarbital (50 mg/kg) for 4 days. Carbon tetrachloride was either administered via a gastric route (*in vivo*) or added to a suspension of microsomes with exogenous NADPH (*in vitro*). Lipids were extracted from purified microsomes, and the fatty acid methyl esters prepared from the purified phospholipids were analyzed by GLC.

(b) Figures represent the fraction of the total fatty acids in the sample extracted before carbon tetrachloride was administered.

(c) Figures represent the percent decrease observed in each fatty acid after $CCl_4$ challenge.

# DISCUSSION

Ultrastructural studies of the rat hepatocyte reveal an extensive network of tubules, vesicles and lamellae known as the endoplasmic reticulum which is present throughout the cytoplasm. The endoplasmic reticulum is responsible for the synthesis and transport of proteins, synthesis of cholesterol and phospholipids and participates in glycogen breakdown (41)

Histochemical, biochemical and immunochemical investigations suggest that a number of enzymes are distributed heterogeneously along the lateral plane of the membrane such as NADPH cytochrome P-450 reductase, cytochrome P-450, NADH cytochrome $b_5$ reductase, cytochrome $b_5$, ATPase and glucose-6-phosphatase on the cytoplasmic side while NDPase, B glucuronidase and glucose-6-phosphatase are localized on the luminal surface (42).

The discovery of the physiological function of the cytochrome P-450 system as the oxygen activating terminal oxidase in the oxygenation reactions of endogenous substrates (43) was further demonstrated to include mixed function oxidase reactions of liver microsomes in drug hydroxylations or oxidative dealkylations (44).

The activity of the drug metabolizing ability of the cytochrome P-450 system and the destruction caused by carbon tetrachloride metabolism in the liver has been well documented (21, 45,46). Less well understood is the correlation of the differences found in the phenobarbital induced endoplasmic reticulum and the

increased tissue destruction reported for carbon tetrachloride-induced cell injury in phenobarbital pretreated rats (19,47). Changes in the phospholipid and fatty acid content of the endoplasmic reticulum with phenobarbital administration may provide insight into the response of this organelle to carbon tetrachloride challenge *in vivo* and *in vitro*.

The study of many endoplasmic reticulum-localized reactions, including carbon tetrachloride-induced cell injury has been carried out *in vitro* and extrapolated to the *in vivo* system. The influence of microsomal preparations on the ability of the monooxygenase system to metabolize carbon tetrachloride with subsequent lipid peroxidation was compared with *in vivo* metabolism.

Cytochrome P-450 electron transport system

The orientation of the cytochrome P-450 mixed function oxidase system within the endoplasmic reticulum is not know, however, several hypotheses have been proposed. One model suggests that the two proteins, cytochrome P-450 and its associated NADPH cytochrome P-450 reductase, exist in the membranes as a functional cluster within which the electron transfer takes place (47,48,49). Kinetic analysis of the reduction of the cytochrome P-450 protein by the reductase which employs NADPH as the source of the reducing equivalents indicates a first (fast) electron transfer and a second (slow) electron transfer phase (50) exists. If the system is organized into a cluster a degree of flexibility of the orientation

of the reductase to the cytochrome P-450 protein exists since the cytochrome and/or the reductase molecules can be reincorporated into microsomes and be enzymatically active and function normally (51). The lateral movement of the reductase and cytochrome molecules is further indicated by enzyme inhibition studies which suggest that the electron transfer is effected to some degree by random translational motion and subsequent collision of the two proteins on the plane of the membrane (52). Data supporting the clustering of the cytochrome P-450 system as well as the lateral movement of the reductase relative to the cytochrome P-450 protein could reflect that data obtained with intact or modified microsomes are complex and the results are ambiguous. Preparation of microsomes for in vitro experiments is disruptive to the endoplasmic reticulum and may influence the orientation of the cytochrome P-450 components and the associated phospholipids such that results from one laboratory may differ from others. It also seems reasonable to assume that the system can exist as an ordered complex in which the reductase and cytochrome P-450 molecules are in close proximity while at the same time translational mobility of one or both components permits the reduction of multiple cytochrome P-450 molecules by a single reductase molecule and permits the incorporation of exogenous cytochrome P-450 molecules into a functionally active system.

Involvement of phospholipids in the cytochrome P-450 system

Electron spin resonance spectra have provided evidence that the reductase protein is enclosed by a halo of the phospholipid matrix which differs from the bulk of the lipid matrix since it demonstrates an abrupt change from the quasi-crystalline state to a fluid state at 32°C, while the bulk lipid is in a more fluid state below 30°C (48).

The actual involvement of phospholipids in the functioning of the cytochrome P-450 system was first reported when a heat stable, organically extractable factor was required for maximal fatty acid hydroxylation activity in a reconstituted cytochrome P-450 system (53). The same factor was found to be required for steroid and carcinogen hydroxylation and the oxidative metabolism of many drugs and exhibited the same $R_f$ values in thin layer and column chromatography as a phosphatidylcholine standard (54). Synthetic phosphatidylcholine was as active as the crude lipid fraction in the reconstituted system but phosphatidylethanolamine could not be substituted for phosphatidylcholine (54).

Phospholipase C treatment of microsomes (10) removed approximately 70 percent of the total phospholipids which resulted in a reduced cytochrome P-450 activity. Subsequent addition of phospholipid did not restore the metabolic activity. Cater (55) found that deoxycholate inhibited cytochrome P-450 mediated metabolism while the addition of phosphatidylcholine could relieve this inhibition. It was suggested that the role of phospholipid could be that of relieving detergent inhibition that was present in the

system of Lu et al. (53). To resolve the question of lipid involvement in cytochrome P-450 activity, lyophilized microsomes were lipid extracted. Both cytochrome P-450 and the reductase were found to be stable when lyophilized prior to extraction (56). With removal of 75 percent of the total phospholipid phosphorous the enzymatic activity of the extracted microsomes was 30-50 percent of the control activity. When total lipid extracts or synthetic phosphatidylcholine (lauroyl glycerol-3-PC) were added to the system, full activity was restored (56). Thus it appears that lipid is an essential part of the microsomal hydroxylation system and that the function of the lipids is unrelated to the use of detergents or phospholipase (58,59).

The purified cytochrome P-450 enzyme has been shown to have some phospholipid residues associated with each polypeptide chain (57). Work on reconstituted cytochrome P-450 systems (60,61) suggests that the phospholipid neither interacts directly at the substrate binding site nor with the heme moiety, rather it provides a proper environment to facilitate conformational transitions of the enzyme. Tsong et al. (61) working with dimyristoyl lecithin (DML) in reconstituted systems found DML to affect the rate of reaction and that this influence is both temperature and concentration dependent. Rates are accelerated only above the gel-liquid crystalline transition temperature. The data indicate that the lipid may form an envelope around the enzyme and depending on the crystalline state of the lipids, the lipids regulate the rate of the substrate indu-

ced conformational changes of cytochrome P-450.

Circular dichroism studies on purified cytochrome P-450 incorporation into dilauroyl glycerol PC liposomes revealed an increase in the α-helical content of the protein (62). The change in the conformation of the protein in the presence of phospholipid may account for increased catalytic rates and an alteration in the substrate-cytochrome P-450 binding. Change in protein conformation upon the addition of phospholipid was not seen for the reductase (62).

The phospholipids of the endoplasmic reticulum may provide a necessary organizational and environmental milieu for the activity of the cytochrome P-450 system.

Influence of phenobarbital administration on the cytochrome P-450 system

A number of investigators have reported on the inductive influence of phenobarbital on the liver resulting in an increase in liver weight (63), microsomal protein (64), total microsomal phospholipids (65) and an increase in the phospholipid:protein ratio of the microsomes (66,67,68) which was confirmed in the present study (Table 1). That the phospholipid:protein ratio remains the same with phenobarbital administration was also reported (65).

Treatment with phenobarbital produced an increase in both smooth and rough endoplasmic reticulum which appeared to parallel an increase in the drug metabolizing enzymes (69,70). Other investigatiors confirmed the relationship between the increase in endoplasmic reticulum and the increase in activity of the drug metabolism of

the cytochrome P-450 system (e.g., 71,72,73)

Subsequently other differences in the phenobarbital induced endoplasmic reticulum have been reported such as the significantly higher turnover rates for phenobarbital induced reductase molecules compared with the uninduced reductase (74). When phenobarbital administration was withdrawn, the induced reductase enzymes were found to turnover more quickly than the controls. They suggested that phenobarbital stimulation produced changes in the way the reductase molecules were organized within the membrane such that the reductase stimulated by phenobarbital administration was not incorporated into the membrane to the same degree as the uninduced moleclues, thereby increasing its turnover rate.

Purified cytochrome preparations from controls and from rats treated with phenobarbital showed different EPR spectra (75), behaved differently in isolelectric focusing (76) and showed different weights in SDS-polyacrylamide gel electrophoresis (77). The microsomal lipids have been reported to undergo more extensive peroxidation in response to carbon tetrachloride administration after phenobarbital induction than control microsomes (78).

Influence of phenobarbital on the phospholipid composition of the endoplasmic reticulum

Phenobarbital administration was found to increase the incorporation of $^{32}P$ into the phosphatidylethanolamine fraction (66) which correlates with an increase in the phosphatidylethanolamine: phosphatidylcholine ratio reported previously (68) and confirmed in

the present study (Table 4). The increase in the phosphatidylethanolamine fraction with phenobarbital administration is thought to be brought about by the decrease in the conversion of phosphatidylethanolamine to phosphatidylcholine (66) due to either decreases in endogenous levels of S-adenosyl methionine or the activity of the transferase.

