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COVALENTLY BOUND ORGANOHALOGEN METABOLITES
TO LIPID COMPONENTS

by

Michael Lee Cunningham

A Dissertation Submitted to the Faculty of the
COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1981
As members of the Final Examination Committee, we certify that we have read
the dissertation prepared by Michael Lee Cunningham
entitled Covalently Bound Organohalogen Metabolites
to Lipid Components

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SIGNED: [Signature]
To my parents

Jack and Helen Cunningham

for their love and support
Aut inveniam viam aut faciam

Charles Percy Snow
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ABSTRACT

Bioactivation of organohalogen xenobiotics produces reactive intermediates which alkylate macromolecules. The activation of carbon tetrachloride, trichloroethylene, and methylene chloride was studied in isolated rat hepatocytes by examining alkylation of lipid, protein, RNA, and DNA. All organohalogen alkylated lipid and protein. Carbon tetrachloride and trichloroethylene, but not methylene chloride, alkylated RNA and DNA. Methylene chloride was more highly activated in an oxygen containing atmosphere by hepatocytes, consistent with a proposed formation of formyl chloride as its reactive intermediate. Trichloroethylene was also shown to be more highly activated in an oxygen containing atmosphere, consistent with a proposed trichloroethylene epoxide reactive intermediate. Carbon tetrachloride was shown to be more highly activated in an oxygen-free atmosphere, consistent with a proposed trichloromethyl free radical reactive intermediate. Hepatocytes from rats pretreated with phenobarbital to induce cytochrome P-450 mixed function oxidase activated carbon tetrachloride and trichloroethylene to alkylating intermediates greater than did hepatocytes from non-induced rats.

The interaction of carbon tetrachloride metabolites with fatty acids was studied in a chemical activation model system. The thermal decomposition of benzoyl peroxide produced free radicals which activated carbon tetrachloride. The resulting trichloromethyl free radicals abstracted a hydrogen from methyl stearate resulting in chloroform and
fatty acid free radicals. Using chemical ionization mass spectrometry, it was discovered that the fatty acid free radical abstracted a chlorine from carbon tetrachloride resulting in chlorinated fatty acid esters. When methyl oleate was used as a substrate in the benzoyl peroxide model system, it was discovered that the trichloromethyl free radical binds covalently, resulting in a fatty acid adduct radical. This radical then abstracted a chlorine to produce chlorotrichloromethyl stearic acid methyl ester, identified by chemical ionization mass spectrometry. Carbon tetrachloride radiolabeled with $^{14}$C or $^{36}$Cl in dual label binding experiments in the benzoyl peroxide model system confirmed the mass spectral data. Methyl stearate bound $^{36}$Cl$^{-}$ and $^{14}$C-carbon tetrachloride in the ratio of approximately 10 to 1, whereas methyl oleate bound in the ratio of approximately 3.5 to 1.

The existence of fatty acid radicals due to hydrogen abstraction or covalent binding by trichloromethyl free radicals was demonstrated in microsomal preparations. In the presence of tritiated water and $^{14}$C-carbon tetrachloride, dual-label analysis demonstrated that the tritium incorporation into microsomal lipids approximately equalled the sum of carbon tetrachloride metabolites bound covalently to microsomal lipids and chloroform production.
INTRODUCTION AND LITERATURE SURVEY

Xenobiotics are compounds that are foreign to biological organisms. Most often they are organic molecules useful in industrial, agricultural, or household applications as solvents, pesticides, cleaning agents, etc. Many xenobiotics are chemically inert compounds that are harmless to living organisms in their parent form. However, they may be altered by enzymes in the smooth endoplasmic reticulum or cytosol and become more toxic to the animal. This process is termed bioactivation if the altered xenobiotic results in increased toxicity to the host compared to the parent compound.

A wide variety of structurally dissimilar compounds can be bioactivated, resulting in short-lived chemical entities termed reactive intermediates which are unstable and capable of alkylating cellular molecules. General classes of reactive intermediates include carbonium ions, carbanions, carbenes, free radicals, or epoxides. The structure of the reactive intermediate depends upon the chemical structure of the parent compound. These highly reactive, electrophilic intermediates may initiate their toxic effects by interacting with critical nucleophilic target sites in cellular macromolecules resulting in covalent attachment of the xenobiotic metabolite to the macromolecule. Target macromolecules may be DNA or RNA in the case of nitrosamines and nitrosamides (Miller, 1970), or protein and lipid in the case of halothane (Sipes, Podolsky and Brown, 1977).
Because of the high reactivity of the intermediates, it is not possible to isolate and quantify their production directly. An indirect method of assessing the formation of reactive intermediates is to determine their covalent binding to tissue macromolecules. For example, the bioactivation of bromobenzene to bromobenzene arene oxide can be measured by the rate of covalent binding of the $^{14}$C-labeled bromobenzene metabolite to liver protein. In addition, the covalent binding to protein can be correlated with bromobenzene-induced centrolobular necrosis of the liver. Similarly, the covalent binding of halothane, carbon tetrachloride, acetaminophen, polycyclic aromatic hydrocarbons and many other substances has been implicated as a mechanism by which they produce tissue injury. Parenthetically, it is becoming an axiom of toxicology that if a metabolite is stable enough to be isolated, it is probably not the agent one is looking for as the cause of tissue damage.

The compounds chosen for this study are the organohalogenes carbon tetrachloride, trichloroethylene, and methylene chloride. These compounds are bioactivated by enzymes in smooth endoplasmic reticulum and bind covalently to tissue macromolecules. The tissue necrosis caused by these compounds is believed to be a result of bioactivation. The study of these compounds has both practical as well as theoretical value, because millions of workers are exposed to them in industrial settings.

Early support for this concept came from the studies of Reid and Gillette. Reid (1973) correlated the amount of covalent binding and
the severity of centrolobular necrosis with halogenated hydrocarbons of varying toxicity such as bromobenzene, chlorobenzene, and iodobenzene. Also, he showed that as the rate of bioactivation was increased by pretreatment with phenobarbital, there was increased covalent binding of radiolabeled metabolites to macromolecules and a potentiation in the toxicity of these compounds. Conversely, decreasing bioactivation capability with piperonyl butoxide or SKF-525A decreased their covalent binding and toxicity.

Gillette's studies (1973) have illustrated the relationship between covalent binding and hepatic necrosis with the over-the-counter pain reliever acetaminophen. At doses below 100 mg/kg, both toxicity and covalent binding are negligible. At higher doses, however, necrosis and binding occur. It was found that as the normally high levels of liver glutathione fall to less than 75% of control, covalent binding of acetaminophen reactive intermediates markedly increases. Subsequently, tissue necrosis parallels covalent binding, and only occurs when GSH levels are depleted. These studies as well as others with chloroform (Ilett et al., 1973) and halothane (Rao, 1977) provide evidence for the role of covalent binding of bioactivated metabolites to tissue macromolecules as a possible mechanism for tissue injury.

Carbon Tetrachloride

Toxicity

Although carbon tetrachloride has been used as a tool to study chemically induced tissue injury for more than a decade, the molecular pathogenesis of acute hepatic damage is still not understood.
There are several theories as to the molecular mechanisms of carbon tetrachloride-induced liver necrosis, but they all agree that metabolic activation to reactive intermediates is a necessary first step. Formed by enzymes present in the endoplasmic reticulum of the liver, these reactive intermediates then interact with liver macromolecules resulting in an impairment of cellular integrity leading to cell death (Reynolds, 1967).

The administration of carbon tetrachloride, a "classic" hepatotoxin, produces well characterized and reproducible liver necrosis. Within a few hours of administration there is an accumulation of fat initially in the centrilobular regions. Swelling and ballooning of the cytoplasm in the hepatocytes surrounding the central vein occurs, while fat accumulation extends into the periportal region. Centrilobular cells may die, leaving the periportal area hepatocytes to perform the functions of the liver and to regenerate new cells. Over a period of several days, regeneration occurs and the liver gradually recovers (Torrjelli, 1978).

The earliest biochemical events due to carbon tetrachloride administration are summarized by Slater (1978). Within 5 minutes of oral dosing with carbon tetrachloride there is easily detectable covalent binding of the carbon tetrachloride metabolite to lipids and proteins. A few minutes later lipid peroxidation begins, reaching its maximum in about 1-2 hours. By 30 minutes, morphological alterations in the endoplasmic reticulum begins to show and a reduction in protein synthesis can be seen. NADPH content decreases and between 1-3 hours
the activities of several enzymes such as glucose-6-phosphatase and cytochrome P-450 are depressed. By 12-18 hours, centrilobular cells have become severely damaged and lysosomal damage has occurred. By 24 hours the centrilobular necrosis has reached its maximum and healing ensues.

Sipes, Krishna, and Gillette (1977) demonstrated that cytochrome P-450 is the enzyme responsible for the bioactivation of carbon tetrachloride. They presented several lines of evidence demonstrating this. Using covalent binding of radiolabeled carbon tetrachloride metabolites to microsomal protein as an index of bioactivation, they demonstrated an 80% reduction in binding as a result of incubation in 80% of carbon monoxide, 20% oxygen as compared to 80% N₂:20% O₂. Similarly, by omitting NADPH, a cofactor for cytochrome P-450 coupled reactions, a 95% reduction in covalent binding was observed. Definitive proof confirming the role of cytochrome P-450 comes from their use of allyl isopropyl acetamide, which reduces only the P-450 cytochrome and led to reduced covalent binding of the carbon tetrachloride metabolite. This treatment did not decrease the level of cytochrome P-450 reductase, proving that this enzyme is not the site of the bioactivation of carbon tetrachloride.

The nature of a reactive intermediate is, by definition, exceedingly difficult to identify due to its reactivity and subsequent short half-life. The half-life of the carbon tetrachloride reactive intermediate formed from carbon tetrachloride has been estimated to be 100 microseconds. The most widely accepted structure of the carbon tetrachloride reactive intermediate is the trichloromethyl radical (•CCl₃). Proof for this structure comes from spin trapping (Lai et al.;
1979) and by the demonstration of the dimerization product hexachloroethylene (Fowler, 1969). The existence of a trichloromethyl radical explains the production of chloroform as the major metabolite of the anaerobic metabolism of carbon tetrachloride. The trichloromethyl radical could abstract a hydrogen radical from a donor molecule (D-H) resulting in chloroform and another radical:

\[ \cdot\text{CCl}_3 + \text{D-H} \rightarrow \text{HCCl}_3 + \cdot\text{D} \]

The oxidative metabolism of carbon tetrachloride produces carbon dioxide and carbon monoxide. Mechanisms accounting for these products postulate two different types of reactive intermediates. Slater's group (Packer, Slater, and Wilson, 1978) proposes a trichloromethylperoxy radical which results from the interaction of trichloromethyl free radical and oxygen:

\[ \cdot\text{CCl}_3 + \text{O}_2 \rightarrow \text{Cl}_3\text{C-O}_2^\cdot \]

The postulate is that this could decompose to phosgene (Cl₂CO) and then to carbon dioxide (CO₂).

Ullrich's group (Ullrich et al., 1979), on the other hand, proposes a carbene intermediate to explain the production of carbon monoxide from carbon tetrachloride (Appendix D, Figure D.1.). They believe that the soret absorption at 450 nm is due to the interaction of carbon tetrachloride and cytochrome P-450 caused by this carbene intermediate binding to cytochrome P-450.

Two of the most extensively studied consequences of carbon tetrachloride bioactivation are lipid peroxidation and covalent
binding of the activated metabolite to cellular macromolecules such as lipid, protein, and nucleic acids. There is considerable evidence in the literature favoring both phenomena as the critical events leading to hepatic necrosis. However, agreement exists only on the requirement for bioactivation as necessary before either event can occur. This is exemplified by the work of Glende (1972) who demonstrated pretreatment with carbon tetrachloride induced protection against a subsequent lethal dose of carbon tetrachloride. He treated rats with a protective dose of carbon tetrachloride of 0.025 ml/rat which destroyed the P-450 enzymes without tissue destruction. This was followed by a challenging lethal dose of carbon tetrachloride (500 microliters) and survival was measured as an index of protection by the first dose. On days 1-3 all the animals survived and the cytochrome P-450 levels greatly decreased. By day 5 the survival following the challenging dose was decreasing as the cytochrome P-450 was resynthesized, demonstrating the requirement for cytochrome P-450 in the necrotic activity of carbon tetrachloride (Glende, 1972). I will review the major arguments for each event being the cause of necrosis, with the caveat that final proof is not available at this time.

Lipid Peroxidation

A characteristic property of unsaturated fatty acids is that oxidative deterioration occurs in the presence of a free-radical initiator and oxygen. This process is called lipid peroxidation and may be a mechanism of cellular injury as a result of the bioactivation of carbon tetrachloride. The homolytic cleavage of carbon tetrachloride
produces chlorine radical (\('\text{Cl}'\)) and trichloromethyl radical (\('\text{CCl}_3'\)); both of which are highly reactive chemical species. These may begin the series of reactions in peroxidation and are called radical initiators. Either of these can abstract a vinylic hydrogen from an unsaturated fatty acid to yield a fatty acid radical. This is followed by the uptake of oxygen to produce a fatty acid peroxy radical. This radical may react with another molecule of fatty acid to yield an unsaturated hydroperoxide and another fatty acid radical which can reinitiate the entire process. A reaction which consumes and produces a radical is termed a propagation reaction. This may continue until a free radical scavenger such as vitamins E, A, or C accepts the radical and prevents the formation of another one. This is a termination reaction.

