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BIOTRANSFORMATION AND NEPHROTOXICITY
OF HALOGENATED ETHYLENES

by
Christopher Donald Hassall

A Dissertation Submitted to the Faculty of the
PROGRAM IN PHARMACOLOGY AND TOXICOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1983
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Christopher Donald Hassall entitled Biotransformation and Nephrotoxicity of Halogenated Ethylenes and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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SIGNED:  Christopher D. Haskell
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Finally I thank my parents for encouraging me to set my life goals high and to persevere in achieving them.
HALOALKENES WERE SHOWN TO REACT WITH CYSTEINE, N-ACETYL CYSTEINE, OR GLUTATHIONE TO FORM HALOGENATED VINYLTHIO (HVT) OR SATURATED CONJUGATES. WHEN HVT WERE ADMINISTERED IV TO RABBITS, ACTIVE TRANSPORT IN THE RENAL TUBULES WAS INHIBITED 50% AT DOSES AS LOW AS 20 mg/kg WITHIN 1 HR AFTER DOsing. THERE WAS SLoughING OF THE RENAL BRUSH BORDER MEMBRANE WITH THE INJURY PROGRESSING TO A SPECIFIC RENAL TUBULAR NECROSIS OF THE S3 SEGMENT. IN VITRO STUDIES WITH RENAL TUBULES FOUND THAT THE HVT PRODUCED A DOSE-RESPONSE RELATED INHIBITION OF ACID/BASE TRANSPORT, WITH COMPLETE INHIBITION OF TRANSPORT OCCURRING AT 1 mM. THE CYSTEINE CONJUGATE SYNTHESIZED FROM TRICHLOROETHYLENE, DCVC, INHIBITED TUBULAR ACTIVE TRANSPORT 60 MIN AFTER IN VIVO DOSING (20-100 mg/kg), 45 MIN AFTER EXPOSURE IN THE ISOLATED PERFUSED KIDNEY (0.01-1 mM) AND 15 MIN AFTER INCUBATION WITH ISOLATED TUBULES (0.01-1 mM). ALL HVT CONJUGATES HAD A SIMILAR POTENCY WITH REGARD TO TRANSPORT INHIBITION IN ISOLATED TUBULES, WITH COMPLETE INHIBITION OCCURRING AT 1 mM WITHIN 15 MIN FOR CYSTEINE CONJUGATES COMPARED TO 45-60 MIN FOR THE N-ACETYL CYSTEINE OR GLUTATHIONE CONJUGATES. THESE LATTER CONJUGATES ARE THOUGHT TO BE BIOACTIVATED TO THE CYSTEINE CONJUGATE PRIOR TO TRANSPORT INHIBITION. INHIBITION OF TUBULAR γ-Glutamyl transpeptidase BY 1 mM AT-125 OR 20 mM SERINE/BORATE PREVENTED THE INHIBITION OF ACID/BASE TRANSPORT BY THE GLUTATHIONE CONJUGATE. IN ADDITION, THE SEQUENTIAL FORMATION OF GLUTAMATE, GLYCINE AND THE VINYL CYSTEINE CONJUGATE AFTER
tubule incubation with the glutathione conjugate provides further evidence for this bioactivation. The cysteine conjugates are thought to be further metabolized in tubules to a toxic intermediate by a brush border localized enzyme, C-S lyase. The inhibitor effect of this intermediate on acid/base transport is reversed in the presence of, or subsequent addition of, 6 mM exogenous glutathione. These studies provide further evidence for the nephrotoxicity of HVT, and formation of the nephrotoxic cysteine conjugates from glutathione and cysteine conjugates. The formation of saturated conjugates from CTFE was also demonstrated. These saturated and/or unsaturated conjugates may be responsible for haloalkene-induced nephrotoxicity.
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INTRODUCTION

Purpose

Halogenated vinyl cysteine conjugates are potent, specific nephrotoxicants in vivo (Parker, 1965; Gandolfi et al., 1981) and in vitro (Bhattacharya and Schultze, 1967); Hassall et al., 1983). However, it is uncertain as to whether these compounds cause the nephrotoxicity observed after exposure to their halogenated ethylene parent compounds. It is thought that halogenated ethylene-induced nephrotoxicity may result from glutathione conjugation in the liver followed by peptidase cleavage in the renal proximal tubules to form the vinyl cysteine conjugate (Lock, 1982). The pars recta region of the proximal tubule is a selective target for this nephrotoxicity. There are several factors which account for this susceptibility. First the cysteine conjugates are formed from the glutathione conjugate in the proximal tubules after cleavage of glutamic acid and glycine by renal tubule-associated peptidase enzymes. Also the cysteine conjugates are concentrated in the tubules prior to excretion from the body; the enzyme responsible for the bioactivation of vinyl cysteine conjugates is located here. This enzyme, C-S lyase, is found in both liver and kidney, with the highest concentration present in the brush border membrane of the renal tubular epithelial cells (Anderson and Schultze, 1965; Bonhaus and Gandolfi, 1981). C-S lyase cleaves halogenated vinyl...
cysteine conjugates to form a reactive thiovinyl intermediate which may react with biomacromolecules to induce nephrotoxicity (Anderson and Schultze, 1965). The objective of this study was to investigate the mechanism of vinyl cysteine conjugate-induced nephrotoxicity, using S-(trans-1,2-dichlorovinyl)-L-cysteine as a prototype cysteine conjugate. In addition, the biotransformation pathway by which these conjugates are formed from the parent halogenated ethylenes and chemically synthesized vinyl glutathione conjugates was examined.

Nephrotoxicity of Halogenated Ethylenes

The haloalkenes, trichloroethylene (TCE), chlorotrifluoroethylene (CTFE), chlorodifluoroethylene (CDFE) and hexachlorobutadiene (HCBD) are structurally similar compounds that have been shown to be toxic in mammalian species (Fishbein, 1976; Jenkins and Anderson, 1978; Berndt and Mehendale, 1979; Potter et al., 1981). Human and animal exposure to TCE may result in central nervous system depression, hepatotoxicity and nephrotoxicity (Browning, 1965). It is uncertain as to whether the liver and kidney injury following both acute and chronic exposure are caused entirely by TCE, or are the result of conditions superimposed on previously susceptible livers or kidneys, or associated with other toxic factors (Browning, 1965). The role of metabolism in TCE-induced toxicity is also a matter for conjecture. There is a direct correlation between the extent of oxidative metabolism of TCE in the liver and its hepatotoxicity (Van Durren and Banerjee, 1976; Moslen et al., 1977; Allemand et al., 1978); however, hepatic metabolism is not thought to account for the nephrotoxicity.
CTFE is a monomer used in the industrial production of the fluoropolymer polychlorotrifluoroethylene, a material used in the synthesis of Teflon-like polymers. Inhalation of CTFE produces a marked focal necrosis in the pars recta region of the proximal tubules of mice (Lock, 1982). Damage was also observed in kidney mitochondria within 30 min of exposure. The mechanism of nephrotoxicity has not been established, however, the increased urinary excretion of fluoride ions resulting from defluorination is not thought to be sufficient to produce the extent and type of damage observed (Potter et al., 1981; Buckley et al., 1981). The renal tubular necrosis following inhalation exposure to CTFE is thought to occur without the influence of hepatic metabolism (Lock, 1982). CDFE, a reductive metabolite of the commonly used inhalation anesthetic halothane, (Maiorino et al., 1981) has also been shown to cause renal damage.

HCBD is a byproduct in the synthesis of perchloroethylene, trichloroethylene and carbon tetrachloride (Davis et al, 1980). It induces lung damage, histological abnormalities within the liver, is toxic to the central nervous system and is perhaps one of the most nephrotoxic aliphatic chlorinated hydrocarbons (Berndt and Mehendale, 1979). HCBD has been shown to impair renal organic anion transport and produce necrosis in the outer medullary region of the cortico-medullary junction (Lock and Ishmael, 1979; Duprat and Gradiski, 1978). A two-year feeding study in rats produced evidence for a dose-dependent HCBD-induced toxicity affecting primarily the kidney at 2 mg/kg or higher.
doses (Kociba et al., 1977). It is widely dispersed in the environment and has proved to be a significant contaminant in the Mississippi River (Laska et al., 1976).

The most prominent toxicological feature resulting from exposure to these unsaturated halogenated aliphatic compounds is nephrotoxicity. At low concentrations of haloalkenes administered by inhalation iv or ip, a decrease in urine concentrating ability may occur without histological evidence of renal injury (Harleman and Seinen, 1979; Potter et al., 1981). At higher doses, extensive tubular degeneration occurs, especially in the pars recta region of the proximal tubules (Potter et al., 1981; Lock and Ishmael, 1982). Degeneration and necrosis of epithelial cells may occur in conjunction with increased cellularity of the epithelial lining. After loss of epithelial cells by necrosis replacement occurs by the division of mature, fully functional epithelial cells (Davis and Kennedy, 1967). Redifferentiation of the proliferating epithelial cells occurs until the second week after damage. Within 2-4 weeks of a single exposure, the tubules are both structurally and functionally normal.

**Biotransformation of Haloalkenes**

Most halogenated hydrocarbons are thought to require biotransformation to produce toxicity. Bioactivation of these compounds may yield reactive electrophilic metabolites which may acylate or alkylate cellular macromolecules resulting in toxicity. Dehalogenation often occurs as a consequence of haloalkene metabolism, although
nephrotoxicity of these compounds is not thought to result from halide release (Buckley et al., 1981). There are two metabolic routes by which haloalkenes may be bioactivated, one by microsomal mixed function oxidase mediated oxidation (Bonse and Henschler, 1976) and the other by conjugation with reduced glutathione (Figure 1). Cytochrome P-450 mediated metabolism of halogenated ethylenes results in the formation of 2 carbon alcohols and acids. Oxidative metabolism is initiated by oxidation to an oxirane which may then undergo several potential reactions in the cell. Toxicity results from electrophilic reactions with essential cellular components (alkylation); whereas, reduction, hydrolysis and conjugation, or rearrangement of the carboxylic compounds are considered to be detoxication mechanisms (Bonse and Henschler, 1976). Subsequent oxidation or reduction of rearrangement products to trichloroacetic acid and trichloroethanol, or hydrolysis of acyl chlorides to the corresponding acids are not thought to result in toxicity (Moslen et al., 1977).

Trichloroethylene is thought to undergo a significant degree of metabolism by oxidation to form a hepatotoxic species (Bonse and Henschler, 1976). However, haloalkene-induced nephrotoxicity is not thought to involve cytochrome P-450 catalyzed bioactivation (Lock, 1982). Hook et al. (1982) also established strong evidence against the role of mixed function oxidases in the bioactivation of HCBD. However, it is possible that glutathione may be involved in the bioactivation of this and other nephrotoxic haloalkenes. Six hrs after ip administration
of HCBD and after 2 hr inhalation exposure to 1% TCE, there was a 50% depletion of hepatic glutathione in the rat, although no depletion of renal glutathione occurred (Moslen et al., 1977; Davis et al., 1980; Lock and Ishmael, 1981). Incubation of HCBD with glutathione-fortified liver subcellular fractions resulted in a HCBD-dependent loss of glutathione and formation of S-(pentachloro-1,3- butadienyl)-glutathione, indicating a direct substitution of glutathione with HCBD (Lock et al., 1982).

A number of xenobiotics undergo S-conjugation with glutathione as the initial step in detoxification by the mercapturic acid pathway. Conjugation occurs primarily in the liver, but may also occur in the kidney, and may occur directly or following activation of xenobiotic in a cytochrome P-450 dependent oxidation (Moldeus et al., 1978). Glutathione conjugation may also occur spontaneously but is generally catalyzed by glutathione S-transferase (Boyland and Chasseaud, 1969). The low substrate specificity for this reaction is explained by the presence of several transferases, such as aryltransferase, epoxide-transferase, alkyltransferase, aralkyltransferase and alkenetransferase, each catalyzing specific transfer reactions (Boyland and Chasseaud, 1969; Ingelman-Sandberg, 1980). The reaction of haloalkenes with glutathione is not thought to require preliminary activation of the haloalkene by cytochrome P-450 catalyzed oxidation, (Hook et al., 1982; Lock, 1982) and may occur by substitution, by an addition/elimination reaction or by an addition. Addition of glutathione to the haloalkene
and subsequent elimination of a hydrogen halide results in formation of an unsaturated vinyl glutathione conjugate as does substitution. These conjugation reactions result in the eventual formation of vinyl cysteine conjugates. An alternative conjugation reaction involves an enzyme-catalyzed nucleophilic attack on the carbon bearing the halogen, without halogen elimination, resulting in formation of a saturated halogenated ethyl glutathione conjugate. This reaction has been shown to occur in rat hepatic cytosolic and microsomal fractions after CTFE exposure (Dohn and Anders, 1982b).

Conjugation with glutathione often results in the detoxification of many xenobiotics. However, this conjugation results eventually in the biotransformation of haloalkenes to potentially nephrotoxic compounds, the cysteine conjugates. The glutathione conjugate formed in the liver may be transferred to the bile and enter the blood circulation after subsequent reabsorption in the small intestine. It may also enter the blood circulation directly and be transported to the kidney for excretion. Here the renal brush border enzymes γ-glutamyl transpeptidase and cysteinylglycine dipeptidase catalyze cleavage of glutamate and glycine to form the nephrotoxic cysteine conjugate (Hughey et al., 1978; Meister et al., 1981).

**Toxicity of S-(trans-1,2-dichlorovinyl)-L-cysteine**

This study principally involved investigation of nephrotoxicity induced by the chemically synthesized cysteine conjugates of the haloalkenes TCE, CTFE, CDFE and HCBD. The cysteine conjugate formed from
TCE, S-(trans-1,2-dichlorovinyl)-L-cysteine (DCVC) was used as the prototype conjugate for this class of compounds. The first indication of nephrotoxicity resulting from exposure to vinyl cysteine conjugates was found in the early 1950s with reports of fatal blood dyscrasias and renal disease in livestock fed on soybean meal defatted with TCE (Pritchard et al., 1956; Rundles, 1958). The toxic factor in TCE-treated soybean meal resulted from the reaction of TCE with amino acid residues, primarily cysteine (McKinney et al., 1959; Schultze et al., 1959). McKinney et al. (1959) chemically synthesized the cysteine conjugate of TCE, that is DCVC, and found that oral administration to calves produced the characteristic hematopoietic and renal toxicity previously seen with TCE-extracted soybean meal (McKinney et al., 1959; Schultze et al., 1959). DCVC also produced a dose dependent focal necrosis of the pars recta region of the renal proximal tubule in rodents and the dog providing further evidence for the nephrotoxicity of the vinyl cysteine conjugate (Parker, 1965; Tarloff et al., 1979).

**Role of C-S Lyase in DCVC Bioactivation**

DCVC has since been shown to be nephrotoxic in all species tested, although susceptibility varies between species (Stonard, 1973; Lock, 1982). Anderson and Schultze (1965) found an enzyme in kidney capable of cleaving DCVC into equimolar amounts of pyruvate, ammonia and a reactive intermediate, and proposed that this cleavage was responsible for the nephrotoxicity of DCVC (Figure 2). The vinyl-sulfur compound formed after cleavage was proposed to be the nephrotoxic species. It
was later determined that this enzyme cleaves a variety of cysteine conjugates, and may function physiologically in the metabolism of certain cysteine conjugates formed during mercapturic acid biosynthesis. Metabolism by this enzyme is unusual in that instead of elimination of cysteine conjugates as a non-toxic mercapturic acid after N-acetylation in the kidney, certain cysteine conjugates may be bioactivated to form toxic metabolites in vivo (Figure 2). Tateishi et al. (1978) further characterized the enzyme with regard to substrate specificity and subcellular distribution (Tateishi and Shimizu 1980) and termed the enzyme cysteine conjugate beta lyase, or C-S lyase. This enzyme catalyzes cleavage of the linkage in the cysteine moiety of cysteine conjugates and results in formation of pyruvate, ammonia and thiols (Dohn and Anders 1980).

C-S lyase is contained in substantial amounts only in the liver and kidney, with other organs containing only trace amounts of the enzyme (Tateishi and Shimizu 1980). In the liver the enzyme is present in the 100,000xg supernatant with no detectable activity in the microsomal fraction (Dohn and Anders 1982a). In the kidney the enzyme is associated with the brush border membrane of the renal proximal tubules (Bonhaus and Gandolfi 1981).

Tateishi and Shimizu (1980) conducted a preliminary survey of potential substrates for C-S lyase and found that S-aryl and a few S-alkyl compounds may be cleaved by the enzyme. It is not known if one or several enzymes are involved in the biotransformation of these different
cysteine conjugates (Dohn and Anders 1982). Although vinyl cysteine
conjugates are substrates for this enzyme, the related glutathione and
N-acetylated cysteine conjugates are not substrates, hence a free amino
group on the cysteine moiety is probably required for activity (Tateishi
et al., 1978). An unsaturated moiety attached to the sulfur, such as an
aromatic group or the electronically similar vinyl group, was also
thought to be required for the enzyme to exert nephrotoxicity (Tateishi
et al., 1978). However Dohn and Anders (1982b) have shown that a
saturated cysteine conjugate of CTFE is nephrotoxic and suggested that
the nephrotoxicity of this conjugate may result from cleavage by C-S
lyase to form a reactive species. Another requirement for the formation
of nephrotoxic intermediates is a halogen atom at the alpha position of
the vinyl cysteine conjugate (Gandolfi et al., 1981).

DCVC is metabolized rapidly by C-S lyase in vivo. Eighty min
after an iv dose in calves no DCVC was detected in blood plasma or lymph
(Derr et al., 1963). After C-S lyase cleavage, the sulfur moiety of
DCVC gives rise to many different compounds including inorganic sulfate,
and one or more of these compounds becomes rapidly associated with the
protein fraction of tissues, primarily in the kidney (Derr et al.,
1963).

In both the calf and rat the highest concentration of $^{35}$S after
administration of $^{35}$S-DCVC was found in the kidney. The liver contained
about 25% of the kidney concentration and other organs approximately
5-7% per unit dry weight (Derr and Schultze, 1963). Between 65 and 80% of
the tissue radioactivity was associated with the protein fraction. Since DCVC only binds to tissues after cleavage by C-S lyase and the distribution of $^{35}$S corresponds closely to organ distribution of C-S lyase (Tateishi and Shimizu, 1980), it seems likely that the susceptibility of the kidneys to DCVC-induced toxicity is determined at least in part by the high concentration of C-S lyase in the kidney, and primarily in the renal proximal tubules.

**Species Susceptibility to DCVC-induced Nephrotoxicity**

After administration of $^{35}$S-DCVC to rats, $^{35}$S was excreted in the urine as inorganic sulfate and N-acetyl DCVC, with 80% of the label excreted within the first 100 min as N-acetyl DCVC (Derr et al., 1963). After administration of DCVC to calves, which are much more susceptible to DCVC-induced toxicity than are rats, no N-acetyl DCVC was found in the urine. Acetylation of DCVC to the mercapturic acid is the major route of metabolism for cysteine conjugates in most species and does not lead to the formation of toxic intermediates (Bhattacharya and Schultze, 1967). This reaction is catalyzed by N-acetyl transferase located in the endoplasmic reticulum of the renal proximal tubules cells (Moldeus et al., 1978). The balance between acetylation of DCVC and cleavage by C-S lyase may account for the species variation in susceptibility to DCVC-induced nephrotoxicity. Terracini and Parker (1965) showed renal tubular necrosis after a single dose of DCVC in the dog was more severe than that produced by equivalent doses in the rat or mouse. Acetylation activity in the dog is low compared to other species (King and Olive,
Therefore, DCVC present in the kidney may be metabolized predominantly by C-S lyase cleavage in the dog. This metabolic step increases susceptibility of the dog to this nephrotoxicant.