While Eriksson and Dallner (79) reported no change in the phospholipid compositon of the microsomes with phenobarbital administration others (80,81) found an increase in the proportion of phosphatidylcholine relative to phosphatidylethanolamine which was attributed to an increased activity in the transferase 12 hours after phenobarbital administration (81). Only a slight increase in phospholipid synthesis was detected by 12 and 24 hours post phenobarbital administration (66) and was not found to be sufficient to account for the marked increase in phospholipid content after several doses of phenobarbital. Data from $^{32}P$ labeled phospholipids indicated that one of the effects of phenobarbital metabolism is a decreased phospholipid catabolism and that this effect became obvious only after 2 or more days of phenobarbital injections (66). Although the activity of the transferase does increase slightly 12 hours after phenobarbital administration the increase is insufficient to effect a change in the phospholipid proportions and does not reflect the steady state influence of phenobarbital on the membrane. Also, the slight increase in phospholipid synthesis with phenobarbital administration was observed in male rats only (66).

The rise in the phosphatidylethanolamine:phosphatidylcholine ratio with phenobarbital administration (Table 2) may reflect the necessity of the bilamellar membrane of the microsome to incorporate more protein. Phosphatidylethanolamine is known to exist as a hexagonal structure under certain conditions (82) and data from model system employing $^{31}P$ nuclear magnetic resonance experiments suggest that the non-lamellar phase of the endoplasmic reticulum, that is, the area which accommodates the cytochrome P-450 system, contains a large amount of phosphatidylethanolamine which is believed to be essential for the accommodation of globular proteins within a bilayer (83). If the model is accurate, phosphatidylethanolamine would exist as inverted micells among the cytochrome P-450 protein molecules in order to facilitate some type of exchange with the outer layers of the membrane (83). The proximity of phosphatidylethanolamine to the cytochrome P-450 molecules implies that any active species issuing from this system would first encounter phosphatidylethanolamine.

Data from kinetic experiments indicate that the phospholipid ratios may have an influence on the kinetic properties of the cytochrome P-450 system (84). Measurements on the reduction of cytochrome P-450 by the reductase indicate that the rate of transfer of the first electron from the reductase to the cytochrome increased as the reductase:cytochrome ratio increased. When the reductase:cytochrome ratio was kept constant but the amount of phosphatidylcholine in the vesicles was varied, the rate decreased significantly as the proportion of phosphatidylcholine was increased. The rate of the first electron donor

is apparently dependent or limited by the number of phospholipid molecules existing between the reductase and the hemeprotein as well as the reductase:cytochrome ratio and would indicate that there is a degree of lateral mobility necessary for the transfer of the reducing equivalents to take place. The presence of a higher phospholipid: protein ratio with phenobarbital administration may influence the reductase rate of the first electron transfer since interactions with the cytochrome P-450 molecule may be decreased.

The role of phosphatidylinositol and phosphatidylserine in membranes has been suggested from work in reconstituted cytochrome P-450 systems using microsomal phospholipids in which there is a decreased proportion of phosphatidylinositol and phosphatidylserine (85). The liposome-cytochrome P-450 compex was found to be thermally labile since the liposome bound enzyme was inactivated by more than 50 percent at 30°C for 30 minutes while the microsomal cytochrome P-450 was resistant to the same thermal exposure (86).

Experimental data from other systems suggests that phosphatidylserine may have an ordering and stabilizing function in membranes. Recent evidence from fluorescent polarization measurements on the interaction of calcium ions with bove brain phosphatidylserine (BBPS) indicates that a calcium-phosphatidylserine complex is formed which preserved acyl chain order at high temperatures (87). Phosphatidylcholine and phosphatidylethanolamine did not appear to be involved in complexing calcium ions since calcium additions were found to have a negligible effect on the organization of these phospholipids in rod outer seg-

ment membranes.

Addition of calcium to phosphatidylserine vesicles in an aqueous saline environment resulted in highly ordered crystalline acyl chains and a high transition temperature (88). Experiments on the Na+-K+ ATPase from rabbit kidney medulla, although no absolute requirement for one of the polar head groups was found, in the absence of negative charges the activity of the enzyme is 44 percent lower than in their presence (89).

If phosphatidylserine, and possibly phosphatidylinositol, are associated with calcium ions in the endoplasmic reticulum these phospholipids may be functioning in the regulation of enzyme activity in the cytochrome P-450 system. From the results of Tsong et al. (61) in which rates of cytochrome P-450 reduction depend on the crystalline state of the lipids surrounding the enzyme the maintenance of an ordered liquid-crystalline state may be necessary for optimal enzyme activity. The involvement of calcium ions in maintaining microsomal integrity is suggested by the report that EDTA added to microsomes increases the amount of the non-lamellar phase present (83). Additions of divalent cations such as calcium and magnesium to microsomes were found to promote greater aggregation of integral proteins. Cation removal was found to favor protein dispersion as the increase of non-lamellar structure occurred. The proportion of phosphatidylserine and phosphatidylinositol decreases with phenobarbital induction (81) (Table 2). The phenobarbital induced microsome, due to decreases in these phospholipids, may be a more labile structure than microsomes

produced from uninduced controls and the lack of acyl order within the membrane could result in a decrease in activity in the phenobarbital induced system.

With phenobarbital administration there is an increase in the number of cytochrome P-450 molecules relative to the reductase present. In the uninduced system the cytochrome:reductase ratio is approximately 10, however this ratio increases to 20 or more (90) with phenobarbital administration. This increased ratio poses a problem of stoichiometry in as much as one reductase molecule must transfer electrons from NADPH to 20 cytochrome P-450 molecules. Recent experiments on the lateral diffusion rates of phospholipids in synthetic and natural membranes (91) demonstrated that there is a decrease in lateral movement in the membrane which correlates with increased fatty acid chain length and increased degree of unsaturation. Phenobarbital-induced endoplasmic reticulum was found to have an increase in linoleic acid (18:2) and decreases in arachidonic (20:4) and docosahexenoic (22:6) fatty acids (Table 3). The influence of phenobarbital on fatty acid composition of the endoplasmic reticulum appears to produce a membrane which increases the potential for lateral diffusion of the phospholipids since the induced membrane has an increased content of shorter chain dienoic fatty acids (18:2) while longer chain polyunsaturated fatty acids (i.e., 20:4 and 22:6) are decreased. The degree of unsaturation in the endoplasmic reticulum may be important in maintaining a certain degree of fluidity since the saturated:unsaturated ratio remains essentially the same with the exception of the small phosphatidylserine component which becomes

more saturated (Table 5). The changes which occur in the fatty acid content of the endoplasmic reticulum with phenobarbital induction may indicate that phenobarbital interfers with the conversion of linoleic acid to longer, more unsaturated fatty acids.

Since the fatty acid composition of the diet was found to be reflected in the fatty acid content of the endoplasmic reticulum (92) the change in the fatty acid profile with phenobarbital administration could reflect dietary influences such as rate-limiting quantities of essential fatty acids, linoleic and linolenic, in the stock rodent food used in the maintenance of the rats. The fat content of the rodent chow was only 4.5 percent (w/w). However, experiments in which rats, maintained on diets containing high concentrations of polyunsaturated fatty acids, were administered phenobarbital demonstrated a decrease in the arachidonic and docosahexenoic fatty acid content of the endoplasmic reticulum when compared with controls similarly fed without phenobarbital injections (92). Thus, providing diets high in polyunsaturates during phenobarbital administration indicated that phenobarbital administration and not the diet determined the fatty acid content of the endoplasmic reticulum. Also, since decreased catabolism rather than increased synthetic rate was found to be primarily responsible for the inductive effect of phenobarbital on endoplasmic reticulum (66) the decreases in arachidonic and docosahexenoic acids with phenobarbital administration in the present study appear to be phenobarbital mediated rather than diet limiting.

Experiments in which both dietary and phenobarbital influences

acid (93) or arachidonic and docosahexenoic (94) acids, cholesterol and vitamin E, which stimulate drug metabolism do not affect the hydroxylation of benzopyrene. The different requirements for dietary lipids may demonstrate that membranes of different structure are required for maximum activity by the two forms of cytochrome P-450 or that fatty acid composition may influence the affinity of the substrate for the active site.

Carbon tetrachloride-induced lipid peroxidation

Since Cameron and Karunaratne (1) described the centrilobular liver necrosis of carbon tetrachloride administration, numerous investigations have been undertaken to correlate the morphologic and biochemical changes produced in the liver. That the disturbances created in the cell by carbon tetrachloride are not merely the result of a solvent effect by a lipophilic agent have been substantiated by several lines of evidence.

After oral administration of carbon tetrachloride high concentrations can be detected in such lipophilic tissues as bone marrow, brain and adipose tissue, while lower concentrations are found in the liver (95); however, extensive necrosis occurs primarily in the liver. If the toxic properties of carbon tetrachloride were solely the result of its lipophilic nature, lipid-rich tissues would be expected to suffer the greatest damage, yet this has not been the case. Additionally, very low doses (0.09 ml/kg body weight) of carbon tetrachloride produce widespread damage such as decreased activity of glucose-6-phosphatase (96) and triglyceride accumulation (97). Where the drug

metabolizing system is incomplete such as in the newborn rat, the necrosis is slight compared with the widespread necrosis which occurs in the adult rat liver in which the drug metabolizing system is completely developed (45,73). Such responses, correlated with a metabolic system, indicate that a more specific type of interaction of carbon tetrachloride with cell constituents is occurring.

Compounds similar to carbon tetrachloride in lipid solubility, density and molecular weight, such as trichlorofluromethane, have been shown to be virtually non-toxic to rat, monkey, dog and guinea pig livers (98). The lack of toxicity is believed to be the result of its greater carbon-halogen bond strength compared with carbon tetrachloride, consequently reducing its ability to be metabolized.