The process of peroxidation results in destruction of polyunsaturated fatty acids. Initially, rearrangement of the double bonds of unconjugated fatty acids radical occurs, resulting in more energetically stable conjugated double bonds. In the presence of oxygen, bond formation may occur to form a peroxy free radical which undergoes intramolecular condensation to form a five-membered oxygen ring which results in destruction of the fatty acid and liberation of malondialdehyde (Appendix E, Figure E.2.). Therefore, in the presence of oxygen and a polyunsaturated fatty acid, a radical initiator such as trichloromethyl free radical may result in the breakdown of fatty acids resulting in a disturbance of the ordered structure of biological membranes.

The peroxidative process may be initiated by and leads to the formation of free radical intermediates which may contribute to
autocatalysis and result in further radical production. The eventual result of chemically induced lipid peroxidation \textit{in vivo} is not understood, but has been implicated as being important in aging phenomena, atherosclerosis, some forms of liver injury, carcinogenesis, and oxygen toxicity (Plaa and Witchi, 1976).

Considerable evidence links lipid peroxidation and the hepatotoxicity produced by carbon tetrachloride intoxication. \textit{In vitro}, carbon tetrachloride has been shown to accelerate lipid peroxidation in rat liver homogenate in association with a loss of glucose-6-phosphatase activity. Lipid peroxidation also occurs \textit{in vivo} in rats after carbon tetrachloride administration. The effects of peroxidation on a membrane may be widespread. Witting (1965) has postulated that lipoperoxidation might lead to a "hole-in-the-membrane" phenomenon which may result in weakening and degeneration of the lipoprotein foundation of cellular and subcellular membranes. This agrees with the postulated role of calcium entry into a cell leading to cell death after exposure to a toxin (Schanne et al., 1979). Some of the effects of the peroxidative decomposition of cellular lipids are red blood cell hemolysis, mitochondrial swelling and disintegration, disintegration of lysosomes and loss of activity for microsomal enzymes (Recknagel and Glende, 1973).

As a result of carbon tetrachloride intoxication there is rapid loss of hepatic protein synthesis and cytochrome P-450, both of which may result from lipoperoxidation. Since both events require intact endoplasmic reticulum membrane, peroxidation of that membrane may well contribute to the loss of those functions. However, since neither of
these functions necessarily result in cell death, their contribution to carbon tetrachloride-induced hepatic necrosis has been questioned (Slater, 1978). In addition, Farber has suggested that the effect of carbon tetrachloride in protein synthesis is not the direct result of damage to the membrane of the endoplasmic reticulum, but may be unrelated to or an indirect effect of the damage to it (Farber, 1979).

Further evidence against lipoperoxidation as an initiating step in the production of carbon tetrachloride-induced hepatic necrosis comes from the work of Stacey and Priestly (1978). They demonstrated that ADP/Fe$^{+3}$ could produce lipid peroxidation without altering membrane integrity as measured by potassium and alanine aminotransferase release from isolated hepatocytes. In addition, they demonstrated carbon tetrachloride induced cell damage with no lipid peroxidation. Thus, the result of lipoperoxidation in carbon tetrachloride induced hepatic necrosis remains unknown.

Covalent Binding

Another important event that occurs due to the bioactivation of carbon tetrachloride is the covalent binding of its metabolites to cellular macromolecules such as protein, lipids, and nucleic acids. Even though a causal relationship between covalent bond formation and toxicity has not been proven this possibility provides an attractive mechanism to explain why relatively low concentrations of chemically reactive metabolites are cytotoxic. The hypothesis is that tissue lesions produced by therapeutic drugs and toxic chemicals may be caused by covalent binding of toxic metabolites to tissue macromolecules.
resulting in a lethal alteration in the cells ability to regulate homeostasis.

The work of Gomez et al. (1975) supports the view that covalent binding to cellular macromolecules is the causative event in carbon tetrachloride-induced liver injury. They demonstrated species differences in carbon tetrachloride-induced hepatotoxicity which show covalent binding of the bioactivated carbon tetrachloride metabolite correlates with the degree of injury, whereas lipid peroxidation does not. Mice showed the highest sensitivity to the necrogenic effect of carbon tetrachloride and showed the highest binding, yet showed no evidence of lipid peroxidation. Guinea pigs, hamsters, and rats demonstrated this correlation, whereas the chicken demonstrated neither binding nor peroxidation, which is consistent with the failure of birds to activate carbon tetrachloride.

Reynolds (1967) demonstrated the recovery of approximately 0.5% of administered $^{14}$C-labeled carbon tetrachloride as non-volatile radioactivity associated with liver fractions. Twenty-five percent of the nonvolatile radioactivity was present in each of the microsomal and cell sap fractions, 40% was present in the residue fraction, and less than 10% was recovered in mitochondria. The macromolecules most heavily labeled by radioactive carbon tetrachloride were lipid and protein. Cholesterol esters, triglycerides, and phospholipids were the most heavily labeled lipid fractions and methionine was thought to be the major amino acid in protein to bind radioactivity covalently. Less than 0.2% of the recovered nonvolatile radioactivity was recovered in
nucleic acids. Other studies using synthetic polynucleotides in vitro did not demonstrate specific binding to any particular nucleic acid.

**Trichloroethylene**

Trichloroethylene (TCE) is an organohalogen compound that has been utilized in a number of different areas. Medically, trichloroethylene has been used in anthelmintic preparations and was widely used as an anesthetic until the advent of safer compounds in the mid-1960s. In the United States use of trichloroethylene as an anesthetic is currently limited to about 60,000 patients a year. Trichloroethylene was used in the food industry as a solvent to decaffeinate coffee. However, the suspected carcinogenicity of trichloroethylene has prompted the Food and Drug Administration to ban trichloroethylene in food processing. The most prolific use of trichloroethylene has been as a solvent for degreasing metal parts prior to painting or electroplating. As much as 90-95% of all trichloroethylene produced in the United States is used in degreasing operations (Warets, Gerstner, and Huff, 1977).

The major organ affected by trichloroethylene intoxication is the kidney. High doses of trichloroethylene have been shown to produce glomerular nephrosis and chronic renal failure. The major concern for human exposure is due to the suspected carcinogenicity of trichloroethylene. The National Cancer Institute document of 1976 reported an increase in the number of hepatocellular carcinomas with metastatic potential in mice of both sexes given intragastric trichloroethylene in high and low dose regimens. No such increase was demonstrated in rats of either sex (USDHEW, 1976).
Trichloroethylene can be bioactivated by the microsomal mixed function oxidase system resulting in a reactive intermediate (Appendix A, Figure A.1.). This is thought to be an epoxide (Henschler and Bonse, 1978) but may also be a chloronium ion (Van Dyke, 1977). These may rearrange and lead to chloral hydrate which is the major metabolite of trichloroethylene. Chloral hydrate can be dehydrogenated to trichloroacetic acid and excreted in the urine, or reduced to trichloroethanol which is excreted in the urine as the alcohol or as the glucuronide.

Due to its structural similarity to the carcinogenic halogenated olefin vinyl chloride, trichloroethylene was expected to be highly reactive toward cellular macromolecules after its bioactivation. Van Duuren and Banerjee (1976) demonstrated irreversible binding to microsomal protein and lipid after metabolic activation. The amount of binding was decreased by 7,8-benzoflavone and SKF-525A, both of which inhibit cytochrome P-450 and increased the binding. The addition of 3,3,3-trichloropropene oxide inhibited epoxide hydrase and increased the binding to lipids and protein. These findings support the view that trichloroethylene is metabolized to an epoxide. These results have been confirmed by two other groups (Uehleke and Poplawski-Tabarelli, 1977; Allemand et al., 1978).

Another study demonstrated irreversible binding to DNA added to microsomal incubations. In this study, salmon sperm DNA was added to microsomal incubations with $^{14}$C-labelled trichloroethylene and isolated at the end of the experiment (Bannerjee and Van Duuren, 1978). The ability of a trichloroethylene metabolite to bind to DNA is important in view of the somatic cell mutation theory which states that in order for
a tumor to develop, a mutated genome must first be produced. DNA binding by this and other chemicals provides a mechanism by which they may affect this change in cellular regulation. In agreement with this view is the finding that trichloroethylene is able to mutate bacteria in the Ames test after bioactivation by rat liver microsomes (Shahin and VonBorstel, 1977).

Methylene Chloride

Methylene chloride (MC) or dichloromethane is a widely used solvent found in paint and varnish removers, metal cleaners, air conditioning refrigerants and as a replacement solvent for trichloroethylene in the food processing industry. Chronic inhalation of this halogenated hydrocarbon at levels of 5,200 ppm for 6 hours has been shown to produce fatty liver in the dog, mouse, and guinea pig, although hepatic necrosis is not a major factor in its toxicity. Acute administration of methylene chloride of greater than 2,600 mg/kg results in death due to cardiac sensitization to circulating catecholamines (Morris, Smith, and Garman, 1979). Methylene chloride has been shown to be non-carcinogenic in species of rats (Chemical and Engineering News, 1978).

The primary metabolite of methylene chloride is carbon monoxide. Human subjects exposed to methylene chloride show elevated blood carboxyhemoglobin levels due to the production of carbon monoxide (Stewart et al., 1972). It is thought that methylene chloride reacts with cytochrome P-450 in the presence of oxygen and NADPH to produce a formyl halide intermediate which decomposes nonenzymatically to carbon monoxide (Kubic and Anders, 1978). Cytosolic metabolism of methylene
chloride has also been demonstrated (Ahmed and Anders, 1978). This is thought to involve catalysis by glutathione and the enzymes formaldehyde dehydrogenase and S-formyl glutathione hydrolase and result in the transformation of methylene chloride to formaldehyde, formic acid and inorganic chloride.

Anders, Kubic and Ahmed (1977) have demonstrated the binding of $^{14}$C-methylene chloride to microsomal protein. This binding requires NADPH and oxygen, and can be increased by pretreatment of the animal with phenobarbital, indicating bioactivation by cytochrome P-450 is required before binding can occur.

The Role of Reactive Metabolites in Toxicity

In studying the toxicity of compounds that require bioactivation, several questions have arisen as to the importance of macromolecular binding in toxicity. An attempt to answer some of these questions was made by Dr. James Gillette in a pair of theoretical articles in 1974 (Gillette, 1974a, 1974b). I will briefly summarize his views below.

Studies of covalent binding to tissue macromolecules have little predictive value in determining whether a compound will evoke toxicity due to the alteration of that macromolecule. Studies correlating binding and necrosis must be done before an investigator may infer cause and effect relationship. Covalent binding to macromolecules is useful in monitoring bioactivation and the formation of reactive intermediates, but demonstrating binding to a particular macromolecule does not prove that the toxicity is mediated by such binding. Indeed, some toxicities may require the covalent binding of the reactive metabolite to DNA,
whereas others may require binding to lipids or to certain enzymes. Highly reactive metabolites may bind to a wide variety of macromolecules yet have toxicity due to binding to any one.

If covalent binding and toxicity are to be correlated, it must be demonstrated that treatments which alter the severity of toxicity are also able to alter the degree of covalent binding. Thus, pretreatment with SKF-525A or cobaltous chloride resulting in decreased cytochrome P-450 activity should decrease both the toxicity and binding of the compound. Also, increasing cytochrome P-450 activity by pretreatment with phenobarbital should result in similar increases in toxicity and binding. Dr. Gillette has also written a theoretical paper on the pharmacokinetics of covalent binding as it concerns the severity of toxicity and the threshold phenomenon of certain drugs. The interested reader is referred to this excellent commentary (Gillette, 1974b).

Objectives

The compounds in this study are bioactivated before they interact with cellular macromolecules. Due to the reactive nature of those intermediates, they are not able to be isolated as such. Rather, the more stable metabolites of the reactive intermediates have been isolated. By use of the structure of the metabolites, inferences were made as to the nature of the reactive intermediates. The objectives of this study were to isolate and characterize the macromolecules to which the reactive intermediates bound covalently. This information would be used to clarify the chemical–biological interactions of the reactive intermediates and describe structural features of the macromolecules
which made them susceptible to attack. Also, identification of the bound adducts would be more complete information concerning the nature of the reactive intermediate. Since isolated are usually stable compounds, they are thought not to be the agents responsible for tissue injury. Therefore, isolating bound adducts on macromolecules would also identify those intermediates which react with cellular constituents and possibly be responsible for causing toxicity.

To study the bioactivation of the organohalogens carbon tetrachloride, trichloroethylene, and methylene chloride a bioactivation system using isolated rat hepatocytes was established. This was used to identify quantitatively which cellular macromolecules each organohalogen could bind covalently.

A chemical activation system was used to study the structural features of fatty acids with which the carbon tetrachloride intermediates could interact, resulting in covalent binding or chloroform production. Finally, a microsomal bioactivation system was used to correlate the results of the chemical activation system. By use of these three activation systems, it was my goal to describe more fully the mechanism of reaction of carbon tetrachloride with fatty acids.
EXPERIMENTAL MATERIALS AND METHODS

Animals

Male Sprague Dawley rats 200-300 g were obtained from Hilltop Vendors (Chatsworth, CA) and maintained on a diet of Standard Purina Rat Chow ad libitum. They were housed at 22°C in environmentally controlled rooms on a normal cycle of 12 hours of light and 12 hours of darkness.

Chemicals

Radiochemicals were purchased from New England Nuclear (Boston, MA). Radiochemical purity was 99% as analyzed by the supplier. The specific activity of the labeled compounds is as follows: carbon tetrachloride 53.0 mCi/m mole; trichloroethylene 5.0 mCi/m mole; methylene chloride 3.9 mCi/m mole.

Chemicals used were reagent grade with the following exceptions. Methylene chloride, chloroform, methanol, and heptane were Distilled in Glass grade solvents purchased from Burdick and Jackson Laboratories Inc. (Muskegon, MI).

