THE COVALENT INTERACTION OF HEPATIC METABOLITES OF THE INSECTICIDE CHLORDANE WITH CELLULAR MACROMOLECULES IN THE RAT AND MOUSE IN VITRO

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Toxicology

Approved:

UTAH STATE UNIVERSITY Logan, Utah

1979
ACKNOWLEDGMENTS

My sincerest thanks are due to Professor Joseph C. Street for providing just the right mixture of teacher, friend and father to get me through the graduate school experience successfully and profitably. I must also thank the Utah State Agricultural Experiment Station for providing funds that helped support me and my family during this work, and Rita Nelson for valuable technical assistance.

I owe a special debt of gratitude to my friend, Dr. James G. Straka and to my wife and friend, Renee. They listened with kind attentiveness to the pontification, the hair-brained schemes and even to the abuse. They, more than anyone, are entitled to say, "I knew you when . . . ."

Alan A. Brimfield
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ABSTRACT

The Covalent Interaction of Hepatic Metabolites of the Insecticide Chlordane with Cellular Macromolecules in the Rat and Mouse In Vitro

by

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Utah State University, 1979

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This investigation addressed several aspects of the covalent interaction of metabolites of the insecticide chlordane with cellular macromolecules in vitro. Microsomal preparations from the liver of mice and rats were used and covalent binding to microsomal protein and RNA or to added calf thymus DNA was studied. Pure $^{14}\text{C}$-labelled cis- and trans-chlordane isomers as well as an isomeric mixture ($^{14}\text{C}$-cis-chlordane plus $^{14}\text{C}$-trans-chlordane 3:1, w:w) were used as substrates for the in vitro system. Biochemical parameters investigated included inhibition of microsomal mixed-function oxidase and epoxide hydratase plus the induction of these enzymes by pretreatment with chlordane or phenobarbital. The effect of these manipulations on covalent binding of the metabolites to the macromolecules was of interest.

Isolation of the protein, RNA and DNA from the in vitro microsomal systems and determination of unextractable radioactivity indicated that the chlordane derived material bound to each of the
macromolecules investigated. The only exception was that mouse liver microsomes did not activate trans-chlordane to a form which bound to DNA in measurable amounts under the conditions employed.

Microsomal epoxide hydratase and aminopyrine demethylase activity were increased in both the rat and the mouse following chlordane pretreatment. The effect of this induction on the macromolecular interaction of chlordane metabolites was variable for both chlordane and phenobarbital pretreated groups. Generally, for the mouse, induction increased binding to protein and DNA but decreased binding to RNA. In the rat, induction decreased binding to each of the macromolecular species. The effect of enzyme inhibition was variable in both species under the different conditions tested except for the binding of cis-chlordane derived material to DNA in the mouse liver system. In that case inhibition of epoxide hydratase clearly reduced the concentration of material covalently interacting with the DNA to unmeasurable levels.

The results indicated little possibility that the primary epoxide metabolite of chlordane, oxychlordane, is involved in the binding. The effects of epoxide hydratase inhibition, however, indicate that some secondary epoxide is involved in the cis-chlordane binding to DNA in the mouse. The possible analogy between the binding behavior of chlordane found in this study and the binding behavior of other well characterized toxic compounds is discussed.
INTRODUCTION

Whatever effects a drug produces in a biologic system must be regarded as ultimate consequences of physicochemical interaction between that drug and functionally important molecules in the living system (1).

This bit of pharmacologic dogma whose origins can be traced to the work of Paul Ehrlich communicates the basic concept from which the theory of the molecular basis for drug action has evolved. If the word "drug" in the quotation is generalized to "chemical", a justification develops for the kind of work described here. Drugs are only a specialized class of chemicals whose biological effects are beneficial within carefully defined limits of dose, dose rate and clinical circumstances. The concept of drug-receptor interaction lies at the foundation of effects produced by all biologically active chemicals whether or not they produce beneficial results.

The quotation, simply stated, says that if there is an effect there must have been a physicochemical interaction. The converse of this statement would be: If there is a physicochemical interaction there must be an effect. It does not take an adventuresome soul to suggest that this statement would be received with considerable skepticism on the part of scientists. Yet it may well be true if one considers that an effect can be long delayed or manifest itself in a way that obscures its origin.

It has been established over the past several decades that a variety of organic compounds are metabolized in the liver to forms which interact covalently with cellular proteins, nucleic acids and/or
phospholipids (2). In fact, it is becoming clear that many of the chemicals that man is exposed to and which are suspected of toxicity share this characteristic. Such a process has been evoked as a step in the molecular mode of action of various hepatotoxins (3), carcinogens (4), mutagens (5) and compounds precipitating allergic reactions (6) and blood dyscrasias (7).

Preliminary experiments with the chlorinated insecticide chlordane showed that after microsomal metabolism a portion of the insecticide remained tenaciously bound to the acid precipitable microsomal residuum. The additional knowledge that chlordane is metabolized to an epoxide and is known to be a carcinogen in rodents indicated that a further investigation of chlordane binding might be a fruitful path of inquiry.

1,1,2,4,5,6,7,8-octachloro-3a-4,7,7a-tetrahydro-4,7-methano-indane.
Chlordane: Background and Administrative History

Background

Chlordane was introduced as an insecticide for agricultural use in 1945 (8). As incorporated into commercial formulations it is a yellow oil commonly referred to as technical chlordane. Recent work has shown that it is a mixture of at least 26 separate compounds of which 11 have been characterized (9). These 11 compounds account for approximately 40% of the material in the oil (10). Significant among these are chlordene, 0.3%; heptachlor, 5.0%; cis-chlordane, 8.3%; trans-chlordane, 9.2% and trans-nonachlor, 3.7% (listed as percent of the technical mixture) (9).

Commercial synthesis of technical chlordane is achieved by the Diels-Alder condensation of cyclopentadiene with hexachlorocyclopentadiene to yield chlordene which is subsequently chlorinated (10). This process yields the heterogeneous oily mixture described above. Pure chlordane is a white crystalline solid consisting of cis- and trans-isomers. Cis-chlordane has a melting point of 106.6-108° and pure trans-chlordane melts in the range 104.6-106° (10). These compounds are virtually insoluble in water but are soluble in a wide range of organic solvents (11). They are extremely lipophilic (11).

Technical chlordane has a relatively low toxicity to mammals on an acute basis. This characteristic plus its resistance to
environmental breakdown make it a valuable insecticide (11). It is a broad spectrum insecticide whose principal use in agriculture is in the control of soil insects attacking root crops and the root systems of field crops. Total use of chlordane for these and other purposes in United States agriculture for 1971 was 1.5 million pounds (11). The second most widespread use of chlordane is in the home owner's market where it finds use as a long lasting barrier to the entry of crawling insects and is widely used to control insects damaging turf, ornamental plantings and home vegetable gardens (11). Chlordane has also found wide use in the prevention of damage to structural timber and forest products by termites and wood boring insects. Its persistence has made it an ideal pest control agent from an economic standpoint because of the infrequent applications needed and the resulting low cost (11).

Administrative history
Federal regulatory officials and environmentalists have also noticed chlordane's environmental persistence. From their point of view persistence was equated to an increased opportunity for the exposure of nontarget species to its toxic effects. Potential for danger to the human population was first recognized officially in 1955 when tolerances of 0.3 ppm were established for chlordane residues on fruits and vegetables under the Federal Food, Drug and Cosmetic Act (12).

This decision was reviewed by the Food and Drug Administration in 1963 (12). But at that time there was no evidence that chlordane caused tumors or any other permanent pathological change in humans.
Neither had there been any indication of deleterious effects on workers engaged in the manufacture or application of chlordane. The 1955 tolerances were allowed to stand (12).

In October of 1974 a petition was filed with the Environmental Protection Agency (EPA) for cancellation of the registration of chlordane and heptachlor by attorneys for the Environmental Defense Fund (EDF) (14). In accordance with the Federal Insecticide, Fungicide and Rodenticide Act, EPA Administrator Russel E. Train issued a Notice of Intent to Cancel Registration for chlordane and heptachlor on November 18, 1974. The EDF petition and the Notice of Intent contended that chlordane and heptachlor were ubiquitous environmental contaminants subject to biomagnification. Additionally, they maintained that chlordane and heptachlor and their metabolites were present in the tissues of 90% of the United States population and that the insecticides and their metabolites had been shown to pose a cancer risk to humans (15, 16).

The Velsicol Chemical Co., the Department of Agriculture (USDA) and several others contested the allegations in the Notice of Intent to Cancel and requested a hearing (14). The request was granted and the hearings began after a lengthy pretrial process (16).

Just after the cancellation hearings had begun, Administrator Train issued a Notice of Intent to Suspend Registration for heptachlor and chlordane based on the principle of imminent hazard (16). Receipt of the results of a review of tissue slides from animal feeding studies, the results of human adipose tissue analyses for 1973 and a study of pesticide residues in the milk of US mothers convinced him that suspension was warranted. The notice was published on August 15,
1975. The cancellation hearing was recessed and a suspension hearing was convened in the same month.

The areas of contention were the same in these hearings as they had been in the cancellation hearings. The registrants challenged the scientific validity of the EPA's principles for evaluating carcinogenic hazard and the interpretation of the tissue slides from the feeding studies. Velsicol's pathologists maintained that the irregularities seen in rodent liver on the slides were not indicative of cancer while EPA's pathologists insisted that they were (14).

The Administrative Law Judge finally issued a decision on December 12, 1975 in which he dismissed the suspension notice. The decision turned on the fact that the differences in interpretation left him no basis on which to find chlordane and heptachlor carcinogenic. The judge felt that he was not competent to find one side or the other in error in their appraisal (17). Administrator Train immediately overturned this decision citing as precedent the principles for the evaluation of carcinogens as they had been applied to other insecticides and his authority under the Federal Insecticide, Fungicide and Rodenticide Act (18).

The cancellation hearings resumed in June of 1976. A year later Velsicol and USDA were finally successful in getting the question of the carcinogenicity of heptachlor and chlordane submitted to a committee of the National Academy of Sciences. The material to be evaluated was sent to the committee on June 15, 1977 (13). The committee was given specific questions to answer. Primary among these was the question of the carcinogenicity of the two pesticides in rats and mice. The reviewing pathologists were of the opinion that chlordane and
heptachlor were carcinogens in the mouse and may have been carcinogens in the rat (7). They informed the judge that they could not make a determination of the risk that these compounds posed for man nor could they say what constituted a safe limit of exposure for man (7).

The cancellation hearings dragged on until the spring of 1978 when a negotiated settlement was reached without an actual verdict.

The point of cancellation of the registration was, by now, a moot one. The registration had already been suspended (18).

Recent Work Linking Chlordane to Cancer and Other Health Problems

Epidemiological evidence linking chlordane to cancer in humans and to certain blood dyscrasias has come to light in the past few years. The evidence seems to correlate with the use of chlordane in the home rather than in agriculture.

Furie and Trubowitz (19) related the case of a 56 year old man who developed refractory megaloblastic anemia after treating his office for termites with a 74% solution of chlordane. The route of exposure seems to have been skin contact and inhalation.

Additional evidence of a link between chlordane and human health problems comes from epidemiological data generated for a second study (6). This relates 5 cases of neuroblastoma reported between 1974 and 1976 in which pre or postnatal exposure to chlordane is felt to have been the primary cause or a contributing cause. Six other cases, unrelated to those with neuroblastoma, were discussed. These dealt with older people who had developed aplastic anemia or leukemia.

Each of the neuroblastoma patients had a history of chlordane
exposure from home extermination processes. In 4 of the 5 cases this was for termite control and the indication was that the treatments were by professional applicators although this was not stated in the text. The children were between the ages of 2 1/2 and 6 years old.

The blood dyscrasia cases were older people and usually followed the use of the insecticide by the patient. The circumstances were similar to those in the Furie and Trubowitz report (19). The age range was from 15 to 68 years old.

In defense of chlordane it should be pointed out that the use of the insecticide by these individuals had been extensive and proper precautions do not seem to have been taken. The link to chlordane was purely circumstantial. In most of the cases there had been exposure to other chemicals as well.

Chemically Induced Pathological Conditions: An Overview

The role of the liver microsomal enzymes

The mammalian organs that lie at the interface with the environment are faced with the task of keeping the body free of harmful chemical substances. In addition to acting as physical barriers to contamination, these organs also possess the enzymatic capacity to neutralize intruding toxic molecules. Although the liver is not at the environmental interface in the strictest sense, it sits astride the incoming blood supply from the gut via the portal vein. Consequently, it has evolved an enzymatic xenobiotic metabolizing repertoire qualitatively similar to that found in the skin and lung but of greater capacity.
The liver system probably arose to neutralize the plant pigments, alkaloids and toxins that are absorbed with the nutritionally useful constituents of food (20). More recently, the use of fossil fuels for energy production and the use of drugs and organic chemical products in consumer goods and agriculture has added to the burden. The phrase "liver xenobiotic metabolism system" has now become synonymous with the group of enzymes that perform hydrolysis, oxidation, reduction and conjugation reactions on a staggering number of food additives, drugs and environmental contaminants as well as on the non-nutritive constituents of food.

The enzymes that provide the cells of the liver with the capacity to transform exogenous chemicals can be placed into two categories corresponding to the tissue fractions from which they are isolated. Esterases, amidases (21), sulfotransferases and acetyltransferases (22) are found in the cytoplasmic fraction. A second group of enzymes is found in the microsomes, which is the term used to describe the isolable remnants of the membranes of the endoplasmic reticulum. The microsomal enzymes are a membrane bound group that consists of several cytochromes with reductive (23, 24, 25) and oxidative (21) capabilities, hepatic epoxide hydratase (HEH) (26) and glucuronyl transferase (21) among others. One function of both of these groups of enzymes is to render foreign molecules suitable for excretion. The process usually leads to detoxification as well.

As a group, the hepatic enzymes are the body's major defense against blood borne toxic chemicals regardless of their origin. The work described here involves the membrane bound group; specifically, the mixed-function oxidases (MFO) and HEH.
In vivo the xenobiotic metabolizing enzymes usually act in concert or sequentially. A hypothetical example might be that of a naphthyl ester. Metabolism might proceed simultaneously by esterase and MFO action to yield, respectively, a carboxylic acid and a naphthol, and an epoxide. The naphthol could be conjugated by glucuronyl transferase for excretion. The epoxide could be cleaved by epoxide hydratase and excreted, or conjugated directly by glutathione-S-epoxide transferase (a sulfotransferase) for excretion as a mercapturic acid. Alternatively, the naphthyl ester could simply be hydroxylated by the mixed-function oxidases prior to glucuronide formation and excretion. Obviously a wide variety of transformations is possible.

The broad spectrum of reactions catalysed by the hepatic enzymes can be a mixed blessing. In the case of some organic molecules, enzymatic transformations may represent toxicant activation rather than detoxication. The phosphorothionates, exemplified by parathion, have no activity toward acetylcholinesterase until they are oxidized to phosphates by the mixed-function oxidases of the liver (27). This is a perfect illustration of the classic "lethal synthesis" found by Peters (28) to account for the devastating acute toxicity of fluoroacetate.

"Toxic synthesis" in the case of short lived compounds of relatively limited availability is a dangerous but controllable situation. It becomes another matter when it involves drugs, long lived environmental contaminants or chemicals used in the home or the workplace. A pattern has been developing in which previously unexplained pathological conditions have been found to result from a series of
events initiated by exposure to a chemical that was thought to be innocuous. The suspected active "agent" is generally a metabolic product of the MFO and represents the product of a toxic synthesis. Some conditions whose etiologies involve this pattern are discussed below.

Chemical carcinogenesis

This is a broad topic involving many toxic chemicals and many target organs. This section will be confined to a discussion of the mechanism of action of the polycyclic aromatic hydrocarbons.

The carcinogenic properties of these derivatives of coal tar have been known since the eighteenth century. Their ability to cause cancer in experimental animals was first reported in 1916 (28). Among the polycyclic aromatic hydrocarbons isolable from coal tar it is only those compounds that contain a phenanthrene nucleus (I, fig. 1) plus one additional benzene nucleus that are carcinogenic (1). The structures of several of these compounds are given in figure 1. The most widely studied of the polycyclic aromatic hydrocarbon carcinogens have been benzo(a)pyrene and dibenzanthracene (II, III, Fig. 1) probably due to the fact that they are constituents of tobacco smoke and tars (1).

The course of chemical carcinogenesis has been shown to involve two phases termed initiation and promotion (1). The first involves enzymatic conversion of the parent compound (procancerogen) to the activated intermediate(s) (proximate carcinogen) (30). This metabolite is the ultimate source of cell damage, a characteristic of initiation, and is a stable alkylating or arylating agent (29). The
Figure 1. Structural formulas for several of the polycyclic aromatic hydrocarbon carcinogens. I Phenanthrene. II Benzpyrene. III Dibenzanthracene. IV 3-Methylcholanthrene.

Lesion itself is felt to be the covalent interaction of this activated intermediate with a site or sites on one or more of the cellular macromolecules (1, 30-33).

Covalent interaction triggers the second or promotion stage. Cells surviving the disruption of their biochemical apparatus by exposure to the activated intermediate are somehow transformed to cancer cells. The specifics of the transformation process up to the point of the appearance of a clone of cancer cells are poorly understood. However, several theories that attempt to explain the process have arisen out of the study of the initiation process. The somatic mutation theory advances the point of view that transformation occurs as a result of a permanent change in the cell genome caused by
irreversible binding of the proximate carcinogen to the cellular DNA (29). That is, the cancer arises from a chemically induced mutation. This is a widely accepted point of view and it gains credibility from the fact that many carcinogens are known to be mutagens as well (5).

A second theory has been advanced that presents a rationalization based on the interaction of the proximate carcinogen with cellular protein without direct involvement of the genetic material (34). Promotion, in this case, is hypothesized to be the result of covalent interaction with proteins that control the expression of the genetic material, the repressor and derepressor proteins. This is based on the fact that transformed cells have been shown to be deficient in certain proteins or groups of proteins.

**Activation and binding of polycyclic aromatic hydrocarbons.** It has been known for some time that benzo(a)pyrene is metabolized in vivo (35, 36) and in vitro (31, 37) by MFO's to compounds that bind covalently to nucleic acids and proteins. Understanding of the activation process and the isolation of the activated intermediates are the result of more recent work (38). This metabolic activation plays such an important role in the initiation process that it will be informative to consider it.

Investigations of MFO metabolism of benzo(a)pyrene have shown that a complicated pattern of eight or more transformation products results (39). The presence of phenolic, quinone and epoxide metabolites has been demonstrated (39, 40) and testifies to the complexity of the process. The presence of dihydrodiols strongly suggests the participation of HEH. This has been verified by the use of inhibitors (39, 40, 41). Early work indicated the direct participation
of the 4,5-epoxide (the K region epoxide) in the initiation process. This theory has largely been supplanted by evidence in favor of one or more diolepoxides that have been isolated as nucleotide adducts (38, 42).

The process of metabolism, then, can be described as one involving a spectrum of reactions. MFO's perform both detoxification and activation reactions when confronted with benzo(a)pyrene. The detoxification reactions give rise directly to phenolic and quinone products. Indirect detoxification results when epoxides spontaneously rearrange to phenols (26). Work has shown that conjugation reactions participate, as well, giving rise to sulfates, premercapturic acids and glucuronides (43). These are primarily detoxifications although there are indications that glucuronides may be cleaved at sites remote from their formation and resume their role as carcinogens (43). The action of glutathione-S-epoxide transferase on epoxide metabolites neutralizes the epoxides to some extent (26). This is illustrated by the fact that inhibitors of this enzyme cause an increase in covalent binding (26, 39-41). The isolation of the diol-epoxide, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene-9,10-oxide, as an adduct of DNA (38) and the study of the production of this compound (42) further testifies to the fact that HEH is an activator. The process (figure 2) involves an initial epoxidation at the 7,8-position by the MFO's followed by hydrolysis to a 7,8-dihydrodiol. The resulting non-aromatic double bond at the 9,10-position is then epoxidized in another cycle through the MFO's leading to the major proximate carcinogen of benzo(a)pyrene (38, 42).

Mechanism of benzo(a)pyrene-DNA adduct formation. The potential
that this diol-epoxide has for damage to nucleic acids and a plausible mechanism to explain its action have been reported by Gamper, et al. (44). The anti-7,8-dihydroxy-7,8-dihydrobenzo(a)-pyrene-9,10-oxide, formed by the MFO's, reacted with RNA and DNA in vitro to give covalent adducts. The reaction with RNA was known to involve the 2-amino group of guanine and C-10 on the hydrocarbon. The product of the arylation of DNA was a hydrocarbon phosphate ester (fig. 2) again involving C-10. This then rearranged to yield a phosphotriester which hydrolysed and led to DNA strand scission (44) (fig. 2).

The devastating effect of this interaction was shown by using E. coli plasmid Col El, a superhelical circular DNA (form I in the terminology of Gamper, et al.). Cleavage of one phosphodiester linkage by diol-epoxide DNA interaction allowed unwinding of the strands and destroyed the circular configuration (form II). Cleavage of a second linkage gave rise to linear fragments (form III). This process was followed by electron microscopy and electrophoresis. The results indicated that the lowest ratio of diol-epoxide to mononucleotide that gave strand cleavage to form II was 0.01. At ratios between 0.5 and 1.0 no form I DNA remained after two hours of incubation (44).