Correlation of the drug metabolizing system with the metabolism of carbon tetrachloride was provided by the observation that prior stimulation of the drug-metabolizing system with phenobarbital (99) resulted in an increased loss of the drug metabolizing ability with carbon tetrachloride administration compared to controls. Pretreatment of animals with SKF-525A which competively inhibits microsomal drug metabolism (8) or subjecting rats to a low protein diet which decreases the amount of endoplasmic reticulum in the liver as well as the activity of the cytochrome P-450 system (21) provided protection from carbon tetrachloride administration (100).

These observations provide evidence that activation of carbon tetrachloride parallels the extent and activity of the cytochrome P-450 system.

Theories on the mechanism of carbon tetrachloride-induced cell injury

Currently there are two theories regarding the destructive effects of carbon tetrachloride metabolism: one theory states that covalent binding of reactive metabolites of carbon tetrachloride to microsomal proteins and lipids is sufficient to cause the disruptive changes seen with administration of the toxin (100) while others propose that lipid peroxidation is an obligatory prerequisite for the destruction observed (8).

Experiments designed to demonstrate the reactive metabolite theory of carbon tetrachloride binding to cell constituents in order to effect liver injury employed $^{14}C$-carbon tetrachloride to follow the addition of the trichloromethyl radical onto cell proteins and lipids (101,102,103). These investigators did find a positive correlation between the amount of radioactivity incorporated into lipids and proteins which they associated with losses in cytochrome P-450 activity and glucose-6-phosphatase activity, although no lipid peroxidation was detected. They suggested that since lipid peroxidation had not occurred, the decreases in enzyme activity were the direct result of the trichloromethyl radical addition onto these proteins.

However, their experimental approach differed in a number of respects from *in vivo* experiments of others, including the one reported here. Carbon tetrachloride was administered by intraperitoneal injections rather than via gastric intubation which is the route generally employed. The absorption of labeled compound metabolized

by the liver administered either by intraperitoneal injections or via gastric intubation resulted in a lower level of substrate present in the liver when injections were compared to gastric routes and the time required for reaching maximal substrate levels was twice as long for injections compared with intragastric administration (104). The time delay for substrates to reach the liver when intraperitoneal injections were used may provide insight into the report that compounds administered by different routes may produce different pharmacological responses (105) as well as being metabolized to different derivatives (106).

Thus, by administering carbon tetrachloride via intraperitoneal injections the experiment could be sufficiently altered so as to produce results quantitatively and qualitatively different from carbon tetrachloride administered by gastric intubation.

Not only is the route of administration important in the development of the cell lesion, the type and extent of liver injury induced with carbon tetrachloride is also dosage dependent (107). The experiments of De Toranzo (101), Castro (102), and Diaz Gomez (103) employed lethal quantities of carbon tetrachloride. Lethal dosages overwhelm the animal with toxin, and since carbon tetrachloride is lipophilic, solvent effects complicate the nature of cell injury which is more easily observed at sublethal doses in which solvent effects are minimal.

As an index of peroxidative damage, diene conjugation is maxi-

mally observed one hour, or sooner, after carbon tetrachloride administration and has been reported to fall sharply after that time (108). Assay for diene conjugates by the proponents of the reactive metabolite addition theory of carbon tetrachloride toxicity was performed 3 or more hours post carbon tetrachloride administration. By allowing such a time lag between administration of carbon tetrachloride and assaying for peroxidative changes, the diene conjugates that may initially be produced could have been metabolized or excreted in 3 hours or longer with no evidence remaining which could be assayed to indicate that peroxidation had occurred. The changes in the experimental design and in the manner in which the assays were performed could result in data which differ in a number of ways from investigators who look at the early events of cell injury following carbon tetrachloride ingestion at sublethal doses.

The theory of lipoperoxidation as a vector in the initiation of cell injury resulting from carbon tetrachloride administration has received more attention by investigators (8,15,109,110). According to the lipoperoxidation theory of cell injury carbon tetrachloride is believed to initiate a lipid peroxidative process in the endoplasmic reticulum which is responsible for subsequent manifestations of carbon tetrachloride administration. The evidence that peroxidation is a major factor in the development of tissue destruction from carbon tetrachloride administration has been accumulating for a number of years.

The theory that carbon tetrachloride toxicity involved lipid

peroxidation depended on the ability of antioxidants to decrease the cell injury due to carbon tetrachloride. Hove (111) reported on the efficacy of vitamin E and other antioxidants in decreasing the lethality of carbon tetrachloride in rats. In order to establish peroxidation as the key event in cell injury it was necessary to demonstrate that peroxidation occurred early in the course of cell injury. Thus additional support for the lipoperoxidative theory came from the observation that microsomal lipids peroxidize very rapidly *in vivo* after carbon tetrachloride administration; peroxidation of microsomal lipids is more than half maximal within 5 minutes (14). Decreases in cytochrome P-450 and glucose-6-phosphatase activities and inhibition of protein synthesis all occur subsequent to the formation of lipid peroxides (30). Furthermore, agents which provide protection from peroxidation such as vitamin E, preserve enzyme activities (22). Glende *et al.*(112) reported that EDTA, when added to microsomes *in vitro*, completely inhibited peroxidation and as long as peroxidation was prevented the enzyme activities of glucose-6-phosphatase and cytochrome P-450 were remarkably stable to large quantities of carbon tetrachloride. When $^{14}C$-carbon tetrachloride was metabolized under anaerobic conditions *in vitro* lipid peroxidation was prevented although extensive incorporation of the $^{14}C$-labeled metabolite could be found in the lipids and proteins of the endoplasmic reticulum while cytochrome P-450 and glucose-6-phosphatase activities were stable (113). The results were interpreted as evidence supporting the lipid peroxidation mode of cell injury; carbon tetrachloride was metabolized with

the consequent addition of reactive metabolites which were not destructive to the enzymes in the absence of lipid peroxidation. Other $^{14}C$-carbon tetrachloride incorporation studies revealed that the metabolites were present in the microsomal lipids in 4-fold higher concentrations than the level found for microsomal protein (13). The incorporation into microsomal protein was similar to the level of incorporation found in mitochondrial protein, however, mitochondrial structure and function remained unimpaired whereas damage to the endoplasmic reticulum is extensive (46).

Covalent binding of the trichloromethyl free radical to protein thiol groups was subjected to close scrutiny (8) and no evidence was found to indicate that reactive metabolites from carbon tetrachloride reacted with protein thiol groups to cause reduced enzyme activity. Recknagel and Glende (8), noting the absence or reduction in cell injury when lipid peroxidation was prevented during carbon tetrachloride metabolism proposed that covalent binding, although it may be disruptive in and by itself, probably does not constitute a mechanism for enzyme loss *in vivo* but rather has a comparatively minor role in cell injury compared to the injury resulting from lipid peroxidation.

That lipid peroxidation is not the only mechanism of carbon tetrachloride-induced cell injury is indicated from studies on hepatocyte cell cultures. The metabolism of carbon tetrachloride by hepatocytes results in the release of lactate dehydrogenase (114). In the presence of glutathione which significantly reduces the production of lipid peroxides, carbon tetrachloride stimulation of lactate dehydro-

genase can be high. They concluded that factors in addition to lipid peroxidation may be important in understanding carbon tetrachloride hepatoxicity.

It has been known for many years that hydrogen atoms on methylene carbons separating double bonds in polyenoic fatty acids are susceptible to free radical attack (115). Free radicals can abstract a methylene hydrogen atom and the resulting free radical, either immediately or following a resonance shift, will react with molecular oxygen to form the peroxy free radical (115). An organic peroxy free radical on any one phospholipid fatty acid side chain would be expected to react via a hydrogen abstraction attack on a methylene hydrogen of a neighboring unsaturated fatty acid (8). Once peroxide formation begins new free radicals appear which set off new chains of peroxide formation leading to decomposition of polyenoic fatty acids (115).

Relating the chain reaction of lipoperoxidative events to the injury resulting from carbon tetrachloride administration provides a mechanism for understanding the devastating effects of very low doses of carbon tetrachloride to the liver. The reactive trichloromethyl radical produced as a consequence of carbon tetrachloride metabolism would abstract a methylene hydrogen atom. Once initiated, a lipoperoxidative chain reaction would be expected to proceed to sites relatively distant from the locus of the initial lethal cleavage. The lipoperoxidation hypothesis links the peroxidative decomposition of the lipids of the endoplasmic reticulum with the pathological phen-

omena caused by this toxic agent.

Carbon tetrachloride-induced lipid peroxidation - evidence for in vivo occurrence

In order to accept a theory of cell injury based on lipid peroxidation, the peroxidative event must be able to be demonstrated to occur *in vivo*. However, the evidence indicating that carbon tetrachloride stimulates lipid peroxidation *in vivo* has proven difficult to obtain. The amount of lipid peroxide formed is small compared with the total lipid present in the liver and the large amount of non-reactive tissue dilutes the peroxidative effect. Malonaldehyde (MDA), an indicator of peroxidation, is rapidly metabolized by liver mitochondria as soon as it is produced (116). By rapidly processing the carbon tetrachloride-treated livers, *in vivo* MDA production was found to increase (117) although the increase produced is small.

Another approach took advantage of a rearrangement of electrons which presumably results when trichloromethyl radicals attack polyenoic fatty acids. The rearrangement of electrons, also referred to as diene conjugation, demonstrates a marked absorption peak at 232-234 nm (118). A time course study by Klaassen and Plaa (119) showed that the maximum increase in diene production occurs 30 minutes to 1 hour after carbon tetrachloride dosing and is followed by a rapid fall in diene conjugate concentration, suggesting metabolism or repair of these by the liver.