Biochemicals

Deoxyribonuclease I (E.C. No. 3.1.4.5) from bovine pancreas containing 2,000 Kunitz units per mg protein, and glucose-6-phosphate dehydrogenase (E.C. No. 1.1.1.49) containing 270 units per mg protein were purchased from Sigma Chemical Company (St. Louis, MO).
Ribonuclease (E.C. 2.7.7.16) containing 68 Kunitz units per milligram protein was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Fatty acids, fatty acid methyl esters, phospholipids, Phospray, glutathione, D-glucose-6-phosphate (disodium salt, lot 48c-5063) and nicotinamide adenine dinucleotide phosphate (NADP, lot 18c-7610) were purchased from Sigma Chemical Company. Silica gel 60 thin layer chromatography plates were also purchased from Sigma Chemical Company. Gas chromatography standards were purchased from Supelco, Inc. (Bellefonte, PA). Collagenase (type II) was purchased from Worthington Biochemical Corp. (Freehold, NJ).

**Preparation of Hepatocytes**

The preparation of the hepatocytes was a joint collaborative effort with Dr. Klaus Brendel, University of Arizona College of Medicine, Tucson, Arizona. An outline of their method is presented below.

The rats were anesthetized with ether throughout the surgical procedure. The abdominal cavity was opened, the bile duct isolated and the cannula of the perfusion apparatus (grooved 15 gauge stainless steel needle) inserted at the general locus of the lineal branch. The perfusion was started (6 ml/min) and the liver transferred from the abdominal cavity to the perfusion apparatus.

The perfusion apparatus was filled prior to the liver isolation with 150 mls of calcium-free perfusion buffer NaCl (130 mM); HEPES (20 mM); KCl (4.6 mM); KH$_2$PO$_4$ (2.3 mM); MgSO$_4$ (4.75 mM) pH 7.5, and equilibrated with oxygen at 37°C. About 25 ml of the perfusate was used to clear the liver of red cells. The liver was then placed into
the perfusion apparatus, the perfusate recirculated, and collagenease added to bring it to a final concentration of 0.05% (w/v). Calcium was also added to bring the final concentration to 5 mM. The perfusion time in the presence of collagenease was 15-20 minutes at a flow rate of 80 mls/min. At the end of the perfusion the liver was disconnected from the cannula, the tissue gently separated by opening scissors, and poured onto a sieve of 60 micron mesh size. The liver cells were washed into a second plastic beaker with buffer and gentle stirring with a soft rubber bulb. The suspensions were then sieved through an 86 micron nylon sieve and the resulting cell suspensions placed in the top of two stacked 50 ml plastic centrifuge tubes. The bottom tubes were prefilled with 45 ml clean oxygenated wash buffer and the upper tubes were conical centrifuge tubes with a 6 mm opening in the bottom. The tubes were centrifuged two minutes at 40xG forming loosely packed pellets which were resuspended in wash buffer, and the washing procedure repeated. The final pellets were gently resuspended in incubation buffer consisting of Gibco medium 199 with 10% fetal calf serum to a concentration of 80 mg/ml or about \(8 \times 10^6\) cells/ml. The yield of cells was 80-90% wet weight of the liver. Viability as determined by trypan blue exclusion was greater than 85%. Some animals were induced with phenobarbital before sacrifice by adding sodium phenobarbital (0.1% w/v) to their drinking water for 10 days, changing to regular tap water on day 11. They were then sacrificed on day 12 by cervical dislocation. To reduce liver glutathione levels, certain rats were injected intraperitoneally 2 hours before sacrifice with 0.6 ml/kg diethyl maleate.
Glutathione levels were measured by the method of Sedlak and Lindsay (1968).

**Incubation Conditions**

Each incubation reaction contained 5 ml cells which were pipetted into 25 ml screw top Erlenmeyer flasks, the bottoms of which had been heated and pushed inward to form a conically raised center which kept the cells from settling in the center of the flask. The opening of the pipet tip used for dispersion of the cells was enlarged to 3 mm and fire polished. Each vial was flushed with N₂ or O₂ by changing the headspace above the cell suspension 5 times of 10 seconds each. The vials were sealed with mini-inert (Supelco) cap containing a Teflon septum. They were preincubated at 37° for five minutes and the incubation begun by injecting ¹⁴C labeled organohalogen (2.0 x 10⁶ dpm) in 2 microliters of ethanol. Reactions were stopped by removing the cap and placing the flask on ice. Aliquots were removed for isolation of macromolecules as described below.

**Macromolecule Isolation**

At the end of incubation, a 0.6 ml aliquot was removed and extracted in 5 ml of a chloroform:methanol (3:1 v/v) solution. The mixture was extracted by shaking for 15 minutes and centrifuged at 3000xG for 15 minutes. The lower organic phase containing lipid material was pipetted away from the upper aqueous layer and protein containing pellet at the interface. The lipid layer was washed with distilled water, evaporated in a tared scintillation vial, resuspended in the corresponding non-labeled organohalogen solvent, dried again and
reweighed. The residue was dissolved in 6 ml Instagel\textsuperscript{R} (Packard) and counted in a Beckman LS8100 liquid scintillation counter. The protein pellet was extracted three times with 5\% trichloroacetic acid at 60\(^\circ\)C, three times with methanol:ether (3:1) at 60\(^\circ\)C, and solubilized in 1 ml 1 N NaOH. An aliquot was removed for quantification of protein by the coomassie dye binding technique (Bradford, 1976). Another aliquot was removed for determination of radioactivity by liquid scintillation counting.

To the remaining cells was added 5 mls of 2\% sodium dodecyl sulfate (SDS). This stopped further metabolism and lysed the cells and nuclei releasing the RNA and DNA. Protein and lipid were extracted from the SDS-cell suspension by three 1 hour extractions with chloroform-isoamyl alcohol (10:2 v/v). The nucleic acids were precipitated from the aqueous layer after the third extraction with 10 mls of 0.4N perchloric acid and sedimented by centrifugation at 10,000\(\times\)G for 20 minutes. The resulting pellet was solubilized in 2.0 ml of 0.1 M Tris-HCl pH 7.4 containing 1 mM EDTA and incubated in the presence of 50 \(\mu\)g RNase (boiled for 30 minutes to destroy DNase activity) for 2 hours at 37\(^\circ\)C. Four ml 0.4N HCl\textsubscript{4} was added and after centrifugation ribonucleotides were recovered in the supernatant. The pellet was solubilized in 2 ml of 0.1 M Tris-HCl (pH 7.5) containing 2 mM MgCl\textsubscript{2} and incubated with 50 \(\mu\)g DNase for two hours at 37\(^\circ\). Four ml 0.4N HCl\textsubscript{4} were added, the mixture centrifuged, and deoxyribonucleotides were removed in the supernatant. Aliquots from each supernatant were counted for bound radioactivity as above and nucleic acids were quantified by measuring absorbance at 260 nm (1 A\textsubscript{260} = 50 \(\mu\)g/ml). DNA
contamination in RNA samples was detected by the diphenylamine reaction (Burton, 1956). RNA was assayed for in the DNA solution as measured by the orcinol reaction (Schneider, 1957) and protein contamination in nucleic acid samples was assayed for by the coomassie dye technique (Bradford, 1976). Covalent binding is expressed as picomoles bound per mg macromolecule. Background binding from cells with no fetal calf serum kept at 5°C for 5 days was subtracted from all values.

Macromolecule Quantification

Total lipids were quantified gravimetrically and protein by the coomassie dye binding technique (Bradford, 1976). Nucleic acids were quantified by measuring absorbance at 260 nm ($A_{260} = 50 \, \mu g/ml$). Contamination of RNA in DNA was determined by the orcinol reaction and DNA in RNA determined by the diphenylamine reaction (Burton, 1956).

Thin Layer Chromatography

Silica gel 60 thin layer chromatography plates were made slightly basic by spraying once with 0.1 M sodium acetate and reactivating at 120° for 2 hours. An aliquot of the $^{14}$C-lipid fraction prepared by chloroform:methanol extraction as described above was applied to the plate. Phospholipid standards (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine) were cochromatographed with the lipid extracts. The plate was developed in chloroform:methanol:acetic acid:water 50:25:8:4 (vols) for approximately 70 minutes. The standards were sprayed with phosphomolybdic acid (10% in ethanol) to locate the resolved lipid components. The areas on the TLC
plate corresponding to the identified standards were scraped and counted for radioactivity.

**Fatty Acid Analysis**

To determine intramolecular distribution of covalently bound radiolabel within the phospholipid molecule, $^{14}$C-labeled phosphatidylcholine was purified by thin layer chromatography as above, scraped off the plate and extracted into chloroform:methanol (3:1). The solvent was evaporated with dry nitrogen and 0.5 ml of 6N HCl was added. This was heated at 100°C in a sealed test tube for 4 hours in order to hydrolyze the ester linkage between the fatty acids and glycerol phosphate backbone. Two hexane extractions (1 ml) separated the non-polar fatty acids from the polar glycerol phosphate backbone. Aliquots of each were removed for liquid scintillation counting. Results were expressed as percent of total binding present in each fraction.

**Partitioning Determination**

The headspace hepatocyte partitioning ratio was determined by analysis of radioactivity distribution in the hepatocyte suspension and in the headspace. Metabolically inactive hepatocytes prepared by serum deprivation were incubated with the radiolabeled compounds. At 15, 30, and 45 minutes, 4 ml of headspace were withdrawn and slowly bubbled into 10 ml Instagel$^R$ scintillation fluid. Six 0.5 ml aliquots of hepatocytes were removed and placed in a scintillation vial containing 1.0 ml Soluene$^R$ tissue solubilizer frozen on dry ice. The vial was capped and allowed to solubilize the hepatocytes at 37°C for four hours. The
vials were refrozen, 6.0 mls of Instagel were added and the vial counted for radioactivity. Total radioactivity in the headspace and hepatocyte suspension accounted for greater than 85% of the administered radiolabel.

**Benzoyl Peroxide Model System**

The components of the model for carbon tetrachloride activation using benzoyl peroxide to initiate the free radical reaction were as described by Gordis (1969). Fifteen milligrams of benzoyl peroxide and 32 mg of fatty acid methyl ester were dissolved in 0.5 ml carbon tetrachloride with approximately 500,000 dpm $^{14}$C- or 350,000 dpm $^{36}$Cl-carbon tetrachloride added. Radical inhibitors were also added in some experiments. The mixture was placed in a vial sealed with an injectable septum and heated at 80°C. The reaction was quenched at the appropriate time by placing in an ice bath. Samples were warmed at 37°C for 25 minutes and 0.5 ml headspace was removed into an airtight syringe for chloroform analysis. Chloroform analysis was performed by gas-liquid chromatography using a Varian model 1440 gas chromatograph, 6 ft. OV 101 column and a flame ionization detector. The column temperature was 160°C and the detector and injector temperature were both 220°C. The area of each peak was calculated using a Minigrator$^R$ integrator and data was expressed as the chloroform-to-carbon tetrachloride ratio.

The vial was then opened and 0.5 ml of the liquid phase was removed and placed in scintillation vials, dried, resuspended in 1.0 ml carbon tetrachloride and dried twice by evaporation. Bound carbon
tetrachloride metabolites were then assayed for by liquid scintillation counting.

**Mass Spectral Analysis**

Mass spectral determination were conducted under the direction of Sai Y. Chang, Ph.D., in the Department of Pharmacology, Arizona Health Sciences Center, University of Arizona, Tucson, Arizona. A Finnigan automated gas chromatograph/mass spectrometer system model 8800 was utilized in these studies. A Finnigan gas chromatograph model 9500 was interfaced to the mass spectrometer with a glass jet separator. For the chemical ionization studies the mass spectrometer was directly interfaced with methane gas. All information was analyzed with a Finnigan data system 6110.

**Dual-Label Data Analysis**

Data computation for dual-label counting experiments was calculated according to the method of Long (1977). In order to determine the actual activity of both isotopes in a dual-label sample, quench curves for both isotopes were constructed. These curves were generated by preparing standards of known activity and adding increasing amounts of quenching agent chloroform. Counting efficiency is calculated by dividing the activity detected by the liquid scintillation counter (CPM) by the known activity of the sample (DPM). Quench curves were constructed by plotting counting efficiency versus quench, or H#/ in the case of the Beckman model LS 8100 liquid scintillation counter used in these studies. The H#/ calculation measures the shift in the compton
edge of a cesium-137 external gamma source induced by quenching. To calculate the contribution of each isotope to the total activity in each channel, quench curves were plotted for each isotope in both channels (four in all) and the counting efficiencies of both isotopes in both channels were used to calculate the activity of each isotope independent of the contribution of the other isotope (Appendix B, Figures B.1. and B.2.). Using the formulas in Figure B.5., the activity of each isotope was calculated without contamination by other isotope. Data processing was performed on a Texas Instruments Programmable 58C calculator.

**Tritium Incorporation into Microsomal Lipids**

Incorporation of tritium (\(^3\)H) from tritiated water into microsomal lipids was postulated to occur if lipid free radicals were formed during reaction of carbon tetrachloride metabolites with fatty acids. Since lipid radicals were postulated to occur due to covalent binding of carbon tetrachloride reactive intermediates as well as proton abstraction by the trichloromethyl free radical resulting in chloroform production, simultaneous determination of covalent binding, chloroform production, and tritium incorporation into microsomal lipids was performed.