The sequence by which this effect is translated into carcinogenesis is unclear. The somatic mutation theory is still in vogue (30). However, considering the fact that gross disruption of the genetic material of the cell usually results in cell death (45) one cannot avoid the conclusion that faulty nucleic acid repair or a change in the mechanism by which gene expression is controlled (46) may be involved in the transformation process. Furthermore, the fact that
Figure 2. The proposed mechanism for benzpyrene-DNA adduct formation. Redrawn from (45).
arene oxides and free radicals are also found as the result of the metabolism of benzo(a)pyrene (32) leaves some doubt about the identity of the proximate carcinogen.

**Hepatotoxicity arising from halogenated hydrocarbons**

A second pathological condition that requires comment in this context is the hepatotoxicity that results from mammalian exposure to some of the halogenated hydrocarbons. The toxic lesion in this case is liver necrosis although compounds in this class have been implicated in other disorders as well (47, 48).

The specific biochemical events leading to necrosis are still not well understood. As is the case with the carcinogens, the compounds that cause necrosis are known to be metabolically activated by the liver microsomal enzymes and to bind to cellular macromolecules (48). Unlike the polycyclic aromatic hydrocarbons, where binding to nucleic acids is felt to be significant, the necrogenic halogenated hydrocarbons seem to interact predominantly with cellular protein (49, 50, 51) and lipids (50, 52). For example, in experiments designed to detect binding to nucleic acids by halogenated benzenes no evidence of interaction with DNA or RNA could be demonstrated (49). The protein and lipid binding activity, however, has been demonstrated both in vivo (49) and in vitro (50). Centrilobular hepatic necrosis has been reported to occur following mammalian exposure to halothane (53), bromobenzene, ortho-dichlorobenzene, iodobenzene (49), trichloroethylene (51) and carbon tetrachloride (50, 52). Definitive work has been carried out using bromobenzene and carbon tetrachloride. The details of the metabolic activation and
mode of action of these two compounds serve to illustrate the behavior of the group.

**Bromobenzene induced hepatotoxicity.** Exposure of rats to single doses of bromobenzene has been shown to initiate a reproducible series of biochemical events. Centrilobular hepatocytes become depleted in glycogen at 12-16 hours. Necrosis follows beginning after 24 hours, becoming maximal at 48 hours and resolving approximately 96 hours after exposure (49). The centrilobular nature of the necrotic reaction suggests the participation of the microsomal xenobiotic metabolizing enzymes since this area of the liver lobule is known to be rich in that type of enzymatic activity (48).

Experiments by Reid and Krishna (49) have made it clear that MFO's are indeed involved. Treatment of rats with piperonyl butoxide of SKF 525-A before administration of bromobenzene blocked the necrotic reaction in vivo. Pretreatment with phenobarbital in the absence of MFO inhibitors had an enhancing effect on necrosis.

When covalent binding to proteins was the effect of interest rather than actual necrosis, similar correlations were seen (53). In vivo binding was blocked by SKF 525-A and enhanced by induction with phenobarbital. The magnitude of protein binding was dose dependent. Sephadex column chromatographic isolation of the protein bound radioactivity indicated that three proteins with molecular weights of 1500, 50,000 and 160,000 were involved. Protein hydrolysis and ligand exchange chromatography indicated that covalent binding occurred at cysteine, cysteic acid and at one unidentified amino acid.

The participation of glutathione in this process was also investigated by this group (49). Liver glutathione levels were depleted by
prior administration of diethyl maleate. Subsequent dosing with $^{14}$C-bromobenzene showed that this treatment accelerated the onset and increased the severity of the centrilobular necrosis. The concentration of covalently bound radioactivity in liver was found to have been more than doubled after this pretreatment.

An indication of the events that account for these effects is found in a study (54) in which it was shown that little covalent binding occurred during the initial phase of $^{14}$C-bromobenzene challenge while large amounts of mercapturic acid were formed. After 90% of the glutathione was depleted, however, considerable covalent binding occurred. The conclusion was that glutathione exerts a protective effect by decreasing the concentration of the hepatotoxic metabolite. This protective action on the part of glutathione has also been reported for the polycyclic aromatic hydrocarbons (vide supra).

Investigation of the metabolism of the halobenzenes (54) led to the discovery of an epoxide metabolite formed by the action of the MFO's. This epoxide was considered to be the activated metabolite that participates in the binding reaction. The finding by Krishna, et al. (53) that this binding involved only three proteins sheds some light on the possible mechanism by which the necrosis is brought about and reinforces the conclusion that a specific biochemical lesion is the cause.

**Hepatotoxicity involving carbon tetrachloride.** Hepatic necrosis as the result of mammalian exposure to carbon tetrachloride is probably the most widely studied of the hepatotoxic effects arising from the halogenated hydrocarbons (55). The topic has been extensively
reviewed (50, 56).

The toxic action of carbon tetrachloride differs considerably from that of the compounds considered so far. Although activation is by the MFO system and necrosis is the end result, this is where the similarity ends. Carbon tetrachloride activation proceeds by a series of reductive steps rather than by oxidation (24, 50).

The events following the activation of carbon tetrachloride are the focus of some disagreement. On the one hand are those who feel that hepatic cell death arises as the result of radical formation and subsequent lipid peroxidation and destruction of membrane integrity (52, 56). On the other hand are those who feel that necrosis is the result of the interaction of a metabolically activated species with tissue proteins (50). Both circumstances have been studied and both seem to correlate with the resultant necrosis. Until more is known about the specific causes of cell death the exact mechanism of the necrogenic activity of carbon tetrachloride will remain in a state similar to that of carcinogenesis.

There has been some disagreement in the past over what constitutes the site of activation of carbon tetrachloride within the microsomal mixed-function oxidase system. Slater (57) proposed that the activation was catalysed by the enzyme NADPH-cytochrome P-450 reductase upstream from cytochrome P-450 in the microsomal electron transport system. This speculation was probably based on the fact that the metabolism was reductive rather than oxidative and has stimulated considerable effort on the part of others.

Work by Sipes, et al. (3) using tissue binding as a means of measuring carbon tetrachloride metabolism, showed that covalent
binding dropped when an in vitro system was pretreated with alylisopropylacetamide. The decrease in binding was proportional to the loss of cytochrome P-450 activity as established by measurement of a reduced rate of ethylmorphine demethylation. Under the effect of alylisopropylacetamide, NADPH-cytochrome P-450 reductase activity remains intact. Work with cobaltous chloride and methyl mercury hydroxide, which have a similar effect on cytochrome P-450 (cyt. P-450), has given similar results (3).

In the same investigation, covalent binding to protein was shown to be inhibited by a carbon monoxide atmosphere and the omission of NADPH from the reaction mixture. Antiserum specific for NADPH-cyt. P-450 reductase reduced covalent binding in proportion to the inhibition of the reductase. Ethylmorphine reductase activity was reduced in the same proportion. It now seems to be generally accepted that the site of reductive, anaerobic, microsomal carbon tetrachloride activation is at cyt. P-450 (3, 24, 50).

The mechanism of carbon tetrachloride activation. The actual mechanism by which carbon tetrachloride is activated appears to be reductive dehalogenation by cyt. P-450. Homolytic cleavage of one of the carbon chlorine bonds under anaerobic conditions results in the production of a trichloromethyl free radical (52). This mechanism was borne out by the discovery that carbon tetrachloride metabolism caused enhanced microsomal lipid peroxidation with the formation of malondialdehyde in vitro (52). Additionally, Uehleke, et al. (50) have described the destruction of cyt. P-450 during carbon tetrachloride metabolism and covalent binding to proteins. Both of these phenomena are felt to be the result of reductive radical production.
Consideration of the products found to result from the anaerobic metabolism of carbon tetrachloride, naturally, sheds some light on the details of the mechanism. Several studies have yielded reports of the production of hexachloroethane (50, and references therein). The best explanation for this is that hexachloroethane represents the termination product of a free radical chain (50). A second metabolite produced under these conditions is chloroform (50). Its presence in in vitro systems is also felt to be the result of the free radical process. The supposition is that chloroform is generated by the interaction of the trichloromethyl radical with the polyenoic lipids of the microsomal membranes. An electron is abstracted and lipid peroxidation within the membranes is initiated (50, 53).

It is characteristic of carbon tetrachloride metabolism in vivo to produce a rapid loss of microsomal enzyme activity including cyt. P-450. Until recently, this had been interpreted to mean that P-450 is being destroyed directly by the action of free radicals on the cytochrome itself or indirectly by their disruption of the membrane matrix (52). The latter point of view is supported by the fact that the activity of other microsomal enzymes is also decreased during carbon tetrachloride metabolism (52). Wolf, et al. (24) have reported that carbon monoxide is a product of anaerobic carbon tetrachloride metabolism in NADPH reduced microsomes. This conclusion was reached after the demonstration of carbon monoxide in post incubation microsomal preparations. Based on these observations and kinetic evidence, this group has concluded that at least part of the loss in cyt. P-450 activity is the result of carbon monoxide binding and subsequent inhibition.
Aerobic metabolism of carbon tetrachloride in microsomal systems has been shown to give rise to phosgene (3). Phosgene is known to be a product of chloroform metabolism as well (ibid.). It could be, then, that it is a secondary metabolic product of carbon tetrachloride arising as a result of chloroform production via free radicals. The suggestion has been put forward (3) that phosgene arises due to the rearrangement of trichloromethanol oxidatively produced by cyt. P-450 during chloroform metabolism. Although phosgene is not a free radical initiator itself, it does participate as a chlorinating and carbonylating reagent via a free radical mechanism after initiation of the chain by other radicals (58). Given this information, it is obvious that phosgene could also be participating in the production of hepatotoxicity caused by carbon tetrachloride. Aside from trichloromethanol and phosgene production, the oxidative metabolism of carbon tetrachloride is known to yield CO₂, certainly a detoxification product (50, 52).

The identification of carbon monoxide as an end product plus the known production of free radicals has led Wolf, et al. (24) to propose a unique mechanism for the reductive metabolism of halogenated methanes by cyt. P-450. They have suggested that in the absence of oxygen the halogenated alkyl compounds react directly with the reduced iron in the heme group of the cytochrome. Here they are transformed by cyclic reduction with electrons from the nucleotide co-enzymes via a carbene mechanism which ultimately yields carbon monoxide. In the case of carbon tetrachloride, for example, transformation proceeds via the trichloromethyl radical to dichlorocarbene which hydrolyses to give two halo acid molecules and carbon monoxide.
This is feasible from an organic chemical point of view because chloroform in the presence of base forms carbon monoxide via a dihalocarbene intermediate (58).

As with carcinogenesis, understanding of the events occurring between cell damage and the appearance of the macroscopic lesion, necrosis, has not progressed far beyond the point of supposition. The exact chemical lesion has not yet been agreed upon. So the question as to how carbon tetrachloride actually initiates its characteristic centrilobular necrosis must await additional work before it can be completely resolved.

**Drug induced blood dyscrasias**

One final pathological condition that must be discussed in this context is the aplastic anemia that develops in a small number of those that are therapeutically exposed to the antibiotic chloramphenicol (59). Only one case in forty thousand is afflicted with this fatal condition but reports of similar dyscrasias resulting from exposure to chlordane suggest that this lesion might fall into the category of those caused by organic chemicals that exert their toxic effects after metabolic activation (6, 19).

Chloramphenicol has been used as an effective treatment in a wide variety of bacterial infections. However, due to the fatal side effect, its usefulness has now been limited to particularly virulent infections or to cases where no other antibiotic offers adequate protection against an otherwise fatal disease (59).

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1Chloramphenicol; (1R, 2R)-(+)1-p-nitrophenyl-2-dichloroacetamido-1, 3-propanediol.
The metabolic activation of chloramphenicol. Work by Pohl and Krishna (60) with $^{14}$C-chloramphenicol showed that metabolic activation in vivo and in vitro results in the covalent binding of radioactivity to tissue proteins. This binding was shown to be enhanced by pretreatment with phenobarbital. This evidence, coupled to the fact that in vitro incubation under N$_2$ or carbon monoxide atmospheres, exclusion of NADPH or treatment with SKF 525-A inhibited binding (61), indicates that this is another case in which a cyt. P-450 mediated activation gives rise to a compound that covalently reacts with tissue proteins.

Identification of the covalently interacting metabolite. Using doubly labelled chloramphenicol, Pohl and Krishna (60) were able to show that the entire molecule was participating in macromolecular binding rather than a fragmentary metabolite. Further, they were able to eliminate the aromatic nitro group as the active site by testing the binding capability of the trifluoroacetamide and unsubstituted acetamide$^1$ analogues as well as dichloroacetamide itself. Binding occurred only in the case of metabolically activated dichloroacetamide indicating that activation and binding were occurring at the dichloroacetamide end of the molecule.

Subsequent investigations (61), using acid and base hydrolysis of chloramphenicol labelled protein and isolation of the drug protein adduct, resulted in the identification of chloramphenicol oxamic acid as the hydrolysis product. This led to the proposal that

$^1$Trifluoroacetamide analogue; (1R, 2R)-1-p-nitrophenyl-2-trifluoroacetamido-1,3-propanediol. Unsubstituted acetamide analogue; 1-p-nitrophenyl-2-acetamido-1,3-propanediol.
chloramphenicol is activated by microsomal hydroxylation of the chlorinated amide carbon. This, in turn, rearranges with the loss of HCl to an oxamyl chloride which reacts with protein at an O, S or N atom or hydrolyses to chloramphenicol oxamic acid. The proposed pathway is given in figure 3.

The Biochemistry of the Microsomal Xenobiotic Metabolizing System

The microsomal mixed-function oxidases

In addition to performing the bioactivations discussed in the preceding section, this group of enzymes has been shown to be involved in the transformation of a variety of other exogenous and endogenous substrates appearing at or arising in lung, intestinal mucosa, kidney and skin as well as the liver (62). Endogenous compounds include steroids, fatty acids, bile acids and heme (63). Compounds of exogenous origin include drugs, carcinogens, antibiotics, insecticides (63) and volatile anesthetics (25) as has been explained above.

The ability of enzymes in hepatic microsomes to metabolize foreign compounds has been established for some time. The first report of this phenomenon occurred in 1949 with the discovery that an enzyme in liver microsomes catalysed the reductive cleavage of amino-azo dyes (64) and their oxidative demethylation (65). Subsequent work showed that a variety of reactions are catalysed by this system. As early as 1955, hydroxylation, deamination and ether cleavage had been recognized (66). The list has now been expanded to include aromatic hydroxylation, sulfoxidation, epoxide formation, desulfuration and dehalogenation (63) as well.
A period of intensive study following the initial discovery of the metabolic activity of hepatic microsomes led to the description of the biochemical conditions necessary for maximal activity of the enzymes. Brodie, et al. (66) showed that aminopyrine demethylation by liver microsomes was maximized by aerobic incubation in the presence of NADP, an NADPH generating system and magnesium ions.

Additional study led to the knowledge that the activity of the microsomal drug metabolizing system was increased by pretreatment with barbiturates (67, 68). This work by Remmer was the first report of the now well-known inducibility of these enzymes. Many compounds are currently known to give rise to this phenomenon including 3-methylcholanthrene, DDT, chlordane and anabolic steroids (63).

It was not until the mid-sixties that the source of the microsomal drug metabolizing activity was shown to reside in a material with the characteristics of a cytochrome (69) that had first been discovered in 1958 (70, 71). This cytochrome was capable of binding
carbon monoxide and gave a reduced versus reduced + carbon monoxide difference spectrum with a characteristic peak at 450 nm (72). As a result, the material has come to be known as cytochrome P-450. Enzymatically it falls into the category of external mixed-function oxidases in that it uses 1 mole of molecular oxygen to produce 1 mole of oxidized substrate and 1 mole of water (73).

The oxidative metabolism of drugs and foreign compounds is not the only enzymatic activity that resides in liver microsomes. A second hemoprotein designated cytochrome b₅ (cyt. b₅) is also present. This material was first reported in 1952 (74). In fact, knowledge of the presence of this cytochrome led to hesitancy in the initial identification of cyt. P-450 in rat and pig liver microsomes. The feeling was that cyt. b₅ might have been the source of the cytochrome-like behavior in the liver microsomes that were under investigation (70, 71).

Cyt. b₅ is now known to be involved in the elongation of saturated fatty acids that occurs in the microsomes. It participates in the desaturation of stearyl CoA and palmitoyl CoA to oleyl CoA and palmitoleyl CoA respectively (75). The terminal enzyme, in this case, is not the cytochrome but an additional non-heme iron protein sensitive to the action of cyanide aptly named the cyanide sensitive factor (76). The source of reducing equivalents for cyt. b₅ is NADH. However, desaturase activity can be maintained using NADPH (75).

Current thought on the sequence of microsomal electron transport and the mechanism of action of cytochrome P-450

Elucidation of the sequence of liver microsomal electron transport from the pyridine nucleotide coenzymes to the terminal electron
acceptors proved to be a thorny path for the researchers who chose to investigate it. Several factors peculiar to microsomal electron transport contributed to the difficulties. Primary among these was the fact that the function of this electron transport system seems to be one of distribution of reducing equivalents rather than the production of energy as is the case in mitochondrial electron transport. This results in a complicated circuit, in the electronic sense, with multiple inputs and outputs and for which the path of electron flow can be altered to fit the metabolic job at hand. Secondly, and more intimately related to the mechanism of action of cyt. P-450, has been the problem of the origin and order of insertion of the electrons reducing the cyt. P-450-substrate complex. The solutions to both of these problems have been partially worked out.

Definitive work on the nature of the sequence of microsomal electron transport to cyt. P-450 was frustrated largely because of the membrane-bound nature of the components. This made them resistant to solubilization in active form by classical means. As a consequence, work progressed slowly. Lu, et al. (63, 77) have succeeded in solubilizing and isolating cyt. P-450 and the membrane-bound components of the associated electron transport system using detergent in the presence of glycerol and separation on DEAE cellulose in the presence of detergent.

Reconstitution experiments with the solubilized components have shown that an active drug metabolizing system requires NADPH, a flavoprotein NADPH-cyt. P-450 reductase, cyt. P-450 and a lipid fraction that has been shown to be phosphatidyl choline. Lu has speculated that the lipid facilitates the transfer of electrons from the
reductase to cyt. P-450 (63).

The nature of electron transport to cyt. b₅ during fatty acid desaturation is similar to that seen with cyt. P-450. A flavoprotein is responsible for the transfer of electrons from NADH to cyt. b₅ and has been termed NADH-cyt. b₅ reductase (78). As mentioned above, electrons are transferred from cyt. b₅ to the terminal desaturase, the cyanide sensitive factor (76).

The situation, then, is one of two chemically parallel electron transport chains involving two cytochromes that operate with maximum efficiency using electrons from different sources. The complexity arises from the fact that there are points of crossover between the parallel pathways. This is reflected in the work of Mueller and Miller in which the xenobiotic metabolizing activity of liver microsomes was first described (64, 65). They were able to demonstrate the metabolism of 4-dimethylaminoazobenzene using both NADH and NADPH as sources of reducing equivalents. This is the case with many other substrates of cyt. P-450 as well; i.e., 3,4-benzpyrene (79), laurate (80), and 0-ethyl-0-(4-nitrophenyl) phenylphosphonothioate (81). In most cases, though, NADPH is the more efficient electron donor with NADH supporting a lesser rate of transformation (82). The converse has also been shown to occur. The 9-desaturation of stearyl CoA, known to be an NADH-cyt. b₅ mediated process, proceeds just as effectively with NADPH as the electron donor (78).

West, et al. addressed the crossover problem as it applies to the hydroxylation of 3,4-benzpyrene and the N-demethylation of benzphetamine, both of which are cyt. P-450 mediated reactions (82). They found that when these reactions were carried out in a reconstituted
system with NADPH as the electron source, cyt. b₅ was not an obligatory component of the electron transport chain. However, when the NADH supported hydroxylation of 3,4-benzpyrene was studied they found that the reaction either ceased or continued at a very reduced rate when NADH-cyt. b₅ reductase or cyt. b₅ was omitted from the reaction mixture. In addition, they were able to show that the hydroxylation of benzpyrene in this system was uneffected by concentrations of cyanide known to inhibit the 9-desaturation of stearyl-CoA confuting the participation of the cyanide sensitive factor in this reaction. These results have led this group to suggest the pathway shown in figure 4 to represent the partial sequence of microsomal electron transport.

![Figure 4](image_url)

Figure 4. Schematic representation of the partial sequence of microsomal electron transport. Redrawn from (82).