More direct evidence for *in vivo* lipid peroxidation has been provided by the finding that ethane, a degradation produce of linolenic

on the activity of the cytochrome P-450 system were considered (93) it was found that a diet high in linoleic acid produced a cytochrome P-450 complex with the highest rate of drug metabolism (93). Following phenobarbital administration the cytochrome P-450 content and activity were optimal in the linoleic supplemented diet when compared with a fat free or lard supplemented diet. They concluded that polyunsaturated fatty acids, primarily linoleic acid, are essential for maximum drug-metabolizing activity and when the diet is supplemented with high concentrations of linoleic acid during phenobarbital administration the endoplasmic reticulum will have optimal cytochrome P-450 content and activity. However, subsequent diets low in linoleic content resulted in a rapid rate of drug metabolism and induction with phenobarbital administration (94) just as the diet containing high levels of linoleic had. Although the diet was low in linoleic acid it contained a large quantity of polyunsaturated fatty acids (94) such as arachidonic and docosahexenoic which may be the end products for which linoleic acid was the precursor. Linoleic acid by itself does not appear to be necessary for maximum activity of the cytochrome P-450 system or for maximal induction of this system by phenobarbital, although linoleic acid may be important as a precursor for other fatty acids which may be essential for optimum cytochrome P-450 activity.

The relationship of fatty acids and the cytochrome P-450 system is complicated by the finding that while oxidative demethylation of drugs and hydroxylation of carcinogens are enhanced by dietary linoleic

acid (120) is exhaled by the intact animal *in vivo* (121). Subsequently it was found that pentane, a peroxidative product of linoleic and arachidonic acids (122) could be used as an indicator of *in vivo* peroxidation when measured in the exhaled breath of the animal (123). The exhalation of ethane and pentane could be markedly reduced by administration of vitamin E to carbon tetrachloride poisoned animals (124). Vitamin E has been shown to prevent lipid peroxidation *in vivo* (111).

The involvement of the cytochrome P-450 protein in the toxic effects of carbon tetrachloride administration was indicated by the discovery that a small dose of carbon tetrachloride administered 24 hours prior to a lethal dose provides protection against the lethal dose (126,127). Depression of the drug metabolizing system was implicated as the mechanism providing protection. After the small initial dose of carbon tetrachloride the activity of the cytochrome P-450 system is only 25-35 percent of controls (12). The decrease in activity of cytochrome P-450 is associated with a decreased metabolism of carbon tetrachloride to its reactive trichloromethyl radical, consequently less liver damage occurs and the animal survives.

It seems reasonable that the cytochrome P-450 molecule could be the site of the pro-oxidant effects of carbon tetrachloride since iron (II) porphrins in heme proteins were found to be oxidized by various alkyl halides to corresponding iron (III) complexes (128).

Inorganic iron is also implicated in *in vivo* peroxidation from the observation that after iron overload there is a small reduction of oxidative demethylation activity and microsomes prepared from these livers demonstrated an increase in lipid peroxidation when incubated *in vitro* (129).

De Matteis and Sparks (130) reasoned that since starvation increased *in vivo* peroxidation (131) and iron overload was found to increase *in vitro* peroxidation (129) a combination of starvation and iron overload might result in a greater increase in *in vivo* peroxidation. A single large dose of iron administered to starved rats led to a further loss of cytochrome P-450 activity over that caused by starvation alone (130). Both starvation and iron treatment stimulated heme biosynthesis without any significant increase in liver porphyrin concentration; such results are compatible with an increased rate of liver heme turnover in both conditions. By employing heme precursors *in vivo* and following the loss of radioactivity caused by starvation or iron overload they concluded that while iron treatment clearly enhanced the degradation of liver heme *in vivo*, the effect for starvation was small and inconclusive. Short term starvation, which has been shown to lower the levels of glutathione (132) results in a small loss of cytochrome P-450 activity but no decrease in glucose-6-phosphatase activity (133). Hogberg (134) reported the presence of small amounts of malonaldehyde in hepatocytes in tissue culture when no exogenous iron was added. This suggests that a limited amount of lipid peroxidation may be of normal occurrence *in vivo*

especially when the cellular levels of antioxidants, like glutathione, are decreased. It has been suggested that the high turnover rate of the cytochrome P-450 electron transport system may reflect the presence of lipid peroxides which occur naturally (135); pro-oxidants may increase the turnover by increasing the levels or rate of lipid peroxidation.

Carbon tetrachloride-induced lipid peroxidation - an in vitro model system

The difficulty of monitoring the carbon tetrachloride-induced peroxidation in the whole liver and the advantages of a purified microsomal system in vitro which did not have the protective and repair devices of the intact liver, appealled to investigators. With further purification the system could be broken up into its component parts in order to obtain a better understanding of the involvement of each component and how each component influenced the cytochrome P-450 system as a whole.

It was observed in vitro that the rate of loss of cytochrome P-450 activity varied according to the species, nutritional state of the animal and correlated with the formation of lipid peroxide; both lipid peroxidation and loss of cytochrome P-450 were greater with either mouse or rat microsomes than rabbit microsomes (136) and both were stimulated by starvation. Since the loss of cytochrome P-450 activity correlated with the formation of lipid peroxides a number of experiments were carried out to find the relationship between them.

Increased lipid peroxidation was found to be associated with a progressive inactivation of cytochrome P-450 during peroxidative damage (137). Where lipid peroxidation and drug metabolism can take place simultaneously in vitro, the intensity of one process is inversely related to that of the other; an increased rate of lipid peroxidation will cause a decrease in drug metabolizing activity (129,138). Conversely, when drug metabolism is stimulated by adding a drug to the system which can be readily metabolized, the extent of lipid peroxidation will be reduced (139). It has been suggested that increased drug metabolism reduces the extent of lipid peroxidation since both processes require reducing equivalents from the same electron transport system and may compete. More recently it has been discovered that some drugs have antioxidant properties either as the parent compound or when they are metabolized (140) which inhibits lipid peroxidation in this system.

Lipid peroxidation was reported to require a loosely bound pool of microsomal iron but drug metabolism does not (129). When microsomes are prepared with EDTA drug metabolism will take place but lipid peroxidation is prevented (138). The addition of ferrous or ferric ions to the system stimulates peroxidation (129). Data from reconstitution studies implies that inorganic iron may not be necessary for initiation of the carbon tetrachloride-induced lipid peroxidation. When purified components of the mixed function oxidase were added to liposomes Masuda and Murano (26) found that iron was not needed in order for peroxidation to occur, however, iron may be a trace contam-

inant in these systems adhering to the flavo- or hemeprotein during purification.

The hemeprotein cytochrome P-450 is also implicated in the decomposition of lipid peroxides (141). When linoleic acid hydroperoxide was incubated with liver microsomes, the hydroperoxide was decomposed, presumably by a free radical mechanism. They hypothesized that linoleic hydroperoxide oxidizes the cytochrome P-450 thiol ligand to form a high spin P-420 state which has 2 to 8 times the peroxidase activity of the P-450 form. Microsomes from phenobarbital pretreated rats were found to have 2.5 times the cytochrome P-450 and showed an enhanced peroxidase activity, presumably the result of more P-420 molecules produced. Radtke and Coon (142) demonstrated that cytochrome P-450 generates lipid peroxides from organic peroxides such as cumene hydroperoxide without the reductase present. The reaction was linear for five minutes; a decrease in activity was associated with heme destruction. When they employed a reconstituted system containing NADPH, cytochrome P-450 reductase, cytochrome P-450 and dilauroyl glycerol-3-phosphatidylcholine, aminopyrine, benzphetamine and ethylmorphine were hydroxylated in the presence of cumene hydroperoxide indicating that the system has a greater affinity for the drug molecules. The phospholipid may increase the affinity of the cytochrome P-450 system for lipophilic drug molecules in lieu of the more polar hydroperoxides.

That lipid peroxidation is the basis for cell injury in the carbon tetrachloride system rather than solvation-related mechanisms

was shown in experiments where lipid peroxidation is allowed to take place in the absence of carbon tetrachloride. In isolated microsomes supplemented with NADPH and in the presence of oxygen (143) or in liver cells incubated with buffer (144) there is a loss of cytochrome P-450 heme and glucose-6-phosphatase activity. Masuda and Murano (145) found that in vitro peroxidation stimulated by carbon tetrachloride was NADPH-concentration dependent. Malonaldehyde production increased almost linearly in the presence of an NADPH-generating system. Experimental data indicates that NADPH is necessary for lipid peroxidation in the enzymatic pathway of carbon tetrachloride metabolism in the cytochrome P-450 system and that lipid peroxidation by itself may be responsible for the loss of cytochrome P-450 and glucose-6-phosphatase activities. However, the effect of carbon tetrachloride addition to a microsomal system with NADPH increases the extent of lipid peroxidation (Tables 17 and 20) as well as producing a more specific locus of destruction (Table 20). In the presence of carbon tetrachloride a destruction of phosphatidylserine occurs, the extent of which is not demonstrated with peroxidation induced by NADPH alone. The destruction involved the loss of stearic acid in addition to losses of arachidonic and docosahexenoic acids. Others (59) reported losses in arachidonic and stearic acids in the phosphatidylcholine fraction with carbon tetrachloride poisoning. The losses reported were only 2 to 3 percent, which agrees with the present data in that phosphatidylcholine was the most resistant phospholipid to carbon tetrachloride-induced peroxidation (Table 7). The metabolism of carbon tetrachlor-

ide apparently contributes a highly reactive metabolite, presumably trichloromethyl radicals, which result not only in the increased peroxidation of unsaturated fatty acids but also in the loss of saturated fatty acids as well. Additionally, phosphatidylethanolamine was lost from the endoplasmic reticulum (Table 6) and from the microsomes (Tables 11 and 12) and this loss was greater in the carbon tetrachloride group. May and McCay (146) found a 50 percent decrease in the phosphatidylethanolamine fraction when microsomes were incubated with NADPH. Concomitantly with a decrease in the phosphatidylethanolamine fraction, they observed a more polar phosphatide on thin layer chromatography. By pooling all the phosphorous determinations they concluded that although the phospholipids in the NADPH-supplemented system were considerably modified, they were not changed to the point of becoming non-extractable with lipid solvents, indicating that extensive lipid-protein polymerication had not occurred. The loss of microsomal protein associated with carbon tetrachloride-induced peroxidation (147) may be a consequence of phospholipid loss in the membrane via a "hole-in-the membrane" mechanism (148). Although May and McCay (146) accounted for all the phospholipid phosphorous in their determinations, it is possible that some phosphates were hydrolyzed from the phosphatidylethanolamine molecule. The phospholipid could have become unextractable by becoming bound to membrane proteins while the free phosphates would indicate to them that all phospholipids had been extracted and accounted for.

