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Title: HEXACHLOROPHENE: ITS ABSORPTION, DISTRIBUTION, EXCRETION, AND BIOTRANSFORMATION IN THE RAT AND RABBIT AND IN VITRO INTERACTION WITH RAT LIVER MICROSOMES

Abstract approved: Redacted for Privacy
Dr. D. R. Buhler

The fate of hexachlorophene, a widely used bactericide, was investigated using both intact male rats and rabbits and liver microsomes from male rats. Hexachlorophene administered intraperitoneally was found to be rapidly absorbed and distributed to all the tissues of the animals. Excretion of the chemical in both species occurred slowly, with 48-83% of the dose appearing in the feces as unchanged hexachlorophene plus a small amount of hexachlorophene glucuronide. The rabbit and rat excreted 21-25% and 4-8% of the dose, respectively, in the urine. A glucuronide conjugate of hexachlorophene accounted for over one-half of the urinary metabolites with the remainder being unchanged hexachlorophene.

In less than four days, about 30-45% of hexachlorophene dose was excreted in the bile as a glucuronide conjugate. Extra-biliary

excretion of hexachlorophene or its metabolites was also suggested. Hexachlorophene was shown to undergo enterohepatic circulation in the rat, which would explain its long half-life in animals and its excretion primarily via the feces. The glucuronide conjugate of the bisphenol excreted in the rat bile and in the rabbit urine was identified as hexachlorophene monoglucuronide.

Hexachlorophene was shown not to be metabolized or conjugated in vitro by the rat liver microsomal enzyme systems. Microsomes were shown to strongly bind large amounts of hexachlorophene. Furthermore, at low concentrations, hexachlorophene inhibited microsomal 0-demethylase, nitroreductase, and glucuronide synthetase activities. Hexachlorophene also caused a decrease in microsomal cytochromes P-450 and b_5 absorbancies, suggesting that interference with these microsomal components may be responsible for the observed in vitro inhibition of microsomal enzyme activity.

Hexachlorophene: Its Absorption, Distribution, Excretion, and Biotransformation in the Rat and Rabbit and *In Vitro* Interaction with Rat Liver Microsomes

by

Allen Jay Gandolfi

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APPROVED:

Redacted for Privacy

Associate Professor of Biochemistry
in charge of major

Redacted for Privacy

Chairman of the Department of Biochemistry

Redacted for Privacy

Dean of Graduate School

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Typed by Cheryl E. Curb for Allen Jay Gandolfi

DEDICATION

To my Parents

for their faith, love, and reassurance

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ABBREVIATIONS AND SYMBOLS

$NADP^+$	- nicotinamide adenine dinucleotide phosphate - oxidized
NADPH	- nicotinamide adenine dinucleotide phosphate - reduced
hexachlorophene-^{14}C	- methylene-^{14}C-hexachlorophene
UDPGA	- uridine 5'-diphosphoglucuronic acid
BHMW	- benzene:hexane:methanol:water (7:3:5:5)
TF	- toluene (saturated with 88% formic acid)
BBA	- n-butanol:benzene:ammonia (1:1:1)
TEA	- toluene:ethyl acetate:acetic acid (5:4:1)
Rf	- distance compound moves/distance solvent front moves
dpm	- disintegrations per minute
cpm	- counts per minute
nm	- nanometers
μCi	- microcurie
mCi	- millicurie
nmole	- nanomoles
μmoles	- micromoles
mmoles	- millimoles
μl	- microliter
ml	- milliliter
mg	- milligram
kg	- kilogram

HEXACHLOROPHENE: ITS ABSORPTION, DISTRIBUTION, EXCRETION, AND BIOTRANSFORMATION IN THE RAT AND RABBIT AND IN VITRO INTERACTION WITH RAT LIVER MICROSOMES

INTRODUCTION

General

Since the initial use of phenol as an antiseptic by Lister in 1867, many unsuccessful attempts were made to incorporate phenolic compounds into soaps. Koch (1881) found that halogenation of the phenol greatly increased the germicidal properties, but that the halophenols were inactive in the presence of organic matter. The inhibition of the germicidal action was probably due to the formation of inactive alkali salts or additions products as seen by Hampil (1928) with alkylphenolic germicides.

The superior antibacterial activity of the halogenated bisphenols over the unchlorinated bisphenols and the chlorinated monophenols was discovered by Bechold and Ehrlich (1906). Following the promising discovery of Harden and Brewer (1937) that brominated 2, 2'-methylenebisphenols retained their germicidal activity in solution at the alkaline pH of their sodium salt, the Givaudan Corporation started synthesizing and testing chlorinated bisphenols. Of the many chlorinated bisphenols tested for antibacterial activity, the compound 2, 2'-methylenebis-(3, 4, 6-trichlorophenol) appeared as one of the most

effective bactericides (Cade and Gump, 1957). Gump applied for a patent on the compound, which was subsequently given the trade name G-11 by Givaudan Corporation and later became known as hexachlorophene or HCP (Gump, 1941).

Once hexachlorophene was found active at alkaline pH, it was incorporated into a two percent germicidal soap that retained almost all of its antibacterial activity (Kuntz and Gump, 1942; Gump and Kuntz, 1950). Subsequent studies showed that hexachlorophene was absorbed from the soap into the skin and remained active for several days (Manowitz and Johnston, 1966). The germicidal activity of hexachlorophene, its specificity for gram positive organisms, and the absence of bacterial resistance has been confirmed by many studies (John, 1949; Greg and Zopf, 1951; Hall, 1969).

Hexachlorophene has been incorporated into many commercial products such as soaps, cosmetics, tooth pastes, antiseptic solutions, deodorants, shaving creams, baby powders, shampoos, and vaginal preparations, since its introduction as an antibacterial agent. Recent reviews have summarized the wide application of hexachlorophene as an antibacterial and antiseptic agent in cosmetics and medicine (Sindar Corp., 1970; Armour-Dial, Inc., 1971).

Hexachlorophene has also been found effective against fungi, mites, flukes, roundworms, and tapeworms. It has been used as an acaricide and as a broad spectrum fungicide and bactericide in

agriculture and for treatment of shoes, laundry, leather goods, paper, air filters, and many other products (Armour-Dial, Inc., 1971; Johnson, 1971). Hexachlorophene has also been used as an antiparasitic agent against liver flukes in rats (Lienert, 1959; Thorpe, 1965), rabbits, cattle (Armour-Dial, Inc., 1971), sheep (Boray, Happich and Andrews, 1967), cats and dogs (Lienert, 1962). Both Chung and co-workers (1963) and Lui and co-workers (1963) have given hexachlorophene to human patients infected with the liver fluke, Cl. sinesis.

Chemistry of Hexachlorophene

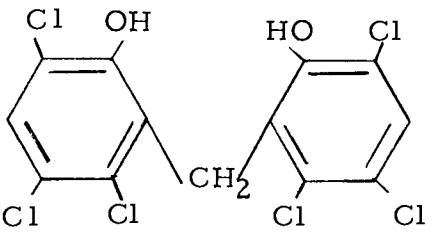


Figure 1. Hexachlorophene, HCP, G-11
2, 2'-methylenebis-(3, 4, 6-trichlorophenol)

Purified hexachlorophene, $C_{13}H_6O_2Cl_6$, is a white crystalline powder with no phenolic or other odor (Figure 1). It has a molecular weight of 406.92 and a melting point of 165-166°C when recrystallized from isopropyl alcohol. It was initially prepared by the condensation of 2, 4, 5-trichlorophenol with formaldehyde in the presence of sulfuric

acid catalyst (Gump, 1941). The pK_a value for one of the phenolic protons is 5.4 making the first proton acidic, while the second pK_a value is 10.85. The second proton dissociation, therefore, occurs only under very basic conditions, probably because of strong intramolecular hydrogen bonding (Mahler, 1954). The two hydroxyl groups also chelate metals and form some stable metal complexes (Adams, 1958).

Hexachlorophene is very hydrophobic, with a solubility in distilled water at room temperature of 0.3 ppm and in 0.1 M phosphate buffer, pH 8, of 45 ppm. Hexachlorophene is soluble in polar organic solvents in the following decreasing order: acetone, alcohol, polyhydric alcohol, ethers, and chlorinated methanes. A saturated corn oil solution contains approximately 40 mg/ml.

While hexachlorophene is fairly stable, it can be photochemically modified to form a variety of products. The primary photodegradation products are dechlorinated derivatives including 2, 2'-dihydroxy-3, 3', 5, 6, 6'-pentachlorodiphenylmethane and 2, 2'-dihydroxy-3, 5, 5', 6, 6'-pentachlorodiphenylmethane with some minor tetrachlorobisphenols and other unidentified derivatives (Shaffer et al., 1971).

The separation and detection of trace amounts of hexachlorophene has required precise methods. Extraction of hexachlorophene from organic tissue is difficult, and lengthy procedures have been developed (Johnston and Porcaro, 1964; Bachmann and Shetlar, 1969).

Hexachlorophene has been readily separated from other bisphenols and germicides by column chromatography (Derry, Holden and Newburger, 1961), thin layer chromatography (Daisley and Oliff, 1969; Graber, Domsky and Ginn, 1969) and electrophoresis (Fogg et al., 1970). Gas chromatography has become the method of choice for hexachlorophene analysis since it is more sensitive and specific. Electron capture gas chromatographic methods have been developed to detect subnanogram levels of methylated hexachlorophene (Gutenmann and Lisk, 1970) and even picogram amounts (Buhler and Rasmussen, 1972).

Radioactive hexachlorophene has been synthesized by Isikow and Gump (1952) using ^{14}C-labeled formaldehyde to produce methylene-^{14}C-hexachlorophene (Figure 2).

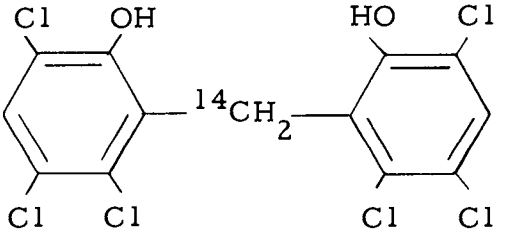


Figure 2. Methylene-^{14}C-hexachlorophene

Toxicology of Hexachlorophene

Despite its extensive use in many commercial products, there have been few studies performed on the toxicity, metabolism, pathology,

and biochemistry of hexachlorophene. A recent review by Kimbrough (1971) and a study by Nakaue, Dost, and Buhler (1972) have described hexachlorophene as being highly toxic both orally and systemically to rats with an age dependent oral LD_{50} ranging from 57.6-87.0 mg/kg and intraperitoneal LD_{50} ranging from 21.8-40.0 mg/kg.

There have been several reports on the toxicity of hexachlorophene to humans (Kimbrough, 1971). The major toxic symptoms in humans were anorexia, nausea, vomiting, abdominal cramps, and diarrhea. An oral dose of 20 mg/kg hexachlorophene for three days caused some of the toxic symptoms in children, while an estimated single oral dose of 250 mg/kg was lethal. Epstein, Wuepper, and Maibach (1968), Baughman (1960), and others have reported hypersensitivity to topically applied hexachlorophene.

Recently, optic nerve atrophy in calves and sheep (Udall and Malone, 1969) and spongy degeneration of brain tissue in rats (Kimbrough and Gaines, 1971) were found after oral administration of hexachlorophene. Curley and co-workers (1971) also showed that dermal absorption of hexachlorophene can occur in infants bathed with a hexachlorophene soap.

The mechanism of action of hexachlorophene has been studied by several investigators in different organisms. Hexachlorophene has shown to strongly bind to bacterial proteins (Gould et al., 1953), animal tissue proteins (Gould, Frigerio and Lebowitz, 1955),

mitochondrial proteins (Caldwell, Nakaue and Buhler, 1972), and erythrocytes and plasma proteins (Flores and Buhler, 1971, 1972). The binding to skin protein has long been noted and believed to be responsible for its long efficacy. A strong hydrogen bond between one of the phenolic protons of hexachlorophene and the oxygen atom of the amide linkage of proteins has been proposed as the mechanism of hexachlorophene binding to proteins (Haque and Buhler, 1972).

Adams (1958) has proposed that the chelating ability of hexachlorophene was related to its mechanism of action. Further studies by Adams and Hobbs (1958) suggested that the antibacterial activity of hexachlorophene was a result of an inhibition of a metal-requiring bacterial enzyme. Hexachlorophene has also been shown to have surface-active or detergent properties, and this was advanced as its mechanism of action against bacteria (Joswick, 1961). The phenolic group, electron attracting groups, ortho to the phenolic group, and a capacity to form intramolecular hydrogen bonds were shown to be mandatory for the tremadocidal action of hexachlorophene (Lienert and Jahn, 1965; Thorsell and Bjorkman, 1966).

Investigations of the effect of hexachlorophene on bacteria led to the discovery of its ability to inhibit enzymes. Gould et al. (1953) showed that hexachlorophene inhibited the succinic, glucose, and lactic dehydrogenases and the cytochrome oxidase system of B. substilis and E. coli with 50% inhibition occurring in the concentration range of

10^{-6} to 10^{-4}M. Gould and co-workers (1955) also investigated succinoxidase, cytochrome c oxidase, and lactic dehydrogenase from the tissues of various animals and found similar results. Thorsell (1967) showed that hexachlorophene inhibited the succinate oxidase of liver flukes both in vitro and in vivo and cholinesterase in vitro, but did not inhibit adenosine phosphatase in either case. Hexachlorophene was also found to inhibit mitchondrial respiration and to uncouple oxidative phosphorylation (Caldwell, Nakaue and Buhler, 1970, 1972).

While its biochemical effects have not been completely elucidated, hexachlorophene appears to exert its primary effect through interaction with proteins. Hexachlorophene seems to bind in variable amounts with all proteins and might inhibit their normal function, cause conformational changes, or disrupt lipoprotein complexes.

Absorption, Distribution, Biotransformation, and Excretion of Xenobiotics

Toxicity of a chemical is determined by a combination of its intrinsic toxicity and the concentration of the chemical that reaches some sensitive site within the organism. The absorption and excretion rates of a foreign compound, termed xenobiotic by Mason (1965), affects its half-life and total body burden, hence its toxicity. Distribution or localization of the xenobiotic in the tissues of an organism can influence the site of toxicity. Biotransformation, the process of

chemical modification of the xenobiotic by the organism, can result in a less toxic metabolite or formation of a more toxic metabolite (Loomis, 1968).

Absorption and excretion of a xenobiotic involves its transport across lipoprotein barrier membranes. The rate of penetration of a xenobiotic across a lipoprotein is dependent on the particular membrane involved and the lipid solubility and degree of ionization of the xenobiotic under physiological conditions. Penetration of xenobiotics into animals may be facilitated by intraperitoneal injection, which permits their rapid absorption by simple diffusion and filtration into the capillaries of the extremely vascular peritoneal cavity (Parke, 1968).

Following absorption, the foreign compounds may be distributed by extracellular body fluids to various organs and tissues. The extent of distribution is dependent on the membrane permeability of the compound and the binding of the compound to proteins in the extracellular fluids. Tissues, such as adipose tissue, can store highly lipid soluble compounds for long periods of time (Butler, 1971).

The main routes of excretion of xenobiotics or their metabolites are via the urine and bile, but elimination may also occur via the lung, glandular secretion (perspiration, saliva, and milk) and gastric secretion (Weiner, 1967). Biotransformation tends to modify xenobiotics into hydrophilic chemical forms that are more readily excreted

by the animal. The kidneys can remove foreign polar compounds from the blood by glomerular filtration, passive tubular transport, and active tubular transport leading to excretion into the bladder. Compounds, secreted in the bile, are excreted into the duodenum where they may be eliminated with the feces or chemically modified by intestinal microflora and returned to the liver by enterohepatic circulation (Scheline, 1968). Enterohepatic recirculation has been proposed as the reason for the long efficacy and slow excretion of several drugs (Smith, 1966). Excretion rates are dependent on the chemical form, membrane permeability, and protein binding ability of the xenobiotic and its metabolites.

The ability of an organism to metabolize foreign substances has long been known and is related to the species, age, sex, health, and environment of the animal (Smith, 1963; Parke and Williams, 1969). Biotransformation of foreign substances in vivo has been divided by Williams (1959) into four main types of changes -- oxidations, reductions, hydrolyses, and syntheses. Intestinal microorganisms in the animal may also catalyze the metabolism of xenobiotics prior to their absorption by the gastrointestinal tract or following secretion into the gastrointestinal tract (Scheline, 1966).

Higher animals are capable of carrying out biotransformations by enzymatic processes primarily localized in the liver and to a lesser extent in the lung and kidney. The soluble and mitochondrial fractions

of hepatic cells contain some enzyme activity, but most of the biotransformations occur with the hepatic microsomes. Microsomes are vesicles formed via a "healing" process from isolated endoplasmic reticulum (ER) membrane fragments that retain the capability of enzymatic biotransformation (Gillette, 1966). Microsomes can be subfractionated by density gradient centrifugation into smooth ER that lacks attached ribosomes and rough ER that has attached ribosomes. The smooth ER membranes are believed to be the most active in catalyzing biotransformation reactions (Fouts and Gram, 1969).

The microsomal enzymes are not only involved in the metabolism of xenobiotics but also in the metabolism of normal endogenous substances such as steroids, fatty acids, and carbohydrates. Consequently, they can influence normal cellular metabolic control of these endogenous substances (Estabrook et al., 1971).

Microsomes contain oxidative, reductive, synthetic, and hydrolytic enzymes involved in biotransformation processes. The oxidative microsomal enzymes require both NADPH (or NADH) and oxygen and have been termed mixed function oxidases by Mason (1957), due to the introduction of a single atom of molecular oxygen into the substrate. Microsomal reduction reactions require NADPH (or NADH) and an oxygen-free atmosphere, since oxygen inhibits the reductive processes. Other appropriate cofactors are necessary for microsomal synthetic reactions such as UDPGA for glucuronide conjugations. Glucuronide

conjugates are formed, like most other conjugates, in a two-step process involving the biosynthesis of the active coenzyme UDPGA, and the transfer of the glucuronic acid from UDPGA to the aglycone by UDP-glucuronyltransferase (Dutton, 1966).

Investigations concerning the composition of the microsomes showed that two cytochromes account for most of the heme in the microsomes and are active constituents of the mixed function oxidase system (Omura et al., 1965). One of the hemoproteins, cytochrome b, is not directly involved in many xenobiotic biotransformation reactions (Oshino, Imai and Sato, 1971; Cohen and Estabrook, 1971). The second cytochrome is the activator of oxygen in the mixed function oxidase system (Estabrook, Cooper and Rosenthal, 1963). This latter cytochrome became known as cytochrome P-450 from the strong absorption of the reduced microsomes at 450 nm after treatment with carbon monoxide (Omura and Sato, 1964a, b). Besides cytochromes b_5 and P-450, the microsomes contain flavoproteins, lipids, and other components that are involved in the reduction of the cytochromes by an electron transport system (Mason, North and Vanneste, 1965).

Polycyclic hydrocarbons, phenobarbital, and many other compounds can cause an in vivo induction of the microsomal enzymes, thus enhancing the metabolism of xenobiotics. In vivo and in vitro microsomal metabolism may also be impaired by some xenobiotics, such as piperonyl butoxide, carbon tetrachloride and SKF-525 A,

possibly by inhibition of cytochrome P-450 or the electron transport system or by reduction in the amount of cytochrome P-450 and other electron transport components. When certain xenobiotics interact with cytochrome P-450, they give characteristic difference spectra either by binding to cytochrome P-450 or by displacement of an endogenous substrate (Gillette, Davis and Sasame, 1972). When treated with salts and detergents in vitro, cytochrome P-450 is converted to its inactive form called P-420 that absorbs at 420 nm after reduction and carbon monoxide treatment, thus inhibiting microsomal metabolism (Imai and Sato, 1967). Cytochrome P-450 can also be converted to P-420 in vitro by phenolic derivatives and other xenobiotics by disruption of the hydrophobic bonding of the heme to the apoprotein (Ichikawa and Yamano, 1967). Increasing the hydrophobicity and degree of chlorination of phenols increases their ability to cause the conversion. Phenols may also inhibit drug metabolism in vitro by altering the flow of electrons in the electron transport system to cytochrome b_5 (Oshino and Sato, 1971).

Absorption, Distribution, Excretion, and Biotransformation of Phenolic Compounds

The widespread use of phenols, their similarity to endogenous substances, their biological activity, and their toxicity, especially that of the chlorinated phenols (Deichmann and Keplinger, 1967), has

led to a fairly extensive investigation of the absorption, distribution, biotransformation, and excretion of simple phenolic compounds. Due to the lipid solubility and weakly acidic nature of phenols, they are rapidly absorbed from the gastrointestinal tract and parenteral injection sites (Shanker, 1971). Chlorination of phenols generally aids in their rapid absorption from the gastroenteric tract, through the skin, and from parenteral sites of injection.

Phenols generally bind reversibly to proteins of the blood and tissues; thus they are well distributed throughout the body in the bound and unbound form. Simple phenols are usually eliminated from the body within 48 hours after administration, primarily via the urine (Knaak and Sullivan, 1966), while polychlorinated monohydric phenols have a half-life of 24 hours (Jakobson and Yllner, 1971). Many phenols of higher molecular weight are known to undergo enterohepatic circulation, which increases their retention in the body and, in some cases, leads to extensive structural modification by the action of intestinal microflora (Smith, 1966). Phenols may also be excreted into the gastroenteric tract by the processes of gastric, intestinal, and salivary excretion (Stowe and Plaa, 1968).

Animals mainly metabolize phenols by conjugation and oxidation (Williams, 1964). Williams (1964) has divided the phenolic molecule into the three parts (Figure 3), each of which may be involved in separate biotransformation reactions: (a) the phenolic group (or

groups); (b) the aromatic ring system; and (c) the substituents (or substituents), R, in any ring position.

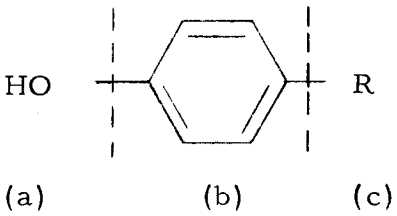


Figure 3. Sites of biotransformation of phenols.

Metabolism of phenols most commonly occurs at the phenolic group. Such biotransformations usually involves conjugation but the phenolic group may also be dehydroxylated or oxidized (Williams, 1964). Three common types of conjugates are formed with phenolic groups: etheral sulfates, glucuronides, or methyl ethers. The biochemical processes yielding these three metabolites are of widespread occurrence in most organisms and are involved in many routes of metabolism of endogenous substances. Glucuronide and etheral sulfate syntheses are the major pathways for the conjugation of phenols. Methylation of foreign phenolic compounds occurs only to a limited extent and primarily with dihydric phenols.

Biotransformation reactions involving the aromatic ring system of phenols are oxidative and generally occur to a lesser extent than the conjugation processes. The ring may be hydroxylated in the positions

ortho or para to the phenolic group to form diphenols. Further biotransformation involves either conjugation of one of the phenolic groups or oxidation of the diphenol to a quinone.

Substituents on the aromatic ring may also be oxidized, reduced, hydroxylated, or conjugated. The extent of substituent biotransformation depends on the type of substituent and the type of phenolic compound, but generally occurs to a lesser extent than conjugation of the phenolic group.

Little work has been done on the absorption, distribution, excretion, and biotransformation of chlorinated phenols possessing isolated benzene rings (Williams, 1964), similar to that of hexachlorophene, and relatively few examples are available for metabolic comparison.

Absorption, Distribution, Excretion, and Biotransformation of Hexachlorophene

Although hexachlorophene has been used extensively for many years, the fate of the compound in animals has received little study. Kok (as cited by Wit and VanGenderen, 1962) initially thought that orally administered hexachlorophene was slowly and incompletely absorbed by the rat. Chung et al. (1963), though, found by methods not described that hexachlorophene was fairly rapidly absorbed through the intestinal wall of animals. Absorbed hexachlorophene is distributed in animals in a manner similar to that of other chlorinated phenols.

Kok (as cited by Wit and Van Genderen, 1962) found that 24 hours after oral administration of methylene-^{14}C-hexachlorophene to rats, the plasma, liver, lungs, and kidneys contained the highest levels of radioactivity. Similar tissue distributions were found in newborn rats that had received daily low level oral doses of hexachlorophene (Ulsamer, Yoder, and Marzulli, 1972). Buhler, Dost and Rasmusson (1972) investigated the distribution of radioactivity in rats at several time periods following either oral or intraperitoneal administration of hexachlorophene-^{14}C and found that six hours after dosing the levels of radioactivity decreased in all tissues except the stomach which increased. The radioactivity in rat tissues from percutaneously absorbed hexachlorophene-^{14}C was distributed in a manner similar to that given orally or intraperitoneally (Nakaue and Buhler, 1972).

Hexachlorophene is slowly excreted from animals mainly via the feces. Almost 80% of an orally administered dose of hexachlorophene to cats appeared in the feces (Chung et al., 1963). Wit and Van Genderen (1962) found that five-seven days after oral administration of hexachlorophene-^{14}C to rabbits, rats, and cows, 30, 6, and 1 percent of the radioactive dose, respectively, appeared in the urine as hexachlorophene, while 63-72 percent of the radioactive dose was excreted in the feces of all three species. St. John Jr. and Lisk (1972) found similar results in the cow using nonradioactive hexachlorophene. No evidence was found in either study for the excretion of hexachlorophene into the milk of

treated cows. Intraperitoneal administration of hexachlorophene-^{14}C to rats resulted in almost twice as much of the radioactive dose appearing in the urine over a seven day period (Figure 4) as compared to orally administered hexachlorophene-^{14}C (Buhler, Dost and Rasmusson, 1972). In guinea pigs, following parenteral administration of hexachlorophene-^{14}C, 95% of the radioactive dose was excreted in 72 hours with only 1% of the dose appearing in the urine (Maibach, 1971). Nakaue and Buhler (1972) have also shown that after 53% absorption of a dermally applied dose of hexachlorophene-^{14}C through the shaved abdomen of the rat, 2% of the applied radioactive dose appeared in the urine during the 24 hour exposure period and almost 15% in the feces.

Only 0.0007-0.125% of an oral dose of hexachlorophene in humans was excreted in the urine in three days and the rest slowly excreted over the next 11 days, mainly in the feces (Chung et al., 1963). Hexachlorophene was shown to be present in the bile fluids of the human subjects both 48 and 72 hours after administration. After parenteral administration of hexachlorophene-^{14}C to humans, between 44-68% of the radioactive dose has been found to be excreted in 5-10 days with only 4.4-8.8% of the dose appearing in the urine (Maibach, 1971). Feldmann and Maibach (1970) estimated the half-life of parenterally administered hexachlorophene in humans to be 48 hours.

There has been considerable speculation as to whether hexachlorophene is metabolized by animals. Kok (as cited by Wit and

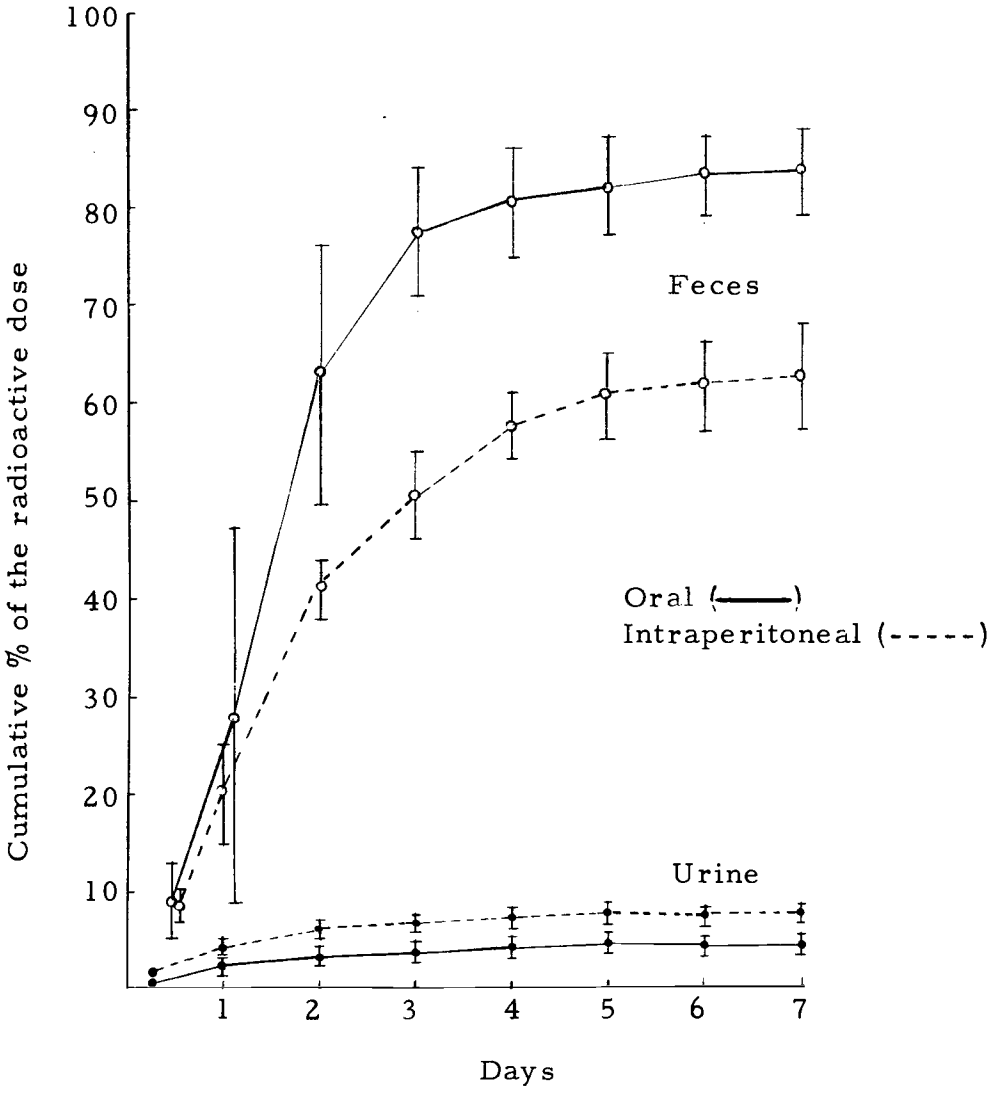


Figure 4. Excretion of radioactivity in the urine and feces of rats receiving 5 mg/kg of hexachlorophene-^{14}C orally or by intraperitoneal injection. o = feces and ● = urine. Each point represents the mean ± standard deviation of four male 200 g rats. (Buhler, Dost and Rasmusson, 1972).