Bioactivation of the vinyl cysteine conjugates is due to cleavage by C-S lyase in what is usually the minor pathway for metabolism. Derr and Schultze (1963) demonstrated that the cleavage product combines irreversibly with proteins or other tissue components containing nucleophilic groups, for example, glutathione and cysteine (Bhattacharya and Schultze, 1967), possibly by alkylation. This fragment, which contains the vinyl carbons and sulfur (Bhattacharya and Schultze, 1967) may initiate the chain of events through which the toxicity of DCVC manifests itself.

Toxicology of the Kidney

Several factors result in the kidney being the primary target tissue where DCVC toxicity is expressed. The kidney is the primary organ involved in the excretion of DCVC (Derr and Schultze, 1963), since at physiological pH DCVC behaves as a zwitterion it may be actively secreted into the proximal tubule. Also halogenated vinyl cysteine conjugates are bioactivated in the proximal renal tubule (Bonhaus and Gandolfi, 1981) resulting in high localized concentrations of the conjugate active metabolite in the proximal tubules.

The kidneys are particularly prone to damage by environmental toxins for several reasons (Finn, 1977). First, they constitute
approximately 0.5-1% of the body weight yet receive 20-25% of the cardiac output (Foulkes and Hammond, 1975). Thus, large fractions of a circulating drug or toxin will quickly reach the kidney. Toxins may also alter renal function indirectly by affecting blood flow to the kidney or the distribution of blood flow within the kidney. Second, even if the toxic agents arrive at the kidney in low concentrations, the process of glomerular filtration, tubular reabsorption and particularly secretion may result in high localized concentrations within the kidney. Third, toxic interactions may occur in the kidney that cannot readily occur in other organs. Fourth, the high rate of metabolic activity of kidney tubular cells results in their vulnerability to the actions of metabolic inhibitors. Finally, the kidney is a site of biotransformation, which may occasionally lead to the formation of toxic metabolites.

The action of toxicants may affect one or more of several functions in the kidney. Changes in renal blood vessel resistance, total renal blood flow and distribution of intrarenal blood flow between different regions may alter cortical and medullary perfusion and consequently affect the urinary concentrating mechanisms (Flamenbaum et al., 1976). Such changes may occur directly or via the localized action of the renin-angiotensin system. Compromised glomerular function and reduced clearance rates may result from: glomerular or hemodynamic pressure changes; tubular blockage; back-diffusion of filtered solute through damaged tubular epithelia; increased glomerular permeability. Tubular obstruction may occur because of deposition of foreign material
or destruction of tubular cells (Foulkes and Hammond, 1975). Specific proximal tubule functions which may be altered are sodium chloride, water, glucose, and amino acid reabsorption and organic ion secretion. Finally, small changes in the fine adjustments that determine the composition of the urine in the distal portions of the nephron and the collecting duct may exert major effects on urinary solute excretion.

The susceptibility of the nephron to renal toxins may result from its role as the major functional unit of the kidney. The nephron consists of a glomerular capillary network surrounded by a Bowman's capsule, proximal tubule, loop of Henle and distal tubule which connects with the collecting duct. The major functions of the nephron are formation of an ultrafiltrate at the glomerulus which is essentially protein-free, active reabsorption of solutes from the glomerular filtrate in the proximal tubule and active secretion of certain solutes into the lumen of both proximal and distal tubules. The cells of the proximal tubules are particularly susceptible to chemical insult, possibly because of their high energy requirement and extensive exposure to toxicants during urine formation (Hook, 1980).

The proximal tubules are the major site of action of many nephrotoxicants, particularly halogenated aliphatic chemicals (Kluwe, 1982). Since many xenobiotics are organic anions or cations binding to serum proteins protects them against filtration but does allow active transport by proximal tubular cells. Such transport may lead to either elevated intracellular concentration or high intraluminal concentrations
Potential nephrotoxicants that undergo filtration can gain access to intracellular organelles through direct penetration of the cell membrane, facilitated transport or pinocytosis (Porter, 1982). Once inside the renal tubular cell injury may occur by mechanisms such as uncoupling of the mitochondrial respiratory chain, modification of critical enzyme activity, interaction with the plasma membrane, or other mechanisms (Porter, 1982). Functionally, energy-dependent secretive and reabsorptive activities in the proximal tubule are depressed (Kluwe, 1982). Cytotoxicity is manifested morphologically as a vacuolar degeneration or necrosis of the proximal tubular epithelium.

There is marked structural, biochemical, histological and functional heterogeneity between and within proximal tubules. Heterogeneity is most marked between superficial tubules found in the outer two-thirds of the cortex and the juxtamedullary tubules. Each proximal tubule is composed morphologically of an initial convoluted (pars convoluta) and a terminal straight (pars recta) segment. Histologically and functionally, proximal tubules are comprised of three distinct areas, $S_1$, $S_2$, and $S_3$. The $S_1$ cells comprise the first portion of the pars convoluta and have thick brush borders, numerous mitochondria and highly folded basolateral membranes. The smaller $S_2$ cells are found in the remainder of the pars convoluta and the initial portion of the pars recta. Cells of the $S_3$ region of the pars recta have a more extensively developed brush border membrane and less...
numerous mitochondria than the $S_1$ and $S_2$ cells, and are the location for many enzymes which may function in the biotransformation of xenobiotics (Maunsbach, 1966; Woodhall et al., 1976).

In addition to its role in processing the primary filtrate, the proximal tubule is the site of enzymes responsible for biotransformation, detoxification and excretion of many xenobiotics. In some cases, this biotransformation activity may surpass that of the liver (Anders, 1980). Unlike the liver, the kidney contains several different cell types which are not homogenously distributed throughout the organ. Renal monooxygenase activities and cytochrome P-450 concentrations are highest in the cortex followed by the medulla and the papilla. This corresponds to the distribution of proximal tubular cells in the kidney (Zenser et al., 1978). Proximal tubular cells, and in particular the smooth endoplasmic reticulum of the $S_3$ cells, possess the highest drug metabolizing capability of the mammalian kidney (Kluwe and Hook, 1980). Concentration of cortical phase I enzymes is low; however, phase II enzymes are present in concentrations equivalent to those in the liver (Kluwe et al., 1978; Kluwe and Hook, 1980; Fry and Perry, 1981). Oxidation of xenobiotics by cytochrome P-450 mediated mixed function oxidase occurs in the cortex and possibly the outer medulla. Although the inner medulla has no detectable cytochrome P-450 activity, cytochrome P-450 independent oxidation of xenobiotic compounds may occur here (Zenser et al., 1979). This reaction exhibits highest activities in the inner medullary portion of the kidney and is arachidonic acid-dependent and mediated via prostaglandin co-oxidation mechanisms (Fry
and Perry, 1981). Other brush border membrane-associated enzymes which may be important in renal biotransformation of xenobiotics are peptidases such as γ-glutamyl transpeptidase and cysteinylglycine dipeptidase and lyases such as C-S lyase.

Detection of Nephrotoxicity

All nephrotoxic agents finally alter the histologic appearance of the kidneys, at sufficiently high concentrations, however, functional tests for detecting renal toxicity may provide an indication for predicting nephrotoxicity than structural changes (Diezi and Biollaz, 1979). Ideally, such tests should indicate the site of renal damage induced by low levels and early in the course of exposure to the toxin. Such tests should also take into account that nephrotoxic effects frequently become evident only after a latent period of several days and may be missed in acute toxicity testing (Peters, 1965). Despite the limitations and lack of precision associated with renal function tests, they are useful in the first evaluation of renal toxicity. The parameters measured in renal function tests in vivo are outlined below. Diezi and Biollaz (1979) and Berndt (1976) provide a more detailed critique of these tests.

1. Glomerular filtration rate. This may be evaluated indirectly using plasma levels of endogenous compounds normally filtered, e.g., urea, creatinine, or by using clearance methods which require simultaneous measurement of urine flow and plasma and urine concentrations of a 'glomerular marker' (a freely filtrable substance thought not to permeate the tubular wall in either direction).
2. Renal plasma and blood flow. Electromagnetic flowmeters may be used to measure renal blood flow in large animals. Renal plasma flow, especially in small animals may be determined using urinary clearance of p-amino hippuric acid. Intrarenal blood flow redistribution may be determined using urinary clearance of PAH. Intrarenal blood flow redistribution may be determined using washout from the kidneys of inert gases injected into the renal artery or the intrarenal distribution of radioactive microspheres.

3. Proteinuria -- An increase in total urinary protein excretion is a sensitive indicator of renal damage but does not provide information concerning the site and nature of the lesion.

4. Urine concentration

5. Urinary acidification

6. Urinary excretion of enzymes -- The usefulness of urinary enzyme determinations in evaluation of renal toxicity is still uncertain. Care must be taken that enzyme(s) are not inactivated in the collection vessel. Also, if enzyme concentration in the urine is low, it may be necessary to concentrate the enzymes without inactivation (Berndt, 1976).

7. Urine flow and electrolyte content

8. Microscopic examination of urine sediment

9. Glucosuria

Each of these renal function tests has limitations. Measurements of renal osmolar concentrations, used to determine concentrating
ability, may be altered by extra-renal functions. A similar problem arises with other tests that rely exclusively on urine analysis. Phenol red excretion or PAH clearance tests are of some use in screening for tubular damage, although the results may reflect circulatory changes in addition to effects on renal tubules.

Another approach to measuring renal function is to evaluate the effects of a nephrotoxin using isolated organs or isolated tissue. This overcomes the problem of delineating between those toxin-induced renal changes which are nephrogenic as opposed to those mediated by extra-renal effects. In particular, the metabolic and excretory intervention of other organs make it impossible to establish in vivo the exact role of the kidney in metabolic clearance from the plasma (Bach and Lock, 1982). Conversely, in vitro systems may lack the metabolic, neural, hormonal or vascular components necessary to produce certain types of toxic response. Comparison of in vivo and in vitro responses may be used to differentiate primary from secondary mechanisms of action.

In vitro systems such as isolated perfused organs or isolated tissues or cells provide economical systems with which to predict animal response to chemical exposure while utilizing fewer animals than would be required for in vivo studies. Also, the interaction between toxicants and tissues at the cellular and molecular levels is often more easily studied in vitro (Kluwe et al., 1982). Therefore in vitro systems were used extensively in this study of haloalkene biotransformation and associated nephrotoxicity.
The isolated perfused organ offers advantages for toxicological studies in that delivery and removal of chemicals proceeds via normal vascular channels without distribution to the peripheral tissue. The preparation functions as an intact organ in which all of the membrane barriers between cells have been maintained. In addition, substances may be investigated over a broad concentration range where upper limits may be detrimental to the whole animal (Bach and Lock, 1982). Disadvantages include short viability since physiological and biochemical integrity can only be maintained for approximately 4 hr (Bach and Lock, 1982). Also changes in renal hemodynamics, including a shunt of blood/perfusate from the outer to the inner cortex (Itskowitz et al., 1973; Gagnon et al., 1974) may produce changes in the concentration and kinetics of toxicants. Finally the limited viability of in vitro preparations generally restricts their use to the analysis of lesions produced acutely by the chemicals in vitro.

Although the isolated perfused kidney preparation enables each animal to serve as its own control, the use of perfused organs is not economical when compared to the numbers of treatments which may be tested using tissue slices or isolated tubules. Also, the complex functions and highly organized structure of the kidney may limit the perfused organ for the investigation of separate functions at the cellular and subcellular level. For this purpose, isolated tissue and cell preparations constitute valuable model systems.
Two of the most widely-used in vitro preparations for renal toxicity studies are the renal cortex slice and the isolated renal tubule. In both preparations, specific transport functions may be studied without the influence of glomerular filtration rate or renal blood flow, with precise control over experimental parameters (Berndt, 1976) and with variation of experimental parameters over a broader range than can be achieved in intact animals (Cross and Taggart, 1950). Changes in tubular active transport may be a useful indicator of nephrotoxicity (Kluwe, 1981) since the proximal tubules are a principle site of nephrotoxin action (Balazs et al., 1963; Hook, 1981), and were used in the present study to investigate nephrotoxicity of haloalkenes and their potential metabolites. Therefore, these in vitro preparations are extremely useful in studying nephrotoxin action, as has been demonstrated for several nephrotoxic halogenated hydrocarbons (Watrous and Plaa, 1972). By dividing tissue from one animal several parameters may be studied while minimizing tissue variability (Hirsch, 1976). Use of slices or isolated tubules also provides an opportunity to compare animal and human material when both are exposed to toxicants under controlled conditions (Bach and Lock, 1982). In both the tubule suspensions and cortical slices intact nephron segments exist, but in the slice normal anatomical configuration persists. A disadvantage of slices compared to isolated tubules is that differences in oxygen tension and solute concentration can occur between cells in slices since the outermost tissue layers may act as a barrier between the bathing
medium and the innermost cells (Burg and Orloff, 1962). In tubule suspensions no such complex anatomical arrangement persists and there is direct contact between tubular cells and the compound studied. Also two discrete compartments, cells and medium, are involved in the transport process and there is rapid equilibration between cells and medium.

Renal Tubule Active Transport

The renal proximal tubule has two major secretory systems for the elimination of xenobiotics, one for organic anions, and the other for organic cations. In mammals, the physiological role of these active secretory processes is accumulation of organic anions which may potentially be metabolized by the renal tubular cell (Berndt, 1982). The physiologic role of cation secretion is not known; however, from a toxicological viewpoint this process is important since many xenobiotics are secreted by these transport processes (Berndt, 1982). Paraaminohippuric acid (PAH) and tetraethylammonium bromide (TEA) are often used as prototype substances to study the organic anion and cation transport systems respectively. These compounds are utilized since they are not normally found in the urine, are secreted avidly and independently of each other; not extensively protein bound, and not metabolized.

Evidence from clearance (Malvin et al., 1958) and in vitro studies indicates separate transport mechanisms for anions and cations (Farah and Rennick, 1956; Holohan et al., 1975). The highest rate of secretion of PAH occurs in the $S_2$ and $S_3$ segments of the proximal tubules (Irish and Grantham, 1980). TEA transport also occurs in the proximal tubule, but the specific location is not known (Berndt, 1982).
Intracellular accumulation of organic anions is energy dependent indicating active transport (Kinsella et al., 1979). Tune et al. (1969) proposed that movement of PAH from the blood into the tubule cells occurs by carrier-mediated active transport across the antiluminal membrane resulting in accumulation in the proximal tubular cell. Net movement of PAH across the luminal membrane then occurs down an electrochemical gradient. Reabsorption is negligible compared to the secretory flux (Moller and Sheikh, 1982). Berner and Kinne (1976) provided evidence that the flux of PAH from the cell to the lumen was by simple diffusion; however, Foulkes (1977) proposed the existence of a second transport step across the luminal membrane involving facilitated transport through pores. It is not known whether antiluminal active transport involves a single carrier with multiple affinities or multiple carriers with different affinities for specific organic anions, although evidence is accumulating to support the latter theory (Irish and Grantham, 1980). Ligandin, a renal organic anion binding protein may function in this transport (Kirsch et al., 1975). Recent studies indicate that PAH may be co-transported with Na+ and transported electroneutrally in a 1:1 complex with Na+ (Sheikh and Moller, 1982). The thermodynamic energy inherent in the Na+ gradient is not thought sufficient to account for intracellular PAH accumulation and other energy sources such as direct utilization of metabolic energy or exchange with endogenous anions may provide energy for this process (Moller and Sheikh, 1982).
Less is known about the secretion of organic cations in the proximal tubules. A carrier for organic cations is thought to exist on the luminal membrane (Kinsella et al., 1979), while antiluminal transport occurs by facilitated diffusion through pores (Holohan and Ross, 1980). In summary, the current concept of transepithelial transport for PAH involves active transport at the antiluminal membrane associated with a carrier and facilitated diffusion through pores at the luminal membrane. The reverse situation occurs for TEA transport (Kinsella et al., 1979).

The renal proximal tubules are the major site at which toxicity of the vinyl cysteine conjugates is expressed (Strafuss and Sautter, 1967; Lock, 1982). All active secretion of organic anions and cations occurs in the proximal tubules and this secretion is sensitive to the effects of nephrotoxins (Kluwe, 1982). Therefore, measurement of active accumulation of anions and cations in tubules was widely utilized in the following studies to assess vinyl cysteine conjugate-induced nephrotoxicity.
Figure 1. Proposed metabolic pathway for the bioactivation of nephrotoxic halogenated ethylenes. Following glutathione conjugation in the liver, peptidase and C-S lyase cleavage in renal proximal tubules result in formation of a toxic metabolite.
Figure 2. Proposed mechanism for the metabolism and bioactivation of halogenated vinyl cysteine conjugates, using DCVC as the prototype conjugate.
METHODS

Materials for Tubule Isolation.

New Zealand white rabbits, 600-800g, were obtained from Blue Ribbon Ranch, Tucson, and maintained for at least 7 days on a 12 hr light, 12 hr dark cycle and fed ad libitum with Lab Blox (Purina Co.). Buffered saline consisted of: 120 mM NaCl; 10 mM CH₃COONa·3H₂O; 5 mM KCl; 1.2 mM MgSO₄·7H₂O; 1.2 mM KH₂PO₄; 2.5 mM CaCl₂·2H₂O; 30 mM NaHCO₃; pH 7.4. 210 micron and 64 micron Nitex monofilament screen sieve were from Tetko Inc. ¹⁴C-para-amino hippuric acid (PAH) (0.9 mCi/mmol), ¹⁴C-tetraethylammonium bromide (TEA) (0.9 mCi/mmol) and ³H₂O (2 mCi/g) were obtained from New England Nuclear (Boston, MA). Silicone fluid, density 1.03, was from Contour Chemical Co. (N. Reading, MA).

Chemical Syntheses.

1. Vinyl cysteine conjugates

S-(trans-1,2-dichlorovinyl)-L-cysteine (DCVC) and S-(pentachlorobuta-1,3-dienyl)-L-cysteine (PCBC) were synthesized via the method of McKinnney et al (1959). This involves reaction of trichloroethylene or hexachloro-1,3-butadiene respectively with cysteine in liquid ammonia using Na as a base. After evaporation of the ammonia the residue was extracted repeatedly with ether to remove residual ethylene, dissolved in water and precipitated with acetic acid. The product was recrystallized from ethanol and water. S-(trans-1,2-difluoro-1-chlorovinyl)-L-cysteine (HVC-1) and S-(1-chloro-2,fluorovinyl)-L-cysteine

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(HVC-2) were also synthesized using this method as described by Gandolfi et al (1981). The parent compounds for these syntheses were chlorotrifluoroethylene and 1-chloro-2,2-difluoroethylene, respectively. The saturated structural cysteine analogue, S(1-hydroxyethyl)-L-cysteine (HEC) was synthesized by the method of Carson and Wong (1964) from 2-bromoethanol (2BE). All compounds were characterized as described by Gandolfi et al (1981).