Three milliliters of 0.05 M Tris-HCl buffer (pH 7.5) were lyophilized and stored at -20°C until reconstituted with three milliliters \(^3\)H\(_2\)O (2.54 x 10^6 dpm/ml; New England Nuclear). Microsomes from phenobarbital pretreated rats were prepared by homogenizing rat liver in 10 volumes cold Tris-HCl buffer followed by centrifugation at
10,000 x G for 30 minutes at 4°C. The supernatant was removed and recentrifuged at 105,000 x G for 30 minutes in a Model TI 50 ultracentrifuge rotor at 4°C. The supernatant was discarded and the microsomal pellet was resuspended in the tritiated water buffer to a final protein concentration of 6 mg/ml. Ten microliters of a generating system composed of glucose-6-phosphate (120 mg), NADP (16 mg), magnesium chloride (220 mg), and glucose-6-phosphate dehydrogenase (50 units) in three milliliters 0.05 M Tris-HCl buffer was added to the microsomes. Aliquots of 0.1 ml were placed in vials and flushed twice with a stream of nitrogen before sealing with an injectable septum. Two microliters of $^{14}$C-carbon tetrachloride in ethanol containing 22.5 nanomoles carbon tetrachloride ($2 \times 10^6$ dpm) were injected into the vials and incubated for various times at 37°C.

At 5, 10, 20, and 30 minutes the reactions were quenched with 1 microliter concentrated HCl. Methylene chloride (1 nanomole in 0.5 µl ethanol) was injected for the internal standard. The vials were incubated at 37°C for 30 minutes and a 0.25 cc aliquot was analyzed for methylene chloride and chloroform by gas-liquid chromatography. Analyses were conducted using a Varian (Palo Alto, California) 3700 gas chromatograph with a nickel electron capture detector. A 6 foot Porpak Q column was used at 140°C. Peak areas were calculated using a Varian CDS 111 automatic integrator. A sample chromatograph is given in Appendix G, Figure G.1. Standard solutions of methylene chloride (1 nanomole per 2 µl ethanol) plus varying chloroform concentrations were incubated 30 minutes or longer in NADPH deficient microsomes. An
aliquot of headspace was removed (0.25 cc) and analyzed for methylene chloride and chloroform. Data was calculated for the standard curve by plotting the chloroform-to-methylene chloride ratio versus chloroform concentration (Figure 25, page 68).

The microsomal suspension was extracted with chloroform: methanol 3:1 (v/v) for removal of total lipids containing bound $^{14}$C-carbon tetrachloride metabolites and tritium from tritiated water. Unbound $^{14}$C and $^3$H was removed by exhaustive solvent extraction as discussed in the macromolecule isolation section. Control samples were treated identically except 2 μl ethanol carrier were injected without carbon tetrachloride. Analysis of the data was conducted on bound radioactivity using the $^3$H/$^{14}$C dual label quench curves in Appendix B, Figure B.2. and the formulas in Figure B.5.
RESULTS

Hepatocyte Study

All three organohalogens became covalently bound to rat hepatocyte lipids and protein when incubated under the appropriate atmosphere. Trichloroethylene (Figure 1) and methylene chloride (Figure 2) were bioactivated to a greater extent when incubated in an oxygen containing atmosphere than an oxygen-free atmosphere whereas carbon tetrachloride bioactivation was favored in an oxygen-free environment (Figure 3). Binding of methylene chloride and trichloroethylene in an oxygen containing environment increased with time for at least 60 minutes and trichloroethylene binding increased for over four hours. Due to decreased hepatocyte viability in an anaerobic atmosphere, carbon tetrachloride binding could be reproducibly shown to increase for only 30 minutes. All binding data are expressed as picomoles of organohalogen bound per milligram macromolecule.

When hepatocytes were isolated from rats pretreated with phenobarbital to increase microsomal cytochrome P-450 levels, the binding of carbon tetrachloride and trichloroethylene to lipids and protein was increased. However, the binding of methylene chloride to these macromolecules was decreased in hepatocytes obtained from phenobarbital treated rats (Table 1). Pretreatment of rats with diethyl maleate decreased the hepatocellular concentration of glutathione (GSH). With a mean reduction of GSH levels to 38% of control, trichloroethylene
Figure 1. Trichloroethylene binding to protein and lipid in isolated rat hepatocytes incubated in oxygen and nitrogen atmospheres.

Four hundred milligrams were incubated with 50 μM trichloroethylene. Protein and lipid were isolated and purified by exhaustive solvent extraction.
Figure 2. Methylene chloride binding to protein and lipid in isolated rat hepatocytes incubated in an oxygen atmosphere.

Four hundred milligrams of hepatocytes were incubated with 79 μM methylene chloride. Protein and lipid were isolated and purified by exhaustive solvent extraction.
Figure 3. Carbon tetrachloride binding to RNA, DNA, protein and lipid in isolated rat hepatocytes incubated in a nitrogen atmosphere.

Four hundred milligrams of hepatocytes were incubated with 10 μM carbon tetrachloride. Protein and lipid were isolated and purified by exhaustive solvent extraction and nucleic acids were isolated and purified by solvent extraction followed by nuclease digestion.
Table 1. Factors that affect the binding of volatile xenobiotics to lipid and protein in isolated rat hepatocytes. -- Hepatocytes were incubated for one hour in sealed flasks. Substrate concentrations were 10, 50, and 73 micromolar for carbon tetrachloride, trichloroethylene, and methylene chloride, respectively. Lipid and protein were isolated and purified by exhaustive solvent extraction.

<table>
<thead>
<tr>
<th></th>
<th>Atmosphere</th>
<th>Glutathione Depletion(^a,c)</th>
<th>Phenobarbital Induction(^b,c)</th>
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<tr>
<td></td>
<td>(O_2/N_2) ratio</td>
<td>Protein</td>
<td>Lipid</td>
</tr>
<tr>
<td>Carbon Tetrachloride</td>
<td>.085</td>
<td>.056</td>
<td>100%</td>
</tr>
<tr>
<td>Trichloroethylene</td>
<td>9.63</td>
<td>5.08</td>
<td>74%</td>
</tr>
<tr>
<td>Methylene Chloride</td>
<td>8.53</td>
<td>11.96</td>
<td>44%</td>
</tr>
</tbody>
</table>

\(^a\) Rat pretreated with diethyl maleate (0.6 ml/kg) two hours prior to sacrifice.

\(^b\) Rat given phenobarbital (1%) in drinking water for 10 days prior to sacrifice.

\(^c\) Expressed as percent of binding of cells from untreated animals.
binding to lipids was decreased by 43% and to proteins by 38% and binding by methylene chloride was reduced to lipids and protein 72% and 58%, respectively. No consistent effects of GSH depletion were observed on the binding of carbon tetrachloride.

Since the xenobiotics used in this study are volatile, experiments were conducted to quantitate the amount of each compound partitioned into the hepatocyte solution in comparison to the amount which distributed into the headspace above the hepatocytes. Partitioning experiments showed the three organohalogens distributed unequally between the hepatocytes and headspace. Over 90% of the radioactivity from the $^{14}$C-labeled organohalogens was recovered in determining partition ratios. Of the administered dose, 17% of the carbon tetrachloride, 28% of the trichloroethylene, and 53% of the methylene chloride was recovered in the hepatocyte suspension. The remainder was recovered in the headspace (Figure 4).

The $^{14}$C-labeled organohalogens were administered undiluted in an ethanol carrier. Due to the different specific activities of the compounds, keeping the disintegrations per minute constant resulted in administering different concentrations. Taking this into account and the different partitioning of each compound, the final concentration in the hepatocyte suspension was 1.7 μM for carbon tetrachloride, 14 μM for trichloroethylene, and 42 μM for methylene chloride.

Since small amounts of protein contamination that may be present in isolated nucleotide preparations would be mistaken for high levels of binding, two methods of protein determination were used in this study.
Figure 4. Partitioning of organohalogens between headspace and hepatocyte suspension.

Radiolabeled organohalogens were injected into sealed flasks containing four hundred milligrams of rat hepatocytes. Aliquots of the headspace and of the hepatocyte suspension were removed and assayed for radioactivity.
Nucleic acids were found to be protein-free by the coomassie dye binding technique and by absorbance ratio of 260/280 nm. Samples with an A260/A280 nm ratio of greater than 2.00 were considered protein-free and were used in binding experiments. These samples were also tested by the coomassie dye binding technique which is sensitive down to 0.1 µg/ml and found to be protein free at that limit of detection.

Deoxyribonucleotide contamination in the ribonucleotide fraction was undetectable using the diphenylamine assay, which is sensitive to 2 µg/ml (Burton, 1956). Ribonucleotide contamination in the deoxyribonucleotide fraction was undetectable using the orcinol reaction which is sensitive to 10 µg/ml (Schneider, 1957).

Radioactivity was found associated with nucleotides after incubation of hepatocytes with carbon tetrachloride (Figure 3) and trichloroethylene (Figure 5). Consistent with the results of binding to lipid and protein, carbon tetrachloride bound greater under anaerobic conditions and trichloroethylene bound greater under aerobic conditions. No binding to nucleic acids was detected in incubations of carbon tetrachloride in an oxygen containing environment or in incubations with trichloroethylene in an oxygen-free environment. Carbon tetrachloride binding to RNA increased with time to a level of 60 picomoles per milligram. The maximal binding of carbon tetrachloride metabolites to DNA was 30 picomoles per milligram.

No radioactivity was detected above background levels in nucleotides from RNA or DNA after hepatocyte incubations with 14C-labeled methylene chloride under aerobic or anaerobic atmospheres.
Figure 5. Trichloroethylene binding to RNA and DNA in isolated rat hepatocytes incubated in oxygen and nitrogen atmospheres.

Four hundred milligrams of hepatocytes were incubated with 50 μM trichloroethylene. RNA and DNA were isolated and purified by solvent extraction followed by nuclease digestion.
Isolation of protein from the same hepatocyte incubation exhibited binding up to 850 picomoles per milligram protein, indicating that bio-activation of methylene chloride had occurred. Since there was no detectable radioactivity associated with the isolated nucleotides in the presence of high amounts of binding to protein it is concluded that: 1) methylene chloride does not bind to RNA or DNA when incubated with isolated hepatocytes; and 2) the method using chloroform:isoamyl alcohol extraction followed by nuclease digestion is a reliable method for the isolation of protein-free nucleotides.

The recovery of nucleotides was quantitated by calculating the nucleotide concentration in the nuclease digest supernatant and multiplying by the volume. Using the nuclease digestion technique, recovery of nucleotides which were present as DNA or RNA accounted for 40-60% of the amount contained in the liver cells. Deoxyribonucleotide yields from hepatocyte DNA ranged from 40-65 micrograms recovered out of the 105 micrograms of DNA that were present in the hepatocyte incubation. Recoveries of ribonucleotides from hepatocyte RNA were 360-560 micrograms per incubation out of 960 micrograms RNA existing in the cells.

Determination of the distribution of covalently bound $^{14}$C in the various hepatocyte lipid classes indicated that carbon tetrachloride and trichloroethylene became largely covalently bound to phospholipids (Table 2). Neutral lipids accounted for 12% and 24% of the recoverable radioactivity after incubation with carbon tetrachloride and trichloroethylene, respectively. The majority of the binding of carbon
Table 2. Lipid fractions binding carbon tetrachloride and trichloroethylene in isolated rat hepatocytes. After incubation with radiolabeled carbon tetrachloride (CCl₄) or trichloroethylene (TCE), hepatocytes were extracted with chloroform:methanol (2:1 by volume). Aliquots of this extract were chromatographed on silica gel thin layer chromatography plates and developed for 70 minutes in chloroform:methanol:acetic acid:water (50:25:8:4 by volume). Phospholipid standards were cochromatographed. Values are means of two determinations.

<table>
<thead>
<tr>
<th></th>
<th>CCl₄</th>
<th>TCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral Lipids</td>
<td>12%</td>
<td>24%</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>88%</td>
<td>76%</td>
</tr>
<tr>
<td>Phosphatidyl-ethanolamine</td>
<td>17%</td>
<td>15%</td>
</tr>
<tr>
<td>Phosphatidyl-choline</td>
<td>30%</td>
<td>13%</td>
</tr>
</tbody>
</table>
tetrachloride was to the phospholipid category, accounting for 88% of the total lipid binding. The two major phospholipids, phosphatidylcholine and phosphatidylethanolamine accounted for 17% and 30% of the total lipid binding for carbon tetrachloride, respectively. These phospholipid classes accounted for 15% and 13%, respectively, for the total trichloroethylene metabolites bound, and the total phospholipid binding accounted for 76% of the recoverable lipid binding by trichloroethylene.

**Benzoyl Peroxide Studies**

In order to study the interaction of the reactive intermediates of carbon tetrachloride with fatty acids, a model activation system was developed. In this system, free radical production by thermal decomposition of benzoyl peroxide (Appendix F, Figure F.1) was used to simulate the bioactivation of carbon tetrachloride by cytochrome P-450 mixed function oxidase. This model system offered the advantages of studying the interaction of the trichloromethyl free radical with individual fatty acids, elimination of lipid peroxidation side reactions, and increased adduct formation (over 100 fold) than was achieved using microsome preparations. The components of the benzoyl peroxide model system for carbon tetrachloride activation are given in Table 3.