VanGenderen, 1962) suggested that the small amount of hexachlorophene that was absorbed by rats from an oral dose was largely metabolized. Chung et al. (1963) found that cats orally dosed with hexachlorophene excreted only the unchanged drug. Thorsell and Applegren (1968) demonstrated that hexachlorophene-^{14}C was not chemically modified or conjugated by the liver fluke. Wit and VanGenderan (1962) investigated some of the aspects of the fate of orally administered hexachlorophene-^{14}C in rabbits, rats, and cattle. They found that most of the radioactivity in the seven day pooled, acid-hydrolyzed urine samples of the rat, rabbit and cow was unchanged hexachlorophene, while in the acid hydrolyzed feces of the rat and rabbit only part of the radioactivity was unchanged and the rest, approximately 30% of the radioactive dose, was unidentified metabolites. All the fecal radioactivity excreted by the cow was unchanged hexachlorophene. Recently, St. John Jr. and Lisk (1972) fed nonradioactive hexachlorophene to dairy cattle and found that after ten days intact hexachlorophene in the feces accounted for 64% of the dose and 0.24% in the urine with the rest of the dose not being detected. These workers stated that there was an absence of conjugates of hexachlorophene in the dairy cow, and demonstrated that in vitro incubation of hexachlorophene with the rumen fluid of the cow for seven hours or with the 10,000 g supernatant of beef liver for one hour did not decompose or conjugate hexachlorophene.

Purpose of the Study

The recent findings that hexachlorophene may cause optic nerve and brain damage in experimental animals emphasizes the need for a thorough understanding of the nature and behavior of hexachlorophene in animals. Hexachlorophene, a useful but highly toxic chemical, is incorporated into a vast number of commercial products that results in a continuous exposure of humans to the chemical. Hexachlorophene can be absorbed through the mucous membranes and skin of humans, and it may cause deleterious effects. Only fragmentary knowledge exists concerning its fate and behavior in humans and other animals, and there is the possibility that the metabolites of hexachlorophene might be the toxic agents. Since the previous limited research concerning the fate and behavior of hexachlorophene in animals has been incomplete or speculative, the present study on the pharmacodynamics and metabolism of hexachlorophene in rats and rabbits and the investigation of the interaction of hexachlorophene with liver microsomes presented in this thesis were undertaken.

METHODS AND MATERIALS

Materials

3, 5-Dichlorosalicylic acid, 5-chlorosalicylic acid, Diazald, 2, 2'methylenebis-(4-chlorophenol), 2, 4-dichlorophenol and 2, 4, 5-trichlorophenol were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). 5-Chlorosalicylaldehyde, and 3, 5-dichlorosalicylaldehyde were bought from Eastman Organics, Inc. (New York, N. Y.). Mazola corn oil (Best Foods, Englewood Cliffs, N. J.) was used as a vehicle to dissolve hexachlorophene for studies in intact animals. Aquasol was obtained from New England Nuclear (Boston, Mass.). Spectrafluor and toluene (Methyl-^{14}C) were purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). Hydroxide of hyamine 10-X, toluene (ring-^{14}C), 2, 5-diphenyloxazole (PPO), and 1, 4-bis-2-(5-phenyloxazolyl) benzene (POPOP) were bought from Packard Instrument Co., Inc. (Downers Grove, Ill.). Aluminum oxide G and silica gel GF-254 were from EM Laboratories, Inc. (Elmsford, N. Y.); florisil was obtained from Fischer Scientific Co. (Fairlawn, N. J.); Bio Sil A came from BioRad Laboratories (Richmond, Calif.); and Amberlite XAD-2 came from Mallinckrodt Chemical Works (St. Louis, Mo.).

Hexachlorophene, 2, 2'-methylenebis-(3, 4, 6-trichlorophenol), U.S.P. grade, was obtained in bulk as a gift from the Givaudan

Chemical Corporation (Clifton, N. J.) and was recrystallized from either isopropyl alcohol-water or from glacial acetic acid. Melting point after recrystallization was 165.0-165.5°C, uncorrected. Purity of the recrystallized hexachlorophene has been checked by gas chromatography in various solvents, mass spectral analysis, and nuclear magnetic resonance and infrared spectra. 2,2'-Methylenebis-(4,6-dichlorophenol), 2,2'-methylenebis-(3,4-dichlorophenol), and 2,2'-dihydroxy-3,3'5,6,6'-pentachlorodiphenylmethane were also gifts from Givaudan Corp.

Methylene-^{14}C-hexachlorophene (1.13-3.52 mCi/mmole) was obtained from Mallinckrodt Nuclear Co. (St. Louis, Mo.) or New England Nuclear Co. (Boston, Mass.) and used as received. The material chromatographed on silica gel thin layer as a single radioactive peak in a variety of solvents. Quantitative gas chromatographic analysis of the dimethylether derivative and measurement of the radioactivity confirmed the quoted specific activities.

Nicotinamide adenine dinucleotide phosphate-oxidized ($NADP^+$), nicotinamide adenine dinucleotide phosphate-reduced (NADPH), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glucuronolactone, D-glucuronic acid, p-nitrophenyl-β-D-glucuronide, uridine 5'-diphosphoglucuronic acid (UDPGA), nicotinamide, diethylamino ethyl cellulose, 1,4-saccharolactone, and Helix pomotia β-glucuronidase-type H-1 were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Patella vulgata β-glucuronidase (with arylsulfatase) was obtained from General Biochemicals Co. (Chagrin Falls, Ohio), and Ketodase (a 5000 unit/ml beef liver preparation) was purchased from Warner-Chilcott Co. (Morris Plains, N. J.).

Glass redistilled water was used for all solutions. All other solvents were obtained from the Department of Agricultural Chemistry, Oregon State University, as reagent or technical grade barrel stock and were redistilled, if necessary, prior to use.

All other chemicals used were reagent grade and purchased from Mallinckrodt Chemical Works (St. Louis), J. T. Baker Chemical Co. (Phillipsbury, N. J.), Eastman Organics, Inc. (New York, N. Y.), Matheson, Coleman, and Bell Manufacturing Chemists (Norwood, Ohio), or Baker and Adamson (New York, N. Y.).

Preparation of Reference Compounds

Reference compounds that could not be obtained from commercial sources were prepared by organic synthesis. The following compounds were chromatographic standards or were considered possible metabolites of hexachlorophene or impurities from the chemical synthesis or ultraviolet degradation of hexachlorophene.

2, 2'-Methylenebis-(3, 4, 6-trichloroanisole). ($C_{15}H_{10}O_2Cl_6$, MW = 434.95) hexachlorophene dimethylether (Prepared by Dr. D. R. Buhler). Hexachlorophene in methanol was treated with an excess of

ethereal diazomethane (prepared from Diazald by second procedure on bottle). An off-white powder was obtained with melting point 95-96°. The proposed structure was confirmed by nuclear magnetic resonance and mass spectral analysis.

2, 2'-Methylenebis-(3, 4, 6-trichloro-1-acetoxybenzene). ($C_{17}H_{10}O_4Cl_6$, MW = 490.97) hexachlorophene diacetate ester. Hexachlorophene was treated with excess acetic anhydride:pyridine (5:1) followed by heating to 70°C for one hour. Recrystallization from ethanol-water yielded 81% of a white crystalline powder with a sharp melting point of 176°C.

3, 5, 6-Trichlorosalicylaldehyde ($C_7H_3O_2Cl_3$, MW = 225.45). The compound was prepared by treating 2, 4, 5-trichlorophenol with chloroform in the presence of sodium hydroxide by the procedure of Hodgson and Jenkinson (1929). Product yield was 31% of yellow material with melting point 115-117°C. The proposed structure was confirmed by mass spectral analysis.

3, 5, 6-Trichlorosalicylic Acid ($C_7H_3O_3Cl_3$, MW = 241.45). 3, 5-Dichlorosalicylic acid was chlorinated with chlorine gas in the presence of 65% fuming sulfuric and a trace of iodine by a combination of the procedures of Richter (1961) and Hanna (1962). The reaction yielded 31% of a white powder with melting point 204-205°C (reference: 206-207°C).

6, 8, 9-Trichloro-1, 3-benzodioxane: ($C_8H_5O_2Cl_3$, MW = 239.48). 2, 4, 5-Trichlorophenol was treated with formaldehyde (37%) in the presence of hydrochloric and sulfuric acids and hydrogen chloride gas by the procedure of Buehler et al. (1941). A white cotton-like product (47% yield) formed that gave a melting point of 112-114°C. Mass and infrared spectral analysis confirmed the proposed structure.

Methyl 2, 3, 4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucuronate: ($C_{13}H_{17}O_9Br$, MW = 397.21). Synthesis of the methyl acetobromoglucuronide derivative, which was used in the synthesis of monomethylhexachlorophene-tri-0-acetyl glucuronate, was performed by the procedures of Bollenback et al. (1955). Glucuronolactone was methylated with sodium methoxide and acetylated with acetic anhydride in the presence of perchloric acid to yield (72%) long white spars after recrystallization from hot 100% ethanol, melting point 179-181°C (reference: 176.5-178°C). The product, methyl tetra-0-acetyl-β-D-glucuronate, was treated with 30% hydrobromic acid in acetic acid to form (93%) tiny white needles of methly 2, 3, 4-tri-0-acetyl-1-bromo-1-deoxy-α-D-glucuronate that melted at 106-108°C (reference: 106-107°C) after recrystallization from ethanol.

2-(Methyl 2, 3, 4-tri-0-acetyl-β-D-glucopyranuronate)-2'-hydroxy-3, 3', 5, 5', 6, 6'-hexachlorodiphenylmethane: ($C_{26}H_{22}O_{11}Cl_6$, MW = 723.18). Following the procedure of Wotiz et al. (1959), 1.5 g of methyl 2, 3, 4-tri-0-acetyl-1-bromo-1-deoxy-α-D-glucuronate was

added to a benzene solution containing 1.5 g of hexachlorophene and 1 g of freshly prepared silver carbonate (silver carbonate prepared by the method of Fieser and Fieser (1967). After standing at room temperature for 48 hours, the silver salts were removed by filtration and the filtrate evaporated in vacuo. The resultant syrup was dissolved in 50% ethanol and allowed to stand in the refrigerator several days until a precipitate formed. The precipitate was isolated by filtration and recrystallized from 50% ethanol to yield (3.7%) pale yellow clusters of fine needles, which first melted at 151°C but after resolidification melted at 168-169°C. Mass spectral analysis of the product showed a six chlorine parent ion pattern at 717 m/e and fragment ion patterns at 404 (hexachlorophene), 316 (methyl aceto-glucuronate), and 196 (major hexachlorophene fragment ion) m/e.

Animals

Random bred Wistar albino rats from a closed colony in the Department of Agricultural Chemistry, weighing between 200-400 grams, were used in all experiments. To avoid any possible inhibitory effects of sexual-steroids in females (Fingl and Woodbury, 1967), only males were used for the metabolic studies.

The rabbits were New Zealand White males that were obtained at approximately two to three months of age (2 kg) and grown to around 3 kg (four months) on Purina Rabbit Chow. The rabbits were

purchased from Smo-Tok Rabbit Ranch, Corvallis, Oregon or Evergreen Rabbitry, Eugene, Oregon.

Collection of Urine and Feces

During metabolism experiments rats were placed in stainless steel metabolism cages (Acme Research Products, Chicago, Ill.) equipped with a baffle system to separate and collect the urine and feces. Urine was collected under toluene to reduce bacterial growth and possible bacterial degradation of metabolites. The feces and urine of rabbits were collected in stainless steel standard small animal cages (Acme Research Product, Chicago, Ill.). The collection pan was fitted with a raised 0.25 inch wire mesh to separate the urine and feces. Periodic dusting of the pan with a few mg of streptomycin sulfate (The Upjohn Company, Kalamazoo, Mich.) was used to retard bacterial growth.

Preparation of Rat Liver Microsomes

All solutions were ice-cold, and all operations were performed at 0-4°C. Male rats (250-350 g) were fasted for 24 hours but received water ad libitum prior to being sacrificed by cervical dislocation. The livers were rapidly excised, washed free of blood with 0.154 M potassium chloride, and homogenized in three volumes of 0.154 M potassium chloride. The homogenates were centrifuged at

10, 000 g for 30 minutes, the supernatant decanted and then centrifuged at 105, 000 g for one hour in a Spinco Model L preparative ultracentrifuge (Beckmann Instruments, Palo Alto, Calif.). The resulting supernatant was decanted, the microsomal pellet was washed once by resuspension in 0. 154 M KCl, and resedimented at 105, 000 g for one hour. The microsomal pellet was finally resuspended in 0. 154 M KCl in such a manner that 1 ml of suspension represented 1 g of liver. The microsomal suspension was kept at 0-4°C until used. Microsomal protein concentration was determined by the method of Lowry et al. (1951).

All assays concerning microsomal enzyme assays were performed with a Gilford Model 2000 (Gilford Instrument, Oberlin, Ohio). Difference spectral analysis of microsomes was performed on a Cary 15 scanning spectrometer (Cary Instruments, Monrovia, Calif.) courtesy of Dr. D. Reed, Department of Biochemistry and Biophysics, Oregon State University.

Radioactivity Counting Techniques

All counting was performed in duplicate in a Packard Tri-Carb liquid scintillation spectrometers models 314-EX or 3375 (Packard Instrument Co., Inc., Downers Grove, Ill.) at optimum settings for measuring ^{14}C.

Aliquots of urine or water and dried aliquots of organic solvents were counted in diotol scintillator (toluene:dioxane:methanol (600:700:420 by volume) containing 146 g of naphthalene and 100 ml of Spectrafluor (10 g PPO and 0.125 g POPOP)]. Aliquots of bile were counted in Aquasol.

Aliquots of feces and tissue (wet or dry) were digested with 60% perchloric acid and 30% hydrogen peroxide (1:2) by the method of Mahin and Lofberg (1966). The digests were counted in 6 ml of cellosolve and 10 ml of toluene-phosphor solution (6 g PPO/liter toluene). To determine if radioactivity was being lost by this digestion procedure, tissues samples treated with known amounts of hexachlorophene-^{14}C were digested and recoveries shown to average 98.5% of the added radioactivity (Table 1). In addition, radioactive fecal samples from animals dosed with hexachlorophene-^{14}C that were solubilized by hyamine hydroxide 10-X or combusted by Schoeniger-type combustion (Kelley et al., 1961), gave identical results to those obtained by the Mahin and Lofberg procedure.

The absolute counting efficiencies of samples counted in the Packard model 314-EX liquid scintillation spectrometer were determined by adding known amounts of toluene-^{14}C as an internal standard and recounting. Counting efficiencies with the model 3375 were obtained by use of the channels ratio method on an external gamma emitting standard (Peng, 1970).

Table 1. Digestion of hexachlorophene-^{14}C treated tissue by the perchloric acid-hydrogen peroxide procedure of Mahin and Lofberg (1966).

mg Tissue (wet)	Volume of hexachlorophene-^{14}C (μl) (8,800 ± 50 dpm/100 μl acetone)	dpm ± standard deviation
--	100	8715 ± 55
90	100	8732 ± 51
50	100	8683 ± 41
100	50	4348 ± 43

Radioactive thin layer chromatograms were scanned under optimum conditions in a Packard Radiochromatogram Scanner (Packard Instrument Co., Inc., Downers Grove, Ill.) equipped with a thin layer plate scanning attachment for 2-π geometry to detect the radioactive zones. For quantification of the radioactive zones, sequential portion from one edge of the streaked plate were transferred to individual counting vials containing diotol and counted.

Extraction and Partition of Radioactivity

Separation of the radioactivity in the urine, bile, and fecal extracts into nonconjugated and conjugated fractions was performed by standard enzymatic hydrolysis and extraction procedures (Bakke and Scheline, 1969). Glucuronides were cleaved to glucuronic acid and aglycones with β-glucuronidase, and aryl sulfatase was used to cleave sulfate conjugates. Acid treatment was used when complete hydrolysis

of all type of conjugates was desired.

A stepwise fractionation of urine and bile or fecal extract via the method of Buhler, Harpootlian, and Johnston (1966) was also employed (Figure 5). The urine was acidified to pH 1 with dilute hydrochloric acid and then extracted three times with 1/3 volume of chloroform to remove the nonconjugated metabolites. This extract was further fractionated into nonconjugated acidic and nonconjugated neutral fractions by a basic wash of the chloroform extract to remove the acidic compounds followed by acidification and re-extraction of the basic wash. The residual aqueous urine was adjusted to pH 5 with sodium hydroxide and buffered with 1/3 volume of 0.1 M acetate buffer, pH 5. β-Glucuronidase was added to a concentration of 500-800 units/ml and then incubated under toluene at 37°C for three days. After incubation, the mixture was again acidified to pH 1 and extracted three times with 1/3 volume of chloroform. The extract was further subfractionated into glucuronide acidic and glucuronide neutral fractions. The aqueous urine residue was again buffered to pH 5 as before and then treated with a combined aryl sulfatase and β-glucuronidase preparation in a concentration of 300-500 units/ml of aryl sulfatase activity. The mixture was incubated at 37°C for three days, acidified to pH 1 with dilute hydrochloric acid, extracted with 1/3 volume of chloroform three times, and separated into sulfate acidic and sulfate neutral fractions.

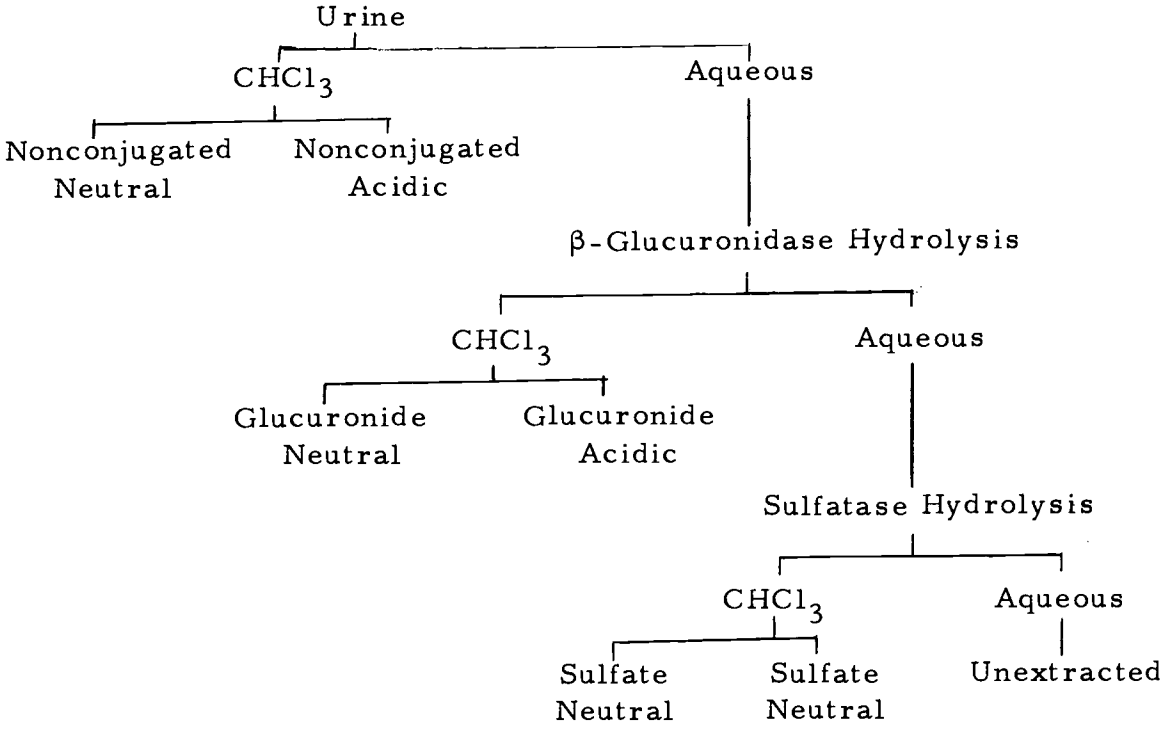


Figure 5. Stepwise fractionation of fecal extract, urine, or bile (from Buhler, Harpootlian and Johnston, 1966).

In certain studies the combined β-glucuronidase and aryl sulfatase preparation was used to cleave both the sulfate and glucuronide conjugates in one step.

Aliquots of certain conjugate samples and fractions were treated with the β-glucuronidase or the β-glucuronidase and aryl sulfatase preparation in the presence of saccharolactone (1×10^{-2} M), a known specific β-glucuronidase inhibitor (Levvy, 1952), to help elucidate the nature of the conjugates. In vitro experiments also showed that 1×10^{-3} M hexachlorophene only slightly inhibited (less

than 5%) the cleavage by β-glucuronidase (5000 units/ml) of p-nitrophenyl-β-D-glucuronide.

In later studies, a nonionic resin, Amberlite XAD-2, was employed to remove hydrophobic compounds from aqueous systems. This latter technique is much simpler and much more efficient than extraction with an organic solvent.

Extraction of Conjugates with Amberlite XAD-2

Amberlite XAD-2 was also used to extract intact radioactive conjugates from urine or bile. Washed resin was added to the bile or urine and stirred overnight at room temperature. The resin was filtered from the urine or bile, added to two bed volumes of methanol, and stirred four hours. The resin was filtered from the methanol and added to a new batch of fresh methanol. The procedure was repeated until no radioactivity was detected in the methanol filtrate of the resin. This procedure removed all the radioactivity from the urine or bile.

Thin Layer Chromatography

Silica gel GF-254 was used almost exclusively as the adsorbent since hexachlorophene was found to bind too tightly to alumina and cellulose. Samples were spotted or streaked onto the plates, and after developing in an appropriate solvent system the plates were dried and examined under short-wave ultraviolet light. Hexachlorophene,

2, 4, 5-trichlorophenol, and hexachlorophene derivatives absorbed ultraviolet light while the chlorinated salicylic acids gave a blue fluorescence. Sometimes the plates were sprayed with diazotized sulfanilic acid to detect phenols or with 0.1% bromphenol blue to detect acidic compounds (Buhler, Harpootlian, and Johnston, 1966). Iodine vapor was also used for general detection of compounds. Radioactive thin layer chromatograms were analyzed by scanning or scraping the plates as previously described.

Hexachlorophene was removed from the thin layer plates as soon as possible because it discolored to a brownish oil when exposed to light. Thin layer chromatography of the brownish oil on silica gel plates developed with toluene (saturated with formic acid) yielded mainly hexachlorophene at 0.80 Rf, a trace amount of a mixture of dechlorinated hexachlorophene components at 0.35 Rf, and a small amount of some unidentified products in a brownish oil at the origin. The mixture of dechlorinated hexachlorophene components was very rapidly discolored by light to a brownish oil. Shaffer et al. (1971) reported a similar degradation of hexachlorophene when it was exposed to ultraviolet light.

The solvent systems used for developing the thin layer plates and the chromatographic mobility of hexachlorophene and related compounds are presented in Table 2. Glucuronides were chromatographed using butanol:acetic acid:water (BAW 4:1:1 or 4:1:5) and isopropyl alcohol:

ammonia:water (PAW 8:1:1) as solvent systems. Glucuronide derivatives were chromatographed with benzene:acetone (9:1), 0.75% methanol in chloroform, and 0.50% methanol in dichloromethane.

Table 2. Silica gel thin layer chromatographic mobility of hexachlorophene and related compounds with various solvent systems.

Compound	Rf value in specific solvent system[a, b]			
	BHMW	TF	BBA	TEA
6, 7, 9-trichloro-1, 3-benzodioxane	.90	--	--	--
2, 4, 5-trichlorophenol	.80	.70	.40	.80
hexachlorophene	.50	.80	.60	.90
5-chlorosalicylic acid	--	.25	--	--
3, 5-dichlorosalicylic acid	.00	.29	.10	.60
3, 5, 6-trichlorosalicylic acid	.00	.35	.00	--
5-chlorosalicylaldehyde	--	.75	--	--
3, 5-dichlorosalicylaldehyde	--	.79	--	--
3, 5, 6-trichlorosalicylaldehyde	.80	.82	--	--

[a] $Rf = \frac{\text{distance compound moves}}{\text{distance solvent moves}}$

[b] Solvent systems abbreviations:
BHMW = benzene:hexane:methanol:water (7:3:5:5)
TF = toluene saturated with 88% formic acid
BBA = butanol:benzene:ammonia (1:1:1)
TEA = toluene:ethyl acetate:acetic acid (5:4:1)

Adsorption Column Chromatography

Extracts of the urine and feces from rabbits were separated on a column of silicic acid (BioSil A 100-200 mesh). The columns were prepared by stirring the silicic acid with the developing solvent and the resultant slurry poured into a 45 x 1.5 (ID) cm glass column equipped with a teflon stopcock. The adsorbent was added to a height of 30 cm and then three bedvolumes of developing solvent passed through it. An aliquot of the extracts was concentrated to dryness on a rotary evaporator, taken up in a minimal amount of dichloromethane, and applied in a thin layer to the top of the column. After applying the extract aliquot, the columns were developed at a rate of 0.5 ml/min and 2.0 ml fractions collected by a Gilson Volumetric Fractionator (Gilson Medical Electronics, Inc., Middleton, Wisconsin). Aliquots (0.1 ml) of the fractions were taken to dryness in individual counting vials, 10 ml of diotol was added, and they were counted in a liquid scintillation spectrometer.

Columns were prepared and developed with either benzene:hexane:methanol:water (BHMW 7:3:5:5) or n-butanol:benzene: ammonium hydroxide (BBA 1:1:1). Figure 6 shows the separation of hexachlorophene, 2, 4, 5-trichlorophenol acid 3, 5-dichlorosalicylic acid by silicic acid column chromatography with BHMW and BBA as developing solvents.

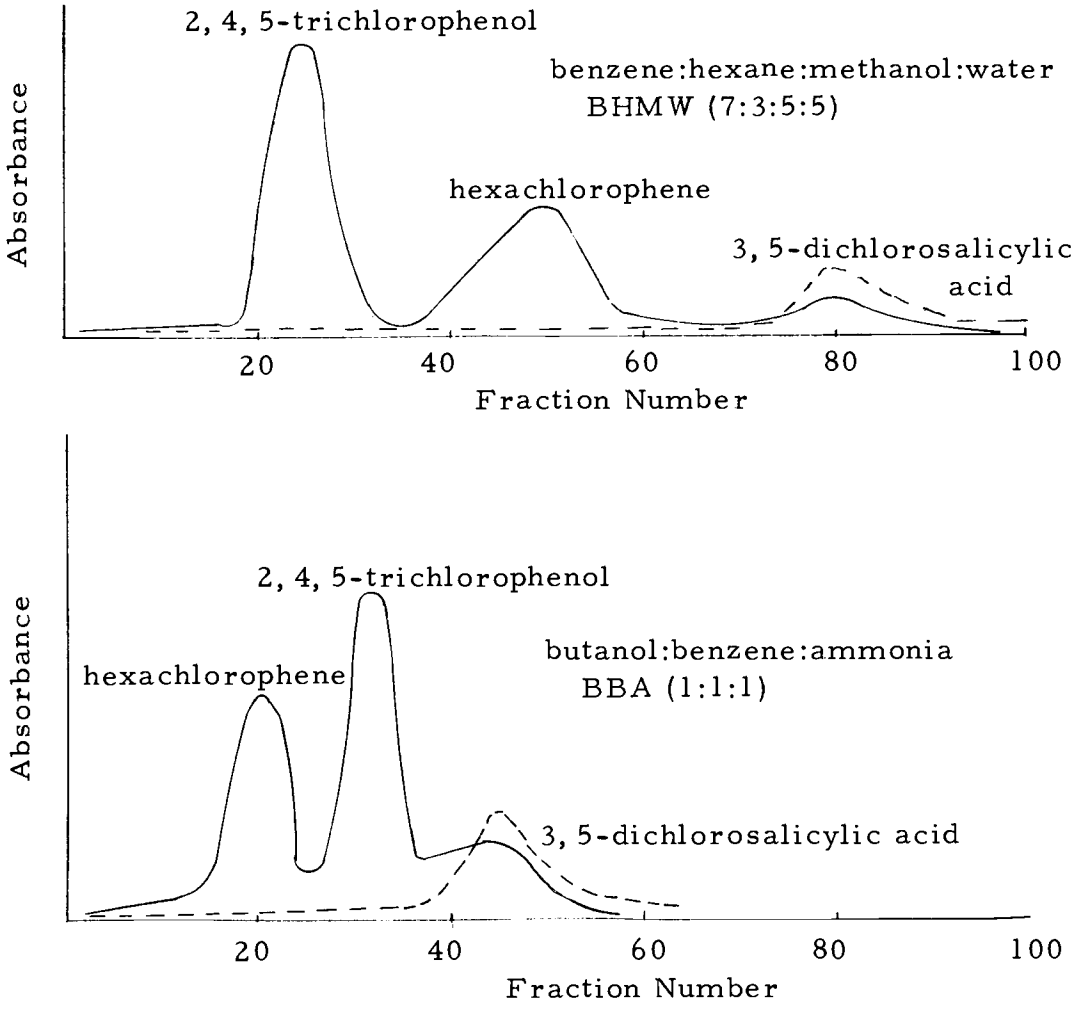


Figure 6. Replica of the separation of approximately 1 mg hexachlorophene, 1 mg 2, 4, 5-trichlorophenol, and 0.5 mg 3, 5-dichlorosalicylic acid by silicic acid column chromatography. Hexachlorophene and 2, 4, 5-trichlorophenol detected by absorption at 295 nm (——), and 3, 5-dichlorosalicylic detected by absorption at 321 nm (-----). Column was developed with benzene:hexane:methanol:water (7:3:5:5) BHMW or n-butanol:benzene:ammonia (1:1:1) BBA.

Gas Chromatography

Electron capture gas chromatography was used for the analysis of hexachlorophene, hexachlorophene metabolites, and ultraviolet photodegradation products of hexachlorophene. A Varian Aerograph model 2100 (Varian Aerograph, Walnut Creek, Calif.) with a Varian A-25 recorder and a 5 ft x 2 mm ID pyrex glass column packed with 7% QF-1 stationary phase on Chromsorb W (Hi Pref) 100/120 mesh support (Varian Aerograph, Walnut Creek, Calif.) was used for analyses. Prepurified nitrogen was used as the carrier gas at 25-30 ml/min flow rate. The detector was a Varian Electron Capture Detector containing a 1 cm^2 cylindrical tritium foil (250 mCi). The injection port was operated at 255°C and the detector at 225°C. The column temperature was operated at optimum conditions for the compound under analysis.