2. Vinyl glutathione and N-acetyl cysteine conjugates

S-(trans-1,2-dichlorovinyl)-L-glutathione (DCVG) and S-{1,2-dichlorovinyl}-L-N-acetyl-cysteine (DCV-NAC) were synthesized by the method of McKinney et al. (1959). Glutathione and N-acetyl cysteine respectively were used as substrates for these syntheses.

3. Saturated CTFE glutathione and cysteine conjugates

The glutathione conjugate of CTFE was synthesized by reacting N,N-dimethyl-formamide (Matheson, Coleman and Bell, Cincinnati, OH), trimethylamine (J.T. Baker, Phillipsburg, N.J.) and glutathione in the presence of CTFE gas (Matheson Gas Products, E. Rutherford, N.J.) at room temperature in a sealed reaction vessel. CTFE was flushed out of the reaction vessel to terminate the reaction. The product, identified by mass spectrometry, was the saturated conjugate S-(2-chloro-1,1,2-trifluoroethyl)-L-glutathione (CTFE-GSH). This method was also used to synthesize the saturated cysteine conjugate, S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine (CTFE-cys), using cysteine instead of glutathione.
4. Substrates for C-S Lase Assays

S-(2-benzothiazolyl)-L-cysteine was synthesized by the method of Dohn and Anders (1982a) from 2-chlorobenzothiazole and cysteine. N-acetyl-S-(6-chloro-2-methoxy-9-acridinyl)-L-cysteine was synthesized from quinacrine and N-acetyl cysteine by reaction at room temperature and pH 7.0 as described by Wild and Young (1965).

Preparation of Isolated Renal Tubule Suspensions.

Tubules were isolated from rabbit kidney cortex using the method of Brendel and Meezan (1975). Young animals (4-6 weeks), with little fibrous tissue in the kidney cortex, are required for this isolation since the technique utilizes tissue homogenization without prior collagenase digestion. Also, the kidneys of rabbits this age are sufficiently large to provide tubules for up to 50 separate incubations. The kidneys of most newborn animals are immature both in structure and function. The ability of rabbit renal cortical tissue to accumulate PAH is low at 2 weeks, increases to a maximum at four weeks then slowly decreases to a plateau (Ecker and Hook, 1974). However, the intrinsic transport capacity for PAH in New Zealand white rabbits is mature at 4 weeks of age (Pegg and Hook, 1975), so animals of this age were used in all studies. Rabbits were killed by a shot at the base of the skull using an air pellet gun. Both kidneys were cannulated via the renal arteries, flushed with buffered saline, pH 7.4 and connected to a perfusion apparatus. The capsule was removed and the kidneys perfused (non-recirculating) for 10 minutes with oxygenated, buffered saline at
37°C. After perfusion the cortex (approximately 5 g) was removed and hand homogenized in 10 volumes of buffered saline using ten strokes in a glass homogenizer with a loose-fitting tapered teflon pestle. The homogenate was filtered through a 210 micron sieve and washed by spraying with 700-800 ml of bicarbonate buffered saline. The filtrate was poured onto a 64 micron sieve and washed by spraying with 300 ml of buffer. The tubules were collected into a corner of the sieve using a stream of buffered saline from a wash bottle, washed into a siliclad coated glass container with buffer containing 1% albumin (Fraction V, Sigma Chemical Co.) and the volume adjusted to 100 ml. The amount of tubules present in a suspension was determined by removing 1 ml aliquots, centrifuging in a Beckman Microfuge 152, precipitate tubules in a pellet, and pellet weight measured. These suspensions contained approximately 95% of proximal tubules, the remaining 5% was comprised of glomeruli and distal tubules (Dr. R.B. Nagle, Pathologist, University of Arizona, pers. comm.).

**Determination of Active Transport in Isolated Tubules.**

Active transport was quantified in isolated renal tubules after exposure to halogenated vinylthio conjugates (HVT) and saturated conjugates *in vivo* and *in vitro*. Acid transport was quantified using PAH and base transport using TEA. Preliminary studies indicated that incubation with 0.01 mM PAH and TEA resulted in maximal tubule to medium (T/M) values. Fifteen μl of 14C-PAH and 15 μl of 3H2O were added simultaneously with cold PAH to quantify acid transport in isolated
tubules, and 15 µl of $^{14}$C-TEA and 15 µl of $^3$H₂O together with cold TEA to quantify base transport. $^3$H₂O was added as an aqueous volume marker. Samples of the tubule suspensions were removed after 15 min, at which time the T/M had plateaued, and placed in microfuge tubes containing 0.3 ml of silicone oil of a density sufficient to separate tubules from the incubation medium. After a 5 second spin at 12,500 rpm (Beckman Microfuge B), tubules formed a pellet at the bottom of the tube while the suspension medium remained on top of the silicone fluid. The $^{14}$C and $^3$H ratio were determined in the tubule pellet and the supernatant medium dissolved in Toulene/Triton X 100/Omnifluor liquid scintillation fluid. Counts were measured in a Searle Analytic 81 Liquid Scintillation system (Searle, Chicago, IL) programmed to count $^{14}$C and $^3$H dual labelled samples. The tissue to medium ratios for PAH and TEA were calculated from these values.

**In Vivo and In Vitro Exposure to Saturated and Unsaturated Haloalkene Conjugates**

**In vivo exposure.** Animals received an intraperitoneal injection with 2 ml of buffered saline (control) or saline containing DCVC or DCVG equivalent to 20, 50, 100 or 200 mg/kg. Animals were terminated 1 hr after injection by a shot at the base of the skull using an air pellet gun. The kidneys were cannulated via the renal arteries, removed perfused with 50 ml of buffered saline. The kidneys were then connected via the cannulae to a perfusion apparatus and perfused for 10 min using non-recirculation. A cortex sample removed and placed in 10% buffered
formalin for later microscopic examination after staining with hematoxylin and eosin. Tubules were isolated from the remaining cortex (within 15 min after death).

**In vitro exposure.** 1. Perfused kidney. Untreated rabbits were killed, both kidneys were cannulated via the renal arteries and flushed with buffered saline, pH 7.4. The kidneys were then connected to a perfusion apparatus powered by a Harvard Apparatus variable speed peristaltic pump, the capsule was removed and the kidneys perfused (non-recirculating) for 10 min with oxygenated, buffered saline at 37°C. Perfusion pressure was maintained at 100-120 mm Hg and corresponded to a flow rate of 5 ml/g tissue/min. The buffer was then replaced with fresh buffer (control) or with saturated or unsaturated haloalkene conjugates (0.01-1 mM) dissolved in buffered saline and the kidneys perfused (recirculating) for 45 min prior to tubule isolation. The perfusate was oxygenated and maintained at 37°C. Every 15 min the perfusate was removed from the perfusion apparatus and replaced with fresh buffer containing the appropriate concentration of the haloalkene conjugate.

2. Isolated tubules. Kidney tubules were isolated from untreated rabbits and suspended in 1% albuminized buffer. Two ml aliquots (8mg wet weight tubules per ml) of the suspension were incubated with buffer (control) or buffer containing 0.01-1 mM HVT in 20 ml siliclad coated glass scintillation vials in a 95% O₂/5% CO₂ atmosphere at 37°C for 45-60 min for glutathione and N-acetyl cysteine conjugates and 15 min for cysteine conjugates prior to the determination of active transport.
To determine the effect of γ-glutamyl transpeptidase inhibition on DCVG-induced inhibition of tubule active transport, tubule suspensions were incubated for 30 min with 2 mM of the irreversible γ-glutamyl transpeptidase inhibitor AT-125 (L(α S,5S)-α-amino-3-chloro-4.5-dihydro-isoazoleacetic acid, Upjohn Co., Kalamazoo, MI) or 20 mM of the rev serine/borate (a reversible γ-glutamyl transpeptidase inhibitor) prior to a 45 min incubation with DCVG.

In Vivo and In Vitro Exposure to TCE

1. In vivo – Two rabbits (850 g, 600 g) were exposed to TCE using slow-release sealed vials surgically implanted under the skin of the neck. One ml glass Hypo vials (Pierce Chemical Company) were filled with TCE, sealed with silicone rubber septa (Pierce Chemical Co., Rockford, IL), and one vial implanted on each side of the neck. For three days prior to implantation the rabbits were housed separately in metabolism cages and provided with water and food ad libitum. Water consumption and urine production were measured at 8:00 a.m. and 8:00 p.m. each day and urine content analyzed as described below. Twenty four hr before TCE implantation hepatic mixed function oxidase activity was decreased in one rabbit by ip injection of 150 mg/kg allyliso-propylacetamide (AIA) (Hoffman LaRoche, Nutley, N.J.) in 2 ml saline. The animal also received 150 mg/kg AIA daily for three days during exposure to TCE.

Prior to implantation rabbits were anesthetized using intramuscular injection of 0.3-0.4 ml of the neuroleptic, acepromazime
maleate (10 mg/ml, Ayerst, New York, N.Y.) and 25 min later with 1.2-1.5 ml of the anesthetic, ketamine hydrochloride injected intramuscularly (50 mg/ml, Parke Davis, Morris Plain, N.J.). After 15 min the neck was shaved and 1.5 cm incisions made through the skin on either side of the neck. The skin was separated from the underlying tissue to form a pouch approximately 4 cm deep and 2 cm wide on each side of the neck and one vial placed septum down in each pouch. The incisions were stitched closed with 000 silk suture (Ethicon, Inc., Somerville, N.J.). To prevent infection, both animals were injected i.m. with 0.05 ml gentamicin sulfate (40 mg/ml) (Schering Corp., Kenilworth, N.J.) in 0.4 ml of buffered saline. Both rabbits were returned to metabolism cages and urine production and water consumption monitored every 12 hr for 60 hr. Animals were then killed by a shot at the base of the skull using an air pellet gun. Renal tubules were isolated and active transport of PAH and TEA determined. The glass Hypo vials were removed, weighed and the amount of TCE released determined.

Urine Analysis. All parameters were determined immediately after urine collection except osmolality and creatine concentrations which were determined in urine stored at -20°C until all samples had been collected. Urine glucose, bilirubin, ketone, protein and blood were measured using Bili-Labstix reagent strips (Ames Division, Miles Laboratories, Inc., Elkhart, IN). Urine osmolality was determined using a 5100B Vapor Pressure Osmometer (Wescor, Inc., Logan, UT). The method of Loken (1954) was used to measure urine creatinine. To 0.25 ml urine
0.75 ml 10% trichloroacetic acid was added, the sample spun at 12,500 rpm for 1 min in a Beckman microfuge. To 0.2 ml of the resulting supernatant, 3 ml of distilled water and 3 ml of working reagent (Hycel, Inc., Houston, TX) were added and OD$_{520}$ measured after 20 min using a Zeiss PM2 DL Spectrophotometer (Carl Zeiss, West Germany).

2. Isolated perfused liver. New Zealand white rabbits (800-900g), either untreated control or treated with 150 mg/kg AIA ip 3 hr prior to liver removal, were anesthetized with ether and the body cavity opened surgically. The hepatic portal vein was isolated and cannula of the perfusion apparatus, a grooved 15 gauge stainless steel needle, inserted distal to the lineal branch. The kidneys were bisected to allow for outflow of perfusate, the liver perfused with approximately 10 ml/min of oxygenated buffered saline, pH 7.4, 37°C, and the vena cava cut to kill the animal. The bile duct was isolated and cannulated with polyethylene tubing, internal diameter 0.015 inches. The liver was removed, the perfusion rate increased to 10 ml/gm/min and perfused for 15 min, non-recirculating, with bicarbonate buffered saline, pH 7.4. The perfusion apparatus was filled with fresh buffer and the liver perfused for 30 min using recirculation. To increase the liver glutathione content, four aliquots of glutathione were added to the perfusate at intervals of 7.5 min. Each bolus of glutathione was sufficient to result in a perfusate concentration of 4 mM. After 30 min the buffer was removed, replaced with fresh buffer containing 1% albumin, and the liver perfused with recirculation for 1 hr. Throughout this 1 hr perfusion,
the liver was exposed to TCE vapor in the 95% O₂/5% CO₂ gas stream used to oxygenate the perfusion buffer. During the 1 hr perfusion 15 ml of TCE, maintained at 50°C to increase vapor pressure, was evaporated. Throughout the perfusion bile was collected in a separate container. After 1 hr the perfusate was removed from the apparatus.

Renal tubules were isolated from a different rabbit and suspended either in the liver perfusate or in 1% albuminized buffer to which the bile collected during liver perfusion was added. Bile concentration was adjusted to that which would have been present in the liver perfusate if the bile had not been collected separately, approximately 2% by volume. After 30 min the tubules were removed, resuspended in fresh 1% albuminized buffer and active PAH and TEA transport determined.

3. Isolated renal tubules. TCE was dissolved in dimethyl sulfoxide (DMSO) to concentrations of 1, 0.1, and 0.01 M. Twenty μl of each stock solution of DMSO was added to 2 ml tubule suspensions (6-8 mg/ml tubule wet weight) in 20 ml glass scintillation vials to give final concentrations of 1, 0.1 and 0.01 mM. The vials were gassed with 95% O₂/5% CO₂, sealed and incubated at 37°C for 20 min in a rotating incubator. Tubules were then resuspended in fresh buffer and active PAH and TEA transport measured.

In Vitro Exposure to CTFE

Active PAH transport. Two ml aliquots of renal tubule suspensions (6-8 mg/ml tubule wet weight) were placed in 25 ml glass
Erlenmeyer flasks with a cone-shaped raised base. The flasks sealed with a screw top, air-tight lid incorporating a two-way stopcock valve. The flasks were gassed with 95% O\textsubscript{2}/5% CO\textsubscript{2} for 1 min, the outlet valve closed and 1, 2.5 or 5 ml CTFE gas injected through the inlet valve using a gas-tight 5 ml glass syringe (Precision Sampling Corp., Baton Rouge, LA). An appropriate volume of air was injected prior to CTFE to insure that total injected gas volume was 5 ml. The sealed flasks were placed in a gyrotory water bath shaker Model G76 (New Brunswick Scientific Co., New Brunswick, N.J.) and incubated at 37°C for 90 min. Tubule suspensions incubated after injection of 5 ml ether or halothane vapor, or air, were used as controls. Some tubule suspensions were incubated with 2 mM of the \gamma-glutamyl transpeptidate inhibitor, AT-125 for 15 min prior to CTFE exposure. After 90 min tubules were removed, resuspended in fresh buffer and active PAH transport measured.

**Nonprotein sulfhydryl.** Five ml tubule suspensions (14 mg/ml tubule wet weight) were sealed in air tight 25 ml Erlenmeyer flasks with a 95% O\textsubscript{2}/5% CO\textsubscript{2} atmosphere. After injection of 5ml CTFE into the flasks tubule suspensions were incubated at 37°C in a gyroratory water bath for 0, 10, 20, 30, 45, 60, 90, and 120 min. Tubules were removed at the appropriate time and non-protein sulfhydryl determined by the method of Sedlak and Lindsay (1968), as described later.

**Gas Chromatographic analysis.** Five ml tubule suspensions (14 mg/ml tubule wet weight) were incubated in 25 ml glass Erlenmeyer flasks
sealed with Tuf-Bond 18 mm Teflon-silicone discs (Pierce Chemical Co., Rockford, IL). CTFE (0.1 or 0.25 ml) was injected into the flasks through the Teflon-silicon disc using a 1 ml air-tight syringe. Separate flasks were used for each time point. After 0, 10, 20, 30, 45, 60, 90 and 120 min 1 ml samples of head-space gas were removed using a 1 ml glass gas-tight syringe, and CTFE concentration determined by gas chromatographic analysis. A Hewlett Packard 5830A gas chromatograph (Hewlett Packard, Avondale, PA), equipped with a flame ionization detector and a Hewlett Packard 1885A GC terminal integrator was used for these measurements. The gas sample was injected into a 1.8 x 2 mm I.D. stainless steel column packed with Poropak Q 100-120 mesh. The injection port was maintained at 220 °C and the detector at 240 °C. Flow rates were 30 ml/min for the nitrogen carrier gas, 300 ml/min for air and 30 ml/min for hydrogen.

Amino acid analysis. Five ml tubule suspensions were incubated with 0.1 ml or 0.25 ml CTFE for 10-120 min in sealed 25 ml glass Erlenmeyer flasks. One ml aliquots were removed from the suspensions, centrifuged for 1 min at 12,500 rpm in a Beckman microfuge and 0.5 ml of the supernatant removed. This was deproteinated by adding 5% trichloroacetic acid and a Beckman Model 121 Automatic amino acid analyzer (Beckman Instrument, Inc., Palo Alto, CA) used to assay the supernatant for the presence of CTFE-GSH. The sodium citrate buffer elution program used in the amino acid analyzer was buffer pH 2.85 from 0-60 min, pH 3.40 60-116 min, pH 4.29 116-162 min, pH 5.30 162-220 min and pH 6.40.
for 220-260 min. Under these conditions a CTFE-GSH standard eluted at approximately 120 min.

**In Vitro Biotransformation of DCVG**

Two ml tubule suspensions in 1% albuminized buffer (20 mg/ml tubule wet weight) were incubated in glass scintillation vials under standard conditions. Five mM DCVG was added and tubules incubated for 0-120 min using a separate suspension for each time point. Every 10 min, 1 ml of suspension was removed and spun at 12,500 rpm for 1 min in a Beckman microfuge. To 0.8 ml of supernatant 0.08 ml of 50% TCA was added and the solution spun at 12,500 rpm to pellet precipitated protein. A 0.25 ml aliquot of supernatant was analyzed for the presence of DCVG, DCVC, glutamate, glycine and ammonia using a Beckman Model 121 automatic amino acid analyzer with conditions set as described previously. Approximate elution times for standards were: Glutamate, 114 min; DCVG, 118 min; glycine, 120 min; DCVC, 160 min; ammonia, 235 min.

**Tissue Histology**

Small pieces of cortex, approximately 1mm by 1mm, were removed from kidneys exposed to DCVC or PCBC in vivo or during perfusion of isolated kidneys. Tissue was placed in buffered formalin, pH 7.4, and later embedded in paraffin wax, cut into 6 µ sections and stained with hematoxylin and eosin. Mounted sections were examined using light microscopy for evidence of necrosis, dilation of tubule lumens, sloughing of tubule brush border membranes, and accumulation of eosinophilic debris in the lumen of tubules.
Renal Tubule Oxygen Consumption

Tubule oxygen consumption was measured by the method of Brendel and Meezan (1973). Consumption measurements were made using a Clark oxygen electrode obtained from Yellow Spring Instrument Company (Yellow Springs, OH) and measurements recorded on a Linear Instruments Corporation chart recorder. Measurements were made in a cell thermostated to 37°C containing 1.9 ml of buffer which had previously been equilibrated with air. To determine the effect of DCVC on O₂ consumption tubule suspensions in 1% albuminized buffer were exposed to DCVC or buffered saline for 30 min at 37°C in a 95% O₂/5% CO₂ atmosphere. Tubules were separated from the suspension by gravity settling for 1 min, removed with a pipette and resuspended in 1.9 ml of aerated buffer in the thermostated cell. This suspension was continuously stirred with a magnetic stir bar to prevent tubules settling. The cell was stoppered and O₂ consumption measured continuously over a 15 minute period. A 1 ml sample of the tubule suspension was then removed, centrifuged at 12,500 rpm for 5 sec and the pellet weighed to relate O₂ consumption per unit time to the wet weight of tubules. A suspension of tubules treated with methanol for 10 min was used as a tubule blank.