The selective loss of phosphatidylethanolamine molecules during

microsomal peroxidation may indicate that a portion of this phospholipid may exist in a different orientation than other phospholipids or that it is located very close to the reactive site. Phosphatidylethanolamine molecules in aqueous environments form hexagonal arrays and appear as sheets (149). It has been suggested that similar arrays of phosphatidylethanolamine may occur in the hydrophilic environment in the membrane (150). With such an orientation phosphatidylethanolamine would be openly exposed to peroxidative insult as active radicals produced by the cytochrome P-450 complex would encounter phosphatidylethanolamine and result in peroxidative breakdown. The current model of the orientation of the cytochrome P-450 complex within the endoplasmic reticulum proposes that phosphatidylethanolamine, by nature of its hexagonal arrangement, forms inverted micells in close proximity to the cytochrome P-450 protein (150).

If phosphatidylethanolamine is close to the locus of free radical production or if the molecule is oriented within the path of the free radical, phosphatidylethanolamine could undergo a drastic change as a result of its interaction with a high energy free radical and thus not be recovered with the other molecules in this fraction. It appears that phosphatidylethanolamine orientation and association in the endoplasmic reticulum differs from other phospholipids since phosphatidylserine was the most highly peroxidized phospholipid with carbon tetrachloride-induced peroxidation both _in vivo_ (Table 9) and _in vitro_ (Tables 17 and 18) yet it was recovered in nearly control amounts (Tables 8 and 14). Reynolds and Moslen (151) also found the

phosphatidylserine fraction to be the most highly peroxidized fraction with carbon tetrachloride-induced lipid peroxidation. They suggested that their results were due to oxidized contaminants of the phosphatidylserine fraction formed during the peroxidative event. Subsequent experiments indicated that the $^{14}C$ metabolites were found with 4 times the frequency in the phosphatidylserine fraction as that found for the phosphatidylethanolamine fraction (152). The fraction of phosphatidylserine containing the highest radioactivity did not migrate with the bulk of the phosphatidylserine on thin layer chromatography and they suggested that these uncharacterized materials may be breakdown products of oxidized lipids that migrate with the phosphatidylserine fraction.

The fact that phosphatidylinositol and phosphatidylserine are difficult phospholipids to separate has made it difficult for investigators to draw conclusions about either phospholipid. Since the combined phosphatidylinositol and phosphatidylserine fraction is a minor component of microsomal phospholipid (14 to 16 percent of the total microsomal phospholipid, see Table 2) it has received little attention in the literature in regard to microsomal peroxidation. In addition to being a minor component, phosphatidylserine is the most labile of the microsomal phospholipids; this is demonstrated by the high levels of endogenous peroxidation found in this fraction (Tables 9, 17 and 18).

In the present study the phosphatidylinositol and phosphatidylserine fractions were separated on high performance liquid chromatog-

raphy (HPLC) and checked for purity with two TLC methods (see Expermental Approach Section) as well as several staining techniques. The phosphatidylserine fraction was shown to have a 2 to 4 percent contamination found to be phosphatidylinositol. It was felt that since the phosphatidylserine fraction was very small, approximately 4 percent of the microsomal phospholipids, further purification would compromise the quantity and quality of this very labile phospholipid.

The increase in the peroxidation of the phospholipid fractions in the untreated controls *in vitro* compared to the *in vivo* controls (Table 9 vs. Table 17) could be the result of a chain reaction of peroxidative events; once initiated they would continue to proceed in washed microsomes while in the endoplasmic reticulum the protective molecules such as vitamin E and glutathione may prevent autocatalysis to some extent. Also, the presence of saturating amounts of carbon tetrachloride in the *in vitro* membrane lipids would not occur *in vivo*. A more generalized peroxidation is indicated *in vitro* by the 3 to 4-fold increase in the specific peroxidation activity of phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine while phosphatidylserine increased peroxidation by a factor of 2 over the *in vivo* levels (Table 17). The fraction of phosphatidylethanolamine which was recovered did not demonstrate a high level of peroxidation while phosphatidylinositol was found to have a higher specific peroxidation activity, in agreement with $^{14}C$ metabolite incorporation (152). The specific activity of phosphatidylcholine was observed to have a higher peroxidative increase in the *in vitro* experiments compared with the *in*

vivo experiments and this increase was much greater for phosphatidylcholine than for the other phospholipids (Tables 9 and 17). The increase in peroxidation for this phospholipid might reflect the changes of phosphatidylcholine orientation to the active site when vesicles are formed to replace the linear pattern of the bilamellar endoplasmic reticulum. Since phosphatidylcholine is approximately equally distributed on both sides of the endoplasmic reticulum (41) formation of microsomes could induce these molecules to flip to the outer surface closer to the reductase-cytochrome interaction. Since the flip-flop motion of phospholipids in erythrocytes was shown to have a half-time of 20 to 30 minutes (153) in the 4 to 5 hours in which microsomes exist for in vitro experiments, a considerable amount of flip-flop can occur.

The more specific attack of phosphatidylserine in vivo may reflect that the in vitro system does not provide much more additional exposure of phosphatidylserine to the free radical, or that there may be only a certain level of peroxidation possible for a molecule of phosphatidylserine and since it is already highly peroxidized, its potential for additional peroxidation is less than the other phospholipids. The assumption that greater peroxidation occurs in the outer membrane i.e., the cytoplasmic surface of the endoplasmic reticulum and the membrane at the buffer interface in microsomes, is supported by the observation that phosphatidylserine and phosphatidylethanolamine are severely affected by peroxidation in this organelle and both phospholipids are components of the outer membrane. The reductase is

located on the outer surface and has been shown to be necessary for lipid peroxidation (154,155) so it would appear that the lipid molecules on the outer surface would have more contact with the active site of radical formation than the inner surface.

The pattern of peroxidation, that is, the various susceptibilities of each phospholipid *in vitro* with the uninduced controls, was similar to the *in vivo* results (Tables 9 and 17), i.e. phosphatidylserine > phosphatidylinositol > phosphatidylethanolamine > phosphatidyl choline. This implies that the *in vitro* system of carbon tetrachloride-induced lipid peroxidation approximates the *in vivo* system. The increase in peroxidation *in vitro* with the greater susceptibility of phosphatidylcholine would probably be the result of flip-flop activity as well as a buffer effect, although the exact cause is not known.

Carbon tetrachloride induced changes in the fatty acid composition of the purified phospholipids

A fatty acid analysis indicates that phosphatidylserine is extensively damaged by peroxidation, especially in the presence of carbon tetrachloride *in vivo* (Table 9) and *in vitro* (Table 17). The loss of stearic acid and the reduction in the amounts of arachidonic and docosahexenoic acids suggests that phosphatidylserine is located close to the site where the free radical is generated. No other phospholipid is so extensively affected (Tables 7 and 20). Carbon tetrachloride is found to be more destructive to phosphatidylserine *in vitro* than the general oxidants NADPH and FeADP (Table 20).

All of the phospholipids had decreases in arachidonic and doco-

sahexenoic acids to a lesser extent than that which occurred in phosphatidylserine (Table 20). In addition, the pattern of fatty acid loss was similar in the in vivo and the in vitro systems (Table 22). On a molar basis phosphatidylethanolamine was found to be more susceptible than phosphatidylcholine to peroxidative damage (Table 22). Previous investigators have reported a greater susceptibility of phosphatidylethanolamine to peroxidation (152) while others reported that losses of unsaturated fatty acids occurred in phosphatidylethanolamine and phosphatidylcholine to the same extent(146)since phosphatidylcholine is at least twice as prevalent as phosphatidylethanolamine in the microsomes this may explain how the number of fatty acids destroyed in the phosphatidylcholine fraction, although equal to the number of fatty acids destroyed in the phosphatidylethanolamine fraction, represent a lower percentage of the more numerous phosphatidylcholine molecules.

