THE METABOLISM AND DISPOSITION OF
HEXACHLORO-1:3-BUTADIENE
IN THE RAT AND ITS RELEVANCE TO
NEPHROTOXICITY

by

John Anthony Nash C Biol, MI Biol

A Thesis submitted to the University of Surrey for
the Degree of Master of Philosophy

1985

Imperial Chemical Industries PLC
Central Toxicology Laboratory
Alderley Park
Macclesfield
Cheshire SK10 4TJ

5901497

ProQuest Number: 10804302

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.

ProQuest 10804302

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

SUMMARY

Following oral administration of a nephrotoxic dose (200mg/kg) of hexachloro-1:3-butadiene (HCBD) to male rats, bile was the principal route of excretion, 17-20% of the dose being eliminated on each of the first two days. Faecal excretion over this period was less than 5% of the dose per day, suggesting enterohepatic recirculation of biliary metabolites. Urinary excretion was small, not exceeding 3.5% of the dose during any 24hr period.

Incubation of HCBD with rat liver microsomes in vitro gave a product which was chromatographically (TLC) similar to the major biliary metabolite of HCBD. The in vitro and major biliary metabolite were identified as S-(1,1,2,3,4-pentachloro-1,3-butadienyl)glutathione which demonstrated a direct conjugation reaction without prior oxidation.

The cysteinylglycine conjugate of HCBD was also found in bile. Evidence has been obtained to show that biliary metabolites of HCBD are reabsorbed and excreted via the kidneys. Whole body autoradiography demonstrated a high concentration of radioactivity in the outer medulla of the kidney on dosing [^{14}C]-HCBD. The glutathione conjugate, its mercapturic acid derivative and bile containing HCBD metabolites are all nephrotoxic when dosed orally to rats. In common with HCBD, these metabolites cause localised damage to the kidney with minimal effects in the liver. Rats fitted with a biliary cannula were completely protected from kidney damage when dosed with HCBD, demonstrating that hepatic metabolites are solely responsible for the nephrotoxicity of this compound.

It is proposed that the hepatic glutathione conjugate of HCB_D was degraded to its equivalent cysteine conjugate which was cleaved by the renal cytosolic enzyme β -lyase to give a toxic thiol which caused localised kidney damage. A urinary sulphenic acid metabolite of HCB_D has been identified which is consistent with this hypothesis. The mode of activation of HCB_D conjugates in the kidney is believed to be analogous to that proposed for S-(1,2-dichloro-vinyl)-L-cysteine.

To Beverley Anne, Claire Louise and Ian Anthony

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisors Dr E A Lock and Dr T Green of Central Toxicology Laboratory and Professor L J King of the Department of Biochemistry, University of Surrey for their helpful advice and encouragement during the course of this work.

I wish to thank Imperial Chemical Industries PLC, Central Toxicology Laboratory, for their support and for allowing me to present this work as an M Phil thesis.

I would like to acknowledge Dr C Rhodes for his help with the biliary toxicity experiment, Dr M D Stonard and Mr C W Gore for the biochemical assays, Mr W Laird for his assistance with the mass spectrometry and Dr J Ishmael for histopathological assessment of livers and kidneys. Mr D Catlow of Imperial Chemical Industries PLC, Pharmaceuticals Division kindly provided the field desorption mass spectra of the glutathione conjugate.

Finally I would like to thank Mrs R J Steele, Miss S A Basford and Miss D L Broome for typing this thesis.

CONTENTS

Page Number

ABBREVIATIONS

1

INTRODUCTION

<u>1.1</u>	<u>HCBD its Uses and the Risk of Human Exposure</u>	2-3
<u>1.2</u>	<u>The Toxicology of HCBD in Animals</u>	3-8
<u>1.3</u>	<u>The Kidney and its Susceptibility
to Chemical Damage</u>	8-15
<u>1.4</u>	<u>Metabolic Considerations and Possible Mechanisms
of Nephrotoxicity</u>	15-25
<u>1.5</u>	<u>Objectives of this Study</u>	25

MATERIALS AND METHODS

26-49

RESULTS

<u>3.1</u>	<u>Excretion and Distribution of HCBD in the Rat</u>	50-57
<u>3.2</u>	<u>Identification of the in vitro Microsomal
Metabolite of HCBD</u>	58-61

CONTENTS - continued

	Page Number
<u>3.3</u> <u>Metabolism of HCBd in the Rat after Oral Dosing</u>	61-80
<u>3.4</u> <u>Toxicity Studies</u>	80-96
 <u>DISCUSSION</u>	 97-117
 <u>REFERENCES</u>	 118-136
 <u>APPENDIX</u>	 137-142

ABBREVIATIONS

BSTFA	-	Bis-trimethylsilyl-trifluoroacetamide
CI	-	Chemical ionisation
DCVC	-	S-(1,2-dichlorovinyl)-L-cysteine
EI	-	Electron impact
GC-MS	-	Gas liquid chromatography - mass spectrometry
GC-RCD	-	Gas liquid chromatography with radiochemical detection
HCBD	-	Hexachloro-1:3-butadiene
HPLC	-	High performance liquid chromatography
PAH	-	p-Aminohippurate
TEA	-	Tetraethylammonium acetate
TMCS	-	Trimethylchlorosilane
TMS	-	Trimethylsilyl

INTRODUCTION

1.1 HCBD its Uses and the Risk of Human Exposure

Hexachloro-1,3-butadiene (HCBD) is a chlorinated aliphatic unsaturated hydrocarbon with the following structure:-

It is a colourless non flammable liquid with the odour of turpentine. It is readily soluble in alcohol, ether, hexane and other organic solvents but sparingly soluble in water (0.0005%) (Gauntley et al 1975). HCBD is produced as a by-product in the manufacture of tetrachloroethylene, trichloroethylene and carbon tetrachloride (IARC, 1979) and is also found in association with the fungicide hexachlorobenzene (Laska et al 1976).

Although HCBD is a by-product it has some commercial uses in the plastics industry and in the recovery of chlorine during the manufacture of hydrochloric acid. In Russia, it has been used as a vineyard fumigant (Laseter et al 1975), to treat grape phylloxera (EPA 1975), and has been suggested for use as an insecticide and herbicide (Laseter et al 1975 and Davis et al 1980).

Due to manufacturing processes and the disposal methods used for

chlorinated compounds HCB_D has been detected as an environmental pollutant in Liverpool Bay (McConnell et al 1975), in the inland lakes fed by the River Rhine (Goldbach et al 1976) and in the lower Mississippi river (Laska et al 1976). Concentrations up to 1.5ppb have been detected in the water, and up to 300ppb in soil samples from the levee of the Mississippi river between Baten Rouge and New Orleans, LA. HCB_D has also been detected in various aquatic organisms. However the Environmental Protection Agency do not believe that there is a sufficient basis to find that the current manufacture, distribution, processing, use or disposal of HCB_D presents an unreasonable risk to the environment (Federal Register 1982). As well as environmental exposure, man could potentially be exposed to HCB_D in his occupational environment when disposing of the "heavies" produced in the production of tri- and tetrachloroethylenes. The suggested maximum permissible level for exposure to HCB_D is 0.0009ppm (0.01mg/m³) (Poteryaeva 1972). Current legislation, the Health and Safety at Work Act, requires that chemicals be tested in animals to enable assessment of human risk. The current literature available on HCB_D is presented in the next section.

1.2 The Toxicology of HCB_D in Animals

Most studies on the effects of HCB_D have been carried out using the rodent species, rat, mouse, hamsters and guinea pigs, although some data is available on cats and avian species. Studies have been carried out using various routes of administration and using acute, subacute or chronic dosing.

Acute

The oral LD100 and LD50 for mice have been reported at 1200mg/kg and 200mg/kg respectively (Poteryaeva 1972). Other studies have reported LD50 values of 110mg/kg and 87mg/kg (Murzakaev 1963), 80mg/kg and 65mg/kg (Gradiski et al 1975) for males and females respectively.

In the study done by Gradiski et al (1975), anatomopathological, haematological and biochemical tests revealed hepatic and renal disorders. The mice showed tubular necrosis, modifications in the number of leucocytes, erythrocytes and total haemoglobin.

Biochemical tests in the female after ip administration of HCBd showed significant changes in serum alanine aminotransferase, aspartate aminotransferase and urea.

The oral LD50 values for rats vary, values have been reported as 580mg/kg in male Sprague-Dawley rats and 200-400mg/kg in female rats (Kociba et al 1977a) whereas LD50 values of 250 ± 30 mg/kg and 270 ± 20 mg/kg have also been reported (Gradiski et al 1975) for males and females respectively.

Intraperitoneal injection of HCBd gave LD50 values of 216 ± 6 and 175 ± 25 mg/kg for male and female rats (Gradiski et al 1975).

HCBd is toxic on acute dermal administration, the LD50 in rats being 4330mg/kg (Chernokon 1971) and in New Zealand rabbits 12000mg/kg. During exposure the rabbits displayed a moderate depression of the

central nervous system. In animals dying within 24hr, macroscopic examination revealed that the main lesions were in the lungs, liver and in the kidneys. Microscopic examination demonstrated that the lesions in the kidney were in the proximal convoluted tubules (hydropic changes and vacuolisation). Those animals surviving longer showed weakness and anorexia, fatty degeneration of the liver, and the cortex of the kidney was soft and pulpy. Within 3 or 5 days the rabbits died from nephrosis. In all animals the skin in the area of exposure was haemorrhagic and necrotic (Duprat and Gradiski 1978). As a skin irritant HCBd has been described as both a mild (Gradiski et al 1975) and medium irritant (Duprat et al 1976) in the rabbit. Gradiski et al (1975) also noted a marked skin reaction on guinea pigs exposed to HCBd. Irritational effects on the ocular mucous membranes of rabbits have also been reported in these studies.

Single inhalational exposures of rats to atmospheres containing 133-500ppm HCBd for 4-7 hours resulted in death of some or all of the exposed animals. Rats survived exposure to 161ppm for 0.88hr or 34ppm for 3.5hr. Guinea pigs and cats were more sensitive and most died on exposure to HCBd at 161ppm for 0.88hr or 34ppm for 3.5hr (Gehring and MacDougall 1971). The LC50 for mice has been reported to be 235ppm for 4 hours (Gage 1970) or 10ppm for 6 hours (Gradiski et al 1978). In this last study, HCBd was ranked as the most toxic of the chlorinated solvents tested.

Sub-acute

Both male and female rats fed HCB_D at 450ppm and 150ppm in diets for 14 days showed a decreased weight gain which was associated with a decreased food consumption. The relative kidney weights increased in males and females at both doses of HCB_D. Histopathological changes were found only in the kidney at 50ppm HCB_D and above. These changes were characterised by degeneration of tubular epithelial cells which occurred in a dose-dependent manner, especially in the straight portion of the proximal tubules in the outer zone of the medulla (Harleman and Seinen 1979). In a different study (Kociba et al 1977b), the kidney was also noted as the organ which was most sensitive to the deleterious effects of HCB_D. An increase in kidney : body weight ratio, tubular degeneration, necrosis and regeneration was observed in rats receiving 30, 65 and 100mg HCB_D/kg/day for 30 days. Other effects observed were decreased food consumption, body weight gain and haemoconcentration at 10mg/kg/day and above and hepatocellular swelling at 100mg/kg/day.

Inhalational exposure to HCB_D at 100ppm for 6hr/day for 12 days produced severe toxicity including respiratory difficulty, decreased weight gain and pathological injury to the tubular epithelium of the kidney (Gage 1970).

Chronic

Rats given daily doses of HCB_D by gavage for 13 weeks showed reduced

body weight gain and increased kidney weight at 6.3 and 15.6mg/kg in both male and females. Degeneration of proximal renal tubules occurred at doses of 2.5 and 6.3mg/kg or more in females and males respectively. At 15.6mg/kg in females, changes were noted in the convoluted and straight portion of the proximal tubules. Epithelial cells of the proximal tubules showed large hyperchromatic nuclei. Hypercellularity of the epithelial lining was the most prominent feature in the straight segment of the proximal tubules, the epithelial cells were small, more basophilic and finely vacuolated. Necrotic cells were observed and nuclear detritus was found in the lumen. The brush border of the epithelial lining was thinner or absent. Calcified casts were found in the loops of Henle and collecting ducts. In males, changes were less pronounced. Urine concentrating ability was reduced in females at greater than 2.5mg/kg HCBd and in males at 15.6mg/kg HCBd. Increased cytoplasmic basophilia of hepatocytes associated with an increased liver weight occurred in males at 6.3 and 15.6mg/kg. An increase in female liver weight was observed only at 15.6mg/kg (Harleman and Seinen 1979).

In a six month study, daily doses of HCBd at 7mg/kg given to rats produced morphological changes in liver, kidney and myocardium in the form of parenchymatous dystrophic lesions and circulation disturbances. Decreased SH groups in blood and cerebral cortex were noted (Murzakaev 1967).

Lifetime ingestion of 20mg/kg/day HCBd in the diet caused multiple toxicological effects in rats. These effects included increased mortality (males) decreased body weight gain, increased urinary

excretion of coproporphyrin and increased terminal kidney weight in males and females. Pathological examination revealed changes in the kidneys, including hyperplasia and neoplasia of renal tubular epithelium. Some of these neoplasia were noted grossly as nodules in the kidneys and were microscopically diagnosed as renal tubular adenomas or adenocarcinomas. 23% of males and 15% of the females at this dose had renal tubular neoplasia, some of which metastasized to the lungs (Kociba et al 1977a; Kociba et al 1977b; Kociba et al 1977c). The lifetime study (Kociba et al 1977a and b) indicated a clear dose-response for HCBP-induced toxicity.

Reproductive studies with HCBP have failed to find any effect on reproduction in terms of percent pregnancy and neonatal survival (Kociba et al 1977a; Schwetz et al 1977) and except for decreased body weight at birth and weaning (Harleman and Seinen 1979) the pregnancies developed normally.

1.3 The Kidney and its Susceptibility to Chemical Damage

Anatomy of the nephron with regard to HCBP induced nephrotoxicity

The nephrotoxin HCBP is known to damage a specific part of the kidney nephron structure, the so called S₃ segment (pars recta) or straight portion of the proximal tubules, situated in the outer stripe of the outer medulla of the rat kidney. The nephron is the functional unit of the kidney and consists of the Malpighian corpuscle (glomerulus and Bowman's capsule), proximal tubule, thin limbs, the distal tubule and the connecting segment or initial

segment of the cortical collecting duct (Fig 1.1). Each nephron possesses a loop of Henle, the length of which depends on the position of its glomerulus in the cortex. The loop of Henle is composed of the straight portion of the proximal tubule (pars recta), the thin limb segment, and the straight portion of the distal tubule (pars recta). In the rat there are two types of nephron, the cortical which have short loops of Henle, and the juxtamedullary nephrons which have long loops of Henle with well developed descending and ascending thin limb segments that enter the inner medulla. Based on the anatomical distribution of regions of the nephrons at various levels in the medulla, it is possible to divide the medulla into an inner and outer zone, with the latter further subdivided into inner and outer stripes. The inner medullary zone contains both descending and ascending thin limbs and large collecting ducts. The inner stripe of the outer medullary zone contains descending thin limbs and ascending thick limbs in addition to the collecting ducts. The outer stripe of the outer medullary zone contains the terminal segments of the proximal tubule, the ascending thick limbs of the distal tubule and collecting ducts.

The initial portion of the proximal tubule, the convoluted segment is usually referred to as the pars convoluta and the more distal straight segment, as the pars recta. In the rat, three distinct morphological segments have been identified (Tisher 1976). The structural features that distinguish these segments have been described in detail by Maunsbach (1964 and 1966). The first segment, S_1 , corresponds to the first portion of the pars convoluta.

Figure 1.1

Diagram of the structural organisation of the mammalian kidney to demonstrate the relationships between the various segment of the nephron and the zones of the kidney.

The second segment, S_2 includes the last part of the pars convoluta and the initial portion of the pars recta. The third segment, S_3 , comprises the remainder of the pars recta and extends to the transition with the thin descending limb of Henle. The epithelium of the pars recta is generally cuboidal; the apical cell surface is convex and is covered by a well developed brush border composed of elongated microvilli measuring up to 4.0μ in length. The brush border is more extensively developed in the S_3 segment than in S_1 or S_2 segments. S_3 cells are smaller in height, number and have smaller more randomly organised mitochondria than in S_1 or S_2 cells. There is a marked absence of lateral interdigitations. Microbodies are increased in size and number in the S_3 cells. The significance of these structural differences is presently unknown, however, it is believed that this portion of the kidney tubule is less involved with sodium and water reabsorption (requiring active transport) than is earlier regions of the proximal tubule, but the rate of energy dependent secretion of organic acids, such as para-aminohippuric acid is greatest in the pars recta.

Susceptibility of the kidney to chemical damage

Maintenance of normal kidney functions, excretion of wastes, regulation of homeostasis, hormone synthesis and biochemical and metabolic processes requires delivery of large amounts of metabolic substrates and oxygen to the kidney, this requires a high blood flow. Although the two kidneys comprise less than 1% of the total bodyweight, they normally receive about 25% of the resting cardiac output.

Because of the high blood flow, any chemical in the circulation will be delivered in high amounts to this organ. Approximately one-third of the plasma reaching the kidney is filtered. As the glomerular filtrate passes down the nephron, approximately 98-99% of the salt and water are reabsorbed, concentrating both the urine and any filtered xenobiotics in the tubular lumen. Thus, a non-toxic concentration of a chemical in the plasma could reach a toxic concentration within the nephron. The high luminal concentration creates a steep concentration gradient between tubular fluid and plasma. Small water soluble molecules or lipid soluble compounds which have high membrane permeability, may then diffuse back into plasma. During passive reabsorption of the xenobiotic chemical from the tubular urine into the blood it will pass through tubular cells in high concentration, potentially leading to intracellular toxicity.

Active transport systems also play a significant role in the excretion of chemicals by the kidney. These systems secrete some chemicals into the tubular lumen and thus increase their excretion in urine. Two major transport systems are primarily responsible for the secretion of foreign chemicals, the organic anion and the organic cation systems (Weiner, 1973). Their major function appears to be the rapid elimination of compounds, either endogenous or exogenous in origin, which might prove toxic to the organism. Both systems transport a broad range of compounds, in general requiring only that the molecule is organic and that it carries a charge at physiological pH. Since so many foreign chemicals and/or their metabolites possess these characteristics, these transport systems

play a dominant role in controlling the rate at which many environmental chemicals are eliminated (Pritchard, 1981). A xenobiotic actively secreted into the tubular urine will pass through the cells of the proximal tubule and could lead to toxicity within these cells.

Normally, plasma binding of xenobiotics renders them unavailable for further disposition however, the active transport secretory systems can effectively strip xenobiotics from plasma proteins (Weiner, 1973) and hence plasma protein bound xenobiotics may become toxic to the kidney.

The kidney is a metabolically competent organ in that it can carry out both phase I and phase II metabolic processes, and therefore it has the potential to metabolise xenobiotics to highly reactive intermediates which may be responsible for the observed acute kidney injury, a point which will be considered in more detail in section 1.4.

Site of injury produced by HCBd in the rat

Pattern of pathological changes: HCBd has been shown to specifically damage the pars recta of the proximal tubule of the rat kidney and in particular the S₃ segment (Ishmael et al 1982).

Administration of HCBd ip at 300mg/kg caused ultrastructural changes as early as one hour after dosing. Mitochondrial swelling was seen in the S₁ and S₂ segments of the proximal tubules after 1-4 hours. By 8 hours, the major pathological changes were largely confined to

the S₃ segment and consisted of loss of brush border, mitochondrial swelling, dilation of the endoplasmic reticulum and cellular necrosis. This was the earliest time that morphological changes were detected by light microscopy. In animals dosed with HCB_D at 200mg/kg ip, several proximal tubules showed necrosis at sixteen hours when a distinct band of necrosis was seen in the outer stripe of the outer medulla. One and two days after dosing proximal tubular necrosis was still evident, whereas distal tubules were unaffected. By three days, vacuolation was observed in the pars convoluta of some tubules, and the pars recta of many of the tubules were filled with amorphous necrotic material while others appeared empty and were lined by flattened cells. Tubular regeneration was apparent by day five. By day fourteen, substantial recovery had occurred.

Effects on renal function: Twenty four hours after a single intraperitoneal injection of HCB_D (200mg/kg) there was a reduction in the renal clearances of inulin (a measure of glomerular filtration rate), the organic cation tetraethylammonium bromide (TEA), the organic anion p-aminohippuric acid (PAH) (indicators of renal active transport mechanisms). The urinary excretion of urea was also reduced and was accompanied by an increase in plasma urea concentration. Urine flow was significantly reduced. These changes suggested a general reduction in renal function (Lock and Ishmael 1979).

A decrease in PAH but not TEA accumulation was seen in renal cortical slices from rats treated with HCB_D 24hr previously, which

demonstrated selective damage to the renal anion transport system without damaging the cation system. The renal anion transport system is located within the proximal tubule, with the most active system being in the straight portion (Tune et al 1969).

Biochemical detection of HCBd induced renal damage: The damage produced by HCBd can be detected using various biochemical markers. Twenty four hours after dosing 100mg/kg HCBd, Lock and Ishmael (1979) noted an increase in plasma urea concentration, an increase in the urinary excretion of alkaline phosphatase, N-acetyl- β -D-glucosaminidase and protein. Urinary glucose has also been shown to be a valuable parameter in assessing renal damage due to HCBd (Gore et al 1981; Berndt and Mehendale 1978).

1.4 Metabolic Considerations and Possible Mechanisms of Nephrotoxicity

Introduction

It is well known that the liver and kidney together serve to eliminate xenobiotics from the body. Expressed simply, xenobiotic molecules too large or too lipid soluble for effective renal excretion can be excreted in the bile. In addition hepatic metabolism has the ability to convert many of the lipid soluble xenobiotics to more polar metabolites, which may then be excreted by the kidney. However, while the role of the kidney as an excretory organ for drugs, chemicals and their polar metabolites is well

described, the involvement of the kidneys in the biotransformation of xenobiotics is relatively poorly understood. The biotransformation of xenobiotics can occur in two steps, termed phase I and phase II (Williams 1959). Phase I reactions, include oxidative, reductive and hydrolytic reactions. Phase II reactions include synthetic (conjugation) reactions including, for example, glucuronidation, sulphation and the formation of glutathione conjugates. These phases reduce the lipophilicity of the parent compounds and in the case of toxic or pharmacologically active compounds this frequently reduces their toxicity. However, examples exist where the reverse is true. The kidney is known to carry out both phase I and II metabolic reactions.

Biological fate of HCB

Limited literature was available on the metabolic fate of HCB when starting this thesis. In order to predict possible routes of metabolism, one could only draw structural comparisons of HCB with compounds upon which metabolic data was available.

The halogenated ethylene series most closely resemble HCB in that, although not possessing a diene structure, they do have an olefinic structure. The examples which will be discussed are vinyl chloride, vinylidene chloride (1,1-dichloroethylene), trichloroethylene and tetrachloroethylene (perchloroethylene).

Nephrotoxic potential of the chloroethylenes

The chloroethylenes are primarily known for their hepatotoxic effects, however some are known to be weak nephrotoxins, producing kidney damage at high doses or on prolonged exposure. Repeated inhalational exposure to vinyl chloride over periods of several weeks produced proximal tubular injury. Exposure of guinea-pigs to 100,000ppm vinyl chloride for 90 days increased total kidney weight and produced severe or moderate epithelial degeneration in the tubular and glomerular epithelium respectively (Prodan et al, 1975). In rats Viola (1970) reported only slight degeneration of the tubular epithelium in males exposed to 30,000ppm vinyl chloride for up to 12 months. However other workers (Torkelson et al 1961) reported tubular changes after 6 months of exposure to 500ppm vinyl chloride. In the case of vinylidene chloride, Prendergast et al (1967) have reported that the nuclei in proximal tubular cells were enlarged following prolonged exposure of rats to 46ppm vinylidene chloride. Vinylidene chloride has also been reported to be injurious to the kidney when administered orally (Jenkins and Andersen 1978). In male rats given 400mg/kg vinylidene chloride vacuolation and necrosis of the renal tubular epithelium as well as generalised tubular dilation was shown. The biochemical markers serum urea nitrogen, creatinine and absolute kidney weight were also elevated.

Tetrachloroethylene at 2.5ml/kg i.p. has been reported to be a weak nephrotoxin in mice (Plaa and Larson, 1965) producing necrosis and swelling of the proximal convoluted tubules and a small increase in urinary protein. Inhalation or ingestion of tetrachloroethylene produces a lesion characterised by degeneration, desquamation,

atrophy and/or necrosis of the renal tubular epithelium (Kluwe, 1981).

Metabolism of the chloroethylenes in relation to HCB

The primary metabolic step in the biotransformation of chlorinated ethylenes is epoxidation by a cytochrome P-450-catalysed oxidative reaction. A general scheme for the biotransformation of chlorinated ethylenes is shown in Fig 1.2. The formation of the epoxides depends on the extent of chlorine substitution in the parent compound. Chemically the rate of formation of epoxides decreases from vinyl chloride to tetrachloroethylene (Henschler, 1977). The stability and reactivity of the epoxides, at least chemically varies widely and also depends on the number of chlorine substitutions and on the relative position of the substituents in the molecule (Greim et al, 1975). Symmetrically substituted epoxides from tetrachloroethylene and isomeric 1,2-dichloroethylene are more stable than vinyl chloride and trichloroethylene epoxide, in which the polarity results in lesser stability and induces intramolecular rearrangement. The most polar and unstable epoxide is that formed from vinylidene chloride (1,1-dichloroethylene).

In addition to the ethylenes, 2-chlorobutadiene a known nephrotoxin (Nystrom, 1948; Von Oettingen et al, 1936), is presumed to form either one or both of the two isomeric epoxides, 2-chloro-1,2-epoxybut-3-ene and 2-chloro-3,4-epoxybut-1-ene. (Bartsch et al, 1979). This assumption is based on observations that the formation of an alkylating intermediate, which has been trapped by 4-(4-nitrobenzyl)pyridine, is dependent on microsomes, cofactors and air or oxygen.

If one speculates that HCB₂D may be activated by cytochrome P-450 to produce a strongly electrophilic epoxide intermediate in a similar manner to those produced from the chloroethylenes or 2-chlorobutadiene which would be capable of alkylating nucleophilic sites in tissue macromolecules, thus causing damage in the kidney. The site-specific nephrotoxicity produced by HCB₂D seemed to argue in favour of this type of mechanism as the kidney possesses the components of the cytochrome P-450 dependent mixed function oxidase system, (Benedetto et al, 1976) which would be necessary for the production of an epoxide. Cytochrome P-450 activity is not homogenous throughout the kidney and P-450 concentrations exhibit a cortical-medullary gradient (highest in the cortex and outer medulla, lowest in the papilla) (Zenser et al, 1978). Anatomically the distribution of mixed function oxidase activities grossly corresponds to the intrarenal distribution of S₃ cells (Fowler et al 1977), the concentration of these cells being greatest in the pars recta of the proximal tubule (Venkatachalam et al 1978). It is this part of the nephron which is most susceptible to HCB₂D-induced damage.

Manipulation of the mixed function oxidase system metabolising the haloethylenes to epoxides has been used extensively and has been shown to alter hepatotoxicity of vinyl chloride (Jaeger et al 1976), vinylidene chloride (Jaeger et al 1974), trichloroethylene and tetrachloroethylene (Moslen et al 1977).

The contribution of the mixed function oxidase system in metabolising HCB₂D to reactive metabolites was investigated by Hook

et al (1982) and by Lock and Ishmael (1981) by modifying hepatic and renal cytochrome P-450's and looking for the extent of liver and kidney injury. Treatment of rats with the hepatic cytochrome P-450 inhibitors, SKF 525A or piperonyl butoxide, did not alter the response of the liver or kidney to injury. Treatment of animals with inducers of cytochrome P-450, phenobarbitone, β -naphthoflavone, or isosafrole also did not alter either liver or kidney injury. However, Arochlor-1254 was reported in one study (Lock et al, 1981) to increase nephrotoxicity of HCBd slightly whilst in the other no such effect could be found. These studies generally indicated that either metabolic activation by cytochrome P-450 was not necessary for the nephrotoxicity of HCBd or that the inducers and inhibitors did not effect the relevant cytochrome P-450 isoenzymes for the metabolism of HCBd.

In addition to alkylating nucleophilic sites of macromolecules, the epoxides of chloroethylenes have another important route of metabolism. They can rearrange by intracellular chlorine migration, to chlorinated aldehydes (eg cis- and trans-1,2-dichloroethylenes, vinyl chloride and trichloroethylene) or acyl chlorides (eg tetrachloroethylene and vinylidene chloride (Henschler, 1977)).

The aldehydes are further metabolised to chloroacids and chloroalcohols. Cis- and trans-1,2-dichloroethylenes give rise to dichloroacetic acid formed by oxidation of the aldehyde and dichloroethanol by reduction of the aldehyde (Henschler, 1977). Vinyl chloride gives chloroacetic acid as a minor metabolite (Green and Hathway, 1975) and has been suggested to arise from

chloroacetaldehyde (Henschler, 1977). The rearrangement product of trichloroethylene, trichloroacetaldehyde (chloral) gives trichloroacetic acid and trichloroethanol.

The acylchlorides are also further metabolised to chloroacids. In the case of tetrachloroethylene, hydrolysis of trichloroacetyl-chloride gives trichloroacetic acid (Bonse et al, 1975; Daniel, 1963). Vinylidene chloride also gives chloroacetic acid from chloroacetyl-chloride.

For vinyl chloride, both the epoxide and chloroacetaldehyde have been proposed as the agents responsible for covalent binding to critical cellular targets, leading to necrosis (and carcinogenesis) (Macdonald, 1982). Trichloroacetyl chloride, derived from tetrachloroethylene has been suggested to be responsible for covalent binding to macromolecules (Henschler, 1977).

Trichloroethylene oxide formation is thought to give rise to the reactive metabolites of trichloroethylene. Proposed reactive metabolites that could bind irreversibly to protein and nucleic acids are trichloroethylene oxide, formyl chloride, glyoxyl chloride and possibly dichloroacetyl chloride (Macdonald, 1982). Similar metabolites derived from HCBd may be responsible for the observed nephrotoxicity.

The rearrangement products of the chloroethylene epoxides (except trichloroethylene a metabolite of which, trichloroethanol is extensively conjugated to glucuronic acid) appear to be conjugated to glutathione. The epoxides per se can also be conjugated with

glutathione, or cysteine and subsequently be transformed according to established metabolic pathways to mercapturic acid derivatives (Henschler and Hoos, 1982). The sulphur-containing metabolites account for a large proportion of the metabolites of vinyl chloride and vinylidene chloride (Green and Hathway 1975 and 1977; Jones and Hathway, 1978a and 1978b) and vinylidene chloride depletes liver non-protein sulphydryl content (Hefner et al 1975; Jaeger et al, 1974).