Metabolism of ¹⁴C-glucose and ¹⁴C-succinate by Isolated Tubules

Measurement of ¹⁴CO₂ from tubules incubated with ¹⁴C-glucose or ¹⁴C-succinate was accomplished using the method and apparatus of Brendel and Meezan (1974). Sixteen vials were incubated simultaneously enabling duplicate incubations for each parameter. ¹⁴CO₂ evolution was
measured for four experimental conditions; buffer with no tubules added; tubule suspension in buffer; tubule suspension in the presence of 0.05 mM DCVC; tubule suspension in the presence of 1 mM DCVC. Tubules were incubated in buffer or DCVC for 45 min prior to addition of glucose or succinate.

Cold and radiolabelled substrated were added at the following concentrations; glucose 5 mM, 14C-glucose 1 μCi/ml. The stoppered vials containing 1 ml of buffer or tubule suspension were incubated at 37°C for 1 hr. Vials containing 1.5 ml of 0.3 N NaOH, used to trap the 14CO2 evolved, were exchanged every 15 min and analyzed for 14C content.

Modification and Assay of Tubule Non-protein Sulfhydryl

Exogenous GSH (6 mM) was added simultaneously with, or 15 min after DCVC exposure in isolated tubules, and immediately after tubule isolation from animals exposed to DCVC in vivo. To study the potential prophylactic effect of GSH, tubules were incubated with 5-15 mM GSH for 15 min prior to incubation with DCVC. Active accumulation of PAH and TEA by tubules was determined after a 15 min incubation with GSH. Other potential sulfhydryl donors, cysteine (1 mM) and cysteamine (0.1 mM) were also utilized in place of GSH.

Endogenous GSH in isolated tubules was depleted by a 10 min pre-incubation in the presence of 0.5 - 2.5 mM diethyl maleate, dissolved in DMSO to a final concentration of 1% DMSO in the incubation buffer or by incubating tubules with 1.5 mM glycidol. The diethyl maleate or glycidol solutions were then replaced with buffered saline or
saline containing DCVC and the tubule preparation incubated for 15 min. After a final 15 min incubation with buffered saline or saline containing 6 mM GSH, PAH and TEA transport were determined.

Tubule non-protein sulfhydryl content (NP-SH) was determined in controls and tubules incubated with CTFE, diethyl maleate or exogenous GSH using the method of Sedlak and Lindsay (1968). An aliquot (0.2 ml) of tubule suspension containing 0.03 ml of tubule packed volume was sonicated in 25 volumes of ice-cold 0.05 M Tris and 1 mM EDTA, pH 7.4. Fifty ul of 5% sulfosalicylic acid was added and the solution spun at 12,500 rpm for 2 min in a Beckman Spinco 152 microfuge. To 1 ml of supernatant and 4 ml of 0.2 M Tris and 10 mM EDTA, pH 8.9 100 µl of Ellman's reagent (99 mg 5', 5' dithiobis-nitrobenzoic acid in 25 ml methanol) was added. The absorbance at 412 nm was determined after 5 min using a Zeiss PM 2 DL Spectrophotometer.

C-S Lyase Assays

Enzyme activity was determined in isolated renal tubules, cortex homogenates and in renal tubule brush border membrane suspensions.

1. Isolated renal tubules. Tubules were prepared from the kidneys of rabbits and suspended in the appropriate buffer for C-S lyase assay at a concentration of 20 mg/ml tubule wet weight.

2. Kidney cortex homogenate. Rabbit kidneys were perfused with approximately 50 ml of bicarbonate buffered saline, pH 7.4, to remove blood. Approximately 2 x 2 mm pieces of cortex were removed with forceps and added to 9 volumes of the appropriate buffer. Twenty ml
aliquots of the buffer/cortex mixture were homogenized for 20 sec with a Brinkmann PT 10-35 Homogenizer and PTA-10TS anaerobic probe generator (Brinkmann Instruments, Westbury, N.Y.) to provide a 10% homogenate (approximately 20 mg/ml protein).

3. Renal brush border membrane. Isolated brush border membrane suspensions were prepared by the method of Malathi et al. (1979). Fresh cortices were homogenized in 30 volumes (w/v) of ice-cold 50 mM mannitol/2 mM Tris-HCl, pH 7.0 in a Brinkmann Homogenizer. One M CaCl$_2$ solution was added to the homogenate to a final concentration of 10 mM and the homogenate stirred for 10 min at 4°C. The homogenate was then centrifuged for 15 min at 3000 xg in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge (Sorvall, Newton, CT). The supernatant was carefully decanted and centrifuged again for 20 min at 43,000xg. The pelleted material, representing brush border membranes, was resuspended in an equal volume of the same buffer and centrifuged again for 20 min at 43,000xg. The brush border membranes were then resuspended at a concentration of 1 ml per g of cortex (4 mg protein/ml) in buffer appropriate for the particular C-S lyase assay.

C-S lyase was assayed in tissue samples using one of the four following methods:

1. Evolution of pyruvate. One ml of renal tubule brush border membrane suspension (5-8 mg/ml. protein) isolated from untreated kidneys or kidneys perfused with 1 mM DCVC for 60 min, were incubated with 0.01 - 1 mM DCVC, used as a C-S lyase substrate in the assay, for 15 min at
37°C. The reaction with DCVC was stopped by the addition of 3 ml perchloric acid (6%). After thorough mixing the solution was centrifuged at 3000 xg for 5 min in a Sorvall GLC-2 bench top centrifuge. The supernatant was decanted into glass test tubes in ice and 0.01 ml of 0.05% methyl orange added as a pH indicator. Potassium carbonate, 5M, was added slowly until the solution became salmon-pink (pH 3.5). After standing for 10 min the solution was centrifuged at 4°C and 3000 g for 3 min and the supernatant decanted. One volume of 0.4 M triethanolamine buffer, pH 7.6, was added to 2 volumes of supernatant and mixed. The resulting solution was placed in a glass cuvette, 0.1 ml of 5 mM reduced β-nicotinamide adenine dinucleotide added (Sigma Chemical Co., St. Louis, MO) and OD₃₄₀ measured with a Beckman Acta V scanning spectrophotometer. Five μl of lactate dehydrogenase, type II (Sigma Chemical Co.), 10 mg/ml, was added to the cuvettes and the change in absorbance measured over a 3 min incubation.

2. Evolution of ammonia. Ammonia evolved from renal brush border membrane suspensions, incubated with DCVC as a C-S lyase substrate, was determined by two methods. Both methods utilized a modification of the Conway microdiffusion technique (Conway, 1947) for ammonia collection. Ammonia was then quantified using Nesslers reagent or by fluorescence. For this assay using Nesslerization brush border membrane suspensions were prepared from untreated tubules, or tubules incubated with 1 mM DCVC for 15 min. One ml of suspension was placed in a 5 ml glass test-tube and the tube sealed with a rubber septum stopper
from which a polyethylene well containing 0.5 ml 0.6 N H₂SO₄ was suspended. DCVC stock solution (0.01 ml) was injected through the rubber septum into the brush border membrane suspension to give a final concentration of 1, 0.1, or 0.01 mM DCVC. The tubes were placed in a gyrotary water bath and incubated for 15 min at 37°C. To terminate the reaction with DCVC, 1 ml of saturated K₂CO₃ was injected through the rubber septum into the brush border membrane suspension and the sealed tubes gently shaken for 2 hr. Tubes were then opened, the H₂SO₄ containing ammonia evolved from DCVC was removed and ammonia content determined by the Nessler technique (Archibald, 1944).

Ammonia evolved from renal brush border membrane suspensions incubated with 0.01-1 mM DCVC or 0.01-0.2 mM S-(2-benzothiazolyl)-L-cysteine was also assayed with a fluorescence technique. O-phthalicdicarboxaldehyde (OPA) (Aldrich Chemical Co., Milwaukee, WI) was used as the acceptor for ammonia in this assay. The OPA assay solution, made fresh daily, contained 1.5 ml of 10 mg OPA/ml in ethanol, 90 ml borate buffer pH 10.4 and 1.5 ml of 2-mercaptoethanol. The assay was similar to that in which ammonia was measured by Nesslerization, but OPA isolation was substituted for H₂SO₄ in the polyethylene well. After 2 hr the OPA solution was removed and fluorescence measured at 345 nm excitation and 455 nm emmission with a Aminco Bowman Spectrofluorometer and an Aminco photomultiplier microphotometer attachment (American Instrument Co., Inc., Silverspring, MD).

3. S-(2-benzothiazolyl)-L-cysteine cleavage. C-S lyase activity was assayed by the method of Dohn and Anders (1982) using
S-(2-benzothiazolyl)-L-cysteine as the enzyme substrate. One ml of a 10% (w/v) kidney cortex homogenate (20 mg/ml protein) in 0.1 M potassium borate buffer was incubated with 1 ml of 4 mM S-(2-benzothiazolyl)-L-cysteine for 15 min. at 37°C. Addition of 0.4 ml 50% trichloroacetic acid terminated the reaction, and protein was precipitated with a 60 sec. spin at 12,500 rpm in a Beckman microfuge. The supernatant OD$_{321}$ was measured with a Zeiss PM2 DL spectrometer. Formulation of the product, 2-mercaptobenzothiazole, was quantified by comparison of OD$_{321}$ with 2-mercaptobenzothiazole (Aldrich Chemical Co.) standards. Enzyme activity was also determined in renal tubules and renal brush border membranes. To characterize this assay standard conditions were varied individually in the following ranges: protein concentration 0-20 mg/ml; incubation time 0-30 min; S-(2-benzothiazolyl)-L-cysteine concentration 0-2.0 mM.

4. N-acetyl-S-(6-chloro-2-methoxy-9-acridinyl)-L-cysteine cleavage. One ml of a 10% kidney cortex homogenate was incubated for 15 min at 37°C with 1 ml of a 0.1 mM solution of the fluorescent substrate dissolved in 0.1 M potassium borate buffer, pH 8.6. Two ml of ethanol were added to terminate the reaction and the mixture spun at 12,500 rpm for 60 sec with a Beckman microfuge. Supernatant fluorescence was measured at 410 nm excitation and 480 nm emission with an Aminco Bowman spectrofluorometer. The assay was characterized by varying the standard conditions as described for S-(2-benzothiazolyl)-L-cysteine, except for fluorescent substrate concentration which was varied from 0-0.1 mM.
C-S lyase activity was determined in untreated and DCVC-treated tissue. To assay for enzyme activity in tubules treated with DCVC, renal tubule suspensions, (4.5 ml containing 25 mg/ml tubule wet weight) were first incubated in bicarbonate buffered saline, pH 7.4. To these suspensions 0.5 ml of buffer or 0.5 ml of 10 mM DCVC dissolved in buffered saline pH 7.4 was added and the suspension incubated for 15 min. Tubules were then removed, washed twice in fresh buffer, resuspended in 0.1 M potassium borate buffer, pH 8.6 and homogenized with a Brinkman Polytron homogenizer. Tubule homogenates (6 mg protein/ml) were incubated with 2 mM S-(2-benzothiazolyl)-L-cysteine for 0, 1, 2.5, 5, 7.5, 10 and 15 min and the formation of 2-mercaptobenzothiazole measured. Isolated tubules were also incubated for 15 min with 0.05-5.0 mM DCVC, tubules washed, homogenized, incubated with S-(2-benzothiazolyl)-L-cysteine and 2-merceptobenzothiazole formation measured.

**Protein Determination**

The protein concentration of kidney cortex homogenates, isolated tubule homogenates and renal brush border membrane suspensions was measured with a fluorometrically using fluorescamine, as described by Bohlen et al. (1973).

**Statistics**

All data were analyzed by one-way analysis of variance and treatment means were compared with Student's t test, p values less than 0.05 were considered significant.
RESULTS

Part 1: Renal Toxicity of Halogenated Vinyl Cysteine Conjugates In Vivo and In Vitro

Renal damage induced by halogenated vinyl cysteine conjugates is localized in the proximal tubules. Therefore an isolated renal tubule suspension assay was developed to study the nephrotoxicity of these conjugates. Four parameters were investigated to ascertain the usefulness of isolated renal tubules for studying vinyl cysteine conjugate nephrotoxicity:

1. The potential inhibition of renal tubular active transport by vinyl and dienyl cysteine conjugates.

2. The equivalence of changes in active transport after exposure to vinyl cysteine conjugates in isolated tubules to those after exposure in the isolated perfused kidney and in vivo.

3. The use of changes in isolated tubule active transport for studying the nephrotoxicity of these conjugates. Five nephrotoxic and non-nephrotoxic conjugates were assayed (Figure 3). The chemically synthesized cysteine conjugates of the structurally similar halogenated ethylenes TCE, CTFE and CDFE have previously been shown to be nephrotoxic in vivo (Gandolfi et al., 1981). The saturated, non-halogenated compound, S-hydroxy-ethyl cysteine was not found to be nephrotoxic. The potential nephrotoxicity of the chemically synthesized cysteine conjugate of hexachlorobutadiene, 5-pentachlorobuta-1,3-dienyl cysteine (PCBC) was also assayed using the isolated tubule suspension.
4. Correlation of other indicators of renal tubular toxicity with changes in active transport. Oxygen utilization, metabolism of succinate and glucose and histological changes were quantified after incubation of isolated tubules with DCVC.

Characterization of tubular active transport. Isolated renal tubule suspensions were incubated with either radiolabeled and cold PAH or TEA. Aliquots were removed from 0-15 min and tissue to medium accumulation determined. Both PAH and TEA tubule to medium concentration ratios reached a plateau within 10 to 15 min after addition to the tubule suspension (Figure 4). Under the defined experimental conditions tissue to medium ratios of 26 ± 7 for PAH and 40 ± 12 for TEA were obtained from untreated animals. Within individual experiments variation was much less than that obtained with suspensions isolated from different animals and was usually within ± 10% of the mean. In subsequent studies active PAH and TEA accumulation was measured at a single time point, 15 min after addition of the transport substrates since active accumulation of PAH and TEA was maximal at this time.

The time course of DCVC-induced inhibition of PAH and TEA active transport in isolated renal tubules was determined by incubating tubule suspension with 1 mM DCVC. Aliquots of the suspension were removed from 0-15 min after addition of DCVC and accumulation of PAH and TEA measured (Figure 5). For both PAH and TEA an initial rapid decrease in transport occurred from 0-4 min followed by a slower steady decrease. At 15 min tissue to medium ratios for PAH and TEA were 1, indicating no active
accumulation of the anion or cation. Since maximal inhibition of transport occurs by 15 min, in subsequent studies tubule suspensions were incubated with DCVC or other cysteine conjugates for 15 min prior to the determination of active transport.

The dose response of tubular active transport to DCVC was determined by incubating isolated tubule suspensions with 0.01-2 mM DCVC for 15 min, then measuring active PAH and TEA transport (Figure 6). As seen in subsequent studies, PAH transport appeared to be more sensitive to transport inhibition by DCVC than was TEA transport. The values in this study are the mean of two determinations; therefore, only an approximate estimate of the ED$_{50}$ for DCVC can be made. For inhibition of PAH transport, the ED$_{50}$ was 0.05 mM, and for TEA transport it was 0.15 mM DCVC. In subsequent transport studies concentrations of 0.01 - 1 mM DCVC were used. These span the effective concentration range of DCVC from little or no transport inhibition at 0.01 mM to complete inhibition at 1 mM DCVC.

In vivo and in vitro exposure to DCVC. 1. Histology: Histological changes were determined after ip administration of DCVC in the rabbit. Renal damage 1 hr after administration of 20-100 mg/kg DCVC was dose-dependent. At 20 mg/kg the tubules appear undamaged apart from a slight accumulation of material in the lumens (Figure 7a). However, at 100 mg/kg there was a focal necrosis of the para recta region of the proximal tubules. Breakdown of tubule epithelial cells, loss of brush border membrane, nuclear karyolysis and accumulation of eosinophillic
debris in the dilated lumens were observed (Figure 7b). No changes were seen in the glomeruli.

Isolated perfused kidneys were exposed to 1, 0.1 and 0.01 mM DCVC for 1 hr using recirculating perfusion, then the cortex analyzed for histological damage. In control animals the kidney tubules were open and showed no swelling and the brush border membranes were intact. At 0.01 mM DCVC approximately 5-10% of tubules appeared swollen and in some tubules the brush border membrane was not well defined. Increased swelling of tubules, sloughing of brush border membrane, appearance of casts within the tubules and an increase in the intracellular space were observed at 0.1 mM DCVC. At 1 mM DCVC there was increased severity of all the damage observed at 0.1 mM DCVC; there was also evidence of broken tubules and protein deposition within the lumen of tubules. The severity of histological damage observed after exposure to DCVC in vivo or in the perfused kidney was directly related to the degree of inhibition of PAH and TEA transport.

2. Active transport: Tissue to medium ratios of both PAH and TEA were significantly decreased in tubules isolated 1 hr after exposure to DCVC in vivo, after 45 min exposure in the perfused kidney and after 15 min exposure in isolated tubules (Figures 8, 9). In vivo exposure to 20, 50 and 100 mg/kg significantly decreased both PAH and TEA transport with complete inhibition of transport occurring at 100 mg/kg. From these dose levels the approximate concentration range to which the kidneys may be exposed after i.p. injection was determined and the
perfused kidney and isolated tubules exposed to these concentrations of DCVC. These concentrations were calculated by assuming distribution of the DCVC in the body water. Approximately 50% of the body weight is whole body water (Goldstein et al., 1974). Therefore in a 1 kg rabbit whole body water is 500 g or 500 ml. A dose of 100 mg/kg DCVC approximates to 100 mg/500 ml. The molecular weight of DCVC is 216, therefore, 100 mg/kg approximates to 1 mM in the total body water. A dose-dependent decrease in transport occurred after exposure to DCVC in both the perfused kidney and isolated tubules. One and 0.1 mM DCVC significantly decreased both PAH and TEA transport (Figures 8, 9) in both preparations, with complete inhibition occurring with 1 mM DCVC. The 45 min additional perfusion required for DCVC exposure in the perfused kidney decreased both PAH and TEA active accumulation by tubules to approximately 14 and 18 times that in the medium respectively. This indicates that perfusion time may be important in determining active transport levels.

Active transport after exposure to cysteine conjugates. To determine whether active transport integrity in isolated tubules could be used to screen for nephrotoxicity of cysteine conjugates, tubule suspensions were incubated with 5 cysteine conjugates (Figure 3). Tissue to medium ratios of both PAH and TEA were significantly decreased below control values (p < 0.05) for tubules exposed for 15 min to 1 and 0.1 mM DCVC, HVC-1, HVC-2 and PCBC (Figure 10). At 1 mM of each of these conjugates T/M ratios were 1 indicating complete inhibition of active
transport. At 0.01 mM T/M ratios were not significantly different from control values for the three vinyl cysteine conjugates and PCBC. Addition of 0.01-1 mM S-hydroxyethyl cysteine to tubule suspension did not reduce active accumulation of PAH or TEA below the control value.