The role of iron in the hemeprotein in the mechanism of peroxidation caused by carbon tetrachloride has been proposed to produce a one electron transfer from Fe(II) to carbon tetrachloride resulting in the trichloromethyl radical (156). It is also known that ferrous or ferric ions in the presence of ascorbate are powerful catalysts for the oxidation of emulsions of pure unsaturated fatty acids (157) and that inorganic iron is considered important in the catalysis of the peroxidation of emulsions of unsaturated fatty acids added to tissue homogenates (158). However, the effect of added iron in the form of FeADP to microsomes is not clearly understood. Hochstein and Ernster (159) first reported that microsomes catalyzed an ADP-activated per-

oxidation of lipids coupled to the TPNH oxidase system. They observed that the oxygen consumption greatly exceeded the disappearance of TPNH and must be accounted for by the oxidation of microsomal material. Peroxidation of lipids was found to be dependent on the presence of contaminating iron compounds in the ADP solution (160). The function of inorganic iron as a catalyst for the peroxidation of unsaturated fatty acids in vivo as well as in vitro has been well documented (157,158, 160,161). It was of interest to determine the pattern of peroxidative attack with FeADP on microsomal phospholipids and fatty acids and also to compare the nature of the peroxidative damage caused by the addition of NADPH alone - would the damage resemble iron catalyzed peroxidation and would these general oxidants produce the same pattern of destruction as induced by carbon tetrachloride.

The general oxidants NADPH and FeADP were found to be less effective as oxidizing agents in vitro compared with carbon tetrachloride (Tables 17 and 18). The loss of phosphotidylethanolamine from these microsomes is not as severe as that which occurs with carbon tetrachloride administration (Tables 11 and 12) and the pattern of fatty acid loss is different (Table 20). Unlike the in vivo or in vitro carbon tetrachloride-induced peroxidation in which phosphatidylserine lost stearic acid in addition to arachidonic and docosahexenoic acids, neither NADPH nor FeADP administration in vitro resulted in the phosphatidylserine fraction losing stearic acid, although arachidonic and docosahexenoic acids were both lost but to a lesser degree (Table 20).

As might be expected with general oxidizers, NADPH and FeADP

increased peroxidation approximately 4-fold over endogenous levels in all the phospholipids (Table 17) although the phosphatidylcholine fraction had a somewhat higher increase which is a general feature of in vitro peroxidation. The fatty acid loss in both NADPH and FeADP/NADPH systems was very similar in amount and pattern to each other (Table 20). Phosphatidylserine was the most affected phospholipid but fatty acid loss was also high in the phosphatidylethanolamine fraction with phosphatidylcholine and phosphatidylinositol relatively unaffected. Microsomes incubated in the presence of NADPH either under normal or hyperbaric oxygen tensions (146) revealed that the only selectivity of the system was primarily toward the polyunsaturated fatty acids and not toward a particular phospholipid group. In the experiments reported here, FeADP/NADPH was shown to be slightly more destructive to phosphatidylinositol and phosphatidylcholine than NADPH alone (Table 17) but the response was unlike that which occurred with carbon tetrachloride administration in which stearic acid was lost from phosphatidylserine. If inorganic iron in the presence of adenine nucleotides is the initiator of peroxidation in microsomes as has been previously indicated (129), the addition of exogenous FeADP/NADPH did not result in a significantly greater increase in peroxidation over that observed by the addition of NADPH alone. The observation may be explained by the possibility that inorganic iron in trace amounts may already be a contaminant of microsomes and that exogenous iron additions would produce little increase in peroxidation over that observed with NADPH alone. The ability of EDTA to inhibit perox-

idation is believed to be the result of its ability to chelate ionic iron (129); EDTA may also be chelating other ions in the system which are important for peroxidation.

The effect of carbon tetrachloride on phenobarbital-induced endoplasmic reticulum, in vivo and in vitro

Phenobarbital induction of the microsomal electron transport system has been reported to enhance carbon tetrachloride hepatotoxicity (19,162,163) and microsomal lipid peroxidation (78,144,164,165).

Garner and McLean (19) demonstrated that after two weeks pretreatment with phenobarbital and $LD_{50}$ dose for carbon tetrachloride is reduced from 3.6 mls $CCl_4$/kg body weight in controls to 0.5 ml/kg in phenobarbital pretreated rats. Microsomes prepared from phenobarbital pretreated rats metabolized 4.3 μm $CCl_4$/g of liver/hour compared with 1.1 μm/g of liver/hour for controls. This aspect of increased metabolic rate of carbon tetrachloride metabolism with phenobarbital pretreatment may have important consequences with respect to the cell's ability to deal with a flood of reactive carbon tetrachloride metabolites released from the cytochrome P-450 system.

The phenobarbital pretreated rats do appear to have more labile lipids in the endoplasmic reticulum since the endogenous peroxidation is higher in this group than in control rats (Table 9). This increased sensitivity to peroxidation has been observed by others (164,166). However, Suarez et al. (165) reported no difference in the microsomal sensitivity to peroxidation with oral carbon tetrachloride administration in phenobarbital pretreated animals (3.0 mls $CCl_4$/kg body weight)

but an increased sensitivity in the microsomal lipids when carbon tetrachloride is inhaled. Their results are difficult to interpret since the oral dosing is absorbed over a shorter period of time and in greater quantities than a dose that has been inhaled over a longer period of time. One would expect that a flood of toxic metabolites from an oral dose would produce a greater lesion than a dose of carbon tetrachloride which has first passed through the lungs before being absorbed into the liver.

The change in fatty acid composition of the endoplasmic reticulum after phenobarbital pretreatment would not appear to offer an explanation for the increased susceptibility for endogenous peroxidation found in this group. Phenobarbital administration results in a decreased amount of the more susceptible polyunsaturated fatty acids, i.e. arachidonic and docosahexenoic, while the content of linoleic acid, which has not been shown to be as susceptible to peroxidation as are the polyunsaturates, increases (Table 3).

Holtzmann (66) established that phenobarbital primarily increases the endoplasmic reticulum content of the hepatocyte by a decrease in phospholipid catabolism. A decrease in catabolism during the initial stages of membrane induction with phenobarbital administration could prevent the normal turnover of peroxidized lipids, resulting in a membrane with a higher degree of endogenous lipid peroxidation as seen in the phenobarbital induced membrane. It has been suggested that lipid peroxidation may have a role in the normal turnover of the membranes of the endoplasmic reticulum (141) and that the degradation of cytochrome P-450 during lipid peroxidation may account for the high rate of turn-

over of the hemeprotein in the liver under normal conditions (167). However, after the endoplasmic reticulum has reached a plateau from phenobarbital induction (168) the turnover rates of the lipid constituents may increase over the turnover rates seen in controls.

The maintenance of an extensive membraneous system in hepatocytes following phenobarbital administration where conditions leading to peroxidation are present, i.e. oxygen radicals and iron, may place a stress on the homeostatic mechanisms in the cell which prevent or minimize endogenous peroxidation. Phenobarbital induced endoplasmic reticulum may not have proper levels of antioxidants, vitamin E for example, and free radical scavengers, such as glutathione to prevent oxidation of the membrane. The animals were fasted 14 to 16 hours prior to experimentation. Fasting has been shown to decrease hepatic glutathione levels (169). A reduction in glutathione levels in a cell containing a two-fold increase in phospholipids with a high percentage of polyunsaturated fatty acids could result in higher endogenous levels of peroxidation than the untreated controls.

The relationship of glutathione to lipid peroxidation is not clear. O'Brien and Little (170) found that glutathione in tissue suspensions decomposed linoleic acid hydorperoxides to a hydroxydienoic acid. Whether or not glutathione functions in this manner <u>in vivo</u> is not known since glutathione was demonstrated to prevent the initial formation of hydroperoxides in microsomes (171). Glutathione peroxidase activity could exert its effect by preventing the initial free radical attack. If the role of glutathione does prevent the

initial free radical attack, then the low levels of lipid peroxidation in the in vivo uninduced controls challenged with carbon tetrachloride may be explained by the presence of glutathione. The phenobarbital induced system may not have sufficient levels of glutathione to protect the membrane from increased levels of hemeprotein present, therefore higher endogenous levels of peroxidation exists in this membrane.

In hepatocyte cell culture (134) iron stimulated lipid peroxide formation when cell glutathione content was depleted with diethylmaleate treatment. This may reflect the in vivo situation in which decreases in cell glutathione content may result in a more peroxidatively-susceptible membrane. The finding that there is a decrease in the proportions of phosphatidylserine and phosphatidylinositol in microsomes derived from phenobarbital pretreated animals may provie some insight as to why there is increased oxidation within the microsomal lipids if these phospholipids are involved in maintaining membrane stability in this system. The diene detection method for quantitation of peroxidative changes which precluded the usage of antioxidants in the preparative stages of microsome purification since the antioxidants would interfer with the UV absorption spectra. Once the lipids were extracted they were stored with antioxidants to prevent further oxidation.

The process of lipid peroxidation is associated with a molecular rearrangement known as diene conjugation which is not dependent on the formation of oxidative breakdown products, many of which have

not been characterized. The molecular rearrangement is of widespread occurrence and is a specific change with an absorption within the narrow range of 232-234 nm. Thus by measuring diene conjugation, a precise chemical change, the peroxidative changes occurring in all unsaturated fatty acid molecules are indicated.

Many investigators use the thiobarbituric acid (TBA) method to quantitate production of malonaldehyde. The TBA test is based on the formation of a chromagen through the condensation of two molecules of TBA with one molecule of malonaldehyde. Malonaldehyde is produced when fatty acids with three or more double bonds are peroxidized (172) while mono- and dienoic fatty acids are presumably not measured. It was important to monitor peroxidative changes in all unsaturated fatty acids because the increase in linoleic (18:2) with phenobarbital administration could influence the extent of peroxidation present however the extent of peroxidation present would not be reflected for linoleic using the TBA method. Chromagen development in the TBA test is dependent on pH, heat and the type of peroxides present (173). In oxidizing systems where proteins are also present, competition of amino acids for the malonaldehyde may occur resulting in lowered color production (174). Meaningful quantitative results from the TBA test can only be obtained by comparing samples of a single material at different stages of oxidation since the number and variety of variables which can influence the TBA color would make this method unsuitable for quantitative measurements.