Glutathione conjugation with foreign compounds is a well documented event, and is considered a detoxification pathway to protect vital nucleophilic sites in tissues from electrophilic attack by alkylating metabolites of xenobiotics (Chasseaud, 1979). A number of glutathione-S-transferases (E.C. 2.5.1.18), catalysing the transfer of glutathione to a wide variety of xenobiotic substrates are known (Hutson, 1976; Chasseaud, 1973), however glutathione can react non-enzymically with some substrates. Any observed depletion of tissue glutathione after the administration of a toxic compound has been taken as indicative of the formation of potentially toxic metabolites (Mitchell et al 1976). Lock and Ishmael (1981) demonstrated that a single i.p. injection of HCBd to male rats at 300mg/kg produced a reduction in the total non-protein sulphydryl content of the liver, the maximum reduction (60% of control) occurring at 6hr after dosing, whereas renal non-protein sulphydryl remained unchanged. This reduction in liver non-protein sulphydryl was dose dependent up to doses of 900mg/kg HCBd.

In an attempt to explain the depletion of liver non-protein sulphydryl on dosing HCBd, if, one presumes that HCBd is metabolised

via a cytochrome P-450-dependent reaction to an electrophilic metabolite (epoxide), in a manner analogous to vinyl chloride and vinylidene chloride, then the epoxide may react directly with glutathione or be conjugated via glutathione S-epoxide transferase (Boyland and Williams, 1965; Fjellstedt et al 1973).

HCBD could also be conjugated to glutathione via glutathione alkene transferase an enzyme which is present in various tissues in the rat (Boyland and Chasseaud, 1967), including liver and kidney.

The role of glutathione in the toxicity of HCBD is not clear because, although HCBD produces a reduction in liver non-protein sulphydryl which would be consistent with the production of a reactive metabolite in the liver and its subsequent detoxification, depletion of liver non-protein sulphydryl by diethylmaleate failed to produce any hepatotoxicity. (Lock and Ishmael, 1982). As the kidney is the target organ for HCBD toxicity failure of kidney non-protein sulphydryl to be depleted on administering HCBD to male rats, seemed to argue against the production of reactive electrophilic metabolites in this organ. This is a difficult concept to understand, as the term "reactive" implies that the metabolite so described is chemically unstable and would quickly react with suitable molecules in close proximity to produce a more stable product. The reactive metabolite would thus have a short half life and formation of the kidney-damaging "reactive" metabolite elsewhere in the body such as the liver is excluded. On the other hand depletion of non-protein sulphydryl with diethylmaleate did increase the toxicity of HCBD to the kidney (Lock and Ishmael,

1982), which suggested that glutathione plays a role in the detoxification of HCBd in this organ.

The role that cytochrome P-450 plays, if any, in the metabolism of HCBd is unclear as is the role of glutathione.

1.5. Objectives of this Study

From the foregoing, it can be seen that although a great deal is known about the toxicology of HCBd, nothing is known about the mechanism of the nephrotoxicity. This mechanism may be related to the metabolism of HCBd. Related chemicals, the chloroethylenes are metabolised by cytochrome P-450 to reactive electrophiles which ultimately produce hepatotoxicity, with very little nephrotoxicity. However in contrast the target organ for HCBd toxicity is not the liver but the kidneys. The literature evidence to date suggests that HCBd may be metabolised differently from the chloroethylenes and this may explain the differing target organ toxicity. The aim of this study was to elucidate the structure of HCBd metabolites, to determine their site of formation and route of excretion and by means of detailed toxicity studies to determine their relevance to the nephrotoxicity of hexachloro-1:3-butadiene.

MATERIALS AND METHODS

MATERIALS

2.1 Animals

Adult male, Wistar derived Alderley Park albino rats, approximately 2 months old weighing 170-200g were used for all studies. The animals were maintained on a standard pellet diet (PCD diet, Special Diet Services Limited, Witham, Essex, UK - for composition see Appendix) and allowed access to water ad libitum unless otherwise stated. A temperature of 22°C and relative humidity of 50% was maintained in the room where the animals were housed. Alternating periods of 12 hours light and 12 hours dark were maintained with artificial light.

2.2 Chemicals

a) Proprietary chemicals

Hexachloro-1:3-butadiene (HCBD) with purity of 99% (Spectrosol grade) was obtained from BDH Chemicals Limited, Poole, Dorset, UK.

Other chemicals were of the highest grade available commercially.

b) Non-proprietary chemicals

Conjugates of hexachloro-1:3-butadiene were synthesised according to the following methods:

i) N-acetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine:
Dry N-acetyl-L-cysteine (50g, 0.3mole) was added to a solution of sodium (0.6mole) in methanol (1 litre). HCBD (88g, 0.33mole) was added slowly with stirring over 30 min. After 90 min., the solution was evaporated to approximately 500ml, 300ml of water was added and the pH adjusted to 5-6. Unchanged HCBD was removed by extracting 4 times with equal volumes of n-hexane. The solution was adjusted to pH 1 with sulphuric acid and extracted 4 times with ethyl acetate. The ethyl acetate extracts were washed twice with water and evaporated to dryness. The residue was recrystallised from acetonitrile after treatment with decolourizing charcoal to yield 38g of product. M.pt.70°C. (Found C, 29.9; H, 2.6; N, 5.7; S, 7.7; Cl, 41.2%, $C_9H_8O_3NSCl_5$ requires C, 27.9; H, 2.1; N, 3.61; S, 8.3; Cl, 45.8%). The probe insertion mass spectrum of the product was consistent with the expected structure (Fig 2.1).

ii) S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione:
This conjugate was prepared from HCBD and glutathione in the presence of sodium and liquid ammonia by a method analogous to that described by McKinney and Biester (1959). The crude product was recrystallised from ethanol/water to give a white crystalline material M.pt.186-187°C. (Found C, 32.0; H, 3.2; N, 7.7; S, 6.3; Cl, 33.1% $C_{14}H_{16}O_6N_3SCl_5$ requires C, 31.6; H, 3.0; N, 7.9; S, 6.0; Cl, 33.4%). The field desorption mass spectrum was consistent with the expected structure (Fig 2.2).

iii) S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-cysteinylglycine:
This conjugate was prepared biologically by treating chemically synthesised S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione

Figure 2.1 Probe insertion mass spectrum of N-acetyl-S-(1,1,1,2,3,3,4-pentachloro-1:3-butadienyl)-L-cysteine.

Figure 2.2

Field desorption mass spectrum of S-(1,1,1,2,3,4-pentachloro-1:3-butadienyl)- glutathione.

(2.5mM) with 20 units of γ -glutamyltransferase from hog kidney (Sigma Chemical Co) in 50mM Tris-HCl buffer pH8.5 for 16hr at 25°C.

2.3 Radiochemicals

i) [^{14}C]HCBD:

[U- ^{14}C]HCBD with a specific activity of 0.16 mCi/mmol (in house synthesis) with a chemical and radiochemical purity exceeding 99% as assessed by gas chromatography, thin layer chromatography and mass spectrometry was used for excretion and tissue distribution studies.

[U- ^{14}C]HCBD with a specific activity of 10.1mCi/mmol was supplied by Imperial Chemical Industries PLC, Physics and Radioisotopes services, (Billingham, Cleveland, UK) in 1mCi batches diluted in 0.5ml ethanol. The chemical and radiochemical purity was checked by gas chromatography with radiochemical detection using a 1.5m x 2mm glass column packed with 20% OV101 on 100-120 mesh supelcoport and exceeded 99% in all cases. All radiolabelled HCBD was stored at -20°C.

ii) [^{35}S]Cysteine hydrochloride:

L-[^{35}S]Cysteine hydrochloride with a specific activity of 68mCi/mmol was supplied by Amersham International Limited, (Amersham, Buckinghamshire, UK) as an aqueous solution containing 0.55mCi in a total volume of 0.11ml.

iii) [^{14}C] S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione:
This conjugate was prepared biologically. [^{14}C]HCBd (2.8mM;5 μCi) was incubated with reduced glutathione (10mM), glutathione reductase (1.2units/ml) and hepatic microsomes (2mg protein/ml) in 0.1M phosphate buffer pH 7.4 for 60 min at 37°C. The final incubation volume was 10ml. At the end of the incubation, the sample was cooled in ice-cold water and unchanged HCBd removed by extraction with diethyl ether (6 x 10ml). The aqueous phase was freeze dried and then redissolved in 1ml water. Aliquots of the clear supernatant were used as the standards.

METHODS

2.4 Excretion and Distribution Studies

a) Preparation of HCBd dosing solutions

Solutions of HCBd in corn oil for dosing to animals were prepared volumetrically. The correct volume of HCBd and [^{14}C]HCBd to prepare a known volume of dosing solution at the required dose was calculated from the specific gravity of HCBd (1.68g/ml). The required volumes of cold HCBd and [^{14}C]HCBd were added to corn oil (Kraft and Wesson Ltd) and made to volume. The dosing solution was checked for radioactivity by liquid scintillation counting prior to dosing.

b) Urinary and faecal excretion of HCBd

A group of six male rats were each given a single oral dose of [^{14}C] HCBd 200mg/kg (46.4 μCi /kg) as a solution in corn oil (5ml/kg). Animals were housed individually in plastic metabolism cages (Forth Tech Ltd) designed for the separate collection of urine and faeces. Urine and faeces samples were collected daily for 5 days. The samples were collected over solid CO_2 (-70°C) and the collecting vessels were covered to reduce exposure to light.

(c) Biliary Excretion of HCBd by Rats after a Single Oral Dose.

(i) Surgical procedure: Surgical anaesthesia was induced with 5%

halothane (Fluothane, ICI PLC, Macclesfield, Cheshire) in oxygen and maintained with 1-2% halothane in oxygen using a Fluotec 3 (Cyprane Keighley, England) and Aldosorber system (J W Turner Ltd, Liverpool). The bile ducts were exposed by a midline abdominal incision (down the linea nigra) and the common bile duct cannulated with polythene tubing (Portex, Hythe, England 0.8mm o.d. 0.4mm i.d) a little below the junction of the right and left hepatic ducts. The cannula was externalised by passing it subcutaneously with the aid of a trochar to emerge through an incision in the dorsal cervical area. The internal midline incision was closed with suture (Mersilk braided silk 3/0, 16mm cutting needle, Ethicon Ltd, Edinburgh, Scotland) and the skin wound closed with surgical clips (9mm Autoclips Clay Adams, Parsippany NJ USA).

- (ii) Animal dosing and sample collection: Three animals were biliary cannulated. On regaining consciousness, rats were dosed orally with HCBd 200mg/kg (250 μ Ci/kg) in corn oil (5ml/kg). Immediately after dosing, the rats were placed in restraining cages (Bollman, 1948) for the collection of bile and urine samples over the time periods 0-24hr and 24-48hr. During the collection of samples, animals had access to limited supplies of pelleted laboratory diet and unlimited drinking water containing 1% dextrose, 0.9% NaCl and 0.05% KCl (w/v).

- (d) Distribution of a single oral dose of HCBd in the rat

(i) The distribution of radioactivity in the rat at 2,4,8 and 16hr after a single oral dose of [^{14}C]HCB D was studied by whole body autoradiography. One animal for each of the time points 2,4, 8hr was dosed at, 200mg/kg (213 $\mu\text{Ci/kg}$) in corn oil (5ml/kg) and one animal for the 16hr time point was dosed at 200mg/kg (250 $\mu\text{Ci/kg}$). Animals were killed at the time points by exposure to excess halothane, and were rapidly frozen at -70°C in a hexane/solid CO_2 mixture. The frozen carcass was embedded in 2% (w/v) carboxymethylcellulose and longitudinal sagittal sections were cut with an LKB PMV type 450 MP cryo-microtome. Apposition autoradiograms were made by pressing the freeze-dried sections against Structurix D7 X-ray film (Agfa Gevaert) in light-tight cassettes (Ullberg, 1962). Autoradiograms were developed after 19 days exposure in the case of the 2, 4 and 8hr time points and after 14 days for the 16hr time point. The amounts of radiolabel in various organs was determined semiquantitatively using a light box and densitometer.

(ii) Determination of the form of radioactivity from [^{14}C] HCB D in the gut:

Four rats were each dosed orally with [^{14}C]HCB D 200mg/kg (2.18 $\mu\text{Ci/kg}$) in corn oil and killed with halothane at 2, 4, 8 and 16 hr after dosing. The small and large intestines were removed, washed externally with water and then homogenised in water with a polytron (Kinematica GmbH, Switzerland). The homogenate was extracted with hexane to remove unchanged

[¹⁴C]HCBd and the aqueous fraction was centrifuged at 10,000g for 30 min (MSE High Speed 18). The aqueous supernatant and the hexane extract were assayed for radioactivity by liquid scintillation counting.

(e) Measurement of radioactivity:

Aliquots of liquid samples such as urine (1ml) and bile (100μl) were admixed with 10ml standard scintillator (Instagel-Packard or FisoFluor-Fisons Ltd) and counted directly. Samples of faeces were freeze dried, weighed, ground to an homogenous powder and samples (100mg) combusted in a Packard tissue oxidiser. [¹⁴C] carbon dioxide from the samples was automatically absorbed in 2-methoxyethylamine and mixed with a toluene based scintillator prior to counting. All counts were corrected for oxidation efficiency determined with [¹⁴C] hexadecane.

All samples were counted using a Tri-carb 460 CD scintillation counter (Packard Instruments Ltd, UK). The values obtained were automatically corrected for background. Counting efficiency was determined using a ²²⁶Ra external standard and the dpm values were derived from appropriate quench curve data stored in the instrument computer.

2.5 Metabolism Studies

(a) Hepatic Microsomal Metabolism of HCBd

(i) Preparation of hepatic microsomal fraction:

Rats were fasted for 24hr prior to preparation of microsomes. The rats were killed by cervical dislocation, the liver removed and a 25% (w/v) homogenate made in ice-cold 0.154M KCl (adjusted to pH 7.4 with 0.1M phosphate buffer) using a Potter homogeniser. The homogenate was centrifuged at 11,000g (av) for 20min at 4°C using an MSE high speed 18 centrifuge. The precipitate was discarded and the supernatant transferred to fresh tubes and centrifuged at 160,000g (av) for 40min at 4°C (MSE Super Speed 75 centrifuge). The fatty layer and the supernatant (cytosolic fraction) were removed and discarded. The microsomal pellet was washed once by resuspending in ice-cold 0.154M KCl and centrifuging at 80,000g (av) for 30 min. Finally, the pellet was resuspended in 20mM Tris-HCl buffer pH 7.4 containing 0.25M sucrose and 5.4mM EDTA to give a protein concentration of 20-30mg/ml.

Protein concentration were determined by the method of Lowry et al (1951).

(ii) Isolation and identification of the microsomal metabolite:

HCBD (5mM), reduced glutathione (4mM), [^{14}C]HCBD (10 μCi) and microsomal protein (7.5mg/ml) were incubated in 0.1M phosphate buffer pH 7.5 for 75 min at 37°C. The total volume of the incubation was 40ml. At the end of the incubation, unchanged HCBD was removed by extracting with hexane (3x40ml). Two volumes of ice-cold ethanol were added to the aqueous

fraction and the precipitated microsomal protein removed by centrifugation at 10,000g for 30 min. The supernatant was decanted and evaporated under reduced pressure at 25°C to 0.5ml. This concentrate was chromatographed on silica gel GF thin-layer plates using n-butanol:acetic acid:water (12:3:5 v/v) as the developing solvent. The major radioactive band (Rf 0.33) was removed and eluted from the silica with water. The eluate was again concentrated (1ml) and hydrolysed with 6M HCl at 100°C for 20hr. Under these conditions, complete hydrolysis of the material with Rf 0.33 occurred to give a single radioactive product, Rf 0.57 in the same system. The hydrolysate was evaporated to dryness and methylated with an ethereal solution of diazomethane. The methylated sample was further chromatographed on thin-layer plates (silica gel GF) using chloroform methanol (9:1 v/v) as the solvent system. The major radioactive band, Rf 0.75, which was strongly UV-absorbing and ninhydrin positive was eluted with ether, concentrated and analysed by GC-RCD using a 1.5m x 2mm glass column which was packed with 5.2% OV101 on 100-120 mesh Supelcoport. The column was operated at 200°C with an Argon:CO₂ (95:5) carrier gas flow rate of 25ml/min. Two radiolabelled mass peaks were identified using an LKB 2091 gas chromatograph EI/CI mass spectrometer operated under the same chromatographic conditions.

(b) Metabolism of HCB₂D in the Rat

(i) Isolation of biliary metabolites:

For the purpose of identification of biliary metabolites, a

male rat was biliary cannulated under halothane-anaesthesia. The rat was allowed to recover from the anaesthetic and then dosed orally with [^{14}C]HCB D , 200mg/kg (250 μCi /kg). The rat was restrained (Bollman, 1948) and bile was collected for 24h.

Bile (14ml) was freeze-dried and extracted with 5ml ethyl acetate:acetic acid:methanol:water (60:15:15:10). The bile extract was compared with an aliquot of the original bile sample by chromatography on thin-layer plates (Silica Gel GF) (20cm x 5cm x 0.25mm) using ethyl acetate:acetic acid:methanol:water (60:15:15:10) and butanol:acetic acid:water (12:3:5). Areas of radioactivity were located using a thin-layer radiochromatogram scanner (Berthold Model LB 2723) and by autoradiography using structurix D7 X-ray film (Agfa Gevaert).

Preparative separation of larger amounts of bile extract was achieved by column chromatography of 1ml samples using a Lobar size B Lichroprep Si 60 column (E. Merck Darmstadt) and eluting with ethyl acetate:acetic acid:methanol:water at 1ml/min. The eluate was monitored with a radiochemical detector (Berthold Model LB503) fitted with a 100 μl flow-cell and 2ml fractions were collected with an LKB Ultrorac fraction collector. Three bands of radioactivity were eluted with retention times of 92, 174 and 204min. Fractions corresponding to each of these bands were pooled and concentrated under reduced pressure. The concentrated bands were compared to the bile extract and a biosynthetic standard HCB D -glutathione conjugate (2.3iii) by TLC on Silica gel

- layers using the two solvent systems described previously. The plates were subjected to radiochromatogram-scanning and autoradiography.

The most polar column fraction (Rt 204 mins), containing the major metabolite as assessed by thin layer chromatograms, was hydrolyzed overnight with 6M HCl at 100°C. The hydrolysate was evaporated to dryness and the methyl ester, N-trifluoroacetyl derivative prepared using ethereal diazomethane and trifluoroacetic anhydride respectively. The derivatised sample was analysed by GC-RCD and by gas chromatography -mass spectrometry (GC-MS).

The material eluting from the column with a retention time of 174min was analysed by thin-layer chromatography alongside an authentic sample of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-cysteinyglycine (2.2biii). Silica gel GF thin-layer plates were used with ethyl acetate: acetic acid: methanol: water and butanol: acetic acid: water solvent systems. Spots on the chromatograms were located under u.v. light (254nm), by spraying with ninhydrin solution or by radiochromatogram scanning.

The material eluting from the column with a retention time of 92min was reconstituted in 2ml ethyl acetate: acetate acid: methanol: water (60:15:15:10) and applied to two preparative silica gel TLC plates (20cm x 20cm x 2mm). The plates were developed in ethyl acetate: acetic acid: methanol: water

(60:15:15:10) and the radioactive bands were located by radiochromatogram-scanning. Two radioactive bands Rf 0.61 and 0.93 were removed and the radioactivity eluted from the silica gel with methanol. The eluates were concentrated by rotary evaporation under reduced pressure. The concentrates were analysed by GC-RCD using a 0.9m x 2mm glass column packed with 5.2% OV101 on 100-120mesh Supelcoport.

- (ii) Identification of sulphur containing metabolites in bile:
Two rats were each dosed orally with [^{35}S]cysteine (23 μg , 10 μCi) in water for five consecutive days and a further rat was dosed similarly with 23 μg /day non-radiolabelled cysteine hydrochloride (Sigma Chemical Co) in the same volume of water. The doses were not adjusted for body weight.

After the cysteine dosing regime, all animals were fitted with an exteriorised biliary cannula as previously described.

Immediately on recovery from the anaesthetic, the [^{35}S]cysteine -dosed rats were given a further 50 μCi (115 μg) [^{35}S]cysteine and the rat previously dosed cysteine hydrochloride was given a further dose, of cysteine hydrochloride (115 μg). Then one [^{35}S]cysteine-dosed rat was dosed i.p. with HCBd (200mg/kg) in corn oil and the other dosed i.p. with corn oil. The rat previously dosed non-radiolabelled cysteine hydrochloride was dosed i.p. with [^{14}C]-HCBd, 200mg/kg (250 μCi /kg). Bile was collected for 24 hr.

Bile was chromatographed on silica gel thin layers using ethyl acetate: acetic acid: methanol: water (60:15:15:10) and butanol: acetic acid: water (12:3:5) systems. TLC plates were examined for radioactive areas by autoradiography.

(iii) Isolation of urinary metabolites:

Observations by Davis et al (1980) and in this laboratory have shown a dose-dependent urinary excretion of HCB_D metabolites. In order to optimise the recovery of radioactivity and mass from treated rats, both high and low doses of HCB_D were used to generate metabolites. Twelve rats were each dosed orally with [¹⁴C]HCB_D, 200mg/kg (50μCi) and a further four rats at a dose level of 20mg/kg (50μCi). Animals were housed individually for the separate collection of urine and faeces. Urine was collected for 24hr (200mg/kg dose group) or 48hr (20mg/kg dose group). The urine samples were combined and filtered through a glass fibre filter (Whatman GF/F) under vacuum. Two volumes of ice-cold ethanol were added to the combined sample and the precipitated protein removed by centrifugation at 10,000g. The supernatant was removed, evaporated under reduced pressure to approximately half volume (390ml), and extracted 4 times with 250ml volumes of diethyl ether at neutral pH. The aqueous fraction was then adjusted to pH 1 with 3M HCl and extracted repeatedly with equal volumes of diethyl ether (11 x 250ml) until no further radioactivity was extracted. The ether extract obtained at pH 1 was evaporated to dryness, reconstituted in distilled water (10ml) and 2ml aliquots chromatographed on a Lobar size B Lichroprep RP-8 reverse phase column with

methanol:water:formic acid (60:40:1 v/v) as the eluting solvent. Radioactivity was monitored in the eluate using a Berthold LB503 Radiochemical detector and fractions collected with an LKB 7000 Ultrorac fraction collector. Three peaks were evident, these were labelled U1, U2 and U3 in order of elution. Fractions corresponding to individual peaks of radioactivity were combined and evaporated to dryness under reduced pressure.

The least polar peak, U3 was analysed underivatized and as its TMS derivative by GC-RCD and GC-MS using 1.5m x 2mm glass columns packed with 20% or 5.5% OV101 on 100-120 mesh Supelcoport. The TMS derivative was also subjected to accurate mass measurement by high resolution GC-MS.

Band U2 was rechromatographed on the preparative RP-8 column using methanol:water (3:7) + 1% formic acid as the eluting solvent for 60min followed by a linear gradient to methanol + 1% formic acid over 60min. The flow rate was 2ml/min and 2ml fractions were collected. Radioactivity was monitored in the eluate. Fractions corresponding to the single radioactive peak were combined and evaporated to dryness under reduced pressure. The dry sample was recovered in methanol. An aliquot of the sample was taken to dryness with a stream of nitrogen dissolved in dry pyridine and treated with Regisil (BSTFA + 1% TMCS, Phase Separations Ltd, UK). The treated sample was analysed by GC-RCD on a 1.5m x 2mm glass column packed with 5.5% OV101 on 100-120 mesh Supelcoport.

A further aliquot (200 μ l) of the methanol extract was taken to dryness in a 25ml round bottomed flask. The flask was attached to a water condenser and 50 μ l dimethyl formamide and 2ml thionyl chloride were added. The resulting solution was refluxed for 2hr. The sample was cooled, diluted with an equal volume of dichloromethane and filtered under vacuum through a GF/F (Whatman) glass fibre filter. Excess thionyl chloride was removed by rotary evaporation under reduced pressure. The residue was dissolved in 50 μ l carbon tetrachloride and subjected to GC-RCD analysis on a 1.5m x 2mm glass column packed with 20% OV101 on 100-120 mesh Supelcoport. This later derivatisation method was adapted from those published by Kirkland (1960) for the preparation of sulphonyl chloride derivatives.

The most polar fraction, U1, was rechromatographed on the preparative RP-8 column using a gradient system of acetonitrile:water (3:7) + 1% formic acid for 70min rising to acetonitrile + 1% formic acid over 120min and maintaining this solvent for 60min. Fractions corresponding to radioactivity peaks were combined and concentrated under reduced pressure. Samples were treated with Regisil and analysed by GC-RCD.

(iv) Gas-liquid chromatography of metabolites:

Radioactive metabolites were analysed by gas chromatography using a Pye series 104 instrument equipped with flame ionisation and radiochemical detection (ESI Nuclear 504 Radiogas Detector). The column effluent was split in the

ratio of 10:1 between the radiochemical detector and the flame ionisation detector. In each case the carrier gas was a mixture of argon/CO₂ (95:5) at a flow rate of 25-30ml/min. Microsomal metabolites were analysed on a 1.5m x 2mm glass column packed with 5.2% OV101 on 100-120 mesh Supelcoport operated at 200°C. The splitter ratio in this case was 1:1. Biliary metabolites were analysed on a 0.9m x 2mm glass column packed with 5.2% OV101 on 100-120 mesh Supelcoport, temperature programmed from 150°C (3 min) to 250°C at 10°C/min, with an injection temperature of 200°C. Urinary metabolites were analysed on a 1.5m x 2mm glass column packed with 5.5% OV101 on 100-120 mesh Supelcoport and on a 1.5m x 2mm glass column packed with 20% OV101 on 100-120 mesh Supelcoport. Both columns were programmed from 100°C (1 min) to 300°C at 15°C/min with an injector temperature of 150°C. Mass peaks corresponding to radioactivity were identified and analysed by GC-MS using the same columns and conditions, but with helium as the carrier gas (20ml/min).

(v) Mass spectrometry of metabolites:

An LKB 2091 EI/CI gas chromatograph-mass spectrometer was used to obtain spectra of metabolites. EI spectra were obtained with the following instrument settings: ion source temperature 240°C, electron energy 70eV, trap current 50µA, accelerating voltage 3500V and scan speed 3. CI spectra were obtained using methane as the reagent gas.

Accurate mass measurement and field desorption spectra were

obtained on a Jeol D300 GC-MS. This instrument was fitted with a 2.7m x 2mm glass column packed with 3% OV101 on 100-120 mesh Supelcoport and programmed from 150°C to 300°C at 15°C/min.

2.6. The Toxicity of Biliary HCB¹²D Metabolites and HCB¹²D Conjugates

(a) The Toxicity of Biliary HCB¹²D Metabolites:

Thirteen rats were fitted with intraperitoneal glass reservoirs under halothane anaesthesia as described by Johnson and Rising (1978). Following recovery from the anaesthetic, the animals were given a single i.p. dose of [¹⁴C]HCB¹²D (200mg/kg) in corn oil. Bile samples were collected for 24hr and stored at -20°C. The volume of each bile sample was determined by weight (1ml=1g) and assayed for radioactivity by liquid scintillation counting. The weight (mg equivalents) of HCB¹²D metabolites was calculated for each residual bile sample. The bile samples were pooled, freeze dried and reconstituted in 10ml of distilled water. A similar volume of control bile obtained from corn oil dosed-rats was concentrated to the same volume. Bile was checked qualitatively by TLC-autoradiography and quantitatively by liquid scintillation counting for possible losses during concentration and recovery.

A group of 3 rats were dosed orally with the reconstituted bile collected from [¹⁴C]HCB¹²D-treated rats at a dose level equivalent to 107mg/kg HCB¹²D, based on the radioactivity

content of the bile. Further groups of 3 rats were dosed with an equivalent amount of control bile, with [^{14}C]HCB (100mg/kg) in corn oil or with corn oil vehicle alone (5ml/kg). The rats were housed individually in metabolism cages for the separate collection of urine and faeces. Twenty four hours after dosing, the rats were sacrificed with halothane and blood and the 24hr urine samples taken for biochemical analysis. Urine was also assayed for radioactivity. Kidneys and liver were removed for the measurement of organ body to weight ratios and water content and for histopathological assessment.

(b) The Toxicity of HCB Conjugates:

The synthesised HCB conjugates, N-acetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine and S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione were examined for in vivo toxicity. A dose-response study was undertaken for each compound in order to determine an effective nephrotoxic dose, followed by a detailed examination of their toxicity using biochemical and histopathological markers.

(i) Dose-response:

Groups of 3 rats were given single oral doses of N-acetylcysteine conjugate of HCB in polyethylene glycol 400 at 10,25,50 and 100mg/kg (5ml/kg), and in a separate study, similar groups of rats were given single oral doses of the glutathione conjugate of HCB as the hydrochloride in water at 50,100,150 and 200mg/kg (10ml/kg). Twenty four hours after dosing, the rats were sacrificed with halothane and blood

samples obtained by cardiac puncture for determination of plasma urea.

(ii) Toxicity:

N-acetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine:

Groups of 6 rats were dosed orally with either N-acetylcysteine conjugate of HCBd 100mg/kg in polyethylene glycol 400 or HCBd 200mg/kg in polyethylene glycol 400. Control animals received polyethylene glycol 400 alone.

S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione

Groups of 6 rats were dosed orally with either the glutathione conjugate of HCBd 138mg/kg as the hydrochloride in water or HCBd 200mg/kg in Polyethylene glycol 400. Control animals received water alone (10ml/kg) or polyethylene glycol 400 (5ml/kg).

Animals were housed individually in plastic metabolism cages for the separate collection of urine (preserved with addition of 50µl 10% aqueous sodium azide) and faeces. After 24hr, the animals were weighed, killed with halothane and blood was sampled by cardiac puncture into Monovette Lithium/Heparin Syringes (Sarstedt W. Germany) for biochemical measurements.

The kidneys were removed and weighed; the right kidney was used for histopathological examination and the left kidney for determination of kidney water content. The livers were removed, weighed and samples taken for histopathology. A

portion of each liver (approximately 1g) was used for the determination of liver water content. Tissue water content was determined as a weight loss on drying to constant weight at 105°C. Urine samples were subjected to biochemical measurements.