**Tubule oxygen consumption and metabolism of glucose and succinate.** DCVC was used as the prototype cysteine conjugate to help elucidate the mechanism of transport inhibition. To determine whether DCVC inhibits active transport by decreasing oxidative phosphorylation, both O\(_2\) consumption and evolution of CO\(_2\) from glucose and succinate were measured in tubules prior to and after exposure to DCVC. Oxygen consumption was approximately linear for the first ten minutes of the incubation hence, consumption rates were calculated over this time. Oxygen consumption rates for tubules exposed to 1-0.01 mM DCVC did not differ significantly from control values (Table 1). Background O\(_2\) loss in the incubation well, determined by using tubules treated previously with methanol, was 0.4 nM/mg/min compared to consumption of approximately 2.4 nM/mg/min for intact tubules. Therefore, tubules used in the oxygen consumption studies were actively respiring. Measurement of the evolution of \(^{14}\)CO\(_2\) from tubules incubated in the presence of \(^{14}\)C-glucose and \(^{14}\)C-succinate was used to determine whether exposure to DCVC decreased active transport by altering the rates of glycolysis or oxidative metabolism. Concentration of 1 mM DCVC or less did not significantly inhibit production of \(^{14}\)CO\(_2\) from \(^{14}\)C-glucose and \(^{14}\)C-succinate (Figure 11).
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<tr>
<th>Treatment</th>
<th>O$_2$ Consumption (nM/mg/min)$^a$,b</th>
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<td>Control</td>
<td>2.37 ± 0.26</td>
</tr>
<tr>
<td>1 x 10$^{-3}$ M DCVC</td>
<td>2.34 ± 0.05</td>
</tr>
<tr>
<td>5 x 10$^{-4}$ M DCVC</td>
<td>2.36 ± 0.08</td>
</tr>
<tr>
<td>1 x 10$^{-5}$ M DCVC</td>
<td>2.16 ± 0.16</td>
</tr>
<tr>
<td>Tubule blank</td>
<td>0.42 ± 0.12</td>
</tr>
</tbody>
</table>

$^a$Dimensions are nmoles O$_2$ consumed/mg wet weight of tubules/min

$^b$Values are means ± SE (n = 3)
Figure 3. Structures of the synthesized vinyl cysteine conjugates; DCVC, HVC-1, HVC-2, the saturated and non-halogenated HEC, and PCBC.
Figure 4. PAH (O) and TEA (0) active uptake with time by isolated renal tubules incubated with 0.01 mM 14C-PAH or 14C-TEA (specific activity 0.9 μCi/mmol). Values are expressed as means (n = 2).
Figure 5. Inhibition of renal tubular active PAH and TEA transport with time by 1mM DCVC. Values are expressed as means, n=2.
Figure 6. Dose response curve for the inhibition of renal tubular active PAH (○) and TEA (●) transport by DCVC. Values are expressed as means, n=2.
Figure 7. Photomicrographs of hematoxylin and eosin stained kidney cortex sections from rabbits 1 hr after an ip injection of DCVC. a) 20 mg/kg, b) 100 mg/kg.
Figure 8. Active PAH accumulation by renal tubules isolated 1 hr after ip administration of 20-100 mg/Kg DCVC (n = 3), 45 min after recirculating perfusion with 0.01 - 1 mM DCVC in the isolated perfused kidney (n=3), and 15 min after incubation of isolated renal tubules with 0.01 - 1 mM DCVC (n = 5). Values are expressed as means ± SE.
Figure 9. Active TEA accumulation by renal tubules isolated 1 hr after ip administration of 20-100 mg/Kg DCVC (n = 3), 45 min after recirculating perfusion with 0.01 - 1 mM DCVC in the isolated perfused kidney (n = 3), and 15 min after incubation of isolated renal tubules with 0.01 - 1 mM DCVC (n = 5). Values are expressed as means ± SE.
Figure 10. PAH and TEA accumulation by isolated renal tubules pre-incubated with the halogenated cysteine conjugates for 30 min followed by incubation with 14C-PAH, 14C-TEA, and 3H2O for 15 min, 1 mM cysteine conjugate, 0.1 mM cysteine conjugate, 0.01 mM cysteine conjugate, control. Values are expressed as means ± SE (n = 5). *Significantly different from control (p < 0.05).
Figure 11. Catabolism of glucose and succinate by isolated renal tubules exposed to DCVC. Catabolism was expressed as production of $^{14}$CO$_2$ from $^{14}$C-glucose and $^{14}$C-succinate. Conditions utilized were: no tubules present, tubules without DCVC added; tubules pretreated for 45 min with 1 mM DCVC; tubules pretreated for 45 min with 0.01 mM DCVC. Values are expressed as means (n = 2).
Part 2: In Vivo and In Vitro Biotransformation of Haloalkenes and Their Glutathione and Cysteine Conjugates

The in vivo formation of nephrotoxic cysteine conjugates from the parent haloalkene has been postulated to account for the nephrotoxicity of several haloalkenes including CTFE and HCBD (Bonhaus and Gandolfi, 1981; Lock, 1982), however, the role of the cysteine conjugate in TCE-induced nephrotoxicity is uncertain. Consumption of TCE defatted soybean meal caused nephrotoxicity in livestock (Pritchard et al., 1956), however, this was not thought to result directly from the action of TCE but indirectly via protein bound DCVC formed from the chemical reaction of TCE with cysteine during the defatting process (McKinney et al., 1957). Nephrotoxicity has not been shown to result from TCE exposure in vivo neither has the formation of DCVC been demonstrated after TCE exposure in vivo or in vitro.

Therefore, the nephrotoxicity of TCE was investigated in vivo and in vitro as was that of CTFE. Also, the validity of the postulated biotransformation of halogenated ethylenes via glutathione conjugation and formation of the vinyl cysteine conjugate was investigated in vitro. Finally, the role of acetylation and deacetylation in the nephrotoxicity of cysteine conjugates of TCE was studied.

In vivo and in vitro exposure to TCE. 1. In vivo. Preliminary studies measuring the penetration of TCE at 22°C and 37°C across a
silicone rubber septum into air indicated that throughout the experiment, the release rate of TCE was constant. The rate of TCE release into rabbits from the implanted vials was therefore assumed to be constant and was calculated by dividing the total amount of TCE released by the implantation time. This was 57 mg/hr for rabbit 1 and 62 mg/hr for rabbit 2. Making the following assumptions, an approximate estimation of the potential amount of TCE required to cause detectable renal damage after metabolism to DCVC may be calculated. The smallest dose of DCVC which inhibits renal tubular active transport after in vivo exposure is 20 mg/kg. Assuming distribution of DCVC in the body water, 20 mg/kg approximates to 0.2 mM in body water. Assuming that 10% of TCE is metabolized by glutathione conjugation (TCE is metabolized primarily by cytochrome P-450 mediated oxidation in the liver) and this eventually forms DCVC, then 2 mM TCE would result in 0.2 mM DCVC in the body water. For a 600g rabbit, this amount of TCE corresponds to 70 mg/300 ml of body water. Release rate of TCE was approximately 60 mg/hr for 60 hr. Therefore this release rate should result in a concentration of DCVC in the rabbit which is sufficiently high to result in detectable nephrotoxicity.

Before and after exposure to TCE in vivo, no glucose, bilirubin, ketone, protein or blood were detected in rabbit urine. Values for other parameters before and after TCE release-vial implantation are summarized in Table 2. Control values are means obtained from three days of urine measurements taken every 12 hours. Values obtained after
implantation are means of two 12 hr measurements for each 24 hr period. For both animals urine total creatinine was significantly decreased (p < 0.05) 24 hr after TCE implantation. No significant change occurred in other parameters.

2. Isolated perfused liver. There was no change in active PAH and TEA transport in isolated renal tubules incubated in perfusate or bile from TCE-exposed livers (Table 3). Bile was added at a concentration of 2% because at higher concentrations bile itself inhibited active transport. In vivo treatment of rabbits ip with 150 mg/kg of the cytochrome P-450 depleting agent AIA, 3 hr prior to liver isolation, did not affect the levels of PAH and TEA transport (Table 3).

3. Isolated renal tubules. Addition of 1 mM TCE in DMSO directly to isolated tubule suspensions significantly decreased active PAH and TEA transport (p < 0.05). Control transport levels were 30 ± 1.5 for PAH and 44 ± 6 for TEA (mean ± SE). Incubation with 1 mM TCE for 20 min reduced PAH and TEA accumulation ratios to 1, indicating complete inhibition of active transport. Incubation with 0.1 and 0.01 mM did not decrease transport.

In vitro exposure to CTFE. Incubation of renal tubule suspensions in sealed vessels containing CTFE resulted in a decrease in tubular PAH transport, with increasing concentrations of CTFE causing greater reductions in transport (Table 4). PAH transport was completely inhibited at the highest concentration of CTFE. Tubules were also pre-incubated for 15 min with the γ-glutamyl transpeptidase inhibitor,
AT-125, prior to CTFE exposure. This resulted in tubules being more refractory to CTFE-induced inhibition of PAH transport. Transport was 61% of control in tubules incubated with AT-125 followed by 223 umol CTFE (5 ml CTFE in a headspace volume of 20 ml), compared to 22% for tubules exposed to this concentration of CTFE alone (Table 4). Active PAH transport was not changed significantly from control after tubule incubation with 5 ml (223 umol) of the inert halogenated hydrocarbons ether or halothane.

The inhibition of tubule PAH transport during incubation in the presence of CTFE occurred slowly. In tubule suspensions incubated in the presence of 5 ml of CTFE gas, the control T/M ratio of 12 was reduced to 11 after 15 min, 6 after 45 min, 3 after 75 min and transport completely inhibited after 120 min (values are means, n = 2). No change in PAH transport occurred in tubule suspensions incubated under the same conditions for 120 min without CTFE present.

Renal tubular non-protein sulfhydryl content decreased progressively throughout the 120 min incubation with CTFE. Levels were significantly reduced below the control (p < 0.05) from 45-120 min (Table 5). After 120 min non-protein sulfhydryl content of tubules incubated without CTFE decreased from 6.3 to 5.6 mM, however, this decrease was not significant.

The concentration of CTFE decreased progressively throughout incubation of renal tubules with 0.1 or 0.25 ml of CTFE gas (Table 5). Concentrations were significantly decreased (p < 0.05) in flasks
containing 0.1 and 0.25 ml CTFE from 20-120 min. There was no decrease in CTFE concentration in flasks containing buffered saline instead of tubule suspension. The saturated glutathione conjugate of CTFE (CTFE-GSH) was detected in tubule suspension medium after 30 and 120 min incubation (Table 5). The amounts of CTFE-GSH are expressed in terms of peak area calculated by the amino acid analyzer integrator.

Incubation of tubule suspensions with the chemically synthesized, saturated glutathione and cysteine conjugates of CTFE for 45 min decreased active PAH transport. One and 0.1 mM CTFE-GSH significantly reduced (p < 0.05) PAH accumulation to 1 and 4, respectively compared to a T/M ratio of 22 ± 4 in untreated tubules. PAH accumulation after incubation with 0.01 mM CTFE-GSH was 25. A 15 min incubation with the saturated cysteine conjugate of CTFE, CTFE-cys, completely inhibited PAH transport at 1.0 mM and significantly reduced the T/M ratio to 2 at 0.1 mM. There was no reduction in the T/M ratio after incubation with 0.01 mM CTFE-cys. All values are means of three determinations.

In vivo and in vitro exposure to DCVG. PAH transport was significantly reduced (p < 0.05) in tubules isolated from rabbits 3 hr after ip injections of 50, 100 or 200 mg/kg of the unsaturated conjugate DCVG (Figure 12). TEA transport was significantly reduced after 100 and 200 mg/kg DCVG in vivo (Figure 12).

Both PAH and TEA T/M ratios were significantly reduced (p < 0.05) in isolated renal tubules incubated for 60 min with 5, 1 and 0.1 mM DCVG, but not with 0.01 mM (Figure 13). PAH and TEA transport was
completely inhibited after 15 min incubation with 1.0 mM DCVC, while complete inhibition of transport by 1.0 mM DCVG did not occur until 60 min (Figure 14).

Incubation of isolated renal tubules with 20 mM serine/borate or 2 mM AT-125 prior to 0.01-1 mM DCVG significantly increased PAH and TEA T/M ratios (p < 0.05) compared to ratios measured in tubules incubated only with DCVG (Figure 15). AT-125 provided better protection against DCVG than did serine/borate.

In vitro biotransformation of DCVG. Incubation of isolated renal tubule suspensions for 120 min with 5 mM DCVG resulted in a steady time-dependent decrease in DCVG concentration to approximately 0.5 mM at 120 min (Figure 16). The concentration of DCVG remained constant at 5 mM throughout a 120 min incubation containing buffer with no tubules, hence disappearance of DCVG was dependent upon the presence of isolated tubules. Concurrent with the decrease in DCVG concentration, glutamate, glycine, DCVC and ammonia appeared in the medium (Figure 16). The order of appearance in the incubation medium was glutamate, glycine, DCVC, ammonia, this also being the order in terms of concentration present in the medium. One exception was at 120 min when DCVC concentration was greater than that of glycine. Tubules were incubated without DCVG to determine the concentrations of glutamate, glycine and ammonia in the medium as a result of tubule metabolism. These values were subtracted from those obtained for tubule incubation with DCVG.
**In vitro exposure to DCV-NAC.** Incubation with 1 mM DCV-NAC resulted in a time-dependent decrease in tubule active PAH transport. After 10, 20, 45 and 60 min incubation PAH transport was 87, 71, 43 and 25% of control respectively. These values are means of four measurements (Table 6). Inhibition of PAH and TEA transport was concentration dependent, with complete inhibition after 60 min incubation with 1 mM DCV-NAC and significant reductions at 0.1 mM \( p < 0.05 \). Transport was not inhibited by 0.01 mM DCV-NAC. Inhibition of PAH and TEA transport by DCVC occurred at equivalent concentrations but occurred within 15 min (Figure 14).
**TABLE 2**

**URINE PARAMETERS AFTER IN VIVO EXPOSURE OF RABBITS TO 60 MG/HR TCE FOR 60 HR**

<table>
<thead>
<tr>
<th>Urine Parameter</th>
<th>Rabbit 1</th>
<th>Rabbit 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0-24 hr&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH</td>
<td>8.3 ± 0.3</td>
<td>7.3</td>
</tr>
<tr>
<td>Urine volume (ml)</td>
<td>39 ± 20</td>
<td>15</td>
</tr>
<tr>
<td>Water consumed (ml)</td>
<td>61 ± 28</td>
<td>78</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>38 ± 13</td>
<td>30</td>
</tr>
<tr>
<td>Total creatinine (mg)</td>
<td>15 ± 5</td>
<td>7</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg)</td>
<td>451 ± 47</td>
<td>560</td>
</tr>
<tr>
<td>PAH transport (T/M)</td>
<td>26 ± 7</td>
<td>-</td>
</tr>
<tr>
<td>TEA transport (T/M)</td>
<td>40 ± 12</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values obtained from 3 days of measurement. Means ± SD (n = 6).

<sup>b</sup>Means (n = 2) except PAH and TEA transport where n = 3.
### TABLE 3

RENAL TUBULE ACTIVE TRANSPORT AFTER INCUBATION IN PERFUSATE OR BILE OBTAINED FROM LIVER EXPOSED TO TCE

<table>
<thead>
<tr>
<th>Tubule Incubation Medium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PAH Transport (T/M Ratio)</th>
<th>TEA Transport (T/M Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (control)</td>
<td>19 ± 3</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Liver perfusate</td>
<td>16 ± 1</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>Buffer + bile</td>
<td>18 ± 4</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>Perfusate/bile mixture from AIA treated animal</td>
<td>16 ± 2</td>
<td>27 ± 5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Tubules incubated for 15 min prior to determination of active transport.

Values are expressed as means ± SD (n = 3).
**TABLE 4**

PAH TRANSPORT IN ISOLATED TUBULES AFTER INCUBATION IN THE PRESENCE OF CTFE

<table>
<thead>
<tr>
<th>Amount of CTFE in Incubation Flask</th>
<th>PAH Transport, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ml</td>
<td>100</td>
</tr>
<tr>
<td>1 ml (45 μmol)</td>
<td>73 ± 7</td>
</tr>
<tr>
<td>2.5 ml (112 μmol)</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>5 ml (223 μmol)</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>5 ml a</td>
<td>62 b</td>
</tr>
<tr>
<td>5 ml air</td>
<td>91 b</td>
</tr>
<tr>
<td>5 ml ether</td>
<td>81 b</td>
</tr>
<tr>
<td>5 ml halothane</td>
<td>100 b</td>
</tr>
</tbody>
</table>

*a* Tubules preincubated for 15 min with 2 mM AT-125 prior to CTFE exposure

*b* Mean, n = 2

Values are expressed as mean ± SE (n = 4).
TABLE 5
DECREASE IN TUBULE NP-SH AND CTFE, AND APPEARANCE OF CTFE-GSH IN INCUBATION SUPERNATANT

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Tubule NP-SH Content(^a)(mM)</th>
<th>CTFE Concentration (^b)((\mu\text{mol}))</th>
<th>CTFE-GSH Formed (peak area)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.3 ± 0.2</td>
<td>4.5 ± 0.1</td>
<td>11.2 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>6.2 ± 0.3</td>
<td>3.9 ± 0.2</td>
<td>11.2 ± 0.2</td>
</tr>
<tr>
<td>20</td>
<td>6.0 ± 0.1</td>
<td>3.1 ± 0.3</td>
<td>8.0 ± 1.2</td>
</tr>
<tr>
<td>30</td>
<td>5.5 ± 0.1</td>
<td>2.0 ± 0.4</td>
<td>9.1 ± 0.2</td>
</tr>
<tr>
<td>45</td>
<td>4.6 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>8.5 ± 1.5</td>
</tr>
<tr>
<td>60</td>
<td>4.3 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>6.8 ± 1.0</td>
</tr>
<tr>
<td>90</td>
<td>3.8 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>5.6 ± 1.5</td>
</tr>
<tr>
<td>120</td>
<td>3.8 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>5.6 ± 1.0</td>
</tr>
</tbody>
</table>

Volume CTFE in Flask (ml) 5 0.1 0.25 0.1 0.25
(4.5 \(\mu\text{mol}\)) (11.2 \(\mu\text{mol}\))

\(^a\)Mean ± SE (n = 3)
\(^b\)Mean ± SE (n = 4)
\(^c\)Single determination.
TABLE 6
PAH AND TEA TRANSPORT IN ISOLATED RENAL TUBULES INCUBATED WITH DCV-NAC

<table>
<thead>
<tr>
<th>Concentration of DCV-NAC (mM)a</th>
<th>PAH Transportb (T/M Ratio)</th>
<th>TEA Transportb (T/M Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0 ± 0</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>0.1</td>
<td>2.4 ± 1</td>
<td>5 ± 0.3</td>
</tr>
<tr>
<td>0.01</td>
<td>25 ± 2</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>0</td>
<td>26 ± 7</td>
<td>40 ± 12</td>
</tr>
</tbody>
</table>

aTubules incubated for 60 min with DCV-NAC.
bValues are means ± SE (n = 3).
Figure 12. Active PAH and TEA transport in renal tubules isolated from rabbits 3 hr after i.p. injection of DCVG. Values are expressed as means ±SE (n = 4). *Significantly different from control (p < 0.05)
Figure 13. Active PAH and TEA transport in isolated renal tubules after 1 hr in vitro incubation with DCVG. Values are expressed as means ±SE (n = 5). *Significantly different from control (p < 0.05).
Figure 14. Time course of DCVC (1 mM) and DCVG (1 mM) induced inhibition of active PAH and TEA transport in isolated renal tubules. Tissue to medium ratios at 15, 30, 45, and 60 min are expressed as a percentage of the control, 0 min (n = 2).
Figure 15. Effect of the γ-glutamyl transpeptidase inhibitors serine/borate (20 mM) and AT-125 (2 mM) on DCVG-induced inhibition of renal tubule PAH and TEA transport in vitro. Tubules were incubated with buffered saline, serine/borate or AT-125 for 30 min followed by DCVG incubation for 45 min prior to the determination of PAH and TEA transport. Values are expressed as means ±SE (n = 3). *Significantly different from control (p < 0.05).
Figure 16. Isolated renal tubule incubation with 5 mM DCVG for 120 min. Disappearance of DCVG and appearance of glutamate, glycine, DCVC and ammonia. Values are single determinations.
Cleavage of vinyl cysteine conjugates by C-S lyase produces a reactive intermediate that binds to subcellular macromolecules. This binding is thought to initiate the events that result in nephrotoxicity (Anderson and Schultze, 1965; Derr and Schultze, 1963). If this reactive intermediate is responsible for the toxicity of DCVC it should be possible to decrease toxicity by the addition of a nucleophilic agent, such as the endogenous radical scavenger glutathione (GSH), to bind with the reactive fragment. Conversely, the depletion of endogenous GSH should enhance the toxicity of DCVC. A major function of GSH is protection of cell proteins and cell membranes against free radicals and electrophiles (Meister and Tate, 1976; Meister, 1981), because of its stability and ubiquitous distribution. GSH is also the most abundant intracellular thiol and is found at concentrations of 0.5-10 mM in virtually all cells (Meister and Tate, 1976). Other nucleophilic agents, cysteamine, cystamine, cysteine, guanidine, lysine and arginine (Figure 17) were also examined as potential protectors against DCVC in isolated tubules.