Phenobarbital induced endoplasmic reticulum has greater peroxidation of lipids after carbon tetrachloride challenge <u>in vivo</u> than

controls. This is not the result of greater enzymatic activity by the reductase and cytochrome but rather a higher level of endogenous peroxidation present initially (Table 9). The increase over the endogenous peroxidation levels is actually less for the phenobarbital induced membrane as that which occurs in controls (Table 9), indicating a decrease in the enzymatic activity of the cytochrome P-450 system. In contrast to the control microsomes in which carbon tetrachloride challenge produced a 3.5-fold increase in peroxidation in the phosphatidylserine fraction and a 2.6-fold increase in the phosphatidylcholine fraction (Table 9), the phenobarbital-induced membrane challenged with carbon tetrachloride resulted in a 2.7-fold increase in peroxidation both in phosphatidylserine and phosphatidylcholine. This seems to indicate that the phenobarbital-induced system demonstrates a more generalized peroxidation in response to carbon tetrachloride challenge than the control microsomes in vivo. In total amounts of peroxidation phosphatidylserine does have the highest level, although much of this is the result of high endogenous levels (Table 9). The resistance of phosphatidylethanolamine to carbon tetrachloride challenge in the phenobarbital pretreated system is reflected by the slight loss of phosphatidylethanolamine in this system compared to controls (Table 6). The resistance to loss and peroxidation of the phosphatidylethanolamine fraction and the increased susceptibility of phosphatidylcholine to carbon tetrachloride challenge may represent some difference in the way phosphatidylethanolamine and phosphatidylcholine are oriented in the phenobarbital-induced system.

Changes in fatty acid composition of the phenobarbital-induced endoplasmic reticulum with carbon tetrachloride challenge in vivo

As in the untreated in vivo control membranes phosphatidylserine was more susceptible to carbon tetrachloride challenge than the other phospholipids in the phenobarbital pretreated rats (Table 9). Phosphatidylserine lost twice the amount of both saturated acid (stearic) and unsaturated acids (arachidonic and docosahexenoic) on a molar basis when compared to uninduced membranes (Table 7). The specific activity for diene conjugates, although higher, does not appear to reflect the more extensive damage occurring in phosphatidylserine (Table 19). The explanation for the lack of a 2-fold increase in diene absorption could be that the loss of fatty acids from the phosphatidylserine molecule would not be present to influence the diene conjugation measurements. Increased fatty acid loss appears to be a separate measurement of phospholipid destruction from the diene measurement of peroxidation. The other phospholipid fractions lost only arachidonic and docosahexenoic acids and the loss, on a molar basis, was less in the phenobarbital pretreated animals than the loss found in the uninduced controls (Table 8).

The data taken together indicate that the phenobarbital-induced system has a higher endogenous peroxidation level while the enzymatically catalyzed NADPH-dependent lipid peroxidative activity is not as high as in controls. The only exception was the 2-fold increased loss of fatty acids in the phosphatidylserine fraction. This may indicate that phosphatidylserine is located very near to the activated

species present in the cytochrome P-450 protein since the reductase activity overall, determined by the extent of peroxidation occurring in excess of initial endogenous levels, was not as high in this system. The phenobarbital-induced system does have higher total specific activities in each phospholipid fraction as compared to controls (Table 9) but this is the result primarily of higher endogenous levels of lipid peroxidation. The lower *in vivo* activity of the cytochrome P-450 system to catalyze lipid peroxidation in the phenobarbital pretreated system may reflect a rate-limiting capacity of the mitochondria to provide necessary reducing equivalents for all of the cell's needs. That this may in fact be the case is suggested by the finding that the ratio of NADPH/NADP+ was only 83 percent of control levels in phenobarbital pretreated animals (175). NADPH is not only required in greater amounts by the increased level of cytochrome P-450 present, it is also used in the synthesis of fatty acids (176) as well as in the desaturation of fatty acids catalyzed by the cytochrome $b_5$ reductase (177). The decreased content of the polyunsaturates arachidonic and docosahexenoic in microsomes of phenobarbital pretreated animals may reflect the rate limiting quantities of NADPH present for the desaturases.

*In vitro peroxidation in phenobarbital induced microsomes with carbon tetrachloride challenge*

*In vitro* the phenobarbital-induced microsomes were much less active in producing enzymatic peroxidation than the controls (Tables 17 and 18). Even with higher endogenous levels of lipid peroxidation the total specific activities of the phospholipid fractions were lower with

the exception of the phosphatidylcholine fraction (Table 18). While the control *in vitro* microsomes showed a 6-fold increase in specific activity for phosphatidylserine and a 5-fold increase in the other phospholipids in response to carbon tetrachloride challenge, the phenobarbital-induced microsomes demonstrated only a 4-fold increase in the specific activity of phosphatidylserine, while the other phospholipids demonstrated a 3-fold increase (Table 18). Phosphatidylcholine, both in uninduced and induced systems, had the highest increase in peroxidation *in vitro* (Tables 17 and 18). This could reflect a greater exposure of phosphatidylcholine to saturating amounts of carbon tetrachloride present in the buffer. *In vitro* controls had an 8-fold increase in specific activity of the phosphatidylcholine fraction with carbon tetrachloride challenge compared to a 5.5-fold increase for the phenobarbital-induced membrane *in vitro*. Although the increase in peroxidation was higher for the controls, the total specific activities were the same for both groups (Tables 17 and 18).

The possible explanation for these observations may be that only a certain amount of peroxidation can occur before the cytochrome P-450 system is inactivated (178). It was suggested that some chain-breaking process operates in the region of the reductase to prevent lipid peroxides produced by the trichloromethyl radical from involving lipid-rich areas away from the initiation site. More recently the increased level of lipid peroxides was found to correlate with decreased activity of the cytochrome P-450 system (15). Whatever the explanation, it appears that a certain amount of peroxidation occurs

before the reaction stops or slows down. It was felt that the concentration of NADPH used in the in vitro studies could be rate limiting. Masuda and Murano (145) found that in vitro carbon tetrachloride -induced lipid peroxidizing systems required less than 200 μm of NADPH for a 20 minute incubation with a protein concentration of 1 mg/ml of buffer for uninduced microsomes. In the experiments reported here the in vitro incubations of control and phenobarbital pretreated microsomes contained the same concentrations of protein per ml of buffer with the same final NADPH concentration, 570 μm. Since the microsomal protein concentrations were the same in phenobarbital and control systems the predominant protein in suspensions made from phenobarbital pretreated animals would be cytochrome P-450 since phenobarbital stimulates the synthesis of the cytochrome P-450 protein 3-fold over control levels, while the reductase increases 2-fold (179). Thus, for the amount of protein present in the incubations there is an increase in the cytochrome P-450:reductase ratio with greater representation of the cytochrome molecule with fewer reductase molecules present. Consequently the reductase reaction becomes rate-limiting with phenobarbital administration (180). It seems reasonable to predict that NADPH is not the limiting factor in this system, although the possibility exists and cannot be excluded that NADPH could be limiting. Since the cytochrome P-450:reductase ratio increases with phenobarbital administration, the ability of the reductase to interact with the cytochrome molecule may be more difficult to effect in this system and consequently may be limiting. If

peroxidation results in producing a more rigid membrane as suggested (181), the reductase:cytochrome interaction would be hindered as peroxidation progressed. Phenobarbital-induced microsomes seem to demonstate that some hindrance may be occurring in this system since carbon tetrachloride challenge in vitro produced a decreased amount of peroxidative destruction (Table 18) compared with control microsomes (Table 17), and the loss of fatty acids from the induced membrane is also less when compared with the loss occurring in control microsomes (Tables 20 and 21). Losses in arachidonic and docosahexenoic fatty acids were only 30 and 50 percent, respectively, of the loses of these polyunsaturated fatty acids lost in control microsomes (Table 22).

In the phosphatidylserine fraction, the phenobarbital-induced microsomes lost almost 50 percent of the stearic acid while the control microsomes lost only 23 percent of this fatty acid. The data indicate that phosphatidylserine, unlike the other phospholipids, is more extensively damaged with carbon tetrachloride challenge in the phenobarbital-induced membrane. The increased destruction to this phospholipid may either be caused by a change in orientation within the cytochrome complex or by a closer association of the more numerous cytochrome molecules with phosphatidylserine.

A further indication that phenobarbital-induced microsomes might differ from control microsomes in their orientation of the protein-lipid interactions is reflected in the loss of the phosphatidylethanolamine fraction. In control microsomes carbon tetrachloride challenge caused a 40 percent loss of phosphatidylethanolamine, while NADPH and

FeADP/NADPH-induced peroxidation caused an approximate 30 percent loss (Table 11). In the phenobarbital-induced membrane *in vitro*, carbon tetrachloride caused a 45 percent loss of phosphatidylethanolamine while NADPH and FeADP/NADPH resulted in a loss of approximately 25 percent of phosphatidylethanolamine (Table 12). The increased loss of phosphatidylethanolamine from the phenobarbital-induced microsomes, while only 5 percent greater than the loss from control microsomes is a significant change from the *in vivo* system in which the phenobarbital-induced endoplasmic reticulum lost only 5 percent of phosphatidylethanolamine with carbon tetrachloride challenge while a 10 percent loss of phosphatidylethanolamine was observed for controls (Table 6). Formation of microsomes from phenobarbital pretreated animals produces a membrane in which phosphatidylethanolamine is more readily lost with carbon tetrachloride administration, while the general oxidants were less effective in removing phosphatidylethanolamine from induced membranes when compared with control membranes (uninduced). Such results suggest that the membrane in the induced system undergoes remodeling during microsomal preparation which changes its response to pro-oxidants from that observed in the *in vivo* situation, unlike the control microsomes in which the *in vitro* results are an enhancement of the *in vivo* experiments.