The instrument could detect as little as 25 picograms of hexachlorophene dimethylether, and under usual operating conditions 100 picograms of hexachlorophene dimethylether gave 30% of full scale deflection. The detector response for a series of chlorinated compounds was proportional to the chlorine content of the compound. Retention times and sensitivity varied with the age and amount of use of the column. Dilutions with benzene were normally employed to adjust the amount injected to be between 20-500 picograms of the compound.

Preparation of Samples for Gas Chromatography

Samples that were to be analyzed as their methyl derivatives on the gas chromatograph were prepared by evaporating an aliquot of the sample to dryness in a rotary evaporator or under a gentle flow of prepurified nitrogen, the dried residue was dissolved in a small volume of methanol or dichloromethane, and 0.5-1.0 ml of etherial diazomethane (0.5 millimole/ml) was added. The mixture was allowed to stand for 15 minutes, evaporated dryness under a gentle flow of nitrogen followed by addition of a measured amount of benzene.

Acetylation of samples was performed by treatment of the dried aliquot with 0.5 ml pyridine:acetic anhydride (1:1) either directly or dissolved in benzene or chloroform. The solutions were allowed to stand for 30-60 minutes , evaporated under a gentle flow of nitrogen, and the dried residue then dissolved in a measured amount of benzene.

Aliquots of samples to be analyzed unmethylated and unacetylated were dried in the same manner, taken up in benzene, and injected into the gas chromatograph.

For quantitative determination of the amount of a compound in an extract solution, standards were also chromatographed, the areas under the peaks measured and a standard curve prepared. The ratio of area under the peak to the picograms of standard injected was used to calculate the amount of material in the unknown sample.

The retention times of hexachlorophene and related compounds at various temperatures are listed in Table 3. The column temperature was varied, depending on which compound was under analysis. Optimum column temperature for analysis of hexachlorophene and hexachlorophene dimethylether was 200-210°C, while 2, 4, 5-trichlorophenol was best analyzed at 130°C.

Mass Spectra

The mass spectra of various compounds were obtained in a Varian MATS CH-7 spectrometer (Varian Aerograph, Walnut Creek, Calif.) equipped with a direct entry probe. Less than microgram quantities of samples were placed in the platinum cup of the probe and the probe was placed in the instrument. The instrument was evacuated and the sample ionized at 70 eV as the temperature was raised in increments to optimum volatility conditions. For glucuronides of hexachlorophene, spectra were taken rapidly at several temperature levels since the glucuronides, like pentachlorophenol glucuronide (Tashiro et al., 1970), decomposed rapidly under mass spectral conditions. Internal standards of perflurodecalin were used as an aid in determining the m/e ratio of the parent and fragment ions.

Compounds containing chlorine give characteristic parent and fragment ion patterns due to the abundance of several chlorine isotopes (Beyon, Saunders and Williams, 1968). Compounds containing six

Table 3. Electron capture gas chromatography retention times of chlorinated phenols, hexachlorophene, and related compounds with respect to column temperature.

Compound (abbreviation)	Retention time (minutes) Column temperature (°C) 100	130	160	210
monophenols				
2, 4-dichlorophenol methyl ether	1.4			
2, 4, 5-trichlorophenol methyl ether	3.6			
2, 4-dichlorophenol		0.6		
2, 4, 5-trichlorophenol		0.9	0.44	
monophenol derivatives				
3, 5, 6-trichlorosalicylaldehyde		2.40	0.82	
5-chlorosalicylic acid, methyl (ester and ether)	2.4	1.04		
3, 5-dichlorosalicylic acid, methyl (ester and ether)	9.2	2.70		
6, 8, 9-trichloro-1, 3-benzodioxane		3.74	1.20	
bisphenols				
2, 2'-methylenebis-(4-chlorophenol) dimethylether				2.15
2, 2'-methylenebis-(4, 6-dichlorophenol) dimethylether				2.50
2, 2'-dihydroxy-3, 3', 5, 6, 6'-pentachlorodiphenylmethane dimethylether				3.1
2, 2'-methylenebis-(3, 4-dichlorophenol) dimethylether				4.3
hexachlorophene dimethylether				5.0
hexachlorophene				7.6
hexachlorophene diacetylester				11.2

chlorines or three chlorines give the m/e ion patterns in Figure 7. The characteristic chlorine patterns made the m/e of hexachlorophene, hexachlorophene derivatives, and their fragment ions relatively easy to detect in the mass spectra.

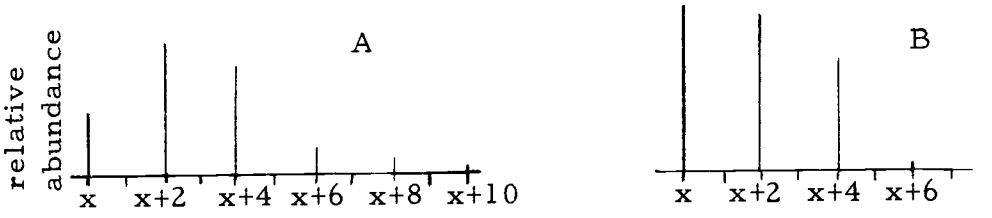


Figure 7. Characteristic m/e ion patterns of compounds containing six chlorines (A) or three chlorines (B).

The complete mass spectrum of hexachlorophene is presented in Figure 8. The major ions are a three chlorine fragment at 196 m/e and two overlapping three chlorine fragments at 280 m/e resulting from the cleavage of the methylene bridge. The six chlorine parent ion is less abundant and starts at 404 m/e.

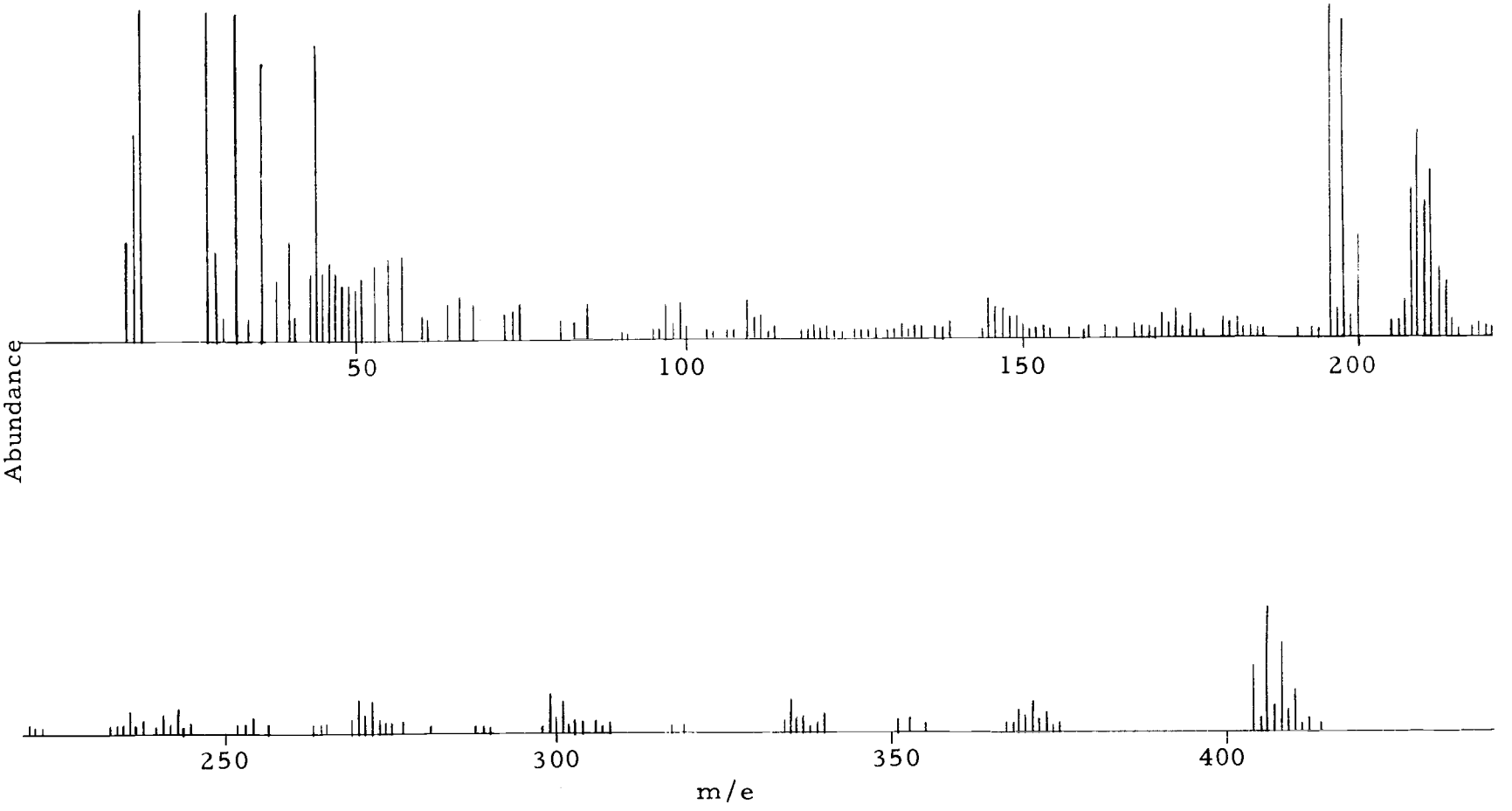


Figure 8. Mass spectrum of hexachlorophene.

RESULTS. PART I: ABSORPTION, EXCRETION, AND BIOTRANSFORMATION OF HEXACHLOROPHENE IN THE RAT

Tritiation of Hexachlorophene

Recognizing that hexachlorophene-^{14}C could be metabolized in the animal by a cleavage of the methylene bridge and a loss of the radioactive label as $^{14}CO_2$ or some other small ^{14}C-labeled molecule, many attempts were made to incorporate a tritium label into the aromatic rings of hexachlorophene. Tritium labeled hexachlorophene would be advantageous for metabolic studies since both fragments of any methylene bridge cleavage process could be detected. Moreover, incorporation of tritium into hexachlorophene would also result in a labeled drug with a higher specific activity.

Prior to using tritium, the exchange methods were tested using deuterium. The products from the various exchange reactions were analyzed at a concentration of 25 mg HCP/ml deuterated chloroform by nuclear magnetic resonance (nmr) in a Varian Model HA-100, nmr spectrometer, Varian Aerograph Co., Walnut Creek, Calif. Since deuterium does not give an nmr signal, the disappearance of the aromatic proton signal at 7.3 ppm was used as a measure of any appreciable incorporation of deuterium.

The following exchange methods were attempted with no detectable incorporation of deuterium into the hexachlorophene molecule:

a. Deuterated trifluoroacetic acid and platinum oxide. Refluxed 21 hours and recrystallized (Garnett, 1962).

b. Same as in (a) but without recrystallization.

c. Phosphoric acid and boron trifluoride (Hilton and O'Brien, 1964). Room temperature and recrystallization.

d. Same as (c), but at 60°C.

e. Fuming sulfuric acid (20%) and deuterated water (Newton, Montgomery and Vernetti, 1963).

f. Dioxane and potassium hydroxide and deuterated water (Jones, 1968).

g. Dimethyl formamide, potassium hydroxide, and deuterated water (Jones, 1968).

h. Sodium ethoxide and deuterated trifluoroacetic acid (Jones, 1968).

i. Sodium ethoxide and deuterated water (Jones, 1968).

Attempts to deuterate precursors in the synthesis of hexachlorophene, 2, 4, 5-trichlorophenol and 1, 2, 4, 5-tetrachlorobenzene by exchange with deuterated trifluoroacetic acid and platinum oxide also proved unsuccessful (Garnett, 1962).

Since nmr spectroscopy could only detect an appreciable incorporation of deuterium, it was decided to try tritiation directly because the use of radioactive label provided a more sensitive means of identifying incorporation into the hexachlorophene molecule. The

following exchange methods were tested with hexachlorophene using tritiated water with a specific activity of 1 mCi/ml:

a. Fuming sulfuric acid (20%) and tritiated water (Newton, Montgomery and Vernetti, 1963). No incorporation.

b. Trifluoroacetic anhydride, tritiated water and platinum oxide. Trace incorporation (0.001%).

c. Tritiated water and platinum oxide (Garnett, 1962). No incorporation.

d. Aluminum trichloride, tritiated water, and dichloromethane (Evans, 1966). No incorporation.

e. Trifluoroacetic anhydride, tritiated water, and platinum oxide in sealed tube and refluxed for four days. Not recrystallized. Slight incorporation (0.05%).

Tritiation of the hexachlorophene molecule by exchange procedures, thus appears to be very difficult. Commercially available hexachlorophene-^{14}C (Figure 2) was, therefore, used throughout this study.

Isolation and Fractionation of Urine and Fecal Radioactivity

Eight male 200 g Wistar rats were given 5 mg/kg oral and intraperitoneal doses of hexachlorophene-^{14}C (0.92 mCi/mmole) dissolved in propylene glycol and the urine and feces collected over a seven day period (Buhler, Dost and Rasmusson, 1972). The 0-2

day frozen urine and feces from this experiment (Figure 4) was thawed and pooled with respect to method of administration. The four pooled fractions contained: intraperitoneal urine 5.80% and feces 41.1% and oral urine 3.41% and feces 63.4% of the administered radioactive dose.

Pooled feces was dried in vacuo over P_2O_5. The dried feces was placed in a soxhelet apparatus and extracted with anhydrous methanol for 12 hours followed by addition of a fresh batch of methanol and an additional 12 hours extraction. This method removed essentially all the radioactivity from the solid fecal residue. The methanol extract was taken to dryness at 30°C in a rotary evaporator, and the evaporated extract then redissolved in water. There was no loss of radioactivity by this process.

The aqueous fecal extracts and the urine were separated into conjugated and nonconjugated fractions by extraction with chloroform and enzymatic hydrolysis with a combined β-glucuronidase and aryl sulfatase preparation. Further fractionation into acidic and neutral compounds gave the fractional distribution of radioactivity shown in Table 4.

The bulk of the radioactivity appeared in the acidic fractions in both the urinary and fecal extracts from rats that received hexachlorophene-^{14}C by either oral or intraperitoneal administration. In both cases, conjugated ^{14}C metabolites of hexachlorophene appeared to be

Table 4. Percent of radioactive dose in 0-2 day pooled urinary and fecal fractions from rats receiving 5 mg/kg methylene-^{14}C-hexachlorophene. Values in parentheses are the percent of the total combined 0-2 day urinary and fecal radioactivity.

Fraction	Percentage of Dose			
	Intraperitoneal Administration		Oral Administration	
	Neutral	Acidic	Neutral	Acidic
Urine				
Nonconjugated	0.09 (0.18)	0.69 (1.47)	0.06 (0.09)	1.13 (1.69)
Conjugated	0.05 (0.11)	2.62 (5.59)	0.04 (0.05)	1.26 (1.89)
Unextracted	2.25 (4.80)		0.65 (0.97)	
TOTAL	5.70 (12.15)		3.14 (4.69)	
Feces				
Nonconjugated	3.01 (6.41)	33.25 (70.93)	8.87 (13.25)	50.72 (75.95)
Conjugated	1.27 (2.70)	3.04 (6.48)	0.25 (0.37)	1.94 (2.90)
Unextracted	nil		nil	
TOTAL	40.58 (86.52)		61.11 (92.47)	

the main radioactive component of urine, while nonconjugated metabolites predominated in the feces. A higher percentage of nonconjugated acidic metabolites were present in the urine of rats receiving orally administered hexachlorophene as compared to the intraperitoneally treated animals. The significant amount of unextracted radioactivity in the urine may be explained by incomplete enzymatic hydrolysis of the conjugates due to naturally occurring inhibitors in the urine or possibly inhibition by hexachlorophene that has been released by hydrolysis of its conjugate. The radioactivity in the neutral fecal fraction could result from ionized hexachlorophene being extracted into chloroform during the fractionation procedure, as has been shown to occur in the tissue extraction studies of Buhler, Dost, and Rasmusson (1972).

Analysis of Urine and Fecal Extraction

Following fractionation of the pooled urine and feces, the various fractions were concentrated in vacuo to a small volume and analyzed by thin layer and gas chromatography and mass spectroscopy.

Thin layer chromatography of the concentrated extracts was performed using TEA and BHMW as the solvent systems. The distribution of radioactivity in the various extract fractions on silica gel thin layer plates after development with TEA is shown in Table 5. This table summarizes the results from a number of thin layer

Table 5. Silica gel thin layer chromatography of urinary and fecal fractions from rats receiving oral or intraperitoneal administration of hexachlorophene-^{14}C. (+ indicates relative level of radioactivity.)

	Relative Level of Radioactivity		
	Rf value[a, b]		
Route/sample	origin-0.2	0.4-0.6	0.8-1.0
Oral/Urine			
unconjugated acidic			+
unconjugated neutral	trace		trace
conjugated acidic			+
conjugated neutral	trace		
Oral/Feces			
unconjugated acidic			+++++
unconjugated neutral			++
conjugated acidic		trace	+
conjugated neutral			trace
Intraperitoneal/Urine			
unconjugated acidic	trace		+
unconjugated neutral			trace
conjugated acidic			++
conjugated neutral		trace	
Intraperitoneal/Feces			
unconjugated acidic	trace	trace	+++++
unconjugated neutral	trace		++
conjugated acidic	trace		++
conjugated neutral			+
Hexachlorophene-^{14}C std.			+++++

[a]All radioactivity in 0.8-1.0 Rf zone identified by gas chromatography as hexachlorophene.

[b]Toluene: ethyl acetate:acetic acid (5:4:1) was the developing solvent system.

chromatographs in which the radioactivity was detected either by use of a radiochromatogram scanner or by scraping the plates and counting the gel in a conventional manner in a liquid scintillation counter. The major radioactive area on the thin layer plates of each fraction has the same mobility as authentic hexachlorophene. Analysis of the fractions by thin layer chromatography in BHMW also showed that the major radioactive component in all fractions had the same mobility as the hexachlorophene standard. In some cases with the BHMW system a small amount of radioactivity was also detected at the origin. It appears that the procedure for the separation of urine and fecal extracts into acidic and neutral fractions was not complete since a component with the mobility of hexachlorophene on thin layer chromatograph was also found in neutral fractions.

The radioactive zones for each fraction of the TEA developed thin layer plates were scraped, eluted and analyzed by gas chromatography. In all cases, analysis of the 0.8-1.0 Rf radioactive areas by gas chromatography after methylation with diazomethane showed only hexachlorophene dimethylether. All other minor radioactive areas on the thin layer plates of the various fractions were also analyzed by gas chromatography for the presence of hexachlorophene, but none was detected. Furthermore, no unique gas chromatographic peak could be associated with any of these minor radioactive components.

The acidic fractions of the urine and feces were pooled with respect to their method of administration and cleaned up by preparative thin layer chromatography with TEA. The radioactive region from each fraction chromatographing as hexachlorophene-^{14}C was eluted with methanol and precipitated by the addition of water, and mass spectral analyses were performed on each of the fractions. In both the oral and the intraperitoneal study, mass spectral analysis yielded a spectrum identical to that of a hexachlorophene standard with the molecular ion having a six chlorine pattern at 404 m/e and the major fragment ions having three chlorine patterns at 196 and 208 m/e. Thus hexachlorophene, either conjugated or nonconjugated, was essentially the only component in the urine and feces of rats dosed by either route of administration.

The occasionally found trace component that chromatographed on silica gel thin layer plates developed with TEA with an Rf of 0.4-0.6 was initially thought to be 3, 5, 6-trichlorosalicylic acid. This compound could result from the hydrolytic cleavage of the methylene bridge of hexachlorophene-^{14}C, which would have yielded the radioactive salicylic acid component and a nonradioactive 2, 4, 5-trichlorophenol or catechol fragment. Mass spectral analysis of the 0.4-0.6 Rf radioactive zones from the thin layer plates did not give a discriminate chlorinated ion pattern either at the m/e of the suspected chlorinated salicylic acid or at any other m/e. Gas chromatographic analysis of

the fractions containing the trace component did not indicate the presence of 2, 4, 5-trichlorophenol, 3, 5, 6-trichlorosalicylic acid, or other known standards. Since there was only trace amounts of the minor radioactive component (0. 08% of dose) present in the urine and fecal extracts, further isolation and analysis of this unknown component were not feasible.

Additional identification of the radioactivity remaining at the origin during thin layer chromatography with either TEA or BHMW was not performed since it too represented only a small trace of the radioactivity on the plate. This radioactivity could represent hexachlorophene conjugates that were not cleaved by the enzyme hydrolysis procedure but which were carried along with the other organic molecules by the extraction process. Both the trace component at the origin and the one found at 0. 4-0. 6 Rf could also be photodegraded hexachlorophene resulting from the interaction of hexachlorophene with light during isolation and chromatography.

Analysis of Urine and Feces from Rats Receiving Repeated Doses of Nonlabeled Hexachlorophene

An attempt was made to increase the extent of hexachlorophene metabolism in the rat by treating animals with repeated doses of hexachlorophene. Male rats were given an intraperitoneal dose of 5 mg/kg of nonlabeled hexachlorophene in corn oil and placed in metabolism

cages. This dose was followed at three-day intervals by two successive 10 mg/kg intraperitoneal doses of nonlabeled hexachlorophene. Control rats receiving only intraperitoneal administration of corn oil were also placed in metabolism cages. Urine and feces were collected continuously from both sets of animals and pooled with respect to control and hexachlorophene treatment. Both sets of feces were extracted and then the fecal extract and the urine were subjected to enzymatic hydrolysis with a combined β-glucuronidase and aryl sulfatase preparation. After hydrolysis, the acidified fecal extract and urine were extracted with chloroform and the chloroform extract concentrated to a small volume.

The concentrated extracts were subjected to thin layer chromatography, and the metabolite zones corresponding in Rf values to those of standard hexachlorophene and 2, 4, 5-trichlorophenol were scraped, eluted, and analyzed by gas chromatography. On methylation of the major zones, only hexachlorophene dimethylether was found. Low temperature gas chromatography did not reveal the presence of any trichlorophenol or other suspected metabolites for which standards had been prepared. Gas chromatography and mass spectroscopy also failed to detect any 3, 5, 6-trichlorosalicylic acid in the urine and fecal extracts.

No evidence was obtained from this study that hexachlorophene induced liver drug metabolizing enzyme activity, resulting in an

increased oxidative metabolism of hexachlorophene.

Analysis of Feces of Rats Fed Hexachlorophene in the Diet

In another attempt at detecting possible trace metabolites of hexachlorophene, feces from rats receiving 100-400 ppm of hexachlorophene in their diet (Nakaue, Dost, and Buhler, 1972) was collected daily and frozen. After a month's collection the feces was thawed and hydrolyzed with hydrochloric acid. The hydrolyzed feces was extracted with chloroform, and the extract separated into acidic and neutral fractions. Thin layer chromatography of the acidic fraction yielded large quantities of a component that chromatographed like hexachlorophene. This material was isolated and recrystallized to yield a product with an identical melting point to that of authentic hexachlorophene. Gas chromatographic analysis of this purified material also confirmed its identification as hexachlorophene. No 2, 4, 5-trichlorophenol was detected upon gas chromatography of the acidic extract. While there were other peaks in the chromatograms, none of them corresponded to known retention times of authentic standards. Mass spectral analysis failed to show the presence of any 3, 5, 6-trichlorosalicylic acid.

The neutral extract fraction was examined by thin layer chromatography and a small amount of hexachlorophene was detected. Many components that absorbed ultraviolet light were also present in the

neutral fraction but none of these substances correspond to known standards.

Prolonged feeding of hexachlorophene in the diet of rats does not appear to result in an increased extent of its metabolism. Hexachlorophene is primarily excreted either as the intact drug or as a conjugate. The feces is the principle route of excretion of administered hexachlorophene from the rat and contains mainly unchanged hexachlorophene with a small amount of hexachlorophene conjugates. A minor excretion of radioactivity in the urine after administration of labeled hexachlorophene is primarily a hexachlorophene conjugate.

Biliary Excretion of Hexachlorophene-^{14}C Radioactivity

The anthelmintic properties of hexachlorophene and the appearance of hexachlorophene mainly in the feces after administration of the bisphenol suggests that a marked biliary excretion occurs in the animal. To examine the extent of biliary excretion of hexachlorophene in the rat, studies were next carried out on bile duct cannulated animals. Male Wistar rats were anesthesized with a 20 mg/kg intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Ill.). The abdomen and the nape of the neck were shaved, and a two centimeter median incision was made on the abdomen. The common bile duct was isolated and tied off at the duodenal end. The bile duct was cut halfway through with an iris scissors, and

the beveled end of a 30 cm piece of polyethylene 10 tubing (Intermedic, Clay-Adams, Parsippany, N. J.) was inserted in the cut, pushed up the bile duct almost to the liver, and tied in place. The other end of the tubing was taken through the side of the abdominal wall and under the skin to the scruff of the rat's neck, where it was pulled out through a small incision in the skin. By passing the cannula out from the nape of the neck, the rat could not reach the tubing and chew through it. The abdominal incision was sutured with surgical silk, and the rat allowed to recover for 12 to 24 hours.

After recovery, the rat was given a 5 or 10 mg/kg intraperitoneal injection of hexachlorophene-^{14}C (72.8 μCi/mmole) in corn oil, placed in a restrictive plexiglass cage, and the bile collected for appropriate time intervals. The rat was allowed food and water ad libitum. The water contained 0.25% (w/v) of sodium chloride to replace the salts removed in the bile.

Aliquots of the collected bile were analyzed for radioactivity and the evidence for biliary excretion of hexachlorophene is presented in Figure 9. For each dose level, two 250 g and two 400 g rats were used. There was no difference in radioactivity excretion rates with respect to the size of the animal. In three days, the animals treated with 5 mg/kg excreted 31% of the radioactive dose via the bile, while the rats given 10 mg/kg excreted substantially more of the radioactive dose (47%) in two and a half days. Animals receiving the higher dose

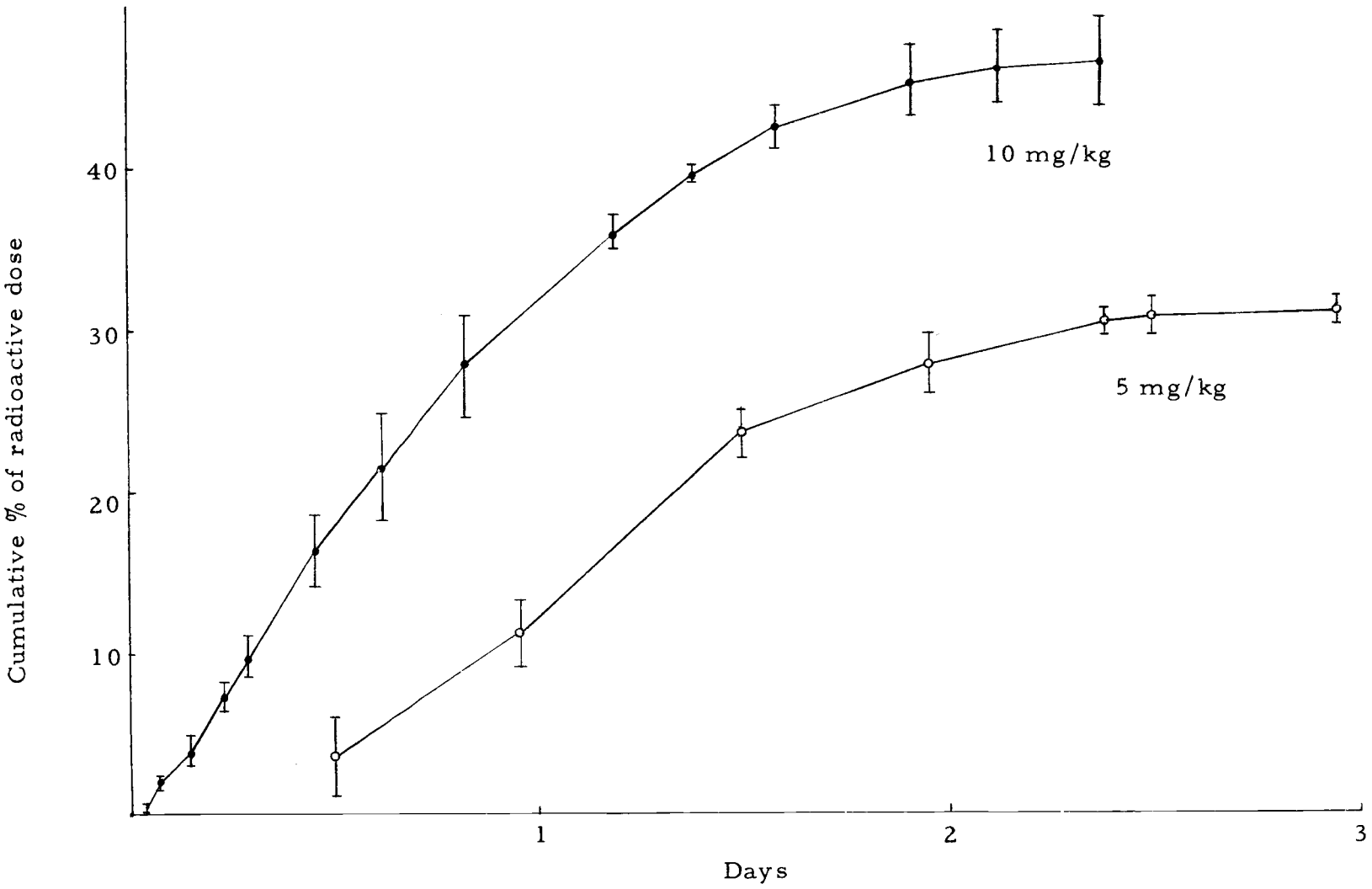


Figure 9. Excretion of radioactivity in the bile of bile cannulated rats receiving intraperitoneal administration of ^{14}C-hexachlorophene. Value represent the mean ± standard deviation of four male rats (two-250 g and two 400 g).

tended to excrete the radioactivity into the bile at a faster rate than the rats given a lower dose. The 31% of the dose appearing in the bile of rats receiving 5 mg/kg does not account for the 63% recovery in the feces of normal rats observed after an intraperitoneal dose of ^{14}C-labeled hexachlorophene (Buhler, Dost and Rasmusson, 1972). Such differences might result from variations in the biliary excretion rates of the surgically treated animals as compared to normal animals, or it might indicate the presence of extra-biliary excretion of the radioactive compounds into the gastroenteric tract. From the preceding metabolic evidence, the radioactive compounds excreted by the bile must either be labeled hexachlorophene or a hexachlorophene conjugate, possibly a glucuronide.