It is uncertain as to whether the toxicity of the DCVC active metabolite is caused by acylation or alkylation of biomacromolecules. Aminothiols, such as those listed above, are potential candidates for both acylation and alkylation and so may bind the active species (Bacq, 1975).
Sulfhydryl protection against DCVC. In a pilot study, simultaneous incubation of isolated tubules with lysine (1 mM), arginine (1 mM) or guanidine (6 mM) together with DCVC (0.1 mM) did not protect against the DCVC induced inhibition of PAH and TEA transport. GSH, cysteine and cysteamine were also evaluated for ability to prevent inhibition of renal tubular active transport induced by 0.1 mM DCVC. One mM cysteine was compared to 6 mM GSH, because cysteine itself inhibited active transport at concentrations greater than 1 mM. A concentration of 0.1 mM cysteamine was utilized since higher concentrations inhibited active transport. Incubation of isolated renal tubules with 0.1 mM DCVC for 15 min reduced PAH and TEA transport to 7.4 ± 1.4% and 10 ± 2% of control, respectively (mean ± SD, n = 3). A subsequent 15 min incubation with 0.1 mM cysteamine did not change PAH or TEA transport. Incubation of DCVC-treated tubules with 1 mM cysteine for 15 min increased PAH transport to 28 ± 6% of control and TEA to 49 ± 6%, while 6 mM GSH increased transport to 112 ± 9% for PAH and 114 ± 8% for TEA transport. Since GSH was the most efficacious in reversing the DCVC-induced inhibition of active transport it was used as the protective agent throughout the remainder of this study.

In vitro exposure to DCVC. GSH was added to tubules simultaneously with, 15 min before, and 15 min after DCVC and acid and base transport measured. Tubules were then incubated with 0.1 mM DCVC and/or 6 mM GSH for 15 min prior to determining the active accumulation of PAH or TEA. Control tubules accumulated PAH to 26 ± 7 times the
concentration present in the medium and TEA to 40 ± 12 times (mean ± SD, n = 4). Incubation with 0.1 mM DCVC completely inhibited the uptake of PAH and reduced TEA accumulation to 4 ± 0.9. Addition of 6 mM GSH to the tubule suspension simultaneously with DCVC maintained active transport of PAH and TEA at the control level (Figure 18). Glutathione alone did not significantly alter the accumulation of PAH or TEA from the control values.

Addition of 6 mM GSH to tubules in which active transport was inhibited by prior incubation with 0.2 mM DCVC allowed recovery of the active transport activity to 50-80% of control (Figure 19) and completely reversed the reduction in tubule NP-SH (Table 7). If the concentration of DCVC was increased to 0.5-1.0 mM there was little, if any, recovery of transport after subsequent incubation with 6 mM GSH (Figure 19).

Tubules preincubated in buffer containing GSH at concentrations 5-15 mM accumulated NP-SH to a concentration equivalent to that of GSH in the medium (Table 8). Subsequent incubation with 0.2 mM DCVC resulted in tubules being more refractory to DCVC-induced inhibition of active transport (Table 8). At all concentrations of GSH, PAH and TEA transport were greater than tubules exposed to DCVC alone, with higher concentrations of GSH providing greater protection against DCVC. Active TEA transport appeared to be protected to a greater extent than PAH transport.

_Exposure to DCVC after GSH depletion._ When the concentration of NP-SH in the isolated renal tubules was reduced by incubating with
diethyl maleate for 15 min (Table 7), the active transport of PAH and TEA was also reduced (Table 7). If the tubules were then exposed to DCVC, the inhibitory effect of DCVC upon active transport was enhanced (Table 9). Enhancement of DCVC toxicity also occurred after preincubation with another GSH depletor, glycidol. The PAH transport in tubules pretreated with 1.5 mM glycidol followed by incubation with 0.5 mM DCVC was $2 \pm 0.4$ compared to $7 \pm 0.6$ for tubules treated with DCVC alone. The respective transport values for TEA were $6 \pm 1$ and $24 \pm 1$. Addition of 6 mM GSH to tubules treated with diethyl maleate and DCVC returned active PAH and TEA transport to control levels (Table 9).

**In vivo exposure to DCVC.** Active transport of PAH and TEA was significantly reduced ($p < 0.05$) in renal tubules isolated from rabbit kidney 1 hr after *in vivo* exposure to 20, 50 and 100 mg/kg of DCVC ip (Figure 20). Subsequent incubation of tubules in 6 mM GSH for 15 min prior to the determination of active transport significantly increased ($p < 0.05$) TEA transport at 50 and 20 mg/kg and PAH transport at 50 mg/kg (Figure 20).

**Non-protein sulfhydryl concentration.** There is an apparent relationship between loss of NP-SH and inhibition of active transport. Incubation of tubules with 1 mM DCVC decreased NP-SH concentration to 51% of normal (Table 7) and almost completely inhibited active PAH and TEA transport (Figure 19). 0.1 mM DCVC reduced NP-SH to 71% of normal and caused a lesser inhibition of active transport, while 0.01 mM DCVC did not change NP-SH or active transport.
Incubation with 0.5 - 2.5 mM diethyl maleate caused a reduction in NP-SH to 56-73% of the normal concentration. Subsequent addition of 1 mM DCVC resulted in a further loss of endogenous NP-SH to 35% of the normal value, and also caused a further reduction in active transport (Table 9).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>NP-SH Content (mM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>0.01 mM DCVC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>0.1 mM DCVC</td>
<td>4.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>1.0 mM DCVC</td>
<td>3.2 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>0.5 mM diethyl maleate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>1.0 mM diethyl maleate</td>
<td>4.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>2.5 mM diethyl maleate</td>
<td>3.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>2.5 mM diethyl maleate followed&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>by 1.0 mM DCVC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 mM DCVC followed&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.8 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>by 6 mM GSH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Tubule suspension exposed to DCVC for 15 min.

<sup>b</sup> Tubule suspension exposed to diethyl maleate for 15 min.

<sup>c</sup> After a 15 min diethyl maleate exposure, tubules were washed and resuspended in medium containing DCVC and incubated for 15 min.

<sup>d</sup> After 15 min, DCVC exposure tubules were washed and resuspended in medium containing 6 mM GSH.

<sup>e</sup> Values are means ± SE of duplicate determinations for three animals.
TABLE 8
REVERSAL OF DCVC-INDUCED RENAL TUBULAR TRANSPORT INHIBITION BY EXOGENOUS GSH

<table>
<thead>
<tr>
<th>Concentration of Exogenous GSH (mM)</th>
<th>Concentration of NP-SH in Tubules (mM)</th>
<th>Active Transport as a % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PAHc</td>
</tr>
<tr>
<td>0</td>
<td>5.7</td>
<td>29</td>
</tr>
<tr>
<td>5.0</td>
<td>7.2</td>
<td>37</td>
</tr>
<tr>
<td>7.5</td>
<td>7.4</td>
<td>41</td>
</tr>
<tr>
<td>10.0</td>
<td>12.8</td>
<td>41</td>
</tr>
<tr>
<td>12.5</td>
<td>14.0</td>
<td>62</td>
</tr>
<tr>
<td>15.0</td>
<td>17.2</td>
<td>66</td>
</tr>
</tbody>
</table>

|                               | TEAc                               |
| 26                              |                                    |
| 75                              |                                    |
| 77                              |                                    |
| 77                              |                                    |
| 97                              |                                    |
| 110                             |                                    |

a Tubule suspension incubated with GSH for 15 min prior to incubation with DCVC.
b Tubules incubated with 0.2 mM DCVC for 15 min.
c Values are means (n = 2).
## TABLE 9

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAH Transport (^d) (T/M Ratio)</th>
<th>TEA Transport (^d) (T/M Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18 $\pm$ 2</td>
<td>52 $\pm$ 2</td>
</tr>
<tr>
<td>0.2 mM DCVC(^a)</td>
<td>17 $\pm$ 1.5</td>
<td>38 $\pm$ 6</td>
</tr>
<tr>
<td>0.5 mM diethyl maleate</td>
<td>6.5 $\pm$ 0.7</td>
<td>26 $\pm$ 6</td>
</tr>
<tr>
<td>0.5 mM diethyl maleate + 0.2 mM DCVC(^b)</td>
<td>1.3 $\pm$ 0.1</td>
<td>1.7 $\pm$ 0</td>
</tr>
<tr>
<td>0.5 mM diethyl maleate + 0.2 mM DCVC + 6 mM GSH(^c)</td>
<td>16 $\pm$ 3</td>
<td>58 $\pm$ 13</td>
</tr>
</tbody>
</table>

\(^a\) Fifteen min incubation.

\(^b\) Fifteen min preincubation with diethyl maleate followed by 15 min with DCVC.

\(^c\) Fifteen min incubation with GSH following diethyl maleate and DCVC treatment.

\(^d\) Values are expressed as means $\pm$ SD (\(n = 3\)).
Figure 17. Structures of potential acceptors for the reactive intermediate formed after DCVC cleavage by C-S lyase.
Figure 18. Protection against DCVC-induced inhibition of renal tubular active transport by simultaneous exposure to 6 mM GSH. Control, 0.1 mM DCVC (15 min), 0.1 mM DCVC and 6 mM GSH (15 min), 6 mM GSH (15 min). Mean ± SE (n=3).
Figure 19. Retroactive protection of renal tubules to DCVC-induced inhibition of active transport by subsequent addition of 6 mM GSH. Active accumulation of TEA and PAH in isolated tubules pre-incubated for 15 min with 0.2, 0.5, or 1.0 mM DCVC, followed by a 15 min incubation in buffer □ or 6 mM GSH □. Mean ± SE (n=3). *Significantly different from tubule suspensions not treated with GSH at an equivalent concentration of DCVC (p < 0.05).
Figure 20. Effect of 6 mM GSH on the active transport in tubules isolated from DCVC-treated rabbits. Active accumulation of TEA and PAH by tubules isolated 1 hr after ip injection of 20, 50 or 100 mg/kg DCVC. TEA and PAH accumulation in tubules incubated in 6 mM GSH after isolation from DCVC-treated rabbits. Mean ± SE. * Significantly different from tubules not treated with GSH at an equivalent concentration of DCVC (p < 0.05).
Part 4: Renal Proximal Tubule C-S Lyase, Assays and Activity

Several in vitro assays have been developed to measure C-S lyase activity, none of which provide an ideal measure of activity. Most assays measure formation of ammonia or pyruvate resulting from the cleavage of C-S lyase substrates. In addition to these assays, when DCVC is used as the substrate disappearance of DCVC may be measured directly at OD$_{258}$ as it is cleaved by the enzyme (Anderson and Schultze, 1965). The cleavage of C-S lyase substrates such as S-(2,4-dinitrophenyl)-L-cysteine (Tateishi et al., 1978) or S-(2-benzothiazolyl)-L-cysteine (Dohn and Anders, 1982), to form colored products may also be used to quantify enzyme activity. Alternatively an indirect assay measures the binding of the DCVC reactive metabolite with the alkylating acceptor 4-(p-nitrobenzyl) pyridine (Bhattacharya and Schultze, 1967).

These and a newly developed assay which measures the cleavage of N-acetyl-S(6-chloro-2-methoxy-9-acridinyl)-L-cysteine were compared to study C-S lyase activity in the kidney cortex. These assays were also used to examine possible suicidal enzyme activity when DCVC is the enzyme substrate.

Pyruvate assay. C-S lyase was detected in brush border membrane vesicle suspensions incubated with the enzyme substrate, DCVC. Pyruvate formation was detected with 1.0 mM DCVC, but not with 0.1 or 0.01 mM DCVC. Enzyme activity was approximately 20 nmol pyruvate/min/mg
protein. No change in enzyme activity was detected in brush border membranes isolated from kidneys perfused with 1 mM DCVC for 60 min prior to brush border membrane isolation.

**Ammonia assay.** With both the Nessler technique and fluorescence detection with OPA, no ammonia liberation was detected from brush border membrane suspensions incubated with the C-S lyase substrates DCVC or S-(2-benzothiazolyl)-L-cysteine. Assuming that all the ammonia formed from DCVC or S-2(benzothiazolyl)-L-cysteine is liberated from the brush border membrane suspension and trapped by the H₂SO₄ or Opa solution, the approximate detection limits are 600 nmol ammonia/min/mg protein for the Nessler technique and 6 nmol ammonia/min/mg protein for the OPA fluorescence assay.

**S-(2-benzothiazolyl)-L-cysteine.** C-S lyase activity was detected in kidney cortex homogenate by measuring the formation of 2-mercaptobenzothiazole from the enzyme substrate S-(2-benzothiazolyl)-L-cysteine (2 mM). Enzyme activity was approximately 1.3 nmol 2-mercaptobenzothiazole/min/mg protein. The dependence of C-S lyase activity on protein concentration, time and S-(2-benzothiazolyl)-L-cysteine concentration is shown in Figure 21. C-S lyase activity was approximately linear over the ranges 0-8 mg protein, 0-15 min incubation time and 0.1-2.0 mM S-(2-benzothiazolyl)-L-cysteine concentrations.

Production of 2-mercaptobenzothiazole was decreased in homogenates prepared from tubules incubated with 1 mM DCVC (Table 10). Inhibition of tubule C-S lyase activity was dependent upon the
concentration of DCVC in which tubules were incubated prior to the assay. Production of 2-mercaptobenzothiazole was not decreased in tubules incubated with 0.05-0.2 mM DCVC (Table 11), however production was significantly decreased (p< 0.05) in tubules incubated with 0.5-5.0 mM DCVC.

**N-acetyl-S(6-chloro-2-methoxy-9-acridinyl)-L-cysteine.** The disappearance rate of substituted acridinyl cysteine was measured to quantify C-S activity. Enzyme activity was 0.25 nmol substituted acridinyl cysteine metabolized min/mg protein. Activity was dependent on protein concentration, time and concentration of the substituted acridinyl cysteine (Figure 21). Metabolism was approximately linear over the range 0-8 mg protein/ml and 0.01-0.1 mM substituted acridinyl cysteine. Metabolism was not linear with time, there appeared to be an initial burst of activity from 0-2 min. before the rate became linear.

Disappearance rate of the substituted acridinyl cysteine was not changed in homogenates prepared from tubules incubated for 15 min. with 1 mM DCVC.
### TABLE 10

DCVC-INDUCED INHIBITION OF C-S LYASE ACTIVITY IN ISOLATED RENAL TUBULES

<table>
<thead>
<tr>
<th>Incubation time with S-(2-benzothiazolyl) cysteine (min)</th>
<th>2-mercapto benzothiazole formed in the 2 ml incubation mixture (nmol)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>0</td>
<td>2.7</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>2.5</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
</tr>
<tr>
<td>7.5</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>79</td>
</tr>
<tr>
<td>15</td>
<td>101</td>
</tr>
</tbody>
</table>

\(^a\)After 15 min incubation of tubules with buffer or 1 mM DCVC tubules were washed, resuspended in 0.1 M potassium borate buffer pH 8.6, and incubated with 2 mM S-(2-benzothiazolyl)cysteine. Means, n = 2.
TABLE 11

EFFECT OF DCVC CONCENTRATION ON INHIBITION OF RENAL TUBULE C-S ACTIVITY

<table>
<thead>
<tr>
<th>DCVC Concentration (mM)</th>
<th>2-mercaptobenzothiazole formed in the 2 ml incubation mixture (nmol)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>0.05</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>0.1</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>0.2</td>
<td>83 ± 1</td>
</tr>
<tr>
<td>0.5</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>1.0</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>2.0</td>
<td>63 ± 8</td>
</tr>
<tr>
<td>5.0</td>
<td>53 ± 2</td>
</tr>
</tbody>
</table>

After a 15 min. incubation with DCVC, tubules were washed, resuspended in 0.1 M potassium borate buffer pH 8.6 (6 mg protein/ml) and incubated with S-(2-benzothiazolyl)cysteine for 15 min. Values are means ± SE (n = 4).
Figure 21. Dependence of C-S lyase activity on protein concentration, incubation time and substrate concentration. C-S lyase activity was quantified by the formation of 2-mercaptobenzothiazole from S-(2-benzothiazolyl)-L-cysteine, or disappearance of N-acetyl-S-(6-chloro-2-methoxy-9-acridinyl)-L-cysteine in kidney cortex homogenates. Except as noted on the abcissas, incubation mixtures contained 2 mM S-(2-benzothiazolyl)-L-cysteine or 0.1 mM N-acetyl-S-(6-chloro-2-methoxy-9-acridinyl)-L-cysteine, 10 mg protein/ml and were incubated for 15 min in a 2 ml incubation mixture. Values are means (n = 2).
DISCUSSION

Several important aspects of the biotransformation and mechanism of haloalkene and vinyl systeine conjugate-induced nephrotoxicity were deduced from the results of this study. These findings are summarized below and discussed in detail later in the Discussion.