(c) The effect of biliary cannulation on HCBd toxicity

Two groups of rats, 3 per group, were fitted with exteriorised biliary cannulae as previously described. One group was dosed orally with HCBd (200mg/kg) as a solution in polyethylene glycol 400 (5ml/kg), the other group received the vehicle alone. A third group of 3 rats underwent part of the surgical procedure in that the abdominal cavity was opened under anaesthesia and immediately closed again with sutures. A biliary cannula was not fitted to these rats, the bile duct was not disturbed and remained fully functional. These rats then received a single oral dose of HCBd (200mg/kg) in polyethylene glycol 400. Animals were restrained and treated as previously except that urine was collected with the addition of 50µl 10% sodium azide. The animals were sacrificed with halothane after twenty four hours and blood sampled by cardiac puncture. The blood and urine samples were submitted for biochemical analysis.

(d) Biochemical assays

Plasma urea was determined by an automated method with a Vitatron PA 800 analyser using SKI kit No. 86245. (Smith

Kline Instruments). Plasma alkaline phosphatase (E.C.3.1.3.1) and plasma alanine aminotransferase (E.C.2.6.1.1) were determined by automated methods with a Vitatron AKES analyser using BCL kits Nos. 123862, 124656 and 124508 respectively (Boehringer Corporation Limited). Urinary glucose was determined using BCL test-combination No 124346. Total urinary protein was determined by a modified method of Pesce and Strande (1973).

The activities of urinary alanine aminopeptidase and γ -glutamyltransferase (E.C.2.3.2.2) were determined by the methods of Mondorf et al (1978), and Szasz (1969), respectively. The activity of urinary alkaline phosphatase was determined by the method of Fernley and Walker (1965) and the activity of N-acetyl- β -D-glucosaminidase (E.C.3.2.1.30) according to Leaback and Walker (1961).

(e) Histopathology

Portions of liver and kidney were fixed in 10% buffered formal saline and paraffin sections (5 μ m) cut and stained with haematoxylin and eosin.

RESULTS

3.1 Excretion and Distribution of HCBd in the Rat.

The excretion profile for each of these routes has been investigated following a single oral nephrotoxic dose of HCBd. The distribution of HCBd in the rat has been examined by whole body autoradiography. The absorption of an oral dose of HCBd has been investigated.

(a) Excretion of [^{14}C]-HCBd

A single oral dose of [^{14}C]HCBd at 200mg/kg produced an excretion pattern in which the faecal route of elimination predominated (Fig 3.1). Over the five day period studied, approx 40% of the dose was excreted in faeces, the bulk of the radioactivity was excreted on days 3-5. The decreased faecal production observed during the first two days may be a contributory factor to this delayed excretion.

Experiments in rats fitted with a biliary cannula demonstrated that approximately 17% of the radioactivity was excreted in both the first 24 hours and in the following 24 hours suggesting that biliary metabolites accounted for most if not all of the radioactivity in faeces. The faecal excretion of radioactivity in the non-cannulated rats for these two periods was low, approximately 1% and 2% respectively of the radioactive dose, suggesting extensive re-absorption and enterohepatic recirculation of biliary metabolites. An estimate of this recirculation can be made from these figures; approximately 15% of the radiolabel excreted in bile over 24hr was re-absorbed from the gut.

The excretion of radioactivity in the urine was low; 10% of the dose excreted over the 5 days, with a maximum of 3.5% of the dose eliminated in any one 24hr period (Fig 3.1).

b) The distribution of a single oral dose of HCBd in the rat

Whole body autoradiograms of rats killed at intervals of 2, 4, 8 and 16hr after a single oral dose of [^{14}C]HCBd at 200mg/kg (250 μCi /kg) demonstrated a similar pattern of distribution at all time points (Fig 3.2). Quantitation of the autoradiograms with a densitometer (Table 3.1) demonstrated that the organs containing most radioactivity were the large and small intestines, kidney and the liver. Where a determination was made the bladder also contained a large amount of radioactivity. The low amount of radioactivity in the stomach, but high amounts in the small intestine indicates either rapid absorption of HCBd from the stomach followed by excretion via the bile into the gut or passage of HCBd directly from the stomach to the small intestine. In order to investigate this further, the gut and contents were extracted with hexane at 2, 4, 8 and 16hr after a single oral dose of [^{14}C]HCBd at 200mg/kg(2.18 μCi /kg). The quantity of hexane-extractable material, assumed to be unchanged HCBd, decreased with time whilst the amount remaining in the aqueous phase, assumed to be metabolites, increased (Table 3.2). Thus the radioactivity shown in the intestine on autoradiograms is due to unchanged [^{14}C]HCBd, both from direct passage of [^{14}C]HCBd from the stomach, and to the presence of [^{14}C]HCBd metabolites from the bile. Biliary excretion of radioactivity was noted as early as 1hr in the bile from rats dosed 100mg/kg HCBd (results not shown).

Figure 3.1 The daily excretion of radioactivity following a single oral dose of [^{14}C] hexachloro-1:3-butadiene (200 mg/kg). The values shown are the means $\pm$ SD.

At 2hr unchanged HCB¹²D accounted for most of the radioactivity, whereas by 16hr the radioactivity was present as water soluble material indicating that absorption of HCB¹²D was virtually complete by this time.

The kidney contained radioactivity at all time points, the greatest amount being noted at 4hr, coinciding with the earliest time noted for HCB¹²D-induced renal necrosis (Ishmael et al 1982). The autoradiograms showed a definite intrarenal distribution of radioactivity, the outer stripe of the outer medulla, the site of damage produced by HCB¹²D, being particularly well labelled (Fig 3.3).

Figure 3.2 Whole body autoradiograms of longitudinal saggital sections of rats showing the distribution of radiolabel after a single oral dose of C-14 hexachloro-1:3-butadiene in corn oil (200 mg/kg : 250 μ Ci/kg) and sacrificed after 2, 4, 8 and 16 hr.

Table 3.1

The relative quantities of radiolabel associated with various tissues in whole body autoradiogram sections as determined by densitometry

Tissue	Light transmission (%) at various times (hr) after dosing			
	2	4	8	16
Adrenal	95	97	97	ND
Bladder	52	ND	ND	ND
Blood	95	98	95	95
Large Intestine	ND	ND	11	10
Testes	ND	98	ND	ND
Myocardium	95	98	96	96
Kidney Cortex	92	88	85	77
Medulla	82	32	57	60
Pelvis	98	95	97	92
Liver	87	87	92	85
Small Intestine	4	10	18	0
Stomach contents	90	92	95	45

Note 100% = No radioactivity

ND = not determined

Table 3.2 The distribution of radiolabel between aqueous and hexane extract phases from the gastrointestinal tract of rats given [^{14}C]HCB and killed at 2, 4, 8 and 16 hr after dosing.

Time (hr)	Radioactivity recovered in each phase expressed as a % of the total radioactivity recovered.	
	Hexane	Aqueous
2	92.8	7.2
4	85.4	14.6
8	58.8	41.2
16	9.5	90.5

Figure 3.3 An autoradiogram of a section of kidney showing the localisation of radioactivity in the cortico-medullary region from an animal given [^{14}C]HCB D , orally 200 mg/kg; 213 $\mu\text{Ci/kg}$ and killed 4 hr after dosing.

3.2 Identification of the *in vitro* Microsomal Metabolite of HCB

This work arose from observations by Dr C R Wolf which demonstrated that HCB caused a marked reduction in glutathione concentration when incubated with male and female hepatic microsomal or cytosolic fractions fortified with glutathione. Studies with the cytochrome P-450 inhibitors, carbon monoxide and SKF 525A, demonstrated that the glutathione depletion was not dependent on cytochrome P-450. Although cytosolic and microsomal fractions could produce a glutathione depletion with HCB, the microsomal fraction was more efficient at producing this effect, and resulted in the formation of a single product (Wolf et al, 1984).

Hexane extractions of the microsomal incubation removed 7.4 μ Ci (9.7mg) of the total 13.1 μ Ci of radioactivity, leaving 2.6 μ Ci (3.4mg) of product in the aqueous phase. Preparative TLC of the aqueous products gave a single radioactive band at Rf 0.33 (butanol: acetic acid: water) which was ninhydrin positive. Acid hydrolysis of this band gave a single ninhydrin staining product with Rf 0.57 (butanol: acetic acid: water). Methylation of this product with diazomethane and subsequent TLC using chloroform:methanol (9:1) gave two radioactive bands, a minor one on the origin and the major one at Rf 0.75 which was strongly u.v absorbing indicating the presence of diene double bonds. GC-RCD analysis of the major band gave two radiolabelled mass peaks in the ratio of 9:1 with retention times of 2.4 and 2.7 min respectively. Both peaks gave identical mass spectra, which were interpreted as that of the methylester of S-(pentachloro-1:3-butadienyl) cysteine. The two peaks were assumed

Figure 3.4 Electron impact mass spectrum of the methyl ester of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine obtained by hydrolysis and derivatisation of the metabolite formed on incubation of [^{14}C]HCBd with male rat hepatic microsomes and glutathione.

Figure 3.5 Chemical ionisation mass spectrum of the methyl ester of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine obtained as in figure 3.4.

to be isomers. The CI and EI spectra are shown for the major isomer Figures 3.4 and 3.5. The original conjugates before hydrolysis were assumed to be isomers of S-(pentachloro-1:3-butadienyl) glutathione. The assignment of the conjugate was S-(pentachloro-1:3-butadienyl) glutathione was subsequently confirmed by comparison of chemically synthesised HCBd-glutathione conjugate on TLC.

3.3 Metabolism of HCBd in the Rat after Oral Dosing.

The isolation and identification of biliary and urinary metabolites is described.

a) Biliary metabolites

The excretion studies showed that the faeces was the major route of excretion of HCBd in the rat. Isolation of metabolites from faeces would have been a difficult task and since excretion data had indicated that the biliary route was the primary source of faecal metabolites it was decided to use this as the source of metabolites. Preliminary investigations were aimed at devising suitable chromatographic methods for the isolation of metabolites from bile. The various methods tried included anion and cation exchange chromatography, TLC, normal and reverse phase HPLC. Out of these methods arose the final method for analysis of HCBd biliary metabolites.

Thin layer chromatography of bile revealed a number of radioactive components (Fig 3.6). The biliary metabolites were isolated by

Figure 3.6

TLC radiochromatogram of 24 hr bile from rats given a single oral dose of [^{14}C] hexachloro-1:3-butadiene (200 mg/kg). Solvent system ethyl acetate : acetic acid : methanol : water (60 : 15 : 15 : 10). Identified metabolites are: X, 5-(1,1,2,3,4-penta-chloro-1:3-butadienyl)-glutathione (R_f 0.46), and Y, S-(1,1,2,3,4,-pentachloro-1:3-butadienyl)-cysteinyl glycine (R_f 0.73).

preparative HPLC methods. The bile sample was freeze dried with no loss of radioactivity and dissolved in ethylacetate: acetic acid: methanol: water (60:15:15:10). This extract was compared with the original bile on TLC using two different solvent systems. The plate scans and autoradiograms demonstrated that the extract contained all the components originally present in bile. Chromatography on a preparative Lobar column fractionated the biliary metabolites into three bands with retention times of 92, 174 and 204min. Chromatographic reproducibility was ± 1 tube. Concentrates of these bands were compared on TLC (Fig 3.7).

The most polar band (Retention time 204min) contained 55% of the radioactivity present in bile as determined by scintillation counting. TLC of this band revealed the presence of two radioactive components (Fig 3.7). The major component (40%) at R_f 0.36 and the minor component (15%) at R_f 0.52 (butanol: acetic acid: water). The R_f of the major component corresponded to the R_f of the major component in the original bile sample. This band was hydrolysed with HCl and methylated with diazomethane. Gas chromatographic analysis of this sample failed to provide a good separation from endogenous materials. The sample was further derivatised and analysis of the methylated and trifluoroacetylated sample by GC-RCD resulted in a good separation of the peak of interest from endogenous materials. The peak of interest had a retention time of 9.0min on the 0.9m x 2mm 5.2% OV101 columns programmed from 150°C (3 min) to 250°C at 10°C/min. GC-MS of this peak gave CI (methane) and EI spectra as shown in Figures 3.8 and 3.9. Interpretation of these spectra indicated this peak to be the methylester of N-trifluoro-

Figure 3.7 TLC radiochromatograms of fractions obtained from preparative column chromatography of bile from rats given [^{14}C]HCB D .

Electron impact mass spectrum of the methyl ester of N-trifluoroacetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine obtained by hydrolysis and derivatisation of the major biliary metabolite.

Figure 3.8

Chemical ionisation (methane) mass spectrum of the methyl ester of N-trifluoroacetyl-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine obtained by hydrolysis and derivatisation of the major biliary metabolite. $M + 1 = 454$.

Figure 3.9

acetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine. Which was consistent with the metabolite prior to hydrolysis being the glutathione conjugate of HCBd.

Synthesis of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)glutathione and subsequent TLC demonstrated that this had an identical R_f to the major biliary metabolite. In addition the in vitro microsomal product also had the same R_f (Fig 3.10 (a) and (b)).

The band eluting from the HPLC column at 174min contained two components (Fig 3.7). The major component at R_f 0.59 accounted for 12% of the radioactivity in bile. The R_f of this component was similar to that for the γ -glutamyltransferase-treated in vitro microsomal metabolite and was therefore tentatively identified as the cysteinylglycine conjugate of HCBd. Hydrolysis with 6M HCl, in an attempt to remove the glycine residue and aid gas chromatographic analysis, gave a sample which when either methylated or methylated and trifluoroacetylated was labile under the chromatographic conditions used. Incubation of the authentic glutathione conjugate of HCBd with γ -glutamyltransferase produced two ninhydrin-positive spots on thin layer chromatograms. The lower spot at R_f 0.18 corresponded to a glutamic acid standard, while the second at R_f 0.60 which was also u.v absorbing, was assumed to be the cysteinyl glycine conjugate of HCBd. Control incubations without γ -glutamyltransferase gave only a single ninhydrin-positive component at R_f 0.37 which corresponded to a freshly prepared authentic standard of the glutathione conjugate of HCBd.

Figure 3.10 TLC radiochromatograms comparing the R_f values of the *in vitro* HCBG-glutathione conjugate with components in bile from rats treated with $[^{14}\text{C}]\text{HCBG}$.

Co-chromatography on TLC in two solvent systems of the γ -glutamyltransferase hydrolysed authentic glutathione conjugate of HCBd with the 174min HPLC band demonstrated a radioactive, ninhydrin-positive spot corresponding to the R_f 0.60 spot in the enzymic hydrolysate, confirming that the major component in the 174min HPLC band was the cysteinylglycine conjugate of HCBd.

Gas chromatographic analysis of the two components in the least polar HPLC band eluting at 92min (Fig 3.7) either underivatised or as methylesters was unsuccessful due to degradation under the chromatographic conditions used.

b) Sulphur-containing metabolites in bile

Thin layer chromatography of bile from rats dosed with [^{35}S]cysteine, [^{35}S] cysteine and 'cold' HCBd and rats dosed with [^{14}C]HCBd gave an autoradiogram as seen in Fig 3.11. In this experiment, corresponding radioactive areas in both the [^{35}S]cysteine/cold HCBd and [^{14}C]HCBd chromatograms with no coincident radioactive area in the [^{35}S]cysteine chromatogram are HCBd metabolites which contain sulphur. Radioactive areas in the [^{14}C]HCBd group with no corresponding area in the [^{35}S]/cold HCBd chromatogram are non-sulphur containing metabolites. It cannot be determined for those radioactive areas which are coincident in all three chromatograms whether the metabolites contained sulphur or not.

Figure 3.11 Determination of sulphur containing metabolites in bile. An autoradiogram of a TLC plate showing the chromatography of samples of bile obtained from rats dosed either $[^{35}\text{S}]$ -cysteine ip or $[^{14}\text{C}]$ -HCBd orally and samples obtained from a rat dosed $[^{35}\text{S}]$ -cysteine ip and non-radioactive HCBd orally. The metabolites which contain sulphur are indicated.

Radioactive areas at Rf's 0.25, 0.30 and 0.40 contained sulphur. The area at Rf 0.40 also chromatographed with the in vitro glutathione-HCBD conjugate standard on this plate and therefore was further evidence for the in vitro formation of a glutathione HCBd conjugate. The faint radioactive areas at 0.26, 0.33, 0.63, 0.67 and 0.72 possibly contained sulphur. No non-sulphur containing metabolites could be identified.

c) Urinary metabolites

A small proportion of the urinary radioactivity (6%) was lost on protein precipitation. Diethyl ether-extraction of urine at neutral pH gave 2% of the total urinary radioactivity. No further analysis was carried out on either of these samples.

Extraction of urine at pH 1 with diethyl ether liberated 30% of the total urinary radioactivity and represented acidic metabolites of HCBd. Fractionation of this extracted material on reverse phase HPLC gave three radioactive bands. The least polar band accounted for 9% of the radioactivity in urine, 1% of the dose of HCBd. GC-RCD of this band on a 20% OV101 column gave a trace with a small radioactive peak associated with the solvent peak, which was probably due to sample breakdown and a large mass peak associated with radioactivity at a retention time of 4.5min. GC-MS analysis of this peak in the CI(methane) and EI modes gave the spectra shown in Figures 3.12 and 3.13. The CI spectrum shows the M+1 ion at 221 with a four chlorine isotope pattern. Assuming from biliary metabolite data that this metabolite was derived from conjugation of

Figure 3.12 Electron impact mass spectrum of the underivatised urinary metabolite showing the major fragment ions and the interpreted structure, that of tetrachlorothiophene.

Figure 3.13 Chemical ionisation mass spectrum of the underivatised urinary metabolite obtained with methane as the reagent gas.
 $M + 1 = 221$.

Electron impact and Chemical ionisation mass spectra of authentic tetrachlorothiophene showing a typical four chlorine pattern and the major fragment ions.

Figure 3.14

HCBD with glutathione, it seemed likely that sulphur would be present. Analysis of the EI spectrum resulted in assignment of the fragment ions as shown in Figure 3.12. The spectrum was interpreted as that of tetrachlorothiophene. Further evidence for this structure was obtained on inspection of the eight peak index of mass spectra (1974). Spectrum D1629 listed the ions 222, 220, 224, 149, 185, 187, 141 and 143 which agreed closely with the most intense ions obtained from this sample: 222, 220, 224, 187, 185, 141, 143, 79, 103 and 71. The structure was confirmed when an authentic sample of tetrachlorothiophene was shown to have the same gas chromatographic retention time and identical mass spectra to that of the biological sample (Fig 3.14).

Tetrachlorothiophene is known to be extremely soluble in ether but not water-soluble. This suggested that this compound was not present in urine as a metabolite of HCBD, but was an artifact produced under the conditions of GC analysis. In addition the urinary metabolite had acidic properties shown by its extraction into ether at pH 1, which were not consistent with it being tetrachlorophene.

In an attempt to prevent sample breakdown, the TMS-derivative was prepared. GC-RCD analysis of the derivative demonstrated a single mass peak with associated radioactivity. It was observed that the derivatised sample was only stable when stored in the derivatising agent. CI (methane) and EI spectra were obtained. CI gave an $M+1$ of 345 (Fig 3.15). The EI spectrum gave $M^+ 344$ with a five chlorine pattern (Fig 3.16). The metabolite was tentatively assigned

Figure 3.15 Chemical ionisation (methane) mass spectrum of the trimethylsilyl derivative of 1,1,2,3,4-pentachloro-1:3-butadienyl sulphenic acid showing the $M + 1 = 345$.

Figure 3.16 Electron impact mass spectrum of the trimethylsilyl derivative of 1,1,2,3,4-pentachloro-1:3-butadienyl sulphenic acid.

the structure 1,1,2,3,4-pentachloro-1:3-butadienyl sulphenic acid. High resolution GC-MS of this sample having a retention time of 12 minutes and gave accurate mass measurements on the M^+ and $M-Cl$ ions which were consistent with this structure.

The computer output for the strongest ions in the 309 and 344 isotope clusters are shown in Table 3.3. The error column shows the deviation from the calculated accurate mass in millimass units based on :-

C = 12.0000
H = 1.00782
N = 14.00307
O = 15.99491
 Cl^{35} = 34.96884
S = 31.97206
Si = 27.97692

For the two Cl^{35} ions at nominal masses 309 and 344 the results were;-

Table 3.3

<u>Calculated Accurate Mass</u>	<u>Measured Mass</u>	<u>Deviation</u>	
		<u>mmu</u>	<u>ppm</u>
308.8896	308.8921	+2.5	+8
343.8585	343.8551	-3.2	-10

Since accurate mass measurements within 10ppm or less are considered to be acceptable at the resolution used (5000) this data supported the assigned structure of the metabolite to be 1,1,2,3,4-pentachloro-1:3-butadienyl sulphenic acid.

The second band obtained on reverse phase HPLC separation of the ether extract represented 5% of the total urinary radioactivity and accounted for less than 1% of the dose. Rechromatography of this band using a gradient RP-8 system failed to achieve any further fractionation of radioactivity. The polarity of this band compared with the least polar band suggested that it could be a stronger acid than the sulphenic acid identified in the least polar band, and may possibly be a sulphinic acid. The sample was therefore treated with BSTFA in an attempt to make it more amenable to GC. GC-RCD analysis of the BSTFA-treated sample failed to produce a detectable radiochemical peak. Attempts to prepare the sulphonyl chloride derivative were more successful in that upon GC-RCD analysis a radiochemical peak was produced. However this radiochemical peak was only associated with a small mass peak which was insufficient for GC-MS analysis. This mass peak had a retention time of 9min and eluted at 140°C from a 20% OV101 column (1.5m x 2mm).

Rechromatography of the most polar fraction from the HPLC separation of the ether extract on a reverse phase gradient system, fractionated the radioactivity into five bands representing 6, 2, 3, 3 and 3% of the total urinary radioactivity. Each band being less than 1% of the dose of HCBd. Although treatment of the samples with

BSTFA in some instances produced material that was amenable to GC analysis and GC-MS analysis, no good mass spectral data was obtained.

3.4 Toxicity studies

Excretion studies have shown bile to be the major route of excretion of radioactivity derived from [^{14}C]HCB. Since the identification of nephrotoxic metabolites of HCB was the primary objective of this study, it was necessary to determine whether the nephrotoxic metabolite(s) or precursor of the nephrotoxic metabolite(s) was excreted in bile. This section is concerned with the demonstration of the nephrotoxic potential of the glutathione-derived biliary metabolites of HCB. Information was also gained on metabolite recirculation.

a) The toxicity of orally administered biliary metabolites of HCB

Qualitative assessment of the biliary metabolite pattern on TLC demonstrated no differences before and after concentration of the bile. Gross quantification revealed no loss of radioactivity. Animals dosed either control bile or corn oil displayed no signs of nephrotoxicity or hepatotoxicity as assessed biochemically or histologically (Tables 3.4 and 3.5). Animals dosed with the ^{14}C -labelled biliary metabolites (107mg/kg equivalents) or HCB (100mg/kg) produced marked changes in kidney:body weight ratio (Table 3.4). The kidney damage produced by HCB was less severe than that produced by dosing of the biliary metabolites as demonstrated by elevated plasma urea concentrations.

This observation was supported by histological data (Table 3.5), which demonstrated tubular necrosis in all metabolite-dosed animals, one of which showed casts and several foci of calcification in the inner medulla of the kidney. The animals dosed HCBd also displayed tubular necrosis but this was less severe than in animals, dosed with biliary metabolites.

The effects on the liver of biliary metabolites was minimal; only the liver:body weight ratio increased over control. This may also have occurred in the HCBd dosed animals. Histological data demonstrated no detectable damage which was consistent with the biochemical data which demonstrated no increase in the liver enzymes aspartate aminotransferase, alkaline phosphatase and alanine aminotransferase.

Animals dosed with either the ^{14}C -labelled biliary metabolites or [^{14}C] HCBd excreted $6.17 \pm 1.52\%$ and $4.62 \pm 0.07\%$ respectively of the administered radioactivity in urine. The appearance of radioactivity in urine of animals dosed with the biliary metabolite is indicative of absorption from the gastrointestinal tract with circulation to and excretion via the kidneys.

b) Nephrotoxicity of Synthetic Conjugates of HCBd

(i) Dose dependency

Single oral doses of N-acetyl-S-(1,1,2,3,4-pentachloro-1,3-butadienyl)-L-cysteine at 10,25 and 50mg/kg failed to produce an elevation in plasma urea, the parameter used to assess kidney

Table 3.4 The toxicity of biliary metabolites of hexachloro-1:3-butadiene

Parameter	[¹⁴ C]HCBd bile (107 mg/kg equiv.)	Control bile ^a	HCBd (100 mg/kg)	Control (Corn oil)
Group				
Plasma urea (mg %)	161.7 ± 5**	65.5	79.3 ± 1.76	57.3 ± 4.2
Kidney water (g H ₂ O/g dry wt)	4.35 ± 0.01**	3.51	4.00 ± 0.1**	3.50 ± 0.1
Kidney:body wt (x 100)	0.45 ± 0.03**	0.37	0.49 ± 0.01**	0.39 ± 0.01
Plasma AST (U/l)	69.0 ± 3.6	75.5	78.6 ± 20.6	74.0 ± 13.1
Plasma ALP (U/l)	364.0 ± 98	195	220.7 ± 52	189.0 ± 24.0
Plasma ALT (U/l)	7.3 ± 1.2	9.5	6.7 ± 2.5	6.7 ± 3.1
Liver water (g H ₂ O/g dry wt)	2.41 ± 0.03	2.32	2.40 ± 0.04	2.31 ± 0.04
Liver:body wt (x 100)	3.7 ± 0.1**	3.1	3.95 ^a	3.03 ± 0.18
Radioactivity in urine (% dose/24hr)	6.2 ± 1.5	-	4.6 ± 0.1	-

Rats were dosed orally with C-14 bile (equivalent to 107 mg/kg HCBd) collected from rats dosed orally with [¹⁴C]HCBd (200 mg/kg). Further groups of rats were dosed with an equivalent amount of control bile, with [¹⁴C]HCBd (100 mg/kg) in corn oil or with corn oil vehicle alone (5 ml/kg). Results are means ± SD (n = 3). a n = 2. ** Significantly different from control animals given corn oil alone, p < 0.001 (t-test).

AST - Aspartate aminotransferase

ALP - Alkaline phosphatase

ALT - Alanine aminotransferase

Table 3.5 The toxicity of biliary metabolites of hexachloro-1:3-butadiene:
Histopathology findings

Rat Number	Group	Kidney	Liver
1	CONTROL (CORN OIL)	NAD	NAD
2		NAD	NAD
3		NAD	NAD
4	CONTROL BILE	NAD	NAD
5		NAD	NAD
7	HCBD 100mg/kg	Tubular necrosis ⁺⁺	minimal hepatocyte vacuolation
8		Tubular necrosis ⁺⁺⁺	NAD
9		Tubular necrosis ⁺⁺	NAD
10	Bile collected from [¹⁴ C]HCBD treated rats 107mg/kg equiv	Tubular necrosis ⁺⁺⁺	NAD
11		Tubular necrosis ⁺⁺⁺	NAD
12		Tubular necrosis ⁺⁺⁺⁺ with casts and several foci calci- fication in inner medulla	NAD

+ - severity of necrosis

NAD - No abnormalities detected

damage. However a dose of 100mg/kg produced a significant rise in plasma urea (Fig 3.17) over the control value.

Single oral doses of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione of 50, 100, 150 and 200mg/kg produced a dose-dependent increase in plasma urea (Fig 3.18). Significant elevations in plasma urea were observed at 100, 150 and 200mg/kg.

These dose responses allowed the selection of an equimolar dose of each compound which would produce a nephrotoxic response and could be used to carry out more detailed comparison of their nephrotoxicities using biochemical and histological parameters. The doses chosen were 100 and 138mg/kg for N-acetyl-S-(1,1,2,3,4-pentachloro-1:3 butadienyl)-L-cysteine and S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione respectively.

Figure 3.17

Nephrotoxicity of single oral doses of N-acetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine in the rat as assessed by the kidney damage marker, plasma urea. Means $\pm$ S.D, n=3 except the control group; n=4. * Significant at $p < 0.02$ (t - test).

Figure 3.18

Nephrotoxicity of single oral doses of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione in the rat as assessed by the kidney damage marker, plasma urea. Means $\pm$ S.D, n=3 except the control group; n=4 and the 50 mg/kg group; n=2.
* Significant at $p < 0.02$ (t - test).

(ii) Toxicity of orally administered synthetic metabolites

A single oral dose of HCB_D (200mg/kg) produced marked changes in all of the biochemical parameters used to assess kidney damage (Tables 3.6 and 3.7). Increases in plasma urea, urinary glucose and protein indicated a significant effect on glomerular filtration and tubular re-adsorption. The concentration of N-acetyl- β -D-glucosaminidase, an enzyme with greatest activity in the renal cortex increased significantly as did urinary alkaline phosphatase and the proximal tubule brush border marker enzymes γ -glutamyltransferase and alanine aminopeptidase. The increases in γ -glutamyltransferase and alanine aminopeptidase were particularly marked. HCB_D also produced a significant increase in kidney:body weight ratios which was associated with an increase in tissue water content. Histological examination of the kidneys showed marked renal tubular necrosis which was seen as a distinct band of damage in the outer stripe of the outer medulla (Fig 3.19), which is associated with the straight portion of the proximal tubules. Liver damage caused by HCB_D was minimal, no significant histological changes could be observed under the light microscope and the only biochemical marker to change was plasma alanine aminotransferase the concentrations of which decreased. The reasons for this decrease are unknown, liver damage usually being indicated by an increase in the plasma concentrations of this enzyme.

When the glutathione and N-acetyl cysteine conjugates of HCB_D were dosed orally to rats at equimolar doses of 138 and 100mg/kg respectively, a remarkably similar pattern of changes to that

observed with HCB_D was seen with all of the kidney biochemical markers (Tables 3.6 and 3.8).

Histological examination of the kidneys from these rats confirmed that the conjugates of HCB_D produced identical kidney damage to HCB_D itself (Tables 3.7 and 3.9 and Figure 3.19). As with HCB_D there was no histopathological or biochemical evidence to suggest any hepatotoxicity.

Control (Polyethylene glycol 400)

HCBD 200mg/kg

Figure 3.19 Rat kidney, 24hr after the administration of polyethylene glycol 400 (5ml/kg p.o.) or HCBD (200mg/kg p.o.). The kidney from the rat given HCBD showed a distinct band of tubular necrosis in the outer stripe of the outer medulla. Haematoxylin and eosin x 40. Cortex left hand side medulla right hand side.

S-(1,1,2,3,4-Pentachloro-1:3-butadienyl)-glutathione

N-acetyl-S-(1,1,2,3,4-Pentachloro-1:3-butadienyl)-L-cysteine

Figure 3.19 cont. Rat kidney, 24hr after the administration of S-(1,1,2,3,4-Pentachloro-1:3-butadienyl)-glutathione (138mg/kg p.o.) or N-acetyl-S-(1,1,2,3,4-Pentachloro-1:3-butadienyl)-L-cysteine (100mg/kg p.o.) showing a distinct band of damage necrosis in the outer stripe of the outer stripe of the outer medulla. Haematoxylin and eosin x 40.