Analysis of the Biliary Radioactivity

The bile samples collected from the bile duct cannulated rats that had been given a 10 or 15 mg/kg intraperitoneal dose of hexachlorophene-^{14}C were pooled with respect to the animal. The pooled bile was extracted with ethyl acetate and the resulting extracts subjected to thin layer chromatography in the TF system. In all cases, a major component containing 95% of the applied radioactivity remained at the origin of the plate and a second component with the remaining radioactivity migrating to the same Rf as authentic hexachlorophene. Since an analysis of the various fractions by thin layer chromatography

indicated that the bile extract from all the animals contained similar mixtures of hexachlorophene metabolites, all the bile fractions were pooled. The pooled extract was treated with Amberlite XAD-2 non-ionic resin to completely remove all radioactivity. The resin was washed thoroughly free from salts with water and the hexachlorophene- and metabolites then eluted with methanol. Thin layer chromatographic analysis of the methanol eluate with either TF or BBA as the solvent showed about 97% of the applied radioactivity remaining at the origin, while the rest had a mobility identical to hexachlorophene. With BAW (4:1:1) as the thin layer solvent only one large area of radioactivity was noted with an Rf of 0.8 and a trace amount at an Rf of 0.2-0.5. The methanolic extract was also analyzed by gas chromatography. Nonmethylated samples showed only a single minor component with a retention time of hexachlorophene. Methylated samples and methylated samples that were acid-hydrolyzed contained a minor component with the retention time of hexachlorophene dimethylether and a second component which is believed to be the monomethyl ether of hexachlorophene on the basis of retention time. The monomethyl derivative of hexachlorophene probably resulted from either the methylation of the single free phenolic group of hexachlorophene monoconjugate with subsequent cleavage by the temperature of the gas chromatograph or the acid hydrolysis to yield the monomethyl product. Acid hydrolyzed samples that were treated with diazomethane after hydrolysis gave

only a hexachlorophene dimethylether component.

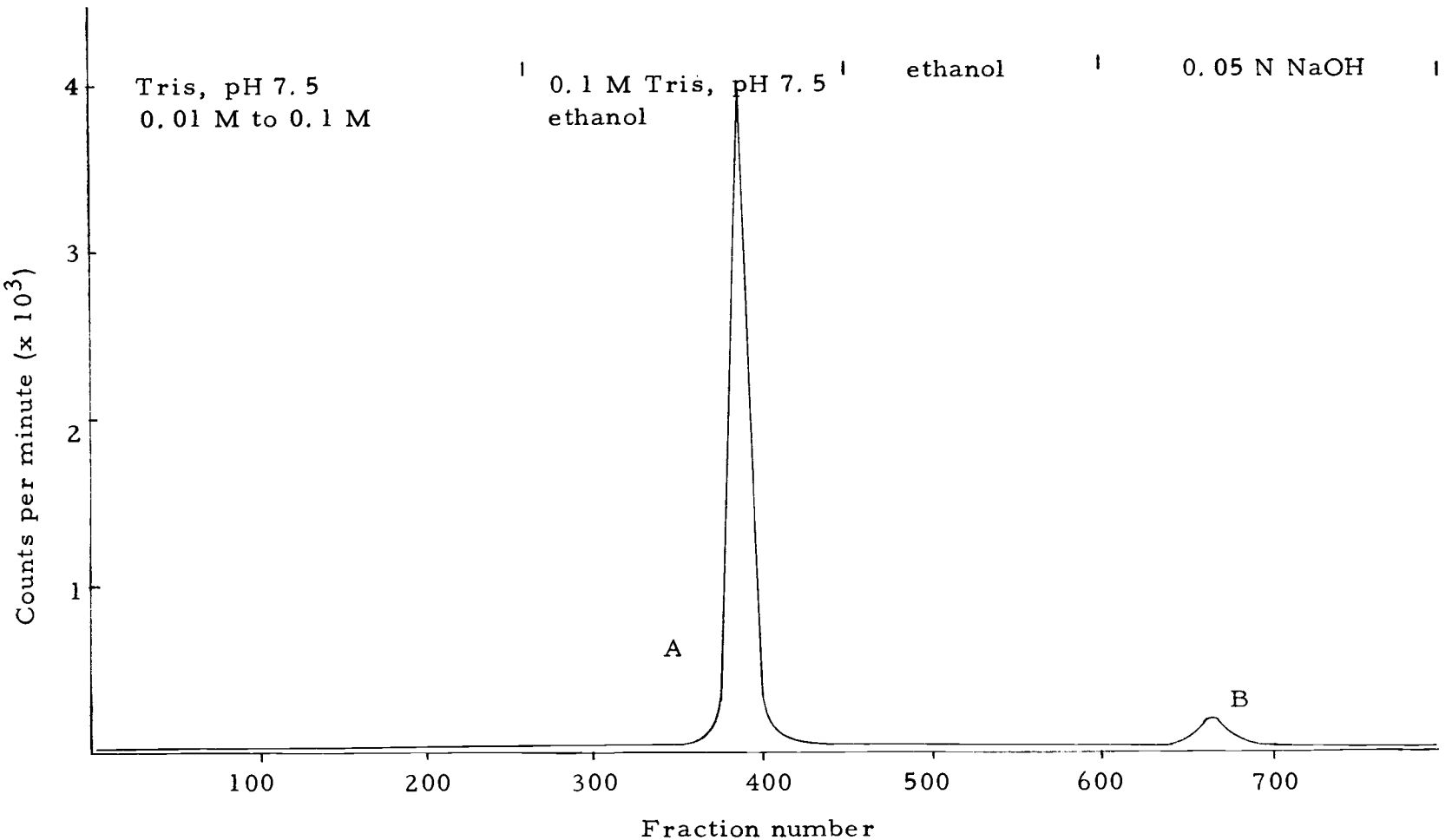
From the thin layer and gas chromatographic analysis of the bile samples, it appears that the major form of biliary radioactivity was not hexachlorophene but an acid labile hexachlorophene conjugate.

DEAE-Cellulose Chromatography of the Bile Extract

All the methanol eluate from the Amberlite XAD-2 resin extraction of the rat bile was concentrated to approximately 1 ml (2×10^6 dpm) and applied to the top of a DEAE-cellulose column, equilibrated with 0.01 M Tris-HCl, pH 7.5. The column was eluted by the procedure of Knaak et al. (1965) with a final 0.05 N sodium hydroxide wash necessary to recover all the radioactivity (Figure 10). Two peaks resulted. Peak A contained 92% of the applied radioactivity and peak B the remaining 8%. The peak fractions were pooled into two fractions, neutralized, and concentrated. The concentrates were treated with Amberlite XAD-2 to remove the radioactivity, and the resin washed with water to remove salts and then eluted with methanol.

Peak B was identified by gas chromatography and mass spectroscopy as hexachlorophene. Aliquots of peak A were subjected to enzymatic hydrolysis by β-glucuronidase and aryl sulfatase preparation with and without the addition of the 1,4-saccharolactone, a specific β-glucuronidase inhibitor (Levvy, 1952). After appropriate incubation,

Figure 10. Chromatography of the Amberlite XAD-2 resin extract of bile from rats treated with hexachlorophene-^{14}C on DEAE-cellulose. The column was prepared and eluted by the procedure of Knaak et al. (1965). The column was made by pouring a slurry of 6 g of DEAE-cellulose in 300 ml of 0.01 M Tris-HCl buffer, pH 7.5, into a 1.5 x 45 cm glass column and allowing it to drain freely. The column was washed overnight with 0.01 M Tris-HCl, pH 7.5, and the effluent checked the following day to see if it was pH 7.5. The extract was concentrated to a small volume and applied to the top of the column in a narrow band. The column was developed with a linear gradient of Tris-HCl, pH 7.5, from 0.01 to 0.1 M. This was followed by a gradient of 0.1 M Tris-HCl, pH 7.5, to 100% ethanol, 100% ethanol for 150 fractions, and finally a 0.05 N sodium hydroxide wash. Four ml fractions were collected, and 0.1 ml aliquots taken every five fractions for measurement of radioactivity.


Tris, pH 7.5
0.01 M to 0.1 M
0.1 M Tris, pH 7.5
ethanol
ethanol
0.05 N NaOH
Counts per minute (x 10³)
4
3
2
1
A
B
100
200
300
400
500
600
700
Fraction number

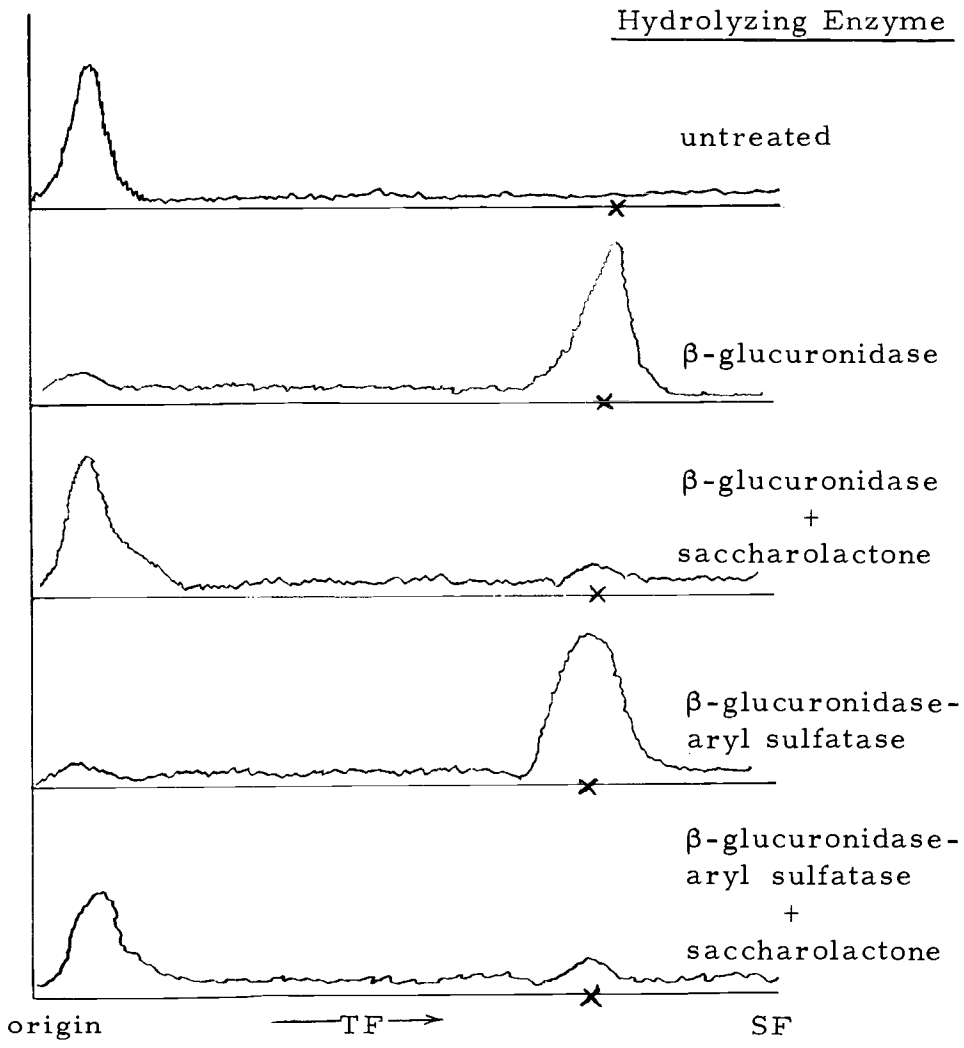


Figure 11. Replica of radiochromatograms from thin layer chromatographic analysis of enzyme hydrolyzed peak A aliquots (Figure 10). X = Rf of hexachlorophene-^{14}C standards. Plates were developed with toluene saturated with 88% formic acid (TF).

the hydrolysates were extracted and analyzed by thin layer chromatography (Figure 11). The hexachlorophene conjugate was apparently a glucuronide, since β-glucuronidase cleaved the conjugate but not in the presence of saccharolactone. Aryl sulfatase, with its associated β-glucuronidase activity inhibited, failed to hydrolyze the conjugate.

The mass spectra of peak A had overlapping chlorine patterns at 579 m/e (Figure 12) and minor fragment ions at 404 (six chlorine pattern), 177, and 175 (unchlorinated patterns) m/e. The spectra has been interpreted to be that of the monoglucuronide of hexachlorophene ($C_{19}H_{14}O_8{}^{35}Cl_6$, MW 580.13). Ionized and unionized forms of the acidic sugar moiety would yield overlapping six chlorine patterns of the parent ion at 579 m/e, while the 404 m/e chlorinated fragment is associated with hexachlorophene, and the fragment ions at 176 and 177 m/e are probably the glucuronic acid ions.

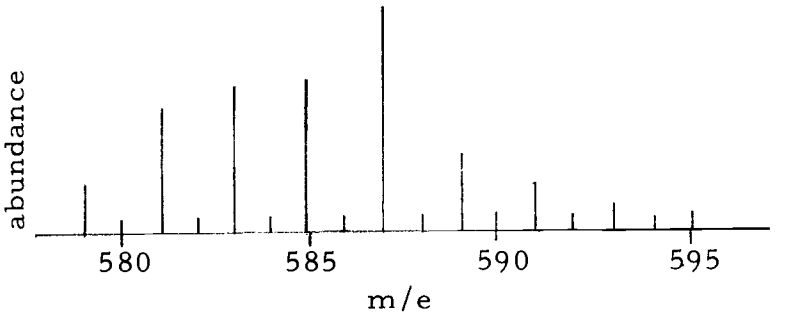


Figure 12. Pattern of peak A parent ion.

Following administration of 5 mg/kg and 10 mg/kg doses of hexachlorophene-^{14}C to the rat, 31 and 47%, respectively, of the initial radioactivity was excreted in the bile in three days. About 92% of the radioactivity recovered in the bile at both dose levels was identified as hexachlorophene monoglucuronide. Thus 28.5 and 43.3% of the radioactive dose, respectively, was eliminated in the bile over a three day period as hexachlorophene monoglucuronide. Neither the feces or urine of hexachlorophene-^{14}C dosed rats have been found to contain this concentration of hexachlorophene conjugate. Evidently, the glucuronide conjugate is cleaved in the gasterointestinal tract by bacterial action prior to its excretion from the animal.

Reabsorption of Biliary Hexachlorophene-^{14}C Radioactivity

Certain phenols related to hexachlorophene are known to undergo enterohepatic circulation (Smith, 1966). To study the possible reabsorption of biliary radioactivity derived from hexachlorophene-^{14}C, two male rats were surgically linked together (Figure 13). The free end of the cannula from a bile cannulated rat (A) was inserted into a small puncture hole in the duodenum of another bile cannulated rat (B) and sutured in place. All tubing was taken under the skin and through an incision in the skin at the nape of the neck, so the animals could not reach the tubing. The incisions were sutured, the rats placed in restrictive cages, and they were allowed to recover for 12-24 hours.

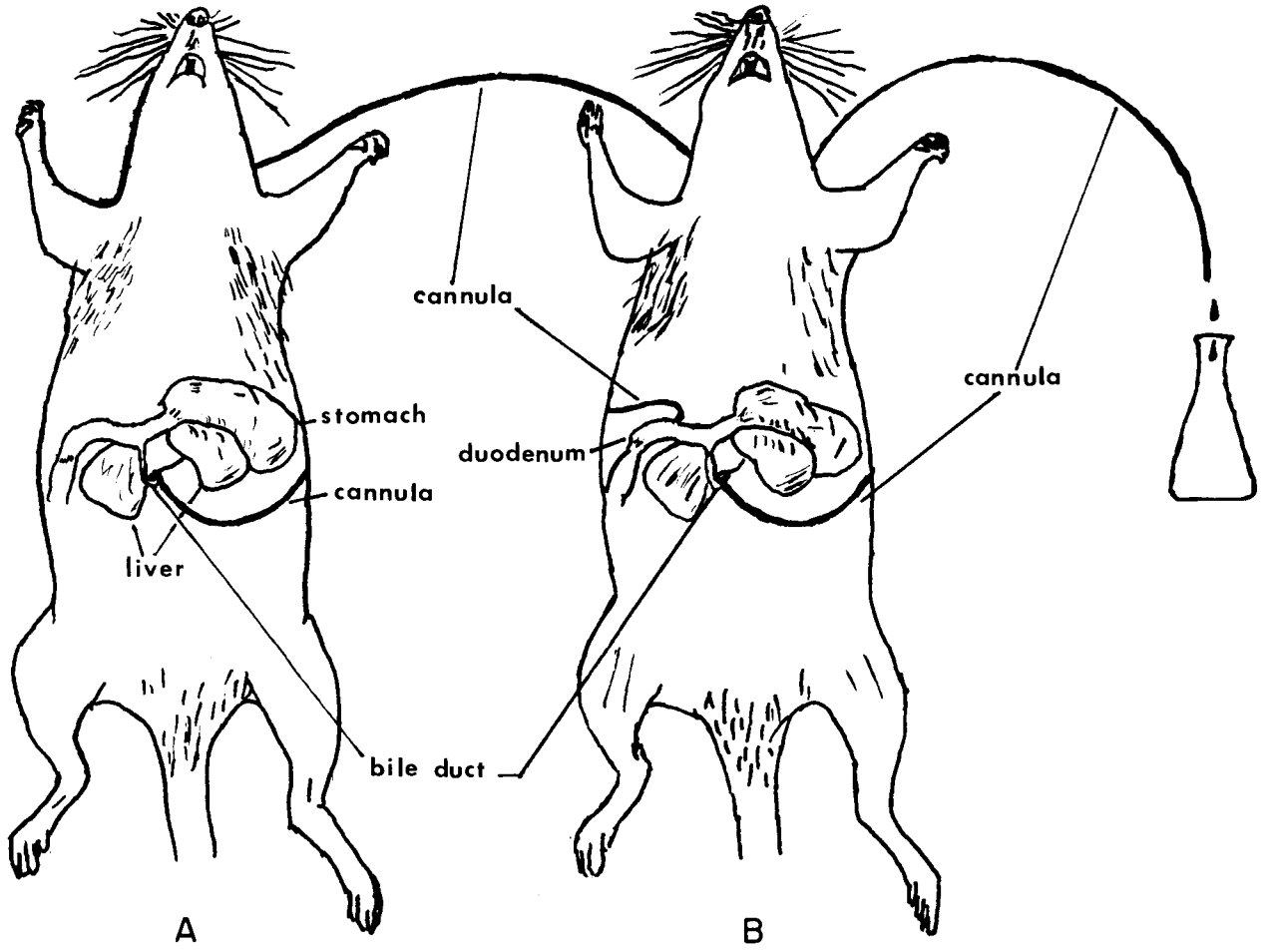


Figure 13. Diagram of the surgical preparation of two rats to study biliary excretion and enterohepatic circulation.

After recovery, a 5 mg/kg intraperitoneal injection of hexachlorophene-^{14}C (72.8 μCi/mmole) in corn oil was administered to rat A. Bile was collected for appropriate time intervals from rat B, and aliquots were removed from the collected fractions and analyzed for radioactivity. The biliary excretion of radioactivity is presented in Figure 14. The dashed line, as determined from a previous study (Figure 9), represents the theoretical amount of the radioactivity that rat A should be excreting into rat B. The biliary excretion curve for rat B was almost identical to the theoretical curve except for a slight time lag.

Approximately 31% of the radioactive dose from rat A should have been excreted into the duodenum of rat B (Figure 9), whereas 28.5% of the original radioactive dose was recovered in the bile of rat B (Figure 14). These results confirm an extensive reabsorption of biliary radioactive components from the gastrointestinal tract.

Amberlite XAD-2 resin extracts of pooled bile fractions from rat B were analyzed and compared to the hexachlorophene monoglucuronide previously isolated. The extract contained only one radioactive component which chromatographed on thin layer plates developed with BAW (4:1:1) and PAW (8:1:1) in a manner identical to that of the hexachlorophene monoglucuronide isolated by the DEAE-cellulose column. Thin layer chromatography of hydrolyzed bile extract showed that the radioactive metabolite was cleaved by only β-glucuronidase,

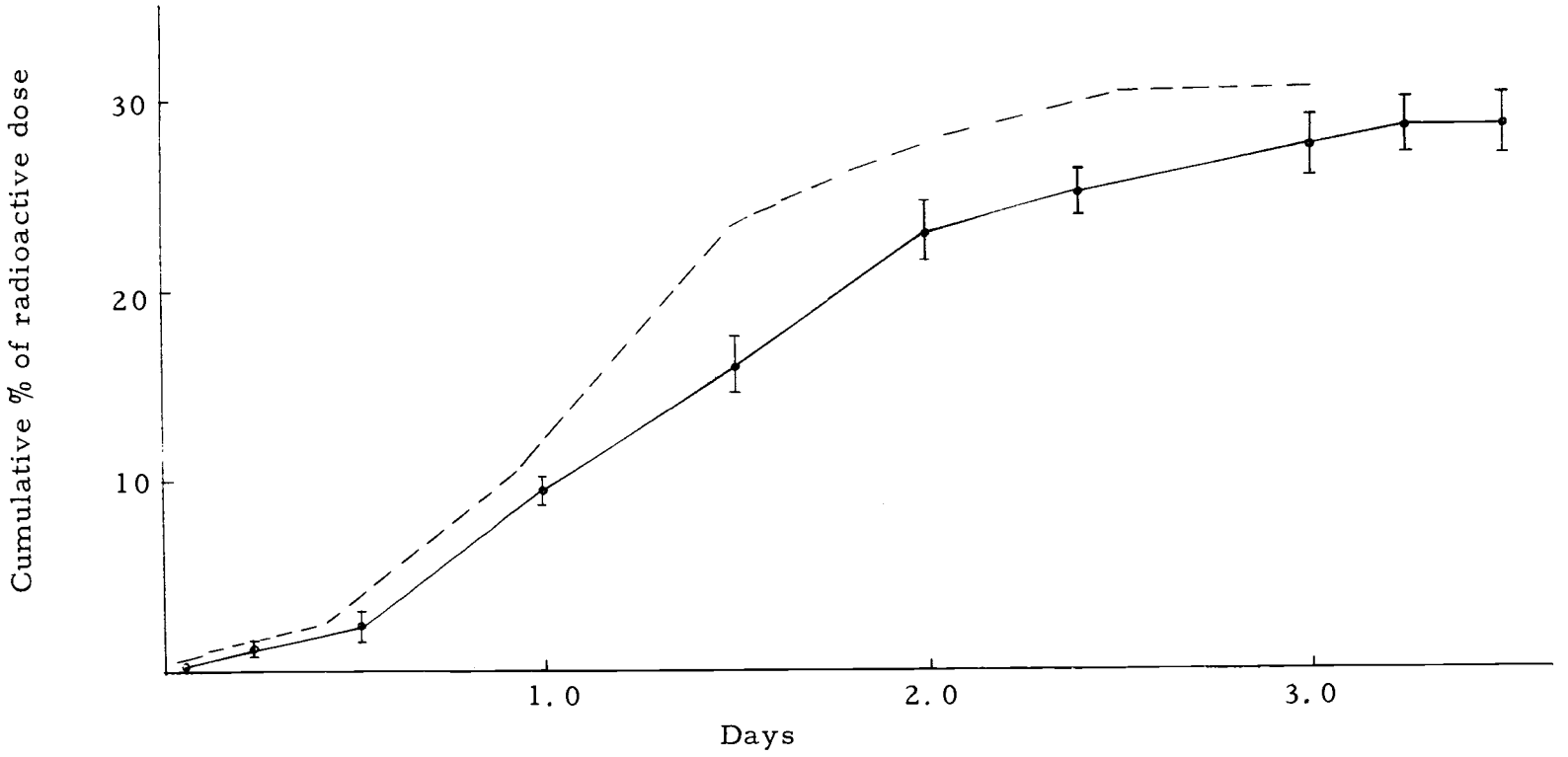


Figure 14. Percent excretion of radioactive dose in the bile of rat B receiving bile from another rat A dosed intraperitoneally with 5 mg/kg of hexachlorophene-^{14}C. Dashed line represents theoretical biliary excretion of radioactivity from rat A. (see Figure 9) Values are the average and range for two sets of 400 g rats.

and that this hydrolysis was inhibited by the presence of 1, 4-saccharolactone. Cleavage with β-glucuronidase yielded a radioactive component with the same chromatographic mobility as authentic hexachlorophene.

After dosing rat A with ^{14}C-hexachlorophene, all of the radioactivity excreted in the bile of rat B was shown to be a glucuronide conjugate of hexachlorophene. No evidence for additional hexachlorophene metabolites was found in the bile even though the phenol was subjected to metabolism in two separate consecutive animals.

Ligation of the Bile Duct Prior to Hexachlorophene-^{14}C Administration

To investigate if routes other than the bile are excreting hexachlorophene metabolites or conjugates into the gastrointestinal tract, bile duct ligated rats were dosed with hexachlorophene-^{14}C. Large male rats (400 g) were operated on as before and the bile duct isolated and tied off at both the duodenal and hepatic ends and in the middle. After recovery, the rats received a 5 mg/kg intraperitoneal injection of hexachlorophene-^{14}C (72.8 μCi/mmole) in corn oil and were placed in metabolism cages. Urine and feces was collected at appropriate intervals and aliquots analyzed for radioactivity.

With the bile duct ligated, rats excreted 68% of the intraperitoneal radioactive dose in five days with 55% of the radioactivity

appearing in the urine (Figure 15). The 13% of the radioactive dose appearing in the feces indicates that there is probably another excretory process occurring that results in the elimination of radioactive hexachlorophene or its conjugate into the gastrointestinal tract. The tissue distribution data of Buhler, Dost and Rasmusson (1972) shows a delayed increase in radioactivity in the stomach. These results along with the appearance of radioactivity in the feces of the bile duct ligated rats suggests that gastric or salivary secretion may occur with hexachlorophene or its conjugates.

Over 90% of the radioactivity in the urine of the bile duct ligated rats was shown to be a glucuronide conjugate of hexachlorophene by thin layer chromatography following analysis of extracts and enzyme hydrolyzed extracts. The remaining urinary radioactivity was unchanged hexachlorophene. Large amounts of hexachlorophene monoglucuronide can apparently be removed from the blood by the kidney, but in the normal animal bile is the preferential route of excretion for this conjugate.

Several attempts were made to administer a normally tolerated dose of 10 mg/kg of hexachlorophene by intraperitoneal injections to bile ligated rats and collect the urine and feces, but in all cases the animals died within a few hours. Apparently removal of hexachlorophene and its conjugate by the liver occurs more rapidly than does

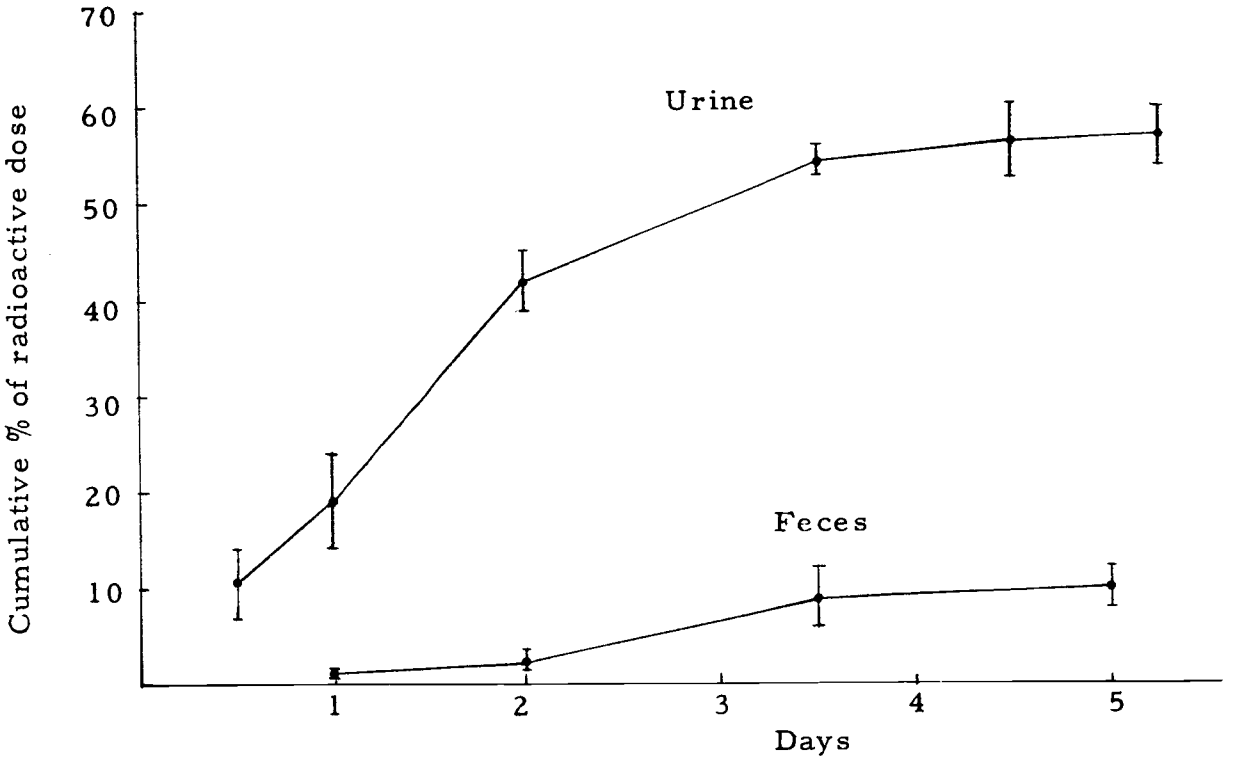


Figure 15. Excretion of radioactivity in the urine and feces of a bile duct ligated rat receiving 5 mg/kg of hexachlorophene-^{14}C intraperitoneally. Values represent the mean $\pm$ standard deviation of four 400 g male rats.

elimination via the kidney. Since renal excretion of hexachlorophene and its metabolites is slow, blockage of biliary excretion causes an accumulation of toxic levels of the bisphenol in the blood.

RESULTS. PART II: ABSORPTION, DISTRIBUTION, BIOTRANSFORMATION, AND EXCRETION OF HEXACHLOROPHENE-^{14}C IN THE RABBIT

Absorption and Excretion

Male New Zealand white rabbits (2.5-3.5 kg) received a 10 mg/kg (60.6 μCi/mmole, 1.0 x 10^7 dpm/3 kg animal) or 15 mg/kg (248 μCi/mmole, 5.4 x 10^7 dpm/3 kg animal) intraperitoneal dose of hexachlorophene-^{14}C dissolved in corn oil. The animals were placed in cages where the urine and feces were collected, periodically removed, and frozen after preparation of suitable counting aliquots. In the 10 mg/kg study the collected urine and feces from all the animals were separately pooled before analysis, while in the 15 mg/kg study the urine and feces were analyzed individually for each animal.