Using $^{14}$C labeled carbon tetrachloride added to the benzoyl peroxide model system, binding of radioactivity to oleic and stearic acid methyl esters was examined. Noncovalently bound radioactivity was removed from the fatty acid methyl esters in the binding experiments by
Table 3. Components of benzoyl peroxide model system for studying carbon tetrachloride activation.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoyl Peroxide</td>
<td>15 mg</td>
</tr>
<tr>
<td>Fatty Acid Methyl Ester</td>
<td>32 mg</td>
</tr>
<tr>
<td>( \text{CCl}_4 )</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

*Incubate in sealed vial under nitrogen atmosphere @ 80°C*
exhaustive solvent extraction as described under the macromolecule isolation section in the materials and methods section. Since non-covalently bound carbon tetrachloride is volatile, repeated washing and evaporation removes all non-bound radioactivity. Figures 6 and 7 show the washout curves for removal of noncovalently bound radioactivity from oleic and stearic acid methyl esters, respectively. The first wash removed the majority of non-bound radioactivity, but three washes were necessary to assure the only source of radioactivity present was due to carbon tetrachloride metabolites bound to fatty acid methyl esters.

The rate of binding of $^{14}$C-carbon tetrachloride metabolites to methyl stearate and methyl oleate in the benzoyl peroxide model system is shown in Figure 8. The binding to the unsaturated fatty acid methyl oleate increased rapidly, reaching a maximum by three hours. The rate of $^{14}$C-carbon tetrachloride metabolites binding to the saturated fatty acid methyl stearate remained constant during the duration of the incubation. Also, the quantity of the binding to methyl oleate was approximately 5 times greater than to methyl stearate.

The rate of incorporation of $^{36}$Cl-labeled CCl$_4$ metabolites to fatty acid methyl esters is shown in Figure 9. When 32 mg each of stearic or oleic acid methyl esters were used, it can be seen that chlorine incorporation increased into both fatty acid methyl esters for two hours before it leveled off. The binding for 18:0 reached a maximum level of binding at about 950 dpm/100 μl, whereas 18:1 reached a maximum at about 2200 dpm/100 μl. This resulted in a $^{36}$Cl binding ratio of about 2.3:1 for 18:1:18:0.
Figure 6. Removal of noncovalently bound radioactivity from methyl oleate by exhaustive solvent extraction after incubation in the benzoyl peroxide model system.

After incubation of 32 mg methyl oleate in the benzoyl peroxide model system, a 10 μL aliquot was removed, 0.5 ml unlabeled carbon tetrachloride was added and evaporated to dryness four times. After each wash a 10 μL aliquot was removed and assayed for radioactivity. Arrow indicates background.
Figure 7. Removal of noncovalently bound radioactivity from methyl stearate by exhaustive solvent extraction after incubation in the benzoyl peroxide model system.

After incubation of 32mg methyl stearate in the benzoyl peroxide model system, a 10 μL aliquot was removed, 0.5 ml unlabeled carbon tetrachloride was added and evaporated to dryness four times. After each wash a 10 μL aliquot was removed and assayed for radioactivity.
Figure 8. Time course for $^{14}$C-carbon tetrachloride metabolites binding to fatty acid methyl esters in the benzoyl peroxide model system.

Thirty-two milligrams of fatty acid methyl esters were incubated with 15 milligrams benzoyl peroxide and 0.5 ml carbon tetrachloride for various times. Aliquots of 100 μL were removed, dried, and resuspended in 0.5 ml unlabeled carbon tetrachloride three times before assaying for radioactivity by liquid scintillation chromatography.
Figure 9. Binding of $^{36}$Cl–carbon tetrachloride to methyl oleate and methyl stearate in the benzoyl peroxide model system.

Fatty acid methyl esters (32 mg) were incubated with benzoyl peroxide (15 mg) in 0.5 ml carbon tetrachloride. At the indicated times an aliquot (100 μL) was placed in a scintillation vial, washed and evaporated three times with 0.5 ml unlabeled carbon tetrachloride before assaying for bound radioactivity.
Dual label experiments in the benzoyl peroxide model system were conducted by adding $^{14}$C- and $^{36}$Cl-carbon tetrachloride together to the reaction mixture and adding that mixture to 18:0 and 18:1 methyl esters. As can be seen in Figure 10, the binding of both $^{14}$C-carbon tetrachloride and $^{36}$Cl-carbon tetrachloride metabolites to methyl oleate increased over a period of several hours. Expressed as picomoles per milligram fatty acid methyl ester recovered, the binding of $^{14}$C-labeled metabolites and $^{36}$Cl-labeled metabolites increased over several hours at about the same rate. The proportion of $^{36}$Cl to $^{14}$C bound was approximately 3.5 at all time points (Figure 11). When methyl oleate replaced methyl stearate in the dual-label benzoyl peroxide model system, a much different binding ratio of $^{36}$Cl- to $^{14}$C-carbon tetrachloride metabolites was found. The ratio was about 9 at all times of incubation (Figure 12). Figure 13 demonstrates the difference in ratios of $^{36}$Cl- to $^{14}$C-carbon tetrachloride binding to methyl oleate and methyl stearate in the benzoyl peroxide model system.

Chloroform is the major metabolite occurring as a result of the free radical activation of carbon tetrachloride. Chloroform was produced in the benzoyl peroxide model system in the presence of methyl stearate and methyl oleate. As shown in Figure 14, the rate of chloroform production increased for four hours during incubation of both methyl esters before leveling off.

In order to demonstrate that the chemical activation of carbon tetrachloride and the subsequent interaction with fatty acid methyl esters in the benzoyl peroxide model system was due to a free radical
Figure 10. Binding of $^{36}$Cl- and $^{14}$C-carbon tetrachloride to methyl oleate in the benzoyl peroxide model system.

Methyl oleate (32 mg) was incubated with benzoyl peroxide (15 mg) in 0.5 ml carbon tetrachloride. Either $^{36}$Cl-carbon tetrachloride (350,000 dpm) or $^{14}$C-carbon tetrachloride (500,000 dpm) was added prior to incubation at 80°C. At the times indicated, an aliquot was placed in a tared scintillation vial and washed repeatedly with unlabeled carbon tetrachloride. After reweighing, the sample was assayed for covalently bound radioactivity. Data were expressed as picomoles $^{36}$Cl- or $^{14}$C-carbon tetrachloride bound per milligram methyl oleate.
Incubation Time (hours)

Figure 11. Ratio of $^{36}$Cl- to $^{14}$C-carbon tetrachloride bound to methyl oleate in the benzoyl peroxide model system.

Methyl oleate (32 mg) was incubated with benzoyl peroxide (15 mg) in 0.5 ml carbon tetrachloride. Both $^{36}$Cl-carbon tetrachloride (350,000 dpm) and $^{14}$C-carbon tetrachloride (500,000 dpm) were added to each incubation before heating at 80°C. At the times indicated an aliquot was placed in a tared scintillation vial and washed repeatedly with unlabeled carbon tetrachloride. After reweighing, the sample was assayed for covalently bound radioactivity. Data were calculated for dual-label counting as discussed in the text and expressed as picomoles $^{36}$Cl-bound per picomole $^{14}$C.
Figure 12. Ratio of $^{36}$Cl- to $^{14}$C-carbon tetrachloride bound to methyl stearate in the benzoyl peroxide model system.

Methyl stearate (32 mg) was incubated with benzoyl peroxide (15 mg) in 0.5 ml carbon tetrachloride. Both $^{36}$Cl-carbon tetrachloride (350,000 dpm) and $^{14}$C-carbon tetrachloride (500,000 dpm) were added to each incubation before heating at 80°C. At the times indicated, an aliquot was placed in a tared scintillation vial and washed repeatedly with unlabeled carbon tetrachloride. After reweighing, the sample was assayed for covalently bound radioactivity. Data were calculated for dual-label counting as discussed in the text, and expressed as picomoles $^{36}$Cl-bound per picomole $^{14}$C.
Figure 13. Ratio of $^{36}$Cl- to $^{14}$C-carbon tetrachloride bound to methyl stearate and methyl oleate in the benzoyl peroxide model system.

Methyl stearate (32 mg) or methyl oleate (32 mg) were incubated with benzoyl peroxide (15 mg) in 0.5 ml carbon tetrachloride. Both $^{36}$Cl-carbon tetrachloride (350,000 dpm) and $^{14}$C-carbon tetrachloride (500,000 dpm) were added to each incubation before heating at 80°C. At the times indicated, an aliquot was placed in a tared scintillation vial and washed repeatedly with unlabeled carbon tetrachloride. After reweighing, the sample was assayed for covalently bound radioactivity. Data were calculated for dual-label counting as discussed in the text and expressed as picomoles $^{36}$Cl/picomoles $^{14}$C bound.
Figure 14. Chloroform production by methyl stearate and methyl oleate in the benzoyl peroxide model system.

Methyl stearate (32 mg) and methyl oleate (32 mg) were each incubated with benzoyl peroxide (15 mg) in 0.5 ml carbon tetrachloride at 80°C. At the times indicated, a 0.5 cc aliquot of headspace was analyzed for chloroform production by gas-liquid chromatography. Data were expressed as the ratio of chloroform to carbon tetrachloride.
process, known inhibitors of free radical chain reactions were added to some incubations. By analyzing chloroform production as an index of carbon tetrachloride activation, it was demonstrated that the free radicals inhibitors butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and N,N-diphenyl-p-phenylene diamine (DPPD) were capable of inhibiting chloroform production in a dose dependent manner (Figure 15). Chloroform production was inhibited 80% by 5 mg of each compound and 95% by 25 mg of each compound.

**Mass Spectral Interpretations**

Chemical ionization mass spectrometry was conducted on fatty acid methyl esters following the addition of carbon tetrachloride activated by the benzoyl peroxide reaction. Prominent molecular ion peaks were observed for three addition reaction products of stearic acid methyl ester and one of oleic acid methyl ester.

A chemical ionization mass spectrum of methyl oleate is presented in Appendix C, Figure C.4. The molecular ion (M) at m/e 298 is indicated by the existence of the collision-stabilized complex due to addition of C₂H₅⁺ (Appendix C, Figure C.2.) at m/e 327 (298 + 29 = 327). Another cluster at m/e 267 is due to loss of a methoxy group (CH₃-O) of molecular weight 31 (M-31 = 267).

The methyl oleate-carbon tetrachloride addition product (Figure 16) exhibited a molecular ion (M) molecular weight (W) of 448 daltons. This was substantiated by the presence of a C₂H₅⁺ adduct ion at MW 477, indicative of the molecular ion at 477 - 29 = 448 daltons. Isotope peaks reflecting the natural abundance of the chlorine
Figure 15. Effect of free radical inhibitors on chloroform production in the benzoyl peroxide model system.

Various amounts of the free radical inhibitors butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), or N,N-diphenyl-p-phenylene diamine (DPPD) were added to the benzoyl peroxide model system containing benzoyl peroxide (15 mg), methyl stearate (32 mg) and carbon tetrachloride (0.5 ml). After incubation for 24 hours a 0.5 cc aliquot of headspace was analyzed for chloroform production by gas-liquid chromatography. Data were expressed as the ratio of chloroform to carbon tetrachloride.
Figure 16. Chemical ionization mass spectrum of the carbon tetrachloride adduct to methyl oleate isolated after incubation of methyl oleate in the benzoyl peroxide model system.
isotopes of molecular weights of 35 and 37 daltons were also observed. These formed the characteristic multiplicity of peaks exhibited by chlorinated compounds (Appendix C, Figure C.1). The abundance distribution on the multiplet was characteristic of a compound containing four chlorines. Other peaks identified in Figure 16 further confirm the proposed structure. A dechlorinated fragment was found at MW 413 daltons (M-35) with a chlorine isotope cluster characteristic of three chlorines. Further dechlorination resulted in clusters at m/e 378 (M-70) and m/e 343 (M-105). Removal of HCl as well as Cl is also apparent in the cluster at m/e 342 (M-106). A totally dechlorinated fragment exists at m/e 306, which occurs as a result of loss of two Cl and two HCl moieties (M-142). These data suggest an elemental composition of C$_{20}$H$_{36}$O$_2$Cl$_4$, which corresponds to the structure in Figure 17.

A chemical ionization mass spectrum of methyl stearate is presented in Appendix C, Figure C.3. The parent molecular peak (M) at MW 298 daltons was indicated by collision-stabilized complexes at m/e 327 (298 + 29 = 327) and at m/e 339 (298 + 41 = 339). The peaks around m/e 267 result from loss of a methoxy group of MW 31 (298 - 31 = 267).

Three stearic acid methyl ester addition products were identified using the benzoyl peroxide activating system. The compound with the lowest boiling point eluting off the column had a molecular ion at MW 332 (M) (Figure 18). Isotope peaks reflected a natural abundance consistent with one chlorine. On this basis the elemental composition
Figure 17. Chloro, trichloromethylstearic acid methyl ester isolated from incubation of methyl oleate in the benzoyl peroxide model system.
Figure 18. Mass spectrum of the methyl monochlorostearate adduct isolated after incubation of methyl stearate in the benzoyl peroxide model system.
was identified as monochlorostearic acid methyl ester (Figure 19). A collision-stabilized complex existed at m/e 361 which indicated that the peak at m/e 332 was the molecular ion (M+29=361). Also, a cluster around m/e 297 was the dechlorination fragment of the monochlorostearic acid methyl ester (M-35=297).

The methyl ester which eluted next had a molecular ion (M) at MW 366 daltons (Figure 20). Isotope peaks reflected a natural abundance consistent with the existence of two chlorines. These data indicated an elemental composition of $C_{19}H_{36}O_2Cl_2$. A collision-stabilized complex existed at m/e 395. This was evidence that the cluster around m/e 366 contained the molecular ion (M+29=395). Thirty-five mass units lower at m/e 331 was a cluster with an isotope cluster characteristic of one chlorine and was identified as a dechlorination fragment (M-35=331). Further dechlorination resulted in a cluster at m/e 296 which had no chlorine isotope cluster (M-70=296). On the basis of these data the compound was identified as dichlorostearic acid methyl ester (Figure 21).