Table 3.6 Assessment of S-(1,1,2,3,4-Pentachloro-1:3-butadienyl)-glutathione for kidney and liver toxicity

Parameter	Mean $\pm$ S.D (n)			
Group	GSH-HCBD 138mg/kg	Aqueous Control	PEG 400 Control	HCBD 200mg/kg
Plasma Urea (mg%)	95.60 $\pm$ 10.92(5)	47.00 $\pm$ 5.25(6)	46.17 $\pm$ 6.97	102.67 $\pm$ 17.95(6)**
Plasma ALP (U/L)	405.80 $\pm$ 64.62(5)	362.67 $\pm$ 54.90(6)	353.00 $\pm$ 38.91(6)	350.17 $\pm$ 38.34(6)
Plasma ALT (U/L)	6.80 $\pm$ 0.45(5)	13.0 $\pm$ 3.16(6)	12.67 $\pm$ 1.51(6)	6.67 $\pm$ 1.51(6)**
Plasma AST (U/L)	35.80 $\pm$ 1.92(5)	40.50 $\pm$ 12.63(6)	37.83 $\pm$ 5.64(6)	41.67 $\pm$ 15.31(6)
Urine Glucose (mg/24h)	48.80 $\pm$ 14.73(5)**	2.27 $\pm$ 0.23(6)	2.25 $\pm$ 0.48(6)	50.12 $\pm$ 10.49(6)**
Urine Protein (mg/24h)	37.28 $\pm$ 6.43(5)**	8.07 $\pm$ 3.29(6)	7.29 $\pm$ 3.88(6)	24.94 $\pm$ 4.27(6)**
Urine ALP (μ mol/h/24h)	17.38 $\pm$ 5.13(5)**	0.75 $\pm$ 0.39(6)	1.15 $\pm$ 0.47(6)	9.97 $\pm$ 3.86(6)**
Urine NAG (μ mol/h/24h)	3.98 $\pm$ 0.70(5)**	1.90 $\pm$ 0.47(6)	1.35 $\pm$ 0.21(6)	2.80 $\pm$ 0.51(6)**
Urine GGT (μ mol/h/24h)	15965.00 $\pm$ 5104.96(5)**	539.73 $\pm$ 55.92(6)	702.00 $\pm$ 167.53(6)	10104.55 $\pm$ 2420.09(6)**
Urine AAP (μ mol/h/24h)	327.28 $\pm$ 88.15(5)**	28.12 $\pm$ 6.69(6)	32.77 $\pm$ 8.44(6)	289.34 $\pm$ 67.85(6) ⁺
Kidney Water (gH ₂ O/g dry wt)	4.71 $\pm$ 0.38(5)**	3.61 $\pm$ 0.12(6)	3.49 $\pm$ 0.18(6)	4.31 $\pm$ 0.21(6)**
Kidney:Body wt x 100	0.98 $\pm$ 0.05(5)**	0.82 $\pm$ 0.03(6)	0.79 $\pm$ 0.02(6)	1.05 $\pm$ 0.02(6)**
Liver Water (gH ₂ O/g dry wt)	2.65 $\pm$ 0.03(5)**	2.55 $\pm$ 0.06(6)	2.58 $\pm$ 0.05(6)	2.75 $\pm$ 0.12(6)*
Liver:Body wt x 100	3.99 $\pm$ 0.33(5)**	5.27 $\pm$ 0.24(6)	5.02 $\pm$ 0.39(6)	4.29 $\pm$ 0.23(6)*

Groups of rats were orally dosed with S-(1,1,2,3,4-Pentachloro-1:3-butadienyl)-glutathione (138mg/kg) in aqueous solution or HCBD (200mg/kg) in PEG 400 as positive control. The two control groups received either water or polyethylene glycol 400. The animals were sacrificed 24hr later and the biochemical parameters measured. Results are means $\pm$ S.D (n). Significantly different from relevant control group at **, p < 0.001; *, p < 0.01; ⁺, p < 0.02.

AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; NAG, N-acetyl- β -D-glucosaminidase; GGT, γ -glutamyltransferase; AAP, alanine aminopeptidase.

Table 3.7 Assessment of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione for kidney and liver toxicity : summary of histopathological findings

Tissue/Finding	GSH-HCBD Conjugate in aqueous solution 138mg/kg (Group 1)	Aqueous solution 10ml/kg (Control Group 2)	PEG 400 5ml/kg (Control Group 3)	HCBD in PEG 400 200mg/kg (Group 4)
<u>Kidney</u>				
Number examined	6	6	6	6
Proximal tubular necrosis (pars recta) in outer stripe of outer medulla	6	0	0	6
Proximal tubular necrosis throughout the cortex	1	0	0	0
Casts	6	0	0	5
Pelvic dilatation	1	0	2	2
Focus of basophilic tubules in cortex	0	0	1	0
<u>Liver</u>				
Number examined	6	6	6	6
Reduced cytoplasmic vacuolation and basophilia	6	0	0	6

Data on individual animals can be found in the appendix

Table 3.8 Assessment of N-acetyl-S-(1,1,2,3,4-Pentachloro-1:3-butadienyl)-L-cysteine for kidney and liver toxicity: Biochemical parameters

Parameter	Mean $\pm$ S.D (n)		
Group	N-acetyl cysteine HCBD Conjugate 100mg/kg	PEG 400 Control	HCBD 200mg/kg
Plasma Urea (mg%)	147 $\pm$ 23 (6)**	51 $\pm$ 15 (6)	108 $\pm$ 23 (6)**
Plasma ALP (U/l)	394 $\pm$ 46 (6)	303 $\pm$ 112 (6)	383 $\pm$ 92 (6)
Plasma ALT (U/l)	7 $\pm$ 2 (6)*	12 $\pm$ 2 (6)	6 $\pm$ 1 (6)**
Plasma AST (U/l)	39 $\pm$ 10 (6)	39 $\pm$ 8 (6)	47 $\pm$ 11 (6)
Urine Glucose (mg/24h)	33.9 $\pm$ 18.8(6)*	2.1 $\pm$ 0.4 (6)	51.5 $\pm$ 11.2(6)**
Urine Protein (mg/24h)	71.8 $\pm$ 19.2(6)**	9.4 $\pm$ 5.2 (5)	62.1 $\pm$ 17.6(6)+
Urine ALP (μ mol/h/24h)	12.61 $\pm$ 6.9 (6)+	0.71 $\pm$ 0.25(6)	17.43 $\pm$ 7.76(6)*
Urine NAG (μ mol/h/24h)	7.67 $\pm$ 1.08(6)**	2.18 $\pm$ 1.09(6)	7.46 $\pm$ 1.87(6)**
Urine GGT (μ mol/h/24h)	17,869 $\pm$ 6879(6)**	647 $\pm$ 377 (6)	12,209 $\pm$ 4787(6)*
Urine AAP (μ mol/h/24)	422.6 $\pm$ 84.3(5)**	32.7 $\pm$ 18.0(5)	329.9 $\pm$ 91.4(6)**
Kidney water (gH ₂ O/g dry wt)	3.93 $\pm$ 0.28(6)**	3.21 $\pm$ 0.23(6)	3.85 $\pm$ 0.18(6)**
Kidney: body wt x 100	1.04 $\pm$ 0.09(6)**	0.79 $\pm$ 0.04(6)	0.96 $\pm$ 0.09(6)*
Liver water (gH ₂ O/g dry wt)	2.43 $\pm$ 0.04(6)	2.37 $\pm$ 0.05(6)	2.62 $\pm$ 0.62(6)
Liver: body wt x 100	4.19 $\pm$ 0.17(6)	4.70 $\pm$ 0.47(6)	4.24 $\pm$ 0.20(6)

Groups of rats were dosed orally with N-acetyl-S-(1,1,2,3,4-Pentachlorobutadienyl)-L-cysteine (100mg/kg) or HCBD (200mg/kg) as positive control. Control rats were given vehicle alone (Polyethylene glycol 400). The animals were sacrificed 24 hr after treatment and the biochemical parameters measured. Results are means $\pm$ S.D(n). Significantly different from control group given PEG 400 alone (t-test) at **,p < 0.001; *,p < 0.01; +,p < 0.02.

AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; NAG, N-acetyl- β -D-glucosaminidase; GGT, γ -glutamyltransferase; AAP, alanine aminopeptidase.

Table 3.9 Assessment of N-acetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L- cysteine for kidney and liver toxicity: summary of histopathological findings

Tissue/Finding	N-Acetylcysteine HCBD conjugate in PEG 400 100mg/kg (Group 1)	PEG 400 5ml/kg (Group 2)	HCBD in PEG 400 200mg/kg (Group 3)
<u>Kidney</u>	6	6	6
Proximal tubular necrosis (pars recta in outer stripe of outer medulla)	6	0	6
Casts	6	0	5
Hydronephrosis	1	1	1
<u>Liver</u>	6	6	6
Single cell necrosis and associated inflammatory cell infiltration (minimal)	0	0	4
Focal mononuclear cell infiltration in portal tracts	1	0	0
Absence of glycogen-type vacuolation of hepatocytes	6	1	5

Data on individual animals can be found in the appendix

c) The effect of biliary cannulation on the nephrotoxicity of HCB
D in the rat

Complete protection from HCB nephrotoxicity was observed in rats that had been fitted with a biliary cannula prior to being given a single oral dose of 200mg/kg HCB (Table 3.10). This group of animals displayed concentrations of glucose, protein, γ -glutamyltransferase and alanine aminopeptidase in their urine which were comparable to the control values of cannulated rats given vehicle alone. The concentrations of these markers in both groups were greatly reduced from values obtained from the non-cannulated HCB (200mg/kg) group.

The surgical procedure for implanting the biliary cannula resulted in abnormally high concentrations of aspartate aminotransferase and plasma urea and precluded their use in this experiment as useful markers in assessing liver and kidney damage. The concentrations of the markers in the sham-operated HCB group were lower than those obtained previously with HCB-dosed animals, the reason for this is not known.

Table 3.10 The effect of biliary cannulation on the toxicity of
hexachloro-1:3-butadiene in the rat

Parameter	Cannulated + HCB (200 mg/kg)	Cannulated + PEG 400	HCB (200 mg/kg)
Plasma ALP (U/l)	275 ± 73	264/437	342 ± 13
Plasma ALT (U/l)	8 ± 3	7/11	8 ± 3
Plasma AST (U/l)	67 ± 17	86/116	57 ± 6
Urine glucose (mg/24hr)	1.0 ± 0.3	0.8 ± 0.3	25.4 ± 13.1
Urine protein (mg/24hr)	2.4 ± 1.7	3.3 ± 3.2	27.7 ± 16.7
Urine GGT (μmol/hr/24hr)	688 ± 272	1139 ± 909	8104 ± 6584
Urine AAP (μmol/hr/24hr)	49 ± 17	37/80	109/187

Rats (n = 3) were biliary cannulated and given a single oral dose of HCB (200 mg/kg). Control rats were cannulated and dosed with the vehicle alone (polyethylene glycol 400). A third group were not cannulated but were given HCB (200 mg/kg) as a positive control. Results are means ± SD or when n = 2 both values are given.

AST, Aspartate aminotransferase; ALP, Alkaline phosphatase;
ALT, Alanine aminotransferase; GGT, γ-glutamyltransferase;
AAP, alanine aminopeptidase.

DISCUSSION

Although the nephrotoxicity of HCBd had been recognised for many years (Chapter 1), little or nothing was known about the metabolic fate of this compound, or the relevance or otherwise of metabolism to the renal toxicity seen in experimental animals. HCBd was known to be metabolised to unknown water soluble metabolites (Davis et al, 1980) which were excreted in urine and faeces. In 1981 Lock and Ishmael observed a dose-dependent depletion of hepatic glutathione in the rat on dosing HCBd. These workers suggested that the depletion may be due to the formation of a reactive metabolite of HCBd, by hepatic metabolism, which is subsequently scavenged by glutathione. The depletion of glutathione in animal tissues after administration of compounds which are known to be toxic is frequently associated with the inactivation of toxic metabolites formed by cytochrome P-450-mediated monooxygenase reactions (Chasseaud, 1979; Mitchell et al, 1976; Jollow et al, 1977).

However, if a toxic metabolite was generated in the liver from HCBd one might expect to see liver damage, (especially if the glutathione pool was depleted) one does not, a point which was not easily explained at the time. The damage produced by HCBd is directed exclusively towards the kidney where paradoxically in male rats no glutathione depletion can be observed (Lock and Ishmael, 1981). The mechanism of HCBd-induced glutathione depletion was investigated in male rat liver preparations in vitro (Wolf et al, 1984) on the assumption that HCBd would be metabolised in a similar manner to analogous chlorinated compounds (see Introduction). HCBd caused a

marked reduction in glutathione concentration when incubated with male (or female) hepatic microsomal or cytosolic fractions fortified with glutathione. In the present study, identification of the microsomal product came from interpretation of the mass spectrum of its acid-hydrolysed and derivatised product as that of the methylester of S-(pentachloro-1:3-butadienyl) cysteine, this being the expected product from hydrolysis of the corresponding glutathione conjugate. Further evidence for the correct interpretation of the spectra and assignment of the original conjugate as that of a glutathione adduct came from dual label experiments (Wolf et al 1984) where a 1:1 ratio of [³H]-glutathione to [¹⁴C]-HCBd was demonstrated in the microsomal product.

HCBd has six chlorine substituents any one of which could be subject to displacement by glutathione. It has been demonstrated that the apparently single microsomal product (by TLC) when subjected to GC-MS analysis as the derivatised acid hydrolysate consists of two isomeric forms, in that two distinct mass peaks gave similar mass spectra. These were assumed to be cis- and trans-isomers formed from original cis- and trans-glutathione conjugates of HCBd. On steric grounds the trans-isomer was expected to predominate.

The evidence suggests that the glutathione depletion observed in vitro by Wolf et al (1984) was due to a direct conjugation reaction of glutathione with HCBd with loss of one atom of chlorine. The reaction was shown not to be cytochrome P-450-mediated because no α -hydroxy substituent was evident and the olefinic bonds remained

intact. The lack of involvement of cytochrome P-450-dependent metabolism, the principle route for structurally related chlorinated ethylenes, was further demonstrated by Wolf et al, (1984) in that the formation of product was not inhibited by carbon monoxide or nitrogen in place of oxygen, and was independent of the presence of NADPH.

The formation of glutathione conjugates by glutathione transferase enzymes is well documented and until recently conjugations of this type have been thought to be mediated by cytosolic transferases, however, Morgenstern et al (1979 and 1980) and Friedberg et al (1980) have now recognised the presence of hepatic microsomal glutathione S-transferases. For most substrates the activity of the microsomal transferases is normally low when compared with cytosolic transferases; less than 10% on a milligram of protein basis. In the case of HCBd this appears not to be true, the rate of HCBd - glutathione conjugation in microsomal fractions being up to twice that found in cytosol (Wolf et al, 1984). One possible explanation for the high microsomal transferase(s) activity may be the lipophilicity of the HCBd molecule favouring partition into the microsomal lipid membranes.

Multiple forms of both microsomal and cytosolic glutathione-S-transferases exist (Friedberg et al, 1980; Habig et al, 1974) and in the case of the cytosolic enzymes these proteins are known to have different substrate specificities. Certain similarities, such as the isoelectric point and immunochemical cross-reactivity between some of the microsomal glutathione S-transferases and those in the

cytosol, are known (Friedberg et al, 1980). However, Morgenstern et al, (1982) have demonstrated that at least one form of microsomal transferase is clearly distinguishable from those in the cytosol. Which transferase(s) is responsible for the conjugation reaction with HCBP is unknown.

The in vivo studies described in this thesis indicated that the main route of excretion of radioactivity after a nephrotoxic dose of HCBP was in the bile, 17% of the dose being excreted in both 24hr and 48hr respectively. In the rat efficient biliary excretion usually requires a molecular weight greater than 325-350 and the presence of a strong polar group (Hirom et al, 1976; Levine, 1978). HCBP (M.W. 261) possessing neither of these characteristics is not excreted in bile unchanged but has been reported to be extensively metabolised and excreted as unknown polar metabolites (Davis et al 1980). In this thesis two metabolites have been identified in bile which possess the necessary characteristics for biliary excretion, S-pentachlorobutadienyl-glutathione (M.W. 531) and also a product of its further metabolism by the mercapturic acid synthesis pathway, S-pentachlorobutadienyl-cysteinylglycine (M.W. 384). Both these conjugates together accounted for over 50% of the radioactivity in bile.

The presence of a glutathione conjugate which is formed in the liver is consistent with the glutathione depletion observed in vivo in male rats (Lock and Ishmael, 1981), and also with the formation and identification of glutathione conjugates in vitro (Wolf et al 1984). The formation of the cysteinylglycine conjugate of HCBP can be explained by the classical degradation pathway for glutathione

conjugates. γ -glutamyltransferase present in the bile duct canniculi in the liver or secreted into the common bile duct by the pancreas via the pancreatic ducts may be responsible for the degradation as demonstrated for methyl mercury glutathione (Hirata and Takahashi, 1981). Cysteinylglycine hydrolase is also present in bile and may further degrade the cysteinylglycine conjugate to S-pentachlorobutadienyl-cysteine.

Additionally, the small intestine also possesses the necessary degradative capability, as does the kidney itself. The degradative capability of the kidney will be discussed later.

Partial degradation of S-pentachlorobutadienyl-glutathione to lower molecular weight conjugates with greater lipophilicity would facilitate reabsorption from the intestine. Comparison of biliary excretion with that in faeces suggested extensive reabsorption and enterohepatic recirculation of HCBd metabolites. The reabsorption of HCBd metabolites has been demonstrated further in these studies. When radiolabelled bile collected from one group of [^{14}C]HCBd-dosed rats was dosed to a second group of rats, six per cent of the dose was excreted in the urine in the first twenty four hours. This experiment was also important in that it demonstrated that in addition to enterohepatic recirculation and faecal excretion, part of the dose eliminated initially in bile, is subsequently excreted by the kidneys. This experiment also established a link between hepatic metabolism, biliary metabolites and kidney damage. The rats dosed with bile containing HCBd metabolites showed marked kidney damage in comparison to rats dosed with control bile, indicating the

hepatic production of a nephrotoxic metabolite. Further experiments demonstrated that, when either the chemically synthesised glutathione conjugate or its mercapturate derivative were dosed to rats they each produced an identical pattern of kidney damage to that found for HCBd itself, without producing liver damage. The absolute role of hepatic metabolism in the toxicity of HCBd was established when biliary cannulated rats dosed with HCBd were completely protected from kidney damage. This experiment clearly established that although the kidney possesses all of the enzymes necessary to synthesise the glutathione and other mercapturic acid pathway metabolites in situ (Chasseaud, 1973) this does not occur at least not to an extent which leads to kidney damage. The conjugation of HCBd with glutathione in the liver is therefore the critical initial event in the production of HCBd nephrotoxicity.

Further evidence supporting the necessary involvement of hepatic-mediated glutathione conjugation in the nephrotoxicity of HCBd came from reassessment of the data of Lock et al (1979) who demonstrated that HCBd when added in vitro to kidney cortical slices failed to produce a decrease in PAH or TEA accumulation (indicators of damage to the kidney anion and cation transport systems respectively). However, renal slices prepared from rats previously dosed in vivo with HCBd showed selective damage to the renal anion transport system without damaging the cation system when tested in vitro. This difference between the in vitro and in vivo treatment with HCBd was suggested at the time to be due to the fact that in vivo tubular cell necrosis could occur as a result of ischaemia and/or as a result of prolonged contact of HCBd with the tubular cells.

However, in the light of current work it is now known that HCB_D requires hepatic metabolism initially to the glutathione conjugate as a critical event in the manifestation of its toxicity, a reaction which the renal cortical slices treated directly with HCB_D in vitro appear unable to undertake to any significant extent.

The exact nature of the reabsorbed HCB_D hepatic metabolites is unknown, but they are thought to be degradation products of the initial glutathione-HCB_D conjugate.

Irrespective of the exact nature of the reabsorbed hepatic metabolites, once delivered to the kidney, the kidney has the ability to degrade all of these conjugates to the cysteine conjugate. For certain glutathione conjugates the kidney is known to play a major role in this metabolism (Meister and Tate, 1976). One such example is the metabolism by isolated kidney cells of paracetamol-glutathione, to the corresponding cysteine and N-acetylcysteine derivatives via the cysteinylglycine intermediate (Moldeus et al; 1978 and Jones et al; 1979). HCB_D-glutathione would be expected to be degraded in a similar manner, by the enzymes γ -glutamyltransferase (γ -glutamyltranspeptidase) and cysteinylglycine hydrolase. Within the kidney, γ -glutamyltransferase is primarily associated with the brush-border membrane of the proximal tubule cells (Glossman and Neville, 1972). The asymmetric orientation of its activity suggests that glutathione metabolism occurs in the tubular lumen (Tsao and Curthoys, 1980). Aminopeptidase is also located on the brush border of the tubular epithelial cells (Okajima et al 1981) and is thought to metabolise cysteinylglycine conjugates

to cysteine conjugates. In addition, a particulate renal dipeptidase which catalyses the hydrolysis of cysteinylglycine has also been recently reported in rat renal brush border membrane (McIntyre and Curthoys, 1982) and may also hydrolyse cysteinylglycine conjugates.

Following the identification of the glutathione and cysteinylglycine conjugates in bile, and the capability of the kidney for glutathione degradation and mercapturic acid synthesis (Hughey et al, 1978) one may have expected the mercapturic acid derivative of HCBd to be present in urine, as was observed for S-(Dichlorovinyl)-cysteine (Derr and Schultze, 1963). Although no direct attempt to identify the mercapturic acid derivative of HCBd was made, synthesis of this metabolite demonstrated that it had extreme water insolubility and therefore may not be excreted in urine.

The glutathione or cysteine conjugates of HCBd per se would not be expected to be reactive species capable of causing kidney damage. Therefore some further metabolic event must be occurring in the 'target organ', the kidney. The cysteine conjugate of HCBd would have a structure analogous to that of the renal toxin S-(trans-1,2-dichlorovinyl)-L-cysteine (DCVC) and the metabolic events which are occurring in the kidney may be similar to those documented for DCVC.

DCVC was first synthesised by McKinney et al (1957) and shown to produce renal tubular necrosis in rats (Schultze et al 1962; Terracini and Parker, 1965) and in several animal species (Terracini and Parker, 1965). In rats a single intraperitoneal injection of

DCVC (100mg/kg) was sufficient to produce necrosis of renal tubules in the inner cortex within 24hr (Terracini and Parker, 1965). The area of damage was defined further as a distinct band in the pars recta of the proximal tubules, the same area in which HCBd-induced damage occurs (Ishmael et al, 1982).

Metabolic studies with DCVC demonstrated that the major components in rat urine were unchanged DCVC, its N-acetyl derivative (mercapturic acid), an unidentified component and inorganic sulphate (Derr and Schultze, 1963a). In vitro work led to the recognition of an additional metabolic step, the enzymatic cleavage of the intracysteine C-S bond of DCVC by bovine liver and kidney preparations (Anderson and Schultze 1965b and Bhattacharya and Schultze, 1967). The enzyme responsible, a β -lyase, was isolated and purified by these workers, and is probably the same enzyme recently isolated and characterised by Tateishi et al (1978) and that isolated by Stevens and Jackoby (1983). β -Lyase activity has been demonstrated in rat liver mitochondrial and cytosolic fractions (Stonard and Parker, 1971a; Stonard, 1973). The products of the cleavage reaction are stoichiometric quantities of ammonia and pyruvate, chloride ions and a reactive fragment which contains sulphur and the vinyl carbon atoms of DCVC.

The liver enzyme was shown to be only active with the L-isomer of DCVC. L-DCVC sulfoxide also served as substrate but the apparent K_m was higher than with L-DCVC (Anderson and Schultze, 1965b). Anderson and Schultze also suggested that formation of a sulfoxide was not a prerequisite for β -lyase cleavage of DCVC. Several other

halo-alkene cysteine conjugates have been shown to be metabolised by this enzyme (Bonhaus and Gandolfi, 1981; Green and Odum, in press). β -Lyase is inactive towards glutathione conjugates or N-acetyl cysteine conjugates, and the free amino group of the cysteine moiety appears to be essential for activity (Tateishi et al 1978; Anderson and Schultze, 1965(b)). HCBD conjugates have also been tested with purified β -lyase. β -lyase failed to metabolise S-pentachlorobutadienylglutathione or N-acetyl-S-(pentachlorobutadienyl)-L-cysteine to yield ammonia and pyruvate, but could metabolise S-(pentachlorobutadienyl)-cysteine. The mercapturic acid conjugate was metabolised by β -lyase after incubation with an acylase enzyme (Green and Odum, in press; Nash et al, 1983). In toxicity experiments where the N-acetyl-cysteine-HCBD conjugate was dosed to animals and produced toxicity, the conjugate was assumed to be deacetylated in the target organ or elsewhere to liberate S-(pentachlorobutadienyl)-cysteine which can serve as a substrate for β -lyase.

Work for this thesis identified a urinary metabolite of HCBD, 1,1,2,3,4-pentachloro-1:3-butadienyl sulphenic acid (Figure 3.12) which is consistent with cleavage of a cysteine - HCBD conjugate by β -lyase. The sulphenic acid metabolite of HCBD is unusual in that it is one of very few stable sulphenic acids known to exist, anthraquinone-1-sulphenic acid and purine sulphenic acids being other examples (Kuhle, 1973; Abraham and Jardine, 1982). A similar although more transient sulphenic acid has been identified in plants following the action of β -lyase on S-(prop-1-enyl) cysteine sulfoxide (Spare and Virtanen, 1963).

Two possible routes by which the sulphenic acid metabolite may arise are shown in Figure 4.1. (1) Cleavage of the HCBd-cysteine conjugate (pentachlorobutadienyl-cysteine) by renal β -lyase to form a thiol followed by oxidation to give the sulphenic acid. (2) Alternatively oxidation of the sulphur to form pentachlorobutadienyl-cysteine sulfoxide, which is subsequently cleaved by β -lyase to produce the sulphenic acid.

Both routes seem equally feasible and will be considered:

(1) All organic thiols and thiol derivatives are quite susceptible to aerobic oxidation yielding a variety of oxy derivatives. The only thiol oxidation reaction to oxy derivatives of general endogenous biochemical significance is that of cysteine to alanine 3-sulphinic acid (cysteine sulphinic acid), the main pathway for the utilisation of cysteine sulphur for sulphate production (Patai, 1974a). Thiols are also known to dimerise (a well known reaction for glutathione), and in the case of pentachlorobutadienyl-cysteine this reaction could give rise to sulphenic acid by subsequent hydrolysis of the disulphide as shown in Figure 4.2 (reaction 1). A reactive thiol generated by β -lyase cleavage of pentachlorobutadienyl-cysteine could more likely react with glutathione to form a mixed disulphide which could give rise to sulphenic acid in a similar manner.

Figure 4.2 also shows that the disulphide can theoretically give rise to a thiosulphinic acid and thiosulphonate, both of which by subsequent hydrolysis can give sulphenic acids (reaction 2 and 3).

Pentachlorobutadienyl - sulphenic acid

Figure 4.1

A scheme showing two possible routes from S-pentachlorobutadienyl-cysteine to pentachlorobutadienyl-sulphenic acid via S-oxidation and β -lyase reactions.

Figure 4.2 Possible reactions giving rise to sulphenic, sulphinic and sulphonic acid derivatives (Capozzi and Modena taken from Patai 1974).

The thiol which may be generated from β -lyase cleavage of pentachlorobutadienyl-cysteine may also undergo S-methylation by methyl transferases present in liver and kidney. This mechanism may be the origin of the many S-methyl metabolites reported for a wide range of compounds in recent years (Miller, 1970; Tateishi and Shimizu, 1976; Jenner and Testa, 1978).

(2) S-oxidation to form sulfoxides is documented for a wide range of sulphur-containing substrates, one example is the S-oxidation of propylcysteine formed from 1-bromopropane (Baines et al, 1977). S-oxidation appears to be catalysed by both cytochrome P-450-dependent monooxygenases and flavoprotein mixed-function amine oxidase (Poulsen et al, 1979). However, which enzyme is responsible for a particular substrate is not entirely clear. Sulfoxides have been shown to be substrates for β -lyase (Anderson and Schultze, 1965(b)).

Regardless of which route to the sulphenic acid is applicable there exists the possibility for further sulphur oxidation products such as sulphinic (RSO_2H) and sulphonic acids (RSO_3H). This type of sequential oxidation has been shown to be enzymic for phenylthiourea and ethylenethiourea. The formation of their sulphinic acid derivatives (phenylformidine- and ethyleneformidine sulphinic acids) being catalysed by microsomal FAD-containing monooxygenase. Further oxidation to the sulphonic acids is at least in part enzymatic (Poulson et al, 1979).

In the case of HCBd two other more polar components were present in urine samples and could have been further oxidation products of the sulphenic acid. These components were derivatised on this assumption but were not identified due to the small quantities of material or failure in attempts to make chromatographically suitable derivatives for mass spectral analysis.

Sulphenic acids are also known to cyclise to thiophenes (a reaction used to identify the sulphenic acid of HCBd). If a similar reaction occurred biologically in the kidney a product, tetrachlorothiophene which is insoluble in water would be produced and might well be reabsorbed, further complicating the biodegradation of HCBd.

HCBd specifically damages the kidney and the pathological lesion produced resembles closely that produced by DCVC. Recent work has shown that DCVC and S-(pentachlorobutadienyl)-cysteine and also the glutathione and mercapturic acid conjugates of HCBd decrease the accumulation of the organic ions PAH and TEA when incubated in vitro with kidney slices. This damage to the renal organic ion uptake system is presumed to be mediated in both cases by a reactive thiol generated by β -lyase cleavage of the cysteine conjugates (Nash et al, 1983). The thiovinyl moiety generated in vitro from DCVC has been shown to be a very reactive alkylating agent in that it will readily alkylate 4-(p-nitrobenzyl)-pyridine (NBP) (Stonard and Parker, 1971a) a widely recognised acceptor for alkyl groups (Epstein et al, 1965). The thiovinyl moiety can combine with proteins (Anderson and Schultze, 1965a), glutathione (Anderson and Schultze, 1965a) and DNA (Bhattacharya and Schultze, 1972). In the

cases of the glutathione and mercapturic acid conjugates of HCBd the kidney appears to degrade them to the cysteine conjugates in order to serve as substrates for renal β -lyase.