The decrease of enzymatically catalyzed peroxidation is also reflected by the presence of lower diene conjugates in the phenobarbital-induced system (Table 18).

Although phenobarbital pretreatment may produce a membrane in

which the fatty acid constituents would favor an increase potential for lateral diffusion, other influences may cancel this effect. The influence of additional cytochrome P-450 molecules may provide some hindrance to the interaction of the reductase with the cytochrome. Longmuir (182) has shown that for the membrane associated with the cytochrome c protein of mitochondria the protein can hinder the lateral movement of phospholipids in the membrane. Additional cytochrome P-450 molecules present in the membrane may lower the lateral mobility of the reductase resulting in a decreased interaction with cytochrome molecules. The system is complex and the decreased activity of the phenobarbital-induced membrane in vitro is most likely the result of a number of factors rather than a single influence.

The presence of higher levels of endogenous lipid peroxidation in phenobarbital microsomes, a higher cytochrome P-450:reductase ratio, a lower percentage of polyunsaturated fatty acids and a greater lipid:protein ratio in the membrane can exert an effect which is reflected in a decrease in peroxidative activity. In addition, the different pattern of peroxidation observed in vitro with induced microsomes may indicate that the presence of each or all of the above differences within the membrane could result in an orientation change of the cytochrome complex to the membrane or between components of the system which are enhanced during the preparative techniques necessary for microsome purification. If phosphatidylinositol and phosphatidylserine are involved in maintaining acyl order and stability

within microsomes, the decrease in these phospholipids with phenobarbital administration may provide some additional insight into possible mechanisms for the changes observed in the induced membrane.

Many investigators have reported on the increased toxicity of carbon tetrachloride administration to animals pretreated with phenobarbital (19,47,178). In a phenobarbital-induced cytochrome P-450 system the administration of carbon tetrachloride would result in an increased production of trichloromethyl radicals over those produced by untreated controls since the metabolic rate of carbon tetrachloride with phenobarbital induction has been shown to increase (19). Thus, on a cellular basis the production of lipid peroxides is accelerated because there is a greater potential for increased quantities of carbon tetrachloride to be metabolized by the increased amounts of cytochrome P-450 present. Since carbon tetrachloride metabolism has been found to parallel lipid peroxidation (8) more lipid peroxides would be produced per cell. The peroxide concentration and the by-products of lipid peroxidation would be higher in phenobarbital pretreated animals compared with controls. The decrease in the $LD_{50}$ dosage with carbon tetrachloride in pretreated animals can in part be attributed to the increased lipid peroxide equivalents produced in the extensive cytochrome P-450 system which exists in the phenobarbital-induced membrane. However, the ratio of lipid peroxides formed to the total microsomal lipid present is not greater in the phenobarbital-induced system than that produced in controls.

These peroxidative changes with phenobarbital administration

and carbon tetrachloride challenge have previously been expressed as the thiobarbituric acid-reactive substances (114,145,165) with the assumption that the protein:phospholipid ratio is the same for phenobarbital-induced membrane as it is for controls. Since the ratio of phospholipid:protein increases with phenobarbital administration the increased amount of peroxidized lipid detected either with thiobarbituric acid or diene absorption spectra would be due to the increased amount of phospholipid present per milligram of protein in the induced endoplasmic reticulum rather than to an increase in the specific activity of peroxidation.

The degree to which lipid peroxides contribute to cell injury and death is not known, although lipid peroxides have been shown to be deleterious to many biological processes. Oxidized unsaturated fatty acids inhibit water soluble enzymes (183), linoleic hydroperoxides have been associated with decreases in the activity of the cytochrome P-450 protein (184) and to have hemolytic properties (185). Studies involved in the quantitation of the hemolytic activity of peroxidized microsomes and their peroxide content revealed that subsequent to an initial peroxidative breakdown of the erythrocyte membrane the production of toxic lipid metabolites appear to be capable of destroying microsomal enzymes in a secondary step (186).

When fatty acids peroxidize their polarity increases, making them more soluble in aqueous media (146). By traversing from their site of origin these toxic substances could be deleterious not only to the functional units of the endoplasmic reticulum but also to other components of the cell. Mitochondria are disrupted when mixed with

peroxidizing microsomes (160), lysosomes lyse (187) and erythrocytes peroxidize (32).

Conversely, when soybean lipoxygenase and linoleic acid were added to microsomal suspensions, a loss of microsomal cytochrome P-450 binding spectra was observed (33) and lipids extracted from peroxidized microsomes, when added to non-peroxidized microsomes, were found to decrease the cytochrome P-450-carbon monoxide binding spectra by more than 50 percent (15).

It is apparent from these experiments that lipid peroxides can migrate from their site of production within the microsome to cause cell injury as well as migrate from the exterior to cause damage within the microsomes itself. Peroxide diffusability has great significance for the peroxidative damage of the cell in vivo; peroxides can apparently cause destruction either by leaving the endoplasmic reticulum or upon entering the endoplasmic reticulum from the cytoplasm.

Phenobarbital administration results in a hepatocyte with an increased membrane enriched with the drug metabolizing system. When such a cell is exposed to carbon tetrachloride, a flood of radicals, presumably trichloromethyl radicals, is generated which have the potential to produce cell injury at the site of production as well as loci removed from the actual site of peroxide production.

The lipoperoxidation theory of carbon tetrachloride-induced cell injury provides a mechanism by which small doses of carbon tetrachloride can produce extensive destruction of tissue mediated by

products of lipid peroxidation which remain to be characterized.

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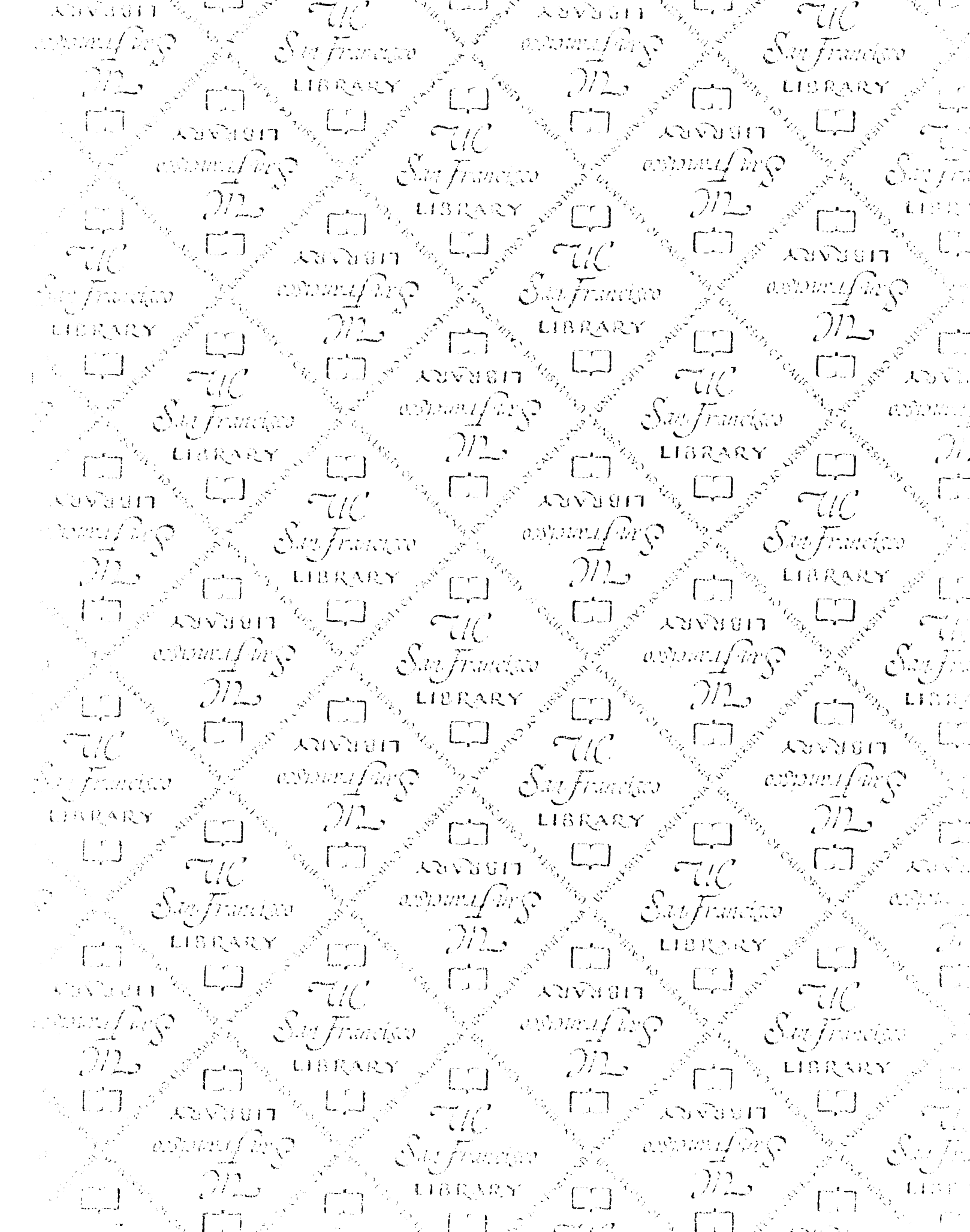
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