The methyl ester eluting last had a molecular ion at MW 400 daltons (Figure 22). The isotope peaks reflected a natural abundance consistent with a molecule containing three chlorines. This indicated an elemental composition of $C_{19}H_{35}O_2Cl_3$. A collision-stabilized complex occurred at m/e 365 (M-35=365). Clusters occurred resulting from the fragmentation of the collision-stabilized complex at m/e 326 and m/e 290. The m/e 326 clusters resulted from loss of two HCl groups (MW 72 daltons) and the methoxy group (MW 31 daltons) from the collision-stabilized complex (M+29-72-31=326). The cluster at m/e 290 resulted
Figure 19. Methyl monochlorostearate isolated after incubation of methyl stearate in the benzoyl peroxide model system.
Figure 20. Mass spectrum of methyl dichlorostearate adduct isolated after incubation of methyl stearate in the benzoyl peroxide model system.
Figure 21. Methyl dichlorostearate adduct isolated after incubation of methyl stearate in the benzoyl peroxide model system.
Figure 22. Mass spectrum of methyl trichlorostearate adduct isolated after incubation of methyl stearate in the benzoyl peroxide model system.
from the loss of three HCl groups (MW 108 daltons) and the methoxy
group (MW 31 daltons) from the collision-stabilized complex
(M+29-108-31=290). These data were consistent with the molecular ion
being identified as trichlorostearic acid methyl ester (Figure 23).

**Tritium Incorporation Studies**

The formation of fatty acid radicals due to the interaction of
carbon tetrachloride reactive intermediates was postulated to result in
incorporation of tritium into fatty acids if tritiated water was used
in place of unlabeled water in microsomal incubations. Tritium
incorporation into microsomal lipids after incubation of microsomes in
tritiated water occurred within minutes after the introduction of carbon
tetrachloride in 2 μl ethanol. Tritium incorporation occurred
simultaneously with the rate of $^{14}C$-labeled carbon tetrachloride
metabolites binding to microsomal lipids. By 30 minutes the rate of
tritium incorporation and $^{14}C$ binding had ceased. The maximum amount
of tritium incorporated into microsomal lipids from phenobarbital
induced rats was approximately 35 picomoles per milligram lipid. The
maximal amount of $^{14}C$ binding of carbon tetrachloride metabolites from
the same animal was approximately 7.5 picomoles per milligram lipid
(Figure 24). Chloroform is a major anaerobic metabolite of carbon
tetrachloride. The quantity of chloroform produced in microsomal
incubations was assayed. Figure 25 is a standard curve for the
calculation of chloroform production in microsomes. The data was
plotted as the ratio of the area under the curve for chloroform (HCCl$_3$)
and the internal standard methylene chloride (H$_2$CCl$_2$) versus the known
Figure 23. Methyl trichlorostearate adduct isolated after incubation of methyl stearate in the benzoyl peroxide model system.
Figure 24. Tritium incorporation and $^{14}$C-carbon tetrachloride metabolites bound to microsomal lipids.

Microsomes were incubated in tritiated water with 25 nanomoles $^{14}$C-carbon tetrachloride. At the times indicated the microsomes were extracted with chloroform:methanol (2:1 vols) and the lipids were washed and evaporated to dryness with that solution. The lipids were weighed and assayed for bound radioactivity by liquid scintillation counting. Data analysis was conducted for dual-label counting as discussed in the text.
Figure 25. Standard curve for measuring chloroform production from carbon tetrachloride in microsomal incubations.

Known amounts of chloroform (HCCl₃) and methylene chloride (H₂CCl₂) were injected into sealed vials containing 100 microliters of a microsomal suspension. A 0.25 cc aliquot of headspace was removed and analyzed by gas-liquid chromatography. Data was expressed as chloroform-to methylene chloride ratio versus the known amount of chloroform in the vial.
amount of chloroform present in the standard. Linear regression analysis gave an \( R^2 \) value of 0.979 in the range of chloroform produced during the microsome incubations with carbon tetrachloride.

Table 4 summarizes the data of the experiments quantifying tritium incorporation into lipids, chloroform production and covalent binding to lipids after microsomal incubation of \(^{14}\)C-carbon tetrachloride in buffered tritiated water. At 5, 10, and 20 minutes of incubation, chloroform production plus covalent binding of carbon tetrachloride metabolites approximately equals the tritium incorporation into the microsomal lipids. At 5 minutes, for instance, the chloroform production of 163±15 picomoles plus the covalent binding of carbon tetrachloride metabolites of 25±2 picomoles approximately equals the tritium incorporation into microsomal lipids of 178±14 picomoles.
Table 4. Tritium incorporation into microsomal lipids, chloroform production from carbon tetrachloride, and covalent binding of carbon tetrachloride metabolites to microsomal lipids. --

Microsomes were incubated in tritiated water with 25 nanomoles $^{14}$C-carbon tetrachloride. At the times indicated, a 0.25 cc aliquot of headspace was analyzed for chloroform by gas-liquid chromatography. The microsomal suspension and the lipid containing layer removed, dried, and assayed for bound tritium and $^{14}$C by liquid scintillation counting. Data analysis for dual-label counting was performed as described in the text.

<table>
<thead>
<tr>
<th>Incubation Time (minutes)</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tritium incorporation into lipids (picomoles)</td>
<td>178 ± 14</td>
<td>250 ± 22</td>
<td>468 ± 60</td>
</tr>
<tr>
<td>Chloroform production (picomoles)</td>
<td>163 ± 15</td>
<td>244 ± 22</td>
<td>474 ± 35</td>
</tr>
<tr>
<td>Covalent binding of $\text{CCL}_4$ metabolites (picomoles)</td>
<td>25 ± 2</td>
<td>38 ± 3</td>
<td>60 ± 5</td>
</tr>
</tbody>
</table>
DISCUSSION

Covalent binding of radiolabeled xenobiotics to hepatocyte lipids and proteins was used in this study as an index of bioactivation. By altering the conditions of the incubation and examining the alteration in the magnitude of binding, inferences can be made about the route of bioactivation of a compound.

Bioactivation of carbon tetrachloride to reactive intermediates which covalently bind to macromolecules was shown to be favored in the absence of oxygen. In the presence of oxygen the binding was almost entirely eliminated (Table 1). Although CCl$_4$ may still be bioactivated in the presence of oxygen, it is thought that the interaction of the trichloromethyl free radical with oxygen results in the formation of a trichloromethyl peroxy free radical (CCl$_3$OO$^\cdot$). It is this radical which is thought to decompose to phosgene (CCl$_2$O), carbon dioxide (CO$_2$), and carbon monoxide (CO) (Packer et al., 1978).

Cell viability was not affected by the absence of oxygen for up to 30 minutes. After that time, however, cell death occurred rapidly. This is the only study to date which has demonstrated anaerobic metabolism in a hepatocyte system.

Substrate concentrations were kept as low as possible in this study of the bioactivation of these compounds to avoid toxicity and loss of cell viability. This was done by using the radiolabeled compounds undiluted by cold parent and using ethanol as vehicle.
Larger amounts of covalent derivatives of reductive intermediates of carbon tetrachloride were bound to lipids and proteins than to nucleic acids. Formation was enhanced by phenobarbital induction prior to sacrifice. Sipes, Krishna, and Gillette (1977) demonstrated that the site of bioactivation of CCl₄ was the cytochrome P-450 mixed function oxidase system, and it was expected that increased levels of that enzyme system brought about by the prior administration of phenobarbital would result in increased bioactivation leading to increased covalent binding by ¹⁴C-carbon tetrachloride equivalents. These data are consistent with that theory. Glutathione depletion by diethyl maleate had inconsistent effects on the binding to lipid and protein by carbon tetrachloride (Table 1). No direct interaction between CCl₄ metabolites and glutathione has been found to date, although chloroform, a metabolite of carbon tetrachloride, has been shown to interact with glutathione (Pohl et al., 1980).

Carbon tetrachloride metabolites alkylated the nucleic acids RNA and DNA in the hepatocyte system. This demonstrates that although the reactive intermediates of CCl₄ are reactive enough to alkylate the ribonucleic acids in the endoplasmic reticulum, they are also stable enough to enter into the nucleus and alkylate nuclear deoxyribonucleic acids. The half-life of the trichloromethyl free radical has been estimated to be 100 microseconds in an aqueous environment (Wilson and Slater, 1975), and it is apparent from this study that this short time span is adequate for a reactive intermediate to traverse the distance from the endoplasmic reticulum into the nucleus and react with DNA.
The formation of covalent derivatives of trichloroethylene bound to lipids and proteins was oxygen dependent. Trichloroethylene has been postulated to accept an oxygen atom from $O_2$ after the oxygen has been activated by the cytochrome P-450 mixed function oxidase system (MFOS) (Uehleke and Poplawski-Tabarelli, 1977). This is thought to result in the formation of an epoxide that, being a highly strained ring structure, is reactive and alkylates nucleophilic molecules such as protein or lipid. That trichloroethylene binding to protein and lipid was increased after pretreatment of the rat with the MFOS inducer phenobarbital is further evidence that the cytochrome P-450 system catalyzes the bioactivation of trichloroethylene.

Trichloroethylene may also be bioactivated by conjugation with the tripeptide glutathione. It is thought that conjugation with the cysteine moiety of the glutathione followed by cleavage of the aspartate and glutamate residues results in trichloroethylene activation to trichlorovinyl cysteine, a compound known to be a highly reactive alkylating agent (Bhattacharya and Schultze, 1972). Pretreatment of rats with diethyl maleate prior to sacrifice resulted in decreased glutathione levels and decreased the level of trichloroethylene equivalents bound to lipid and protein. The results of these studies are in agreement with the dual activation pathways postulated for trichloroethylene of MFOS activation to an epoxide and glutathione conjugation resulting in trichlorovinyl cysteine.

The formation of covalent derivatives of methylene chloride to lipids and protein was also oxygen dependent. It was demonstrated in a
series of papers by Anders' group (Kubic and Anders, 1978; Ahmed and Anders, 1978; Anders et al., 1977) that MFOS metabolized methylene chloride to carbon monoxide. It was also shown that glutathione is involved in the formation of formaldehyde and formate from methylene chloride. When the activity of the MFOS was increased by phenobarbital pretreatment the binding of $^{14}$C-methylene chloride metabolites to lipid and protein was decreased. This indicated that the biotransformation pathway resulting in carbon monoxide does not produce reactive intermediates capable of binding to lipid and protein. The decreasing of hepatic glutathione content with diethyl maleate prior to sacrificing the rat also reduced the binding of methylene chloride to protein and lipid, indicating that the activation of methylene chloride through glutathione conjugation to formaldehyde may be the major pathway of bioactivation leading to covalent binding.

These studies demonstrated that isolated rat hepatocytes may be useful in examining bioactivation of volatile xenobiotics. They were capable of producing reactive intermediates from three organohalogen compounds resulting in alkylation of hepatocyte macromolecules. Hepatocytes offer the advantages of duplicating the morphology of \textit{in situ} liver cells in respect to the relative location of the bioactivation enzymes and subcellular organelles. This is especially important in the case of the cytochrome P-450 mixed function oxidases in the smooth endoplasmic reticulum and nuclei containing the genetic information molecule, deoxyribonucleic acid (DNA). The somatic cell mutation hypothesis states that a permanent, heritable alteration in
nucleic acid base sequences must occur as the initiating event in neoplastic transformation (Miller, 1970). It has been shown in the cases of polycyclic aromatic hydrocarbons, aflatoxin, and acetylaminofluorine that a correlation exists between the ability of a chemical to bind covalently to DNA and the carcinogenic potency of that compound (Miller, 1970). Compounds bioactivated by the MFOS must travel across the cytosol and through the nuclear membrane before they can interact with nuclear DNA to initiate carcinogenesis. Since the cellular morphology of liver is maintained in hepatocyte preparations, the results using hepatocytes to study xenobiotic bioactivation more closely resemble the in vivo situation than artificial reconstituted system such as microsomes with added DNA or added nuclei.

This study showed that the volatile organohalogens carbon tetrachloride, trichloroethylene, and methylene chloride were bioactivated by hepatocytes resulting in alkylation of tissue macromolecules. The carcinogenic solvents carbon tetrachloride and trichloroethylene alkylated the macromolecules lipid, protein, and the nucleic acids RNA and DNA. Methylene chloride was shown in this study to be bioactivated by hepatocytes to intermediates which alkylated lipid and protein macromolecules but not nucleic acids.

These results are in agreement with the somatic cell mutation hypothesis. This suggests that these compounds may be able to initiate their carcinogenic activity through covalent binding to DNA after bioactivation. By using hepatocytes as a model system I demonstrated that reactive intermediates of both these compounds exist in the cell long
enough to migrate into the nucleus and once there are reactive enough to attack nucleic acids. Both were also able to alkylate RNA and may also produce cellular injury by decreasing the fidelity of the translation process.

Methylene chloride did not alkylate either RNA or DNA, although it did alkylate lipid and protein. This is consistent with a study demonstrating no significant increase in cancer frequency after prolonged methylene chloride exposure (Anonymous, 1978). Although absence of evidence is not evidence of absence, the results indicating that methylene chloride is non-carcinogenic and does not alkylate nucleic acids are consistent with the somatic cell mutation hypothesis.