As the data above is similar for HCBd and DCVC it is suggested that the biochemical target for HCBd within the kidney S_3 cells may be the same as that proposed for DCVC (Stonard, 1973). Biochemical studies with DCVC have shown that DCVC inhibited both DNP-stimulated (DNP; 2,4-dinitrophenol, an agent uncoupling oxidative phosphorylation) and unstimulated respiration of rat liver and kidney mitochondria in vitro. When DCVC (50mg/kg) was injected intraperitoneally into rats DNP-stimulated respiration of rat kidney mitochondria was substantially inhibited 2-4hr after administration (Parker, 1965), and also delayed stimulation of ATP hydrolysis, both in vivo and in vitro (Stonard and Parker, 1971). These effects were thought to be related to production of a reactive metabolite. Like liver and kidney fractions, rat liver mitochondrial fractions have the ability to degrade DCVC to pyruvic acid and ammonia and at least one other unidentified metabolite, which has the characteristics of an alkylating agent (Stonard, 1971; Stonard and Parker, 1971b). The sites of action of DCVC are thought to be the multienzyme complexes, 2-oxoacid dehydrogenases (Stonard and Parker, 1971b). It has been shown that lipoyl dehydrogenase (NADH_2 : lipoic acid oxidoreductase E C 1.6.4.3) an enzyme present in both the pyruvate and 2-oxoglutarate dehydrogenase complexes and glutathione reductase (an extramitochondrial cytosolic enzyme) are inhibited in vitro (Stonard, 1973). The active sites of lipoyl dehydrogenase and glutathione reductase are both thought to contain FAD and a redox-

disulphide grouping (Massey 1963). A mechanism for the action of DCVC is shown in Figure 4.3.

The inhibition of mitochondrial events may lead to the observed mitochondrial swelling observed with HCBd (Lock and Ishmael 1982) and eventually to cell death. A scheme for the hepatic conjugation and renal activation of HCBd is shown in Figure 4.4.

Figure 4.3 Mechanism for the renal activation of S-(trans-1,2-dichlorovinyl)-L-cysteine

Figure 4.4 Proposed mechanism for hexachloro-1:3-butadiene elicited nephrotoxicity.

Work contained in this thesis has established a number of important principles for HCBd-induced nephrotoxicity which may be applicable to other haloethylene-produced nephrotoxicities.

Conjugation of HCBd with glutathione in the liver is the initial crucial event and probably accounts for the reduction in glutathione concentrations seen in vivo (Lock and Ishmael, 1981) on dosing HCBd to rats. Experiments using biliary cannulation techniques have demonstrated indirectly that the kidney cannot produce glutathione conjugates of HCBd at least not to an extent which can cause nephrotoxicity. Although other workers have synthesised various glutathione and cysteine conjugates (Bonhaus and Gandolfi (1981), Gandolfi et al, (1981), Green and Odum (1985), in order to demonstrate their nephrotoxicity in vitro or occasionally in vivo the present study provides the first evidence for the production of a metabolite in vivo which leads to nephrotoxicity. It has been demonstrated conclusively that the glutathione conjugate is eliminated in bile and that degradation and reabsorption of subsequent metabolites occurs. It is the glutathione conjugate which is a precursor of the ultimate nephrotoxic metabolite produced by β -lyase cleavage of the proximate nephrotoxin S-(pentachlorobutadienyl)-cysteine. The identification of the sulphenic acid metabolite of HCBd constitutes the only published evidence to date of the identification of a β -lyase cleavage product from a halocarbon-cysteine conjugate.

HCBd is therefore an example of a glutathione conjugation reaction which subsequently leads to a toxic metabolite rather than a non-

toxic, less biologically active metabolite. The specific region of damage produced in the kidney nephron is probably a consequence of the kidney possessing all the necessary enzymes for glutathione conjugate degradation in close proximity to the enzyme β -lyase. In this case, the enzymes are used for xenobiotic degradation yielding a reactive metabolite. No evidence has been obtained for the production of toxicity via epoxide formation as seen with chloroethylene -induced liver damage.

REFERENCES

Abraham R T and Jardine I (1982). Purine-6-sulfenic acid. Eighth European Workshop on Drug Metabolism. Abstract 301.

Anderson P M and Schultze M O (1965a). Interaction of S-(1, 2-dichlorovinyl)-L-cysteine with proteins. Arch Biochem Biophys 109 615-621.

Anderson P M and Shultze M O (1965b). Cleavage of S-(1,2-dichlorovinyl)-L-cysteine by an enzyme of bovine origin. Arch Biochem Biophys 111 593-602.

Baines P J, Bray H G and James S P (1977). Mercapturic acid formation in the developing rat. Xenobiotica, 7 (11) 653-663.

Bartsch H, Malaveille C, Barbin A and Planche G (1979). Mutagenic and alkylating metabolites of halo-ethylenes, chlorobutadienes and dichlorobutenes produced by rodent or human liver tissues: Evidence for oxirane formation by P-450-linked microsomal mono-oxygenases. Arch Toxicol 41 249-277.

Benedetto C, Slater T F, Dianzani M U (1976). NADPH-cytochrome C reductase, cytochrome P-450 and NADPH linked lipid peroxidation in microsomal fractions obtained from rat tissues. Biochem Soc Trans 4 1094-1097.

Berndt W O and Mehendale H M (1978). Effect of hexachlorobutadiene on renal function in the rat. Toxicol Appl Pharmacol 45 296-297.

Bhattacharya R K and Schultze M O (1967). Enzymes from bovine and turkey kidneys which cleave S-(1,2-Dichlorovinyl)-L-cysteine. Comp. Biochem. Physiol 22 723-735.

Bhattacharya R K and Schultze M O (1972). Properties of DNA treated with S-(1,2-Dichlorovinyl)-L-Cysteine and a lyase. Arch Biochem Biophys 153 105-115.

Bollman J L (1948). A cage which limits the activity of rats. J Lab Clin Med 33 1348.

Bonhaus D W and Gandolfi A J (1981). Conjugation and bioactivation of chloro-trifluoroethylene. Life Sci 29 2399-2405.

Bonse G, Urban T H, Reichert D and Henschler D (1975). Chemical reactivity, metabolic oxirane formation and biological reactivity of chlorinated ethylenes in the isolated perfused rat liver preparation. Biochem Pharmacol 24 1829-1834.

Boyland E and Chasseaud L F (1967). Enzyme-catalysed conjugates of glutathione with unsaturated compounds. Biochem J 104 95-102.

Boyland E and Williams K (1965). An enzyme catalysing the conjugation of epoxides with glutathione. Biochem J 94 190-197.

Capozzi G and Modena G (1974). Oxidation of thiols. In the chemistry of the thiol group. Part 2 Chapter 17 p785-839. Ed Saul Patai. John Wiley and Sons, London.

Chasseaud L F (1973). Distribution of enzymes that catalyse reactions of glutathione with alpha-beta unsaturated compounds. *Biochem J* 131 765-769.

Chasseaud L F (1979). The role of glutathione and glutathione-S-transferases in the metabolism of chemical carcinogens and other electrophilic reagents. *Adv Cancer Res* 29 175-274.

Chernokan V F (1971). Toxicity of hexachlorobutadiene during its intake into an organism through the skin. *Chem Abs* 74 (19) 97218.

Curthoys N and Hughey R P (1979). Characterization and physiological function of rat renal γ -Glutamyltranspeptidase. *Enzyme* 24 383-403.

Daniel J W (1963). The metabolism of ^{36}Cl -labelled trichloroethylene and tetrachloroethylene in the rat. *Biochem Pharmacol* 12 795-802.

Davis M E, Berndt W O and Mehendale H M (1980). Disposition and nephrotoxicity of hexachloro-1:3-butadiene. *Toxicology* 16 179-191.

Derr R F and Schultze M O (1963). The metabolism of ^{35}S -(1,2-Dichlorovinyl)-L-cysteine in the rat. *Biochem Pharmacol* 12 465-474.

Duffel M W and Jakoby W B (1982). Cysteine S-conjugate N-Acetyltransferase from rat kidney microsomes. *Molecular pharmacology* 21 444-448.

Duprat P, Delsaut K and Gradiski D (1976). Effects of the principle aliphatic chlorinated solvents on skin and conjunctiva of the rabbit. Eur J Tox 9 (3) 171-177.

Duprat P and Gradiski D (1978). Percutaneous toxicity of Hexachlorobutadiene. Acta pharmacol et toxicol 43 346-353.

Epstein J, Rosenthal R W and Ess R J (1955). Use of -(4-Nitrobenzyl) pyridine as analytical reagent for ethylenimines and alkylating agents. Analyt Chem 27 1435-1439.

Eight peak index of mass spectra (1974). 2nd Ed. Pub Mass spectrometry data centre, Awre, Aldermaston, Reading, UK.

Environmental Protection Agency (1975). Survey of industrial processing data - Task 1. Hexachlorobenzene and Hexachlorobutadiene pollution from chlorocarbon processes. Final report EPA 56013-75-003.

Federal Register (1982) 47 (250) 58029.

Fernley H N and Walker P G (1965). Kinetic behaviour of calf-intestinal alkaline phosphate with 4-methylumbelliferyl phosphate. Biochem J 97 95-103.

Fjellstedt T A, Allen R H, Duncan B K and Jakoby W B (1973). Enzymatic conjugation of epoxides with glutathione. J Biol. Chem 248 (10) 3702-3707.

Fowler B A, Hook G E R and Lucier G W (1977). Tetrachlorodibenzo-p-dioxin induction of renal microsomal enzyme systems: Ultrastructural effects on pars recta (S₃) proximal tubule cells of the rat kidney. J Pharmacol Exp Ther 203 712-721.

Friedburg T, Bentley P, Stasiecki P, Glatt H R, Raphael D and Oesch F (1980). Identification, solubilisation and characterisation of microsomal associated glutathione-S-transferases. J Biol Chem 254 12028-12033.

Gage J C (1970). The subacute inhalation toxicity of 109 industrial chemicals. Br J Ind med 27 1-18.

Gandolfi A J, Nagle R B, Soltis J J and Plescia F H (1981). Nephrotoxicity of halogenated vinyl cysteine compounds. Res Commun Chem Path Pharmacol 33 249-261.

Gauntley P, Magadur J L, Morel G, Chaumont P and Canel F (1975). Dosage de l'hexachlorobutadiene dans les milieux biologiques. European Journal of Toxicology 8 (3) 152-158.

Gehring P J and MacDougall D (1971). Review of the toxicity of Hexachlorobenzene and Hexachlorobutadiene. Dow Chemical Co, Midland Mich as cited in Kociba et al 1977a.

Glossman H and Neville D M (1972). γ -glutamyltransferase in the kidney brush border membranes. FEBS Lett 19 340-344.

Goldbach R W, VanGenderen H and Leeuwangh P (1976). Hexachlorobutadiene residues in aquatic fauna from surface water fed by the River Rhine. The Science of the Total Environment 6 31-40.

Gore C W, Hughes K P, Mahler D, Simpson M, Smith I K, Taylor M L and Stonard M D (1981). Personal communication.

Gradiski D, Bennet P, Raoult G and Magadur J L (1978). Toxicite aigue comparee par inhalation des principaux solvants aliphatiques chlores. Archives des maladies professionnelles de medecine des travail et de Securite Sociale (Paris) 39 no 4-5 249-257.

Gradiski D, Duprat P, Magadur J L and Fayein E (1975). Etude toxicologique experimentale de l'hexachlorobutadiene. Eur J Toxicol 8 180-187.

Green T and Hathway D E (1975). The biological fate in rats of vinyl chloride in relation to it oncogenicity. Chem Biol Interactions 11 545-562.

Green T and Hathway D E (1977). The chemistry and biogenesis of the S-containing metabolites of vinyl chloride in rats. Chem Biol Interactions 17 137-150.

Green T and Odum J (1985). The nephrotoxicity and mutagenicity of cysteine conjugates of haloalkanes. Chem Biol Interactions (in press).

Greim H, Bonse H, Radwan Z, Reichert D and Henschler D (1975).

Mutagenicity in vitro and potential carcinogenicity of chlorinated ethylenes as a function of metabolic oxirane formation. Biochem Pharmacol 24 2013-2017.

Habig W H, Pabst M J, Jakoby W B (1974). Glutathione S-Transferase (The first enzymatic step in mercapturic acid formation). J Biol Chem 249 7130-7139.

Harleman J H and Seinen W (1979). Short term toxicity and reproduction studies in rats with Hexachloro(1,3)-butadiene - Toxicol Appl Pharmacol 47 1-14.

Hefner R E, Watanabe P G and Gehring P J (1975). Preliminary studies of the fate of inhaled vinyl chloride monomer in rats. Ann N Y Acad Sci 246 135-148.

Henschler D (1977). Metabolism of chlorinated alkenes and alkanes as related to toxicity. J Environmental Pathology and Toxicology 1 125-133.

Henschler D and Hoos R (1982). Metabolic activation and deactivation mechanisms of di, tri and tetrachloroethylenes. In Adv Exp Med and Biol 136A. Biological reactive intermediates II p 659-666. Plenum Press, London.

Hirata E and Takahashi H (1981). Degradation of methyl mercury glutathione by the pancreatic enzymes in bile. Toxicol Appl Pharmacol 58 483-491.

Hirom P C, Millburn P and Smith R L (1976). Bile and urine as complementary pathways for the excretion of foreign organic compounds. *Xenobiotica* 6 55-64.

Hook J B, Rose M S and Lock E A (1982). The nephrotoxicity of hexachloro-1:3-butadiene in the rat: Studies of organic anion and cation transport in renal slices and the effect of monooxygenase inducers. *Toxicol Appl Pharmacol* 65 373-382.

Hughey R P, Rankin BB, Elce J S and Curthoys N P (1978). Specificity of a particulate rat renal peptidase and its localisation along with other enzymes of mercapturic acid synthesis. *Arch Biochem Biophys* 186 211-217.

Hutson D H (1976). Glutathione conjugates. In *Bound and Conjugated Pesticide Residues*. 29 p103-131 ACS Symposium Series.

IARC (1979). IARC Monographs on the evaluation of carcinogenic risk of chemicals to man, 20. Some halogenated hydrocarbons, Lyon, 179-193.

Ishmael J, Pratt I S and Lock E A (1982). Necrosis of the pars recta (S3 segment) of rat kidney produced by hexachloro-1:3-butadiene. *J Path* 138 99-113.

Jaeger R J, Conolly R B and Murphy S D (1974). Effect of 18hr fast and glutathione depletion on 1,1-dichloroethylene induced hepatotoxicity and lethality in rats. *Exp Mol Pathol* 20 187-198.

Jaeger R J, Mullen F E, Coffman L J and Murphy S D (1976). Vinyl chloride hepatotoxicity and its alteration by modifiers of hepatic biotransformation in the rat. Proc Eur Soc Toxicol 17 301-308.

Jenkins L J Jr and Anderson M E (1978). 1,1-dichloroethylene nephrotoxicity in the rat. Toxicol Appl Pharmacol 46 131-141.

Jenner P and Testa B (1978). Review article. Novel pathways in drug metabolism. Xenobiotica 8 1-25.

Jollow D J, Kocsis J J, Snyder R and Vainio H (1977). Biological reactive intermediates. Plenum Press, London.

Jones B K and Hathway B K (1978a). The biological fate of vinylidene chloride in rats. Chem Biol Interactions 20 27-41.

Jones B K and Hathway D E (1978b). Differences in metabolism of vinylidene chloride between mice and rats. Br J Cancer 37 411-417.

Jones D P, Moldeus P, Stead A H, Ormstad K, Jornvall H and Orrenius S (1979). Metabolism of glutathione and a glutathione conjugate by isolated kidney cells. J Biol Chem 254 2787-2792.

Kirkland J J (1960). Analysis of sulphonic acids and salts by gas chromatography of volatile derivatives. Anal Chem 32 1388-1393.

Kluwe W M (1981). The nephrotoxicity of low molecular weight halogenated alkane solvents, pesticides and chemical intermediates p179-226. In Toxicology of the kidney. Ed. J B Hook, Raven Press, New York.

Kociba R J, Schwetz B A, Keyes D G, Jersey G C, Ballard J J, Dittenber D A, Quast J F, Wade C E and Humiston C G (1977a). Chronic toxicity and reproduction studies of hexachlorobutadiene in rats. Environ Health Perspect 21 49-53.

Kociba R J, Keyes D G, Jersey G C, Ballard J J, Dittenber D A, Quast J F, Wade C E, Humiston C G and Schwetz B A (1977b). Results of a two year chronic toxicity study with hexachlorobutadiene in rats. Am Ind Hyg Assoc J 38 589-602.

Kociba R J, Keyes D G, Jersey G C, Ballard J J, Dittenber D A, Quast J F, Wade C E, Humiston C G and Schwetz B A (1977c). Results of a two year chronic toxicity study with hexachlorobutadiene (HCBD) in rats. Tox App Pharm Abs 173 41 204.

Kuhle E (1973). Reactions of sulfenyl halides. In the chemistry of the sulfenic acids, p43, Georg Thieme, Stuttgart.

Kupiecki F P, Virtanen AI (1960). Cleavage of alkyl cysteine sulfoxides by an enzyme in onion (*Allium cepa*). Acta Chem Scand 14 1913-1918.

Laseter J L, Bartell C K, Laska A L, Holmquist D G, Condie D B, Brown J W and Evans R L (1975). EPA report 506/6-76-010.

Laska A L, Bartell C K and Laseter J L (1976). Distribution of Hexachlorobenzene and Hexachlorobutadiene in water, Soil and selected aquatic organisms along the Lower Mississippi River, Louisiana. Bulletin of Environmental Contamination and Toxicology 15 (5) 535-541.

Leaback D H and Walker P G (1961). Studies on glucosaminidase, the fluorimetric assay of N-acetyl- β -glucosaminidase. Biochem J 78 151-156.

Levine W G (1978). Biliary excretion of drugs and other xenobiotics. Ann Rev Pharmacol Toxicol 18 81-96.

Lock E A (1982). Renal necrosis produced by halogenated chemicals. In nephrotoxicity assessment and pathogenesis p396-408. Ed PH Bach, F W Bonner, J W Bridges, E A Lock. Wiley Heyden, London.

Lock E A and Ishmael J (1979). The acute toxic effects of hexachloro-1:3-butadiene on the rat kidney. Arch Toxicol 43 47-57.

Lock E A and Ishmael J (1981). Hepatic and renal non-protein sulphydryl concentration following toxic doses of hexachloro-1:3-butadiene in the rat: The effect of Aroclor 1254, phenobarbitone and SKF525A treatment. Toxicol Appl Pharmacol 57 79-87.

Lock E A and Ishmael J (1982). Hepatotoxicity and nephrotoxicity of Hexachlorobutadiene. *Advances Pharmacol and Therapeutics* 5 87-96.

MacDonald T L (1982). Chemical mechanisms of halocarbon metabolism *CRC Critical Reviews in Toxicology* 11 (2) 85-120.

Massey V (1963). Lipoyl Dehydrogenase. In the enzymes 2nd Ed. Vol 7 Ch 13 275-302. Ed P D Boyer, H Lardy and K Myrback. Academic Press, New York.

Maunsbach A B (1964). Ultrastructure of different segments within the rat renal proximal tubule. *Anat. Rec* 148 387.

Maunsbach A B (1966). Observations on the segmentation of the proximal tubule in the rat kidney. Comparision of results from phase contrast, fluorescence and electron microscopy. *J Ultrastruct. Res* 16 239.

McConnell G, Ferguson D M, and Pearson C R (1975). Chlorinated hydrocarbons and the environment. *Endeavour* 34 13-18.

McIntyre T and Curthoys N P (1982). Renal catabolism of glutathione - Characterisation of a particulate rat renal dipeptidase that catalyses the hydrolysis of cysteinylglycine. *J Biol Chem.* 257 (20) 11915-11921.

McKinney L L and Biester H E (1959). Possible toxic factor of trichloroethylene extracted soybean oil meal. *J Amer Chem Soc* 81 909-915.

- McKinney L L, Weakly F B, Eldridge A C, Campbell R E, Cowan J C, Picken J C, Biester H E (1957). S-(Dichlorovinyl)-L-cysteine: An agent causing fatal aplastic anemia in calves. J Am Chem Soc 79 3932-3933.
- Meister A and Tate S S (1976). Glutathione and related γ -glutamyl compounds: Biosynthesis and utilisation. Ann Rev Biochem 45 559-604.
- Miller J A (1970). Carcinogenesis by chemicals: An overview. Cancer Res 30 559-576.
- Mitchell J R, Hinson J A and Nelson S D (1976). Glutathione and drug induced tissue lesions. In Glutathione: Metabolism and Function, ed by I M Arias and W B Jakoby, pp 357-367, Raven Press, New York.
- Moldeus P, Jones D P, Ormstad K and Orrenius S (1978). Formation and metabolism of a glutathione-S-conjugate in isolated rat liver and kidney cells. Biochem Biophys Res Commun 83 195-200.
- Mondorf A W, Breirer J, Hendus J, Scherberich J E, Mackenrodt G, Shah P M, Stille W and Schoeppe W (1978). Effect of aminoglycosides on proximal tubular membranes of the human kidney. Eur J Clin Pharmacol 13 133-142.
- Morgenstern R, De Pierre J W and Ernster L (1979). Activation of microsomal glutathione-S-transferase activity by sulphhydryl reagents. Biochem Biophys Res Commun 87 657-663.

Morgenstern R, Guthenberg C and Depierre J W (1982). Microsomal Glutathione S-Transferase purification, initial characterization and demonstration that it is not identical to the cytosolic glutathione S-transferases A, B and C. Eur J Biochem 128 243-248.

Morgenstern R, Meijer J, De Pierre J W and Ernster L (1980). Characterisation of rat liver microsomal glutathione-S-transferase activity. Eur J Biochem 104 167-174.

Moslen M T, Reynolds E S and Szabo S (1977). Enhancement of the metabolism and hepatotoxicity of trichloroethylene and perchloroethylene. Biochem Pharmac 26 369-375.

Murzakaev F G (1967). Action exerted by low hexachlorobutadiene doses on the activity of the central nervous system and morphological changes in animals so poisoned. Gig Tr Prof Zabol 11 (3) 23-8 (Chem Abstr 67 31040 1967).

Murzakaev F G (1963). Toxicity data for hexachlorobutadiene and its intermediates. Farmakol i Toksikol 26 (6) 750. (Chem. Abstr 60 13776b 1964).

Nash J A, Odum J and Green T (1983). The biotransformation of hexachlorobutadiene and the role of sulphur containing metabolites in its nephrotoxicity. Proc Symp on Sulphur in Xenobiotics.(Birmingham) 151.

Nystrom A E (1948). The effect of chloroprene on the kidney. Acta Med Scand 219 (5) 66-68.

Okajima K, Inoue M and Morino Y (1981). Topology and some properties of the renal brush border membrane-bound peptidase(s) participating in the metabolism of S-carbamidomethyl glutathione. *Biochem Biophys Acta* 675 379-385.

Parker V H (1965). A biochemical study of the toxicity of S-Dichlorovinyl-L-cysteine. *Fd Cosmet Toxicol* 3 75-84.

Patai S (1974a). Biochemistry of the thiol group. In the chemistry of the thiol group Part 2 13 p597. John Wiley and Sons, London.

Patai S (1974b). Oxidation of thiols. In the chemistry of the thiol group Part 2 7 p786. John Wiley and Sons, London.

Pesce M A and Strande C S (1973). New micromethod for the determination of protein in cerebrospinal fluid and urine. *Clin Chem* 19 1265-1267.

Plaa G L and Larson R E (1965). Relative nephrotoxic properties of chlorinated methane, ethane and ethylene derivatives in mice. *Toxicol Appl Pharmacol* 7 37-44.

Poteryaeva G E (1972). Substantiating the maximum permissible concentration of hexachlorobutadiene in the air of industrial premises. *Gig Sanit* 37 (2) 32-6 (Chem Abstr 76 (20) 117219 1972).

Poulson L L, Hyslop R M and Ziegler D M (1979). S-oxygenation of N-substituted thioureas catalyzed by the pig liver microsomal FAD - containing monooxygenase. *Arch Biochem Biophys* 198 78-88.

Prendergast J A, Jones R A, Jenkins L J and Siegel J (1967). Effects on experimental animals of long-term inhalation of trichloroethylene, carbon tetrachloride, 1,1,1-trichloroethane, dichlorodifluorethane and 1,1-dichloroethylene. *Toxicol Appl Pharmacol* 10 270-289.

Pritchard J B (1981). Renal handling of environmental chemicals. In *Toxicology of the kidney*, Edited by J B Hook, Raven Press, New York. 99-116.

Prodan L, Suciu I, Pislaru V, Ilea E and Pasecu L (1975). Experimental chronic poisoning with vinyl chloride (monochloroethane). *Ann N Y Acad Sci* 246 159-163.

Schmidt-Nielsen B and O'Dell R (1961). Structure and concentrating mechanism in the mammalian kidney. *Am J Physiol* 200 1119.

Schultze M O, Derr R F, Mizuno N S, Joel D D and Sautter J H (1962). Effect of phenylalanine on toxicity of S-(dichlorovinyl)-L-cysteine in the rat and the calf. *Proc Soc exp Biol, NY* 111, 499.

Schwetz B A, Smith F A, Humiston C G, Quast J F and Kociba R J (1977). Results of a reproduction study in rats fed diets containing hexachlorobutadiene. *Toxicol Appl Pharmacol* 42 387-398.

Spare C G and Virtanen A I (1963). On the lachrymatory factor in onion (*Allium Cepa*) vapours and its precursor. *Acta Chem Scand* 17 641-650.

- Stevens J and Jakoby W B (1983). Cysteine Conjugate β -lyase. Mol Pharm 23 761-765.
- Stonard M D (1971). Ph D thesis, University of London, London.
- Stonard M D (1973). Further studies on the site and mechanism of action of S-(1,2-dichlorovinyl)-L-cysteine and S-(1,2-dichlorovinyl)-3-mercaptopropionic acid in rat liver. Biochem Pharmacol 22 1329-1335.
- Stonard M D and Parker V H (1971a). Metabolism of S-(1,2-dichlorovinyl)-L-cysteine by rat liver mitochondria. Biochem Pharmacol 20 2428-2437.
- Stonard M D and Parker V H (1971b). 2-oxoacid dehydrogenases of rat liver mitochondria as the site of action of S-(1,2-dichlorovinyl)-L-cysteine and S-(1,2-dichlorovinyl)-3-mercaptopropionic acid. Biochem Pharmacol 20 2417-2427.
- Szasz G (1969). A kinetic photometric method for serum γ -glutamyl transpeptidase. Clin Chem 15 124-136.
- Tateishi M and Shimizu H (1976). A new metabolic pathway of bromazepam involving attachment of a methylthio group. Xenobiotica 6 431-439.
- Tateishi M, Suzuki S and Shimizu H (1978). Cysteine conjugate β -lyase in rat liver. J Biol Chem 253 8854-8859.

Terracini B and Parker V H (1965). A pathological study on the toxicity of S-Dichlorovinyl-L-cysteine. Fd Cosmet Toxicol 3 67-74.

Tisher C C (1976). Anatomy of the kidney. In The Kidney vol 1

Brenner B M and Rector F C. Eds Saunders Company Philadelphia, USA.

Torkelson T R, Oyen F, and Rowe V K (1961). The toxicity of vinyl chloride as determined by repeated exposure of laboratory animals. Am Ind Hyg Assoc J 22 354-361.

Tsao B and Curthoys N P (1980). The absolute asymmetry of orientation of γ -glutamyl transpeptidase and aminopeptidase on the external surface of the rat renal brush border membrane. J Biol Chem 225 7708-7711.

Tune B M, Burg M B, Patlak C S (1969). Characteristics of p-aminohippurate transport in proximal renal tubules. Am J Physiol 217 1057-1063.

Ullberg S (1962). Autoradiographic localisation in the tissues of drugs and metabolites. Biochem Pharmacol 9 29-37.

Venkatachalam M A, Bernard D B, Donohoe J F and Levinsky N G (1978).

Ischemic damage and repair in the rat proximal tubule: Differences among the S₁, S₂, and S₃ segments. Kidney Int 14 31-49.

Viola P L (1970). Pathology of vinyl chloride. Med Lav 61 174-180.

Von Oettingen W F, Hueper W C, Deichmann-Gruebler W, and Wiley F H (1936). 2-chlorobutadiene (Chloroprene): Its toxicity and pathology and the mechanism of its action. J Ind Hyg Toxicol 18 240-270.

Weiner I M (1973). Transport of weak acids and weak bases. In Handbook of Physiology, Renal physiology, p521-554, Ed J Orloff and R W Berliner, American Physiological Soc, Washington, D C.

Williams R T (1959). In Detoxication Mechanisms 2nd Ed p734-739. Wiley and Sons, New York.

Wolf C R, Berry P N, Nash J A, Green T and Lock E A (1984). The role of microsomal and cytosolic glutathione-S-transferases in the conjugation of hexachloro-1:3-butadiene and its possible relevance to toxicity.

J Pharmacol Exp Ther 228 202-208.

Zenser T V, Mattammal M B and Davis B B (1978). Differential distribution of the mixed function oxidase activities in rabbit kidney.

J Pharmacol Exp Therap 207 719-725.

APPENDIX

Assessment of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione for kidney and liver toxicity: Histopathological findings (Continued)

Pathology No	Animal No	Group	Sex	Pathological Findings in Kidney	Pathological Findings in Liver
03214/82	6	1	M	Proximal tubular necrosis (pars recta) in outer stripe of outer medulla +++. A few basophilic granular casts in the medulla.	Reduced cytoplasmic vacuolation (glycogen-type) and cytoplasmic basophilia.
03215/82	23	1	M	Proximal tubular necrosis (PTN) in outer stripe +++. A few hyaline casts in medulla. Moderate pelvic dilatation.	As above.
03216/82	3	1	M	PTN outer stripe +++. A few hyaline casts and several intratubular basophilic (calcified) deposits in medulla.	As above.
03217/82	8	1	M	Severe PTN involving the outer stripe of the outer medulla and extending throughout the cortex +++. Large numbers of hyaline casts in the medulla.	As above.
03218/82	24	1	M	PTN outer stripe +++. Several basophilic (calcified) deposits in medulla.	As above.
032119/82	7	1	M	PTN outer stripe +++ with some extension into the medullary rays. Small numbers of hyaline and basophilic casts in medulla.	As above.

continued:

Assessment of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione for kidney and liver toxicity : Histopathological Findings (continued)

Pathology No	Animal No	Group	Sex	Pathological Findings in Kidney	Pathological Findings in Liver
03220/82	5	2	M	NAD	NAD (glycogen-type vacuolation)
03221/82	11	2	M	NAD	NAD (glycogen-type vacuolation)
03222/82	17	2	M	NAD	NAD (glycogen-type vacuolation)
03223/82	10	2	M	NAD	NAD (glycogen-type vacuolation)
03224/82	12	2	M	NAD	NAD (glycogen-type vacuolation)
03225/82	20	2	M	NAD	NAD (glycogen-type vacuolation)
03226/82	14	3	M	NAD	NAD (glycogen-type vacuolation)
03227/82	18	3	M	Small focus of basophilic tubules in cortex. Moderate pelvic dilatation.	NAD (glycogen-type vacuolation)
03228/82	13	3	M	Moderate pelvic dilatation.	NAD (glycogen-type vacuolation)
03229/82	4	3	M	NAD	NAD (glycogen-type vacuolation)
03230/82	21	3	M	NAD	NAD (glycogen-type vacuolation)
03231/82	16	3	M	NAD	NAD (glycogen-type vacuolation)
03232/82	19	4	M	PTN outer stripe +++. A few hyaline casts in medulla and in distal tubules in outer cortex. Slight pelvic dilatation.	NAD apart from loss of glycogen type vacuolation.