Excretion of radioactivity with respect to time by the rabbits after a single dose of labeled hexachlorophene is shown in Figure 16. The radioactivity appeared primarily in the feces, while the urine contained 20-25% of the radioactive dose. The animals receiving the 15 mg/kg level of hexachlorophene were partially intoxicated as evidenced by their lethargic appearance and lack of appetite, and they excreted less of the radioactive dose in the four day time period than did unstressed animals given the lower dose level.

Wit and Van Genderen (1962) reported that the rabbit treated orally with hexachlorophene-^{14}C excreted essentially all the

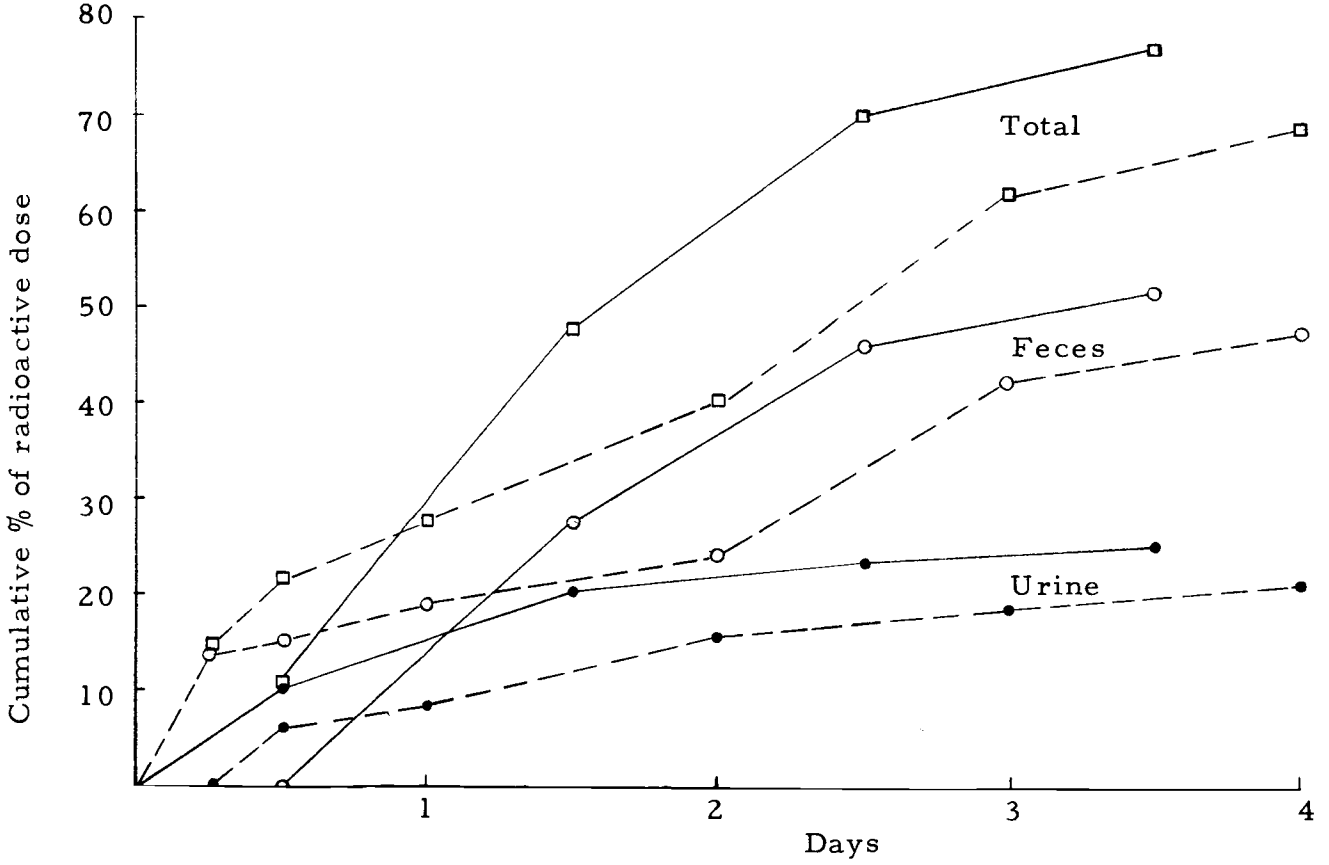


Figure 16. Excretion of radioactivity in the urine and feces of rabbits receiving a 10 mg/kg (——) or 15 mg/kg (-----) intraperitoneal administration of hexachlorophene-^{14}C. o = feces, ● = urine, and □ = total. The values for the 15 mg/kg dose are the mean from two rabbits (5.4×10^7 dpm/animal) and the 10 mg/kg dose values are the pooled samples of six rabbits (1.01×10^7 dpm/animal).

radioactive dose in five days with approximately 30% appearing in the urine. In the present study only 70-78% of the administered radioactivity was recovered in 3.5-4 days from the intraperitoneally treated rabbits. Somewhat greater recoveries of radioactivity in urine and feces have also been found with rats following an oral dose of hexachlorophene-^{14}C as compared to an intraperitoneal dose (Buhler, Dost and Rasmusson, 1972). The large amount of radioactivity found in the feces of intraperitoneally dosed rabbits indicates the presence of biliary excretion of radioactivity, which was previously shown in this study to occur with rats.

In a continuation of the 10 mg/kg study, the same rabbits (six) were dosed a second time with 10 mg/kg unlabeled hexachlorophene a week after treatment with labeled hexachlorophene, and the urine and feces collected over a four day period. After another three days (one week after the second dose) the rabbits were given a third 10 mg/kg intraperitoneal dose of hexachlorophene in the form of the ^{14}C-labeled compound. The urine and feces was collected over a 3.5 day time period and analyzed for each individual animal. There was considerable variation in the cumulative recovery of radioactivity in both the urine and feces between the individual animals after the third dose with urine values ranging between 25-38% (mean 31 $\pm$ 5%) and the fecal values between 41-74% (mean 52 $\pm$ 12%). There appeared a slight increase in the amount of urinary radioactivity after the third dose,

as compared to the urinary radioactivity after the first dose, while the feces remained approximately the same.

For use in hexachlorophene glucuronide studies to be described later, the rabbits also received a fourth 10 mg/kg dose of nonradioactive hexachlorophene a week after the previous radioactive dose, and the urine and feces again collected over a four day period.

The remaining 22-30% of the radioactive dose that was not recovered in the urine and feces from rabbits that received 10 or 15 mg/kg intraperitoneal doses of hexachlorophene-^{14}C was assumed to be either retained in the animals or possibly metabolized to volatile radioactive components. If enterohepatic circulation of hexachlorophene or its metabolites occurs in the rabbits as was shown to occur in the rat, an extensive distribution of the radioactivity in the animals' tissues and a long retention time would be expected.

Tissue Distribution

Two male rabbits (3 kg) were sacrificed and the distribution of radioactivity in the tissues determined four days after the animals received a 15 mg/kg intraperitoneal dose of hexachlorophene-^{14}C (248 μCi/mmole, 5.4×10^7 dpm/3 kg animal). The specific activities are presented in Table 6 along with the radioactive dose remaining in the whole tissues. These values have been calculated directly or by using the approximations of Donaldson (1924) for rodents to determine

Table 6. Tissue distribution of radioactivity in rabbits four days after intraperitoneal administration of 15 mg/kg hexachlorophene-^{14}C (248 μCi/mmole). Values are the mean of two 3 kg male rabbits. The average radioactive dose to an animal was 5.4×10^7 dpm.

Tissue	Specific Activity 10^3 dpm/g dry weight	Percent Radioactive Dose
Blood cells	0.26	0.001
Bladder	5.26	0.010
Bladder contents	15.65	0.160
Brain	0.20	0.001
Caeca	1.55	0.011
Caeca contents	6.51	0.306
Eyes	0.66	0.005
Fat	0.21	0.094
Gall bladder and bile	16.93	0.011
Heart	1.29	0.003
Intestines - small	1.44	0.017
Intestines - large	1.33	0.009
Intestinal contents - small	5.21	0.079
Intestinal contents - large	4.08	0.087
Kidney	2.70	0.020
Liver	7.79	0.435
Lung	3.31	0.012
Muscle	0.29	0.160
Plasma	19.03	0.070
Spleen	0.44	0.001
Stomach	0.89	0.006
Stomach contents	4.03	0.087
Testes	1.42	0.026
TOTAL		1.612

the weight of blood, fat, and muscle in the whole animal. The highest specific activities were found in the plasma, bladder contents, and gall bladder, while the brain, fat, and muscle were among the lowest. The highest percent of the dose (0.435%) was recovered in the liver with appreciable levels being found in the intestines, caeca contents, bladder contents, fat, muscle, and the stomach contents. The presence of radioactivity in the stomach contents might suggest the possibility of gastric or salivary secretion of hexachlorophene and its metabolites as previously suggested for the rat. A direct penetration of hexachlorophene-^{14}C into the gastrointestinal tract following a bathing of the stomach and intestines by the corn oil solution of hexachlorophene-^{14}C may also explain the radioactivity in the stomach contents.

Estimated total percentage of the radioactive dose recovered in the rabbit tissues examined four days after intraperitoneal administration of hexachlorophene-^{14}C was 1.61%, which does not account for all the remaining unexcreted radioactive dose. The radioactivity might be localized in a tissue or small area in the animal that was not analyzed or representatively sampled. Previous studies with the digestion technique used to measure the tissues have shown the technique to be reproducible and to account for all the radioactivity present. Following intraperitoneal administration of labeled hexachlorophene dissolved in corn oil, some of the bisphenol might have also

precipitated out of the corn oil solution and not have been distributed or detected. Formation of a volatile metabolite might explain the missing dose, but this has already been shown not to occur in the rat (Buhler, Dost and Rasmusson, 1972). If salivary secretion of hexachlorophene or its metabolites occurs, another possible loss of radioactivity might result when the rabbits groom themselves, dispersing some of the administered radioactivity onto the fur of the animals.

Respiration Studies

To test the possible conversion of hexachlorophene-^{14}C to $^{14}CO_2$ or other volatile radioactive metabolites, four rabbits (3 kg) were given an intraperitoneal injection of 10 mg/kg of hexachlorophene-^{14}C (70.7 μCi/mmole, 1.2×10^7 dpm/animal). Animals were placed individually in an all glass sealed chamber equipped with a wire mesh raised floor, and the expired air and excreted urine were monitored for 48 to 72 hours. The expired air from the animal and air bubbled through the collected urine was separately drawn by a vacuum pump at 500 ml/minute through a continuous gas analyzer [Cary model 31 vibrating reed electrometer (Monrovia, Calif.); Hewlett Packard Input Scanner, Digital Voltmeter, and Digital Recorder (Palo Alto, Calif.); RIDL Computer (Melrose Park, N.J.); and Dymec VFC (Palo Alto, Calif.)] followed by a monoethanolamine:ethanol (1:1) trap, and then returned to the continuous gas analyzer for further monitoring. The

monoethanolamine:ethanol traps were changed every four hours and aliquots of the trapping solutions were counted in diotol in a liquid scintillation counter. Near the end of the experiments, the collected urine was acidified to pH 1 with concentrated hydrochloric acid and monitored for several hours in the preceding manner.

No radioactive carbon dioxide or labeled volatile metabolites were detected in the respired air of any of the rabbits or in the air bubbled through the collected urine through 72 hours of monitoring by the continuous gas analyzer. The monoethanolamine:ethanol traps, which would collect and accumulate $^{14}CO_2$ and slowly expired acidic metabolites, also contained no radioactivity. Analysis of the acidified urine by the gas analyzer and the monoethanolamine:ethanol traps showed there was no ^{14}C-labeled volatile product released upon acidification. This experiment, which would have measured as little as 0.025 μCi of ^{14}C or a 0.05% conversion of the radioactive dose in ten minutes by the continuous gas analyzer or 0.0007 μCi (0.0014% of radioactive dose) in the four hour collection period for the monoethanolamine:ethanol traps, detected absolutely no volatile radioactive metabolites derived from hexachlorophene-^{14}C. Thus metabolism of hexachlorophene-^{14}C by the rabbit, by the gut microflora in the rabbit, and bacterial organisms in the feces or urine, or release of volatile labeled compounds by acidification of the urine did not occur.

Since hexachlorophene-^{14}C did not undergo appreciable conversion to volatile radioactive metabolites, all of the dose administered intraperitoneally to the rabbit should be either recovered in the urine and feces or remain in the tissues of the animals. The combined total of radioactivity remaining in the tissues sampled and that excreted in the urine and feces failed to account for the entire administered dose.

Isolation and Fractionation of Urine and Fecal Radioactivity

The frozen urine collected from 0-4 days from the two rabbits that received 15 mg/kg intraperitoneal doses of hexachlorophene-^{14}C (248 μCi/mmole) was thawed, and the urine from each collection period acidified and extracted with benzene or ethyl ether with a liquid-liquid extractor into conjugated and non-conjugated fractions. The cumulative percent of the dose found in the conjugated and non-conjugated portions of the urine for each collection period is presented in Figure 17 with respect to time. Conjugated metabolites appeared to predominate in the urine and also tended to increase in percentage with respect to time.

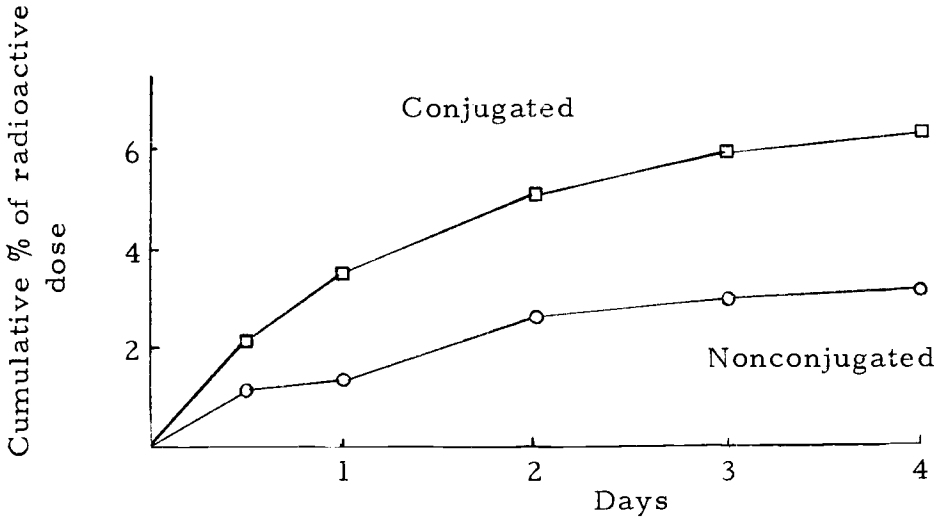


Figure 17. Excretion of conjugated and nonconjugated radioactive metabolites with respect to time in pooled urine from two rabbits receiving a 15 mg/kg intraperitoneal dose of hexachlorophene-^{14}C.

Upon separation of the urine into conjugated and nonconjugated fractions by continuous liquid-liquid extraction a considerable amount of the radioactivity was not recovered. The apparent loss of radioactivity might be explained by a volatilization of radioactive compounds during the liquid-liquid extraction step [hexachlorophene forms complexes with ethers (Armour-Dial, 1971)], photopolymerization of the chlorinated bisphenol, or some destruction of radioactive metabolites during the heated liquid-liquid extraction with benzene. Such a loss of radioactivity in urine or fecal samples did not occur in the earlier rat studies or in subsequent rabbit experiments when the liquid-liquid extraction process was not used.

All conjugated urine fractions from the various collection periods (6. 5% of radioactive dose) following treatment of the two rabbits with

15 mg/kg hexachlorophene-^{14}C were pooled after removal of the non-conjugated compounds by liquid-liquid extraction. The combined conjugate fraction was separated into glucuronide and sulfate fractions after enzymatic hydrolysis with β-glucuronidase and aryl sulfatase and subsequent extraction with chloroform. Further separation of the resulting sulfate and glucuronide extracts along with the pooled non-conjugated fractions from the same animals into acidic and neutral compounds gave the fractional distribution of radioactivity shown in Table 7. The primary form of radioactivity in the urine was acidic in nature, which suggests the presence of either unchanged hexachlorophene, acidic metabolites, or possibly extractable acidic conjugates. Most of the acidic material in the urine was present as a conjugate, almost entirely in the form of glucuronides. An appreciable amount of radioactivity could not be extracted from the urine after enzyme treatment perhaps as a result of an incomplete hydrolysis of the urinary glucuronides and sulfates due to the presence of endogenous inhibitors of the hydrolytic enzymes (Marsh, 1962). There was also a slight amount of radioactivity in the neutral extract and in the acidic sulfate fraction. In addition to the obvious possibility of neutral metabolites or sulfate conjugates, radioactivity in these fractions might result from extraction of ionized hexachlorophene into the organic phase during the neutral-acidic separation. Since the aryl sulfatase preparation used also contained β-glucuronidase activity, the discovery

of radioactivity in the sulfate fractions could have resulted from the incomplete cleavage of glucuronides during the previous β-glucuronidase hydrolysis step.

Table 7. Percent of the radioactive dose in 0-4 day pooled urinary fractions from two rabbits receiving a 15 mg/kg intraperitoneal administration of hexachlorophene-^{14}C. Values in parentheses are the percent of the total radioactivity present in the urine extracts.

Urinary Fraction	Percent of the Dose	
	Neutral	Acidic
Nonconjugated	0.03 (0.3)	2.75 (28.8)
Glucuronide	0.33 (3.5)	5.26 (52.5)
Sulfate	0.20 (2.0)	0.54 (3.7)
Acid Hydrolyzed	0.03 (0.2)	0.13 (1.3)
Unextracted	0.65 (7.6)	
TOTAL	9.92 (100)	

The validity of the fractionation procedure used for the rabbit urine is in question due to the considerable loss of radioactivity during the fractionation of the conjugated and nonconjugated material by the liquid-liquid extraction. Whether the fractions in Table 7 are representative of the original urine is dependent on the nature of the radioactive compounds that were lost. A later study, however, demonstrated a similar distribution of radioactivity in nonconjugated extracts from urine of six rabbits receiving 10 mg/kg intraperitoneal doses of hexachlorophene-^{14}C. In this study, to be described subsequently,

all of the radioactivity originally present in the urine was accounted for in the extracts after separation of the urine by extraction with ether:ethyl acetate into conjugated and nonconjugated fractions containing 58% and 42% of the total urine radioactivity, respectively.

Frozen feces from the two rabbits treated intraperitoneally with 15 mg/kg of hexachlorophene-^{14}C was also thawed, pooled, and extracted with methanol:water (19:1) three times, a procedure which removed 75% of the radioactivity from the fecal residue. The combined filtrate was concentrated on a rotary evaporator at 30°C until only water remained, and the acidified aqueous phase then separated into conjugated and nonconjugated fractions by extraction with chloroform. The distribution of radioactivity in the conjugated chloroform extract, nonconjugated aqueous fecal extract, and the amount remaining in the fecal residue is shown in Table 8. Approximately 97% of the radioactivity in the extract of the 0-4 day feces or 35.1% of the radioactive dose was in the nonconjugated form. The nature of the radioactivity remaining in the fecal residue, which might represent more polar metabolites or conjugates of hexachlorophene or compounds similar to that found in the methanol:water extract, is not known since an exhaustive extraction to recover all the fecal radioactivity was not performed.

Table 8. Percent of the radioactive dose in pooled fecal fractions from 0-4 days from two rabbits receiving a 15 mg/kg intraperitoneal administration of hexachlorophene-^{14}C. Values in parentheses are the percent of the total 0-4 day fecal radioactivity.

Fecal Fraction	Percent of the Dose
Nonconjugated	35.06 (78.0)
Conjugated	0.76 (1.7)
Fecal residue	9.06 (20.1)
TOTAL	44.88 (99.8)

Analysis of Urine Fractions

After enzymatic hydrolysis and extraction, the nonconjugated, sulfate, and glucuronide fractions of the urine from two rabbits treated intraperitoneally with 15 mg/kg hexachlorophene-^{14}C (Table 7) were concentrated *in vacuo* to a small volume and analyzed by thin layer, column, and gas chromatography and mass spectroscopy.

Thin layer chromatographic analysis of the concentrated extract fractions was performed using TF as the solvent system. The distribution of radioactivity in the various fractions after silica gel chromatography in the TF system is shown in Table 9. This table summarizes the results from a number of thin layer chromatograms in which the radioactivity was detected by use of either a radiochromatogram scanner or by scraping the plates and counting the gel in a liquid scintillation counter in the conventional manner. The

major radioactive zone in all fractions examined had the same chromatographic mobility as that of authentic hexachlorophene. Similar results were obtained upon thin layer chromatography of the various fractions in the BBA system. In some cases with the BBA and TEA solvents, a small amount of radioactivity was also detected at the origin of the plate. This was not investigated further, since it was assumed to be only small amounts of glucuronide or sulfate conjugates that were not hydrolyzed and were extracted along with the hydrolyzed material by the organic solvent.

Table 9. Silica gel thin layer chromatography of urinary fractions from rabbits receiving intraperitoneal administration of hexachlorophene-^{14}C (15 mg/kg). The relative level of radioactivity is indicated by the number of plusses (+).

	Rf [a]		
Urine Fraction	Origin - 0.2	0.3-0.6	0.7-0.9
Nonconjugated			
acidic	trace		+++
neutral			trace
Glucuronide			
acidic	+	trace	+++++
neutral			trace
Sulfate			
acidic	trace		trace
neutral	trace		
Hexachlorophene-^{14}C (standard)			+++++

[a] Toluene (saturated with 88% formic acid) was used as the solvent system.

The major radioactive component in the urine extracts was isolated from each fraction following preparative thin layer chromatography in the TF system. In all cases, analysis of the eluted material by gas chromatography after methylation with diazomethane showed only hexachlorophene dimethylether. Gas chromatographic analysis of the other radioactive regions was not performed due to the trace amount present.

In order to separate larger amounts of radioactive material for more ready identification of metabolites, silicic acid column chromatography of the concentrated metabolites fractions represented in Table 7 was next performed. Since the thin layer chromatography had shown that the "neutral" fractions contained hexachlorophene, all rabbit urine acidic and neutral fractions (Table 7) were pooled and evaporated to a small volume at room temperature. One-fifth of the pooled concentrate (2.23×10^6 dpm) was applied to a silicic acid column and developed with BHMW. Fractions were collected, and analysis of aliquots of the fractions for radioactivity yielded the results presented in Figure 18. Rechromatography of peak A, fractions 26-40, on another column developed with BBA gave a single symmetrical radioactive peak. The peak fractions from the second column were pooled and concentrated to dryness in vacuo. Aliquots of this material were analyzed by thin layer chromatography with TF and showed to contain a single radioactive component with the same

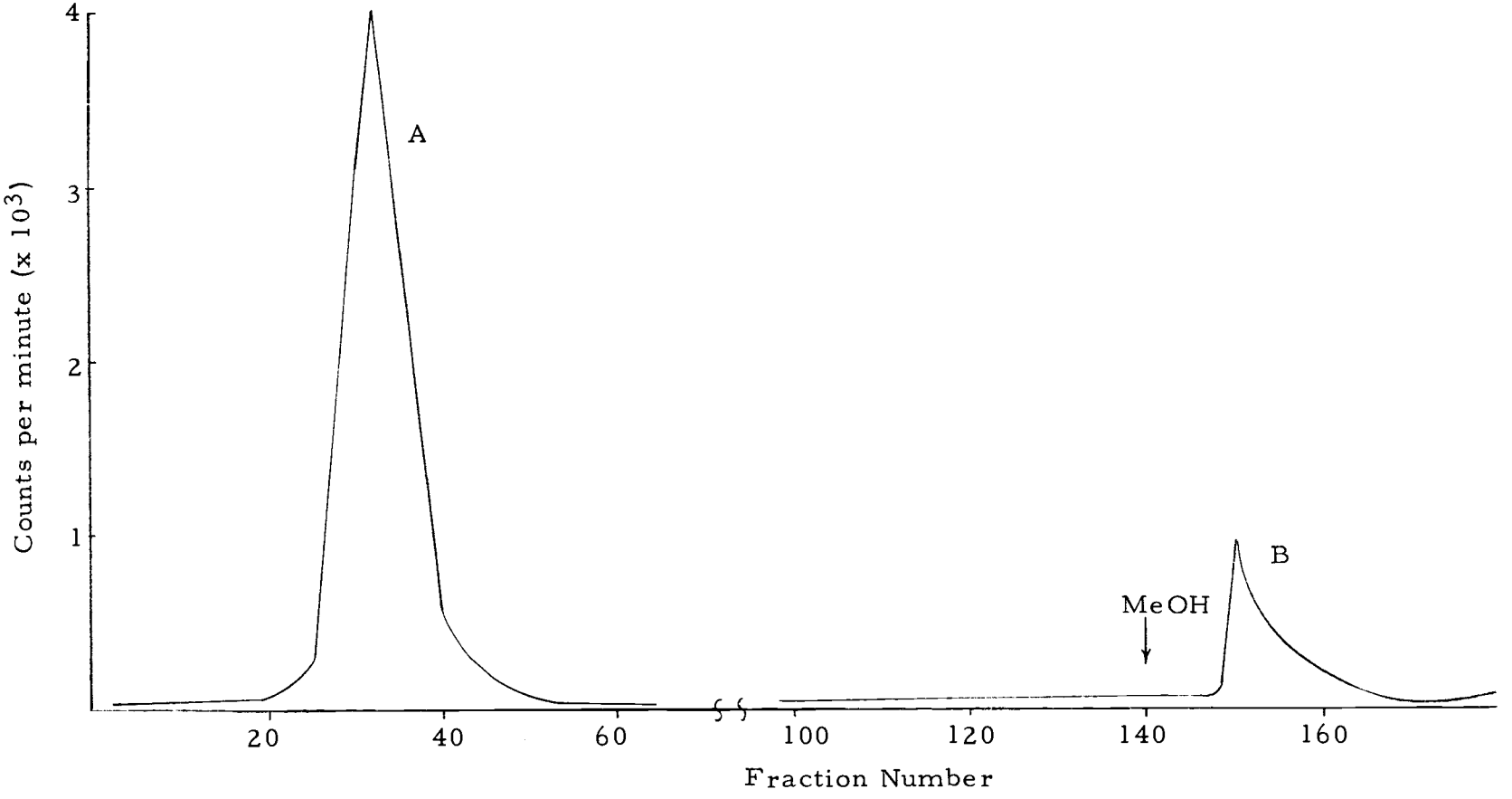


Figure 18. Silicic acid column chromatography of one-fifth of the pooled concentrated rabbit urine fractions (Table 7) from rabbits receiving 15 mg/kg.hexachlorophene-^{14}C intraperitoneally. Column was developed with benzene:hexane:methanol:water (7:3:5:5) followed by methanol.

chromatographic mobility as hexachlorophene. In addition, only hexachlorophene dimethylether was detected by gas chromatography after methylation of the purified fraction. Mass spectral analysis of the purified fraction containing the major urinary component yielded a spectrum identical to that for authentic hexachlorophene.

The pooled shoulder fractions (fractions 20-25, 40-52) of peak A (Figure 18) were rechromatographed on another silicic acid column and shown to contain a minor radioactive component (0.07% of the total urine extract radioactivity), that was tentatively identified as 2, 2'-dihydroxy-3, 3', 5, 6, 6'-pentachlorodiphenylmethane on the basis of gas chromatographic retention time. This compound was probably formed by photodegradation of hexachlorophene during the isolation of urinary metabolites, since this chlorinated bisphenol is reported to be the main product of ultraviolet degradation of hexachlorophene (Shaffer et al., 1971).

The fractions of peak B (Figure 18) were also pooled and concentrated to give an oil. Thin layer analysis of fraction B in the TF system revealed a single radioactive component at the origin of the plate. This material apparently consisted mainly of hexachlorophene glucuronide since further treatment of fraction B with β-glucuronidase released a second radioactive component (65% of the total fraction radioactivity) with a thin layer mobility identical to that of hexachlorophene. Subsequent treatment of the hydrolyzed fraction B with a

combined aryl sulfatase and β-glucuronidase preparation did not form any additional radioactive components or release any additional quantities of hexachlorophene. Consequently, sulfate conjugates did not appear to be present in fraction B. The identity of the radioactive compound released after hydrolysis of the glucuronide was confirmed as hexachlorophene by gas chromatography of the methylated material following preparative thin layer chromatography. The radioactivity remaining in fraction B after β-glucuronidase and aryl sulfatase hydrolysis failed to migrate upon thin layer chromatography in the TF system. Further analysis of this material was not performed. It probably represented either hexachlorophene glucuronide not completely hydrolyzed upon incubation with β-glucuronidase because of the presence of endogenous inhibitors or possibly the very polar unknown substance found by Shaffer et al. (1971) from the photodegradation of hexachlorophene.

The urinary metabolites of hexachlorophene in the rabbit after intraperitoneal injection were thus found to consist of hexachlorophene glucuronide (58.6%), nonconjugated hexachlorophene (29.1%), and 7.6% of unextracted material and acid hydrolyzed or possibly sulfate conjugates of hexachlorophene. No other acidic metabolites of hexachlorophene were found upon column chromatography of the combined urine extracts even though the presence of other minor components had been suggested in the initial thin layer chromatography. While

there was some evidence for the occurrence of sulfate conjugates in the release of extractable radioactivity (3.6%) after incubation of the urine with a combined aryl sulfatase and β-glucuronidase preparation, this material could also represent the additional cleavage of glucuronides.

Analysis of the Fecal Fractions

The conjugated and nonconjugated extract fractions from the feces of rabbits dosed with 15 mg/kg hexachlorophene-^{14}C intraperitoneally (Table 8) were also analyzed by thin layer gas, and column chromatography and by mass spectroscopy.

Aliquots of the pooled conjugated fraction were treated with β-glucuronidase and the hydrolyzed material then chromatographed on silica gel thin layer plates developed with TF. Almost all the applied radioactivity (87%) chromatographed as hexachlorophene while a small amount of radioactivity also remained at the orgin of the plate. After ethyl ether extraction of the hydrolyzed conjugate fraction, the major radioactive component was identified as hexachlorophene upon gas chromatography of the methylated extract. No further attempts were made at characterization of the material remaining at the orgin of the thin layer plates after enzymatic hydrolysis, since it represented only 0.09% of the dose and contained a large amount of oily nonradioactive impurities.