The major error occurring in studies of these kind is failure to purify the nucleic acids of protein and lipid contamination. Since the amount of binding to nucleic acids is usually in the picomole/mg range and the levels of radioactivity are only several times background, scrupulous care must be made to assure contamination free-nucleic acids. The results showing methylene chloride binding to protein and lipid while demonstrating no detectable binding to nucleic acids verifies the enzymatic hydrolysis method of preparation of nucleotides and substantiates the positive results for binding to nucleic acids by carbon tetrachloride and trichloroethylene. That methylene chloride did not bind to DNA or RNA but did bind extensively to protein and lipid verifies the methodology for isolation and purification of nucleic acids by enzymatic hydrolysis.
It is clear that electrophilic reactivity per se does not automatically confer mutagenic, carcinogenic, or other toxic properties upon a compound. The chemical structure and reactivity of the reactive intermediate must be considered in assessing the potential toxicity of a compound. In this study, the trichloromethyl free radical from carbon tetrachloride had sufficient reactivity to interact with nucleic acids, as did the trichloroethylene oxide intermediate from trichloroethylene. However, no reactive intermediate of methylene chloride had sufficient reactivity to alkylate nucleic acids. Indeed, methylene chloride is less mutagenic in the Ames test after bioactivation than before bioactivation (Shahin and VonBorstel, 1977).

The finding that a compound is mutagenic in bacteria yet is not a mutagen in mammals, and by association a carcinogen, is not unique to methylene chloride. The insecticide and antihelminetic dichlorvos (dichlorovinyl dimethyl phosphate) has also been shown to be mutagenic or carcinogenic in mammalian test systems (Wright, Hutson, and Wooder, 1979). Dichlorvos is bioactivated to reactive intermediates which alkylate protein in vivo and can methylate guanine when combined with isolated RNA or DNA. However, methylation of guanine by dichlorovos proceeds along normal biosynthetic pathways through 1-carbon transfer reactions through tetrahydrofolate and results in the functional base 7-methylguanine.

Therefore, the presence of alkylated macromolecules is not sufficient evidence to judge the potential toxicity of a compound. Both methylene chloride and dichlorovos are capable of alkylating
macromolecules and producing mutations in eukaryotes (Wright et al., 1979; Shahin and VonBorstel, 1977) but neither are able to induce genetic damage in mammalian cells (Cunningham, et al., 1979; Wright, et al., 1979).

The postulated interactions of trichloromethyl free radical prior to this study are presented in Appendix H, Figure H.1. The only lipid components thought to interact with the trichloromethyl free radical were the polyunsaturated fatty acids attached to phospholipids (Slater, 1978). Hydrogen abstraction resulting in chloroform production was believed to occur only at the vinylic carbon. This allowed the double bond to shift forming a conjugated diene radical (Appendix H, Figure H.1.). This radical was then postulated to a) interact with a trichloromethyl free radical to form an adduct; b) interact with oxygen to form a peroxide; or c) interact with an unsaturated fatty acid to form a dilipid radical (Slater, 1978). None of these postulated reactions products has been identified to this authors knowledge.

Radical addition of the trichloromethyl free radical was thought to occur across a double bond of a polyunsaturated fatty acid resulting in the lipid adduct radical shown in Appendix H, Figure H.1. This lipid adduct radical was thought to interact with a) a trichloromethyl free radical resulting in an unsaturated fatty acid with two trichloromethyl adducts; b) oxygen to form an unsaturated trichloromethyl fatty acid peroxy free radical; or c) another unsaturated fatty acid to form an unsaturated trichloromethyl fatty acid dimer to form an
unsaturated trichloromethyl fatty acid dimer free radical (Appendix H, Figure H.1.) (Slater, 1978). None of the postulated adducts have been demonstrated to date.

The use of the benzoyl peroxide system to generate reactive intermediates of carbon tetrachloride has proven most useful in quantitatively comparing the interaction of different fatty acids with carbon tetrachloride reactive intermediates. The intermediates produced in this system were the result of the decomposition of the free radical generator benzoyl peroxide activating carbon tetrachloride, and were therefore quantitatively and qualitatively equivalent in all incubations. Biological systems such as microsomes enriched in saturated or unsaturated fatty acids, or reconstituted cytochrome P-450 systems with dioleoyl- or distearoyl-phosphatidylcholine suffer from quantitatively altering the production of reactive intermediates due to alterations in the lipid environment critical to the activity of the activating enzymes. Therefore, model systems such as the benzoyl peroxide one used in this study can be useful in studying the interactions of reactive intermediates with isolated biological macromolecules.

The use of chemical ionization mass spectrometry as a research tool in this study is a unique and little used application of this technique. The usual ionization mechanism in mass spectrometry is bombardment with electrons at very low pressures (10^-5 Torr), resulting in characteristic fragmentation patterns due to metastable ion fragments. Chemical ionization, on the other hand, uses an ionized reactant gas such as methane or isobutane at higher pressures (1 Torr) to interact
with the sample molecules to produce charged species with less resultant fragmentation. Ionizing samples in these less drastic conditions results in greater stability of molecules of larger mass-to-charge ratio (m/e) and therefore easier identification of parent molecules.

The purpose of using higher pressures around 1 Torr is to alter the character of the intermolecular interactions occurring in the gas phase at the ionization source. At low pressure (10⁻⁴ Torr), the ions formed at the ionization source are CH₄⁺, CH₃⁺, and CH₂⁺. As the pressure is increased, these primary ions interact with neutral CH₄ molecules to give secondary and higher-order ionic products such as CH₅⁺, C₂H₅⁺, C₂H₄⁺, C₃H₇⁺, and C₃H₅⁺ (Appendix C, Figure C.2). These ionized the sample molecule by addition of H⁺ (by CH₅⁺ or C₃H₇⁺) or by abstraction of H⁺ (by C₂H₅⁺ or C₃H₅⁺). Ionized sample molecules are then separated and focused for analysis at the ion collector.

In addition to the reactions between secondary reactant ions resulting in the above reactions, reactant ions may react with electron-rich compounds to form collision-stabilized complexes, commonly referred to as adduct ions. These complexes form molecular ions of higher molecular weight than the parent molecule by 57 mass units if the carrier is isobutane or by 29 and 41 mass units if the carrier is methane. Detection of peaks with m/e of 29 and 41 higher usually indicate a parent molecular ion at m/e 29 and 41 lower. Fragmentation of the collision-stabilized complexes may occur and must be considered when analyzing fragmentation patterns.

A distinguishing feature in analyzing the mass spectral data is the identification of chlorine isotope clusters. Isotope clusters are
formed in mass spectra due to the existence of stable isotopes of certain atoms. A second isotope makes an especially prominent appearance in a spectrum if it is more than one unit higher in mass than the most abundant isotopic species (McLafferty, 1973). This is true in the case of chlorine which forms characteristic isotope clusters, the pattern of which is determined by the number of chlorine atoms in the molecule. One chlorine on the molecule imparts an easily recognizable cluster of two peaks corresponding to the parent fragment containing the $^{35}$Cl isotope (A) and an A+2 peak containing $^{37}$Cl. The peak heights reflect the natural abundance of chlorine isotopes in the abundance of 68.5\% $^{35}$Cl and 32.5\% $^{37}$Cl. A molecule containing two chlorines will have an isotopic cluster of three peaks. This is due to molecules containing combinations of $^{35}$Cl and $^{37}$Cl atoms resulting in a prominent peak containing two $^{35}$Cl atom and one $^{37}$Cl atom, and a third peak four mass units higher than the first containing two $^{37}$Cl atoms. In proportion to the natural abundance of the two chlorine isotopes, these three peaks will have peak heights in the ratio of 1.00:0.47:0.226 for the molecules containing $^{35}$Cl + $^{35}$Cl:$^{35}$Cl + $^{37}$Cl:$^{37}$Cl + $^{37}$Cl. A molecule containing more than two chlorines will exhibit more complex isotope clusters (Appendix C, Figure C.1).

The mass spectral identification of the 9,10-chloro, trichloro-methyl adduct across the double bond of methyl oleate in the benzoyl peroxide system indicates that the initial attack of the activated carbon tetrachloride occurs by an addition reaction of the trichloro-methyl free radical to one carbon containing the double bond. This
occurs by pairing one electron of the pi bond of the oleic acid methyl ester with the unshared electron of the trichloromethyl radical. The other pi electron remains unpaired in the sp\(^3\) orbital of the opposite carbon of the double bond resulting in a lipid adduct radical (Figure 26). This lipid radical intermediate was confirmed by the use of spin trapping methods to be the result of trichloromethyl attachment to unsaturated fatty acids in microsomal incubations (Poyer et al., 1980).

The lipid adduct radical, being electrophilic in nature, attacks an electron-rich molecule, carbon tetrachloride, in the model system. Attack upon a molecule of carbon tetrachloride results in the homolytic cleavage of a carbon-chlorine bond, attachment of the chlorine to the chlorine to the carbon of the lipid radical and production of trichloromethyl radical (Figure 26). This results in the chloro, trichloromethyl fatty acid methyl ester identified by mass spectrometry after incubation of oleic acid methyl ester in the benzoyl peroxide model system.

The lipid adduct is proposed to follow the above sequence with the trichloromethyl radical addition preceeding the addition of chlorine. If a chlorine radical added initially to the double bond the resulting lipid adduct radical would not contain a trichloromethyl group, but would contain another chlorine derived from homolytic attack on carbon tetrachloride. No evidence for a dichloro adduct to oleic acid methyl ester was found. Since a dichloro adduct was found after the incubation with stearic acid methyl ester, I believe no dichloro adduct was produced in measureable amounts in the incubation of oleic acid methyl ester in the benzoyl peroxide model system.
Figure 26. Postulated reaction of the trichloromethyl free radical with methyl oleate in the benzoyl peroxide model system.
The isolation and identification by mass spectral analysis of mono-, di-, and trichlorostearic acid methyl esters in the benzoyl peroxide model system indicate that the initial attack of activated carbon tetrachloride occurs by abstraction of a hydrogen resulting in a fatty acid methyl ester radical (Figure 27). The resulting lipid radical causes a homolytic cleavage of a carbon-chlorine bond of a carbon tetrachloride molecule, resulting in chlorinated fatty acid methyl esters and a trichloromethyl free radical which could abstract a hydrogen and reinitiate the sequence of events. This is a propagation type free radical reaction since it produces a free radical for each one it consumes.

Three chlorinated fatty acid methyl esters were identified by mass spectrometry after incubation of methyl stearate in the benzoyl peroxide model system (Figures 19, 21, and 23). The existence of mono-, di-, and trichlorinated fatty acid methyl esters after incubation with carbon tetrachloride indicates that multiple hits occurred upon individual fatty acids. Due to the length of the stearic acid chain it is not surprising that multiple events occur resulting in several sites of chlorination.

An adduct indicating the binding of a trichloromethyl radical was not found. Although there was a small amount of $^{14}$C bound to the isolated methyl stearate, no evidence for a methyl stearate adduct containing an additional carbon or carbon and chlorine was found. Indeed, a mechanism of addition of the carbon nucleus to a lipid radical would be difficult to justify chemically, since that would involve the
Figure 27. Postulated reaction of the trichloromethyl free radical with stearic acid methyl ester in the benzoyl peroxide model system.
lipid radical attack on the carbon and not on a chlorine. Since the carbon atom on carbon tetrachloride is shielded by four highly electron rich chlorines, it would be very difficult for the lipid radical electron to avoid interaction with the chlorine electrons before it could reach the electrons of the carbon atom. Also, a chlorine atom is about the size of a methyl group and stearic hindrance would shield the carbon atom from attack.

Substitution of the trichloromethyl radical in place of the methyl ester group was also not indicated by the mass spectral analyses. These three adducts were identified after using stearic acid in the benzoyl peroxide system and then transmethylating with HCl-methanol as well as in the experiments beginning with stearic acid methyl ester. If adduct formation occurred at the ester group then the transmethylation would have removed adducts attached there. Even after transmethylation all three adducts were identified. Also, a fragment in the mass spectrum of the trichlorostearic acid methyl ester resulted from loss of three chlorines and loss of the methoxy group (Figure 22). This is further evidence that the ester function was not chlorinated.

These data demonstrate that the trichloromethyl free radical can interact with unsaturated sites on fatty acids resulting in adduct formation. Interaction with saturated sites on fatty acids may result in proton abstraction resulting in chloroform production and a lipid radical which may then interact with other molecules. Although the work presented here demonstrates two pathways for the interaction of the
trichloromethyl free radical and fatty acid components, it does not contradict previously postulated pathways nor does it eliminate the possibility that they may also occur.

As was discussed in the introduction, the mechanism of cellular injury due to the interaction of carbon tetrachloride metabolites with cellular macromolecules has not been established. The presence of lipid peroxidation in carbon tetrachloride induced hepatic necrosis has been well documented (Rechnagel and Glende, 1973) as well as the phenomenon of covalent binding of carbon tetrachloride metabolites to macromolecules (Gomez et al., 1975). Recent information from two independent laboratories has been presented favoring the covalent binding theory of toxicity. DeGroot and Haas (1980) demonstrated a decline in the activity of cytochrome P-450 in anaerobic microsomal incubations after the addition of carbon tetrachloride. In this experiment, lipid peroxidation did not occur due to the lack of oxygen and the addition of EDTA. They conclude that the inactivation of cytochrome P-450 is due to the interaction of the carbon tetrachloride metabolites with the cytochrome P-450 itself and not due to secondary lipid peroxidation processes (DeGroot and Haas, 1980).

Strubelt and Breining (1980) demonstrated increased liver damage in rats exposed to carbon tetrachloride in an atmosphere of 6% O₂:94% N₂ compared to rats exposed to carbon tetrachloride in air (18% O₂:82% N₂). Hypoxia alone or hypoxia plus paracetamol, allyl alcohol, bromobenzene, or thioacetamide did not enhance the hepatotoxicity of these compounds. Hypoxia enhances the reductive metabolism of carbon
tetrachloride and increases the extent of covalent binding of carbon
tetrachloride metabolites to macromolecules (Cunningham et al., 1979).