NAD - no abnormalities detected, PTN - Proximal tubular necrosis, Pathological findings moderate (+) to severe (+++).

Group 1 - GSH/HCBD conjugate 138 mg/kg, Group 2 - Aqueous solution (Control) 10 ml/kg, Group 3 PEG 5 ml/kg,
Group 4 - HCB 200 mg/kg

Assessment of S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione for kidney and liver toxicity : Histopathological findings

Pathology No	Animal No	Group	Sex	Pathological Findings in Kidney	Pathological Findings in Liver
03233/82	2	4	M	PTN outer stripe +++ extension into the medullary rays.	Absence of glycogen-type vacuolation.
03234/82	22	4	M	PTN outer stripe +++. A few hyaline and basophilic casts in medulla. Moderate pelvic dilatation.	Absence of glycogen-type vacuolation and decreased cytoplasmic basophilia.
03235/82	9	4	M	PTN outer stripe +++. A few casts in cortex and medulla.	Absence of glycogen-type vacuolation and decreased cytoplasmic basophilia.
03236/82	15	4	M	PTN outer stripe +++. A few casts in medulla.	Reduced cytoplasmic vacuolation and decreased basophilia.
03237/82	1	4	M	PTN outer stripe +++ with some extension into the medullary rays. A few casts in medulla.	Absence of glycogen-type vacuolation and decreased cytoplasmic basophilia. Minimal focal mononuclear cell infiltration.

NAD - no abnormalities detected, PTN - Proximal tubular necrosis, Pathological findings moderate (+) to severe (+++).

Group 1 - GSH/HCBD conjugate 138 mg/kg, Group 2 - Aqueous solution (Control) 10 ml/kg, Group 3 PEG 5 ml/kg,
Group 4 - HCB 200 mg/kg

Assessment of N-acetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine for kidney and liver toxicity : Histopathological findings

Pathology No	Animal No	Group	Sex	Pathological Findings in Kidney	Pathological Findings in Liver
00913/82	20	1	M	Proximal tubular necrosis (PTN - pars recta) in outer stripe of outer medulla +++. Hyaline casts in medulla and outer cortex ++.	NAD apart from absence of glycogen-type vacuolation.
00914/82	17	1	M	PTN outer stripe +++. Hyaline casts in medulla and outer cortex ++.	As above.
00915/82	4	1	M	PTN outer stripe +++. Hyaline casts in medulla ++. Moderate hydronephrosis.	As above.
00916/82	9	1	M	PTN outer stripe +++. Hyaline casts in medulla +.	As above.
00917/82	12	1	M	PTN outer stripe +++. Hyaline and basophilic granular casts in the medulla.	As above.
00918/82	19	1	M	PTN outer stripe +++. Hyaline casts in the medulla +++. .	Absence of glycogen-type vacuolation. Minimal focal mononuclear cell infiltration in portal tracts

NAD - no abnormalities detected, PTN - Proximal tubular necrosis, Pathological findings moderate (+) to severe (+++).

Group 1 - 100 mg/kg N-acetylcysteine/HCBD conjugate, Group 2 - PEG 400, Group 3 - 200 mg/kg HCBd.

Continued

Assessment of N-acetyl-S-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine for kidney and liver toxicity : Histopathological findings - (Continued)

Pathology No	Animal No	Group	Sex	Pathological Findings in Kidney	Pathological Findings in Liver
00919/82	24	2	M	NAD	Absence of glycogen-type vacuolation.
00920/82	1	2	M	Slight hydronephrosis.	NAD
00921/82	23	2	M	NAD	NAD
00922/82	2	2	M	NAD	NAD
00923/82	16	2	M	NAD	NAD
00924/82	5	2	M	NAD	NAD
00925/82	11	3	M	PTN outer stripe +++ Basophilic granular casts in medulla +.	Absence of glycogen-type vacuolation.
00926/82	8	3	M	Slight hydronephrosis.	NAD
00927/82	21	3	M	PTN outer stripe +++ Basophilic and hyaline casts in medulla +.	Minimal single cell necrosis and inflammatory cell infiltration. Absence of glycogen-type vacuolation.
00928/82	18	3	M	PTN outer stripe +++ Hyaline casts in medulla +.	Minimal single cell necrosis and inflammatory cell infiltration. Absence of glycogen-type vacuolation.
00929/82	6	3	M	PTN outer stripe +++ Hyaline casts in cortex and medulla ++.	As above.
00930/82	13	3	M	PTN outer stripe +++ Basophilic (clacified) casts in medulla ++.	Minimal single cell necrosis and inflammatory cell infiltration. Absence of glycogen-type vacuolation.
				Hyaline casts in cortex +.	

NAD - no abnormalities detected, PTN - Proximal tubular necrosis, Pathological findings moderate (+) to severe (+++).

Group 1 - 100 mg/kg N-acetylcysteine/HCBD conjugate, Group 2 - PEG 400, Group 3 - 200 mg/kg HCBd.

The Metabolism and Disposition of Hexachloro-1:3-butadiene in the Rat and Its Relevance to Nephrotoxicity

JOHN A. NASH, LAURENCE J. KING,* EDWARD A. LOCK, AND TREVOR GREEN¹

Biochemical Toxicology Section, Central Toxicology Laboratory, Imperial Chemical Industries PLC, Alderley Park, Macclesfield, Cheshire SK10 4TJ, England, and *Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, England

Received August 31, 1983; accepted November 3, 1983

The Metabolism and Disposition of Hexachloro-1:3-butadiene in the Rat and Its Relevance to Nephrotoxicity. NASH, J. A., KING, L. J., LOCK, E. A., AND GREEN, T. (1984). *Toxicol. Appl. Pharmacol.* 73, 124-137. Following po administration of a nephrotoxic dose (200 mg/kg) of hexachloro-1:3-butadiene (HCBD) to male rats, the principal route of excretion was biliary, 17-20% of the dose being eliminated on each of the first 2 days. Fecal excretion over this period was less than 5% of the dose per day, suggesting enterohepatic recirculation of biliary metabolites. Urinary excretion was small, not exceeding 3.5% of the dose during any 24-hr period. The major biliary metabolite was a direct conjugate between glutathione and HCBD itself. The cysteinylglycine conjugate of HCBD has also been found in bile. Evidence was obtained to show that biliary metabolites of HCBD are reabsorbed and excreted via the kidneys. The glutathione conjugate, its mercapturic acid derivative, and bile containing HCBD metabolites were all nephrotoxic when dosed orally to rats. In common with HCBD, these metabolites caused localized damage to the kidney with minimal effects in the liver. Rats fitted with a biliary cannula were completely protected from kidney damage when dosed with HCBD, demonstrating that hepatic metabolites were solely responsible for the nephrotoxicity of this compound. It is proposed that the hepatic glutathione conjugate of HCBD was degraded to its equivalent cysteine conjugate which was cleaved by the renal cytosolic enzyme β -lyase to give a toxic thiol which caused localized kidney damage. A urinary sulphenic acid metabolite of HCBD has been identified which is consistent with this hypothesis. The mode of activation of HCBD conjugates in the kidney is believed to be analogous to that proposed for *S*-(1,2-dichlorovinyl)-L-cysteine.

Hexachloro-1:3-butadiene (HCBD) is used commercially in small quantities as a vineyard fumigant and in the recovery of chlorine gas in the chemical industry. More significant amounts of HCBD are found in industrial wastes, generated mainly from the manufacture of tetra- and trichloroethylene and carbon tetrachloride (I.A.R.C., 1979). The toxicity of HCBD is directed almost exclusively toward the kidney (Gradiski *et al.*, 1975; Kociba *et al.*, 1977; Lock and Ishmael, 1979), and in the rat it specifically damages the pars recta of the proximal tubule (Ishmael *et al.*, 1982).

Although HCBD is metabolized in rats to unidentified water soluble metabolites (Davis *et al.*, 1980) which are excreted in urine and bile, the relevance of these metabolites to the toxicity of HCBD is unknown. The dose-dependent depletion of hepatic glutathione levels observed in male rats dosed with HCBD (Lock and Ishmael, 1981) is consistent with the possible formation of reactive electrophilic epoxides in common with the metabolism of other chloro-alkenes (Henschler and Greim, 1976). However, the toxicity of HCBD *in vivo* does not seem to be affected by inducers or inhibitors of hepatic and renal mixed function oxidases (Lock and Ishmael, 1981; Hook *et*

¹ To whom all correspondence should be addressed.

al., 1982), the enzymes associated with epoxide formation, nor do these observations explain the organ specificity of HCBd. Recent work in this laboratory with renal and hepatic *in vitro* preparations (Wolf *et al.*, 1984) has shown that depletion of hepatic glutathione is a result of a direct glutathione *S*-transferase mediated reaction between HCBd and glutathione. The same study failed to find any role for cytochrome *P*-450 in the hepatic metabolism of HCBd *in vitro*. These observations formed the basis of this *in vivo* investigation of the mechanism of activation of HCBd to toxic, organ specific metabolites. We carried out experiments to elucidate the structure of HCBd metabolites, to determine their site of formation, and by means of detailed toxicity studies, their relevance to the nephrotoxicity of hexachloro-1:3-butadiene.

METHODS

Materials. Hexachloro-1:3-butadiene (Spectrosol grade, >99% purity) was obtained from BDH Chemicals Ltd, Poole, Dorset, UK. [^{14}C]Hexachloro-1:3-butadiene, specific activity 10.1 mCi/mmol, was supplied by Physics and Radioisotope Services, Imperial Chemical Industries

PLC, Billingham, Cleveland, UK. The chemical and radiochemical purity which exceeded 99% was assessed by gas chromatography with radiochemical detection and by gas chromatography-mass spectrometry. All other chemicals and reagents were obtained from commercial sources at the highest purity available.

Synthesis of conjugates of hexachloro-1:3-butadiene. *N*-Acetyl-*S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine: Dry *N*-acetyl-L-cysteine (50 g, 0.3 mol) was added to a solution of sodium (0.6 mol) in methanol (1 liter). Hexachloro-1:3-butadiene (88 g, 0.33 mol) was added slowly with stirring over 30 min. After 90 min, the solution was evaporated to approximately 500 ml, 300 ml water was added, and the pH was adjusted to 5–6. Unchanged hexachloro-1:3-butadiene was removed by extracting four times with equal volumes of *n*-hexane. The aqueous solution was then adjusted to pH 1 with sulfuric acid and extracted 4 times with ethyl acetate. The ethyl acetate extracts were washed twice with water and evaporated to dryness. The residue was recrystallized from acetonitrile after treatment with decolorizing charcoal to yield 38 g of product; the melting point was 70°C. (Found C, 29.9; H, 2.6; N, 5.7; S, 7.7; Cl, 41.2%, $\text{C}_9\text{H}_5\text{O}_3\text{NSCl}_5$ requires C, 27.9; H, 2.1; N, 3.61; S, 8.3; Cl, 45.8%). The probe insertion mass spectrum of the product was consistent with the expected structure (Fig. 1).

S-(1,1,2,3,4-Pentachloro-1:3-butadienyl)-glutathione. This conjugate was prepared from hexachloro-1:3-butadiene and glutathione in the presence of sodium and liquid ammonia by a method analogous to that described by McKinney and Biester (1959). The crude product was

FIG. 1. Probe insertion mass spectrum of *N*-acetyl-*S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine.

recrystallized from ethanol/water to give a white crystalline material with a melting point of 186–187°C. (Found C, 32.0; H, 3.2; N, 7.7; S, 6.3; Cl, 33.1%, $C_{14}H_{16}O_6N_3SCl_5$ requires C, 31.6; H, 3.0; N, 7.9; S, 6.0; Cl, 33.4%). The field desorption mass spectrum was consistent with the expected structure (Fig. 2).

Animals. Male, Alderley Park (Wistar derived) rats, 170 to 200 g body weight, were housed in temperature (22°C) and humidity (50%) controlled rooms equipped with a 12-hr lighting cycle. Feed (PCD diet, Special Diet Services Ltd, Witham, Essex, UK) and water were provided *ad libitum* throughout all experiments unless otherwise stated.

Excretion studies. Six rats were dosed orally with [^{14}C]HCBD (200 mg/kg) as a solution in corn oil (5 ml/kg). Each dose contained 9.28 μ Ci of radiolabeled HCBD. The rats were housed singly in plastic metabolism cages designed for the separate collection of urine and feces. Excreta were collected frozen in dry ice at 24-hr intervals for 5 days and analyzed for radioactivity. Aliquots of urine (1 ml) were mixed with 10 ml of standard liquid scintillation fluid (Fisofluor, Fisons Ltd.) and assayed by liquid scintillation counting with a Packard Tri-Carb Model 460 CD spectrometer. Fecal samples were freeze dried and ground to a homogeneous powder; samples (100 mg) were quantified as $^{14}CO_2$ in a Packard tissue oxidizer and liquid scintillation spectrometer.

Additional studies were carried out with three rats with exteriorized bile flow. Following recovery from the surgical implantation of a biliary cannula, each rat was given a single po dose of [^{14}C]HCBD (200 mg/kg; 50 μ Ci) as a solution in corn oil (5 ml/kg). The rats were restrained for the collection of bile and urine (Bollman, 1948), but had access to pelleted laboratory diet and drinking water containing 1% w/v dextrose, 0.9% w/v sodium chloride, and 0.05% w/v potassium chloride. Bile and urine samples were collected at 24-hr intervals for 2 days. Aliquots of bile (0.1 ml) and urine (1 ml) were mixed with a standard scintillation fluid and assayed for radioactivity.

Distribution studies. The tissue distribution of radio-

activity was studied 2, 4, 8, and 16 hr after a single po dose of [^{14}C]HCBD (200 mg/kg; 42.6 μ Ci) as a solution in corn oil (5 ml/kg). A single rat was killed at each time point by exposure to excess halothane BP, and rapidly frozen in a hexane/solid CO_2 mixture. The frozen carcass was embedded in 2% w/v carboxymethylcellulose, and 20- μ m longitudinal sagittal sections cut with an LKB PMV type 450 MP cryomicrotome. Apposition autoradiograms were prepared from the freeze-dried sections (Ullberg, 1962).

Identification of biliary metabolites. A 24-hr bile sample from a rat dosed with [^{14}C]HCBD (200 mg/kg; 50 μ Ci) was freeze dried; the residue was dissolved in ethyl acetate:acetic acid:methanol:water (EAMW 60:15:15:10) and chromatographed on thin layer plates (Silica Gel GF) with the same solvent system (Fig. 6). Areas of radioactivity were located by a thin-layer radiochromatogram scanner (Berthold Model LB 2723). Preparative separation of larger amounts of bile was achieved by column chromatography with a Lobar size B Si 60 column (E. Merck, Darmstadt) with the same solvent system at a flow rate of 1 ml/min. The eluate was monitored with a radiochemical detector (Berthold Model LB 503) and collected in 2-ml fractions. Three bands of radioactivity were eluted with retention times of 92, 174, and 204 min. Fractions corresponding to each of these bands were pooled and concentrated under reduced pressure. The most polar fraction (RT 204 min) containing the major metabolite on thin layer chromatograms (R_f 0.46) was hydrolyzed overnight with 6 M HCl at 100°C. The hydrolysate was evaporated to dryness, and the methyl ester, *N*-trifluoroacetyl derivative was prepared with ethereal diazomethane and trifluoroacetic anhydride successively. The derivatized sample was analyzed by gas chromatography and by gas chromatography-mass spectrometry (GC-MS).

The material eluting from the column with a retention time of 174 min was analyzed by thin-layer chromatography alongside an authentic sample of *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-cysteinylglycine. Silica gel GF thin layer plates were used with either the previously described solvent system (EAMW) or a mixture of *n*-butanol:acetic acid:water (BAW 12:3:5 v/v). The standard was prepared by treating *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl) glutathione (2.5 mM) with γ -glutamyl transpeptidase (20 units) in Tris-HCl buffer (50 mM, pH 8.5) for 16 hr at 25°C. Spots on chromatograms were located under uv light, by spraying with ninhydrin solution or by radiochromatogram scanning.

Identification of urinary metabolites. Observations by Davis *et al.* (1980) and in this laboratory have shown a dose-dependent urinary excretion of HCBD metabolites. In order to optimize the recovery of radioactivity and mass from treated rats, both high and low doses of HCBD were used to generate metabolites. Twelve rats were dosed po with [^{14}C]HCBD (200 mg/kg; 50 μ Ci) and a further four rats at a dose level of 20 mg/kg; 50 μ Ci. Urine was collected for 24 hr (200 mg/kg) or 48 hr (20 mg/kg), and

FIG. 2. Field desorption mass spectrum of *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)glutathione.

the total urine samples were combined. Two volumes of ice-cold ethanol were added to the combined urine sample, and the precipitated protein was removed by centrifugation at 10,000g. The supernatant fraction was removed, evaporated under reduced pressure to approximately half volume, and extracted four times with equal volumes of diethyl ether at neutral pH. The aqueous fraction was then adjusted to pH 1 with 3 M HCl and extracted repeatedly with equal volumes of diethyl ether until no further radioactivity was extracted. The pH 1 ether extract was evaporated to dryness; the residue was dissolved in distilled water and chromatographed on a Lobar size B Lichroprep RP8 reverse phase column with methanol:water:formic acid (60:40:1 v/v) as the eluting solvent. Fractions corresponding to individual peaks of radioactivity were combined and evaporated to dryness under reduced pressure; their silyl derivatives were prepared with a mixture of BSTFA/1% TMCS in dry pyridine. Samples were analyzed by radiochemical gas chromatography and by high-resolution gas chromatography-mass spectrometry.

Gas chromatography. Radioactive metabolites were analyzed by gas chromatography with a Pye series 104 instrument equipped with flame ionization and radiochemical detection (ESI Nuclear 504 Radiogas Detector). The column effluent was split in the ratio of 10:1 between the radiochemical detector and the flame ionization detector. In each case the carrier gas was a mixture of Argon/CO₂ (95:5) at a flow rate of 30 ml/min. Biliary metabolites were analyzed on a 0.9 m × 2 mm glass column packed with 5.2% OV101 on 100–200 mesh Supelcoport, temperature programmed from 150°C (3 min) to 250°C at 10°C/min. Urinary metabolites were analyzed on a 1.5 m × 2 mm glass column packed with the same material and programmed from 100 to 300°C at 15°C/min. Mass peaks corresponding to radioactivity were identified and analyzed by GC-MS with the same columns and conditions, but with a helium carrier gas (20 ml/min).

Mass spectrometry. An LKB 2091 EI/CI gas chromatograph-mass spectrometer was used to obtain spectra of metabolites. Accurate mass measurement and field desorption spectra were obtained on a Jeol D300 GC-MS. This instrument was fitted with a 2.7 m × 2 mm glass column packed with 3% OV101 on Supelcoport (100–120 mesh) and programmed from 150 to 300°C at 15°C/min.

The toxicity of biliary HCBd metabolites and HCBd conjugates. Bile was collected from 13 rats fitted with biliary cannulae for 24 hr after a single po dose of [¹⁴C]HCBd (200 mg/kg). The bile samples were pooled, freeze dried, and reconstituted in a small volume of distilled water (10 ml). Control bile obtained from corn oil dosed rats was concentrated in the same way.

A group of three rats was dosed po with the reconstituted bile collected from [¹⁴C]HCBd-treated rats at a dose level equivalent to 107 mg/kg HCBd based on the radioactivity content of the bile. Further groups of three rats were dosed

po with an equivalent amount of control bile, with [¹⁴C]HCBd (100 mg/kg) in corn oil, or with corn oil vehicle alone (5 ml/kg). The rats were housed individually in metabolism cages for the separate collection of urine and feces. Twenty four hours after dosing, the rats were killed with halothane, and blood and the 24-hr urine samples were taken for biochemical analysis. Urine was also assayed for radioactivity. Kidneys and liver were removed for the measurement of organ body weight ratios and water content and for histopathological assessment. Portions of liver and kidney were fixed in 10% formol saline, and paraffin sections (5 µm) were cut and stained with hematoxylin and eosin.

The toxicity of equimolar doses of the *N*-acetyl cysteine and glutathione conjugates of HCBd (100 and 138 mg/kg, respectively) was assessed in groups of six rats following single po doses of the test compounds. A third group of six rats received po doses of HCBd (200 mg/kg). The conjugates of HCBd were found to be insoluble in corn oil, the vehicle chosen for the earlier studies with HCBd itself. It was considered preferable to dose these conjugates as solutions in polyethylene glycol 400 (PEG) or water rather than as suspensions in corn oil. When direct comparisons with HCBd were made, this administration was also dosed in PEG. The *N*-acetyl cysteine conjugate and HCBd were therefore dosed as solutions in polyethylene glycol 400 (5 ml/kg); the glutathione conjugate was dosed as the hydrochloride in aqueous solution. Control rats received polyethylene glycol alone. Twenty-four hour blood and urine samples and liver and kidneys were taken for the procedures described above.

The effect of biliary cannulation on HCBd toxicity. Two groups of rats, three per group, were fitted with exteriorized biliary cannulae as previously described. One group was dosed with HCBd (200 mg/kg) as a solution in polyethylene glycol 400 (5 ml/kg); the other group received the vehicle alone. A third group of three rats underwent part of the surgical procedure in that the abdominal cavity was opened under anesthesia and immediately closed again with sutures. A biliary cannula was not fitted to these rats; the bile duct was not disturbed and remained fully functional. These rats then received a single po dose of HCBd (200 mg/kg) in PEG 400. Twenty-four hour blood and urine samples were taken for biochemical analysis and liver and kidney for histopathology as described above.

Biochemical assays. Plasma urea, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and urinary glucose were all determined by commercially available methods (Smith Kline Instruments; Boehringer Corporation Limited) in conjunction with Vitatron Analyzers. Urinary protein was determined by a modification of the method of Pesce and Strande (1973). The activities of urinary alanine aminopeptidase and γ-glutamyl transferase were measured by the methods of Mondorf *et al.* (1978) and Szasz (1969), respectively. Urinary alkaline phosphatase was determined by the method of Fernley

and Walker (1965) and *N*-acetyl- β -D-glucosaminidase by the method of Leback and Walker (1961).

Tissue Distribution of Radioactivity

RESULTS

Excretion and Tissue Distribution

Over the 5-day period studied, the major route of elimination of a single po dose of 200 mg/kg of [14 C]HCBD was in the feces (39%), the bulk of the radioactivity being excreted on Days 3 to 5 (Fig. 3). Experiments with rats fitted with a biliary cannula demonstrated that 35% of the dose was excreted in bile during the first 48 hr (Fig. 3), suggesting that biliary metabolites accounted for most if not all of the radioactivity in feces. The fecal excretion over this period was low (5.4%) suggesting extensive reabsorption and enterohepatic recirculation of biliary metabolites. However fecal output on Days 1 and 2 was reduced, and this reduced output may contribute to the low excretion of radioactivity over this period. The excretion of radioactivity in urine was low, a maximum of 3.5% of the dose being eliminated in any 24-hr period over the 5 days (Fig. 3).

Autoradiograms of longitudinal sagittal sections taken sequentially through whole animals dosed po with [14 C]HCBD at 200 mg/kg and killed after various times were qualitatively similar at all time points and only the 4-hr whole body autoradiogram is presented as being representative (Fig. 4). Quantitation with a densitometer demonstrated low levels of radioactivity in the stomach, but higher levels in the small intestine indicated either rapid absorption of HCBD followed by excretion via the bile into the gut or passage of HCBD directly from the stomach to the intestine. Extraction of the gut and its contents with hexane at this time point indicated that both hexane (85%) and water (15%)-soluble forms of radioactivity were present suggesting that some metabolism had occurred but that most of the radioactivity was present as unchanged HCBD (85%). Similar experiments at 8 and 16 hr showed that by 16 hr absorption was virtually complete and that the radioactivity present in the gut at this time was mainly due to water-soluble metabolites. The auto-

FIG. 3. The daily excretion of radioactivity following a single po dose of [14 C]hexachloro-1:3-butadiene (200 mg/kg). The values shown are the means $\pm$ SD.

FIG. 4. Whole body autoradiogram of a longitudinal sagittal section showing distribution of radiolabel in the rat after a single po dose of [^{14}C]hexachloro-1:3-butadiene in corn oil (200 mg/kg; 250 $\mu\text{Ci/kg}$) and killed after 4 hr.

radiogram showed a definite intrarenal distribution of radioactivity, the outer medulla, the site of damage produced by HCBBD being particularly well labeled.

Biliary Metabolites

Preparative column chromatography of a freeze dried extract of bile fractionated the biliary metabolites into three bands, the most polar of which (RT 204 min) contained over half of the radioactivity (55%) in bile. Thin layer chromatograms of this band revealed the presence of a major component (40%) at R_f 0.46 which corresponded to synthesized *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl) glutathione, together with a minor component (15%) at R_f 0.64. Hydrolysis, methylation, and trifluoroacetylation of the major component gave a product which was identified by GC-MS as the *O*-methyl ester of *N*-trifluoroacetyl-*S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine. Figure 5 shows the electron impact mass spectrum together with the interpreted structure.

Incubation of the glutathione conjugate of hexachloro-1:3-butadiene with γ -glutamyl transpeptidase produced 2 ninhydrin-positive spots on thin-layer chromatograms. The spot at R_f 0.18 (BAW system) corresponded to glutamic acid while the second at R_f 0.60, which was uv absorbing, was assumed to be the cysteinyl glycine conjugate of HCBBD. When this conjugate was cochromatographed in two solvent systems (BAW and EAMW) with the column chromatography band with a retention time of 174 min, the cysteinyl glycine conjugate of HCBBD was shown to be the major component of this band, accounting for 12% of the radioactivity in bile. The remaining biliary metabolites (Fig. 6) accounting for approximately 45% of the radioactivity in bile have not been identified.

Urinary Metabolites

Ether extraction of pooled urine samples from [^{14}C]HCBBD dosed rats yielded 1.6% of the total urinary radioactivity at neutral pH and 29.5% at pH 1. When the pH 1 ether

FIG. 5. Electron impact mass spectrum of the *O*-methyl ester of *N*-trifluoroacetyl-(1,1,2,3,4-pentachloro-1;3-butadienyl)-L-cysteine obtained by hydrolysis and derivatization of the major biliary metabolite.

extract was chromatographed on a reversed phase column, three bands of radioactivity were isolated. Gas chromatography of the trimethylsilyl derivative of the least polar of these bands gave a single radiolabeled peak eluting at 240°C with a retention time of 9.4 min. This metabolite was subsequently identified by mass spectrometry as the trimethylsilyl derivative of 1,1,2,3,4-pentachloro-1:3-butadienyl sulphenic acid (Fig. 7) and accounted for 8.9% of the radioactivity in urine, 1% of the dose of HCB. The structure was confirmed by accurate mass measurement of the molecular and $M - Cl$ ions (Fig. 7). If this metabolite were not stabilized by esterification, it readily cyclized in the gas chromatograph to give tetrachlorothiophene, which was identified by comparison of its mass spectrum with that of an authentic sample. The identification of this cyclization product is consistent with the urinary metabolite being the sulphenic acid structure assigned from the high-resolution mass spectrum of its trimethylsilyl ester. The two remaining ether extractable bands representing 15.9 and 4.7% of the radioactivity in urine and the non-ether-soluble material (70%) are the subject of further investigation.

The Toxicity of HCB and HCB Conjugates

A single po dose of hexachloro-1:3-butadiene (100 to 200 mg/kg) produced marked changes in all of the parameters used to assess kidney damage 24 hr after dosing (Tables 1 and 2). Increases in plasma urea, urinary glucose, and protein indicated a significant reduction of glomerular filtration and tubular

FIG. 6. TLC radiochromatogram of 24-hr bile from rats given a single po dose of [^{14}C]hexachloro-1:3-butadiene (200 mg/kg). Solvent system ethyl acetate:acetic acid:methanol:water (60:15:15:10). Identified metabolites are X, *S*-(1,1,2,3,4-penta-chloro-1:3-butadienyl)-glutathione (R_f 0.46), and Y, *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-cysteinyl glycine (R_f 0.73).

FIG. 7. Electron impact mass spectrum of the trimethylsilyl derivative of 1,1,2,3,4-pentachloro-1:3-butadienyl sulphenic acid. Mass measurement of the M^+ and $M - Cl$ ions by high resolution mass spectrometry gave

Calculated Accurate Mass	Measured Mass	Deviation (ppm)
308.8896	308.8921	+8
343.8585	343.8551	-10

reabsorption (Table 2). The high urinary glucose levels which are believed not to be associated with high plasma glucose levels (Gradiski *et al.*, 1975) also indicated damage to the proximal tubule as did the marked increase in the enzymes alkaline phosphatase and γ -glutamyl transferase. Hexachloro-1:3-butadiene also produced a significant increase in kidney to body weight ratio (26%) which was associated with an increase in kidney water content (Table 1). There was no histopathological or biochemical evidence (Table 2) to suggest any hepatic damage.

A single po dose of bile containing radio-labeled HCBd metabolites also caused marked renal toxicity (Table 1). No increase in liver enzyme markers was seen, the only significant change was an increase in liver/body weight

ratio (Table 1). Control bile showed no signs of toxicity to either kidney or liver in these experiments. It was shown by thin-layer chromatography that the freeze drying and reconstitution in distilled water produced no qualitative or quantitative changes in the radioactivity content of the original bile samples. Rats given bile containing radioactive HCBd metabolites excreted 6% of the radioactivity in urine compared to 4.6% in rats given [^{14}C]HCBd (Table 1).