There are several pathways by which the haloalkenes and their metabolites may be biotransformed, with nephrotoxicity primarily resulting after glutathione conjugation and peptidase cleavage to form the vinyl cysteine conjugates. Competing pathways of biotransformation may determine the toxicity of the haloalkenes or their chemically synthesized glutathione or cysteine conjugates. In the liver the haloalkenes may undergo cytochrome P-450 mediated oxidation or conjugation with glutathione, with only the latter route leading to formation of a vinyl cysteine conjugate. In addition to biotransformation to the nephrotoxic vinyl cysteine conjugate, it is possible that haloalkenes may undergo conjugation with GSH to form a saturated conjugate. This was demonstrated when isolated renal tubules were incubated in the presence of CTFE and an ethyl glutathione conjugate formed. Also the chemically synthesized saturated glutathione and cysteine conjugates of CTFE were shown to be nephrotoxic in vitro. Another determinant of nephrotoxicity is the degree to which the vinyl cysteine conjugates are metabolized in the renal tubules by C-S lyase or N-acetylase. C-S lyase cleavage results in the formation of a nephrotoxic intermediate while
N-acetylation leads to excretion of the conjugate as a non-toxic mercapturic acid. Deacetylation of N-acetyl vinyl cysteine conjugates was also shown to occur in the proximal tubule, resulting in the reformation of the vinyl cysteine conjugate. Therefore the balance between N-acetylation and deacetylation may also determine the severity of vinyl cysteine conjugate-induced nephrotoxicity. Another variable which may determine species susceptibility is the metabolism of haloalkenes to vinyl cysteine conjugates by gut microflora. Gopal Krishna (pers. comm.), at the National Institutes of Health, dosed calves orally with TCE and demonstrated that the resultant nephrotoxicity was caused by DCVC formed by the gut microflora.

In addition to competing pathways of biotransformation, the severity of vinyl cysteine-induced nephrotoxicity is determined by the type of lesion produced in the proximal tubule. One result of the nephrotoxic response is inhibition of active anion and cation transport in the proximal tubules. Although toxic to isolated mitochondria, DCVC did not inhibit oxygen consumption or oxidation of glucose and succinate in isolated proximal tubules, indicating that inhibition of active transport is probably not caused by inhibition of oxidative phosphorylation. Transport inhibition was accompanied by a decrease in NP-SH and was partially reversed by subsequent addition of GSH to the medium. Since tubule active transport and cell membrane integrity are dependent upon GSH concentration, it is possible that the lesion produced by the DCVC reactive metabolite results from binding initially
to the subcellular radical scavenger, GSH. This depletes endogenous GSH and results in a partially reversible decrease in transport. Once available intracellular GSH is depleted the metabolite may then bind to cell membrane nucleophilic groups and irreversibly damage the cell membrane.

Initial studies involved the development of an isolated renal tubule system to study vinyl cysteine conjugate-induced nephrotoxicity, and biotransformation of haloalkene and glutathione conjugates to form the vinyl cysteine conjugates. These studies indicate that a site of action of the vinyl cysteine conjugate is the proximal tubule, and that the tubules are able to bioactivate DCVC to a toxic species. In addition to necrosis of the proximal tubules after in vivo exposure, active PAH and TEA transport in renal tubules was inhibited after in vivo or in vitro exposure to DCVC, with the isolated tubules responding in a similar manner to this nephrotoxicant as did the perfused kidney and the whole animal. Since DCVC produces acute renal necrosis in all species tested (Lock, 1982) with necrosis occurring specifically in the proximal tubules, isolated tubules provide a well defined system in which to study the mechanism(s) of this toxicity. Isolated tubules have been used for many in vitro studies of renal function and metabolism since isolation is simple and many samples may be obtained from one animal (Hirsch, 1976). Also, many of the difficulties inherent in tissue slice studies are eliminated (Burg and Orloff, 1962) and concentration of sub­startes or toxin to which all tubules are exposed is uniform (Cross and Taggart, 1950).
The localization of renal damage in the proximal tubules of rabbits is in agreement with previous studies utilizing different species and both parent halogenated ethylenes and chemically synthesized cysteine conjugates (Terracini and Parker, 1965; Klaassen and Plaa, 1966; Gandolfi et al, 1981). Localization of necrosis at the corticomedullary junction may be due to concentration of DCVC in this region as a result of its secretion. In addition to being the site of secretion and reabsorption of many materials, the proximal tubules are the major site of biotransformation in the kidney, with most drug metabolizing capabilities occurring in the $S_3$ cells (Rush and Hook, 1982). Such metabolism may occur via cytochrome P-450 mediated mixed function oxidases or by non-oxidative metabolism catalyzed by peptidases and lyases such as C-S lyase located in the brush border membrane region (Bonhaus and Gandolfi, 1981). The nephrotoxicity of DCVC is thought to result from bioactivation to a toxic intermediate after cleavage by C-S lyase (Anderson and Schultze, 1965). The inhibition of renal active transport caused by DCVC in the perfused kidney or isolated renal tubule suspensions indicates that the bioactivation of DCVC does not require hepatic metabolism and that the enzymes involved with bioactivation are present in the isolated tubule preparation.

The four proven nephrotoxic conjugates, DCVC, HVC-1, HVC-2 and PCBC (Figure 11) inhibited active accumulation of PAH and TEA by isolated kidney tubules while the non-nephrotoxic hydroxyethyl cysteine (Figure 11) did not inhibit transport at a concentration of 1 mM. The
results of the present in vivo study are in agreement with those obtained from the in vivo exposure of mice to these compounds in which a focal necrosis of the pars recta of the proximal tubules was produced for all compounds except hydroxyethyl cysteine (Gandolfi et al., 1981). This indicates that renal function as measured by tubular active transport may provide a rapid indication of the nephrotoxicity of these conjugates.

PCBC also inhibited PAH and TEA transport. The nephrotoxic parent of PCBC, hexachloro-1,3-butadiene (HCBD) inhibits renal transport in the rat (Berndt and Mehendale, 1979; Lock and Ishmael, 1979) and causes degeneration of the pars recta region of the proximal tubule (Harleman and Seinen, 1979; Jaffe et al., 1983). HCBD inhibits PAH and TEA transport after in vivo exposure (Lock and Ishmael, 1979) but only PAH transport decreases if renal tissue is exposed in vitro (Lock and Ishmael, 1979; Berdt and Mehendale, 1979). In the present study, PCBC inhibited both PAH and TEA transport in vitro. HCBD itself is thought to have little effect on the transport of either PAH or TEA (Lock and Ishmael, 1979; Berndt and Mehendale, 1979). Addition of 0.1 mM HCBD to renal cortical slices caused no decrease in PAH or TEA accumulation (Lock and Ishmael, 1979) while in the present study 0.1 mM PCBC caused a significant decrease in both PAH and TEA transport, indicating that this chemically synthesized metabolite is nephrotoxic while the parent compound per se is not.

The nephrotoxicity and biotransformation of the haloalkenes TCE and CTFE were studied in vivo and in vitro to further elucidate the
bioactivation of the haloalkenes to the cysteine conjugate. TCE did not produce nephrotoxicity in rabbits. After 60 hr in vivo exposure to 60 mg/kg/hr TCE the only detected change in renal function was a decrease in total urinary creatinine, this probably being a result of anesthesia and surgery rather than TCE exposure. In previous studies TCE has produced liver necrosis in vivo but no evidence of renal damage has been found (Plaa and Larson, 1965; Lock, 1982). However, there have been numerous reports of renal damage caused by the cysteine conjugate of TCE, DCVC, after in vivo exposure (McKinney et al., 1959; Schultze et al., 1959; Parker, 1965; Tarloff et al., 1979).

The lack of nephrotoxicity after in vivo TCE exposure may result from low level, or no formation of DCVC as a result of the oxidative metabolism. Chlorinated ethylenes may be extensively metabolized in the liver via a cytochrome P-450 mixed function oxidase mediated oxidation to form a reactive epoxide (Bonse and Henschler, 1976). The epoxide may rearrange to form the major oxidative metabolite of TCE, chloral hydrate (Moslen et al., 1977a, 1977b; Rush and Hook, 1982) or may bind to liver macromolecules. Lock (1982) concluded that TCE was metabolized primarily by oxidation in the liver and hence nephrotoxicity did not occur after in vivo exposure. Another possibility for the lack of nephrotoxicity is that prolonged low-level exposure to DCVC formed in vivo from TCE may result in binding of the reactive metabolite to C-S lyase, resulting in inactivation of the enzyme. If C-S lyase is inactivated the reactive metabolic of DCVC
would not be formed. A similar inactivation of C-S lyase was suggested to occur after exposure of the rat to low levels of CTFE (Buckley et al., 1982).

The major site of haloalkene glutathione conjugation is the liver. Since TCE was not shown to be nephrotoxic in vivo, the potential formation of the TCE-glutathione conjugate, DCVC, was investigated in the perfused liver. Liver perfusate and bile were tested for the presence of DCVG using renal tubule PAH and TEA transport integrity as a bioassay. Haloalkene-glutathione conjugates have a molecular weight of 350-400 and should be secreted from the liver primarily in the bile (Chasseaud, 1976). However, both bile and perfusate were tested in the bioassay to ensure that any conjugates formed would be detected. To enhance the potential formation of the glutathione conjugate the isolated liver was perfused with 4 mM glutathione prior to TCE exposure, since organ perfusion may substantially reduce endogenous glutathione concentration (Leibach et al., 1974). The glutathione perfusion should ensure that the liver contained sufficient glutathione to bind with TCE. To reduce the oxidative metabolism of TCE, a rabbit was injected with AIA prior to liver isolation. In vivo administration of AIA destroys the heme moiety of hepatic microsomal cytochrome P-450 (Deloria et al., 1980; Bonkowsky et al., 1980), with maximal inhibition 2-12 hr after injection. Incubation of renal tubules with liver perfusate or bile collected from AIA, glutathione and TCE exposed livers did not inhibit tubule active transport. This indicates that DCVG was not formed by the liver after TCE exposure at a concentration sufficient to inhibit transport.
Although glutathione S-transferases are present primarily in the liver, these enzymes are also present in the renal tubules (Boyland and Chasseaud, 1969). Therefore, since tubules are potentially capable of forming DCVC from TCE, the potential toxicity of TCE in isolated renal tubule suspensions was investigated. Incubation with 20 mM TCE for 20 min inhibited tubule active transport. Because of the short time required for TCE to inhibit active transport, it is unlikely that inhibition results from formation of DCVG with subsequent bioactivation to DCVC. DCVC inhibits active transport within 15 min while the additional bioactivation required for DCVG increases this time to 45 min. Therefore if inhibition of active transport after TCE exposure resulted from DCVG formation this inhibition should not occur for at least 45 min. Transport inhibition may be caused directly by TCE and result from interference with membrane integrity since TCE is a highly lipophilic solvent (Browning, 1965). This possibility was not verified, since tubules were not incubated with structurally similar but inert solvents such as those used in the investigation of CTFE-induced nephrotoxicity.

In contrast to TCE, CTFE inhibited tubule active transport after in vitro exposure. Incubation of renal tubule suspensions with CTFE also resulted in a progressive decrease in CTFE concentration, a concomitant decrease in tubule NP-SH and appearance of CTFE-GSH in the suspension medium. Prior incubation of tubule suspensions with the γ-glutamyl transpeptidase inhibitors AT-125 or serine/borate (Meister et al., 1981) significantly protected against the decrease in active transport resulting from subsequent incubation with CTFE. The
protection against CTFE conferred by these inhibitors of glutamate from CTFE-GSH is required before toxicity is expressed. This seems logical since the sequential cleavage of glutamate and glycine is required to form the cysteine conjugate.

Inhibition of tubule active transport after incubation with CTFE was not caused by a direct effect of CTFE on tubule membranes since incubation with the lipophilic gases, ether and halothane, did not inhibit transport. It is possible that the decrease in transport may result from the decrease in renal tubule NP-SH since active PAH and TEA transport is dependent on glutathione. However, addition of the γ-glutamyl transpeptidase inhibitor, AT-125, significantly protected active transport indicating that the decrease in NP-SH alone is not responsible for the decrease in tubule active transport. The chemically synthesized saturated conjugates, CTFE-GSH and CTFE-cys also inhibited the PAH transport. Maximal inhibition occurred at 45 min for CTFE-GSH and 15 min with CTFE-cys. A similar time difference was noted for tubule transport inhibition by DCVG and DCVC, and may be related to bioactivation of the glutathione conjugate to the cysteine conjugate.

Glutathione conjugation may occur non-enzymatically in the presence of glutathione (Boyland and Chasseaud, 1969). However, the accelerated tubule-dependent disappearance of CTFE and formation of the saturated conjugate, CTFE-GSH, is in agreement with the enzymatic formation of this compound postulated by Dohn and Anders (1982). This conjugate, originally thought to be the unsaturated S-vinyl product, was
assumed to exert nephrotoxicity via formation of the vinyl cysteine conjugate, HVC-1, and subsequent cleavage by C-S lyase. However, the chemically synthesized, nephrotoxic, glutathione conjugate was characterized as the saturated halogenated ethyl-glutathione conjugate, CTFE-GSH. The conjugate formed by incubation of isolated tubules with glutathione co-eluted with the chemically synthesized saturated conjugate, CTFE-GSH, on the amino acid analyzer and was therefore assumed also to be the saturated conjugate. This finding is in agreement with Dohn and Anders (1982b) who demonstrated formation of the saturated conjugate after incubation of rat hepatic cytosol with CTFE. It is possible that CTFE-GSH is a substrate for C-S lyase although high substrate specificity for C-S lyase is conferred by aromatic (Tateishi and Shimizu, 1980), or vinyl groups, which have an electronic configuration similar to aromatic groups. Anders, M. W. (pers. comm.) proposed that the saturated conjugate may also rearrange to yield a nephrotoxic episulfonium ion, although the role of this ion in CTFE-GSH toxicity is not certain.

The chemically synthesized, unsaturated glutathione conjugate of TCE, DCVG, inhibited renal active PAH and TEA transport after in vivo and in vitro exposure in the rabbit. The potency of DCVG was similar to DCVC; however, maximal inhibition of transport in vitro occurred after 60 min compared to 15 min for DCVC. This indicates that DCVG may require additional biotransformation, such as cleavage of glutamate and glycine from the glutathione residue, for nephrotoxicity to be
expressed. This scheme has been postulated to account for the bio-
activation of HCBD and CTFE to their respective cysteine conjugates
after glutathione conjugation in the liver (Lock, 1982). Formation of
the cysteine conjugate from the glutathione conjugate may occur in
isolated renal tubules since γ-glutamyl transpeptidase and cysteinyl-
glycine dipeptidase are both present in the proximal tubule (Hughey et
al., 1978; Meister et al., 1981). Two lines of evidence demonstrate
that DCVG exerts nephrotoxicity only after metabolism to DCVC. First,
incubation of tubules with the irreversible (AT-125) and reversible (L-
serine in the presence of borate) γ-glutamyl transpeptidase inhibitors
(Meister et al., 1981) decreased the inhibition of active transport
produced by subsequent incubation with DCVG. AT-125 was the most
effective in protecting against DCVG. The other line of evidence for
DCVC being responsible for the DCVG-induced transport inhibition was
provided by the in vitro incubation of tubules with DCVG. Throughout
the incubation, DCVG disappeared while there was a concurrent and
sequential appearance of glutamate, glycine, DCVC and ammonia in the
incubation medium. Glutamate and glycine were assumed to be products of
DCVG peptidase cleavage, and ammonia to result from cleavage of DCVG by
C-S lyase (Anderson and Schultz, 1965). The delayed appearance of
ammonia, and consequent formation of the reactive metabolite, corre-
sponds with the late onset of tubule transport inhibition. Ammonia pro-
duction is only an approximate indication of DCVC cleavage by C-S lyase
because in addition to producing ammonia (which was corrected for in
control studies), renal tubules can also consume ammonia (Pitts, 1968).
Unlike C-S cleavage which activates DCVC, N-acetylation leads to excretion of DCVC as the non-toxic DCV-NAC since the N-acetyl conjugate is not a substrate for C-S lyase (Tateishi et al., 1978). This may be formed physiologically in a reaction catalysed by an endoplasmic reticulum-associated N-acetylase (Tate, 1980; Moldeus et al., 1978). Since DCV-NAC is non-toxic, inhibition of tubule active transport by DCV-NAC may occur as a result of deacetylation in the kidney to reform DCVC. Enzymes that deacetylate N-acetylated xenobiotics have been found in the liver and kidney of many species (Dauterman, 1980), with considerable variation in activity between species, strains and individuals. The role of acetylation in xenobiotic excretion is determined by the balance between the two competing steps, acetylation and deacetylation. This competition may be important in determining species susceptibility to DCVC-induced nephrotoxicity. The dog, a species highly susceptible to DCVC-induced renal damage (Terracini and Parker, 1965), has low N-acetylase and high deacetylase activity (Dauterman, 1980). Hence, in the dog, DCVC would be metabolized only to a small extent by acetylation and would therefore be available for cleavage by C-S lyase. The rabbit, a species less susceptible to DCVC-induced renal damage (Terracini and Parker, 1965) has high N-acetylase and lower deacetylase activity (Dauterman, 1980). Therefore relatively less DCVC would be metabolized by C-S lyase. The inhibition of PAH transport in isolated tubules by DCV-NAC indicates that deacetylation can occur in the tubules, although the slow onset (45-60 min) of transport inhibition
may result from low deacetylase activity in the proximal tubules. If deacetylation of DCV-NAC to DCVC is responsible for transport inhibition, tubules isolated from species having high deacetylase activity would be expected to show an increase susceptibility to DCV-NAC induced toxicity, however in this study toxicity was studied only in the rabbit.

Although these studies have further defined the pathway of haloalkene biotransformation, the mechanism by which renal tubular toxicity is induced by exposure to vinyl cysteine conjugate is not known. The decreased active transport after in vivo or in vitro exposure to the vinyl cysteine conjugates did not appear to be caused by inhibition of mitochondrial oxidation since incubations in the presence of 1 mM halogenated vinyl cysteine did not inhibit tubule O2 utilization or production of 14C02 from 14C-glucose or 14C-succinate. Farah and Rennick (1956) suggested that TEA transport derives its main energy supply from an oxidative mechanism which may not be dependent upon the integrity of the Krebs cycle, while PAH transport is fully dependent upon the Krebs cycle. Depression of gluconeogenesis (Yoshida and Mekoff, 1968), alteration of mitochondrial structure (Goyer, 1968) and impairment of mitochondrial oxidative phosphorylation (Goyer, 1971) have all been implicated in inhibition of tubular active transport. DCVC is a known kidney and liver mitochondrial poison in vivo (Stonard and Parker, 1971a) and in vitro (Stonard and Parker, 1971b). Oxygen utilization after in vivo (4 hr after 55 mg/kg, ip) or in vitro (0.1 mM) exposure was irreversibly decreased by approximately 30%. In the present study
no difference was seen between control and DCVC treated tubules in \( O_2 \) utilization or production of \( ^{14}C_2 \) from \( ^{14}C \)-glucose and \( ^{14}C \)-succinate. Lack of inhibition of \( ^{14}CO_2 \) production from succinate indicates that there is no inhibition of the citric acid cycle activity by DCVC while the results of the glucose incubation indicate that DCVG does not decrease glycolysis or oxidation of glucose-derived metabolites in the citric acid cycle. Thus, DCVC does not appear to inhibit renal tubular mitochondrial oxidation at the concentrations used in these studies. The lack of inhibition of mitochondrial respiration in rabbit renal tubules may be due to the inability of DCVC to reach the renal tubular mitochondria or due to species variation in metabolism since the studies of Stonard and Parker (1971b) utilized mitochondria isolated from rats. The reactive nature of the toxic product formed after cleavage by the cell membrane associated C-S lyase would presumably prevent access to the inner regions of the cell. Isolated mitochondria may metabolize DCVC to a reactive species, with biotransformation occurring in either the matrix or inter-membrane space (Stonard and Parker, 1971a). However, for DCVC to inhibit mitochondrial oxidation it must penetrate one or both mitochondrial membranes as the parent molecule.