The fecal extract containing the nonconjugated metabolites was concentrated in a rotary evaporator at room temperature to yield 8.9 g of a dense brown oil. An aliquot of the oil was dissolved in methanol and chromatographed on silica gel thin layer plates in the BBA, BHMW, and TF systems. All three solvent mixtures separated the nonconjugated fraction into two radioactive components, one major zone with the mobility of hexachlorophene (95% of the applied radioactivity) and a second minor zone at the orgin of the plate. A minor radioactive component with an Rf of 0.4 was also noted on chromatography in the TF system.

Since there was a large amount of nonradioactive contaminants in this fraction, counter current distribution in phosphate buffer pH 7-8 against hexane, iso-octane, benzene, or ethyl acetate was tried in an attempt to purify the radioactive components, but this technique failed due to the low solubility of the oil in the aqueous partition phase. A 400 mg aliquot of the oil was next subjected to column chromatography on silicic acid developed with BBA as shown in Figure 19.

Fractions for peak A (containing 96% of the applied radioactivity) were pooled and evaporated to dryness to give approximately 60 mg of material. Fraction A was analyzed by thin layer and gas chromatography and mass spectroscopy and shown to contain hexachlorophene as the only radioactive component. Preparative thin layer chromatography with BHMW and TF of an aliquot of peak A residue gave a

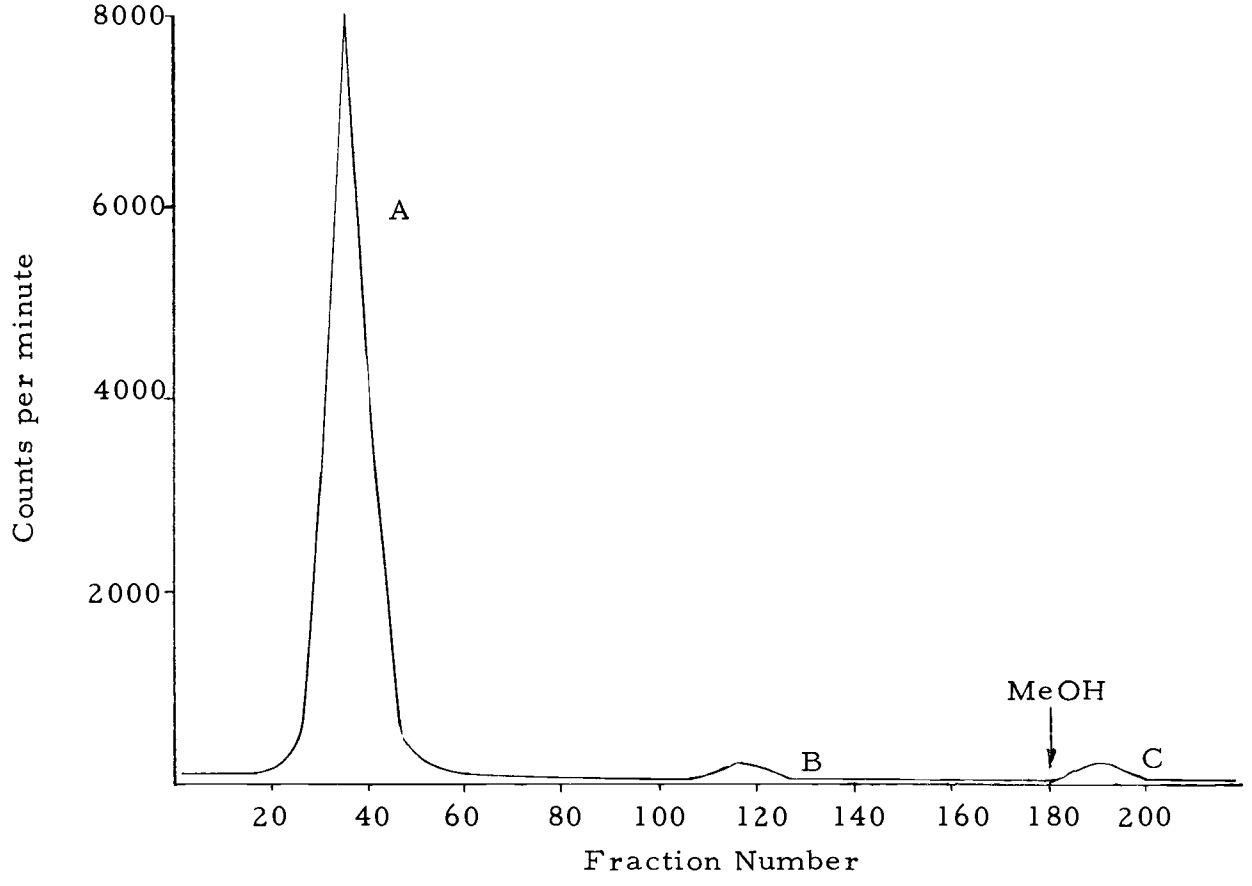


Figure 19. Silicic acid column chromatography of 600 mg aliquot of concentrated fecal nonconjugated fraction (Table 8) from rabbits receiving 15 mg/kg hexachlorophene-^{14}C intraperitoneally. Column developed with n-butanol:benzene:ammonia (1:1:1) followed by methanol.

purified hexachlorophene with a specific activity of 225 μCi/mmole hexachlorophene (concentration of hexachlorophene measured by gas chromatography), in good agreement with the specific activity of the administered hexachlorophene-^{14}C (248 μCi/mmole).

Pooled fraction C (with 2% of the applied radioactivity) was also evaporated to dryness to yield an oily residue. An aliquot of fraction C was treated with β-glucuronidase, and the hydrolysis mixture then examined by thin layer chromatographic analysis in the BBA, BHMW, and TF systems. Two radioactive zones were detected. A majority (70%) of the applied radioactivity did not move from the orgin of the plate while the remaining radioactivity (30%) was concentrated in a second zone with a thin layer mobility similar to that of authentic hexachlorophene. It is not clear whether the major radioactive component (70%) in the β-glucuronidase treated fraction was present as unhydrolyzed hexachlorophene glucuronide, other hexachlorophene conjugates or metabolites, or photodegradation products of hexachlorophene.

Fractions from peak B (Figure 19) were pooled (20% of applied radioactivity) and evaporated to dryness to yield a brown oil. This fraction was rechromatographed on another silicic acid column developed with BHMW (Figure 20). Two radioactive peaks resulted, peak B-I containing 10% of the applied radioactivity and peak B-II with the remaining 90%. The radioactive compound present in peak B-I was

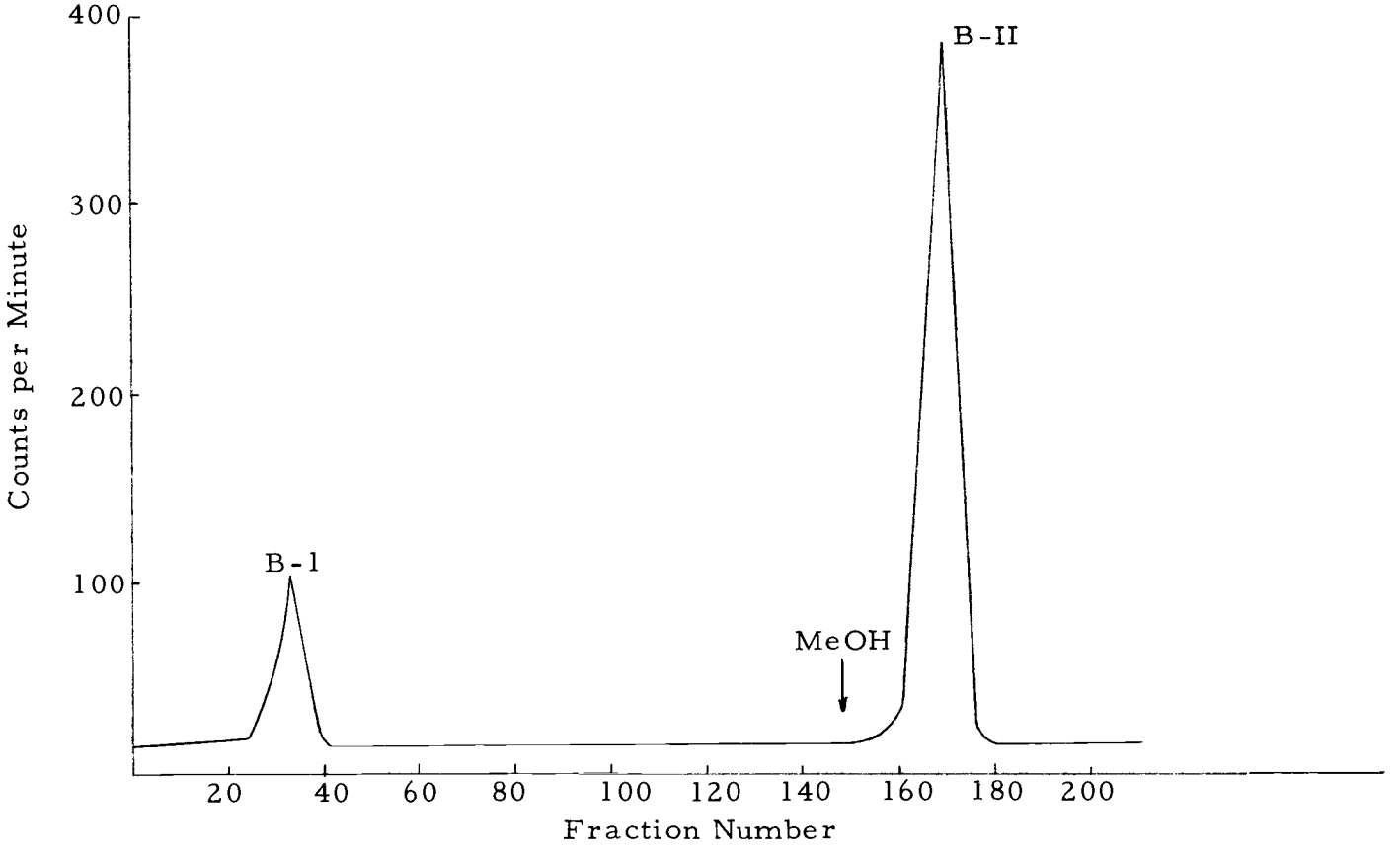


Figure 20. Silicic acid column chromatography of peak B from Figure 19 from fecal extract from rabbits receiving 15 mg/kg intraperitoneal administration of hexachlorophene-^{14}C. Column was developed with benzene:hexane:methanol:water (7:3:5:5) followed by methanol.

identified as hexachlorophene by gas chromatography and mass spectroscopy after initial purification of the pooled fractions by thin layer chromatography. Incubation of the pooled and evaporated fractions from peak B-II with β-glucuronidase or a combined β-glucuronidase and aryl sulfatase preparation converted 70% of the fraction radioactivity to free hexachlorophene. The radioactivity remaining in the β-glucuronidase treated fraction (30%) did not migrate from the orgin during thin layer chromatography and was, therefore, probably unhydrolyzed hexachlorophene glucuronide or other polar conjugates or complexes of hexachlorophene.

Silicic acid chromatography of the fecal extract (Figure 19), therefore, yielded three radioactive peaks: the major radioactive component (96% of the total radioactivity) hexachlorophene (peak A); a minor fraction with 2% of the total radioactivity containing primarily hexachlorophene glucuronide (peak C); and a mixture (2% of total radioactivity) of hexachlorophene, hexachlorophene glucuronide, and possibly other polar components (peak B). The presence of a third peak (B) containing a mixture of free hexachlorophene and hexachlorophene glucuronide may have resulted from an entrainment of these radioactive components in the large amount of oily residue from the silicic acid column in fractions 105-125. Application of larger aliquots (1.8-2.1 g) of the fecal nonconjugated fractions to silicic acid columns tended to prove this premise since multiple small peaks that contained

hexachlorophene and hexachlorophene glucuronide were found to be eluted from the column.

The primary radioactive metabolite recovered in the feces from rabbits treated with hexachlorophene was identified as unchanged hexachlorophene (93% of the radioactivity in the fecal extract) with a small amount of hexachlorophene glucuronide (6%) and a small quantity of some unidentified polar material.

Isolation and Identification of Hexachlorophene Glucuronide

Urine from six New Zealand white rabbits receiving intraperitoneal administration of alternating 10 mg/kg doses of labeled and unlabeled hexachlorophene, as previously described, was collected over a 3.5 day interval following each dose. Almost 25% of the first radioactive dose was excreted via the urine in 3.5 days and 31% of the dose in the urine in 3.5 days after the second treatment with hexachlorophene-^{14}C (third dose).

The radioactive urine collected between 0.5-2.5 days following the first dose of labeled hexachlorophene was pooled (5.54×10^6 dpm) and isolation of the glucuronide conjugates attempted using the basic lead acetate method of Kamil et al. (1951). The method did not work for hexachlorophene since all of the radioactivity precipitated with the particulate material in the urine in the normal lead acetate stage. Hexachlorophene glucuronide was probably bound to the particulate

matter in the urine and precipitated with it. This approach was discarded and the radioactivity almost completely recovered (5.43 x 10^6 dpm) by suspending the precipitate, treating it with hydrogen sulfide to precipitate the lead, filtering and washing the lead precipitate with a small amount of methanol, and combining the filtrate and the methanol washings. The recovered urinary radioactivity from the first dose of labeled hexachlorophene was combined with the 0.5-2.5 days radioactive urine from the third dose (12.3 x 10^6 dpm - combined total), and the pooled urine except for a small portion then extracted by the procedure of Polakova et al. (1971). In this method, the urine was adjusted to pH 10 by the addition of sodium carbonate and extracted with ethyl acetate three times. To remove conjugates, the urine was made pH 1 by the addition of dilute hydrochloric acid and extracted with ethyl acetate-ether mixtures until no radioactivity remained in the aqueous phase. The pH 10 extract from the 0.5-2.5 days pooled radioactive urine contained 42% of the pooled radioactivity, and the remaining 58% was recovered in the pH 1 extract. This same separation procedure was applied to the 0.5-2.5 days pooled nonradioactive urine from the two treatments of the rabbits with nonlabeled hexachlorophene (doses 2 and 4) after addition of the remaining small portion of the pooled 0.5-2.5 days radioactive urine to serve as a tracer. In this case 40% of the urine radioactivity appeared in the pH 10 extract and 60% in the pH 1 fraction.

Thin layer and gas chromatography demonstrated that the pH 10 extract of the 0.5-2.5 days pooled radioactive urine contained mainly nonconjugated hexachlorophene. Analysis of the pH 1 extract of this same urine showed that almost all of the radioactivity (more than 95%) was in the form of a polar component that did not migrate upon thin layer chromatography in the TF system plus a minor amount (less than 5%) of nonconjugated hexachlorophene contaminant. Treatment of aliquots of the pH 1 extract with β-glucuronidase or a combined β-glucuronidase and aryl sulfatase preparation yielded in both cases more than 95% of a component with the thin layer chromatographic mobility of hexachlorophene and a small amount of unhydrolyzed conjugate or other material that failed to move in the thin layer chromatographic systems.

The pH 1 extracts from the radioactive and nonradioactive urines (with tracer) were combined. This combined pH 1 extract was evaporated to dryness, dissolved in a small amount of water, and treated with Amberlite XAD-2 resin to recover hexachlorophene metabolites. After washing with water, the resin was eluted with methanol, and the eluate concentrated to yield 28 g of oil that contained 7.12×10^6 dpm (5.5% of the radioactivity present in the two doses of hexachlorophene-^{14}C). Approximately 23.5 g (6.05×10^6 dpm) of the oil was partially purified by chromatography on a large silicic acid column developed with increasingly polar solvents (petroleum ether-

acetone-methanol). A single radioactive peak was found, and the resulting fractions were pooled and evaporated to an oil (14.9 g; 5.55×10^6 dpm).

Derivation of the oil was performed to aid in isolation and purification of the glucuronides. The oil was treated with ethereal diazomethane and allowed to stand overnight. After removal of the ether by evaporation, the gum was treated with acetic anhydride-pyridine (1:1) and allowed to stand for five days. The excess acetic anhydride-pyridine was removed in vacuo to give an oil. This derived material was further purified by chromatography on a florisil colum and then, after concentrating the fractions from the single radioactive peak, on an alumina F-120 column, both developed with increasingly polar solvents (hexane-benzene-acetone-methanol). The peak fractions from the major radioactive peak on the second column were combined and evaporated to give an oil (1.6 g; 2.0×10^6 dpm). During the various steps in the clean-up process, no other hexachlorophene metabolites or conjugates were detected by thin layer chromatographic analysis of minor radioactive fractions. The purified fraction (1.6 g; 2.0×10^6 dpm) was then applied to a long silicic acid column (80 cm) and eluted with increasingly polar solvents as shown in Figure 21. Three radioactive components were observed to be present.

The fractions making up peaks A and B were individually pooled and evaporated to form oils weighing 380 mg (9.9×10^5 dpm) and

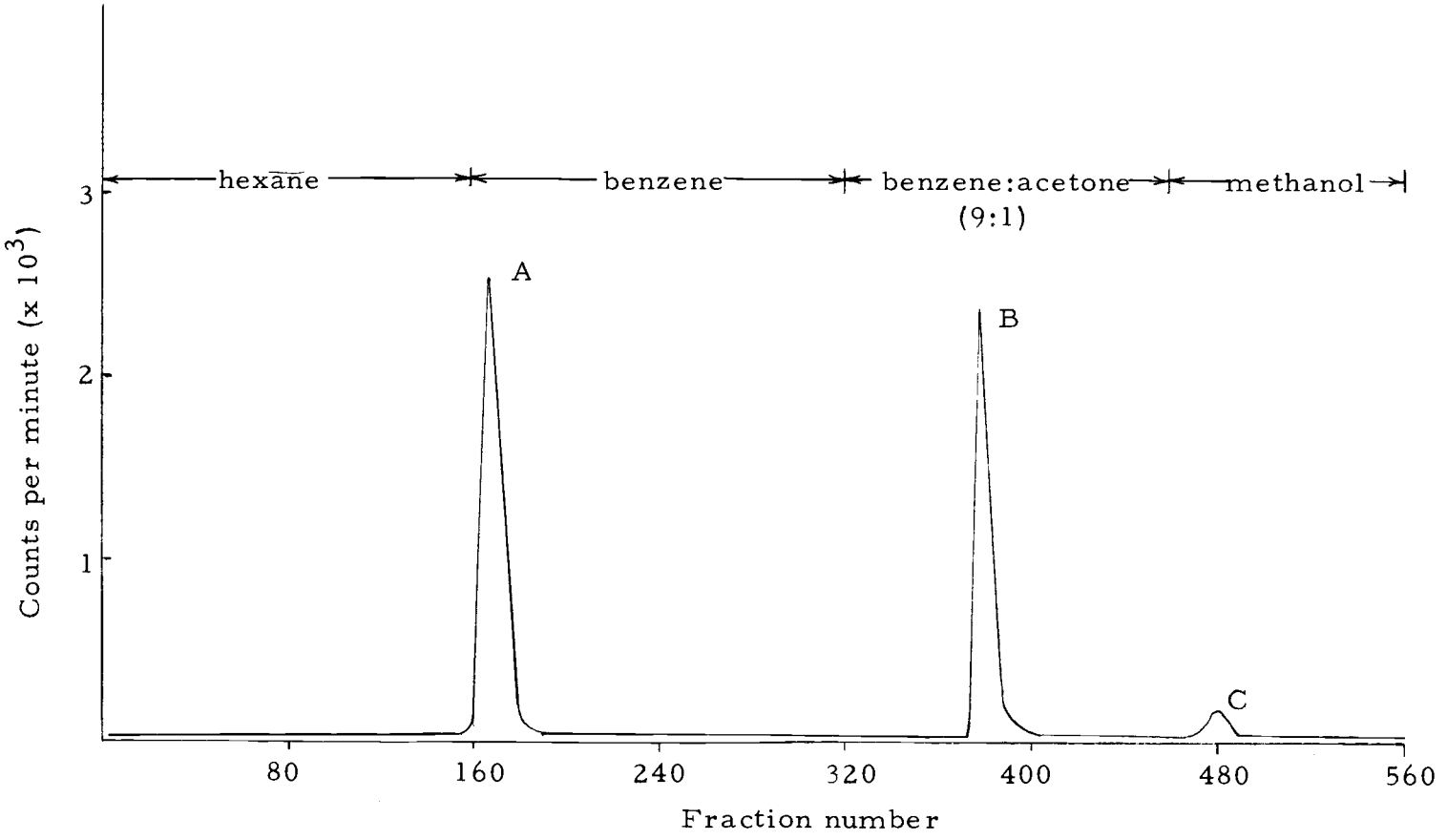


Figure 21. Silicic acid column chromatography of major radioactive peak from florisil and alumina column chromatography of methylated and acetylated oil from pH 1 extract of urine from rabbits receiving hexachlorophene-^{14}C.

505 mg (9.4×10^5 dpm), respectively. Aliquots of the combined fraction A were chromatographed on silica gel thin layer plates developed with benzene:acetone (9:1) and were shown to contain a single radioactive component with an Rf value identical to that of synthetically prepared hexachlorophene-mono(methyl tri-0-acetyl-glucuronate) after the latter was treated with diazomethane. Aliquots of fraction B were also shown to contain a single radioactive component with the same chromatographic mobility as that of synthetically prepared hexachlorophene-mono(methyl tri-0-acetyl-glucuronate) following treatment of the latter with acetic anhydride-pyridine. After dissolving aliquots of fraction A in 10% hydrochloric acid and allowing to stand for one hour to afford a gentle acid hydrolysis of the conjugate, the hydrolysis mixture was extracted. Gas chromatography showed that two components were present in the acid hydrolyzed fraction A extract, free hexachlorophene (60%) and another component which, on the basis of retention times, appeared to be the monomethylether of hexachlorophene. Similar acid hydrolysis of an aliquot of fraction B yielded a major component (80%) with the retention time of hexachlorophene and a minor component (20%) with a retention time similar to that estimated for the monoacetyl ester of hexachlorophene. Attempts to synthesize the monoether and monoester of hexachlorophene have failed so conclusive chromatographic evidence is not available. Mass spectral analysis of fraction A gave a six chlorine pattern at 418 m/e (hexachlorophene

monomethylether) and a nonchlorinated ion at 316 m/e (methyl acetoglucuronate fragment). Mass spectral analysis of peak B yielded two six chlorine patterns, one at 446 m/e (hexachlorophene monoacetyl ester) and the other at 404 m/e (hexachlorophene), and a nonchlorinated ion occurring at 316 m/e (methyl acetoglucuronate fragment). From these data, fraction A can be identified to be hexachlorophene monomethylether mono(methyl tri-0-acetyl glucuronate) (Figure 22a) and fraction B as hexachlorophene monoacetylester mono(methyl tri-0-acetyl-glucuronate) (Figure 22b).

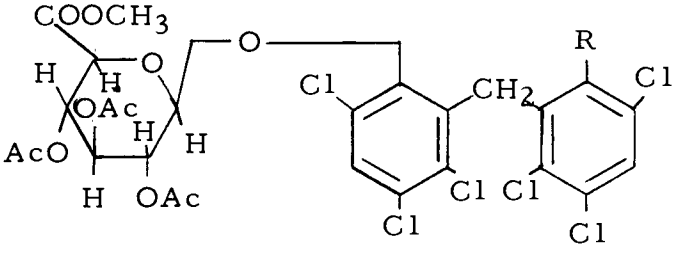


Figure 22. 2-(methyl 2, 3, 4-tri-O-acetyl-β-D-glucopyranuronate)-2'-R-3, 3', 5, 5', 6, 6'-hexachlorodiphenylmethane. Ac = acetyl. R = methoxy (a); = acetoxy (b).

Apparently hexachlorophene monoglucuronide was not completely methylated during derivation and as a result, some hexachlorophene monoglucuronide was later acetylated in the free phenolic position where methylation should have occurred (Figure 23). Incomplete methylation has also been found by Buhler and Rasmusson (1972) when

hexachlorophene is methylated by diazomethane in certain tissue extracts.

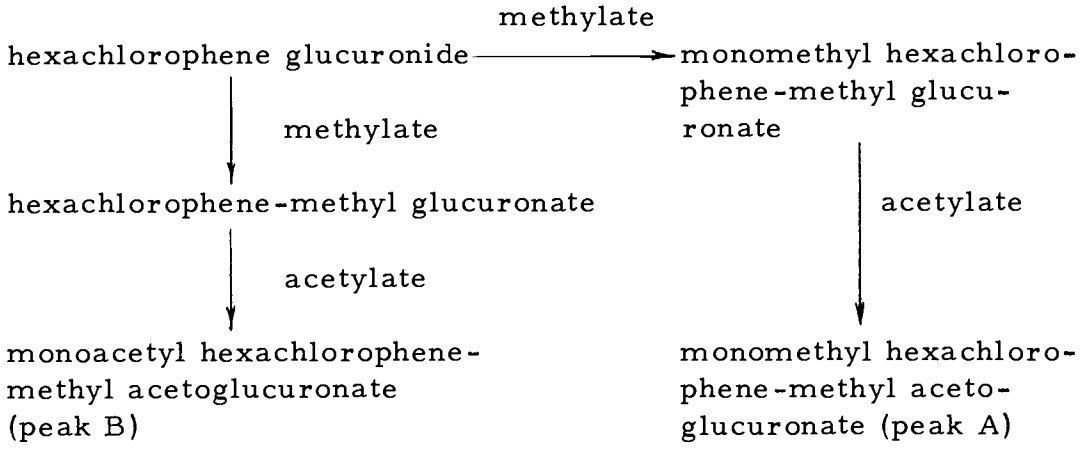


Figure 23. Formation of the monomethyl and monoacetyl hexachlorophene-methyl acetoglucuronate during derivation of hexachlorophene monoglucuronide.

The last peak, fraction C (Figure 21), has not been identified. It did not migrate from the orgin upon silica gel thin layer chromatography in any of the solvent systems used in these studies. Since treatment of the material with diazomethane or acetic anhydride-pyridine did not modify its chromatographic mobility, it is not non-derived hexachlorophene monoglucuronide. The material could be another hexachlorophene conjugate that does not chromatograph in the solvent systems used and cannot be derivatized, or it might be photochemically degraded hexachlorophene (Shaffer et al., 1971) formed during the lengthy isolation of the glucuronide.

The conjugated radioactive material appearing in the urine of rabbits dosed with hexachlorophene-^{14}C was shown to be

hexachlorophene monoglucuronide. No evidence for a double conjugate or sulfate conjugate was found with the isolation and purification procedures used, hence they were not present in significant amounts.

RESULTS. PART III: IN VITRO INTERACTION OF HEXACHLOROPHENE WITH RAT LIVER MICROSOMES

Incubation of Hexachlorophene-^{14}C With a Rat Liver Oxidative Microsomal Incubation System

St. John Jr. and Lisk (1972) recently reported that hexachlorophene did not undergo metabolism or conjugation when incubated with the 10,000 g liver supernatant fraction from the cow. To investigate if hexachlorophene could be directly oxidized by rat liver microsomes, hexachlorophene-^{14}C (250-5000 nmoles) in 50 µl of acetone was added to an incubation system consisting of 5-15 mg of microsomal protein, 0.1 µmoles of nicotinamide, 25 µmoles magnesium chloride, 2 µmoles $NADP^+$, 6 µmoles glucose-6-phosphate, and 2 units glucose-6-phosphate dehydrogenase in 3-5 ml of 0.1 M phosphate buffer, pH 7.4. The incubation mixture was shaken for 1-3 hours at 37°C in a Dubnoff-type metabolic shaker. After incubation, 10% trichloroacetic acid was added to precipitate the protein, and the mixture then extracted three times with ethyl acetate. No radioactivity was found in the protein precipitate after extraction. Thin layer and gas chromatographic analysis of the extract showed only unchanged hexachlorophene and no other radioactive components. Control experiments utilizing p-nitroanisole demonstrated that 0-demethylation was not affected by addition of 50 µl of acetone. On a basis of these results, it appears that hexachlorophene was resistant to oxidation by microsomal enzymes.

Reaction of Hexachlorophene With a Model Microsomal System

To investigate further the possibility of oxidative metabolism of hexachlorophene, the bisphenol was reacted in the model chemical oxidative system of Udenfriend et al. (1954). This system is thought to simulate biological oxidations and is therefore often used as a model system. The reaction mixture contained 7.1 μmoles sodium ascorbate, 0.75 μmoles ferrous sulfate, 4.0 μmoles disodium ethylenediaminetetraacetate, and 250 nmoles sodium phosphate buffer, pH 6.0 in a total volume of 5 ml. After addition of an acetone solution of hexachlorophene-^{14}C (3.7-12.3 nmoles) to the reaction mixture, the solution was vigorously oxygenated for 0.5-1.0 hours at room temperature. The radioactivity was then extracted with ethyl ether and analyzed. Chromatography of the ether extract on thin layer plates yielded only one radioactive component, and gas chromatographic analysis indicated a quantitative recovery of hexachlorophene. These experiments confirmed the resistance of hexachlorophene toward oxidation using a model chemical system.

Attempts to Biosynthesize Hexachlorophene Glucuronide

A microsomal glucuronide conjugating system was prepared by the procedure of Knaak and Sullivan (1966) from rat liver to use in an attempt at biosynthesizing hexachlorophene glucuronide, the major

in vivo metabolite. Fresh liver microsomes (10-15 mg protein), 5-9 μmoles UDPGA, 25 μmoles magnesium chloride, and 5 μmoles 1, 4-saccharolactone were added to 5 ml of 0. 1 M phosphate buffer, pH 7. 4. The mixture was incubated at 37°C in a metabolic shaker for 1-3 hours with hexachlorophene-^{14}C (500-5000 nmoles added in 50 μl of acetone or polyethylene 400). In several experiments, hexachlorophene-^{14}C was applied to porous polyethylene discs (0. 25 cm diameter), which were then added to the biosynthetic system. Previous studies with phosphate buffer, pH 7. 4, showed the hexachlorophene impregnated polyethylene discs leached hexachlorophene slowly into the buffer to yield a sustained saturated hexachlorophene solution in one hour. At the end of the incubation period the biosynthetic reaction was stopped by the addition of 10% trichloroacetic acid, and the radioactivity completely extracted from the incubation mixture with ethyl acetate. No evidence for the formation of the glucuronide conjugate was found from thin layer and gas chromatographic or mass spectral analysis of the recovered radioactive material. Although hexachlorophene glucuronide is formed in vivo in the rat, the rat liver microsomal glucuronide synthesis system was not capable of converting hexachlorophene to the glucuronide form. In control experiments, this same system was shown to conjugate p-nitrophenol with glucuronic acid in the presence of 50 μl of acetone.