Evidence from other work (78) supports this scheme for microsomal electron transport and adds an additional level of complexity. The addition of organic peroxides to preparations of microsomes causes the rapid oxidation of both cyt. b₅ and cyt. P-450. The peroxides are reduced to an alcohol and water. The reaction is catalysed by cyt. P-450 but proceeds with either NADH or NADPH as a source of electrons. The use of antibodies to cyt. b₅ and NADH-cyt.
b₅ reductase have shown conclusively that both the cytochrome and the reductase are required for the NADH supported reaction. NADPH dependent hydroperoxidase activity operates independently of cyt. b₅ and its flavoprotein reductase. In fact, the addition of cyt. b₅ was inhibitory in a reconstituted P-450 system metabolizing cumene hydroperoxide at the expense of NADPH. Cyt. b₅ has also been shown to play a part in the hydroxylation of laurate and chlorobenzene.

It seems, then, that in some cases cyt. P-450 mediated oxidations proceed via electron flow through cyt. b₅. It is possible that several cytochromes P-450 exist and that some of these are obligated to accept electrons from NADH through cyt. b₅ as well as from NADPH. The latter suggestion draws credibility from the fact that a different MFO, cyt. P-448, is known to be induced by polycyclic aromatic hydrocarbons in rodents and is specific for their hydroxylation (79). Additionally, electrophoretic studies have shown the presence of multiple forms of cyt. P-450 (83).

The current view of liver microsomal electron transport, then, is of a system supplied by electrons from both NADH and NADPH. These electrons run along two interconnecting pathways to the point where they are either directed to the cyanide sensitive factor or to one of several terminal oxidases of the cyt. P-450 group. The ultimate fate of the electrons in the system is determined by the substrate at hand and, presumably, by the state of induction of the tissue in which these events are occurring.

The mechanism of cyt. P-450 catalysed reactions has been the subject of intensive investigation since Omura, et al. showed that it was the enzyme responsible for liver microsomal drug metabolism (84,
General agreement has been reached on the overall sequence of events involved in the cyclic reduction and oxidation of the cytochrome. Basically, the sequence can be visualized as shown in figure 5. The figure is a composite from the work of Coon (84) and Estabrook (85).

Step A depicts the reaction of ferric cyt. P-450 with substrate. This is a rapid reaction and, according to Coon (84) may or may not precede the initial electron transfer as shown in the figure at step B. No matter what the sequence, the result of the transfer of an electron from NADPH is the reduction of the cytochrome heme-iron to the ferrous state. Molecular oxygen joins in step C to give the ternary ferrous cyt. P-450, substrate, oxygen complex. It is at this point that carbon monoxide can react with the cyt. P-450-substrate complex to give the characteristic 450 nm absorption peak seen in the ultraviolet difference spectrum. Step D represents an intermolecular electron transfer. An electron is passed from the ferrous heme-iron to the bound molecular oxygen yielding a bound superoxide anion and ferric cyt. P-450.

It is generally agreed that from this point to the liberation of oxidized substrate and water the reaction becomes concerted. A second electron, originating either from NADPH or from NADH via cyt. b5, is transferred to the ternary complex. Estabrook (85) feels that this electron immediately rereduces the cytochrome iron as shown in the figure at E. (However, recent work by Coon's group (85, 86, 87) indicates the possibility of another mechanism not shown in the figure. This will be discussed below.) Reduction of the iron is
followed in concerted fashion by intramolecular electron transport to give fully activated oxygen which attacks the substrate and simultaneously draws two protons from the medium to yield hydroxylated substrate, water and oxidized cyt. P-450.

The controversy still surrounding this sequence involves two main points. As mentioned above, there is still confusion regarding the origin of the second electron; the electron entering the cyclic reduction at e. Additionally, there is still an argument involving the form taken by molecular oxygen during its transient relationship with iron as the activating electron transfer occurs. In this context this is actually a moot point. For the sake of completeness, however, it will be explained briefly.

Uncertainty over the electron transport sequence has its origins in two areas. Electron transfer from NADPH is known to involve two
electrons (73) and stoichiometric work with cyt. P-450 has shown that one mole of NADPH is indeed oxidized for each mole of water and oxidized substrate produced (84). The transfer of electrons to cyt. P-450, however, is a staggered sequential process passing through a flavoprotein reductase. Flavoproteins are known to be able to transfer only one electron at a time (73).

The search for an explanation of how two electrons generated at the same time can participate in sequential reductions has given rise to the work on the crossover of electrons between the NADPH and NADH pathways. There does not seem to be much doubt that in some cases the second electron comes from NADH via cyt. b5. This, however, still leaves the fate of both electrons from NADPH unexplained.

As indicated above, Coon's group have recently published work that sheds some light on this problem (85, 86, 87). Briefly, they have been able to isolate cyt. P-450 and the flavoprotein reductase in highly purified form from the liver of rabbits induced with phenobarbital. The purified reductase has been shown to contain one molecule of FAD and one molecule of FMN per protein chain. This is consistent with an earlier report of the same phenomenon (88). The presence of two flavins associated with one enzyme may partially explain the problem of sequential transfer. Under these conditions the reductase has the capability, in theory at least, of accepting two electrons. This leaves only the sequential transfer of the two electrons from the reductase to the cyt. P-450 to be rationalized.

In the same work (85, 86, 87) there is an indication that iron is not the only electron acceptor on the cyt. P-450 molecule. Experiments with purified rabbit liver cyt. P-450 indicates the presence of
a sulfhydryl electron acceptor. The indication is that within cyt. P-450 there is a two step intramolecular electron transfer. In the course of catalysis the second electron is passed from a redox active sulfhydryl to ferric iron and then to oxygen. This work is no cause for the theory of second electron transfer from cyt. b5 to be discontinued, however. The evidence for that mechanism as reviewed by Schenkman appears to be too strong (78). The experimental evidence probably should be interpreted to mean that it is entirely possible for the second electron to arise by either mechanism and that the ultimate determinate of the source of this electron may be the substrate at hand.

A final facet of this interesting problem involves the existence of more than one form of MFO. In their work with purified cyt. P-450, Coon's group titrated their pure liver microsomal cytochrome with dithionite and found that it accepted two electrons (86). The experiment carried out with purified cyt. P-450 from the bacterium P. putida showed the uptake of only one electron per cyt. P-450 molecule indicating a basic mechanistic difference between these two cytochromes (85). Work mentioned above (83) has shown that multiple forms of cyt. P-450 exist in liver microsomes. It is very possible that one or more of these forms depends on cyt. b5 as a source for its second electron.

The second area of controversy within the context of mechanism of action of cyt. P-450 involves the transient form of oxygen during second electron transfer and its attack on substrate. The confusion surrounds whether oxygen-substrate interaction occurs before the second electron is passed from ferrous iron to oxygen (while the
oxygen is still in the superoxide state) or after the transfer of the second electron when a ferric hydroperoxide exists (85). Actually, there is an additional possibility. Hamilton (89) has noted the similarity between reactions involving carbene transfer in the transition state (carbenoid reactions) and the products of cyt. P-450 catalysed reactions. The transfer of the active oxygen species has been termed the oxenoid mechanism by analogy. A case has been made for the formation of an intraenzyme peracid as the source of the oxene formed as the result of nucleophilic of iron bound peroxide on an enzyme carbonyl. These mechanistic areas of controversy will only be resolved by future study as has been the case in other areas of the chemistry of this highly complex group of enzymes.

Hepatic epoxide hydratase

A second source of hepatic microsomal xenobiotic transforming activity lies in the enzyme cleaving the oxirane rings of epoxides, hepatic epoxide hydratase (HEH). This enzyme is responsible for the hydrolytic production of trans-dihydrodiols from exogenous epoxides and from epoxides generated from unsaturated compounds by the cyt. P-450 system (26). Like the MFO's, HEH has been implicated in the activation of epoxides to forms which bind covalently to cellular macromolecules (26, 38) and in the detoxification of epoxides potentially harmful in their own right (90, 91). Oesch and his collaborators are primarily responsible for the isolation and biochemical characterization of these enzymes (90-96). The topic has been reviewed in detail by Oesch (26).

HEH activity has been detected in all species tested.
Furthermore, distribution is wide within the individual organism. Activity has been found in liver microsomes from rat, mouse, pig, guinea pig, rabbit, rhesus monkey (92) and humans (26). In rat and guinea pig, activity is high in liver, intermediate in kidney and intestinal mucosa and absent in brain, heart, spleen and muscle (92). Activity has been detected in skin from the mouse (97) and rat (98).

Investigation of the behavior of these enzymes indicated that their activity is increased by pretreatment with a variety of organic compounds. Pretreatment of male rats with phenobarbital, 3-methylcholanthrene and benzene gave mixed results. Phenobarbital induced HEH activity 230-270%. Activity was increased to 120-145% of controls with 3-methylcholanthrene while benzene caused no increase in enzyme activity (99). In an unrelated investigation, feeding 25 ppm dieldrin was found to increase HEH activity to a maximum of 250% of controls in rats (100). Recent work has identified trans-stilbene oxide as a potent and selective inducer of HEH giving levels of induction up to 300% of controls with no parallel increase in the activity of the MFO's (101). The increased activity, in this case at least, was due to an increase in enzyme concentration rather than an activation of pre-existing protein. The analogy to the microsomal MFO's is a tempting one. However, HEH has been shown to be under separate genetic control (100) and HEH activity has been isolated free of the MFO's (97).

Solubilization and purification of HEH from guinea pig liver made biochemical characterization possible (94). HEH activity was found to reside in a protein distinct from squalene oxidocyclase which is also found in liver microsomes (95). Activity was
independent of metal ions and NADPH and was unaffected by inhibitors of the MFO's. The enzyme was not inhibited by carbonyl reagents but a slight and significant inhibition arose following treatment with sulphydryl reagents. High substrate concentrations were inhibitory while product concentration had no effect (27). The pH optimum was sharp at pH 9.0 (94).

The chemistry of the enzymatic cleavage of oxiranes was investigated using microsomal preparations and naphthalene-1,2-oxide as a substrate (27). Attention to the origin of entering oxygen with \( \text{H}_2^{18}\text{O} \) showed that trans-dihydrodiol formation involved incorporation of \(^{18}\text{O} \) at the 2-position of the naphthalene, leaving the oxirane oxygen at carbon 1. The product diol invariably had the trans-configuration.

Further work with microsomes using naphthalene itself rather than the 1,2-oxide has shown that oxygen from air is incorporated into the 1-position of the trans-dihydrodiol resulting from the ring cleavage (27). This result would be expected following epoxide formation by the MFO's. As indicated above, the oxygen at the 2-position originated with water. The same was found to be true for the alkyl epoxide formed from styrene providing proof of the intermediate nature of the epoxide in the microsomal metabolism of these two compounds.

**Involvement of epoxide hydratase in metabolism of the chlorinated cyclodiene insecticides**

The way that HEH is involved in the metabolism of chlordane itself is not yet clear. The pathway for chlordane metabolism proposed by Brimfield, et al. (102) assumes the participation of HEH
but only on the basis of circumstantial evidence. The conclusion reached in their investigation, that oxychlordane is not a substrate for HEH per se, is shared by Street and Blau (103). They reasoned that oxychlordane was a terminal storage residue at least in rats. Some light is shed on this problem in the following discussion.

Work with the cleavage of the oxirane rings of chlorinated cyclodienes such as dieldrin, endrin and heptachlor epoxide antedates the investigations of Oesch, et al. None of the following studies is directly concerned with chlordane but the compounds above are closely related to chlordane and, in some cases, have been shown to arise during chlordane metabolism (102).

Working with the epoxides of chlorinated cyclodienes and their analogues, Brooks has shown that these compounds are transformed to trans-dihydrodiols in preparations of mammalian liver microsomes (90, 104). Initially two compounds were studied (90). The first, HCE (VII, figure 6) is analogous to the insecticide dieldrin but has no methylene bridge on the non-chlorinated cyclohexane ring. The second, chlordene epoxide (I, figure 6) is a six chlorine containing analogue of heptachlor epoxide and oxychlordane. Both HCE and chlordene epoxide resemble the methanoindanes in that they are asymmetric and they are presented to HEH as a racemic mixture.

The point of interest in this study was the action of pig liver microsomes on these epoxides in the absence of MFO activity. With no NADPH added to the microsomal incubation system an optically active trans-diol was produced representing roughly half of the racemic substrate added. An unmetabolized residue of optically active epoxide remained. Significantly, metabolism in the form of epoxide ring
Figure 6. Structures for the cyclodienes used in the study of the interaction of epoxides from this group with HEH. I, Chlordene exo-epoxide; II, Chlordene endo-epoxide; III, Chlordene 2-endo-3-exo-dihydrodiol; IV, Chlordene 2-exo-3-endo-dihydrodiol; V, Heptachlor exo-epoxide; VI, Heptachlor endo-epoxide; VII, HCE; VIII, HCE trans-diol; IX, Heptachlor 2-endo-3-exo-dihydrodiol; X, Heptachlor 2-exo-3-endo-dihydrodiol.
cleavage ceased (HCE) or proceeded at a very reduced rate (chlordene epoxide) when this point had been reached (90).

The optically active epoxides recovered from microsomal incubation had the same IR spectra after purification as the racemic mixture originally added. Resubmission of the recovered purified epoxides to microsomes in the absence of NADPH produced no significant metabolism in the case of HCE and a much reduced rate of metabolism in the case of chlordene epoxide. Addition of NADPH yielded oxidative metabolites of each of the recovered epoxides (90).

Since the stereoselectivity of the MFO's was not investigated, any answer to the question as to whether or not a racemic mixture of epoxide enantiomers is produced when non-epoxide starting materials are employed remains speculative. But work reported by Oesch with naphthalene suggests that the epoxides are synthesized with low stereoselectivity (25). The pattern, then, seems to be production of racemic epoxide enantiomers (or, at least, mixed enantiomers) followed by stereoselective ring hydration to yield optically active trans-diols and optically active residual epoxide. Further epoxide metabolism then proceeds by oxidative mechanisms.

This phenomenon was studied in greater detail in a subsequent investigation (104). The results confirmed Brooks' earlier work outlined above. The action of pig, rabbit and rat liver microsomes on racemic epoxides in the absence of NADPH produced optically active trans-diols and residual epoxides. There were two main points made in the second investigation that are of interest here. The effect on epoxide ring hydration of exo and endo epoxide groups in the context of the methanoindanes and the effect of increasing cyclopentane ring
chlorination on this process were established.

Four compounds were employed as substrates for microsomal preparations. Two stereoisomeric chlordene epoxides (I & II, figure 6) and two stereoisomeric heptachlor epoxides (V & VI, figure 6) were used. The result of the microsomal metabolism of chlordene exo-epoxide (I, figure 6) was the same as that seen in the previous study. An optically active trans-diol of chlordene (III, figure 6) was produced. The hydroxyl groups were 2 endo, 3 exo indicating attack at the least sterically hindered 2-position by OH$^-$. When the endo epoxide of chlordene (II, figure 6) was submitted to microsomal metabolism two trans-diols were found. The major metabolite was 2-exo-3-endo-hydroxy (IV, figure 6) indicating OH$^-$ attack from above at the 2-position. The minor trans-diol was 2-endo-3-exo (III, figure 6), the same configuration seen in the metabolite of exo-chlordene epoxide. In the cases of both the endo and exo-chlordene epoxides chemical hydrolysis produced the same compounds as did hepatic microsomes with the same quantitative relationships.

When the experiment was repeated using the stereoisomeric heptachlor epoxides the results were similar. The exo-epoxide yielded one trans-diol and the endo-epoxide gave two. The interesting point that arose during the course of this experiment was that the heptachlor epoxides were much more resistant to enzymatic hydrolysis than the simpler chlordene epoxides. Additionally, the major metabolite of the endo-epoxide was a 2-endo-3-exo-trans-diol (IX, figure 6) which is the opposite configuration to that found with the endo-chlordene epoxide. Obviously, the chlorine at position 1 of the endo-heptachlor epoxide directs OH$^-$ attack to the 3-carbon instead of to carbon 2 as
was the case with the chlordene epoxide. Again, chemical hydrolysis of the heptachlor epoxides produced the same results as those found using microsomes.

Brooks concluded from this work (90, 104) that epoxide hydration effectively competes with post epoxide oxidative metabolism for the favored enantiomer of these asymmetric pairs. He also concluded that the epoxide hydrolysing enzymes were more stereoselective than the oxidative enzymes. This stereoselectivity was found in microsomes from the pig, the rabbit and the rat although the rabbit and rat enzymes were much more sluggish than those from the pig.

Brooks further concluded that the facility with which the epoxide hydratase enzymes transform the methanoindane cyclodiene epoxides is effected by the bulk of the hexachloronorbornene nucleus and its proximity to the sites of enzymatic attack. This steric effect is modified by increasing chlorination of the cyclopentane portion of the molecule. Increasing chlorination directs epoxide hydratase attack toward those sites more sterically hindered by proximity to the hexachloronorbornene nucleus and slows the process of epoxide cleavage considerably.

When these concepts are applied to considerations of the 8 chlorine containing oxychlordane it is small wonder that no transdiol of this compound has appeared in investigations of chlordane metabolism. The work of Brooks supports the view that oxychlordane is a terminal residue (103).

Mammalian Biotransformation of Chlordane

One of the prerequisites for the study of xenobiotic
transformation is, of course, that the starting materials be as pure and homogeneous as possible. In the study of chlordane metabolism this has meant the use of pure chlordane rather than the technical mixture that results from commercial chlordane synthesis. Since chlordane is made up of two isomers, metabolic studies have routinely been carried out in parallel using pure cis-chlordane and pure trans-chlordane. Interestingly, the indications have been that the metabolism of the two isomers differs qualitatively (102, 105) and quantitatively (105, 106) both in vivo (105, 106) and in vitro (102).

One of the significant manifestations of these differences has been found in the production of the epoxide metabolite oxychlordane (V, figure 7). Street and Blau (103, 107) exposed pure cis and trans-chlordane isomers (I, II, figure 7) to metabolism by liver post-mitochondrial supernatent from rats pretreated with DDT. They were able to measure the recovery of unmetabolized parent compounds and two metabolites, the epoxide and dichlorochlordene (IV, figure 7) gas chromatographically. The results showed that 37.7% of the trans-isomer was converted to oxychlordane while only 14.1% of the cis-isomer underwent this conversion. Measurement of the recovery of the unmetabolized parent compounds showed that 39% of the trans-chlordane remained indicating that most of it had been transformed to the epoxide. Only 18% of the cis-isomer was left unaltered leaving a large portion of the metabolized material unaccounted for. Based on this they concluded that although both isomers are oxidatively metabolized to the epoxide, a larger portion of the resultant epoxide metabolite arises from trans-chlordane than from cis. They also reached the conclusion that metabolic paths other than epoxide
Figure 7. The proposed routes for cis- and trans-chlordane metabolism by rats in vivo. The solid lines indicate the active routes and the broken lines represent weak metabolic routes. The thickness of solid lines indicates relative metabolic activity. Adapted from (106).
formation account for roughly 70% of cis-chlordane metabolism and only 20% of the transformation of trans-chlordane. Additional work has confirmed these conclusions (105, 108).

The results of Street and Blau also led them to propose a metabolic route for the production of oxychlordane from either of the isomers through the common unsaturated intermediate dichlorochlordene (107). One experiment showed that rabbit liver performed the chlordane to dichlorodene conversion with less facility than rat liver in vitro but was equally capable of converting preformed dichlorochlordene to the epoxide. Based on this, they proposed that the desaturation was the rate limiting step in oxychlordane production.

Evidence from several studies (103, 105, 109) indicates that the epoxide metabolite oxychlordane is a terminal fat storage residue of chlordane rather than a metabolic intermediate. In vitro work (103) showed no significant degradation of oxychlordane when it was exposed to the action of rat liver homogenate. No oxychlordane was found in the feces of rats fed either a 25:75 mixture of trans- and cis-isomers or the pure isomers alone although other metabolites were present (109). In a related study, however, animals given a single oral dose of this epoxide excreted it intact in feces. Balba and Saha (105) isolated no oxychlordane from the urine of rabbits fed radio labelled pure isomers. But fat analysis in conjunction with this study confirmed the earlier work of Street and Blau (107) showing that more oxychlordane was produced from trans-chlordane than from the cis-isomer and that this dichotomy was reflected in the amount of epoxide found stored in fat. An explanation for this difference in relative amounts of epoxide product have had to await
the application of more sophisticated analytical techniques.

Direct evidence for pathways other than epoxide production

The extent to which oxidative metabolism contributes to chlordane biotransformation by paths other than epoxide production became further evident with the report of hydroxylated metabolites found in the urine of rabbits fed $^{14}$C-labelled trans-chlordane (110). Two compounds were found. One was identical to one of the two chlorohydrins derived synthetically from chlordene (XIII, figure 8). Based on the polarity it exhibited during thin layer chromatography, this compound was assigned the structure 1-hydroxy-2-chlorodihydroheptachlor (VII, figure 7). Brooks (107), reviewing an earlier description of this work, concluded on steric grounds that this compound would have an exo-hydroxyl group. The second metabolite was thought to be 1,2-dihydroxydihydrochlordene (XVII, figure 8) although confirmation of this was not pursued. It was assumed that this diol was the product of the hydrolytic removal of both chlorines.