When the glutathione or *N*-acetyl cysteine conjugates of HCBd were dosed po to rats at equimolar doses of 138 and 100 mg/kg, respectively, a remarkably similar pattern of changes was seen in all of the biochemical markers (Table 2). Each parameter was affected with a clear quantitative correlation be-

TABLE 1

THE TOXICITY OF BILIARY METABOLITES OF HEXACHLORO-1:3-BUTADIENE ^{a,b}

Parameter	[¹⁴ C]HCBd bile (107 mg/kg equiv.)	Control bile ^c	HCBd (100 mg/kg)	Control (Corn oil)
Plasma urea (mg%)	161.7 ± 5**	65.5	79.3 ± 1.76	57.3 ± 4.2
Kidney water (g H ₂ O/g dry wt)	4.35 ± 0.01**	3.51	4.00 ± 0.1**	3.50 ± 0.1
Kidney: body wt (× 100)	0.45 ± 0.03**	0.37	0.49 ± 0.01**	0.39 ± 0.01
Plasma AST (U/liter)	69.0 ± 3.6	75.5	78.6 ± 20.6	74.0 ± 13.1
Plasma ALP (U/liter)	364.0 ± 98	195	220.7 ± 52	189.0 ± 24.0
Plasma ALT (U/liter)	7.3 ± 1.2	9.5	6.7 ± 2.5	6.7 ± 3.1
Liver water (g H ₂ O/g dry wt)	2.41 ± 0.03	2.32	2.40 ± 0.04	2.31 ± 0.04
Liver: body wt (×100)	3.7 ± 0.1**	3.1	3.95 ^c	3.03 ± 0.18
Radioactivity in urine (% dose/24 hr)	6.2 ± 1.5	—	4.6 ± 0.1	—

^a AST, Aspartate aminotransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase.^b Rats were dosed po with ¹⁴C bile (equivalent to 107 mg/kg HCBd) collected from rats dosed po with [¹⁴C]HCBd (200 mg/kg). Further groups of rats were dosed with an equivalent amount of control bile, with [¹⁴C]HCBd (100 mg/kg) in corn oil or with corn oil vehicle alone (5 ml/kg). Results are means ± SD (*n* = 3).^c *n* = 2.** Significantly different from control animals given corn oil alone, *p* < 0.001 (*t* test).

tween the conjugates and HCBd itself, suggesting that hepatic glutathione conjugates of HCBd were involved in HCBd-mediated nephrotoxicity. The conjugates were also similar to HCBd in that they showed no histopathological or biochemical evidence of liver

TABLE 2

THE TOXICITY OF SYNTHESIZED HEXACHLORO-1:3-BUTADIENE CONJUGATES ^{a,b}

Parameter	Control ^c	HCBd (200 mg/kg)	Glutathione HCBd conjugate (138 mg/kg)	<i>N</i> -Acetyl cysteine-HCBd conjugate (100 mg/kg)
Plasma urea (mg%)	51 ± 15	108 ± 23**	95 ± 11**	147 ± 23**
Plasma ALP (U/liter)	303 ± 112	383 ± 92	406 ± 65	394 ± 46
Plasma ALT (U/liter)	12 ± 2	6 ± 1**	7 ± 0.5**	7 ± 2*
Plasma AST (U/liter)	39 ± 8	47 ± 11	36 ± 2	39 ± 10
Urine glucose (mg/24 hr)	2.1 ± 0.4	51.5 ± 11.2**	48.8 ± 14.7**	33.9 ± 18.8*
Urine protein (mg/24 hr)	9.4 ± 5.2	62.1 ± 17.6*	37.3 ± 6.4**	71.8 ± 19.2**
Urine ALP (μmol/hr/24 hr)	0.7 ± 0.25	17.4 ± 7.76*	17.4 ± 5.1**	12.6 ± 6.9*
Urine NAG (μmol/hr/24 hr)	1.9 ± 0.47	7.56 ± 1.87**	4.0 ± 0.7**	7.77 ± 1.08**
Urine GGT (μmol/hr/24 hr)	647 ± 377	12209 ± 4787*	15965 ± 5105**	17869 ± 6879**
Urine AAP (μmol/hr/24 hr)	33 ± 18	330 ± 91.4**	327 ± 88**	422 ± 84**

^a ALP, Alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NAG, *N*-acetyl-β-D-glucosaminidase; GGT, γ-glutamyltransferase; AAP, alanine aminopeptidase.^b Rats were dosed po with equimolar doses of *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione (138 mg/kg) or *N*-acetyl-*S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine (100 mg/kg). A further group was dosed HCBd (200 mg/kg) in polyethylene glycol 400 as positive control.^c The control group received polyethylene glycol 400. Results are means ± SD (*n* = 6).* *p* < 0.01 (*t* test).** Significantly different from control, *p* < 0.001.

damage. Histopathological examination of the kidneys from these rats confirmed that the conjugates of HCBd caused an identical lesion to that already described for HCBd itself (Ishmael *et al.*, 1982).

Complete protection from HCBd nephrotoxicity was observed in rats that had been fitted with a biliary cannula before being given a single po dose of 200 mg/kg HCBd (Table 3). Each of the parameters measured was comparable to control values from cannulated rats given the vehicle alone. Because of the surgical procedures involved, only a limited number of animals were used in this study and statistical evaluation of the results was not meaningful. However, the marked differences in glucose, protein, GGT, and AAP levels (Table 3) between cannulated and non-cannulated HCBd treated rats clearly indicate that cannulation and loss of bile prevents HCBd-mediated nephrotoxicity. Although the enzymes, AST, ALP, and ALT, used to detect liver toxicity were unchanged, the surgical procedure for implanting the biliary cannula resulted in abnormally high levels of plasma urea in rats given the vehicle alone and precluded the use of this marker in assessing kidney damage. The third group of rats which had undergone part of the surgical procedure but had been left with the bile duct intact displayed typical kidney damage when

given a single po dose of HCBd (Table 3). No evidence was found for the dosing vehicle having an effect on any of the parameters measured in these studies (Tables 1, 2, and 3).

DISCUSSION

The organ selectivity of HCBd suggests that metabolism may be a prerequisite for the nephrotoxicity of this chemical. A direct interaction between HCBd and target tissue would be less selective and hence unlikely to account for the organ and site specificity of the damage (Ishmael *et al.*, 1982). In common with most xenobiotics, the principal site of metabolism of HCBd would be expected to be the liver rather than the kidney. One of the objectives of this study was to investigate whether hepatic metabolites of HCBd had a role in kidney damage or whether unique metabolism of HCBd in the kidney was the critical event.

It has been known for several years that HCBd causes a depletion of hepatic glutathione levels in the rat (Lock and Ishmael, 1981). More recent *in vitro* studies in this laboratory have shown this depletion to be a result of direct conjugation of HCBd with glutathione mediated primarily by a microsomal glutathione transferase (Wolf *et al.*, 1984), an observation now confirmed *in vivo*. The same

TABLE 3

THE EFFECT OF BILIARY CANNULATION ON THE TOXICITY OF HEXACHLORO-1:3-BUTADIENE IN THE RAT^a

Parameter	Cannulated + HCBd (200 mg/kg)	Cannulated + PEG 400	HCBd (200 mg/kg)
Plasma ALP (U/liter)	275 ± 73	264/437	342 ± 13
Plasma ALT (U/liter)	8 ± 3	7/11	8 ± 3
Plasma AST (U/liter)	67 ± 17	86/116	57 ± 6
Urine glucose (mg/24 hr)	1.0 ± 0.3	0.8 ± 0.3	25.4 ± 13.1
Urine protein (mg/24 hr)	2.4 ± 1.7	3.3 ± 3.2	27.7 ± 16.7
Urine GGT (μmol/hr/24 hr)	688 ± 272	1139 ± 909	8104 ± 6584
Urine AAP (μmol/hr/24 hr)	49 ± 17	37/80	109/187

^a Rats (*n* = 3) were biliary cannulated and given a single po dose of HCBd (200 mg/kg). Control rats were cannulated and dosed with the vehicle alone (polyethylene glycol 400). A third group were not cannulated but were given HCBd (200 mg/kg) as a positive control. Results are means ± SD or when *n* = 2 both values are given.

study failed to find any evidence for cytochrome *P*-450-dependent metabolism, the principal route for structurally related chlorinated ethylenes (Henschler and Greim, 1976). The identification of a glutathione conjugate *in vitro* (Wolf *et al.*, 1984) and the known high biliary excretion of HCBd metabolites (Davis *et al.*, 1980) suggested that the po route of administration was the most appropriate for this current study. The use of this route when dosing chemically synthesized biliary metabolites more closely represents the way in which biliary metabolites are introduced into the small intestine *in vivo*, than does the ip route used by other workers (Davis *et al.*, 1980; Lock and Ishmael, 1979, 1981). Intraperitoneal injection is also unsuitable for whole body autoradiography studies and hence the po route was used throughout.

In the present study, hepatic metabolites of HCBd are excreted mainly in the bile, 17% of the dose being excreted on each of the first 2 days. In the rat, biliary excretion is usually associated with the formation of polar conjugates with a molecular weight greater than 300 (Hirom *et al.*, 1976) and would be the preferred route of elimination of the glutathione conjugate of HCBd (molecular weight > 500). This conjugate and its cysteinyl glycine derivative accounted for over 50% of the radioactivity in bile.

A comparison of biliary excretion of radioactivity over 2 days with that in feces over 5 days (Fig. 3) suggests extensive reabsorption of biliary metabolites and enterohepatic recirculation. Reabsorption frequently occurs as a result of hydrolysis or partial degradation of conjugates by enzymes in the bile duct or small intestine. Evidence for partial degradation has been obtained by the identification of the cysteinyl-glycine conjugate of HCBd in rat bile. This metabolite which accounts for 12% of the radioactivity in bile is possibly produced by the action of γ -glutamyl transferase of pancreatic origin as demonstrated for methyl mercury glutathione by Hirata and Takahashi (1981), or by the action of this enzyme in the liver.

Degradation of the glutathione conjugate of HCBd to either the cysteinylglycine or cysteine conjugate will markedly increase the lipophilicity of the molecule, thus favoring reabsorption. A significant quantity of reabsorbed metabolites particularly of reduced molecular weight may be excreted via the bloodstream and the kidneys. Experiments in this study support this view, and in addition to demonstrating that biliary metabolites are reabsorbed and passed to the kidney, it has also been demonstrated that such metabolites are toxic to the kidney. In these experiments bile collected from HCBd-treated rats was dosed to a second group of rats. Six percent of the dose was excreted in urine in the first 24 hr and the rats showed marked kidney damage in comparison to rats dosed with control bile. These experiments established a link between hepatic metabolism, biliary metabolites, and kidney damage. Subsequently, when either the glutathione conjugate or its mercapturate derivative was dosed to rats, they each produced an identical pattern of kidney damage to that found for HCBd (Table 2). Thus it appears that the glutathione conjugate of HCBd or one of its degradation products excreted in bile and reabsorbed from the gut is responsible for the renal toxicity of HCBd. This thought was confirmed when biliary-cannulated rats dosed with HCBd were completely protected from kidney damage, an observation essential in establishing the role of hepatic metabolism in the nephrotoxicity of this compound. Although kidneys possess all of the enzymes necessary to synthesise these conjugates *in situ* (Chasseaud, 1973), this study has established that this action does not occur, at least not to an extent that leads to kidney damage. Thus, although only just over 50% of hepatic biliary metabolites have been identified, evidence has been obtained to support the findings of Wolf *et al.* (1984) that the principal metabolic pathway for HCBd is by direct conjugation with glutathione.

Reabsorption of the glutathione conjugate of HCBd from the gut is more likely to occur after degradation to the lower molecular

weight, more lipophilic, cysteinylglycine, or cysteine conjugates. However, irrespective of the exact nature of the reabsorbed metabolites, the kidney has the potential to degrade all of them to the cysteine conjugate by means of the enzymes γ -glutamyl transferase and cysteinylglycinase present in the brush border (Hutson, 1976). Following *N*-acetylation in renal tubular cells, the mercapturic acids are then excreted in urine. Work with the renal toxin *S*-(1,2-dichlorovinyl) cysteine (DCVC) has led to the recognition of an additional enzyme C-S or β -lyase, which metabolizes cysteine conjugates of several halo-alkenes (Anderson and Schultze, 1965a; Bonhaus and Gandolfi, 1981). The enzyme will cleave cysteine but not glutathione or *N*-acetyl cysteine conjugates to form ammonia, pyruvate, and a reactive fragment containing sulfur which is believed to be a thiol (Anderson and Schultze, 1965b). Because of the substrate specificity of β -lyase, we presume that in our experiments the glutathione and *N*-acetyl cysteine conjugates of HCBd are metabolized to the free cysteine conjugate in order to become substrates for this enzyme.

Evidence has been obtained to suggest that

the reaction of the thiol fragment derived from DCVC with dithiols and sulfhydryl groups in proteins, and with glutathione itself, may lead to renal toxicity (Anderson and Schultze, 1965a; Stonard and Parker, 1971; Stonard, 1973).

A scheme for the hepatic conjugation and renal activation of HCBd is shown in Fig. 8. The structure of the cysteine conjugate of HCBd is analogous to that of DCVC suggesting a similar mechanism of action. Furthermore dichlorovinyl cysteine and HCBd (or its conjugates) both produce a lesion to the pars recta of the proximal tubule in the rat (Terracini and Parker, 1965; Ishmael *et al.*, 1982), an area of the renal tubule which contains high concentrations of peptidase and cysteinyl glycinase enzymes (Hughey *et al.*, 1978) and presumably β -lyase. We have identified a urinary metabolite which is consistent with cleavage of a cysteine-HCBd conjugate by β -lyase. This metabolite, 1,1,2,3,4-pentachloro-1:3-butadienyl sulphenic acid (Fig. 8) is the first evidence to be presented for this type of cleavage occurring *in vivo*. Oxidation of the sulfur atom of this metabolite may have occurred prior to cleavage since cysteine sulf-

FIG. 8. Proposed mechanism for hexachloro-1:3-butadiene elicited nephrotoxicity.

oxide conjugates are also known to be substrates for β -lyase (Anderson and Schultze, 1965b). Alternatively oxidation of a thiol to a sulphenic acid and subsequently to sulphinic and sulphonic acids is likely to occur and may constitute a detoxification pathway for this type of thiol. Compounds of this type are also known to cyclize to thiophenes and to dimerize to thiosulfinates (Spare and Virtanen, 1963). During the identification of the sulphenic acid metabolite of HCB, it was found that unless this metabolite was stabilized by ester formation, it readily cyclized to give tetrachlorothiophene. Similar reactions occurring in the kidney would give products insoluble in water which would readily be reabsorbed. This type of reaction may partly account for the proliferation of HCB metabolites found while there is apparently only a single metabolic event occurring in the liver. The sulphenic acid metabolite of HCB is unusual in that it is one of very few stable sulphenic acids known to exist, anthraquinone-1-sulphenic acid and purine sulphenic acids being the other examples (Kuhle, 1973; Abraham and Jardine, 1982). A similar although more transient sulphenic acid has been identified in plants following the action of β -lyase on *S*-(prop-1-enyl) cysteine sulfoxide (Spare and Virtanen, 1963).

We suggest that thiols generated by cleavage of cysteine conjugates by β -lyase could account for the stable thiol metabolites found for chemicals such as hexachlorobenzene (Koss *et al.*, 1976). Subsequent methylation of thiols by hepatic methyl transferases may be related to the formation of the *S*-methyl metabolites that have been found for a wide range of chemicals (Miller, 1970; Tateishi and Shimizu, 1976; Jenner and Testa, 1978).

It is concluded from this study that depletion of glutathione in the liver is not a response to the formation of a reactive metabolite of HCB but results from a simple conjugation reaction that is not associated with hepatotoxicity. It is only when this conjugate is degraded to its cysteine equivalent that it becomes a substrate for renal β -lyase and a toxic

metabolite is formed (Fig. 8). Although this enzyme is widely distributed in the rat, the highest concentrations are found in the kidney (Tateishi *et al.*, 1978; Tateishi and Shimizu, 1981), the principal organ responsible for the formation and excretion of cysteine conjugates and hence the target organ for these chemicals.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. C. Rhodes for his help with the biliary toxicity experiment, Dr. M. D. Stonard and Mr. C. W. Gore for the biochemical assays, Mr. W. Laird for his assistance with the mass spectrometry, and Dr. J. Ishmael for histopathological assessment of the livers and kidneys. Mr. D. Catlow of Imperial Chemical Industries PLC, Pharmaceuticals Division, kindly provided the field desorption mass spectrum of the glutathione conjugate.

REFERENCES

- ABRAHAM, R. T., AND JARDINE, I. (1982). Purine-6-sulphenic acid. Eighth European Workshop on Drug Metabolism. Abstract 301.
- ANDERSON, P. M., AND SCHULTZE, M. O. (1965a). Interaction of *S*-(1,2-dichlorovinyl)-L-cysteine with proteins. *Arch. Biochem. Biophys.* **109**, 615-621.
- ANDERSON, P. M., AND SCHULTZE, M. O. (1965b). Cleavage of *S*-(1,2-dichlorovinyl)-L-cysteine by an enzyme of bovine origin. *Arch. Biochem. Biophys.* **111**, 593-602.
- BOLLMAN, J. L. (1948). A cage which limits the activity of rats. *J. Lab. Clin. Med.* **33**, 1348.
- BONHAUS, D. W., AND GANDOLFI, A. J. (1981). Conjugation and bioactivation of chlorotrifluoroethylene. *Life Sci.* **29**, 2399-2405.
- CHASSEAUD, L. F. (1973). Distribution of enzymes that catalyze reactions of glutathione with alpha-beta unsaturated compounds. *Biochem. J.* **131**, 765-769.
- DAVIS, M. E., BERNDT, W. O., AND MEHENDALE, H. M. (1980). Disposition and nephrotoxicity of hexachloro-1:3-butadiene. *Toxicology* **16**, 179-191.
- FERNLEY, H. N., AND WALKER, P. G. (1965). Kinetic behaviour of calf-intestinal alkaline phosphatase with 4-methylumbelliferyl phosphate. *Biochem. J.* **97**, 95-103.
- GRADISKI, D., DUPRAT, P., MAGADUR, J. L., AND FAYEIN, E. (1975). Etude toxicologique experimentale de l'hexachlorobutadiene. *Eur. J. Toxicol.* **8**, 180-187.
- HENSCHLER, D., AND GREIM, H. (1976). Carcinogenic potential of chlorinated ethylenes, tentative molecular rules. *Inserm Symp. Ser.* **52**, 171-176.

- HIRATA, E., AND TAKAHASHI, H. (1981). Degradation of methyl mercury glutathione by the pancreatic enzymes in bile. *Toxicol. Appl. Pharmacol.* **58**, 483-491.
- HIROM, P. C., MILLBURN, P., AND SMITH, R. L. (1976). Bile and urine as complementary pathways for the excretion of foreign organic compounds. *Xenobiotica* **6**, 55-64.
- HOOKE, J. B., ROSE, M. S., AND LOCK, E. A. (1982). The nephrotoxicity of hexachloro-1:3-butadiene in the rat: studies of organic anion and cation transport in renal slices and the effect of monooxygenase inducers. *Toxicol. Appl. Pharmacol.* **65**, 373-382.
- HUGHEY, R. P., RANKIN, B. B., EKE, J. S., AND CURTHOYS, N. P. (1978). Specificity of a particulate rat renal peptidase and its localisation along with other enzymes of mercapturic acid synthesis. *Arch. Biochem. Biophys.* **186**, 211-217.
- HUTSON, D. H. (1976). Glutathione conjugates. In *Bound and Conjugated Pesticide Residues. ACS Symp. Ser.* **29**, 103-131.
- I.A.R.C. (1979). IARC Monographs on the evaluation of carcinogenic risk of chemicals to man, 20. Some halogenated hydrocarbons, Lyon, 179-193.
- ISHMAEL, J., PRATT, I. S., AND LOCK, E. A. (1982). Necrosis of the pars recta (S3 segment) of rat kidney produced by hexachloro-1:3-butadiene. *J. Pathol.* **138**, 99-113.
- JENNER, P., AND TESTA, B. (1978). Review article. Novel pathways in drug metabolism. *Xenobiotica* **8**, 1-25.
- KOCIBA, R. J., SCHWETZ, B. A., KEYES, D. G., JERSEY, G. C., BALLARD, J. J., DITTENBER, D. A., QUAST, J. F., WADE, C. E., AND HUMISTON, C. G. (1977). Chronic toxicity and reproduction studies of hexachlorobutadiene in rats. *Environ. Health. Perspect.* **21**, 49-53.
- KOSS, G., KORANSKY, W., AND STEINBACH, K. (1976). Studies on the toxicology of hexachlorobenzene. II. Identification and determination of metabolites. *Arch. Toxicol.* **35**, 107-114.
- KUHLE, E. (1973). Reactions of sulphenyl halides. In *The Chemistry of the Sulphenic Acids*, p. 43. Georg Thieme, Stuttgart.
- LEABACK, D. H., AND WALKER, P. G. (1961). Studies on glucosaminidase. 4. The fluorimetric assay of *N*-acetyl- β -glucosaminidase. *Biochem. J.* **78**, 151-156.
- LOCK, E. A., AND ISHMAEL, J. (1979). The acute toxic effects of hexachloro-1:3-butadiene on the rat kidney. *Arch. Toxicol.* **43**, 47-57.
- LOCK, E. A., AND ISHMAEL, J. (1981). Hepatic and renal non-protein sulphhydryl concentration following toxic doses of hexachloro-1:3-butadiene in the rat: The effect of Aroclor 1254, phenobarbitone and SKF525A treatment. *Toxicol. Appl. Pharmacol.* **57**, 79-87.
- MCKINNEY, L. L., AND BIESTER, H. E. (1959). Possible toxic factor of trichloroethylene extracted soybean oil meal. *J. Amer. Chem. Soc.* **81**, 909-915.
- MILLER, J. A. (1970). Carcinogenesis by chemicals: An overview. *Cancer Res.* **30**, 559-576.
- MONDORF, A. W., BREIRER, J., HONDUS, J., SCHERBERICH, J. E., MACKENRODT, G., SHAH, P. M., STILLE, W., AND SHOEPPE, W. (1978). Effect of aminoglycosides on proximal tubular membranes of the human kidney. *Eur. J. Clin. Pharmacol.* **13**, 133-142.
- PESCE, M. A., AND STRANDE, C. S. (1973). New micro-method for the determination of protein in cerebrospinal fluid and urine. *Clin. Chem.* **19**, 1265-1267.
- SPARE, C. G., AND VIRTANEN, A. I. (1963). On the lachrymatory factor in onion (*Allium Cepa*) vapours and its precursor. *Acta Chem. Scand.* **17**, 641-650.
- STONARD, M. D. (1973). Further studies on the site and mechanism of action of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2-dichlorovinyl)-3-mercaptopropionic acid in rat liver. *Biochem. Pharmacol.* **22**, 1329-1335.
- STONARD, M. D., AND PARKER, V. H. (1971). Metabolism of *S*-(1,2-dichlorovinyl)-L-cysteine by rat liver mitochondria. *Biochem. Pharmacol.* **20**, 2428-2437.
- SZASZ, G. (1969). A kinetic photometric method for serum γ -glutamyl transpeptidase. *Clin. Chem.* **15**, 124-136.
- TATEISHI, M., AND SHIMIZU, H. (1976). A new metabolic pathway of bromazepam involving attachment of a methylthio group. *Xenobiotica* **6**, 431-439.
- TATEISHI, M., AND SHIMIZU, H. (1981). Cysteine conjugate β -lyase. *Methods Enzymol.* **77**, 253-256.
- TATEISHI, M., SUZUKI, S., AND SHIMIZU, H. (1978). Cysteine conjugate β -lyase in rat liver. *J. Biol. Chem.* **253**, 8854-8859.
- TERRACINI, B., AND PARKER, V. H. (1965). A pathological study on the toxicity of *S*-(1,2-dichlorovinyl)-L-cysteine. *Food Cosmet. Toxicol.* **3**, 67-74.
- ULLBERG, S. (1962). Autoradiographic localisation in the tissues of drugs and metabolites. *Biochem. Pharmacol.* **9**, 29-37.
- WOLF, C. R., BERRY, P. N., NASH, J. A., GREEN, T., AND LOCK, E. A. (1984). The role of microsomal and cytosolic glutathione-S-transferases in the conjugation of hexachloro-1:3-butadiene and its possible relevance to toxicity. *J. Pharmacol. Exp. Ther.* **228**, 202-208.

Role of Microsomal and Cytosolic Glutathione S-Transferases in the Conjugation of Hexachloro-1:3-Butadiene and Its Possible Relevance to Toxicity

C. ROLAND WOLF,¹ PAMELA N. BERRY, JOHN A. NASH, TREVOR GREEN and EDWARD A. LOCK

Biochemical Toxicology Section, Central Toxicology Laboratory, Imperial Chemical Industries PLC, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK

Accepted for publication September 30, 1983

ABSTRACT

The mechanism of hexachloro-1:3-butadiene (HCBd)-induced glutathione depletion in male and female rats has been investigated in rat liver and kidney preparations *in vitro* in order to characterize the enzymes involved and to study the relationship between this effect and the nephrotoxic action of this compound. HCBd caused a marked reduction in glutathione concentration when incubated with male or female hepatic microsomal or cytosolic fractions fortified with glutathione. In contrast with that reported for other halo-olefins, the depletion of glutathione in the microsomal fraction is not related to the formation of metabolites via cytochrome P-450. The microsomal rate of depletion appeared to be due to a direct reaction catalyzed by a microsomal glutathione S-transferase. A glutathione adduct of HCBd was isolated by thin-layer chromatography and mass spectral

analysis strongly indicates the structure to be as S-(1,1,2,3,4-pentachloro-1,3-butadienyl)glutathione, confirming a direct substitution reaction without prior oxidation. This conjugate was formed at a faster rate by the hepatic microsomal fraction than by the cytosolic fraction suggesting a major role for the microsomal glutathione S-transferases in the disposition of this compound. A second more polar glutathione-dependent adduct which may be a double conjugate was formed with cytosol. Glutathione adducts were also formed by male and female kidney cytosol and microsomal fractions but at a slower rate than in liver fractions. It is suggested that the glutathione conjugate of HCBd may be converted to the cysteine derivative, the structure of which is similar to that of S-dichloro-vinyl-L-cysteine and therefore may be nephrotoxic by a similar mechanism.

It has been known for some time that glutathione plays an important role in the elimination of potentially toxic foreign compounds (Chasseaud, 1979). Many exogenous chemicals are known to be metabolized by the cytochrome P-450-dependent monooxygenase system (present predominantly in the liver, but also in other organs, *e.g.*, kidney and lung) to electrophilic products, *e.g.*, epoxides (Jollow *et al.*, 1977) and these potentially toxic products then either react with glutathione directly or are conjugated via a reaction catalyzed by the glutathione S-transferases. Such reactions represent a detoxification pathway. As a consequence of these studies, any observed depletion of tissue glutathione after the administration of a toxic compound has been taken as indicative of the formation of potentially toxic metabolites (Mitchell *et al.*, 1976). Although there are possible alternative explanations for a decrease in tissue glutathione concentration, the correlation between glutathione depletion and toxic metabolite formation for many compounds has been reasonably good (Mitchell *et al.*, 1976).

HCBd is a relatively potent nephrotoxin in rodents (Lock and Ishmael, 1979; Berndt and Mehendale, 1979) but has little effect on the liver (Lock *et al.*, 1982b). It is therefore interesting that a marked depletion of hepatic glutathione is observed in male rats after HCBd administration (Lock and Ishmael, 1981), whereas in the kidney no depletion can be measured. In contrast, in female animals which are more susceptible to the nephrotoxic effects of this compound (Hook *et al.*, 1983) the depletion of hepatic glutathione is not as marked in the liver, but significant depletion in the kidney can be measured. Depletion of hepatic glutathione with diethylmaleate before HCBd administration did not produce any hepatotoxicity (Hook *et al.*, 1982). Therefore, there seemed to be no direct correlation between glutathione depletion and toxicity. We have investigated 1) *in vitro* whether HCBd-mediated glutathione depletion is due to the formation of reactive, potentially toxic metabolites and 2) the relative roles of cytochrome P-450 and the glutathione S-transferases in this reaction. The relationship between these reactions and the mechanism of HCBd-induced nephrotoxicity is discussed. Preliminary observations on this work have been reported by Wolf *et al.* (1982) and Lock *et al.* (1982a).

Received for publication May 2, 1983.

¹ Present address: Imperial Cancer Research Fund, Medical Oncology Unit, Western General Hospital, Edinburgh, EH4 2XU, Scotland, UK.

ABBREVIATIONS: HCBd, hexachloro-1:3-butadiene; av, avoidupois.

Materials and Methods

Materials. HCBd (spectroscopic grade > 99% pure), 1-chloro-2,4-dinitrobenzene and 5,5'-dithiobis(2-nitrobenzoic acid) were purchased from BDH Chemicals Ltd. (Poole, Dorset, UK). Glutathione ($[^3\text{H}]$ glycine labeled), specific activity 1 mCi/mmol, was purchased from New England Nuclear (Boston, MA). HCBd, uniformly $[^{14}\text{C}]$ -labeled was synthesized by Physics and Radioisotope Services, Imperial Chemical Industries PLC (Billingham, UK). The specific activity was 9.9 mCi/mmol and the radiochemical and chemical purity > 99%. All other reagents were obtained from commercial sources and were of the highest purity available.

Preparation of hepatic and renal microsomal and cytosolic fractions. Hepatic and renal cytosolic and microsomal fractions were prepared from male or female Alderley Park (Wistar-derived) rats of 170 to 210 g b.wt. which had been fasted for 24 hr before use. The rats were killed by cervical dislocation, tissues were removed and a 25% w/v homogenate made in ice-cold 0.154 M KCl (adjusted to pH 7.4 with 0.1 M phosphate buffer) using a Potter type homogenizer. The homogenate was then centrifuged at $11,000 \times g$ (av) for 20 min at 4°C, the supernatant was transferred to fresh tubes and centrifuged at $160,000 \times g$ (av) for 40 min at 4°C. The fatty layer on the top of the samples was discarded and the supernatant (cytosolic fraction) removed and stored at -70°C. The microsomal pellet was washed once by resuspending in ice-cold 0.154 M KCl and then centrifuged at $80,000 \times g$ (av) for 30 min. Finally the pellet was resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 5.4 mM EDTA at a protein concentration of 20 to 30 mg/ml. Samples were either used immediately or for some experiments stored at -70°C for up to 2 weeks. Protein concentrations were determined by the method of Lowry *et al.* (1951). A sample of hepatic or renal cytosol from each rat was dialysed overnight at 4°C against 0.1 M phosphate buffer (pH 7.4), to reduce the glutathione concentration to < 0.1 mM.