Both PAH and TEA transport systems are thought to be mediated by protein-like carriers. Foulkes and Miller (1959) first postulated that PAH transport is mediated by such a carrier and more recently indirect evidence has been provided for the existence of plasma membrane protein carriers which regulate both organic anion and cation transport into
tubule cells (Holohan et al., 1975). Thus, it is possible that the susceptibility of active transport to DCVC exposure may result from covalent binding of the DCVC generated reactive intermediate to nucleophilic groups such as the sulfhydryl groups associated with membrane transport proteins. A less specific binding to membrane associated sulfhydryl groups would also reduce active anion and cation transport by altering membrane integrity.

Derr and Schultze (1963) demonstrated that the electrophilic product of DCVC cleavage by C-S lyase combines irreversibly with proteins or other tissue components containing nucleophilic groups, possibly by alkylation. Of the potential electrophilic acceptors investigated as potential protective agents against DCVC-induced transport inhibition, only the sulfhydryl-containing nucleophiles reduced the degree to which DCVC inhibited renal tubular active PAH and TEA transport. The sulfhydryl compounds cysteamine and cystamine did not afford significant protection against DCVC, although both agents have been used as radical scavengers in the treatment of drug poisonings such as that induced by paracetamol (Strubelt et al., 1974; Labadarios et al., 1977). Cystamine is thought to be rapidly reduced in vivo to the thiol form, this being the active radical scavenger. Under the conditions prevalent in the isolated tubule suspension, it is possible that cysteamine is rapidly oxidized to cystamine, which may explain its lack of protection against DCVC. Addition of a reducing agent such as dithiothreitol to the suspension would maintain cysteamine in the reduced form, but was not used in this study.
Glutathione and cysteine protect isolated renal tubules against the DCVC active metabolite, with GSH being the most efficacious. Glutathione was shown not only to protect against decreased active transport when added simultaneously with DCVC, but also reversed the decrease in transport caused by prior exposure to DCVC. The degree of this recovery decreased as the concentration of DCVC increased. Also, pre-incubation of tubules with GSH made them more refractory to DCVC-induced inhibition of acid and base transport. The increase in NP-SH above normal levels in tubules incubated with GSH is in agreement with previous studies indicating that the kidney is able to actively accumulate GSH (Fonteles et al., 1976; Leibach et al., 1974; Bartoli et al., 1978; Meister, 1975). Finally, when renal tubules were isolated from rabbits exposed to DCVC, the reduced tubular active transport exhibited by these animals could be partially restored to normal values by treating the tubules with buffer containing GSH.

In the isolated tubule suspension, cells accumulated NP-SH to a concentration equivalent to that in the incubating medium (Table 3). This is consistent with the current concept of renal GSH transport which indicates that the kidney is able to transport GSH across the renal tubular brush border membrane (Rankin and Curthoys, 1982). The current concept of this transport states that the tripeptide is hydrolyzed in the extracellular space of the renal tissue prior to uptake into the renal tubular cells (Hahn et al., 1978; Meister, 1983). The initial degradation results from the action of γ-glutamyl transpeptidase.
(γ-glutamyl transferase) which is located in the brush border membrane of the kidney tubules (Meister et al., 1981; Silbernagl et al., 1978; Welbourne and Dass, 1982). The oxidized form of GSH, glutathione disulfide (GSSG) and not GSH, is the preferred substrate for γ-glutamyl transpeptidase (Jones et al., 1979; Ormstad, 1982) and oxidation to GSSG may be the initial step in metabolism of exogenous GSH in kidney (Jones et al., 1979). The resulting cysteinylglycine is further cleaved to cysteine and glycine by cysteinylglycine dipeptidase is localized in the same subcellular compartment of renal tissue as γ-glutamyl transpeptidase and is assumed to act at the outer surface of the cell (Hahn et al., 1978). Hence, complete hydrolysis of GSH can occur in the lumen of the kidney tubule. The constituent amino acids formed from extracellular cleavage of GSH may then be transported into the cell by active transport (Meister, 1973), where GSH may be resynthesized in an ATP-utilizing reaction (Orlowski and Wilk, 1976).

To further examine the possible involvement of GSH in the protection against nephrotoxicity produced by DCVC, endogenous tubular GSH was depleted using diethyl maleate. The depletion of GSH results from nonenzymic and enzyme catalyzed conjugation of the tripeptide with diethyl maleate (Chasseaud, 1976b). Diethyl maleate produces a transient depletion of GSH. In cells isolated from rabbit kidneys preperfused with 0.004 mM diethyl maleate for 6 min intracellular GSH is depleted to approximately 30% of control values (Ormstad, 1982). Replenishment of GSH occurred within 35-40 min in the presence of the
precursor amino acids, glutamic acid, glycine and cysteine (Ormstad et al., 1980). Depletion of GSH by various pretreatment such as diethyl maleate has been shown to potentiate toxicity of a number of compounds. This potentiation may be at least partially reversible. For example, GSH depletion increased acetaminophen induced hepatic toxicity, while treatment with cysteine, a precursor of GSH, decreased both necrosis and covalent binding (Mitchell et al., 1976). Although diethyl maleate itself decreases the active transport of PAH and TEA, it appears to augment the inhibitory effect of DCVC, possibly by reducing endogenous GSH concentration and allowing more of the reactive intermediate to bind to subcellular macromolecules. The degree of DCVC-toxicity potentiation by diethyl maleate (Table 4) appeared to coincide with the cumulative depletion of tubular NP-SH when tubules were incubated with DCVC after pretreatment with diethyl maleate (Table 2). Diethyl maleate and GSH probably do not inhibit the metabolism of DCVC; therefore, they apparently alter renal toxicity by their effect on GSH availability.

There are two possible causes for the depletion of tubule GSH after incubation with DCVC. First, the DCVC metabolite may be conjugated with GSH, thus depleting the endogenous GSH level if the rate of GSH synthesis is lower than the rate of conjugation. This has been shown with other in vitro systems. The concentration of endogenous GSH in bromobenzene treated hepatocytes decreases initially during conjugate formation since the rate of conjugation exceeds that of GSH synthesis (Thor et al., 1978). In chloroform treated hepatocytes activity of
γ-glutamyl cysteine synthetase is decreased resulting in inhibition of GSH synthesis (Ekstrom et al., 1982). Secondly, GSH depletion may result from a DCVC-induced decrease in GSH synthesis. The two sequential enzymatic steps in GSH synthesis in which ATP is required (Orlowski and Wilk, 1976; Orrenius and Jones, 1978) may be susceptible to DCVC toxicity since DCVC inhibits mitochondrial oxidation of certain key intermediates in the isolated mitochondrial preparation (Stoner and Parker, 1971). However, mitochondrial respiration in isolated tubules is not decreased after DCVC exposure (Chapter 2), hence, it is unlikely that the ATP supply would be decreased.

A possible mechanism to explain the role of GSH in protecting against DCVC-induced decreased active transport is that a minimal subcellular concentration of GSH is required to maintain the integrity of tubular active transport (Zehavi-Willner et al., 1971; Fonteles et al., 1976). After exposure to low concentrations of DCVC, NP-SH is depleted in the renal tubules. Since GSH represents over 95% of the acid soluble NP-SH of most tissues (Hewitt et al., 1974) it is possible that the decrease in NP-SH is caused by depletion of intracellular GSH which acts as a nucleophile to bind the electrophile metabolite. In addition to this depletion there is a decrease in active PAH and TEA transport. Partial depletion of GSH at low concentrations of DCVC may result in reversible membrane damage caused by one or more of the following: diminution of surface change, possibly involving loss of sialic acid groups (Kosower and Kosower, 1974); changes in ion transport
rates, some of which may be due to oxidation of membrane thiol groups (Meznick, 1970; Carter, 1973); lipid alteration, destruction and loss due to auto oxidation reactions induced by free radicals and other oxidizing agents (Kosower and Kosower, 1974). At higher concentration of DCVC, much of the available cystosolic GSH may be utilized in binding the DCVC reactive metabolite and the excess toxic metabolites may then react with macromolecular nucleophiles within the cell. The decrease in active transport resulting from this binding is not reversed by addition of GSH to the tubules.

Cation and anion transport systems in kidney tubules are distinct and independent, and the difference in susceptibility to vinyl cysteine conjugates may result from the proximity of C-S lyase to these transport sites. C-S lyase has been demonstrated in renal brush border preparations (Bonhaus and Gandolfi, 1981), but activity in the tubule antiluminal membrane has yet to be investigated. However, for the reactive product to inhibit active anion and cation transport, it seems likely that C-S lyase should also be present at the antiluminal membrane of the tubule cells in addition to the brush border membrane, since active organic anion transport has been reported to occur across the antiluminal membrane while active organic cation transport is thought to occur across the luminal membrane (Kinsella et al., 1979). To verify the location of C-S lyase in the proximal tubule cell an accurate assay is required to measure enzyme activity at different subcellular locations.
Since all substrates are cleaved by C-S lyase to form ammonia and pyruvate, measuring formation of these cleavage products should provide a universal assay for C-S lyase, regardless of the substrate used. However, current assays for ammonia and pyruvate lack accuracy and sufficient sensitivity to measure C-S lyase in vitro, unless a purified enzyme preparation is used. No consistent and reproducible ammonia production was detected in renal tubule brush border membrane with the Nessler technique or fluorescence assay. Both DCVC and S-(2-benzothiazolyl)-L-cysteine (Figure 22) were used as the enzyme substrate in these assays. Low sensitivity may result when ammonia is measured as a gas evolved from the incubation. However, high background ammonia concentration in the deproteinized incubation supernatant precluded ammonia measurement in the incubation medium. Also, ammonia production and utilization in renal tissue (Pitts, 1968) increases the inherent inaccuracy of this assay.

C-S lyase was detected in brush border membrane suspensions by measuring pyruvate evolution from DCVC. As with ammonia measurements, accuracy of the pyruvate assay is decreased by the potential for further anabolic and catabolic reactions. To improve the accuracy and sensitivity of both assays, purified enzyme preparations should be used. (Dr. E. A. Lock, ICI, personal communication). C-S lyase activity was low in rabbit brush border membrane suspensions, 19 nmol pyruvate/min/mg protein compared to a value of 92 nmol/min/mg protein in rat brush border membrane (Bonhaus and Gandolfi, 1981). The low value may result from
species variation in C-S lyase activity (Tateishi and Shimizu, 1980), or greater sensitivity of the rabbit enzyme to inactivation by the reactive metabolite formed from DCVC cleavage. To avoid problems with enzyme inactivation, substrates which did not produce reactive intermediates after enzyme catalyzed cleavage were used in subsequent C-S lyase assays.

C-S lyase activity was detected in kidney cortex homogenates by measuring disappearance of the fluorescent substrate, N-acetyl-S(6-chloro-2-methoxy-9-acridinyl-L-cysteine. The activity was 0.25 nmol substrate metabolized/min/mg protein; therefore, affinity of this substrate for C-S lyase is approximately 20% of that for S-(2-benzothiazolyl)-L-cysteine and 1-2% of that for DCVC. No decrease in fluorescence occurred when this substrate was incubated with isolated tubules or renal brush border membrane suspensions. A free amino group on the cysteine moiety or cysteine conjugate is required for these conjugates to be substrates for C-S lyase cleavage (Tateishi et al., 1978). However, the fluorescent substrate is N-acetylated and must be deacetylated prior to C-S lyase cleavage (Figure 23). Deacetylases are cytosolic (Dauterman, 1980); therefore the substrate may only be deacetylated, and be a substrate for C-S lyase, in preparations such as the cortex homogenate where cellular structure is disrupted and the deacetylase freely available.

The activity of C-S lyase in rabbit kidney cortex homogenates, measured by the production of 2-mercaptobenzothiazole from
S-(2-benzothiazolyl)-L-cysteine was 1.3 nmol/min/mg protein. This compares with a value of 6.2±1 nmol/min/mg protein in rat kidney cortex homogenate obtained by Dohn and Anders (1982a). The difference between these values may result from differences in homogenate preparation, incubation time, homogenate protein concentration, or may reflect a species variation. Tateishi and Shimizu (1980) reported C-S lyase activity is rabbit liver 105,000 g supernatant was approximately twice that in the rat, but no comparison of kidney cortex levels was made. C-S lyase activity, determined by the S-(2-benzothiazolyl)-L-cysteine assay, was decreased to approximately 60% of normal in isolated renal tubules incubated with 5 mM DCVC for 15 min prior to the enzyme assay. However, no decrease was detected in homogenate prepared from tubules incubated with DCVC. The DCVC reactive species binds irreversibly with nucleophilic groups of biomacromolecules including the C-S lyase complex. In isolated tubules, the intact brush border membrane structure in the vicinity of the membrane-associated C-S lyase, results in the enzyme complex being the closest available nucleophilic group; hence, the reactive species may bind irreversibly with C-S lyase decreasing activity (Anderson and Schultze, 1965; Bhattacharya and Schultze, 1967; Buckley et al., 1981). However, in homogenates prepared from tubules incubated with DCVC there is no ordered brush border membrane structure and nucleophilic groups other than those of the C-S lyase complex would be present in close proximity to the enzyme to bind the reactive species. Therefore, C-S lyase activity would be decreased
to a lesser extent in the cortex homogenate than in the tubule preparation after incubation with DCVC.

Alternatively, the lack of C-S lyase inhibition in the homogenate could result from the DCVC reactive metabolite binding reversibly by acylation with C-S lyase in isolated tubules. Subsequent homogenization of tubules would increase the availability of other potential acceptors for the reactive metabolite. The metabolite may then acylate other nitrogen centers such as amines and amino acids resulting in liberation of the bound enzyme. Binding of the DCVC reactive intermediate with nucleophilic groups by acylation contradicts the current dogma of irreversible binding by alkylation. Several lines of evidence point to an alkylation reaction, the intermediate reacts with sulfydryl compounds such as glutathione and cysteine (Bhattacharya and Schultze, 1967), with the alkyl acceptor 4-(p-nitrobenzyl)pyridine (Bhattacharya and Schultze, 1967) and with proteins including C-S lyase (Anderson and Schultze, 1965). This alkylating species is thought to contain vinyl carbon and sulfur but no chlorine (Bhattacharya and Schultze, 1967). Therefore, cleavage by C-S lyase yields pyruvate, ammonia and chloride in a 1:1:2 ratio in addition to the reactive fragment (Anderson and Schultze, 1965). To react with biomacromolecules by acylation, the reactive intermediate must be formed by the pathway shown in Figure 24. No direct experimental evidence is available to support this pathway; however, it is presented as a biochemically viable alternative to the classical cleavage of DCVC. Following C-S lyase
catalyzed cleavage of DCVC, a β-elimination occurs and pyruvate, ammonia and dichlorovinyl mercaptane are formed. Dichlorovinyl mercaptane may then undergo rearrangement to form chlorothioacetic acid chloride, a potentially toxic species. Hydrolysis results in formation of the relatively inactive chloroacetic acid, while reaction with biomacromolecules by an acylation may result in toxicity. Unlike the classical pathway for DCVC cleavage, only 1 mole of chloride would be evolved for every mole of DCVC cleaved.

The C-S lyase assays utilizing S-(2-benzothiazolyl)-L-cysteine and N-acetyl-S-(6-chloro-2-methoxy-9-acridinyl)-L-cysteine allow detection of C-S lyase in kidney cortex homogenates. The S-(2-benzothiazolyl)-L-cysteine assay may also be used successfully with other in vitro preparations such as isolated tubule and brush border membrane suspensions while, the fluorescence assay has the disadvantage of only functioning in systems where the deacetylase is freely available.

Development of more sensitive versions of these or other assays, utilizing colorimetric or fluorescence detection, would allow determination of the specific location of C-S lyase in the proximal tubule cell and, in particular, determine if the enzyme is associated with the antiluminal as well as luminal proximal tubule cell membrane. Such an assay would also be useful in further defining C-S lyase substrate specificity.

In addition to answering many of the questions posed at the outset of this study, the findings pose many new and intriguing
questions. Further defining the location of C-S lyase in the proximal tubule cells may provide insights into the mechanisms involved in the inhibition of both PAH and TEA transport by the vinyl cysteine conjugates. It has been demonstrated that the saturated ethyl glutathione conjugate may be formed when isolated renal tubules are incubated in the presence of CTFE. Also the chemically synthesized saturated, ethyl glutathione and ethyl cysteine conjugates inhibit active transport in isolated tubules. Further work is required to define the role of these saturated conjugates in haloalkene-induced nephrotoxicity and the mechanism by which they result in the formation of a nephrotoxic species. The specific action of the vinyl cysteine conjugate reactive metabolite at the cell membrane, although resulting in transport inhibition in vitro and proximal tubule necrosis in vivo, is not known. Answering these questions will not only aid in elucidating the mechanism by which the reactive metabolite elicits toxicity in the proximal tubule, but should also provide many useful insights into the study of mechanisms of nephrotoxicity.
Figure 22. Chemical synthesis of S-(2-benzothiazolyl)-L-cysteine and C-S lyase catalyzed metabolism to 2-mercaptobenzothiazole. Pyruvate and ammonia.
Figure 23. Chemical synthesis and C-S lyase catalyzed cleavage of N-acetyl-S-(6-chloro-2-methoxy-9-acridinyl)-L-cysteine.
Figure 24. Schematic representation for C-S catalyzed formation of a nephrotoxic species from DCVC.
GLOSSARY

TCE  Trichloroethylene
CTFE Chlorotrifluoroethylene
CDFE Chlorodifluoroethylene
HCBD Hexachloro-1,3-buta diene
DCVC S-(trans-1,2-dichlorovinyl)-L-cysteine
PCBC S-(pentachlorobuta-1,3-dienyl)-L-cysteine
HVC-1 S-(trans-1,2-difluoro-1-chlorovinyl)-L-cysteine
HVC-2 S-(1-chloro-2-fluorovinyl)-L-cysteine
HEC S-(1-hydroxyethyl)-L-cysteine
2BE  2-bromoethanol
DCVG S-(trans-1,2-dichlorovinyl)-L-glutathione
DCV-NAC S-(trans-1,2-dichlorovinyl)-L-N-acetyl-cysteine
CTFE-GSH S-(2-chloro-1,1,2-trifluoroethyl)-L-glutathione
CTFE-cys S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine
PAH  Para-aminohippuric acid
TEA  Tetraethylammonium bromide
HVT  Halogenated vinylthio conjugate
AT-125 L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazole-acetic acid
AIA  Allylisopropylacetamide
DMSO Dimethylsulfoxide
Diamide Diazenedicarboxylic acid bis (N,N-dimethylamide)
REFERENCES