Similar compounds have been identified in two additional studies involving metabolism of chlordane in different systems. Barnett and Dorough (109) identified a hydroxylated dihydroheptachlor with a 1-chloro-2-hydroxy configuration (VII, figure 8) as a component in extracts of the feces of rats fed a mixture of 75% cis-chlordane and 25% trans-chlordane. Configuration, in this case, was based on comparison with the behavior of a reference sample with the same structure. Acetylation of the experimental material showed that it consisted of equal concentrations of two compounds. The second corresponded to a minor component of the acetylated reference sample. This
Figure 8. The proposed routes of metabolism for cis- and trans-chlordane in microsomal preparations from induced rat liver. Structure I-II represents the complete cis- and trans-chlordane molecules. Metabolites are shown only as the five-membered portion of the molecule with the hexachloronorbornene nucleus understood. Metabolites proposed, but not actually found in this or other work, are designated with a question mark (?).
second compound could be identical to the 1-hydroxy-2-chloro metabolite found in the work of Poonawalla and Korte. Brimfield and Street (108) identified the 1-chloro-2-hydroxydihydroheptachlor (VII, figure 8) as a metabolite of trans-chlordane formed by rat liver post-mitochondrial supernatant. Structural determination, in that case, was based on thin layer chromatographic comparison with a synthetic standard characterized by nuclear magnetic resonance.

Additional light has been shed on the metabolic fate of chlordane exclusive of the dichlorochlordene to oxychlordane conversion by the work of Barnett and Dorough (109). Although they made no attempt to outline a pathway to account for their results, they were able to partially characterize four previously unreported metabolites of chlordane. These compounds were a hydroxylated chlordane (III, figure 8), a dihydroxydihydroheptachlor having a molecular weight of 404 (X, figure 8), the cis- and/or trans-diol of dihydrochlordene (XVII, figure 8) and a triply hydroxylated dihydrochlordene (XX, figure 7). Excreted conjugates of hydroxychlordane and the trihydroxy species were also observed, which constituted the first report of chlordane generated conjugates.

The results of the work of Poonawalla and Korte (110), Barnett and Dorough (109) and Brimfield and Street (108) lend further credibility to the conclusion reached by Street and Blau (107). Considerable oxidative metabolism of both chlordane isomers occurs exclusive of epoxide formation.

The overall pathway of chlordane metabolism: comparison of in vitro and in vivo investigations

The in vitro investigation. The possibility of finding a
mechanistic explanation for the production of different amounts of oxychlordane from cis- and trans-chlordane isomers led Brimfield, et al. to restudy the primary hepatic metabolism of these isomers in vitro (102). It was felt that working in vitro would help to eliminate the uncertainty that often arises in establishing the origin and mode of production of metabolites due to the effects of digestive tract physiology and microflora.

The results of this work led to the proposal of the pathway shown in figure 8. The pathway was an attempt to explain the metabolic dichotomy with respect to oxychlordane production found by Street and Blau in a manner consistent with this and previous work on cyclodiene metabolism.

Briefly, it was proposed that a molecule of chlordane can follow one of three courses during hepatic microsomal metabolism. It can be hydroxylated at the 3 position to give hydroxychlordane (III, figure 8), presumably prior to conjugate formation per (109). Alternatively, desaturation to dichlorochlordene (IV, figure 8) can occur followed by the formation of oxychlordane (V, figure 8). Or, finally, it can proceed through reductive dehalogenation to give metabolites with increasingly fewer chlorines on the cyclopentane ring (VI and XI, figure 8). These dehalogenated molecules can then participate in the pathways of hydroxylation or desaturation and epoxidation that parallel the formation of dichlorochlordene and oxychlordane from chlordane. The major route taken by a given chlordane isomer is probably determined by whether the chlorine at position 2 is exo (cis) or endo (trans). The trans-isomer is predominately desaturated or hydroxylated while the cis-isomer is dehalogenated as its major mode
of biotransformation (102).

An additional metabolic transformation indicated by the scheme in figure 8 is the hydrolytic cleavage of epoxide rings by HEH. As mentioned above, this reaction has been shown to be stereospecific always yielding trans-dihydrodiols and is independent of molecular oxygen and NADPH (104, 111). No evidence was found in this work to indicate an interaction of HEH with oxychlordane. However, the induction of rats with a 25:75 (w:w) of trans- and cis-chlordane caused an increase in microsomal HEH activity (112). Additionally, the cleavage of heptachlor epoxides, chlordene epoxide and the epoxides of various of the methanonaphthalene derivatives is well documented (104, 111). The finding of a dihydrodiol of heptachlor (X, figure 8) from cis-chlordane (102) as well as the report of a six-chlorine containing diol from the work of Barnett and Dorough (109) provided evidence that HEH may be involved in chlordane metabolism at some level other than that of oxychlordane where it seems to be inactive.

The in vivo investigation. The most current investigation of the metabolism of pure cis- and trans-chlordane isomers to be found in the literature is that of Tashiro and Matsumura (106). This was an in vivo study using rats in which a small amount of confirmatory in vitro work was carried out. The research included feeding pure chlordane isomers and collecting urine and feces for metabolite recovery, qualitative identification and quantitation, the determination of relative excretion rates using $^{14}$C-labelled cis- and trans-chlordane and, finally, the determination of the relative insecticidal activity of isolated metabolites using a mosquito larvae
bioassay.

The conclusions reached as a result of this investigation are in general agreement with the proposals of Street and Blau (103, 107) and the results of the in vitro metabolism work of Brimfield and Street (108). They found that while both cis- and trans-chlordane are metabolized to oxychlordane via the common intermediate dichlordene, more oxychlordane was generated in the metabolism of trans-chlordane. Tashiro and Matsumura also found that the major metabolic route for cis-chlordane involves forms of oxidative metabolism other than epoxide formation leading to more complete metabolism of cis-chlordane and a higher excretion rate for cis-chlordane derived material. Agreement between this investigation and the study of Brimfield, et al. (102) lies in the identification of heptachlor as a chlordane metabolite. The results of this in vivo work were incorporated into a proposed pathway for the biotransformation of the chlordanes in rats. This pathway is reproduced in figure 7.

In spite of the general agreement between the in vitro work of Brimfield, et al. and this in vivo investigation, correlation breaks down to some extent when the details of the two pathways are compared. Specifically the sequence of events proposed to account for the various metabolites identified is considerably different.

The identification of a hydroxylated chlordane as a metabolite of trans-chlordane in the in vitro paper (102) marks a major departure from the Tashiro and Matsumura pathway. This compound was neither among the direct metabolic products identified from extracts of urine and feces or the aglycones generated as a result of the action of \(\beta\)-glucuronidase on isolated glucuronide conjugates from the in vivo
work. Based on the results of Barnett and Dorough this might have been an expected source of the hydroxylated chlordane (109). Brimfield, et al. felt that the fact that this material arose as a product of the metabolism of trans-chlordane only made mechanistic sense from the point of view that the relatively facile desaturation of the trans-isomer indicates a tendency toward oxidative metabolism not shared by cis-chlordane. They were not able, unfortunately, to back up this point of view with the results of Barnett and Dorough because the latter did not study the metabolism of pure isomers but used a mixture. The absence of hydroxy-chlordane in Tashiro and Matsumura's investigation may be explainable on the basis of hepato-biliary circulation of this metabolite or its conjugation product leading to "remetabolism" and excretion as the dihydroxy or trihydroxy chlordenes. Both of these compounds were identified (106).

Significantly, the production of heptachlor or a heptachlor-like substance is reported in both the investigations (VIII, figs. 7 & 8) leading to the offering of overall pathways of metabolism. Tashiro and Matsumura found that heptachlor arises as the result of the metabolism of both cis-chlordane and trans-chlordane. Brimfield, et al. detected it only in extracts of the microsomal metabolism of trans-chlordane although they indicate the probability that it arises as the result of the metabolism of both isomers in the pathway. The point of contention with respect to heptachlor production, in the two metabolic schemes, is where it arises in overall chlordane metabolism.

Tashiro and Matsumura have placed heptachlor in the position of a terminal residue undergoing no additional metabolism (fig. 7). Brimfield, et al. on the other hand, speculate that heptachlor
belongs in the position of a metabolic intermediate (fig. 8). In the course of their study Tashiro and Matsumura subjected the heptachlor isolated from in vivo metabolism to metabolism by rat liver postmitochondrial supernatant. Their results show only the production of a small quantity of 1,2-dihydroxy-dihydrochlordene although this is not reflected in the final pathway. It is undoubtedly this dearth of metabolic products that led them to place heptachlor in a terminal position. The literature shows that heptachlor is, indeed, a metabolically labile compound both in vivo (113) and in vitro (114) yielding heptachlor epoxide and a hydroxy epoxide. Neither Brimfield, et al. or Tashiro and Matsumura found that these compounds arise during the metabolism of chlordane. But it is possible that a heptachlor epoxide does arise as a result of chlordane metabolism and is rapidly hydrolysed to the diol or converted to the hydroxy-epoxide and then bound to tissue macromolecules in the manner of the hydroxy-epoxides formed from the carcinogenic polycyclic aromatic hydrocarbons (38). The report by Brimfield, et al. (102) of a dihydrodiol of heptachlor from the metabolic transformation of cis-chlordane and the production of a 6 chlorine containing diol in the in vivo investigation by Barnett and Dorough (109) support the former point of view. The binding theory may have some merit, though, in the light of a report of the carcinogenic activity of heptachlor in mouse liver as the result of feeding heptachlor (115). This is clearly an area where further work is called for.

The report of Brimfield, et al. (102) of a cis-chlordane derivative with a fully saturated cyclopentane ring having only 1 chlorine (VI, figure 8) marks another point of disagreement between the result
of the in vivo and the in vitro investigations. There is no precedent for this type of metabolic product in any of the studies of chlordane metabolism under discussion here. This, in fact, may be the kind of easily overlooked detail on which a case can be built for studying metabolism both in vivo and in vitro before drawing conclusions about metabolic phenomena. The obvious conclusion is that this compound does not show up in urine or feces.

The assumption from the presence of this metabolite in the in vitro system was that reductive dehalogenation is involved in the hepatic metabolism of the chlordanes; primarily the cis-isomer (102). Although this is at odds with the results of the in vivo studies, it is not an entirely novel suggestion. For example, it has been known for some time that DDT is converted to its reduced dehalogenated derivative DDD in rat liver homogenate (116). Recently, Lay, et al. (117) described the reductive dechlorination of the aldrin metabolite dihydroaldrin dicarboxylic acid in liver microsomes. Although Tashiro and Matsumura did not find this metabolite per se, a glance at their pathway indicates that dehalogenation is one of the dominant modes of cis-chlordane metabolism (IV, V, VII, figure 7).

Confirmation of these results of Brimfield, et al. will have to await additional in vitro work. For the time being, however, the obvious conclusion is that the dihydroheptachlor (VI, figure 8) is rapidly and completely converted to other metabolites so that it does not appear in urine or feces.

Summary

What has been presented here, then, has been information concern-
ing the metabolism, molecular mode of action and symptomology of a widely disparate group of compounds. The characteristic that is shared by all of them is that they covalently interact with tissue macromolecules after metabolic activation to produce pathological conditions in mammals.

Chlordane has now been shown to produce cancer in rodents and, epidemiologically, to be associated with various anemias and cancers in humans that resemble the conditions produced by chloramphenicol and the polycyclic aromatic hydrocarbon carcinogens. It has, as well, been shown that chlordane is acted upon by the same enzyme systems that are responsible for the metabolic activation of many of the carcinogens, the necrogens and chloramphenicol. Additionally, the transformation of chlordane seems to proceed by similar mechanisms.

Evidence that chlordane participates in covalent binding to cellular macromolecules has, so far, been lacking. This leaves some doubt whether chlordane fits the activation-binding-effect model shown for the other compounds. The results of the dissertation work presented here help to dispel that doubt.
STATEMENT OF THE DISSERTATION PROBLEM

Research Objectives

This investigation was undertaken to determine whether or not chlordane interacts covalently with various cellular macromolecules during hepatic microsomal metabolism. Upon finding covalent interaction, the objective was to sufficiently characterize its biochemical detail to allow a comparison of chlordane with other chemicals whose toxic action seems to depend on macromolecular binding. This aspect of the investigation included a determination of the effect of various pretreatments on MFO and HEH induction and a brief look at the interaction of oxychlorodane with HEH. The hope was that work such as this would allow the formation of conclusions about how chlordane binding to macromolecules comes about and by analogy to other carcinogens, what the expected consequences of this binding would be. Work such as this will help direct future research into the significance of chlordane as a long lived environmental contaminant.

The intended comparisons were to the polycyclic aromatic hydrocarbons and the halogenated methanes. These groups are known to be metabolized in microsomes to forms which interact covalently with cellular macromolecules. The details of the interaction have been well characterized in the cases of these two groups and the reactions have been correlated with cancer and centrilobular hepatic necrosis respectively.

A third objective was the determination of whether an isomeric
preference was at work. Did cis-chlordane metabolism produce a
greater concentration of covalently bound material than trans-chlor-
dane or vice versa? This was an important question based on the
evidence for qualitative differences in the mechanism of metabolism
of cis- and trans-chlordane.

Late in the work an additional objective was included. A
National Cancer Institute study showed that chlordane was a liver
carcinogen in the mouse but not in the rat. Based on the equivocal
nature of the results from the rat experiments it was felt that the
mouse might be a more sensitive organism and yield more clear cut
results. At this point, then, the experimental species was changed
and this change has permitted the drawing of some conclusions about
the correlation between in vitro studies of binding behavior and the
production of cancer in whole animals. This situation may represent
a model for the study of species specific toxicity; i.e., the
tendency of different species to develop different pathological
lesions when confronted with the same toxic compound.
MATERIALS AND METHODS

Animals and Materials

Animals

Male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, Ca.) were used in the experiments involving the rat and male C57 black/6J Fl hybrid mice (same source) in the mouse work. Weights of the animals at the beginning of an experiment were 100-120 g for the rats and 20 g for the mice.

Feed additives and feed

The pure cis- and trans-chlordane (1-exo, 2-exo, 4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindane and 1-exo, 2 endo, 4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindane) added, individually or as a mixture to the experimental feed, were analytical reference standards of defined purity given to us by the Velsicol Chemical Co., Chicago, Ill. The cis-isomer was 100% by infrared spectrometry and the trans-isomer was 99.8% pure as checked at the source by the same method. Both were recrystallized from benzene and analyzed by electron capture gas chromatography before use. The cis-chlordane contained no electron capturing contaminants. The trans-chlordane appeared to be contaminated by a small quantity of heptachlor (less than 1%) as established by gas chromatographic retention time. Phenobarbital, used as an inducer, was USP grade and was obtained from a local pharmacy.

The feed used for both experimental and maintenance diets was
Lab Blox® (Allied Mills, Inc., Chicago, Ill.). During maintenance periods this was used as received in pelleted form. For experimental purposes, the pellets were ground into a coarse powder.

**Reagents and biochemicals**

**Enzyme substrates and inhibitors.** The $^{14}$C-labelled cis-rich chlordane used as a substrate for microsome preparations during the methods development work and in the initial experiments was a gift of the Velsicol Chemical Co. This was a mixture of 25% trans-chlordane and 75% cis-chlordane uniformly labelled on the norbornene nucleus. This material had a specific activity of 10.9 mC/mMole and was made available as a 7.52 mg sample representing 0.2 mC.

Thin-layer chromatography of this mixture showed a halogenated contaminant with an $R_f$ below that of cis and trans-chlordane. It was not determined whether this spot was radioactive. An attempt at additional purification by neutral alumina column chromatography with electron capture gas chromatographic monitoring of the column effluent was carried out. No substantial change in the gas chromatographic profile of the mixture was seen after passage through the neutral alumina column. No further purification was attempted due to the fact that additional quantities of this labelled chlordane were no longer available.

Pure samples of uniformly ring labelled $^{14}$C-cis-chlordane and $^{14}$C-trans-chlordane were also used in this work. Like the cis-rich mixture mentioned above, these were a gift of the Velsicol Chemical Co. The pure $^{14}$C-cis-chlordane had a specific activity of 10.9 mC/mMole and consisted of 1.88 mg dissolved in benzene with a total activity of
0.05 mC. The $^{14}$C-trans-chlordane had a specific activity of 6.26 mC/mMole and consisted of 3.27 mg dissolved in benzene with a total activity of 0.05 mC. These were available in very limited quantity and were again irreplaceable so no additional purification was attempted. Thin-layer chromatography showed no detectable halogenated contaminants in either sample.

Oxychlordane (1,2,4,5,6,7,8,8-octachloro-2,3-exo-epoxy-2,3,3a,4,7,7a-hexahydro-4,7-methanoindane) analytical reference grade, used in the methods development work and as a gas chromatographic standard was a gift of the Velsicol Chemical Co. This material was of 98% purity as tested at the source.

Tritiated styrene oxide, the material used as substrate in the assay of hepatic epoxide hydratase activity, was purchased from New England Nuclear Corp., Boston, Mass. The specific activity as received was 15.5 mC/mMole in a sample containing 6.71 mg. For use as a substrate in the enzyme assay, this material was diluted with unlabelled styrene oxide (ICN, K&K Laboratories, Inc., Plainview, N. Y.) that had been purified by vacuum distillation. The activity as used (labelled + unlabelled styrene oxide) was 0.036 mC/mMole. This provided 110,000 dpm in 1.4 micromole/sample.

Aminopyrine used as a microsomal substrate to evaluate microsomal demethylase activity was purchased from Matheson, Coleman and Bell Corp., Norwood, Ohio. This material was used as received with no further purification.

Trichloropropene oxide (TCPO) (1,2-epoxy-3,3,3-trichloropropane) (Aldrich Chemical Co., Milwaukee, Wis.) was vacuum distilled before use. This was used to inhibit the activity of hepatic epoxide
hydratase in those parts of the work where the metabolism of chlordane without epoxide cleavage was of interest.

Other biochemicals. Ribonucleic acid from calf liver, deoxyribonucleic acid from calf thymus (DNA), nicotinamide adenine dinucleotide phosphate (NADP). D-glucose-6-phosphate (G-6-P), ribonuclease A. glucose-6-phosphate dehydrogenase (G-6-PDH) and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Calf thymus DNA was deproteinized before use by the method of Kay, et al. (118).

Chemicals used in scintillation counting. PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-2-(4-methyl-5-phenyloxazoylbenzene)] scintillation grade, were purchased from Packard Instrument Co., Downers Grove, Ill. Naphthalene, reagent grade, was obtained from J. T. Baker Chemical Co., Phillipsberg, N. J. Tissue solubilizers An-Ti-Sol I and An-Ti-Sol II and the detergent Triton X-100 were purchased from ICN Radiochemicals Division, Irvine, Ca. Solvents used in the scintillation counting mixtures were obtained from normal sources and were reagent grade or better.

Materials for the colorimetric assay of nucleic acids. Diphenylamine, used in the determination of DNA concentration, was obtained from the Aldrich Chemical Co., San Leandro, Ca. This was recrystallized before use according to the method of Schneider (119). Glacial acetic acid used in this procedure was purified to remove carbonyl impurities by refluxing over excess KMnO₄ for 1.5 hours and then distilled. The concentrated H₂SO₄ used was Fisher A-300 suitable for cholesterol analysis (Sargent-Welch Scientific Co., Denver, Colo.). This was the only grade of H₂SO₄ that was found to be suitable for this method. Others caused the formation of a precipitate when mixed
with the other components of the color reagent. Acetaldehyde was obtained from the Aldrich Chemical Co., Milwaukee, Wis. and was redistilled before use.

Orcinol (3,5-dihydroxytoluene) used in the determination of RNA ribose was purchased as technical grade from Eastman Organic Chemicals, Rochester, N. Y. This material was recrystallized three times (119) before a usable white crystalline product was obtained. Concentrated HCl and ferric chloride used in this determination were reagent grade or better and were obtained through standard chemical supply houses.

Material used in the deproteinization of calf thymus DNA. Sodium lauryl sulfate used was a product of Sargent-Welch Co., Skokie, Ill. and was used as received. Absolute ethanol used in this procedure and throughout this work was Rossville Gold Shield Brand®, IMC Chemical Group, Inc., Agnew, Ca.

Material used in the determination of inorganic phosphate. Inorganic phosphate, used as an alternative method for determining nucleic acid concentration, was quantitated by the method of Fiske and Subbarow (120). The chromogenic reagent, 1-amino-4-naphthol sulfonic acid, was purchased from Eastman Organic Chemicals, Rochester, N. Y. Before use the reagent was recrystallized (120). Sulfuric acid, sodium sulfite and sodium bisulfite were reagent grade. Reagent grade monobasic potassium phosphate used as a phosphate standard was heated overnight at 110° before weighing and prepared as described by Fiske (120).
Animal care and housing

Upon arrival, the rats were held six to a cage in stainless steel cages with wire mesh bottoms. Feeding during this period was ad libidum with pelleted commercial laboratory diet and water. The mice were held ten per cage in standard polycarbonate rat cages with synthetic bedding throughout the acclimation and experimental periods. Feed was the same as that for the rats and was provided ad libidum. Temperature in the animal facility was kept at a constant 72° with a 12 hour photoperiod.