Determination of glutathione depletion. The interaction of HCBd with glutathione was assessed by measuring the loss of sulphhydryl group reactivity with 5,5'-dithiobis(2-nitrobenzoic acid) by the following procedure. Incubations were carried out in 0.1 M phosphate buffer (pH 7.4) containing hepatic microsomes (1–2 mg of protein per ml) or dialysed cytosol (5–9 mg of protein per ml), reduced glutathione (10 mM), glutathione reductase (1.2 U/ml) and for some experiments a NADPH generating system (NADP, 6 mM; MgCl_2 , 12 mM; glucose-6-phosphate, 7.5 mM and glucose-6-phosphate dehydrogenase, 1 U/ml). The final incubation volume was 10 ml. The depletion of glutathione was followed after the addition of $[^{14}\text{C}]$ HCBd (2.8 mM and 1 $\mu\text{Ci}/\text{ml}$) as a solution in ethanol (0.05 ml). Incubations were carried out at 37°C in air and samples were removed (0.3 ml) at 30, 60, 90 and 120 min. The reaction was stopped by the addition of ice-cold 0.6 M trichloroacetic acid (0.3 ml). Glutathione concentration was determined on the protein-free solution by the method of Sedlak and Lindsay (1968). For some experiments the enzyme concentration (microsomal protein) and substrate concentration(s) (HCBd or glutathione) were varied to determine the kinetics of the reaction.

Measurement of glutathione conjugate formation. Liver glutathione conjugate formation was determined on the samples obtained from the glutathione depletion studies. Kidney glutathione conjugate formation was determined by incubating microsomal or dialysed cytosolic fractions at a protein concentration of 0.9 to 1.5 or 2.2 to 2.7 mg/ml, respectively, under the conditions described for measuring hepatic glutathione depletion. At the end of 2 hr incubation samples were quickly cooled in ice-cold water and the remaining unchanged HCBd removed by extraction with diethyl ether (6×10 ml). The aqueous phase was then freeze dried and the microsomal fraction reformed in 1 ml of water and the cytosolic fraction in 1.2 ml. Aliquots of the clear supernatant (40–60 μl) were then applied to silica gel GF thin-layer chromatography plates (20 $\times$ 5 cm) and the plates developed in *n*-butanol-acetic acid-water mixture (12:3:5). Areas of radioactivity were determined quantitatively using a Berthold model II thin-layer chromatograph scanner linked to a PET Commodore computer. For some

hepatic experiments 1-chloro-2,4-dinitrobenzene (1 mM), a competitive substrate for glutathione transferases, was present as a solution in ethanol at a final concentration of 0.5% v/v. In other experiments, $[^3\text{H}]$ glutathione (1 $\mu\text{Ci}/\text{ml}$) was used to replace the radiolabeled HCBd to help identification of the conjugate. The dependence of the reaction on cytochrome P-450 and molecular oxygen was measured in samples gassed with CO-O_2 (80:20) or nitrogen for 1 min before HCBd addition, these latter experiments were done in stoppered tubes.

Identification of the glutathione conjugate. To enable isolation and identification of the HCBd metabolite the incubation conditions were modified as follows: HCBd (5 mM), reduced glutathione (4 mM), $[^{14}\text{C}]$ HCBd (10 μCi) and microsomal protein (7.5 mg/ml). The total incubation volume was 40 ml and the incubations were carried out for 75 min at 37°C. At the end of the incubation unchanged HCBd was removed by extracting 3 times with hexane (40 ml). Two volumes of ice-cold ethanol were then added to the aqueous fractions and the precipitated microsomal protein removed by centrifugation at $10,000 \times g$ for 30 min. The supernatant was decanted and evaporated under reduced pressure at 25°C to a volume of 0.5 ml. This concentrate was then chromatographed on silica gel GF thin-layer chromatography plates using *n*-butanol-acetic acid-water (12:3:5 v/v) as the developing solvent. The major radioactive band (R_f 0.33) was removed and eluted from the silica with water. The eluate was again concentrated (1 ml) and hydrolyzed with 6 M HCl at 100°C for 20 hr. Under these conditions complete hydrolysis of the material with R_f 0.33 occurred to give a single radioactive product R_f 0.57 in the same system. The hydrolyzate was evaporated to dryness and methylated with an ethereal solution of diazomethane. The methylated sample was further chromatographed on thin-layer plates (silica gel GF) using chloroform-methanol (9:1 v/v) as the solvent system. The major radioactive band R_f 0.75 which was strongly UV absorbing and ninhydrin positive was eluted with ether, concentrated and analyzed by gas chromatography. The gas chromatograph (Pye Series 104) was fitted with flame ionization and radiochemical detection (ESI Nuclear 504 Radiogas detector). The effluent from the 5 foot $\times$ 2 mm glass column which was packed with 5.2% OV101 on 100 to 120 mesh Supelcoport was split equally between the two detectors. The column was operated at 200°C with an Argon- CO_2 (95:5) carrier gas flow rate of 25 ml/min. Two radiolabeled mass peaks were identified using an LKB 2091 EI/CI gas chromatograph mass spectrometer operated with the same chromatographic conditions.

Synthesis of glutathione and cysteine conjugates of HCBd. 1. *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine. HCBd was reacted with the sodium salt of *N*-*t*-BOC-*S*-benzyl-L-cysteine in liquid ammonia at -70°C for 1 hr. The ammonia was allowed to evaporate and the residue treated with a solution of approximately 1 M HCl in ethyl acetate for 30 min at room temperature. The resulting white solid (m.p. 180–182°C) was removed by filtration and shown to be *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-L-cysteine by mass spectrometry and by $[^{13}\text{C}]$ NMR ($-\text{COOH}$, 168.156 ppm; $-\text{CH}$, 51.680 ppm; $-\text{CH}_2\text{S}$, 32.882 ppm; $-\text{C}=\text{C}-\text{C}=\text{C}-$, 133.159, 125.358, 124.383 and 119.724 ppm).

2. *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)-glutathione. This conjugate was prepared from HCBd and glutathione by a method analogous to that described by McKinney and Biester (1959). The crude product was recrystallized to give a white crystalline material m.p. 186–187°C. Microanalysis, the field desorption mass spectrum and $[^{13}\text{C}]$ NMR spectrum were consistent with the expected structure.

Results

HCBd-mediated glutathione depletion by hepatic microsomal and cytosolic fractions from male and female rats. Initial experiments were designed to establish suitable incubation conditions for investigating HCBd-mediated glutathione depletion *in vitro* and were based on the premise that a cytochrome P-450-dependent activation reaction was required before conjugation would occur. Male rat liver microsomal

samples, fortified with glutathione and an NADPH generating system, were incubated with HCBd and a time-dependent loss of glutathione measured (fig. 1). A small reduction in glutathione occurred in samples incubated in the absence of HCBd or with heat-inactivated microsomes (fig. 1). Glutathione depletion catalyzed by HCBd was proportional to microsomal protein concentration and was linear up to 4 mg of protein per ml. The rate of depletion was also dependent on the concentration of glutathione (fig. 2A) and a K_m of 290 μ M and a V_{max} of 1.38 nmol/min/mg of microsomal protein was obtained. The K_m value for HCBd was 550 μ M with a V_{max} of 0.91 nmol/min/mg of microsomal protein (fig. 2B). Incubations carried out in the absence of the NADPH generating system produced a similar rate of HCBd-mediated glutathione loss to that in the presence of NADPH (fig. 1; table 1). When male rat liver cytosol was used instead of microsomes a lower rate of loss was measured (fig. 1; table 1). The depletion of glutathione by microsomes or cytosol from female liver was similar to that obtained with male samples (table 1).

Experiments using the above incubation system with kidney subcellular fractions were not feasible due to the rapid loss of added glutathione in the presence and absence of HCBd. This is presumably due to the presence of high concentrations of glutathione metabolizing enzymes.

Fig. 1. HCBd-mediated glutathione loss by male rat liver microsomes and cytosol. A, rat liver microsomes (10–12 mg of protein) or B, cytosol (50–90 mg of protein) were incubated in 0.1 M phosphate buffer (pH 7.4) containing glutathione (10 mM), glutathione reductase (12 U) and were indicated an NADPH generating system and HCBd (2.8 mM). Samples were removed from the incubation at the times shown and glutathione measured in a protein-free extract. For some experiments the microsomes were heated to 100°C for 5 min before use (○) or alternatively the reaction contained no HCBd (○). With NADPH (●). Minus NADPH (▲).

Fig. 2. Double reciprocal plot of the rate of HCBd-mediated glutathione depletion vs. glutathione or HCBd concentration using male hepatic microsomal fractions. A, glutathione. Incubations were made in 0.1 M phosphate buffer (pH 7.4, 10 ml) containing male rat liver microsomal protein (11 mg), glutathione reductase (12 U), HCBd (2.7 mM) and glutathione at the concentrations shown. Incubations were for 2 hr at 37°C with samples taken at several time points to determine the initial reaction rate. B, HCBd. Incubations were made in 0.1 M phosphate buffer (pH 7.4; 1 ml) containing rat liver microsomal protein (4 mg), glutathione reductase (0.25 U), glutathione (2.5 mM), an NADPH generating system and HCBd at the concentrations shown. Incubations were for 30 min at 37°C. Results are shown as the mean of two experiments for each substrate.

TABLE 1

Rate of HCBd-mediated glutathione depletion by male and female hepatic microsomal and cytosolic fractions

Rat liver fractions were incubated as described under "Materials and Methods" in the presence of glutathione (10 mM), [¹⁴C]HCBd (2.8 mM and 1 μ Ci/ml), glutathione reductase (12 U) and an NADPH generating system where indicated. Samples (0.3 ml) were removed at 30, 60, 90 and 120 min for glutathione measurement and the rate of loss calculated. Values are shown as mean $\pm$ S.E. with the number of animals in parentheses.

Fraction	Glutathione Depletion nmol/min/mg protein
Male	
Microsomes + HCBd	0.592 $\pm$ 0.038 (5)
Microsomes + NADPH + HCBd	0.524 $\pm$ 0.034 (5)
Microsomes alone	0.006 $\pm$ 0.006 (4)
Cytosol + HCBd	0.349 $\pm$ 0.020 (6)
Cytosol alone	0.061 $\pm$ 0.032 (3)
Female	
Microsomes + HCBd	0.604 $\pm$ 0.047 (6)
Cytosol + HCBd	0.368 $\pm$ 0.012 (5)

Formation of HCBd-mediated glutathione conjugates by microsomal and cytosolic fractions from male and female rat liver and kidney. To establish whether the hepatic glutathione depletion due to HCBd was the result of the formation of a glutathione conjugate, radiolabeled HCBd was incubated with microsomal fractions fortified with gluta-

thione. The deproteinized incubation medium was then chromatographed on thin-layer plates and the results of a typical experiment are shown in figure 3. A radioactive metabolite (R_f 0.33) was observed on scanning the thin-layer chromatogram. The formation of this product was independent of the presence of NADPH but dependent on the presence of reduced glutathione and an enzymatically active microsomal preparation. When [^3H]glutathione was used instead of [^{14}C]HCBd a peak of radioactivity with an identical R_f value to the [^{14}C]HCBd metabolite was observed (data not shown). The material of R_f 0.33 which was isolated and characterized by gas chromatography and mass spectrometry after hydrolysis and methylation gave a mass spectrum consistent with that of the methyl ester of *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)cysteine (fig. 4). Two radiolabeled mass peaks in the ratio of 9:1 were detected with retention times of 2.4 and 2.7 min. Both peaks gave identical mass spectra and are therefore assumed to be the *cis*- and *trans*-isomers of this conjugate. The identification of this cysteine derivative, after hydrolysis, using conditions known to hydrolyze glutathione (Hutson, 1976) is consistent with the metabolite R_f 0.33 being the corresponding glutathione conjugate. This is further supported by 1) experiments using [^3H]glutathione which gave an identical product to that isolated, 2) chemically synthesized *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl) glutathione had an identical R_f value in the thin-layer chromatographic system to the metabolite and 3) the mass spectrum of the chemically synthesized methyl ester of the cysteine conjugate was identical to the derivatized metabolite. The [^{13}C]NMR spectrum of the synthesized product showed the structure to be *S*-(1,1,2,3,4-pentachloro-1:3-butadienyl)cysteine. As well as substantiating the structure of the metab-

Fig. 3. Thin-layer chromatography scan of radiolabeled metabolites resulting from the incubation of [^{14}C]HCBd and glutathione with male hepatic microsomes or cytosol. Incubations were carried out as described in figure 1. After incubation samples were cooled to 4°C, centrifuged and 40 μl of the supernatant run on silica gel plates in butanol-acetic acid-water (12:3:5). Plates were scanned using a radiochromatogram scanner. Scan: 1, microsomes plus NADPH; 2, microsomes minus NADPH; 3, cytosol; 4, microsomes minus GSH; 5, microsomes boiled at 100°C for 5 min; 6, microsomes plus oxidized glutathione to replace GSH.

TABLE 2

Rate of HCBd-glutathione conjugate formation by male and female hepatic microsomal and cytosolic fractions

Rat liver fractions were incubated as described in the legend to table 1. After 2 hr the reaction was stopped, the excess HCBd extracted into diethyl ether and a sample of the aqueous phase taken for radioactivity determination and for thin-layer chromatography. The amount of conjugate formation was calculated from the area under the radiochromatogram scans. Results are mean $\pm$ S.E. with the number of animals shown in parentheses.

Fraction	Conjugate Formation nmol/min/mg protein
Male	
Microsomes + HCBd	0.715 $\pm$ 0.055 (4)
Microsomes + NADPH + HCBd	0.576 $\pm$ 0.036 (6)
Microsomes alone	0.025 $\pm$ 0.008 (5)
Cytosol + HCBd	0.290 $\pm$ 0.014 (6)
Cytosol alone	0.034 $\pm$ 0.009 (6)
Female	
Microsomes + HCBd	0.575 $\pm$ 0.021 (5)
Microsomes + NADPH + HCBd	0.601 $\pm$ 0.037 (6)
Cytosol + HCBd	0.168 $\pm$ 0.009 (6)

TABLE 3

Effect of 1-chloro-2,4-dinitrobenzene on the formation of HCBd-glutathione conjugates by male rat liver microsomal and cytosolic fractions

Male rat liver microsomal fraction (9 mg of protein) or cytosol fraction (25 mg of protein) was incubated with glutathione (4 mM) and [^{14}C]HCBd (1 mM and 1 $\mu\text{Ci/ml}$) as described under "Materials and Methods" for 60 min at 37°C in the presence or absence of 1-chloro-2,4-dinitrobenzene (CDNB) (1 mM). The reaction was then cooled to 4°C, centrifuged and an aliquot of the supernatant chromatographed on a thin-layer chromatogram and the conjugate formation calculated from the area under the radioactive peaks. A, the product with R_f 0.14 and B, the pentachlorobutadienyl-glutathione conjugate with an R_f 0.33. Results are shown as mean $\pm$ S.E. of three animals.

Fraction	Conjugate Formation nmol/min/mg protein
Microsomes	0.309 $\pm$ 0.047
Microsomes + CDNB	0.231 $\pm$ 0.027
Cytosol	0.098 $\pm$ 0.010
Cytosol + CDNB	0.053* $\pm$ 0.005
	0.286 $\pm$ 0.033
	0.210* $\pm$ 0.016

* Significantly different from control. $P < .05$ using Student *t* test.

olite these data also demonstrate that the product of R_f 0.33 does not contain positional isomers of the HCBd-glutathione adduct.

To quantitate the rate of metabolite formation the thin-layer plates were scanned using a computer-linked radiochromatogram scanner and the peak areas were determined. The results for male and female hepatic microsomal and cytosolic fractions are shown in table 2. In addition to the major metabolite (R_f 0.33) a second smaller and more polar metabolite (R_f 0.14) was observed in the incubation with female hepatic microsomes and with male and female hepatic cytosolic fractions (fig. 3). The ratio of the metabolite at R_f 0.14 compared with R_f 0.33 was 0.4 to 0.5. Incubation of male hepatic microsomes plus NADPH under an atmosphere of carbon monoxide or nitrogen did not reduce the rate of HCBd-mediated glutathione conjugate formation [control, 100 $\pm$ 18% (3); 20% O_2 in CO, 95 $\pm$ 18% (3); nitrogen, 123 $\pm$ 8% (3)]. 1-Chloro-2,4-dinitrobenzene, a substrate for glutathione *S*-transferase enzymes reduced the formation of the HCBd-conjugate when present at an equimolar concentration to HCBd (1 mM) in male microsomal and cytosolic fractions (table 3).

When male and female kidney microsomal fractions were incubated with [^{14}C]HCBd and glutathione a radioactive product was formed which had an R_f of about 0.35 on the thin-layer chromatography plates. This peak was not observed in incu-

Fig. 4. Mass spectrum of the derivatized metabolite formed on incubation of HCBd with male hepatic microsomes and glutathione. The incubation conditions and derivatization conditions are described under "Materials and Methods."

TABLE 4

Rate of HCBd-glutathione conjugate formation by male and female renal microsomal and cytosolic fractions

Rat kidney fractions were prepared as described under "Materials and Methods" and incubated at 37°C for 2 hr in the presence of glutathione (10 mM), HCBd (2.8 mM) and glutathione reductase (12 U). Protein concentrations were microsomal (8–13 mg) and cytosol (21–27 mg). An aliquot was then chromatographed on a thin-layer plate and the plate scanned using a radiochromatogram scanner. Results are shown as mean $\pm$ S.E. with the number of animals in parentheses.

Fraction	Conjugate Formation nmol/min/mg protein
Male	
Microsomes + HCBd	0.089 $\pm$ 0.008 (6)
Microsomes alone	0.009 $\pm$ 0.003 (3)
Cytosol + HCBd	0.049 $\pm$ 0.010 (5)
Cytosol alone	<.005 (3)
Female	
Microsomes + HCBd	0.122 $\pm$ 0.010 (5)
Cytosol + HCBd	0.143 $\pm$ 0.026 (6)

bations made in the absence of glutathione. The rate of formation of this product was similar for male and female microsomal fractions (table 4). Incubation of male or female renal cytosolic fractions with radiolabeled HCBd and glutathione led to the formation of two radiolabeled products which on thin-layer chromatography had R_f values of 0.14 and 0.33, similar to that found with liver cytosol. The ratio of these metabolites was about 1:1. The rate of formation of these radiolabeled products was dependent on glutathione and was

about 3 times faster in cytosol from female kidney compared with male kidney (table 4).

Discussion

The depletion of glutathione in animal tissues after the administration of compounds which are known to be toxic is usually associated with the inactivation of toxic metabolites formed by cytochrome P-450-mediated monooxygenase reactions (Chasseaud, 1979; Mitchell *et al.*, 1976; Jollow *et al.*, 1977). It has been proposed that when the concentration of glutathione became too low to effectively deactivate these metabolites, they react with proteins and other macromolecules which results in cellular damage (Mitchell *et al.*, 1973; Jollow and Smith, 1977). We have shown previously that HCBd administration to rats causes a marked depletion in hepatic nonprotein sulphhydryl content (mainly glutathione) in males (Lock and Ishmael, 1981), whereas in female animals both hepatic and renal glutathione levels are reduced (Hook *et al.*, 1983).

In the present study we have examined this effect *in vitro* and shown that HCBd does cause glutathione depletion and that the product formed is as a result of direct conjugation of HCBd with glutathione. The reaction is catalyzed by microsomal glutathione S-transferases and is not related to the formation of metabolites *via* cytochrome P-450. The rate of formation of the conjugate in microsomes compared closely with the rate of glutathione depletion, supporting a 1:1 stoichi-

ometry of HCBd with glutathione. Gas chromatography-mass spectrum indicates the presence of two isomeric forms of this conjugate, one of which predominates. The two isomers could not be distinguished by their mass spectra, but on steric grounds the *trans*-isomer might be expected to predominate.

The presence of hepatic microsomal glutathione *S*-transferase(s) has only recently become apparent (Morgenstern *et al.*, 1979, 1980; Friedberg *et al.*, 1980). Multiple forms of both microsomal and cytosolic glutathione *S*-transferases exist (Friedberg *et al.*, 1980; Habig *et al.*, 1974) and in the case of the cytosolic enzymes these proteins are known to have different substrate specificities. Certain similarities, for example isoelectric point and immunochemical cross reactivity between some of the microsomal glutathione *S*-transferases and those in the cytosol, are known (Friedberg *et al.*, 1980). However, Morgenstern *et al.* (1982) have clearly demonstrated that at least one form of microsomal transferase is clearly distinguishable from those in the cytosol. However, in the case of HCBd, these enzymes appear to play a major role, the rate of formation of the HCBd-glutathione conjugate in the microsomal fraction being up to twice that found in cytosol. The activity of these enzymes with a variety of substrates under normal conditions is low compared with the activity of the hepatic cytosolic glutathione *S*-transferase(s), less than 10% on a milligram of protein basis (Glatt and Oesch, 1977; Morgenstern *et al.*, 1980; Friedberg *et al.*, 1980). Therefore, the microsomal enzyme(s)

did not appear to play an important role in the conjugation of most foreign compounds.

The major microsomal glutathione conjugate of HCBd also appeared to be formed by rat liver cytosol, but at a slower rate. This suggests that either the enzyme(s) responsible for HCBd conjugation in the cytosol are different, present at a lower concentration or that HCBd preferentially partitions into the lipid membranes of the microsomes and results in more substrate being available to the microsomal transferase(s) than to those in the cytosol. A second more polar metabolite (R_f 0.14) has not been identified but some support for a double conjugate is the finding that more glutathione is consumed than product formed, suggesting a stoichiometry of greater than 1:1. The formation of an HCBd glutathione conjugate by the microsomal transferase will result in a significantly less lipophilic metabolite than HCBd itself. A water soluble conjugate of this nature is unlikely to be retained by the lipophilic membranes thus reducing the opportunity for a second conjugation with glutathione. In the case of the transferases present in the cytosol, although less substrate (HCBd) is available the conjugate when formed is likely to be a better substrate for the soluble enzymes than the membrane bound transferases. Thus, conjugation with 2 mol of glutathione is likely to be favored in the cytosol fraction if such an event is occurring.

The overall activities of the male and female hepatic microsomal and cytosolic enzymes were not significantly different to

Fig. 5. Conjugation of HCBd with glutathione (GSH) and its possible nephrotoxic mechanism by analogy with S-dichlorovinyl-L-cysteine and S-difluorochlorovinyl-L-cysteine.

explain convincingly why HCBd-mediated glutathione depletion is more marked in males (Hook *et al.*, 1983).

Kidney microsomal and cytosolic fractions from both male and female animals contain enzymes capable of conjugating HCBd with glutathione, although their activity is lower than that of the liver. Female kidney cytosol appeared to be about 3 times more active than male kidney cytosol and this may in part account for the marked glutathione depletion observed in female but not male kidney after HCBd administration (Hook *et al.*, 1983).

Unlike other halo-olefins such as trichloroethylene or tetrachloroethylene which are almost exclusively metabolized *via* cytochrome P-450 (Henschler and Bonse, 1977), HCBd is not. This is substantiated by the finding that the formation of the major metabolite in microsomes was independent of the presence of NADPH and was not affected by carbon monoxide, a potent cytochrome P-450 inhibitor, or by anaerobiosis, and is in agreement with the *in vivo* studies in which prior treatment of rats with inducers or inhibitors of cytochrome P-450 did not modify the nephrotoxicity of HCBd (Lock and Ishmael, 1981; Hook *et al.*, 1982).

We have isolated the glutathione conjugate of HCBd and evidence is presented indicating a direct substitution reaction, with the loss of a chloride ion and without the incorporation of oxygen. This glutathione conjugate formed in the liver or directly in the kidney will be converted by conventional pathways in the kidney to give a cysteine conjugate (Bray *et al.*, 1959). The structure of the HCBd-cysteine conjugate would be analogous to that of *S*-dichlorovinyl-L-cysteine, a known nephrotoxin (Terracini and Parker, 1965). *S*-dichlorovinyl-L-cysteine is metabolized by the enzyme C-S or β -lyase, which is present in high concentrations in the kidney, to yield a reactive alkylating moiety, pyruvate and ammonia (Anderson and Schultze, 1965). Activation by this C-S lyase enzyme has also been proposed to account for the nephrotoxicity of the cysteine conjugates of chlorotrifluoroethylene and chlorodifluoroethylene (Gandolfi *et al.*, 1981). Thus, by analogy we suggest that HCBd may exert its nephrotoxicity *via* a similar mechanism (fig. 5). This hypothesis is supported by the finding that the *N*-acetylcysteine conjugate of HCBd is nephrotoxic to rats and the finding that a glutathione conjugate equivalent to that identified here is excreted in the bile after HCBd administration to rats (Green *et al.*, 1982).

Acknowledgments

The authors acknowledge the valuable technical assistance of Mr. E. F. Howard.

References

- ANDERSON, P. M. AND SCHULTZE, M. O.: Cleavage of *S*-(1,2-dichlorovinyl)-L-cysteine by an enzyme of bovine origin. *Arch. Biochem. Biophys.* **111**: 593-602, 1965.
- BERNDT, W. O. AND MEHENDALE, H. M.: Effects of hexachlorobutadiene (HCBd) on renal function and renal organic ion transport in the rat. *Toxicology* **14**: 55-65, 1979.
- BRAY, H. G., FRANKLIN, T. J. AND JAMES, S. P.: The formation of mercapturic acids. 2. The possible role of glutathione. *Biochem. J.* **77**: 690-696, 1959.
- CHASSEAUD, L. F.: The role of glutathione and glutathione-S-transferases in the metabolism of chemical carcinogens and other electrophilic reagents. *Adv. Cancer Res.* **29**: 175-274, 1979.
- FRIEDBERG, T., BENTLEY, P., STASIECKI, P., GLATT, H. R., RAPHAEL, D. AND OESCH, F.: Identification, solubilization and characterization of microsome associated glutathione-S-transferases. *J. Biol. Chem.* **254**: 12028-12033, 1980.
- GANDOLFI, A. J., NAGLE, R. B., SOLTIS, J. J. AND PLESCIA, F. H.: Nephrotoxicity of halogenated vinyl cysteine compounds. *Res. Commun. Chem. Pathol. Pharmacol.* **33**: 249-259, 1981.
- GLATT, H. AND OESCH, F.: Inactivation of electrophilic metabolites by glutathione-S-transferases and limitation of the system due to subcellular localization. *Arch. Toxicol.* **39**: 87-96, 1977.
- GREEN, T., NASH, J. A. AND KING, L. J.: The *in Vivo* Metabolism of Hexachloro-1:3-Butadiene in Relation to Its Nephrotoxicity, 8th European Workshop on Drug Metabolism, September 5-9, Abstract 152, Liege, 1982.
- HABIG, W. H., PABST, M. J. AND JAKOBY, W. B.: Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**: 7130-7139, 1974.
- HENSCHLER, D. AND BONSE, G.: Metabolic activation of chlorinated ethylenes: Dependence of mutagenic effect on electrophilic reactivity of the metabolically formed epoxides. *Arch. Toxicol.* **39**: 7-12, 1977.
- HOOK, J. B., ISHMAEL, J. AND LOCK, E. A.: Nephrotoxicity of hexachloro-1:3-butadiene in the rat: The effect of age, sex and strain. *Toxicol. Appl. Pharmacol.* **67**: 122-131, 1983.
- HOOK, J. B., ROSE, M. S. AND LOCK, E. A.: The nephrotoxicity of hexachloro-1:3-butadiene in the rat: Studies of organic anion and cation transport in renal slices and the effect of monooxygenase inducers. *Toxicol. Appl. Pharmacol.* **65**: 373-382, 1982.
- HUTSON, D. H.: Glutathione conjugates in bound and conjugated pesticide residues. *ACS Symp. Ser.* **29**: 103-131, 1976.
- JOLLOU, D. J., KOCIS, J. J., SNYDER, R. AND VAINIO, H.: Biological Reactive Intermediates, Plenum Press, London, 1977.
- JOLLOU, D. J. AND SMITH, C.: Biochemical aspects of toxic metabolites formation, detoxification and covalent binding. In *Biologically Reactive Intermediates*, ed. by D. J. Jollow, J. J. Kocsis, R. Snyder and H. Vainio, pp. 42-59, Plenum Press, New York, 1977.
- LOCK, E. A. AND ISHMAEL, J.: The acute toxic effects of hexachlorobutadiene on the rat kidney. *Arch. Toxicol.* **43**: 47-57, 1979.
- LOCK, E. A. AND ISHMAEL, J.: Hepatic and renal non-protein sulphhydryl concentration following toxic doses of hexachloro-1:3-butadiene in the rat: The effect of Aroclor 1254, phenobarbitone or SKF 525A treatment. *Toxicol. Appl. Pharmacol.* **47**: 79-87, 1981.
- LOCK, E. A., NASH, J. A., GREEN, T. AND WOLF, C. R.: Hexachloro-1:3-butadiene-mediated glutathione depletion: The role of liver microsomal and cytosolic S-transferases. *Pharmacologist* **24**: 136, 1982a.
- LOCK, E. A., PRATT, I. S. AND ISHMAEL, J.: Hydrophobic change in rat liver induced by hexachloro-1:3-butadiene. *J. Appl. Toxicol.* **2**: 315-320, 1982b.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. AND RANDALL, R. J.: Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275, 1951.
- MCKINNEY, L. L. AND BIESTER, H. E.: Possible toxic factor of trichloroethylene extracted oil meal. *J. Am. Chem. Soc.* **81**: 909-915, 1959.
- MITCHELL, J. R., HINSON, J. A. AND NELSON, S. D.: Glutathione and drug-induced tissue lesions. In *Glutathione: Metabolism and Function*, ed. by I. M. Arias and W. B. Jakoby, pp. 357-367, Raven Press, New York, 1976.
- MITCHELL, J. A., JOLLOU, D. J., POTTER, W. Z., GILLETTE, J. R. AND BRODIE, B. B.: Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.* **187**: 211-217, 1973.
- MORGENSTERN, R., DEPIERRE, J. W. AND ERNST, L.: Activation of microsomal glutathione-S-transferase activity by sulphhydryl reagents. *Biochem. Biophys. Res. Commun.* **87**: 657-663, 1979.
- MORGENSTERN, R., GUTHENBERG, C. AND DEPIERRE, J. W.: Microsomal glutathione S-transferase. *Eur. J. Biochem.* **128**: 243-248, 1982.
- MORGENSTERN, R., MEIJER, J., DEPIERRE, J. W. AND ERNST, L.: Characterization of rat liver microsomal glutathione-S-transferase activity. *Eur. J. Biochem.* **104**: 167-174, 1980.
- SEDLAK, J. AND LINDSAY, R. H.: Estimation of total protein bound and non-protein sulphhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* **25**: 192-205, 1968.
- TERRACINI, B. AND PARKER, V. H.: A pathological study on the toxicity of *S*-dichlorovinyl-L-cysteine. *Food Cosmet. Toxicol.* **3**: 67-74, 1965.
- WOLF, C. R., BERRY, P. N., NASH, J. A., GREEN, T. AND LOCK, E. A.: The role of rat liver microsomal and cytosolic glutathione S-transferases in hexachloro-1:3-butadiene-mediated glutathione depletion. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **319**: R17, 1982.

Send reprint requests to: Dr. C. R. Wolf, Imperial Cancer Research Fund, Medical Oncology Unit, Western General Hospital, Edinburgh, EH4 2XU, Scotland, UK.