Binding of Hexachlorophene-^{14}C to Microsomes

Hexachlorophene-^{14}C (0.5 μmole) was added to a typical microsomal incubation mixture to determine the extent of binding of hexachlorophene to the microsomes. The microsomal system contained: 5 mg rat liver microsomal protein, 10 μmoles nicotinamide, 25 μmoles magnesium chloride, 2 μmoles $NADP^+$, 6 μmoles glucose-6-phosphate, and 2 units glucose-6-phosphate dehydrogenase (3 μg protein) in 5 ml of 0.1 M phosphate buffer, pH 7.4. After incubation of the mixture for one hour at 37°C, it was diluted to 10 ml with the phosphate buffer and centrifuged at 105,000 g for one hour. The supernatant was decanted and an aliquot analyzed for radioactivity. The microsomal pellet was resuspended in 10 ml of 0.1 M phosphate buffer, pH 7.4, and resedimented at 105,000 g for one hour. The supernatant was again decanted and counted. The microsomal pellet was resuspended in 1 ml of phosphate buffer, and aliquots were digested and counted for radioactivity by the procedure of Mahin and Lofberg (1966). The distribution of the radioactivity in the supernatants and the microsomal pellet is shown in Figure 24. The results indicate that 89% of hexachlorophene-^{14}C was recovered in the microsomal pellet. Hexachlorophene is, therefore, strongly bound to the microsomal lipoprotein membranes since the associated radioactivity is not lost after washing and resedimentation.

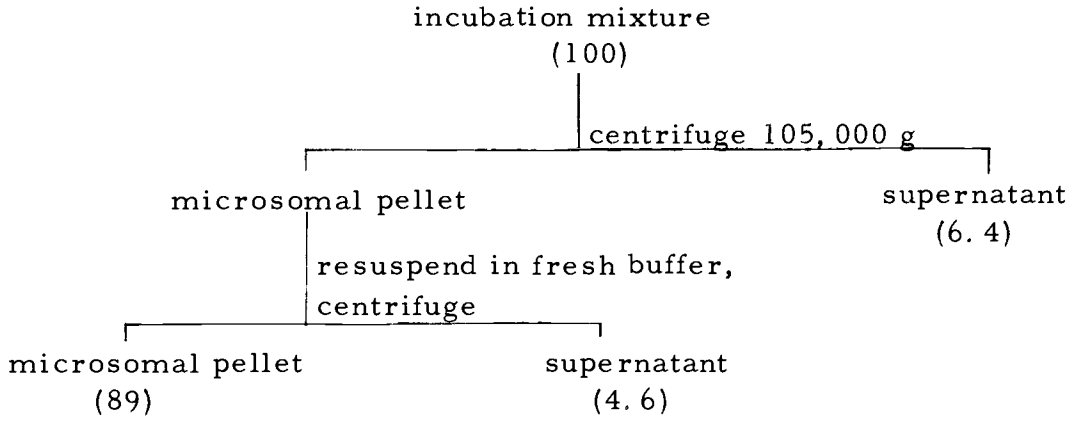


Figure 24. Flow scheme of radioactivity in washed microsomes after isolation from a microsomal incubation mixture treated with hexachlorophene-^{14}C. Values in parentheses are the percent of initial radioactivity.

The capacity of the microsomes to bind hexachlorophene was determined by equilibrium dialysis experiments at 4°C. Aliquots of a fresh rat liver microsomal suspension in 0. 1 M phosphate buffer, pH 7. 4, were diluted with the phosphate buffer to a total volume of 1. 5 ml. After addition of hexachlorophene-^{14}C (160 nmoles) in 10 µl acetone, the suspensions were then shaken in a Dubnoff shaker for 15 minutes at room temperature. A 1 ml aliquot from each mixture was sealed in a bag made from cellulose tubing (Union Carbide Corp., Chicago, Ill.), and the bags dialyzed together at 4°C for 24 hours against a stirred 2 liter solution of 0. 154 M potassium chloride. The KCl solution was changed after 24 hours and stirred for an additional 24 hours. The dialysis bags were removed, the contents

drained into a 10 ml of Aquasol, and the radioactivity determined in a liquid scintillation counter. The nmoles of hexachlorophene-^{14}C present in the bag after dialysis were determined and divided by the concentration of the protein in the bag to yield the amount of hexachlorophene bound per mg protein. Similar dialysis procedures were performed on fragments of smooth and rough endoplasmic reticulum from rat liver obtained from Dr. L. Davis, Department of Agricultural Chemistry, Oregon State University. The amount of hexachlorophene-^{14}C bound to the various forms of membranes is presented in Figure 25. Control dialysis bags containing no microsomal protein retained less than 0.20 mmoles hexachlorophene after dialysis. No radioactivity was detected adhering to the bags after dialysis.

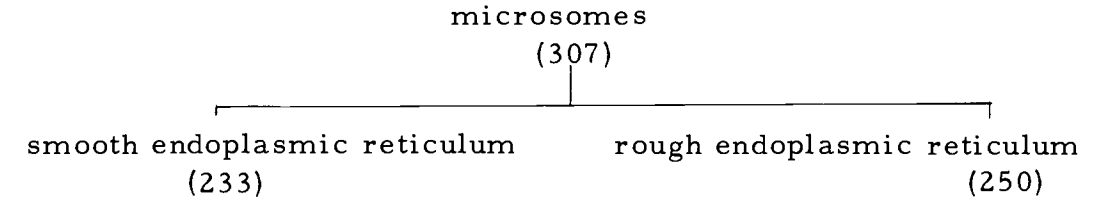


Figure 25. Amount of hexachlorophene-^{14}C bound to microsomal membranes. Values in parentheses are the nmoles of hexachlorophene bound per mg protein determined by equilibrium dialysis.

The microsomal membranes appear to have a large capacity for binding hexachlorophene. The smooth and rough ER bound somewhat less hexachlorophene than the unfractionated microsomes probably due to a slight change in the membranes during the separation process, but there was no preferential binding of hexachlorophene to smooth or rough membrane. The large binding capacity of isolated membranes for hexachlorophene has previously been shown by Flores and Buhler (1972) with isolated red blood cell membranes.

Effect of Hexachlorophene on the Production of NADPH by Microsomal Incubation Mixtures

Wang and Buhler (1972) have shown that purified glucose-6-phosphate dehydrogenase is inhibited by low concentrations of hexachlorophene. Glucose-6-phosphate dehydrogenase is a common component added to microsomal incubation mixtures to supply the microsomes with NADPH. Accordingly, the production of NADPH in a microsomal incubation mixture containing added glucose-6-phosphate dehydrogenase was determined in the presence of hexachlorophene by measuring the change in absorbancy at 340 nm. The results of the incubation (Figure 26) showed that under the conditions of incubation, hexachlorophene did not inhibit the production of NADPH by the NADPH generating system. The small amount of glucose-6-phosphate dehydrogenase that was present (0.25 μg) was probably protected from

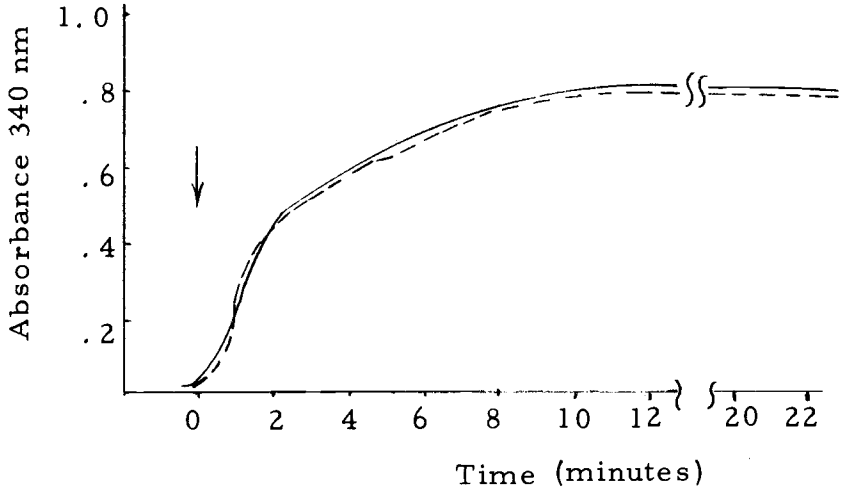


Figure 26. Production of NADPH by microsomal incubation mixture in the presence of hexachlorophene. Formation of NADPH measured by the increase in absorption at 340 nm. Solid line is control and dashed line is with the addition of hexachlorophene. Arrow indicates initiation of the reaction. Assay conditions: microsomes (1 mg protein), NADPH (0.26 μmoles, magnesium chloride (2.5 μmoles), nicotinamide (1 μmole), and glucose-6-phosphate dehydrogenase (0.2 units, 0.25 μg protein). Hexachlorophene (30-90 nmoles) in 10 μl of acetone was added with the control receiving only 10 μl acetone. Both control and hexachlorophene treated incubation mixtures were pre-incubated five minutes. Glucose-6-phosphate (1 μmole) was added to start the reaction, which was run at 37°C and continuously monitored at 340 nm.

specific inhibition by hexachlorophene because of the large binding capacity of microsomal protein for this chlorinated bisphenol. A similar protein protective effect was reported by Caldwell, Nakaue and Buhler (1972) when they added excess bovine serum albumin to bind hexachlorophene and thus decrease the inhibition of mitochondrial respiration by hexachlorophene.

In vitro Effect of Hexachlorophene on Microsomal Enzymatic Activity

The large binding capacity of the microsomes, the inhibitory effect of hexachlorophene on some enzymatic reaction (Gould et al., 1953; Caldwell, Nakaue and Buhler, 1972), and the absence of metabolism of hexachlorophene by the microsomes, prompted a study of the possible inhibitory action of hexachlorophene on microsomal enzymatic activity. The enzymatic capacity of microsomes in the presence of hexachlorophene was investigated by measuring the O-demethylation activity by the production of p-nitrophenol from p-nitroanisole, the nitroreductase activity by the production of p-aminobenzoic acid from p-nitrobenzoic acid, and the uridine diphosphate glucuronyltransferase activity by the consumption of p-nitrophenol. The results, shown in Tables 10, 11, and 12, show that low concentrations of hexachlorophene strongly inhibited the three liver microsomal enzymes tested. The oxidative O-demethylation process was the most sensitive to added hexachlorophene, while the

Table 10. In vitro inhibition of microsomal p-nitroanisole demethylase activity by incubation with hexachlorophene.[a] Each value represents the mean ± standard deviation of the number of determinations, which are in parentheses.

nmoles hexachlorophene added / mg microsomal protein	nmoles p-nitrophenol formed / mg protein/hour	Percent Inhibition
control	48.9 ± 5.7 (6)	--
0.35	42.4 ± 4.4 (4)	14
0.74	33.0 ± 4.5 (6)	33
1.68	34.5 ± 6.5 (6)	29
2.48	25.9 ± 2.9 (6)	47
7.43	20.9 ± 5.8 (4)	57
37.10	9.4 ± 5.0 (4)	81

[a]Incubation media of Hoffman, Worth and Anderson (1968): microsomal protein (2.2-4.4 mg), nicotinamide (10 μmoles), NADP+ (1.3 μmoles), magnesium chloride (25 μmoles), glucose-6-phosphate (5.6 μmoles), glucose-6-phosphate dehydrogenase (2 units), in 1.5 ml of 0.1 M phosphate buffer, pH 7.4. The incubation mixture was preincubated with hexachlorophene (2-80 nmoles added in 50 μl acetone) for five minutes. Controls received only 50 μl acetone and were also preincubated for five minutes. p-Nitroanisole (1.7 μmoles) was added and the mixture incubated at 37°C for one hour in a metabolic shaker. p-Nitrophenol was assayed by the method of Kinoshita, Frawley and DuBois (1966): 2.5 ml of ice-cold acetone was added to stop the reaction. 0.2 ml of 0.5 M glycine-sodium hydroxide buffer, pH 9.5, was added and the mixture centrifuged for five minutes. The optical density of the supernatant was read at 410 nm.

Table 11. In vitro inhibition of microsomal p-nitrobenzoic acid reductase activity by incubation with hexachlorophene.[a] Each value represents the mean ± standard deviation of the number of determinations, which are in parentheses.

nmoles hexachlorophene added / mg microsomal protein	nmoles p-aminobenzoic acid formed / mg microsomal protein/hr	Percent Inhibition
control	7.6 ± 0.6 (4)	--
1.24	7.0 ± 0.2 (4)	8
12.38	5.7 ± 0.8 (4)	25
49.50	3.7 ± 0.5 (2)	48
123.8	2.1 ± 0.4 (4)	73
1237.6	-0-	100

[a]Incubation media of Heitbrink and DuBois (1964): microsomal protein (2.4-6 mg), magnesium chloride (50 μmoles), nicotinamide (10 μmoles), $NADP^+$ (1.3 μmoles), glucose-6-phosphate (5.6 μmoles), glucose-6-phosphate dehydrogenase (2 units) in 3 ml of 0.1 M phosphate buffer, pH 7.4. Hexachlorophene (3-3000 nmoles) in 50 μl acetone was added and the mixture preincubated for five minutes at room temperature. Controls received only 50 μl acetone and were also preincubated for five minutes. 3 μmoles p-nitrobenzoic acid was added and the mixture incubated in a metabolic shaker for one hour at 37°C under nitrogen. Assay for p-aminobenzoic acid by the method of Bratton and Marshal (1939): 3 ml of 10% trichloroacetic acid was added to stop reaction and the mixture centrifuged. 4 ml of the supernatant was reacted with 1 ml of 0.1% sodium nitrite followed three minutes later by 1 ml of 0.5% ammonium sulfamate. After three more minutes 1 ml of 100 mg% N-1-naphthylethylenediamine dihydrochloride was added and the mixture allowed to stand ten minutes. Two ml of 95% ethanol was added and the absorbance recorded at 545 nm.

Table 12. In vitro inhibition of microsomal uridine diphosphate glucuronyltransferase activity by incubation with hexachlorophene. p-Nitrophenol was used as substrate.[a] Each value represents the mean ± standard deviation for six determinations.

nmoles hexachlorophene added / mg microsomal protein	nmoles p-nitrophenol consumed / mg protein/20 minutes	Percent Inhibition
control	9.2 ± 0.6	--
0.5	9.2 ± 0.5	0
1.0	8.3 ± 0.7	10
5.0	8.0 ± 0.7	13
9.9	7.8 ± 0.7	15
49.5	7.3 ± 0.5	20
99.0	6.7 ± 0.4	27

[a]Incubation mixture of Storey (1965a): microsomal protein (3-6 mg) and UDPGA (98 μmoles) in 0.1 M phosphate buffer, pH 7.4 (3 ml). Hexachlorophene (1.5-600 nmoles) in 50 μl acetone was added and preincubated at room temperature for five minutes. Controls received only 50μl acetone and were also preincubated for five minutes. p-Nitrophenol (42 μmoles) was added to start reaction and incubated at 37°C for 20 minutes. Assay for consumption of p-nitrophenol by method of Isselbacher (1956): Reaction stopped by placing in an ice-bath and adding 0.5 ml of 0.2 N trichloroacetic acid. The mixture was centrifuged and 0.5 ml of the supernatant was added to a cuvette containing 1 ml of water and 0.1 ml 2 N sodium hydroxide. The absorbance at 400 nm was measured.

glucuronide conjugation process was the least affected. Both the oxidative and reductive enzymatic processes were 50% inhibited at hexachlorophene concentrations (nmoles/mg protein) almost ten times less than the maximum binding capacity of the microsomes for hexachlorophene. The conjugation process was considerably less sensitive being 25% inhibited at a concentration three times lower than the maximum binding capacity.

In vitro Effect of Hexachlorophene on Microsomal Components

Ichikawa and Yamano (1967) demonstrated that in vitro incubation of rabbit liver microsomes with chlorinated phenols caused the conversion of cytochrome P-450 to its inactive P-420 form. The ability of a phenol to convert P-450 to P-420 was directly related to the number of chlorine substituents and its hydrophobic character, with pentachlorophenol being the strongest agent tested. Ichikawa and Yamano theorized that the conversion resulted from the interaction of the phenolic agent with the hydrophobic groups binding the heme to the apoprotein.

The effect of hexachlorophene on the cytochrome P-450 of rat liver microsomes was investigated at 37°C along with pentachlorophenol as a reference compound, by the procedure of Ichikawa and Yamano (Table 13). From Table 13 it was determined that approximately 100 nmoles of hexachlorophene or 140 nmoles of

Table 13. Decrease in cytochrome P-450 absorbancy by in vitro incubation with hexachlorophene or pentachlorophenol.[a] Each value represents the mean of three determinations.

nmoles added		After incubation	
mg microsomal proteins		nmoles P-450/	Percent
hexachlorophene	pentachlorophenol	mg protein	Decrease
control		1.08	--
4.95	--	1.02	5
49.50	--	0.81	25
495	--	0.18	83
--	48.90	0.79	27
--	489	0.30	72
--	978	0.16	85

[a]Incubation and assay procedure of Ichikawa and Yamano (1967): microsomes (6 mg protein) were incubated with hexachlorophene or pentachlorophenol (30-6000 nmoles in 10 µl acetone) in 3 ml of 0.1 M phosphate buffer, pH 7.0 at 37°C for ten minutes in a metabolic shaker. Controls received only 10 µl acetone and were similarly incubated and assayed. After incubation approximately 1 mg of sodium dithionite was added and 1 ml of the solution placed in a reference cuvette and another 1 ml placed in the sample cuvette. Carbon monoxide was bubbled for 15 seconds through the sample cuvette while air was bubbled through the reference cuvette. The spectra from 500 to 400 nm was recorded. The difference in absorption between 450 nm and 500 nm was divided by the extinction coefficient for P-450, 91 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964b) and the concentration of protein to yield the nmoles P-450/mg microsomal protein.

pentachlorophenphenol per milligram protein caused a 50% decrease in cytochrome P-450 absorbancy, thus hexachlorophene is a somewhat more potent agent than pentachlorophenol. Since a complimentary peak of P-420 appeared as the P-450 peak decreased, the action of the two phenols was believed to represent a conversion of P-450 to P-420, and not a result of the phenols binding to the cytochrome and making it unavailable for analysis (Figure 27).

The conversion of P-450 to its inactive P-420 form by hexachlorophene could explain the inhibitory effects of hexachlorophene on oxidative and reductive microsomal enzyme activity. If a concentration of 100 nmoles hexachlorophene per mg microsomal protein causes 50% of the P-450 to be converted to P-420 in ten minutes, much lower concentrations of hexachlorophene could also inhibit the oxidative and reductive enzyme systems during longer incubation periods, as seen previously with the 0-demethylase and nitroreductase systems (Tables 10 and 11).

Incubation of hexachlorophene with rat liver microsomes was also shown to decrease the absorbancy of reduced cytochrome b_5 (Figure 28). The amount of hexachlorophene added per miligram microsomal protein to cause the decrease in cytochrome b_5 absorption was similar to the amount added to convert cytochrome P-450 to P-420 (Table 14). The decrease in absorption of cytochrome b_5 might result from the denaturation of cytochrome b_5 by the detergent properties of hexachlorophene (Joswick, 1961) or from an inhibition of

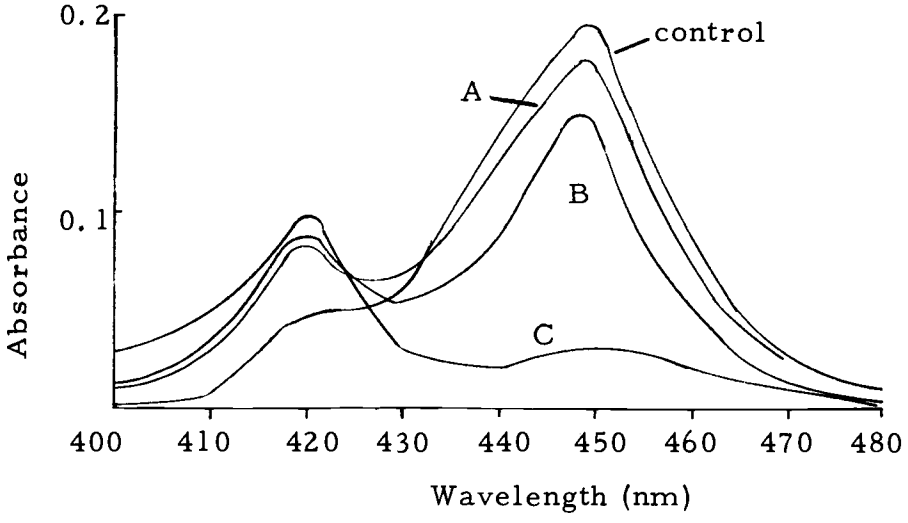


Figure 27. Cytochrome P-450 carbon monoxide difference spectra of microsomes after incubated ten minutes at 37°C in the presence of hexachlorophene. Assay conditions are identical to those in Table 13. Nanomoles hexachlorophene added per milligram microsomal protein in incubation mixture: A = 4.95, B = 49.5, C = 495.

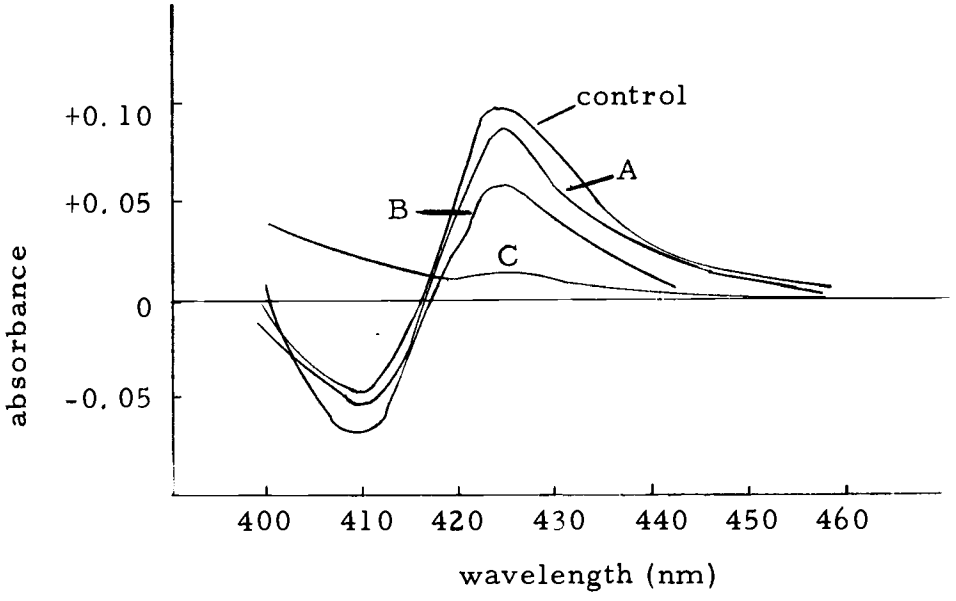


Figure 28. Cytochrome b_5 difference spectra of microsomes after incubated for ten minutes at 37°C in the presence of hexachlorophene. Assay conditions are identical to those in Table 14. Nanomoles hexachlorophene added per milligram microsomal protein in incubation mixture: A = 4.95, B = 49.5, C = 495.

Table 14. Decrease in cytochrome b_5 absorbancy by *in vitro* incubation of microsomes with hexachlorophene.[a] Each value represents the mean of two determinations.

nmoles hexachlorophene added / mg microsomal protein	After incubation: nmoles cytochrome b_5/ mg protein	Percent Decrease
control	0.98	--
4.95	0.85	13
49.50	0.56	43
495	0.12	88

[a]Incubation and assay procedure of Mazel (1971): microsomes (4 mg protein) were incubated with hexachlorophene (20-2000 nmoles in 10 µl acetone) in 2 ml 0.1 M phosphate buffer, pH 7.4, for ten minutes at 37°C in a metabolic shaker. Control received only 10 µl acetone and were similarly incubated and assayed. After incubation, 0.7 ml aliquots of the mixture were placed in the sample and reference cuvettes. A few mg sodium dithionite was added to the sample cuvette only and the spectra recorded from 500-400 nm. The difference in absorption between 423 nm and 500 nm was divided by the extinction coefficient, 171 $mM^{-1}cm^{-1}$ and the protein concentration and yielded the nmoles cytochrome b_5/mg microsomal protein.

cytochrome b_5 reduction by hexachlorophene.

A few inhibitors of microsomal enzymes have been shown to interact with cytochrome P-450 and yield a characteristic difference spectra (Gillette, Davis and Sasame, 1972). Hexachlorophene (15-495 nmoles in 10-100 μl of acetone-phosphate buffer) was added to a cuvette containing microsomes (3-4 mg protein) in 1-3 ml of 0.1 M phosphate buffer, pH 7.4. The spectra (0.1 OD full scale) from 500-380 nm was recorded against an identical microsomal suspension receiving only 10-100 μl of acetone-phosphate buffer. No difference spectra could be obtained at concentrations lower than 50 nmoles hexachlorophene per mg protein. At higher concentrations of hexachlorophene, the samples became slightly turbid, and the spectra that resulted had a slow steady increase in the absorption to 0.04 OD as the wavelength decreased. Since hexachlorophene converted P-450 to P-420 at fairly low concentrations, a difference spectra could not be readily obtained. The Cary 15 spectrometer used for these difference spectral studies may also not have detected a small change in absorbance due to the design of the machine.

DISCUSSION

Hexachlorophene is rapidly absorbed and then slowly excreted by both the rat and rabbit. Within 0.5 hours after intraperitoneal administration of hexachlorophene-^{14}C to rats, appreciable amounts of radioactivity appeared in the bile (Figure 9), indicating that hexachlorophene was rapidly absorbed from the peritoneal cavity of these animals and then quickly eliminated via the liver into the bile. Rapid absorption of the chlorinated bisphenol and accumulation by the liver would explain the prompt appearance of toxicologic symptoms immediately following administration of hexachlorophene, such as the marked hyperthermia from uncoupling of oxidative phosphorylation within 15 minutes after intraperitoneal administration of hexachlorophene to rats (Nakaue, Dost and Buhler, 1972). In addition, radioactivity in bile from a rat given an intraperitoneal dose of hexachlorophene-^{14}C was shown to be rapidly and almost completely reabsorbed from the intestinal tract of a second rat, apparently after hydrolysis of hexachlorophene conjugates by the gut microflora to release the free bisphenol (Figure 14). These results suggest that a rapid and almost complete absorption of oral doses of hexachlorophene should occur. Buhler, Dost and Rasmusson (1972) have confirmed comparably high rates of hexachlorophene absorption in the rat following either oral or intraperitoneal doses of the labeled bisphenol with

equivalent levels of radioactivity appearing in the tissues within two hours after either route of administration. The appearance of 8-10% of the radioactive dose in the urine of rabbits 12 hours after intraperitoneal administration of hexachlorophene-^{14}C (10 or 15 mg/kg) suggests a rapid absorption of the administered drug also occurs in the rabbit.

Following intraperitoneal administration of labeled hexachlorophene to rabbits and rats, the primary route of excretion of the radioactivity was via the bile and into the feces. The rabbit was found to excrete 45-52% of the radioactive dose in the feces and 20-25% in the urine following intraperitoneal administration of 10 or 15 mg/kg of the hexachlorophene-^{14}C (Figure 16). Similar results were found by other investigators after administration of labeled hexachlorophene to rats, rabbits, cows, guinea pigs, and man, with the feces the major route of excretion (Wit and Van Genderen, 1962; Buhler, Dost and Rasmusson, 1972; Maibach, 1971).

In the present study, rabbits eliminated between 20-30% of the administered radioactive dose in the urine within four days. All other species excreted less than 10% of the dose in the urine over comparable time periods. A greater urinary excretion of radioactivity by the rabbit might result from a lower rate of biliary excretion of xenobiotics by this species thus resulting in a larger total amount of the material that may be removed from the body by the kidney (Williams, 1971).

Evidence that hexachlorophene undergoes biliary excretion and probably enterohepatic circulation has been available for some time (Thorpe, 1969). Biliary excretion of conjugated metabolites of the related phenols, bithionol (Meshi, Yoshikawa and Sato, 1970) and pentachlorophenol (Jakobson and Yllner, 1971) also have been shown. In addition, the molecular weight of conjugated hexachlorophene would make it likely to be excreted via the bile rather than the kidneys (Stowe and Plaa, 1968).

In rats, 31 and 45%, respectively, of the radioactivity from a 5 mg/kg and 10 mg/kg intraperitoneal dose of hexachlorophene-^{14}C was shown to be excreted into the bile. This extent of biliary excretion is comparable to that of many of the steroidal hormones and the structurally related synthetic estrogen, diethyl stilbesterol (Smith, 1966). Up to 90% of the administered dose of diethyl stilbesterol to rats is excreted in the bile as the monoglucuronide, and between 60-100% of the diethyl stilbesterol monoglucuronide eliminated in the bile was hydrolyzed by gut microflora, reabsorbed, and reexcreted in the bile by enterohepatic circulation (Smith, 1966). With hexachlorophene, over 92% of the biliary radioactivity, which was mainly in the form of the monoglucuronide conjugate of hexachlorophene, was reabsorbed from the intestinal tract and reexcreted in the bile suggesting a marked degree of enterohepatic circulation. The extensive reabsorption of radioactivity from the gut of the rat indicated that the monoglucuronide

conjugate of hexachlorophene is hydrolyzed by intestinal microflora to release hexachlorophene, which is then reabsorbed, since absorption of highly polar acidic compounds, like glucuronides, by the gut is known to be limited (Smith, 1966). Enterohepatic circulation of hexachlorophene could explain the long retention of the bisphenol and its metabolites in the body.

Ligation of the bile duct in rats treated with hexachlorophene-^{14}C intraperitoneally greatly increases the excretion of radioactivity via the urine. The urinary metabolites are mainly in the form of the glucuronide conjugate of hexachlorophene, which is preferentially excreted in the bile in normal rats. The low excretion of hexachlorophene glucuronide via the urine observed in normal rats might be explained by low glucuronide levels in the blood, strong binding of the conjugate to plasma proteins, or reabsorption of the glucuronide by the renal tubules. In bile duct ligated animals, the amount of unbound glucuronide in the blood is probably much higher and apparently then available for removal by the kidney.