At the start of the "induction period" ground commercial laboratory diet adulterated with inducer (100 ppm) was substituted for the pelleted food. The treated feed and water were given ad libidum. Control animals received ground laboratory diet that contained no chlordane. The duration of the induction period was 10 days. During this time the feces trays (rat cages) or bedding (mouse cages) were cleaned and fresh water was provided daily. Food was replenished as needed.

Feed preparation

Pelleted commercial laboratory diet was ground in a burr mill to the consistency of coarse sand. Unlabelled pure cis-chlordane, trans-chlordane, phenobarbital or a mixture of 75% cis-chlordane and 25% trans-chlordane was weighed out in a quantity sufficient to yield 100 ppm by weight. The chlordane was dissolved in 50-100 ml of diethyl ether and added to the ground feed. This mixture was agitated in a commercial mixer until the odor of ether was no longer detectable;
usually one half hour. Feed for control animals (uninduced) was made up by the same procedure omitting only the inducer compound. The mixer blade and bowl were washed in detergent and rinsed with acetone and hexane between batches to prevent the carry-over of chlordane into control feed.

The inducing feed was formulated so that the inducer compound corresponded exactly to the intended $^{14}$C-labelled microsomal substrate with the exception of the feed containing phenobarbital. Feed for three animals for 10 days consisted of 1.0 to 1.5 kg in the case of the rats and 0.5 to 0.75 kg for 10 mice. Feed was stored at room temperature in hexane washed polyethylene containers with push on tops.

**Sacrifice of the animals and excision of the livers**

**Rats.** At the end of the 10 day induction period, feed was removed from the induced and control animals for 24 hours. The rats were killed with ether and the livers quickly removed and blotted on wet paper toweling to remove excess blood. The hilus was dissected out and the bulk of the liver was weighed, minced with scissors into an ice cold homogenizer jar and homogenized in cold 1.15% KCl at 4°C in a ratio of 1 g of liver to 4 ml of KCl solution. Homogenization was in a Lourdes homogenizer operated at 70% maximum speed for 1.5 minutes in an ice bath. Prior to further processing, like homogenates were pooled and packed in ice. Three induced rats and three control rats provided more than enough liver homogenate for an experimental run.

**Mice.** The mice, like the rats, were killed with ether. The livers were excised and the gall bladder, removed with the liver in
this case, was carefully dissected away. The lobes of the livers were cut away from the hilus, weighed, pooled according to pretreatment and homogenized. Ice temperatures were maintained throughout this procedure. Ten mouse livers yielded between 36 and 50 ml of homogenate depending on the pretreatment.

Preparation of microsomes

Rats. Rat microsomes were prepared from the respective pooled liver homogenates basically by the method of Claude (121). Crude homogenate was spun in a refrigerated centrifuge at 10,000 x g for 15-20 minutes to sediment large cell fragments including those the size of mitochondria. Lipid rising to the top of the tube during centrifugation was removed with suction and the supernatant containing microsomes and soluble enzymes (hereafter referred to as post-mitochondrial supernatant) was decanted, pooled and its volume recorded. The measured volumes of the pooled homogenates were used as guides in the reconstitution of the microsomes later in the procedure.

Post-mitochondrial supernatant was centrifuged for 1 hour at 85,000 x g in a Beckman Model L preparative ultracentrifuge equipped with a 40 head to sediment the microsomal fraction. The supernatant was discarded and the pellet was resuspended in a convenient volume of 1.15% KCl using a Potter-Elvehjem homogenizer equipped with a Teflon pestle. This suspension was resubmitted to ultracentrifugation under the same conditions as those used for the post-mitochondrial supernatant. The resultant washed microsomal pellets were suspended in 1.15% KCl and pooled according to origin (control or induced).
The final volumes were adjusted to equal those of the original post-mitochondrial supernatants yielding two microsomal suspensions at concentrations equivalent, in one case, to 250 mg of induced liver per milliliter and, in the other case, to 250 mg of control liver per milliliter. One milliliter of the appropriate suspension was used as the source of microsomal MFO activity in the final incubation mixtures. Additional aliquots were removed as needed for the determination of microsomal protein concentration, aminopyrine demethylase activity, HEH activity and the concentration of cyt. P-450.

**Mice.** Preparation of the mouse liver microsomes directly paralleled the procedure described for the rat through the initial ultracentrifugation. The final ultracentrifugation of the mouse liver microsomes was carried out in 0.5 M Tris, pH 7.4, that was 0.25 M with respect to sucrose rather than in the 1.15% KCl that had been used in the rat procedure. The purpose for this was to enable the microsomes to be frozen for use at a later time. Freezing in Tris-sucrose is a better method for preserving enzyme activity in stored microsomes than freezing in KCl (122).

Subsequent to the final ultracentrifugation, the microsomal pellet was resuspended in the Tris-sucrose buffer at high protein concentration (> 30 mg/ml) and frozen until needed (approximately 12 days). The frozen microsomes were thawed at refrigerator temperature when needed and resuspended with the Potter-Elvehjem homogenizer after appropriate dilution (122). Final protein concentration in all cases was 3.0 mg/ml.
In vitro oxidative metabolism of $^{14}$C-chlordane

An acetone solution (50 microl) of $^{14}$C-labelled pesticide substrate was added to 25 ml incubation flasks. In the work with the $^{14}$C-cis rich chlordane mixture this corresponded to the addition of 1.54 micromoles of chlordane (labelled plus unlabelled carrier) having a specific activity of 0.97 mCi per millimole and yielding an activity of $3.35 \times 10^6$ dpm per flask. In the experiments where the pure chlordane isomers were used the acetone substrate solution added to each flask also contained 1.54 micromoles of pesticide. The final specific activity in the case of trans-chlordane was 0.2 mCi per millimole and for the cis-isomer was 0.8 mCi per millimole. The activity per flask in the case of the pure trans-chlordane experiment was $6.8 \times 10^5$ dpm while that in the pure cis-chlordane experiment was $2.7 \times 10^6$ dpm. The discrepancy in the specific activities reflects the limited amount of labelled pure isomers available.

Following the addition of the substrate solution, the acetone was driven off with $N_2$ and 0.2 ml of ethoxyethanol and a large glass bead were added to each flask. The incubation volumes were 4.7 ml per flask. This consisted of 0.5 ml of 0.07 M glucose-6-phosphate in a 0.24 M solution of magnesium sulfate, 0.5 ml of 0.1 M potassium phosphate buffer at pH 7.4 containing 2 units of glucose-6-phosphate dehydrogenase and 1.5 ml of phosphate buffer containing NADP, 3.67 mM. DNA, dissolved in cold phosphate buffer overnight, was added to each flask at the rate of 1.0 ml containing 3.0 mg of DNA. In those flasks where inhibition of HEH was desired, the contents were made $5 \times 10^{-4}$ M with respect to TCPO by adding 0.2 ml of a TCPO-ethoxyethan-
ol solution, 2.3 mg TCPO per ml, in place of pure ethoxyethanol.

The flasks, so charged, were placed in a metabolic shaking incubator at 37° with O₂ gassing for 5 minutes. At the end of this period the incubation was started by adding 1.0 ml of the appropriate microsomal preparation to each flask. The incubation time in all cases was 30 minutes.

The procedure was altered somewhat in the control groups where the reduced NADP generating system was omitted from the flasks to inactivate cyt. P-450 and prevent oxidative metabolism. In this case no NADP, G-6-P or G-6-PDH were added. Instead, volumes of buffer and magnesium sulfate solution corresponding to the volumes carrying these components were added in their place. All other additions remained the same. TCPO, of course, was not added to the control flasks.

Treatment of the post-incubation microsomal supernatant and the isolation of DNA

Separation of the soluble DNA form the RNA and protein contained in the microsomes was carried out according to the method of Speier and Wattenberg (123) with slight modifications. Incubations were stopped by cooling the flasks in an ice bath. While maintaining the ice temperature, the contents of each flask were added to an ultracentrifuge tube and diluted to volume with 0.1 M phosphate buffer, pH 7.4. This was followed by ultracentrifugation at 85,000 x g for 1 hour. The use of 1.15% KCl as a diluant at this stage was strictly avoided because it caused considerable precipitation of the DNA.

Following centrifugation, the supernatant containing the DNA was decanted from the microsomal pellet into 50 ml glass stoppered centrifuge tubes. The contents of each tube were made 0.57 M with
respect to NaCl and 2 volumes (roughly 25 ml) of cold absolute ethanol were added. The tubes were capped and inverted several times causing the DNA to form a stringy, gelatinous precipitate. The precipitate was removed by winding it around the tip of a Pasteur pipette and transferred to another 50 ml tube containing an additional 10 ml of cold absolute ethanol. The DNA was allowed to soak overnight at refrigerator temperature.

On the following day the DNA precipitates were spun down and the ethanol pooled with the microsomal supernatant and ethanol mixture from which it was originally precipitated. These aqueous ethanol fractions (a separate fraction for each incubate) were refrigerated for later extraction. The precipitated DNA was dissolved in 5 mM citrate buffer at pH 7.4 (123).

The recovered DNA was deproteinized as described by Sevag (124). The tubes containing the DNA were shaken vigorously in the presence of chloroform:isoamyl alcohol (24:1, v:v) for 30 minutes and centrifuged to break up any resulting emulsion. The aqueous phase was pipetted away without disturbing the chloroform-protein emulsion at the interface. This procedure was used twice or until no emulsion formed at the interface. The DNA containing aqueous phase was recovered and the chloroform was discarded.

Each aqueous, deproteinized DNA sample was extracted with diethyl ether until scintillation counting of the extracts showed a constant level of radioactivity. The DNA was then precipitated as outlined above, washed with ethanol and acetone and dried.

The dried DNA samples were dissolved in 1.0 ml of distilled water and hydrolysed by adding an equal volume of 1.0 N perchloric
acid and heating in a 70° water bath for 15 minutes (125). Tests of DNA concentration were performed on appropriate aliquots of this solution. One milliliter aliquots of these solutions were added to scintillation vials and counted.

**Treatment of recovered microsomes**

Microsomal pellets recovered from the ultracentrifugation of the incubation flask contents were loosened from the bottom of the tubes and rinsed into 15 ml conical tubes with 2 ml of standard saline citrate (126). Bovine pancreatic ribonuclease (1% in 0.15 M NaCl, pH 5) was added to each tube to give a final concentration of 50 micrograms per ml. Each tube was covered with a marble to prevent evaporation and incubated overnight at 37°.

At the end of the incubation period an equal volume of 0.5 N perchloric acid was added to each tube. The contents were mixed, allowed to sit for 5 minutes and spun down. The enzymatically hydrolysed RNA remained in the supernatant and the perchloric acid-insoluble protein formed a pellet on the bottom of the tube. The supernatant was immediately decanted into 50 ml glass-stoppered centrifuge tubes. The protein was washed with 2 ml of 0.5 N perchloric acid and recentrifuged. The wash was added to the perchloric acid fraction containing the hydrolysed RNA.

The RNA fractions were extracted with aliquots of diethyl ether to remove non-covalently bound 14C-labelled chlordane and its metabolites. Extraction was repeated until a constant level of radioactivity appeared. Routinely, 20 to 30 extraction cycles had to be performed before constant radioactivity was attained.
Residual ether was removed from the RNA fractions with a stream of \( \text{N}_2 \) and they were split for the determination of RNA content and bound radioactivity. RNA ribose was measured by the orcinol method (125).

The perchloric acid-insoluble pellet was made up of denatured microsomal protein. Residual chlordane was removed by exhaustive extraction. The protein, with unbound radioactivity removed, was solubilized by treatment with 1.0 N \( \text{NaOH} \) (127). Fifty microliters of this was then assayed for protein concentration in the presence of \( \text{NaOH} \) as described by Lowry, et al. (127). The standard used was \( \text{NaOH} \) solubilized bovine serum albumin. The remainder was assayed for bound radioactivity by scintillation counting.

**Scintillation counting of RNA, DNA and protein recovered from microsomal incubations**

Scintillation counting was performed on a Packard Tricarb scintillation spectrometer. Quench correction was by the channels ratio method after calibration with chloroform quenched samples containing 0.1 ml \( ^{14} \text{C}-\text{toluene} \) (4.31 x 10\(^5 \) dpm) in a toluene based cocktail. To increase the accuracy of these quenched standards, 1.0 ml of 0.5 N perchloric acid was added and neutralized with 1.0 N \( \text{NaOH} \) prior to the addition of the scintillation cocktail and the \( ^{14} \text{C}-\text{toluene} \).

DNA and RNA samples containing perchloric acid were prepared for counting by adding the appropriate aliquot to the scintillation vial, neutralizing the acid with 1.0 N \( \text{NaOH} \) (to pH paper) in the vial and then adding 14 ml of a toluene based cocktail. The cocktail consisted of 5.0 g PPO, 0.5 g dimethyl POPOP, 333 ml of Triton X-100 and toluene to make 1 liter (128). Protein solubilized with 1.0 N \( \text{NaOH} \)
was prepared for counting by the same method but neutralization was with glacial acetic acid rather than base. The samples were counted three times for 30 minutes.

**Enzyme assay procedures**

Evaluation of the metabolic activity of the microsomal preparations used in the binding studies was required to make the results meaningful. Two specific assays of enzymatic activity and cyt. P-450 analysis were performed on control and induced microsomes.

Cytochrome P-450 was determined as outlined by Omura and Sato (72). This method allows the cyt. P-450 to be determined in the presence of other cytochromes by taking advantage of the characteristic absorbance at 450 nm when it is binding carbon monoxide while in the reduced state. The results are reported as the change in optical density at 450 nm upon binding carbon monoxide per milligram of microsomal protein. Microsomal protein was measured by the method of Lowry, et al. (127).

The activity of the microsomal MFO's was evaluated as the ability to demethylate aminopyrine as described by Mazel (129). The procedure, designed for use with post-mitochondrial supernatant, was modified for use with microsomes by the inclusion of glucose-6-phosphate dehydrogenase (2 units per incubate) in the incubation mixture. Activity, in this case, is reported as moles of formaldehyde produced per milligram of microsomal protein.

Microsomal HEH activity was determined by the method of Oesch, et al. (93). This method utilizes the fact that $^3$H-styrene oxide is converted to the more water soluble $^3$H-styrene dihydrodiol by HEH. The
dihydrodiol is extracted almost exclusively into ethyl acetate after removal of unreacted styrene oxide with petroleum ether. Results are expressed as moles of styrene dihydrodiol formed per milligram of microsomal protein.

Statistical analysis

Results were analysed by analysis of variance using a factorial design as described by Winer (130). Significance was tested using an F-test after partitioning the treatment sum of squares according to planned comparisons. Significance was assumed when \( P \) equalled 0.05 or less.

In the case of the rat data several samples were lost or otherwise altered so that their usefulness was destroyed. This resulted in treatment groups of unequal sizes and made data analysis by the classical analysis of variance technique impossible. In this case, the data were analysed by the method of unweighted means (130) in which unequal group sizes are compensated for by the use of the harmonic mean instead of the actual group mean. Indications to this effect are given in the tables where this applies.

In the mouse data, where very few samples were lost, a missing value was compensated for by the use of the group mean instead of the missing value. In these cases a degree of freedom was sacrificed in the calculation of the error mean square to compensate.
RESULTS

The planning and execution of this work were guided by one factor more than any other. A comparison of the binding behavior of the chlordane isomers had to be made to tie this work to previous investigations in which the metabolism of cis- and trans-chlordane had been compared and found to be different. A comparative study, of course, necessitated the use of radioactively labelled pure chlordane isomers. And while these were on hand, the quantities were sufficient for only one major experiment.

Fortunately, a considerable quantity of a radioactively labelled cis-chlordane rich mixture of the pure isomers was also on hand. This was a gift of the Velsicol Chemical Corp. and consisted of 25% trans-chlordane and 75% cis-chlordane. The decision was made to proceed with the investigation using this mixture in preliminary trials and methods development work and the small quantity of the labelled pure isomers for a final experiment. For this reason the experiments in which rats were used were carried out with the labelled cis-chlordane rich mixture as substrate. The labelled pure isomers were used only in the experiment in which the mouse was the experimental species.

Enzyme, Substrate and Induction Effects on Covalent Binding of $^{14}$C-Chlordane to Protein, RNA and DNA in the Rat and Mouse in vitro

The rat

The experiments under discussion here were of factorial design.
This allowed the simultaneous evaluation of the effects of a variety of biochemical manipulations. The results of the experiment in which rat liver microsomes were used are presented in table 1 in such a way that comparison of the group means for a given treatment is possible. The effects of chlordane induction and the inhibition of MFO and HEH on the binding of chlordane to protein, RNA and DNA were investigated.

**Factorial analysis of the rat liver binding results.** The analysis of variance for the rat experiment is shown in table 6 (Appendix). As is evident from the table, the only statistically significant effect in this case was induction and this occurred only in the protein group. The slight significance appearing in the interaction mean square for protein arose as the result of the strong effect of pretreatment carried over into the interaction mean square.

**Enzyme effects.** This category included the effect of MFO's and the effect of HEH inhibition on binding. Deficiencies in the design of the experiment made it necessary to combine all of the enzymatic manipulations into a single category called enzyme effects.¹

No significance was found to be attached to the enzyme effects category in the analysis of variance for any of the macromolecules. However, removal of the enzymatic effects from the influence of pretreatment by the use of the t-test on means within the two pretreatment groups enabled some significance to surface.² In the induced group, MFO inhibition exerted a slight but significant protective effect (95% level) against chlordane binding to protein.³ In the

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¹ See footnote c, Appendix table 6.

² See Appendix table 7A, comparisons 2 and 3.

³ See Appendix table 7A, comparison 2, protein column.
Table 1. Summary of the effects of pretreatment and hepatic microsomal enzyme activity on the covalent interaction of $^{14}$C-chlordane with microsomal protein and RNA, and calf thymus DNA during metabolism by rat liver in vitro.$^{a}$

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MFO</th>
<th>HEH</th>
<th>Pretreatment$^{c}$</th>
<th>(nmoles chlordane bound/mg. protein)$^{d}$</th>
<th>(nmoles chlordane bound/mg. RNA)$^{e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Interaction with protein.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$25%$ trans- $75%$ cis-chlordane mixture.</td>
<td>+</td>
<td>+</td>
<td></td>
<td>7.542 ± 0.485</td>
<td>1.151 ± 0.042</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>8.906 ± 0.817</td>
<td>1.001 ± 0.139</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>8.572 ± 1.221</td>
<td>1.052 ± 0.063</td>
</tr>
<tr>
<td>B. Interaction with RNA.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$25%$ trans- $75%$ cis-chlordane mixture.</td>
<td>+</td>
<td>+</td>
<td></td>
<td>5.061 ± 0.692</td>
<td>0.908 ± 0.039</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td></td>
<td>5.503</td>
<td>1.032</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>3.726 ± 0.212</td>
<td>0.895 ± 0.041</td>
</tr>
<tr>
<td>Substrate</td>
<td>MFO</td>
<td>HEH</td>
<td>Pretreatment&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----</td>
<td>-----</td>
<td>--------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Interaction with DNA.</td>
<td>+</td>
<td>+</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25% trans- 75% cis-chlordane mixture.</td>
<td>+</td>
<td>-</td>
<td>Chlordane</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
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<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>Pretreatment&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tr>
<td></td>
<td>None</td>
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<td></td>
<td>Chlordane</td>
</tr>
</tbody>
</table>

<sup>b</sup>MFO = mixed-function oxidases. HEH = hepatic epoxide hydratase. Enzyme inhibition is indicated by a (-). Details of the inhibitions are given in note<sup>a</sup>.

<sup>c</sup>Pretreatment was with a mixture of cis and trans chlordane (3 to 1 by weight) at 100 ppm in feed for 10 days. Unpretreated animals were given unadulterated feed.

<sup>f</sup>(nmoles chlordane bound/mg. DNA-P)<sup>f</sup>

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>Chlordane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.086 ± 0.043</td>
<td>0.064 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>0.083 ± 0.011</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>0.068 ± 0.030</td>
<td>0.066 ± 0.003</td>
</tr>
</tbody>
</table>

<sup>a</sup>Incubation conditions: to a 25 ml flask were added in order; 1.54 micromoles of a mixture of chlordane isomers (75% cis:25% trans) with a specific activity of 0.97 mC/m mole, 0.2 ml of ethoxyethanol, 0.5 ml of a 0.25 M solution of MgSO<sub>4</sub>·7H<sub>2</sub>O 0.07 M with respect to glucose-6-phosphate, 0.5 ml 0.1 M potassium phosphate buffer pH 7.4 containing 2 units of glucose-6-phosphate dehydrogenase, 1.5 ml phosphate buffer 3.67 mM with respect to NADP and 3 mg DNA dissolved in 1.0 ml phosphate buffer. In those flasks where inhibition of hepatic epoxide hydratase was desired, the contents were made 0.5 mM with respect to TCPO. Inhibition of mixed-function oxidases was by omission of NADP, G-6-P and G-6-PDH from the incubation mixture. Final volume was 4.7 ml. Incubation was for 30 min with O<sub>2</sub> gassing and was begun by the addition of the microsomes.