These data indicate that the reductive metabolism results in
deactivation of cytochrome P-450 in microsomes and increased hepato-
toxicity in vivo. Also, Stacey and Priestly (1978) demonstrated that
lipid peroxidation due to oxidative metabolism of carbon tetrachloride
in isolated hepatocytes did not result in decreased cell viability.
Therefore, the reductive metabolism of carbon tetrachloride resulting
in chloroform production and covalently bound adducts may be the
metabolic pathway resulting in carbon tetrachloride toxicity rather
than oxidative destruction of lipid components.

The mechanisms I propose to account for the adducts identified
by mass spectroscopy require the formation of reactive intermediate
lipid radicals. These occur either by addition of the trichloromethyl
free radical across a site of unsaturation or by abstraction of a
hydrogen atom from a saturated site. In both these cases an unstable
chemical reactive intermediate, the trichloromethyl free radical,
interacted with a fatty acid molecule producing an unstable biological
reactive intermediate, a fatty acid reactive intermediate. In the
benzoyl peroxide system, this biological free radical interacted with
the chemical environment surrounding it to abstract a chlorine atom
and become stabilized.

In order to demonstrate that these reactions occur in vivo,
evidence for the formation of lipid radicals was sought. This was
accomplished using rat hepatic microsome preparations, since these
contain the cytochrome P-450 enzymes which bioactivate carbon tetrachloride as well as phospholipids with which the carbon tetrachloride metabolites interact. If the postulated lipid radicals were indeed formed in vivo, microsome preparations should offer an ideal biological model to demonstrate the formation and interactions of the lipid radicals.

In the biological milieu, lipid radicals can interact with many different types of molecules. Proteins, nucleic acids, other lipids, glutathione, vitamins, cofactors, and inorganic compounds are all present and may be potential targets for interaction with lipid radicals. However, it seemed that the molecule with the most likely chance of being detected interacting with a lipid radical would have to be abundant, possess an ionizable hydrogen or an easily abstracted functional group, and be available as a radioactive isotope. For these reasons the interaction of lipid radicals with water was chosen for study. Microsomes, like all biological tissues, are surrounded by water; water possesses ionizable hydrogens; and radioisotopically labeled water is readily available as $^3\text{H}_2\text{O}$.

The hypothesis being tested in these following experiments was the following: If lipid radicals were formed by interaction with carbon tetrachloride metabolites, then they will abstract hydrogen atoms from water or tritium atoms from tritiated water and result in lipids that contain tritium after incubation with carbon tetrachloride and tritiated water.
In order to test this hypothesis, the microsomal incubations were conducted in tritiated water with a high specific activity and lipids were assayed for bound tritium. The tritiated water available contained \(2.54 \times 10^6\) dpm per milliliter which was not high enough to simply add to an incubation. Therefore, the buffer was lyophilized to remove non-radiolabeled water and reconstituted with tritiated water of the specific activity indicated above. By use of the lyophilization step, adequate specific activity was obtained to perform the experiments.

The results of the tritium binding experiments (Figure 24) indicated that the magnitude of the tritium incorporated was greater (7 fold) than the covalent binding of carbon tetrachloride metabolites. Therefore other phenomena must also be responsible for the tritium incorporation into microsomal lipids after bioactivation of carbon tetrachloride. Chloroform production in the benzoyl peroxide model system was postulated to result in a lipid radical after attack or a saturated fatty acid by the trichloromethyl free radical. Therefore, the quantity of chloroform produced in microsomal incubations with carbon tetrachloride was analyzed in comparison to the amount of tritium incorporated into microsomal lipids.

The data in Table 4 demonstrates that chloroform production approximately equals the amount of tritium incorporation into microsomal lipids. Tritium incorporation was approximately 44 picomoles per milligram of microsomal lipid, and each incubation contained about 11 milligrams lipid. Total tritium incorporation was approximately 468 picomoles per incubation. Total chloroform production after
incubation for twenty minutes was about 474 picomoles. This value for chloroform production from carbon tetrachloride was similar to the amount obtained by Reiner et al. (1972). They obtained chloroform production at levels of approximately 11 nanomoles/milligram protein/5 minutes and the results obtained in this study were equivalent to 0.20 nanomoles/milligrams protein/5 minutes. The difference is due to the lower substrate concentration used in this study (25 nanomoles) compared to their study (1000 nanomoles).

Although the covalent binding of carbon tetrachloride metabolites to unsaturated sites may contribute to the observed magnitude of tritium incorporation (Figure 28), it is not sufficient to account for all tritium incorporation demonstrated. Therefore the majority of the tritium incorporation appears to be a result of the trichloromethyl free radical abstracting a hydrogen from a fatty acid resulting in a fatty acid radical and chloroform. The fatty acid radical abstracts a tritium from tritiated water resulting in the tritiated microsomal lipid which was formed after incubation in tritiated water in the presence of carbon tetrachloride (Figure 29).

Prior to this study, the vinylic proton was thought to be the only hydrogen capable of being abstracted by the trichloromethyl free radical. The energy for dissociation of this bond is approximately 88 Kcal/mole. The bond dissociation energy to break the carbon-hydrogen bond of a saturated hydrocarbon such as stearic acid is approximately 98 Kcal/mole (Morrison and Boyd, 1973). The difference in energy to abstract a hydrogen from a saturated site is 11% greater
Figure 28. Postulated interaction of the trichloromethyl free radical with unsaturated sites on fatty acids in microsomes.
Figure 29. Postulated interaction of the trichloromethyl free radical with saturated sites on fatty acids in microsomes.
than the energy needed to abstract a hydrogen from a vinylic site. This difference is obviously not sufficient to prevent the trichloromethyl free radical from attack at saturated sites.

The significance of the results of the experiments in the benzoyl peroxide model system and the microsomal incubations in tritiated water lies in understanding more fully the interactions of the reactive intermediates of carbon tetrachloride with fatty acids. Prior to this study, saturated fatty acids were thought to be inert to attack by the trichloromethyl free radical. This study has demonstrated that there exists an interaction of the trichloromethyl free radicals with saturated fatty acids resulting in proton abstraction and chloroform production. The resulting lipid radical abstracted a chlorine from carbon tetrachloride in the benzoyl peroxide model system and may be a site for tritium abstraction in microsomes incubated in tritiated water.

Saturated fatty acids comprise 41% of the total fatty acid pool in mammalian cells (Weber et al., 1976). By demonstrating that these fatty acids may be targets of attack by activated xenobiotics, a more fully integrated concept of the total reaction of reactive intermediates is possible.

The mass spectral interpretations from the benzoyl peroxide model system indicated that lipid radicals were formed during the interaction of the trichloromethyl free radical with saturated or unsaturated sites on fatty acids. This was also indicated by the results of the microsomal studies in tritiated water which demonstrated
tritium incorporation into lipids during carbon tetrachloride bioactivation. Although the abstraction of a tritium (or proton) is not in itself potentially toxic, the resulting hydroxyl radical (·OH) may be. Hydroxyl radical have been shown to interact with the nucleic acids adenine, thymine, uracil, and guanine as well as producing single strand breaks in DNA (Willson, 1978). Amino acids and proteins are also susceptible to attack by hydroxyl radicals. The enzymatic activities of lysozyme and ribonuclease have been shown to be inhibited by hydroxyl radicals (Willson, 1978).

Therefore, the production of hydroxyl radicals may be the toxic reactive intermediates formed after carbon tetrachloride intermediates react with fatty acids. As demonstrated by Reiner et al. (1972), the rate of chloroform production from carbon tetrachloride is higher in rat liver microsomes incubated without oxygen than those incubated with oxygen. In vivo exposure of rats to carbon tetrachloride and reduced oxygen tension resulted in greater hepatotoxicity than rats exposed to carbon tetrachloride and normal oxygen tension (Strubelt and Breining, 1980). Presumably, the chloroform production would also be greater in the case of reduced oxygen tension. The resulting production of fatty acid radicals and hydroxyl radicals would be increased, possibly enhancing the toxicity of carbon tetrachloride.
APPENDIX A

POSTULATED METABOLISM OF TRICHLOROETHYLENE
Figure A.1. Postulated biotransformation of trichloroethylene resulting in the stable metabolites trichloroacetic acid, trichloroethanol, and trichloroethanol glucuronide.
APPENDIX B

DUAL LABEL LIQUID SCINTILLATION COUNTING EXPERIMENTS

Quench curves, isotope emission spectra, and formulas for dual-label data analysis for calculation for $^{14}\text{C}$ and $^{36}\text{Cl}$ or $^{3}\text{H}$ and $^{14}\text{C}$ dual label liquid scintillation counting experiments.
Figure B.1. Quench curves for use in $^{14}$C/$^{36}$Cl dual-label liquid scintillation counting.

Each isotope was counted in both windows with increasing amounts of the quenching agent chloroform. H number is a measure of the degree of quench in a sample calculated from an external standard.
Figure B.2. Quench curves for use in $^3$H/$^{14}$C dual-label liquid scintillation counting.

Each isotope was counted in both windows with increasing amounts of the quenching agent chloroform. H number is a measure of the degree of quench in a sample calculated from an external standard.
Figure B.3. Spectra of isotope emission intensities for $^{14}\text{C}$ and $^{36}\text{Cl}$. 
Figure B.4. Spectra of isotope emission intensities for $^3$H and $^{14}$C.
1. \( \text{CPM 1} = \text{DPM 1} \times \text{Eff 11} + \text{DPM 2} \times \text{Eff 21} \)

2. \( \text{CPM 2} = \text{DPM 1} \times \text{Eff 12} + \text{CPM 2} \times \text{Eff 22} \)

Where:

- \( \text{Eff 11} \) = counting efficiency of Isotope 1 in channel 1
- \( \text{Eff 12} \) = counting efficiency of Isotope 1 in channel 2
- \( \text{Eff 21} \) = counting efficiency of Isotope 2 in channel 1
- \( \text{Eff 22} \) = counting efficiency of Isotope 2 in channel 2
- \( \text{CPM 1} \) = Total count rate in channel 1
- \( \text{CPM 2} \) = Total count rate in channel 2

Figure B.5. Formulas for dual-label data analysis.

Samples containing \(^{14}\text{C}\) and \(^{36}\text{Cl}\) or \(^{3}\text{H}\) and \(^{14}\text{C}\) were counted in both isotope channels and the data calculated using formulas 1 and 2 in order to determine the counts per minute due to each isotope without overlap by the other isotope.
APPENDIX C

SUPPLEMENTARY INFORMATION FOR MASS SPECTRAL DATA ANALYSIS
Figure C.1. Isotopic clusters resulting from combinations of chlorine atoms.
\[ \text{CH}_4^+ + \text{CH}_4 \rightarrow \text{CH}_5^+ + \text{CH}_3^- \]
\[ \text{CH}_3^+ + \text{CH}_4 \rightarrow \text{C}_2\text{H}_5^+ + \text{H}_2 \]
\[ \text{CH}_3^+ + 2\text{CH}_4 \rightarrow \text{C}_3\text{H}_7^+ + 2\text{H}_2 \]
\[ \text{CH}_2^+ + 2\text{CH}_4 \rightarrow \text{C}_3\text{H}_5^+ + 2\text{H}_2 + \text{H}^- \]
\[ \text{CH}_2^- + \text{CH}_4 \rightarrow \text{C}_2\text{H}_4^+ + \text{H}_2 \]
\[ \text{CH}_2^- + \text{CH}_4 \rightarrow \text{C}_2\text{H}_3^+ + \text{H}_2 + \text{H}^- \]

Figure C.2. Reactions of methane at the ion source in chemical ionization mass spectrometry.
Figure C.3. Chemical ionization mass spectrum of methyl stearate.
Figure C.4. Chemical ionization mass spectrum of methyl oleate.
APPENDIX D

REACTIONS OF DICHLOROMETHYL CARBENE
Figure D.1. Postulated reactions of the dichloromethyl carbene in aqueous media.
APPENDIX E

STRUCTURE OF PHOSPHOLIPIDS AND THEIR REACTIONS WITH FREE RADICALS
Figure E.1. The general structure of phospholipids.

X is choline in phosphatidylcholine, serine in phosphatidylserine, and ethanolamine in phosphatidylethanolamine.
Figure E.2. Postulated reactions of free radicals (R *) and oxygen (O2) with polyunsaturated fatty acids resulting in lipid peroxidation.
APPENDIX F

CHROMATOGRAPH OF HEAD SPACE ABOVE MICROSONES
METABOLIZING CARBON TETRACHLORIDE
Microsomes (6 mg/ml) were incubated with 22.5 nanomoles carbon tetrachloride in a sealed vial at 37°C. After quenching with 1 microliter concentrated HCl, 1 nanomole methylene chloride was injected as the internal standard. A 0.25cc aliquot of the headspace was analyzed for chloroform and methylene chloride by gas-liquid chromatography using a Varian 3700 gas chromatograph and a nickel electron capture detector. A six-foot Porpak Q column was used at 140°C. Peak areas were calculated using a Varian CDS 111 automatic integrator.
APPENDIX G

FREE RADICAL PRODUCTION BY BENZOYL PEROXIDE
Figure G.1. Degradation of benzoyl peroxide at elevated temperature to produce free radicals.
APPENDIX H

REACTIONS OF THE TRICHLOROMETHYL FREE RADICAL WITH FATTY ACIDS
Figure H.L. Postulated interactions of the trichloromethyl free radical with fatty acids prior to this study.
REFERENCES


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Strubelt, O., and Breining, H., Toxicology Letters, 6:109, 1980.