The plasma, bladder contents, and gall bladder contained the highest C-14 specific activities four days after intraperitoneal administration of hexachlorophene-^{14}C to rabbits, while the fat, muscle, and brain were among the lowest (Table 6). The largest percent of the dose remaining in the tissues of the animal was found in the liver with appreciable amounts also present in the intestines, bladder

contents, caeca contents, fat, muscle, and stomach contents. No extensive degree of storage was noted among the tissues examined. The slow excretion of radioactivity from the rabbit, mainly via the feces, together with the concentration of radioactivity in the gall bladder suggests that in the rabbit, as in the rat, the bisphenol and its metabolites are mainly excreted via the bile and that hexachlorophene probably also undergoes enterohepatic circulation. The finding of high plasma radioactivity levels four days after dosing implies that hexachlorophene and its biotransformation products also firmly bind to blood proteins. Such strong binding, together with an extensive enterohepatic circulation, probably results in the long retention of radioactivity by the rabbit and in the low urinary excretion of hexachlorophene and its metabolites. The distribution of radioactivity in tissues of rabbits dosed with hexachlorophene-^{14}C was similar to that found in rats given the labeled bisphenol either orally or intraperitoneally (Wit and Van Genderen, 1962; Buhler, Dost and Rasmusson, 1972).

Appreciable radioactivity still remained in the stomach contents of rabbits four days after intraperitoneal administration of hexachlorophene-^{14}C, and radioactivity also was present in the feces of bile duct ligated rats dosed intraperitoneally with the labeled bisphenol. The distribution data of Buhler, Dost and Rasmusson (1972) indicate a delayed appearance of peak radioactivity levels in the stomach after

intraperitoneal administration of hexachlorophene-^{14}C to rats. Extra-biliary excretion of hexachlorophene or its metabolites into the gastrointestinal tract is, therefore, suggested. Gastric excretion of hexachlorophene or its metabolites, which occurs with the related chlorinated phenol, pentachlorophenol (Jakobson and Yllner, 1971), would explain these results. The apparent extra-biliary excretion of hexachlorophene and its metabolites may also result from salivary excretion, which is known to occur with a number of acidic compounds in species with basic saliva (Stowe and Plaa, 1968). An additional possibility might be the direct penetration of radioactive hexachlorophene from the peritoneal cavity into the entire gastrointestinal tract. The latter explanation seems unlikely since the specific activities (dpm/g dry tissue) of the stomach and intestinal contents from dosed rabbits were almost equal (Table 6). The amount of radioactivity in the intestines could be expected to be much higher from biliary excretion alone than that appearing in the stomach as a result of diffusion from the peritoneal cavity.

Explanation of the observed optic nerve atrophy (Udall and Malone, 1969) and spongy degeneration of brain tissue (Kimbrough and Gaines, 1971) in hexachlorophene treated animals on a basis of a specific accumulation of the bisphenol or its metabolites in nervous tissue does not appear to be probable because of the low levels of radioactivity found in rabbit brain tissue four days after administration

of hexachlorophene-^{14}C. One could postulate, however, that degenerative changes could result from a transitory localization of hexachlorophene or its metabolites in nervous tissue shortly after dosing.

Only 70-78% of the radioactive dose of hexachlorophene-^{14}C administered to rabbits was recovered in the urine and feces. No radioactivity was detected in air expired by the treated rabbits, and the estimated radioactivity remaining in the tissues of the animals (1.61% of the dose after four days) failed to account for the remainder of the administered dose (22-30%). A possible explanation for the incomplete recovery of radioactivity would be an inaccurate assessment of the radioactivity which remains in the tissues of the dosed animals. Samples from all tissues, such as bone, were not taken for measurement of radioactivity content and, in the case of more widely distributed tissues (muscle, fat, and blood), only a small and perhaps nonrepresentative sample from a single location was removed for analysis. It is also possible that hexachlorophene precipitated out of the corn oil solution after injection (perhaps after a more rapid absorption of the corn oil), and a small amount of the radioactive precipitate then remained lodged at a site in the peritoneal cavity where it is not readily absorbed and hence not detected by tissue distribution analysis. Rabbits that were dosed repeatedly with hexachlorophene still did not eliminate the entire dose in the urine and feces during the collection period and showed only a slight increase

in urine and no increase in fecal recoveries. These results suggest that the site of retention of the unexcreted radioactivity is not readily saturated.

Bithionol sulfoxide, a sulfur analogue of hexachlorophene, has been shown to be excreted and distributed in a manner similar to that of hexachlorophene (Takahashi, Yoshikawa and Sato, 1969). The ^{35}S-labeled thiobisphenol was found to be rapidly absorbed from the gastrointestinal tract, with over 70% of a dose being absorbed from the ligated loop of a rat's intestine in four hours. Radioactivity was well distributed throughout the rat with highest levels occurring in the plasma, liver, and lung and the lowest in the brain. Following oral administration, the amounts of radioactivity excreted in six days via the urine and feces were 6% and 65%, respectively. An incomplete recovery of the radioactive dose and strong binding of the parent compound to plasma proteins also were noted, results comparable to those found with hexachlorophene in the present study.

The nature of the radioactivity in the urine and feces of rabbits and rats dosed with hexachlorophene-^{14}C was quite similar. In the urine of both species, acidic metabolites of hexachlorophene predominated, occurring mainly in the form of conjugates. The percent of the dose excreted in the urine of rabbits was significantly higher than that present in rat urine although similar proportions of conjugated and nonconjugated metabolites were present in the urine of both

species. Apparently both animals metabolize hexachlorophene in a similar manner, differing only in the relative amounts excreted in the urine.

Metabolites of hexachlorophene present in the feces of both species were likewise quite comparable. Nonconjugated acidic metabolites of hexachlorophene predominated in the feces. Less than 10% of the fecal radioactivity in the rat and rabbit was in the form of conjugated metabolites.

Wit and Van Genderen (1962) analyzed the urine and feces of rats, rabbits, and cattle for the amount of hexachlorophene excreted following treatment of the different species with an oral dose of hexachlorophene-^{14}C. In their work radioactivity in urine was not determined, but feces was plated on planchets and counted in a flow counter. After hydrolysis of conjugates by refluxing with acid, the acid hydrolyzed radioactive urine and fecal extract were treated with sodium hydroxide to make the solution basic, unlabeled hexachlorophene added, and the hexachlorophene precipitated from the solutions by acidification. The precipitate was analyzed for hexachlorophene radioactivity on planchets at infinite thickness by an end window counter. From these procedures, Wit and Van Genderen found that a major portion of the radioactive dose (63-72%) in the species examined was recovered in the feces. In the cow all the fecal radioactivity was identified as unchanged hexachlorophene, while in the rat and rabbit,

approximately 30% of the dose was not recovered from the feces by the methods used and was speculated to be metabolites of hexachlorophene. This radioactivity could also have been lost during the isolation and precipitation of hexachlorophene, since measurements of radioactivity were apparently not taken during the isolation procedures. These researchers also found 1, 6, and 30% of the radioactive dose, respectively, appearing in the urine of the cow, rat, and rabbit as unchanged hexachlorophene. Since the total radioactivity in the urine was not determined, other radioactive metabolites of hexachlorophene might have been present but not detected. St. John Jr. and Lisk (1972) found similar results in cows treated with unlabeled hexachlorophene after analysis of urine and feces by a gas chromatographic method specific for hexachlorophene. They reported 63% of the hexachlorophene fed to cattle was excreted in the feces as unconjugated hexachlorophene, and that less than 1% of the dose was found in the urine also as unchanged hexachlorophene. Other metabolites of hexachlorophene could have been present but not detected by their gas chromatographic method. There was an apparent absence of conjugate formation of hexachlorophene in the cow, since acidic hydrolysis of the urine and feces did not release additional amounts of hexachlorophene as determined by gas chromatographic analysis. In neither the study by Wit and Van Genderen nor the study by St. John Jr. and Lisk was an attempt to further investigate the presence of metabolites of

hexachlorophene.

My studies identified essentially all the radioactive components in the extracts of urine and feces from both rats and rabbits as either free hexachlorophene or its glucuronide conjugate. Some unextracted radioactive material remained in the feces in the rabbit studies, but this incomplete recovery probably resulted from the rapid extraction procedure employed. All of the radioactivity in rat feces was extracted by a more exhaustive procedure and shown to be hexachlorophene or hexachlorophene conjugates. The radioactivity in the urine of rats and rabbits was shown in this study to be over half in the form of hexachlorophene conjugates with the remainder as unchanged hexachlorophene. After acid hydrolysis, Wit and Van Genderen detected quantities of hexachlorophene in the urine of the two species similar to those found in the present studies.

The methods used in this study followed the radioactivity excreted in both the urine and feces, and assays were made during procedures to locate all the radioactivity. The nature of the radioactivity in the urine and feces was investigated at many stages of isolation with and without hydrolysis. The thin layer and gas chromatography and radioactive assay procedures used in this study had limits of analysis far lower than those previously used and could detect minute amounts of degraded or metabolized hexachlorophene. The possible adherence of hexachlorophene to glassware was not

investigated but, in all cases, multipled samples were assayed, and the transfer of most solutions was accompanied with a methanol rinse of the previous container.

The major and probably only metabolite of hexachlorophene excreted by rats and rabbits is the glucuronide conjugate of hexachlorophene. This conjugate has been isolated and identified as hexachlorophene monoglucuronide in rabbit urine and rat bile (Figure 29). If the conjugate of hexachlorophene in the urine and feces of hexachlorophene treated rats (Table 4) can also be assumed to be hexachlorophene monoglucuronide, the distribution of hexachlorophene and hexachlorophene monoglucuronide shown in Table 15 is present after oral or intraperitoneal administration of hexachlorophene-^{14}C.

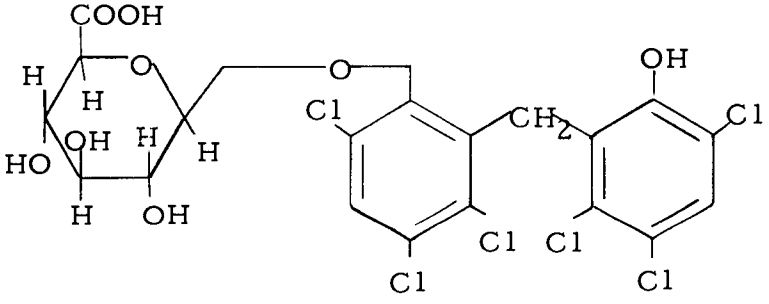


Figure 29. Hexachlorophene monoglucuronide; 2-(β -D-glucopyranuronate)-2'-hydroxy-3, 3', 5, 5', 6, 6'-hexachlorodiphenylmethane.

Table 15. Percent of hexachlorophene and hexachlorophene monoglucuronide in the pooled 0-2 day urine and feces of rats administered 5 mg/kg of hexachlorophene orally or intraperitoneally.

	Percent of total urine and fecal radioactivity[a]	
0-2 day pooled samples	Hexachlorophene	Hexachlorophene monoglucuronide
Intraperitoneal dose		
Urine	1.7 (0.78)	5.7 (2.67)
Feces	77.4 (36.29)	9.2 (4.31)
TOTAL	79.1 (37.07)	14.9 (6.98)
Oral dose		
Urine	1.8 (1.19)	1.9 (1.30)
Feces	89.2 (59.59)	3.3 (2.19)
TOTAL	91.0 (60.78)	5.2 (3.49)

[a]Values in parentheses are percent of radioactive dose.

A C-14 distribution similar to that of rats was found in the urine and feces from rabbits intraperitoneally treated with hexachlorophene-^{14}C. The data presented in Table 16 is the amount of hexachlorophene and hexachlorophene monoglucuronide found during isolation of the monoglucuronide conjugate from rabbit urine and the results from the fractionation study presented in Table 7.

Table 16. Percent of hexachlorophene and hexachlorophene monoglucuronide in the urine and feces of rabbits dosed intraperitoneally with hexachlorophene-^{14}C.

	Percent of total urine and fecal radioactivity		
0-4 day pooled samples	Hexachlorophene	Hexachlorophene monoglucuronide	Other
Urine	10-13	16-18	0-5[a]
Feces	53	3	14[b]
TOTAL	63-66	19-21	0-19

[a]Summation of sulfate, acid hydrolyzed, and unextracted fractions. In later studies, when extraction of the urine was performed with Amberlite XAD-2, these fractions were not present. Hence, the radioactivity in these fractions probably represented a mixture of unhydrolyzed glucuronide and unextracted hexachlorophene.

[b]Represents the amount of radioactive material remaining in the fecal residue after nonexhaustive extraction of feces with methanol:water.

Because extraction of the radioactivity from the urine of rats and rabbits dosed with hexachlorophene-^{14}C (Tables 4 and 7) was not complete, the calculated distribution of hexachlorophene and hexachlorophene monoglucuronide (Tables 15 and 16) may vary depending on the nature of the unextracted radioactivity. The residual urine radioactivity in the initial rabbit studies was later shown to be hexachlorophene and hexachlorophene monoglucuronide, and probably the same would be true in the rat studies. In any case, the small quantities of unextracted radioactivity in the urine of either species would probably not affect the total metabolite distribution. The fecal radioactivity of the rabbit was not completely extracted by the methanol:water technique, while all the radioactivity in the feces of the treated rats

was extracted and shown to be hexachlorophene or hexachlorophene glucuronide. Therefore, the radioactivity remaining in the feces of the rabbit was also probably hexachlorophene or hexachlorophene monoglucuronide. An exhaustive extraction of the feces with a heated soxhelet apparatus was not performed for fear of a loss of radioactivity during the heated process as happened during the previous liquid-liquid extraction process with the rabbit urine.

The loss of radioactivity during liquid-liquid extraction of the urine of hexachlorophene-^{14}C treated rabbits might have resulted from reasons already postulated. In any case, the loss resulted in almost a 50% decrease in the radioactivity remaining in the total urine after liquid-liquid extraction to remove the nonconjugates. The relative abundance of radioactive components in the urine could be greatly influenced, depending on the nature of the missing radioactivity. However, further experiments with rabbit urine during the glucuronide isolation showed that the radioactive metabolites in the urine were similar to those found in the initial study, and the lost material was, therefore, assumed to be the same as the remaining radioactivity.

It became evident from the tentative identification of an ultraviolet degradation product of hexachlorophene, 2,2-dihydroxy-3,3',5,6,6'-pentachlorodiphenylmethane, and some unidentified radioactive polar material (Shaffer et al., 1971), that studies with hexachlorophene must consider the possible nonenzymatic degradation of the compound

by environmental interactions.

The fate of hexachlorophene is similar to that of other diphenols. A sulfur analogue of hexachlorophene, bithionol, is excreted mainly as bithionol glucuronide with this conjugate accounting for 50% of the urinary radioactivity and 97% of the radioactivity in the bile (Meshi, Yoshikawa and Sato, 1970). Bisphenol A, an unchlorinated bisphenol, is excreted almost entirely as a glucuronide conjugate in the urine and as one-third of the dose present in the feces (Knaak and Sullivan, 1966).

Conjugation of acidic phenols such as hexachlorophene or pentachlorophenol was initially thought not to occur, especially with sulfate (Dodgson, Smith and Williams, 1950). Recently, though, an acid hydrolyzable conjugate (Jakobson and Yllner, 1971) and a glucuronide conjugate (Tashiro et al., 1970) of pentachlorophenol and a glucuronide conjugate of hexachlorophene isolated in the present studies have proven otherwise.

All phenols with a diphenylmethane hydrocarbon structure were also believed to be incapable of forming sulfate conjugates (Williams, 1959). The apparent absence of significant amounts of sulfate conjugates of hexachlorophene in the urine, feces, and bile of rats and rabbits is in line with this theory. The possible occurrence of a small amount of sulfate or other conjugates of hexachlorophene, however, cannot be completely eliminated. It is possible that in the blood and tissues of the treated animals other types of conjugates other than

glucuronides may be formed and then hydrolyzed prior to elimination, but this possibility has not been investigated. Moreover, if other highly polar conjugates of hexachlorophene were present and were resistant to acid cleavage, they would not have been detected in the urine or feces.

Formation of hexachlorophene monoglucuronide rather than a diglucuronide conjugate probably occurs because monoconjugation with glucuronic acid (or sulfate) forms a relatively acidic compound. Since very acidic compounds have been shown to not be extensively conjugated with glucuronic acid, further conjugation of the acidic monoconjugates would probably not occur and then would tend to be excreted in the monoconjugate form (Parke, 1968). Steric hindrance might also be a factor restricting formation of a diconjugate.

The toxic effects of hexachlorophene, such as uncoupling of oxidative phosphorylation, have been shown to occur immediately after administration of hexachlorophene (Nakaue, Dost and Buhler, 1972). If one assumes that a majority of the hexachlorophene did not undergo conjugation until a later time as shown in Figure 17, any toxic effects observed prior to that time would probably then result from the parent compound. In the studies with bile duct ligated rats, the conjugate that is normally excreted mainly in the bile was eliminated via the kidney. If conjugation were to continue at the same rate in the ligated rat as in the cannulated rat, it may be assumed that the levels of

glucuronide conjugate were higher in these animals under conditions where the animals appeared to be relatively unstressed. In bile duct ligated rats given a higher dose of hexachlorophene the animals died soon after administration of the compound, but under these circumstances no significant conjugation probably occurred in the short time period prior to death. In any case, the glucuronide conjugate is probably not significantly more toxic than the parent compound.

The formation of hexachlorophene monoglucuronide and the elimination of the conjugate in the bile represents a detoxification mechanism. The aglycone, which is known to have deleterious effects on liver function, was modified and presumably removed from its site of action. A glucuronide conjugate tends to be intrinsically less toxic than its parent compound (Williams, 1959). Glucuronide conjugates are also less likely to cross the membranes of the body and be distributed into the cells due to the large hydrophilic ionized carbohydrate moiety (Mandel, 1971), thus lowering the amount present in tissues like the brain and decreasing the possibility of the glucuronide being a toxic agent.

Further in vivo studies with the isolated hexachlorophene monoglucuronide from the rat bile or the isolated material from rabbit urine was not attempted, due to an inability to obtain a large amount of the purified glucuronide. The isolated hexachlorophene monoglucuronide was an oil containing many impurities, which could not be

further purified to any appreciable extent. Attempts were made to remove methoxy and acetyl groups from the synthetic hexachlorophene glucuronide derivative, but only an impure oil resulted with some possible cleavage of the glycosidic bond.

While the glucuronic acid conjugation process would be expected to greatly decrease the toxicity of hexachlorophene and aid in its removal from the body, hexachlorophene monoglucuronide excreted in the bile is apparently hydrolyzed by the gut microflora, and the released hexachlorophene is then reabsorbed from the intestinal tract. This enterohepatic circulation, coupled with a strong binding of hexachlorophene or hexachlorophene monoglucuronide to plasma proteins, no doubt greatly influences the toxicity of the bisphenol by increasing its biological half-life.

In the investigation of the possible in vitro oxidative metabolism of hexachlorophene, both microsomal oxidative metabolism and a model chemical oxidative system (Udenfriend et al., 1954) failed to oxidize hexachlorophene. These results confirmed the earlier in vivo studies with rats and rabbits that hexachlorophene was resistant to oxidative metabolism. St. John Jr. and Lisk (1972) have also found that hexachlorophene was neither oxidized or conjugated by either the 10,000 g beef liver supernatant fraction or the rumen fluid of the cow. These workers also reported the absence of conjugates of hexachlorophene in the urine and feces of cows dosed with the chlorinated bisphenol.

While my attempts failed to demonstrate conjugation of hexachlorophene in vitro by rat liver microsomes, rats were shown to form hexachlorophene glucuronide in vivo. The most likely explanation for this difference would be that in vivo hexachlorophene is selectively localized in the liver cells in such a way that it is readily available for conjugation. It probably is bound to cellular constituents with a phenolic group accessible for conjugation, while in vitro hexachlorophene is preferably bound strongly to the microsomal membranes themselves. Since UDP-glucuronyl transferase protein represents but a small amount of the total microsomal protein and is of low specific activity (Mandel, 1971), hexachlorophene may not selectively interact with the transferase and undergo appreciable conjugation. Hexachlorophene was also demonstrated to be an inhibitor of in vitro microsomal UDP-glucuronyl transferase activity (Table 12). Possibly the amount of hexachlorophene added to the microsomal system poisoned the transferase enzyme, but this should not have occurred when hexachlorophene was added applied to a polyethylene disc where it was slowly released to the enzyme system. While it is also possible that liver might not be the site of conjugation of hexachlorophene, this tissue has the highest level of UDP-glucuronyl transferase activity (Mandel, 1971).

In view of the lack of hexachlorophene oxidation in vivo and the large binding capacity of hepatic microsomes for hexachlorophene, the

effects of the bisphenol on microsomal enzyme activity was investigated. Hexachlorophene was found to inhibit the 0-demethylase, nitroreductase, and UDP-glucuronyl transferase activities of the microsomes (Figure 30). Hexachlorophene caused an inhibition of microsomal oxidation and reduction systems at concentrations of added hexachlorophene per milligram microsomal protein far below the maximum binding capacity (305 nmoles hexachlorophene/mg protein) of the microsomes for hexachlorophene. The concentration of hexachlorophene required for in vitro inhibition of the oxidative and reductive microsomal enzymes is similar to that necessary for inhibition by other known microsomal inhibitors, SKF-525 A, piperonyl butoxide, sesamex, and tropital (Anders, 1968). Hexachlorophene inhibited the glucuronic acid conjugation process at somewhat higher concentrations, in a manner analogous to that of competative phenolic inhibitors (Storey, 1965b). The extent of inhibition of the in vitro glucuronic acid conjugating system by hexachlorophene is also comparable to the inhibition by several insecticide synergists, which are also known mixed function oxidase inhibitors (Mehendale and Dorough, 1971).

The mechanism for inhibition of microsomal metabolism in vitro could involve interaction of the inhibitor with either a common component of the microsomal enzyme system resulting in its destruction or reduced concentration or the microsomal membranes resulting

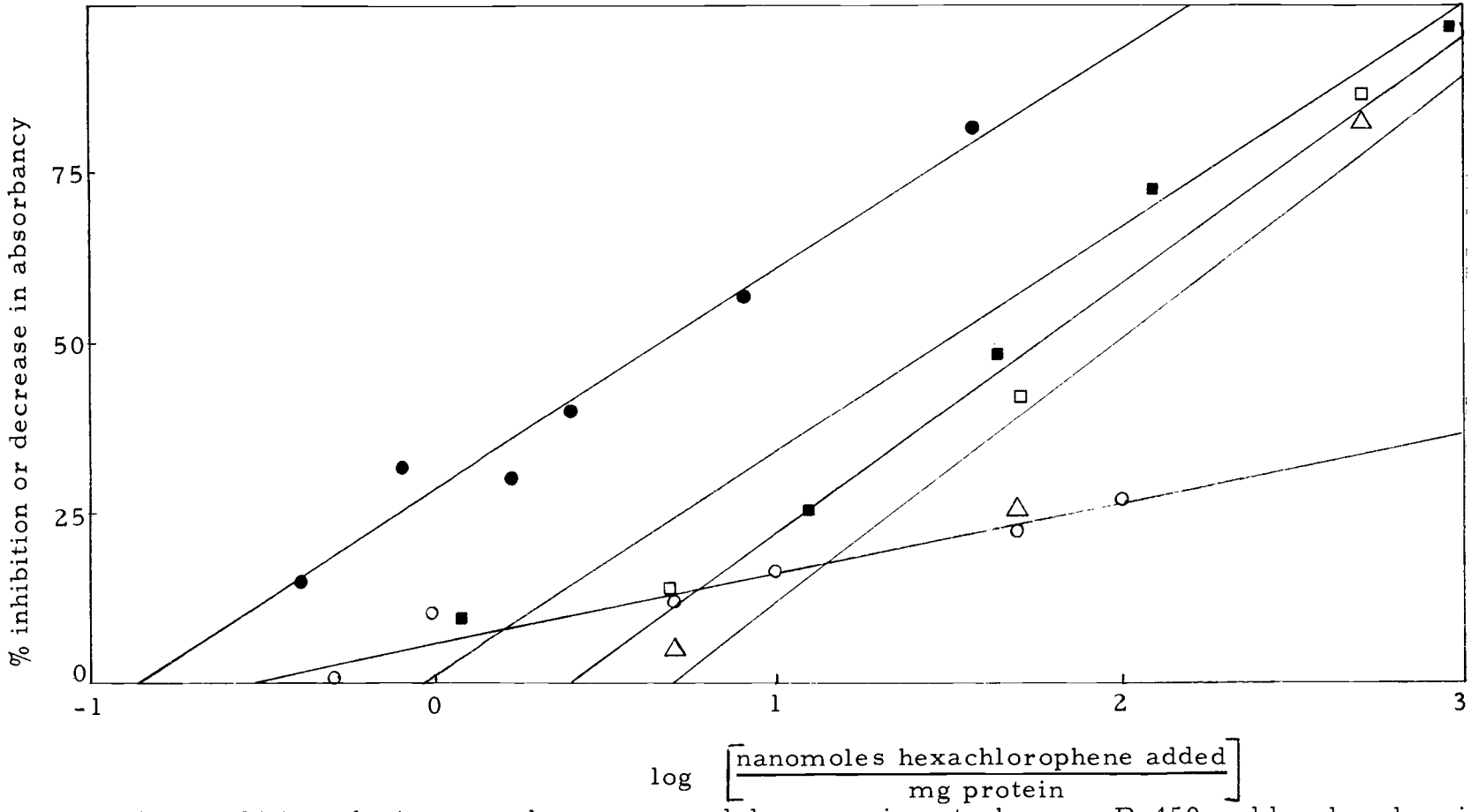


Figure 30. Inhibition of microsomal enzymes and decrease in cytochromes P-450 and b_5 absorbancies by in vitro incubation with hexachlorophene. ● = p-nitroanisole demethylase, ■ = p-nitrobenzoic acid reductase, o = p-nitrophenol conjugation with glucuronic acid, Δ = decrease in cytochrome P-450 absorbancy, P-420, and □ = decrease in cytochrome b_5 absorbancy. Conditions of assays in text.

in physiochemical effects and changed permeability to substrates (Anders, 1971). Cytochrome P-450 was found to interact with hexachlorophene, causing a decrease in P-450 absorbancy with its possible conversion to P-420 (Figure 30). Destruction of a common component, such as cytochrome P-450, by hexachlorophene could be expected to inhibit most of the microsomal enzymatic processes. Hexachlorophene was also shown to exhibit detergent-like properties with bacterial membranes (Joswick, 1961), suggesting that it might be capable of modifying microsomal membrane permeability. The absorbancy of microsomal cytochrome b_5 was also decreased by incubation of microsomes with hexachlorophene (Figure 30), possibly because of the detergent action of the bisphenol. However, the concentration of hexachlorophene necessary to cause a decrease in absorbancy of the two cytochromes is higher than that required for inhibition of the microsomal enzyme systems. It might be possible, though, for hexachlorophene to produce an alteration in cytochrome function at a lower concentration and upon a longer incubation period, thus eventually inhibiting various microsomal enzyme systems (Figure 30).

Many of the agents that inhibit microsomes both in vitro and in vivo interact with the ligands of cytochrome P-450 and cause characteristic difference spectra. Hexachlorophene did not yield a difference spectra with P-450, possibly, due to the conversion of cytochrome P-450 to P-420, thus destroying the active chromaphore.

Other possible mechanisms for the inhibition of the microsomal enzymes by hexachlorophene may stem from the ability of hexachlorophene to chelate metals, as suggested by Adams and Hobbs (1958) for its mode of action with bacterial enzymes. If the iron of the heme group of cytochromes P-450 and b_5 could chelate with hexachlorophene, this would undoubtedly inhibit the activation of oxygen by the cytochrome and decrease the enzymatic activities of systems involving these cytochromes. Mason, North and Vanneste (1965) have reported the conversion of cytochrome P-450 to P-420 by chelating agents specific for copper and iron, possibly by acting through a chelating mechanism.

The similarity in the slope of the lines in Figure 30 suggests that, except for the conjugating process, a common mechanism is responsible for the effect of hexachlorophene on the oxidative and reductive microsomal systems. The inhibition of microsomal enzymes by hexachlorophene was also shown to occur at concentrations similar to those necessary for the inhibition of mitochondrial cytochrome c oxidase (Caldwell, Nakaue and Buhler, 1972). Since Flores and Buhler (1971) found that 50% of the hexachlorophene bound to treated red blood cells was associated with hemoglobin, hexachlorophene may commonly interact with hemoproteins in the animal systems. The concentration of hexachlorophene necessary for uncoupling of oxidative phosphorylation, which is thought to occur via an effect on the mitochondrial membrane, was shown, though, to be much lower than that

necessary for the inhibition of cytochrome c oxidase and microsomal enzymes.

Some of the in vitro effects of hexachlorophene on microsomes might not occur in vivo with the endoplasmic reticulum but other inhibitors have been shown to effect drug metabolism similarly under both conditions (Anders, 1971). Nakaue (1972) has recently demonstrated inhibition of p-nitroanisole demethylase activity of liver homogenates from rats receiving an oral dose of hexachlorophene and of smooth and rough endoplasmic reticulum from rats receiving hexachlorophene in their diet. This same author has also found a 44% increase in hexobarbital sleep time in rats following an oral dose of hexachlorophene.

This study has shown that at the dose levels administered to rats and rabbits, hexachlorophene was detoxified to its monoglucuronide conjugate, and this conjugate was excreted in the urine and bile. No evidence was found for the activation of hexachlorophene that would cause the observed toxicological responses in animals. Thus the toxic agent appears to be the unmodified bisphenol. Some of the delayed toxicological responses, such as optic nerve atrophy (Udall and Malone, 1969) and spongy degeneration of brain tissue (Kimbrough and Malone, 1971) may result from the slow excretion of the administered dose of hexachlorophene due to the strong plasma binding and enterohepatic circulation of the drug.

During normal use of products containing hexachlorophene, the

small amount of the bisphenol that is absorbed should be readily metabolized by conjugation and eliminated slowly from the body. However, if there is a continual exposure to low levels of hexachlorophene or an accidental ingestion of a large dose of the bisphenol, inhibition of normal metabolism of drugs and endogenous steroid hormones by the liver endoplasmic reticulum and other toxicological responses could occur. These data on the fate of hexachlorophene in rats and rabbits and the *in vitro* inhibitory effect of hexachlorophene on rat liver microsomes should also be useful in explaining any toxicological responses that hexachlorophene may cause in humans.

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