<sup>b</sup>Values given are the means ± the S. E. for 5 samples. (Values with no S. E. are derived from only 1 sample as the result of a laboratory accident.)
Table 1. Continued.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MFO</th>
<th>HEH</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chlordane</td>
</tr>
</tbody>
</table>

*0.56 mg microsomal RNA added per incubate as determined by average post incubation recovery.
(Values with no S. E. are derived from only 1 sample as the result of a laboratory accident.)
*3.0 mg "buffered" calf thymus DNA added per incubate.
(Values with no S. E. are derived from only 1 sample as the result of a laboratory accident.)
unpretreated group, HEH inhibition produced a slight but significant protection against binding to RNA (95% level).\(^1\) Only these two of the twelve comparisons made showed significance. There was no significance attached to any of the t-tests comparing the effect of enzyme inhibition versus no inhibition on chlordane-DNA interaction when pretreatment was held constant. The indication was that inhibition of the enzymes studied had no effect on the covalent binding of chlordane derived material to DNA in the \textit{in vitro} rat liver system.\(^2\)

**Pretreatment.** Table 7B (Appendix) presents a more detailed evaluation of the effect of pretreatment than that shown in the analysis of variance. t-Tests comparing the group means representing no inhibition allowed only the effect of pretreatment to show. The results show that chlordane pretreatment significantly reduced chlordane binding (99% level) to protein and RNA but had no effect on binding to DNA.\(^3\)

**The mouse**

The mouse was used in the final experiment of this investigation to which the labelled pure isomers were committed. Table 2 gives the results of this experiment.

The investigation of pretreatment effects was expanded in the mouse work due to the fact that pure isomers were used as substrate instead of the mixture used with the rat. This necessitated the use of two different chlordane pretreatments; one with each isomer.

\(^1\)Appendix table 7A, comparison 3, RNA column.

\(^2\)Appendix table 7A, comparisons 1-4, DNA column.

\(^3\)Appendix table 7B, comparison 5.
Table 2. Summary of the effects of pretreatment, substrate, and hepatic microsomal enzyme activity on the covalent interaction of $^{14}$C-chlordane isomers with microsomal protein and RNA, and calf thymus DNA during metabolism by mouse liver in vitro.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Microsomal enzyme activity</th>
<th>Pretreatment\textsuperscript{c}</th>
<th>(nmoles chlordane bound/mg protein)\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFO</td>
<td>HEH</td>
<td>None</td>
</tr>
<tr>
<td>A. Interaction with protein.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cis</td>
<td>+</td>
<td>+</td>
<td>1.710 ± 0.142</td>
</tr>
<tr>
<td>Trans</td>
<td>+</td>
<td>+</td>
<td>1.743 ± 0.138</td>
</tr>
<tr>
<td>Cis</td>
<td>+</td>
<td>-</td>
<td>2.494 ± 0.283</td>
</tr>
<tr>
<td>Trans</td>
<td>+</td>
<td>-</td>
<td>2.337 ± 0.110</td>
</tr>
<tr>
<td>Cis</td>
<td>-</td>
<td>+</td>
<td>3.000 ± 0.244</td>
</tr>
<tr>
<td>Trans</td>
<td>-</td>
<td>+</td>
<td>1.878 ± 0.047</td>
</tr>
<tr>
<td>B. Interaction with RNA.\textsuperscript{e}</td>
<td></td>
<td></td>
<td>(nmoles chlordane bound/mg RNA)\textsuperscript{d}</td>
</tr>
<tr>
<td>Cis</td>
<td>+</td>
<td>+</td>
<td>0.339 ± 0.076</td>
</tr>
<tr>
<td>Trans</td>
<td>+</td>
<td>+</td>
<td>0.068 ± 0.038</td>
</tr>
<tr>
<td>Cis</td>
<td>+</td>
<td>-</td>
<td>0.350 ± 0.081</td>
</tr>
<tr>
<td>Trans</td>
<td>+</td>
<td>-</td>
<td>0.013 ± 0.013</td>
</tr>
</tbody>
</table>
Table 2. Continued

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Microsomal enzyme activity</th>
<th>Pretreatment&lt;sup&gt;c&lt;/sup&gt;</th>
<th>(nmoles chlordane bound/mg DNA-P)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFO</td>
<td>HEH</td>
<td>None</td>
</tr>
<tr>
<td>Cis</td>
<td>-</td>
<td>+</td>
<td>0.291 ± 0.084</td>
</tr>
<tr>
<td>Trans</td>
<td>-</td>
<td>+</td>
<td>0.006 ± 0.006</td>
</tr>
<tr>
<td>C. Interaction with DNA.&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cis</td>
<td>+</td>
<td>+</td>
<td>0.017 ± 0.010</td>
</tr>
<tr>
<td>Trans</td>
<td>+</td>
<td>+</td>
<td>0.000</td>
</tr>
<tr>
<td>Cis</td>
<td>+</td>
<td>-</td>
<td>0.000</td>
</tr>
<tr>
<td>Trans</td>
<td>+</td>
<td>-</td>
<td>0.000</td>
</tr>
<tr>
<td>Cis</td>
<td>-</td>
<td>+</td>
<td>0.040 ± 0.014</td>
</tr>
<tr>
<td>Trans</td>
<td>-</td>
<td>+</td>
<td>0.000</td>
</tr>
</tbody>
</table>

<sup>a</sup>Incubation conditions: As in note<sup>a</sup> Table 1 but mouse liver microsomes added at the rate of 3 mg of microsomal protein per incubate. Substrates in this case were the pure isomers rather than the cis-trans mixture used in the rat work. Chlordane concentration was 1.54 micromoles/flask with specific activities of 0.2 mC/mM (trans) and 0.8 mC/mM (cis).

<sup>b</sup>MFO = mixed-function oxidase. HEH = hepatic epoxide hydratase. Enzyme inhibition is indicated by a minus (-). Details of inhibition are given in note<sup>a</sup> Table 1.

<sup>c</sup>Pretreatment was with the indicated material at 100 ppm for 10 days in feed. In the case of chlordane pretreatment, the inducer corresponds to the isomer used as substrate.
Table 2. Continued

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MFO</th>
<th>HEH</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Chlordane</td>
<td></td>
<td></td>
<td>Chlordane</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td></td>
<td></td>
<td>Phenobarbital</td>
</tr>
</tbody>
</table>

Substrate activity

![Substrate activity table]

Values given are the mean S. E. for 3 observations.

0.35 mg microsomal RNA added per incubate determined by average post incubation recovery.

3.0 mg calf thymus DNA per incubate. See note f Table 1.
Phenobarbital pretreatment was added to the design in an attempt to stimulate binding in analogy to the halogenated methanes. Other factors under investigation were the effect of substrate, MFO inhibition and HEH inhibition. The dependent variables observed included binding to microsomal protein, microsomal RNA and added calf thymus DNA, as in the work with rat liver described above.

Factorial analysis of mouse liver mediated binding. The analysis of variance table generated from the data produced in the mouse experiment is given in Appendix table 8. Again, the effects of enzyme inhibition had to be combined under a single heading: enzyme effects. This category showed no significance in the analysis of protein binding results mainly due to the great variability within the group. The significance arising in the RNA column (95% level) was difficult to explain. The RNA group means themselves (table 2) certainly did not indicate any pattern and testing of the individual group means showed no significant differences for either MFO or HEH inhibition when all of the other variables were held constant. Perhaps the slight significance indicated for enzyme effects on binding to RNA is an indication of the sensitivity of the analysis of variance technique over the t-test.

Enzyme effects also showed no significance in the DNA binding data when the effect of MFO inhibition on groups in which cis-chlordane was the substrate were analyzed. Inhibition of HEH reduced the binding of cis-chlorldane to DNA to zero (table 2). For this reason, the effect of HEH on cis-chlordane binding to DNA could not be evaluated statistically. The effect, however, is clear. The cis-

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1Appendix table 9A and 9B, comparisons 1-12.

2Appendix table 9B, DNA column.
chlordane derived material arising during microsomal metabolism in mouse liver preparations is activated by a HEH mediated process.

As is evident from the analysis of variance (Appendix table 8) the effect of pretreatment was strong in the case of protein and DNA. Substrate was highly significant in protein and RNA as shown by its main effect mean square in table 8. The effect of substrate on binding to DNA could not be evaluated, as with HEH inhibition, due to the values of zero for trans-chlordane binding. But the nature of the data again forces one to conclude that it was very significant.

The interactions of the main effects in the mouse data bear closer scrutiny. The mean square for substrate-pretreatment interaction in the analysis of variance table (Appendix table 8) in protein does not show significance in spite of the fact that the main effect of each of these was very significant in its own right. The indication is, then, that whatever effects the enzymatic manipulations (enzyme effects) had on binding were the same for both substrates. Looking back at the mean square for enzyme effects, it becomes evident that this is true. Enzyme inhibition had no effect on any of the pretreatment groups. The significance of the substrate-enzyme effects interaction and pretreatment - enzyme effects interaction in the protein data simply reflect the significance of pretreatment in the former and substrate in the latter interactions. The nucleic acid groups showed no significant interaction effects.

Enzyme effects. As was true with the rat data, this treatment category was a composite of the effect of both MFO and HEH inhibition because of a failure to include an experimental group in which both enzymes were inhibited. This deficiency destroyed the balanced
factorial design of the experiment and made it impossible to isolate a main effect of MFO and a main effect of HEH during the partitioning of the treatment sum of squares. Table 9 (Appendix) gives a detailed analysis of the effect of MFO inhibition on $^{14}$C-chlordane binding based on t-tests within treatment groups. Two of the protein treatment groups showed significant effects due to MFO inhibition. Both of these lie in that portion of the experiment in which cis-chlordane was used as substrate. The statistical analysis showed that in uninduced microsomes, inhibition of MFO activity substantially increased binding by cis-chlordane to mouse liver microsomal protein.

Cis-chlordane binding to microsomal protein was also significant when phenobarbital was the inducer. In this case, MFO inactivation had a protective effect on the protein much the same as one would expect if the microsomal MFO's were performing an activation on chlordane. Analysis of the nucleic acid binding data yielded no indication of any significant effect on binding due to MFO inhibition.

Some of the most interesting results arising in this investigation were those that resulted from the effect of HEH inhibition on binding to macromolecules in the mouse liver microsomal system. In the protein group only the non-induced microsomes mediated any increase in binding when the HEH inhibitor was added. Binding to protein by material derived from both isomeric substrates was increased under

1 Appendix table 9A, comparisons 1 and 3, protein column.
2 Appendix table 9A, comparison 3.
3 Appendix table 9.
4 Appendix table 9B, comparisons 7 through 12.
these conditions. For some reason the induced groups showed little or no effect on binding to protein due to the inactivation of HEH.\(^1\) This was true under the influence of both substrates and for microsomes from mice pretreated with cis-chlordane, trans-chlordane or phenobarbital.

HEH inhibition had no apparent effect on binding to RNA. With DNA, however, the results were striking. With cis-chlordane as substrate, inhibition of HEH reduced binding to unmeasurable levels. In one of the three pretreatment groups, phenobarbital, there was slight binding in the presence of HEH inhibition but it was significantly lower (95% level) than binding in the presence of active enzyme.\(^2\) The indication is that HEH somehow contributes to the activation of cis-chlordane to a form which binds to DNA. No formal statistical comparisons were possible with the trans-chlordane group because trans-chlordane binding to DNA was either unmeasurable or nonexistent under all of the treatment conditions.

**Pretreatment.** The effect of pretreatment on binding is analysed in Appendix table 9, part C. No significance was found for binding to nucleic acids using the t-test. Unpretreated group means were compared with pretreated group means within the RNA and DNA data.\(^3\) Similar comparisons showed significance when the means of the results of chlordane binding to protein were evaluated. The binding of cis-chlordane derived material to protein was more than tripled by 

\(^1\)Appendix table 9B, comparisons 8, 9, 11 and 12.

\(^2\)Appendix table 9B, comparison 9, DNA column.

\(^3\)Appendix table 9C, comparisons 13 through 16, RNA and DNA columns.
phenobarbital pretreatment.\textsuperscript{1} Likewise, trans-chlordane binding to protein was increased by pretreatment with trans-chlordane\textsuperscript{1}. Effects such as these are what would be expected if binding to protein was dependent on the MFO activation of chlordane. But the experimental significance of a difference due to chlordane pretreatment on the one hand and phenobarbital pretreatment on the other is a bit elusive. It may be important to note in this context that in the case of binding to protein as it is effected by pretreatment, the tests of cis-chlordane pretreated microsomes acting on cis-chlordane versus non-pretreated microsomes and the test of phenobarbital pretreated microsomes acting on trans-chlordane versus non-pretreated microsomes fell just short of significance at the 95\% level.\textsuperscript{2} The indication is that the isolation of the two significant effects in the protein results may only be the result of variability and small group size. It is probable that under better experimental conditions and with larger groups pretreatment would have been significant in all of the protein groups. The nucleic acids, on the other hand, showed no tendency toward significance due to pretreatment effects.\textsuperscript{3}

\textbf{Substrate.} The results of the analysis of variance of the mouse experiment indicate that the effect of substrate was the most significant factor tested (Appendix table 8). Differences in the concentration of bound material arising from the two labelled chlordane isomers were significant at the 99\% level by F-test in the protein

\textsuperscript{1}See table 2, part A and Appendix table 9C, comparisons 14 and 15, protein column.

\textsuperscript{2}Appendix table 9c, comparisons 13 and 16.

\textsuperscript{3}Appendix table 9C.
and RNA data. A stark difference appeared between cis- and trans-chlordane binding to DNA. Trans-chlordane did not interact while cis-chlordane did. It is because of this that no substrate effect analysis was run on the DNA binding data in the analysis of variance.\(^1\) The difference was maintained regardless of pretreatment or manipulation of the microsomal enzymes. This strong effect of substrate required a closer look isolated from the other variables. Again, this was carried out using the t-test.\(^2\)

The chlordane-protein covalent interaction was examined in this manner. In spite of the strong significance of substrate in the overall analysis of variance, the magnitude of the binding to protein found when cis- and trans-isomers were compared was different in only 2/3 of the treatment groups.\(^3\) For example, in the groups where there was no enzyme inhibition\(^4\) or where HEH was inhibited,\(^5\) only the phenobarbital pretreated microsomes mediated a significant difference with less binding by trans-chlordane derived material than by that from cis-chlordane. In microsomes where MFO had been inactivated the pattern was reversed.\(^6\) In this case phenobarbital pretreatment caused no difference in the concentration of bound material between the two isomers while in chlordane pretreated and non-pretreated microsomes there was

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\(^1\) See Appendix table 8, footnote c.

\(^2\) Appendix table 9D, comparisons 17 through 25.

\(^3\) Appendix table 9D, protein column.

\(^4\) Appendix table 9D, comparisons 17, 18 and 19, protein column.

\(^5\) Appendix table 9D, comparisons 20, 21 and 22, protein column.

\(^6\) Appendix table 9D, comparisons 23, 24 and 25, protein column.
less binding by material derived from trans-chlordane.

In contrast to protein, RNA bound radioactivity differed significantly with substrate in nearly every treatment group.\(^1\) This seems to carry over into the DNA results as well. The indication is that a different mechanism is at work in the processing of chlordane for binding to nucleic acids than that involved in processing prior to protein binding. In the results of binding to RNA every significant difference due to substrate involved greater binding by cis-chlordane than by trans-chlordane.\(^2\) The only instance where there was no significant difference between the means in the RNA results was in the chlordane pretreated, HEH inhibited groups.\(^3\)

The Effect of Induction and Other Factors on the Activity of Microsomal Constituent Enzymes

Characterization of the effect of induction on mouse and rat liver microsomes

Experimental microsomes were assayed for the effect of pretreatment on MFO activity (as aminopyrine demethylase), HEH activity and the level of cyt. P-450. A summary of the results for both rat and mouse liver microsomes is given in table 3. In the case of the rat, pretreatment for 10 days with a mixture of 25% trans-chlordane--75% cis-chlordane at 100 ppm in feed caused an increase in all three of the microsomal parameters measured. A similar general increase in activity was seen in the mouse although different pretreatments were

\(^1\)Appendix table 9D, comparisons 17 through 25, RNA column.

\(^2\)See table 2, part B.

\(^3\)Appendix table 9D, comparison 21, RNA column.
Table 3. The effect of pretreatment on microsomal enzymatic activity expressed as percent of control.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Species\textsuperscript{b}</th>
<th>Pretreatment\textsuperscript{c}</th>
<th>Aminopyrine demethylase\textsuperscript{d}</th>
<th>Cytochrome P-450\textsuperscript{e}</th>
<th>Hepatic epoxide hydratase\textsuperscript{f}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Phenobarbital</td>
<td>141</td>
<td>137</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td>Cis-chlordane</td>
<td>204</td>
<td>156</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>Trans-chlordane</td>
<td>178</td>
<td>171</td>
<td>456</td>
</tr>
<tr>
<td>Rat</td>
<td>25% trans-chlordane mixture</td>
<td>128</td>
<td>228</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>75% cis-chlordane mixture</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Non-pretreated control = 100% . Because of the manner in which the data were collected, they did not lend themselves to statistical evaluation.
\textsuperscript{b}Male Sprague-Dawley rats and male C57 black/6J F1 hybrid mice.
\textsuperscript{c}Pretreatment was with 100 ppm of the appropriate compound in feed for ten days.
\textsuperscript{d}Calculated as mg Ch$_2$O formed per mg microsomal protein.
\textsuperscript{e}Calculated as millimoles P-450 per mg microsomal protein.
\textsuperscript{f}Calculated as micromoles styrene dihydrodiol formed per mg microsomal protein.

used. In comparing the responses of the rat and the mouse to pretreatment it is evident from table 3 that the profiles differ. The order of increasing activity in induced rat liver microsomes can be expressed as: MFO < HEH < cyt. P-450. By contrast, the mouse liver microsomes roughly show the order: Cyt. P-450 < MFO < HEH. These differences are probably partially responsible for the differences seen in binding behavior between the two species.

The differences between the pretreatment groups in the mouse data is also of interest. The chlordanes seem to be more potent inducers of the parameters tested than the barbiturate. This is especially interesting in the case of epoxide hydratase which is known to respond
very substantially to induction by phenobarbital.

The enhancement of epoxide hydratase activity by pretreatment with the chlordane mixture was investigated in some detail during the early phase of this work. Figure 9 shows the enhancement effect graphically at one level of microsomal protein. Clearly, pretreatment with chlordane increases the concentration of total epoxide hydrolysed in the standard assay for HEH activity. Unfortunately, this was not repeated with mouse liver or to evaluate the effect of pretreatment with pure chlordane isomers.

The effect of oxychlordane on rat liver microsomal HEH activity

Oxychlordane was added to HEH assay mixtures at three concentrations to test the possibility that it was an inhibitor of the enzyme. The results of this experiment are given in table 4.

Table 4. The effect of added oxychlordane on the activity of hepatic epoxide hydratase in rat liver microsomes.

<table>
<thead>
<tr>
<th>Oxychlordane concentration</th>
<th>Micromoles of styrene diol mg microsomal protein</th>
<th>t-value</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X 10^{-5} M</td>
<td>0.318 ± 0.126</td>
<td>0.3961</td>
<td>108</td>
</tr>
<tr>
<td>5 X 10^{-4} M</td>
<td>0.358 ± 0.018</td>
<td>7.6504**</td>
<td>122</td>
</tr>
<tr>
<td>5 X 10^{-3} M</td>
<td>0.328 ± 0.020</td>
<td>3.0964*</td>
<td>112</td>
</tr>
<tr>
<td>None</td>
<td>0.239 ± 0.010</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

*a Standard epoxide hydratase assay using uninduced male Sprague-Dawley rats. Pooled microsomes from two rats were used. Epoxide hydratase substrate concentration 0.8 micromoles tritiated styrene oxide per assay. 

*b 0.2 mg microsomal protein were added to each incubate. Values given are the mean ± standard deviation of three observations.

*c Determination of "t" with 5 degrees of freedom. *Indicates significance at the 95% level. **Indicates significance at the 99% level.
Figure 9. The effect of induction with chlordane on the activity of microsomal hepatic epoxide hydratase in the rat.
As is evident from the results, the presence of oxychlordane did not have an inhibitory effect on the enzyme. At the two higher concentrations a slight but significant enhancing effect was evident. This data, of course, is only indicative of what happens in the presence of styrene oxide the HEH substrate in the assay system. No attempt was made to generalize these results to other substrates of HEH.

The Comparison of Relative Binding to Protein, RNA and DNA in Rat and Mouse Liver Systems

Reference to tables 1 and 2 shows that binding to a given macromolecule varied in the order: protein > RNA > DNA. This was true for both species used. When the same data are compared for rat and mouse, generally more chlordane derived material seems to have bound to protein, RNA and DNA in rat liver microsomes than with those from the mouse.

The difference in binding profiles must also be considered in the light of the relative concentrations of the individual macromolecules available for participation in binding reactions. Table 5 shows the amounts of protein, RNA and DNA in an incubation system by macromolecule and species.

It is clear that the difference in protein content between the rat and mouse liver systems may have had an effect on the concentration of bound material when the results from tables 1 and 2 are compared. The rat, with only half as much available protein present, shows a consistently greater concentration of bound radioactive material. The situation is reversed with respect to RNA. Table 5 shows that a milliliter of rat liver microsomes contained a higher
Table 5. Average total amounts of protein, RNA and DNA present in the in vitro mouse and rat liver microsomal incubate.$^a$

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>1.50 mg</td>
<td>0.56 mg</td>
<td>3.00 mg</td>
</tr>
<tr>
<td>Mouse</td>
<td>3.00 mg</td>
<td>0.35 mg</td>
<td>3.00 mg</td>
</tr>
</tbody>
</table>

$^a$Values given were derived from measurement of microsomal protein content by the Lowry method, measurement of average recovered RNA using the orcinol reaction and the concentration of the solution of calf thymus DNA added to each incubation flask. Incubate volume was 4.7 ml.

concentration of RNA than an equal quantity of mouse liver microsomes. When the experimental results are examined in this case, though, the relationship developed with protein is still with us; i.e., a greater concentration of bound radioactivity is found in RNA after chlordane metabolism by the rat system than after metabolism by the mouse. Obviously, the dichotomy between the rat and mouse is not totally a function of the relative concentrations of the macromolecules under investigation.

The hypothetical correlation between macromolecule concentration and the concentration of bound radioactive material breaks down even further when the DNA results from rat and mouse (tables 1 and 2) are compared. In the case of DNA the concentration within all of the incubates was the same. Yet the now familiar pattern of rat $>$ mouse for the amount of chlordane derived material bound to DNA persists. Based on this argument, it seems reasonable to conclude that the differences between the rat and mouse are not primarily the result of the different relative concentrations of the macromolecular species added to the in vitro system in each case.
DISCUSSION

Based on the results above it becomes possible to determine whether the hypotheses formulated as research objectives were correct. The original purpose of this investigation was to determine if chlordane metabolites bound to cellular macromolecules after processing by liver and if so was there an isomeric preference in the binding that correlated to differences that had been seen in the liver microsomal metabolism of the isomers of chlordane. Questions were also raised as to whether chlordane binding behavior paralleled that of other compounds known to exert their toxic effects following a process of metabolic activation and macromolecular binding and whether in vitro binding behavior in rat and mouse could be correlated with the known carcinogenicity of chlordane in the mouse but not in the rat.

Binding to Cellular Macromolecules

It is obvious from the results that one or both of the isomers of chlordane interacted with each of the macromolecules in both species investigated. Furthermore, the level of binding was sensitive to the experimental conditions in some cases. At times, binding was seen to vary with the state of activity of the microsomal MFO's and HEH, species, microsomal enzyme induction and substrate isomer.

Isomeric Preference

It was impossible to discern any isomeric preference in the results from the rat because of the mixture of isomers used as
substrate. In the mouse, where the individual isomers were used, this route of inquiry was fruitful. Calf thymus DNA showed a clear preference for interaction with the material derived from cis-chlordane in that binding by trans derived material was so low that it was either nonexistent or unmeasurable. Additionally, the data in table 2 show that when RNA was the target molecule binding by trans-chlordane was consistently lower than binding by the cis-isomer.

The study of the interaction of chlordane with mammalian systems has consistantly shown an isomeric dichotomy. Cis-chlordane is less toxic to male rats on an acute basis than trans-chlordane (107) and cis-chlordane is metabolized to oxychlordane to a lesser extent than trans-chlordane (106, 107). There are, as well, indications that reductive metabolism plays a more prominant role in cis-chlordane metabolism than in the metabolism of the trans-isomer in the rat (102). So the fact that differences appear in the covalent interaction of the chlordanes with nucleic acids is really not surprising. This phenomenon probably arises because of the different metabolic patterns attributable to the different isomers. Confirmation of this, however, has to await a study of the isomers' comparative metabolism in the mouse.

There is little to be gained by speculation about the identity of the molecular species binding to mouse liver nucleic acids at this point. The mouse has not been used in any of the existing studies of the metabolism of chlordane. Additionally, the reactivity of the metabolite of interest makes it unlikely that it would be identified in such a study. The methods normally used in the study of xenobiotic metabolism are not designed to dislodge and recover covalently bound
metabolites for identification.

Concerning the metabolism of chlordane in the mouse and the identity of the activated species, however, two conclusions can be stated with certainty. Based on the result of this investigation, one can safely assume that the mouse, like the rat, metabolizes the isomers of chlordane by different pathways. Otherwise the differences in binding seen with the nucleic acids would not have appeared. Additionally, based on what is known about the production of oxychlordane in other species and the characteristics of its interaction with epoxide hydratase shown here, it is safe to say that oxychlordane is not the material that is covalently interacting with the nucleic acids in this study. Information from investigations of the rat and rabbit (102, 103, 106, 107, 109) indicates that if oxychlordane were involved in the binding, the isomeric preference would have been reversed. More oxychlordane evolves from the mammalian metabolism of trans-chlordane than from cis-.

The isomeric pattern seen with the nucleic acids in the mouse work breaks down to some extent when one considers binding to microsomal protein. Comparison of the corresponding values for cis- and trans-chlordane binding to protein from table 2 shows that the cis-trans pattern seen with the nucleic acids is not evident in the case of protein. For example, the amount of bound radioactivity derived from each of the isomeric substrates was approximately equal when fully active, unpretreated microsomes were used (Table 2, part A). This is a reversal of the effect seen with DNA (Table 2, part C).

It is difficult to explain this loss of isomeric preference as one goes from the nucleic acids to the protein results for the mouse.
liver system. It is tempting to say that different metabolites are involved. One metabolite prefers protein as the target macromolecule and is produced from both isomers. The variability of the effect of HEH inhibition on the protein binding by this metabolite indicates that it is something other than an epoxide (Table 2, part A). The other metabolite prefers nucleic acid as a binding site, is primarily a cis-chlordane derivative and behaves, to some extent, as if it arises from an epoxide precursor dependent on HEH for activation (Table 2, parts B and C).

Aside from the question of isomeric preference, perhaps a consideration of the preference of chlordane derivatives for a particular macromolecule is warranted at this point. The results from both the rat and the mouse systems indicate that protein is the preferred target molecule. RNA and then DNA follow based on the relative amounts of radioactivity bound per unit weight of macromolecule (Tables 1 and 2).

One's first impression is that this reflects differences in the total quantities of the macromolecules present in the in vitro system. In the presence of a constant quantity of activated metabolite the concentration of the target molecules would become the limiting factor. The target molecule present in lowest concentration would exhibit the highest rate of radioactive decay on a weight basis assuming equal reactivity toward all three macromolecules on the part of the binding species and the absence of proximity effects. If this were true, however, the amount of radioactive material bound to RNA would have exceeded that bound to both protein and DNA since RNA was present in the lowest concentration (Table 5). This was not so in either the rat
or the mouse. In both cases the concentration of bound material on protein and RNA were at least triple the concentration bound to DNA. Yet the protein > RNA > DNA relationship held. It was quite clear that this difference is either real or based on some factor other than target macromolecule concentration.

It is more conceivable that a proximity effect was at work and contributed to the preference of the active species for the one macromolecule over another. Protein and RNA, the sites of the greatest binding, were of microsomal origin and, as such, were in closer proximity to the activating enzymes than the exogenous calf thymus DNA. Assuming such an effect, the difference between the amounts of chlordane derived material bound to protein and RNA would be a true reflection of relative reactivity. The low concentration of material bound per unit weight of DNA phosphorus, on the other hand, would be explained by the remoteness of DNA from the site of chlordane activation. Therefore, no direct comparison can be made, with respect to reactivity at least, between protein and RNA on the one hand and DNA on the other.

The fact that DNA is not a component of microsomes does not make this effect any less real, however. The low concentration of DNA at the site of metabolic activation and the subsequent paucity of binding may be an artifact of working in vitro but it also mimics the actual situation within the cell. The site of metabolic activation in vivo (the endoplasmic reticulum) does not coincide with the highest concentration of DNA. So the relationship of the DNA to sources of metabolic activation in the in vitro system may be a better approximation of reality than was originally apparent.
Chlordane Binding Behavior Compared to That of Other Compounds that Bind After Metabolic Activation

Polycyclic aromatic hydrocarbon carcinogens, halogenated benzenes and halogenated methane necrogens and chloramphenicol were discussed above as examples of compounds producing a well characterized toxic effect whose mode of action is felt to involve covalent binding to cellular macromolecules. There are other compounds that fulfill these criteria, of course, but those above are representative. Chlordane behavior, overall, does not fit well with the pattern seen in any of the groups mentioned. But there are some illuminating negative correlations.

Cis-chlordane binding to DNA

The halobenzenes and halomethanes do not bind to nucleic acids (49). In this sense, then, chlordane cannot be considered to behave as these two groups do. Yet there is a factor in the biochemistry of carbon tetrachloride activation that was not investigated in this work with chlordane. The activation of carbon tetrachloride to its protein binding free radical(s) is felt to be a reductive process (50). The result of recent work with chlordane metabolism suggests that cis-chlordane is also partially metabolized by reductive processes (102). The metabolism of carbon tetrachloride under reducing conditions leads to increased binding (49). If chlordane metabolism were to be investigated under similar circumstances an analogy between its activation and that of carbon tetrachloride might still appear.
The behavior of chloramphenicol has not been investigated vis-à-vis nucleic acid binding (60, 61) so no comparison can be made between it and cis-chlordane in this case. The PAH carcinogens, however, do interact with DNA and MFO activation and epoxide cleavage are involved.

Superficially, the comparison of cis-chlordane's interaction with DNA and that of the PAH carcinogens seemed promising. In addition to the production of epoxide activation products by both chlordane and the PAH's, chlordane and several other cyclodiene insecticides are reputed to cause cancer in rodents (7, 115). The similarity seems to fade considerably, though, when one compares the effect of HEH inhibition on the covalent interaction of chlordane and benzpyrene with DNA. Benzpyrene binding to calf thymus DNA in rat liver microsomal systems has been reported to increase when epoxide hydratase is inhibited (41). In our system the binding of cis-chlordane was found to decrease under those conditions.

Considering the evidence that the active intermediate in benzpyrene binding to DNA is a diol epoxide (38, 44) processed by both mixed-function oxidases and epoxide hydratase, the effects of epoxide hydratase inhibition on benzpyrene binding mentioned above seem odd. One would expect to see a reduction in binding when the hydratase was inhibited if it participated in activation. Clearly, more than one compound must be interacting with DNA as the result of the MFO activation of benzpyrene. Some epoxide metabolite must also interact. Cis-chlordane, on the other hand, behaves as if HEH were indeed an activator. When the enzyme is inhibited measurable binding to DNA drops to zero.
Cis-chlordane binding to protein

The covalent interaction of cis-chlordane with microsomal protein offers no clear cut analogy to any of the model systems either. Pretreatment increases binding to some extent (See table 2, part A) but there is no dramatic increase due to phenobarbital induction like that seen with the halogenated methanes (50) and the halobenzenes (49). Phenobarbital pretreatment was used in the mouse work for the purpose of investigating this phenomenon. I hoped that a large increase in binding due to the pretreatment with the barbiturate would indicate a kindred mechanism at work in both the model compounds and the chlordanes. In this sense (binding to protein), therefore, binding by cis-chlordane differs from that of the model compounds. Whether this difference is due to the mechanism of activation or to some variation in the response of the mouse to phenobarbital induction as compared to the species used in the CCl₄ work is unknown. The bromobenzene investigations (49, 53, 54) were conducted partially in the same mouse so the indication, in that case at least, is that the activation mechanism differs.

The effect of mixed-function oxidase inhibition on cis-chlordane binding to protein by eliminating the passage of reducing equivalents to the terminal oxidase (cofactor omission) was interesting. In the unpretreated mouse microsomes this inhibition had the effect of doubling binding of cis-chlordane derived material to microsomal protein. The effect was highly significant (P < 0.01, Appendix table 9, comparison 1). A similar trend was observed with trans-chlordane binding but in that case the increase in chlordane protein interaction was not significant. The magnitude of the binding simply remained
unchanged from that seen in the fully active microsomes.

One's first impression on being confronted with these results is to conclude that the inhibition by elimination of the cofactors necessary for the generation of reducing equivalents was unsuccessful. The fact cannot be denied, however, that the mixed-function oxidases cannot function either reductively or oxidatively in the absence of electrons from NADPH. It may be possible that another microsomal enzyme is participating in the activation of chlordanes for binding to protein. This phenomenon deserves further attention.

Some of the results from the investigation of the binding of chlordane to protein in the rat (Table 1, part A) are germane to this discussion. The departure of the rat results from the published work on CCl₄ and bromobenzene is greater than that seen in the case of the mouse. Although phenobarbital pretreatment was not used in the rat experiments, the effect of chlordane pretreatment was to cause a decrease in the binding in all of the rat experimental groups. This indicates, at least for the rat, that the role of mixed-function oxidase activity is one of inactivation rather than activation of the binding metabolite.

**Trans-chlordane binding to protein**

The data on the binding of trans-chlordane derived material to mouse liver microsomal protein presents a more coherant picture (Table 2, part A) than the cis-chlordane data. Pretreatment caused an increase in binding to protein in fully active microsomes. Inhibition of hepatic epoxide hydratase caused a general increase in binding over the corresponding fully active system indicating a
protective role for this enzyme and the possible participation of an epoxide. In this sense the binding of trans-chlordane to protein is analogous to what would be expected with bromobenzene (55) where an epoxide is definitely involved in the protein interaction. However, in the pretreated, hepatic epoxide hydratase inhibited system the binding was of the same magnitude as that for the epoxidase inhibited, non-pretreated liver. This indicates that the induced increment of epoxide hydratase had little effect on the binding and leads one to believe that the increased binding in the fully active enzyme group was due primarily to mixed-function oxidase induction.

Mixed-function oxidase inhibition showed very little effect overall when trans-chlordane was the microsomal substrate. In this sense this work departs from the reports of protein binding from the model compounds where significant reductions in binding were noticed (50, 54). It may be significant that in those investigations the mixed-function oxidases were actively inhibited with, for example, piperonyl butoxide or SKF 525a. In this work mixed-function oxidase inhibition was by cofactor omission as was mentioned above. It is possible that sufficient activity was maintained by the presence of cofactors carried over from the tissue isolation so that the omission effect was masked.

As was the case with cis-chlordane binding to protein, there was a statistically significant (P < 0.01) but not dramatic phenobarbital mediated increase in binding to protein when trans-chlordane was the substrate (Table 2, part A). This illustrates a departure from the halobenzene and halomethane compounds similar to that seen in the cis-chlordane protein binding results. It may be appropriate to
conclude, then, that although trans-chlordane binding to protein is more closely analogous to the bromobenzene model system than cis-chlordane, there was not sufficient evidence generated in this study to allow one to state that they behave the same. Epoxide hydratase seemed to be involved in trans-chlordane binding but not that portion of the activity that is inducible. Phenobarbital induction generally increased binding to protein by the trans-isomer but the increase was unaffected by the inhibition of the mixed-function oxidases.

The Effect of Chlordane Pretreatment on Hepatic Epoxide Hydratase Activity

There have been no investigations reported in the literature in which the effect of chlordane induction on microsomal hepatic epoxide hydratase activity was studied. The closest anyone seems to have come is the work of Bellward, et al. (100) in which it was reported that dieldrin contaminated feed increased activity in rats considerably. Faced with this dearth of information and the well documented metabolism of chlordane to one or, possibly, more epoxides I felt that the effect of chlordane pretreatment on the activity of this inducible enzyme had to be investigated before meaningful conclusions could be reached about binding by chlordane derived material.

Table 3 shows the results of chlordane pretreatment on epoxide hydratase activity in the mouse and the rat. The difference in the rate of metabolism of styrene oxide with induced and noninduced rat liver is shown in figure 9. The results are quite clear. Both the rat and the mouse show an increase in liver microsomal epoxide hydratase activity following 10 days of induction with 100 ppm of
chlordane in feed. Probably significant in this context is the fact that the mouse shows a much greater degree of induction than the rat. The results showed 2.3 to 3.2 times the amount of induction found in the rat depending on the isomer used in the pretreatment. Rat pretreatment, of course, was with the cis-trans (3:1) mixture already mentioned so a comparison on an isomer basis was not possible.

Based on the variability of the results of the binding it is difficult to say what effects these differences produced in the actual metabolism of the chlordanes in the two species studied. In virtually all cases induction caused a drop in binding in the rat (Table 1). Since the pretreatment also induced mixed-function oxidase activity, it is impossible to conclude what increment of this reduction was due to epoxide hydratase activation or whether increased epoxide hydratase activity had any effect on binding at all. It is clear from the results shown in table 1, part A that inhibition of epoxide hydratase had the effect of increasing binding to protein in the rat in both induced and noninduced groups. So the enzyme must have been involved in a metabolic deactivation toward protein binding in this species. The same comparison in the mouse (Table 2, part A) is confounded by pretreatment and substrate effects. Inhibition also had a striking, but opposite, effect on binding to DNA in the mouse system as was already discussed (vide supra).

It may be that the activating effect of the hydratase toward DNA binding in the mouse plus the higher levels of epoxide hydratase induction in the mouse contribute to the difference in relative carcinogenicity of chlordane in the rat and in the mouse.
Suggested Areas for Further Investigation

The results of this study have suggested several areas for further investigation. Some are the result of what are felt to be shortcomings in the present investigation while others are of a more basic nature. Most of the areas suggested for further study on this problem and related areas will have to wait until the labelled pure isomers again become available.

There were several shortcomings in these experiments that should be examined in more detail pending the availability of the labelled isomers. The species differences in chlordane carcinogenicity found in the National Academy of Sciences study might be explained if the binding work with rat liver microsomes, reported here, could be repeated using the isomers instead of a cis-trans mixture. It would be particularly interesting to know if the inactivity of trans-chlordane derived material toward DNA is universal or a phenomenon only associated with the C57 black/6J F1 hybred mouse. Clarification of this may show that the chlordane isomers in concert with this mouse and the rat, offer a system for the study of species variation in chemical carcinogenesis.

Because of the doubts that have arisen in this work as a result of the use of a passive method of mixed-function oxidase inhibition, this work could be profitably repeated using a more specific method for the inhibition. Binding in the presence of mixed-function oxidase inhibition and the failure to include a group in which both the mixed-function oxidases and epoxide hydratase were inhibited, have caused some doubt to arise as to just what enzyme or enzymes are participating in the activation of chlordane for binding. The use of a more
specific inhibitor of cytochrome P-450 along with trichloropropene oxide would clear up this confusion to some extent.

Another area that might prove fruitful for additional research is the participation of glutathione in the process that has been under investigation here. There is strong evidence from the work with both the necrogens and the carcinogens that the presence of glutathione, in a system like the one used in this work, has a very great modulating effect on toxic events. Rerunning these experiments using known glutathione concentrations in the incubation mixtures and using microsomes from animals dosed with a glutathione depleting substance would clarify the details of its involvement in the binding of chlordane metabolites to cellular macromolecules. The only concession that was made to the possibility of glutathione participation in the present work was the washing of the microsomes during their preparation. The incubation system was never actually assayed for its presence.

Another area that needs attention is the metabolism of the chlordane isomers in the C57 black/6J mouse. Knowledge of the metabolic patterns for chlordane in this animal would obviously have been extremely helpful in the evaluation of the results generated in this work. It would be no less valuable applied retroactively. Also, this is work that could be carried out with unlabelled pure isomers which eliminates the unavailability of the radioactive material as an impediment and an expense.

Finally, there are two aspects of chlordane metabolism that have not been studied. The first is the effect of in vitro metabolic activation under anaerobic conditions. Work with the halogenated anesthetics (25) and the halomethanes (50, 56) as well as work with
chlordane has shown that reductive processes are involved in the metabolism of these compounds. In the case of the anesthetics and the methanes, it has been shown that binding to protein is increased by in vitro metabolism under anaerobic conditions. A study of the metabolism and binding of the chlordanes under these conditions could have very interesting results.

The second aspect of chlordane that should be investigated is its interaction with the blood forming tissues. The suspicions of Furie and Trubowitz (19) and Infante and Epstein (6) should be tested. It may be that although chlordane has not been found to be toxic to man in the fashion that it is to rodents, the toxicity has eluded us because we have been looking in the liver and nervous system rather than the bone marrow, which appears to be its site of action based on the evidence from the epidemiologic studies.
SUMMARY

This investigation addressed several aspects of the covalent interaction of metabolites of the insecticide chlordane with cellular macromolecules in vitro. Microsomal preparations from the livers of mice and rats were used and covalent binding to microsomal protein and RNA and added calf thymus DNA was studied. Pure $^{14}$C-labelled cis- and trans-chlordane isomers as well as an isomeric mixture (3:1, cis-to trans-, $^{14}$C) were used as substrates for the in vitro system. Biochemical parameters investigated included inhibition of microsomal mixed-function oxidase and epoxide hydratase plus the induction of these enzymes by pretreatment with chlordane or phenobarbital. The effect of these manipulations on covalent interaction of the metabolites with the macromolecules was of interest.

Isolation of the protein, RNA and DNA from the in vitro microsomal systems after 30 min. incubations and scintillation counting indicated that the chlordane derived material bound to all of the macromolecules investigated. The only exception was the trans-isomer's interaction with DNA in the mouse liver system. Mouse liver microsomes do not activate trans-chlordane to a form which binds to DNA in measurable amounts under the conditions employed here.

Results from this work indicated that microsomal epoxide hydratase and aminopyrine demethylase activity are increased in both the rat and the mouse following chlordane pretreatment. The effect of this induction on the macromolecular interaction of metabolites was variable for both chlordane and phenobarbital pretreated groups.
Generally, for the mouse, induction increased binding to protein and DNA and decreased binding to RNA. In the rat induction decreased binding to all of the macromolecular species. The effect of enzyme inhibition was variable in both species under all of the conditions tested. However, in the mouse epoxide hydrolase inhibition reduced binding by cis-chlordane to DNA to unmeasurable levels.

The results indicate little possibility that the epoxide metabolite of chlordane, oxychlordane, is involved in the binding. The effects of epoxide hydrolase inhibition, however, indicate that some epoxide is involved in cis-chlordane binding to DNA in the mouse.

The possible analogy between the binding behavior of chlordane found in this study and the binding behavior of other well characterized toxic compounds is discussed.
LITERATURE CITED


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Appendix A

Tabular Results of the Statistical Analyses
Table 6. Covalent interaction of $^{14}$C-chlordane with cellular macromolecules during metabolism by rat liver microsomes. Analysis of variance applied to the data summarized in table 1 using the method of unweighted means.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Protein</th>
<th>RNA</th>
<th>DNA $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>1</td>
<td>0.3813**</td>
<td>0.0028</td>
<td></td>
</tr>
<tr>
<td>Enzyme effects$^c$</td>
<td>3</td>
<td>0.0125</td>
<td>0.0010</td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>3</td>
<td>0.0488*</td>
<td>0.0014</td>
<td></td>
</tr>
<tr>
<td>Within cells variation</td>
<td>32</td>
<td>0.0110</td>
<td>0.0014</td>
<td></td>
</tr>
<tr>
<td>(Error)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aBecause of unequal treatment group sizes due to pipetting error and a laboratory accident the usual analysis of variance technique could not be applied here. Unweighted means analysis provides an alternative method for analyzing data such as these when the unequal group sizes arise randomly as they did here.

*bThe DNA analysis is separated from protein and RNA because 2 additional DNA samples were lost leading to fewer within cells (error) degrees of freedom.

*cThe experimental design was conceived to allow separate analysis of the effects of the inhibition of mixed-function oxidase and epoxide hydratase. Biochemical good sense and the hypothetical model indicated that inhibition of both at the same time was unnecessary. Therefore, no treatment group was included in which both enzymes were inhibited. This destroyed the symmetry of the factorial design and made it necessary to combine these into one category.

*Indicates significance at the 95% level.  **Indicates significance at the 99% level.
Table 7. Values of $t$ and indications of significance for various comparisons of group means in the data from the rat.$^a$

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Values of $t^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Number Pretreatment</td>
<td><strong>MFO</strong></td>
</tr>
<tr>
<td></td>
<td>Target macromolecules</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>A. Tests of the significance of enzymatic manipulation.$^c$</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>Chlordane</td>
<td>+</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>Number Pretreatment</td>
<td>Microsomal enzyme activity</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td></td>
<td>MFO</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>5. Chlordane</td>
<td>+</td>
</tr>
</tbody>
</table>

B. Test of the significance of chlordane pretreatment.\(^d\)

- Comparisons shown are for groups of varying sizes and were carried out using the results of the analysis of variance by the unweighted means technique. Values of $t$ were calculated using the group means arising from the unweighted means analysis. T-test variance used error mean square and $N$ for each group mean.
- Numbers of degrees of freedom in t-tests for protein and RNA were 32 and for DNA 24.
- Enzymatic manipulation refers to the inhibition of mixed-function oxidases (MFO) and hepatic epoxide hydratase (HEH). A minus (-) indicates inhibited enzyme.
- Pretreatment was carried out with the cis-rich chlordane mixture (25% pure trans-chlordane and 75% pure cis-chlordane on a weight to weight basis) in feed for 10 days at a concentration of 100 ppm.
- Indicates significance at the 95% level. ** Indicates significance at the 99% level.
Table 8. Covalent interaction of $^{14}\text{C}$-chlordan with cellular macromolecules during metabolism by mouse liver microsomes. Analysis of variance applied to the data summarized in table 2.a

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Mean squares for the factor analyzed</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Proteinb</td>
<td>RNA</td>
<td>DNA</td>
</tr>
<tr>
<td>Treatment</td>
<td>17</td>
<td>0.0314**</td>
<td>0.0496*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>1</td>
<td>0.1183**</td>
<td>0.6805**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>2</td>
<td>0.0406**</td>
<td>0.0031</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme effects</td>
<td>2</td>
<td>0.0007</td>
<td>0.0286*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate x pretreatment</td>
<td>2</td>
<td>0.0208</td>
<td>0.0202</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate x enzyme effects</td>
<td>2</td>
<td>0.0272*</td>
<td>0.0068</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment x enzyme effects</td>
<td>4</td>
<td>0.0364*</td>
<td>0.0074</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>4</td>
<td>0.0229*</td>
<td>0.0040</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>35</td>
<td>0.0062</td>
<td>0.0080</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Treatment                         | 5                  | 0.00064                             |          |          |          |
| Pretreatment                      | 2                  | 0.00110*                            |          |          |          |
| Enzyme effects                    | 1                  | 0.00060                             |          |          |          |
| Pretreatment x enzyme effects     | 2                  | 0.00030                             |          |          |          |
| Error                             | 12                 | 0.00028                             |          |          |          |

a. Analysis of variance was according to a completely randomized factorial design.
b. The values for the group variances in the protein groups deviated from normality so a log transformation was applied prior to analysis of variance. RNA values required no transformation.
c. DNA values were transformed to square roots to compensate for non-normality of group variances. A substrate comparison within the DNA data was impossible. Trans-chlordane binding was unmeasurable or nonexistent leading to values of 0 for binding. Zero values violate the variance normality assumption necessary for the use of the analysis of variance technique.

*Indicates significance at the 95% level.  **Indicates significance at the 99% level.
Table 9. Values of t and indications of significance for various group mean comparisons in the data from the mouse.

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Microsomal enzyme activity</th>
<th>Values of t</th>
<th>Target macromolecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Pretreatment</td>
<td>Substrate</td>
<td>MFO</td>
</tr>
<tr>
<td>1.</td>
<td>None</td>
<td>Cis</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Cis</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cis</td>
<td>Cis</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Cis</td>
<td>Cis</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Phenobarbital</td>
<td>Cis</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
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A. Tests of the significance of mixed-function oxidase inhibition.
Table 9. Continued

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<th>Substrate</th>
<th>Microsomal enzyme activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Values of &lt;sup&gt;c&lt;/sup&gt;</th>
<th>Target macromolecules</th>
</tr>
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<tbody>
<tr>
<td></td>
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B. Tests of significance of epoxide hydratase inhibition.

|        |              | Cis       | +                                |                      | 3.2276*               |
|        |              | Cis       | +                                |                      | 0.0995                |
| 7.     | None         | Cis       | +                                | +                    |                      |
|        | None         | Cis       | +                                | -                    |                      |
|        | Cis          | Cis       | +                                | +                    | 1.3359                |
| 8.     | Cis          | Cis       | +                                | -                    | 0.2575                |
|        | Phenobarbital| Cis       | +                                | +                    | 1.3670                |
|        | Phenobarbital| Cis       | +                                | -                    | 1.3111                |
| 9.     | Phenobarbital| Cis       | +                                | +                    | 3.5697*               |
|        | None         | Trans     | +                                | +                    | 3.3484*               |
| 10.    | None         | Trans     | +                                | -                    | 1.3819                |
Table 9. Continued

<table>
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<th>Substrate</th>
<th>Microsomal enzyme activity&lt;sup&gt;b&lt;/sup&gt;</th>
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C. Tests of the significance of pretreatment.

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<th>Values of t&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Target macromolecules</th>
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<table>
<thead>
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<th>Number</th>
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<th>HEH</th>
<th>Values of t&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Target macromolecules</th>
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<td>D. Tests of the significance of substrate.</td>
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<td>+</td>
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<td>4.7191**</td>
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<td>2.0479</td>
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Table 9. Continued

<table>
<thead>
<tr>
<th>Number</th>
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<th>Substrate</th>
<th>Microsomal enzyme activity</th>
<th>Target macromolecules</th>
<th>Values of t&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td>MFO</td>
<td>HEH</td>
<td>Protein</td>
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<td></td>
<td></td>
<td></td>
<td>DNA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values of t represent comparisons of the means of the 3 observations from each treatment group.

<sup>b</sup> Microsomal enzyme activity refers to the state of mixed-function oxidase (MFO) and hepatic epoxide hydratase (HEH). A minus (-) indicates inhibited enzyme.

<sup>c</sup> Values of t with 4 degrees of freedom.

<sup>d</sup> Binding to DNA by trans-chlordane derived material was unmeasurable or nonexistent and yielded a value of 0. For this reason, evaluations of statistical significance were impossible. Additionally, HEH inhibition led to values of 0 for binding of cis-chlordane derived material to DNA in comparisons 7 and 8. No t-test was possible on these values either.

* Indicates significance at the 95% level. ** Indicates significance at the 99% level.
Appendix B

Preliminary Experimental Work and Methods Development

The first indications that chlordane participated in binding to cellular constituents arose during the investigations of Street and Blau (103, 107). They found that total recovery of pure chlordane isomers from in vitro metabolism was incomplete and consistently varied with the isomer used as substrate. Possible explanations for this included covalent binding so an experiment was carried out in 1974 to test this possibility.

Briefly, pure $^{14}$C-labelled cis- and trans-chlordane isomers were incubated in vitro with microsomes prepared from the livers of rats pretreated with DDT. The individual incubates were extracted with hexane after an appropriate incubation period to remove organic soluble parent compound and metabolites. The aqueous fraction was then acidified to pH 2.0 precipitating the microsomal protein. The aqueous fraction plus precipitated protein was then exhaustively extracted (24 hours) with diethyl ether. The proteinaceous residuum was collected by millipore filtration, submitted to oxygen flask combustion and bound radioactivity was determined by scintillation counting of the evolved $^{14}$CO$_2$.

The results of this work are shown in table 10. The indications were that binding was occurring at some site in the acid precipitable proteinaceous remainder and that an isomeric preference for material derived from trans-chlordane was at work in the DDT induced rat liver system.
Table 10. The results of the preliminary investigation of covalent binding by chlordane derived material in rat liver microsomes.a

<table>
<thead>
<tr>
<th>Substrate isomer</th>
<th>Inducer</th>
<th>Inhibitor</th>
<th>Nanomoles of chlordane\textsuperscript{e} mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis DDT</td>
<td>None</td>
<td>TCPO</td>
<td>0.197</td>
</tr>
<tr>
<td>trans DDT</td>
<td>None</td>
<td>TCPO</td>
<td>0.486</td>
</tr>
<tr>
<td>cis DDT TCPO</td>
<td>0.172</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trans DDT TCPO</td>
<td>0.099</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} In vitro incubation performed with microsomes prepared from the livers of female Sprague-Dawley rats. 4.7 mg microsomal protein per incubate. Four repetitions of each treatment were made but incubates were pooled before the protein was collected and combusted so no index of variability has been included.

\textsuperscript{b} The substrate concentration was 1.2 X 10^{-5} M.

\textsuperscript{c} Animals were induced with 150 mg/kg DDT in corn oil i.p. once a day for three days.

\textsuperscript{d} An inhibitor of hepatic epoxide hydratase, 1,1,1-trichloropropene oxide, was included at a concentration of 1.3 X 10^{-5} M.

\textsuperscript{e} These weights are based on the oxygen flask combustion of the recovered dried proteinaceous residuum.

Following this preliminary experiment and prior to the start of the experimental work, several questions had to be addressed. These revolved mainly around two points in the proposed dissertation work.

Firstly, there was a need to determine that the exogenous DNA being added to the in vitro system was free from protein. Additionally, the purity of the nucleic acids and protein reisolated after in vitro incubation had to be established. It was imperative that cross contamination be kept to a minimum. Without these assurances, the credibility of a report of binding to any one of the macromolecules of interest would be questionable at best.

Secondly, certain changes had to be made in the in vitro chlordane metabolizing system used in previous work to accommodate added
DNA and enzyme inhibitors and to allow for the isolation of the macromolecular components. The integrity of the chlordane metabolic system had to be tested in the presence of these changes and the effect of the components of the in vitro system on the added DNA had to be established.

Pretreatment of calf thymus DNA prior to use in binding studies

Sodium DNA from calf thymus (Type I) purchased from Sigma Chemical Co. was assayed at the source by the Lowry method and reported to contain 2.3% protein. A confirmatory protein determination by the same method in our laboratory showed a protein content of 6.2% (average of two samples). It was felt that this was too much contamination for this work so the DNA was further deproteinized by the method of Kay, et al. (118).

After deproteinization, a second protein determination was carried out. This showed that protein contamination had been reduced to 3.3% (average of two samples). The once purified DNA was again subjected to the Kay procedure and the level of protein was determined as before. The twice deproteinized DNA was found to contain less than 1% protein.

The DNA used in the bulk of the experimental work was sodium DNA (Type II) from calf thymus purchased from Sigma Chemical Co. at a later date. Upon arrival, this material was subjected to the Kay procedure immediately. The only difference between the treatment of this DNA and that purchased earlier was that the type II DNA was carried through an additional protein precipitation step. Assay of this material showed less than 0.9% protein (average of five samples).
This was considered to be adequate purity.

Tests of the purity of DNA recovered from in vitro metabolism by the method of Speier and Wattenberg (123)

The purity of the DNA recovered from the in vitro microsomal metabolic system was also of concern. Contamination at this point was an indication of incomplete separation of $^{14}$C-labelled DNA from microsomal RNA and protein containing bound $^{14}$C-chlordane residues. To test the purity of recovered DNA nine incubation mixtures, complete with the exception of $^{14}$C-labelled chlordane, were incubated at 37° for 30 minutes. The DNA was separated from the microsomes by ultracentrifugation and decantation as outlined in the methods section. Protein determinations run on appropriate aliquots of this recovered DNA showed an average of 1% contamination.

Two subsequent changes in the procedure for the treatment of DNA recovered from the microsomal incubation mixtures substantially reduced this contamination. As is indicated in the methods section, the Sevag procedure (124) was inserted at one point. Additionally, the use of DNAase to solubilize recovered DNA was dropped in favor of hot perchloric acid hydrolysis (119) followed by collection of acid insoluble material by centrifugation. This eliminated the necessity for adding further protein contamination in the form of DNAase to the already highly purified DNA preparation.

Optimization of the conditions for the epoxide hydrase assay

Early trouble with the determination of hepatic microsomal epoxide hydrase activity indicated the necessity for establishing optima for microsome concentrations, incubation time and inhibitor
concentration in the assay mixture. To establish proper incubation
time and microsomal protein concentration, an experiment was run in
which these two factors were varied. The results are shown in table
11. As a result of this experiment, the incubation time used in the
experimental assays was extended from the 5 minutes called for in the
procedure (92) to 15 minutes and the protein level used in the exper-
imental assays was approx. 0.48 mg/sample. This concentration was at
the high end of the range called for in the Oesch procedure (92) and
corresponded to the addition of 0.2 ml of the 1:4 (w:v) microsomal
suspension used in the chlordane incubations.

Optimization of the concentration of TCPO for
inhibition of epoxide hydrase activity

A diversity of claims in the literature for the effectiveness of TCPO in inhibiting epoxide hydrase activity (26, 126) led to some con-
fusion about the quantity that should be added to give metabolically
significant and measurable inhibition of the enzyme. A trial was set
up in which a series of concentrations were used that covered the
range of those reported in the literature. The results of this trial
are presented in table 12.

The decision was made to use the middle value. This was based on
three factors: 1) The fact that this concentration (5 X 10^{-4} M) was
reported by Oesch (26) as an acceptable level for inhibition.
2) There was no significant increase in inhibition to be gained in
going to the next higher concentration (5 X 10^{-3} M). 3) Concentra-
tions of the inhibitor had to be kept as low as possible to avoid un-
wanted biochemical effects such as competition with substrate for cyt.
P-450 in the complete in vitro chlordane metabolizing system.
Table 11. Optimization of microsomal protein concentration and incubation time in the assay of hepatic epoxide hydratase activity (See page 74 for details of the assay procedure).\textsuperscript{a}

<table>
<thead>
<tr>
<th>Microsomal protein concentration (mg)</th>
<th>Incubation time (min.)</th>
<th>Micromoles styrene glycol per mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.48</td>
<td>5</td>
<td>0.26</td>
</tr>
<tr>
<td>0.48</td>
<td>10</td>
<td>0.35</td>
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<tr>
<td>0.48</td>
<td>30</td>
<td>0.81</td>
</tr>
<tr>
<td>0.48</td>
<td>60</td>
<td>0.90</td>
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<td>2.38</td>
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<tr>
<td>2.38</td>
<td>10</td>
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<td>2.38</td>
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<td>2.38</td>
<td>60</td>
<td>0.18</td>
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<td>4.76</td>
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<td>4.76</td>
<td>10</td>
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<tr>
<td>4.76</td>
<td>30</td>
<td>0.09</td>
</tr>
<tr>
<td>4.76</td>
<td>60</td>
<td>0.12</td>
</tr>
</tbody>
</table>

\textsuperscript{a}One sample per incubation time. Substrate styrene oxide concentration 2.0 mM.

Table 12. Studies on the optimal concentration for the inhibition of hepatic epoxide hydratase with 1,2-epoxy-3,3,3-trichloropropane (TCPO).\textsuperscript{a}

<table>
<thead>
<tr>
<th>TCPO Concentration</th>
<th>Micrograms $^{14}$C-styrene glycol\textsuperscript{b}</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>35.2 ± 1.2</td>
<td>0</td>
</tr>
<tr>
<td>$5 \times 10^{-5}$ M</td>
<td>22.3 ± 3.5</td>
<td>37</td>
</tr>
<tr>
<td>$5 \times 10^{-4}$ M</td>
<td>19.5 ± 17.8</td>
<td>45</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$ M</td>
<td>19.1 ± 17.8</td>
<td>46</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Assay conditions: 0.2 ml of an uninduced microsomal preparation from rat liver containing an average of 0.231 mg of protein, 2.0 mM with respect to substrate styrene oxide concentration, 15 minute incubations at 37°.

\textsuperscript{b}Each value represents the average of 3 samples ± standard deviation.
Tests of the effect of the presence of oxychlordane on the activity of epoxide hydrase

To assess the effect of oxychlordane generated during chlordane metabolism in vitro, an experiment was conducted that was similar to the one just described. Three oxychlordane solutions were made up in acetonitrile such that the addition of a suitable aliquot would render the epoxide hydrase assay mixture either $5 \times 10^{-5}$, $5 \times 10^{-4}$ or $5 \times 10^{-3}$ molar with respect to oxychlordane. The substrate concentration in the assay mixture is $2 \times 10^{-3}$ molar. The results are presented in table 13. They indicate that there was no inhibition of epoxide hydrase at any of the oxychlordane concentrations tested. The effect, in fact, appears to be a slight enhancement of epoxide hydrase activity.

Table 13. The effect of various concentrations of oxychlordane on hepatic epoxide hydrase activity in rat liver microsomes.a

<table>
<thead>
<tr>
<th>Oxychlordane concentration$^b$</th>
<th>Micrograms $^{14}$C-styrene glycol$^c$</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>35.2 ± 1.2</td>
<td>0</td>
</tr>
<tr>
<td>$5 \times 10^{-5}$ M</td>
<td>36.9 ± 14.0</td>
<td>less than 0</td>
</tr>
<tr>
<td>$5 \times 10^{-4}$ M</td>
<td>43.0 ± 2.1</td>
<td>less than 0</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$ M</td>
<td>39.4 ± 2.4</td>
<td>less than 0</td>
</tr>
</tbody>
</table>

$^a$Assay conditions: 0.2 ml of an uninduced microsomal preparation from rat liver containing an average of 0.231 mg of protein, 2.0 mM with respect to substrate styrene oxide concentration, 15 minute incubations at 37°C.

$^b$Oxychlordane used was an analytical reference standard supplied by the Velsicol Chemical Co. 98% purity.

$^c$Values are an average of 3 samples ± standard deviation.
VITA

Alan A. Brimfield

Candidate for the Degree of

Doctor of Philosophy

Dissertation: The Covalent Interaction of Hepatic Metabolites of the Insecticide Chlordane with Cellular Macromolecules in the Rat and Mouse In Vitro

Major Field: Toxicology

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Personal Data: Born at Camden, New Jersey, April 13, 1945, son of Edwin L. and Muriel Dougherty Brimfield; Married Renee Marcia Becker June 10, 1972; one child - Whitney Marie.

Education: Attended elementary school at Pennsauken, New Jersey; graduated from Pennsauken High School in 1963; received the Bachelor of Arts degree from Rutgers University, College of South Jersey with a major in Biology in 1968; did graduate work in Toxicology at Utah State University receiving the Master of Science degree in 1976.

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